Old Herborn University Seminar Monograph

17. POSSIBILITIES FOR ACTIVE AND PASSIVE VACCINATION AGAINST OPPORTUNISTIC INFECTIONS

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OLD HERBORN UNIVERSITY SEMINAR MONOGRAPH

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MEETING SUMMARY: POSSIBILITIES FOR ACTIVE AND PASSIVE VACCINATION AGAINST OPPORTUNISTIC INFECTIONS*

RICHARD I. WALKER (Conference Chairman),
THOMAS BLANCHARD, JAN MATTHIAS BRAUN, JOHN J. CEBRA,
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IAN ALAN HOLDER, J. HUEBNER, RUTH MATTHEWS, GERALD B. PIER,
LUIGINA ROMANI, B.U. VON SPECHT, and MATTHIAS TRAUTMANN
(Conference Faculty)

INTRODUCTION

Opportunistic pathogens present an ever-growing threat to mankind in spite of numerous medical advances. This event is a consequence of an increase in bacterial resistance to commonly used antibiotics all over the world as well as a greater survival of individuals immunocompromised by chronic and acute diseases or injuries. To meet this challenge, the International Study Group for New Antimicrobial Strategies (ISGNAS) took a fresh look at the possibilities for vaccination against opportunistic infections. Although this approach to control these infections has been a goal for many years, to date there are no licensed products available for this purpose. Based on the results presented at an ISGNAS-sponsored meeting in Herborn, Germany, June 23-25, 2003, there is now reason to hope that this situation could change. This volume of the Old Herborn University Seminar Monographs constitutes the report of that meeting. The meeting consisted of one day of formal reports followed by discussion among the conference faculty. The major points presented during this meeting are summarised below.

WHAT IS AN OPPORTUNISTIC PATHOGEN?

Colonisation of skin and mucosal surfaces with microorganisms begins at birth such that a generally beneficial symbiotic relationship is established that lasts throughout life. The importance of some members of this microbial community is illustrated by research presented from Dr. Cebra's laboratory, which provides insight into the role of intestinal microorganisms in the development and maintenance of the intestinal humoral immune system. Infections referred to collectively as "opportunistic" are those resulting from otherwise commensal organisms, either resident or hospital acquired, when the normal state is disturbed by factors which damage mucosal surfaces or mediate immune defects, such as antibiotic use, intravenous catheters, mucosal breakdown or HIV infection. Dr. Romani suggests another piece to this equation. Her work with *Candida* suggests a model where changes in the microorganism promote an alteration in the response of host dendritic cells (DC), which effectively

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transforms the relationship from a commensal state to a disease state. It is with this in mind that the term "opportunistic pathogen" should not only include the traditional culprits (i.e. *Pseudomonas, Staphylococcus,* etc), but other organisms normally colon ising host surfaces, such as *Helicobacter pylori*. These observations raise important unanswered questions about the regulation of the host immune system with respect to the restoration and maintenance of a commensal state with its microflora.

USE OF PASSIVELY-ADMINISTERED ANTIBODIES

Over 20 years ago the passive infusion of antibodies directed against a conserved region of the lipopolysaccharide (LPS) of Gram-negative bacteria was reported to be effective against sepsis. This approach was not further developed, however, as consistent efficacy was not obtained. At this meeting Dr. Cross reported that a subunit vaccine composed of detoxified J5 LPS complexed to group B meningococcal outer membrane protein provided both active and passive immunity and protection in animal models. A phase I study with this material showed it to be safe and immunogenic. These data suggest that further studies with this approach are now warranted.

Specific prevention of *Klebsiella* infections by passive immunotherapy has also received more attention recently. Dr. Trautmann's report focused on the generation of O serogroup-specific antisera in animals. O antigen specific antibodies were able to opsonise non-encapsulated *Klebsiella* strains, while fully encapsulated bacteria were resistant against O antibody-mediated opsonisation. *In vivo* experiments, however, demonstrated a prophylactic effect on *Klebsiella* bacteraemia in mice. Dr. Trautmann's work suggests that O antigen-specific antibodies may be useful to supplement K antigen-specific hyperimmune globulins for passive immunoprophylaxis of *Klebsiella* infections.

In another approach involving passive immunisation, Dr. Matthews reports that recombinant antibodies can be used synergistically with an antimicrobial agent to control disease. Her work showed that patients with invasive candidiasis, being treated with amphotericin B, showed a close correlation between recovery and antibody to the immunodominant heat shock protein 90. Human recombinant antibody to this protein has synergistic antifungal activity with amphotericin B and is now the subject of a clinical trial. The combination of antimicrobial agents and specific antisera against other opportunistic pathogens merits examination as a strategy to better control infection.

CANDIDATES FOR ACTIVE VACCINATION AGAINST OPPORTUNISTIC PATHOGENS

Progress has also been realised towards the goal of active immunisation against many of the major opportunistic pathogens. This approach is facilitated by the facts that compromised patients respond immunologically to active vaccination and the fact that 65 percent of surgeries are elective, which would indicate that at-risk populations could be identified for vaccination. Further reason

for optimism with this approach is the discovery and application of new antigens and techniques, particularly conjugate vaccine technology.

Pseudomonas aeruginosa is an opportunistic pathogen responsible for often life-threatening complications. Based on an extensive number of approaches that have been studied to vaccinate against this pathogen, reviewed in Dr. Holder's presentation, it is now possible to begin to focus on pseudomonas antigens that seem most promising. Of these, the type III translocation protein (PcrV), LPS-O-polysaccharide, OMP and flagellar antigens are noteworthy. In fact, Dr. von Specht described a recombinant OMP vaccine that was safe and immunogenic in burn patients. New data from others show that O antigen or capsular polysaccharide could offer useful vaccine antigens for *Escherichia coli* and the polysaccharide-based vaccine approach may also be applicable to development of a vaccine against *Cryptococcus*.

Polysaccharide conjugate vaccines also offer a promising approach for vaccines against Gram-positive organisms. Dr. Fattom reported clinical trial results with a capsular polysaccharide vaccine against *Staphylococcus aureus* capsular types 5 and 8, which together comprise over 80% of the clinical isolates worldwide. A trial with this vaccine in haemodialysis patients found that efficacy could be observed at 40 weeks post immunisation as vaccination reduced the number of staphylococcal bacteraemias by 57%. Dr. Pier reported that the genes for biosynthesis of certain capsular polysaccharide adhesins of *S. aureus,* the poly-N-acetyl glucosamine (PNAG) molecules, are present in virtually all strains of this pathogen. Immunisation of mice with PNAG elicited opsonic and protective antibodies against multiple isolates of staphylococcus. High titred opsonic antibody was produced to multiple strains of *S. aureus* and *S. epidermidis* when this antigen was coupled with diphtheria toxin to produce a conjugate vaccine.

Enterococci are one of the most common causes of hospital-acquired infections and many strains have developed resistance to all known antibiotics. Dr. Huebner's group has found that enterococci also possess polysaccharide-containing capsules with features of teichoic acids, which may provide vaccine candidates. One of these polysaccharides is expressed by both *E. faecalis* and *E. faecium* and the antigen is a target for opsonic antibodies. Rabbit antibodies raised against this purified polysaccharide were effective as a therapeutic agent in mice even when the administration of antisera was initiated up to 48 hours after challenge with live bacteria. Dr. Fattom's group has also been looking at the possibility of polysaccharide conjugate vaccines for enterococcus. Their vaccine polysaccharide is conserved on the surface of most enterococcal isolates and antibodies to it mediate in vitro opsonic killing and in vivo protection against *E. faecalis* challenge.

Clostridium difficile is a major cause of hospital-acquired infectious diarrhoea and colitis following antibiotic administration and subsequent loss of the protection afforded by intestinal flora. Dr. Giannasca reported that a toxoid vaccine being evaluated in the clinic is well tolerated and very immunogenic. Anti-toxin A IgG titres were found to far exceed the level associated with protection. The utility of the vaccine to generate a hyper-immune globulin for passive protection in acute care settings remains to be determined.

Its possible that in some cases commensal bacteria with cross-reactive antigens to those of a pathogen may be exploited for immunisation. Dr. Braun showed that both *Neisseria lactamica*

and *Moraxella catarrhalis* isolates bound antibodies to epitopes on the meningococcal LPS (epitopes associated with L3,7,9). His studies provided evidence that blood group like glycoconjugate antigens found on some commensal species might be involved in natural immunity to meningococcal endotoxins during childhood. It should be considered that natural antibody to commensal opportunistic pathogens may benefit the response to specific vaccination. Such a vaccine could in essence be viewed as a booster inoculation. In the future, immunomodulatory techniques could be developed which could also boost natural antibodies of interest.

IMMUNE RESPONSES TO OPPORTUNISTIC PATHOGENS

Evidence obtained with most bacterial opportunistic pathogens shows the importance of circulating antibodies in protection. Although many of the organisms colonise normal mucosal surfaces, it was noted that the problem arises when the host defences are altered such that the organisms get to other sites they do not usually inhabit (i.e. the bloodstream). Further, since it is difficult to dislodge organisms when they exist in a commensal mode on mucosal surfaces (i.e. nasal carriage of *S. aureus*), induction of circulating opsono-phagocytic antibodies rather than local immunity offers the most promising strategy for immunological control of bacteraemia. As indicated above, numerous antigen candidates are now available to induce protective immune responses against opportunistic pathogens. The search for conserved protective antigens is an important element of this vaccination strategy because of the relatively large number of pathogens under consideration and the many serotypes which might be clinically relevant. It is not known at present whether common antigens will provoke sufficient immunity compared to type-specific immunity. Combined vaccines for a number of major opportunistic pathogens, such as those described here, should be sought in the future. Whether immunologic interference will be a problem in vaccine combinations remains to be determined.

A better understanding of the relationship between antibody responses to specific pathogens and protection is needed. This information will be required as vaccines move toward licensure. For example, what comprises a surrogate marker for protection? Is a calculated protective antibody level a reasonable surrogate marker for protection and equivalency measure in other populations than the one in which an efficacy trial was run? Perhaps in vitro functional equivalency could be used to make the case of antibody levels equivalency more acceptable as a surrogate marker. *In vivo* protection studies in animals may be able to help interpret the significance of antibody responses. A difficulty here is the need for an animal model which closely mimics the population expected to develop an infection.

H. pylori colonises about half of the human population and, for as yet unknown reasons, in some, is associated with symptomatic gastritis, peptic ulcer disease and an increased risk for gastric adenocarcinoma. This opportunistic pathogen may differ from the others considered in this meeting because cellular immunity seems to be the key to control rather than humoral immunity. Dr. Blanchard proposed a model of *H. pylori* pathogenesis in which the pathogen induces local inflammatory and

immune responses that are limited by a population of regulatory T cells in the stomach. Consequently, immunisation might be better achieved by activation of *H. pylori*-specific T cells in peripheral lymph nodes that are capable of promoting either a qualitatively or quantitatively different inflammatory response when recruited into the stomach.

Dr. Romani suggested, based on her work with opportunistic fungi, that optimally effective immunities may be achieved by targeting specific receptors on dendritic cells *in vivo*. Her studies showed that DCs phagocytose fungal components through distinct recognition receptors which translated into disparate downstream signalling events, ultimately affecting cytokine production and co-stimulation. This was responsible for Th polarisation of patterns of susceptibility or resistance to infection. Her research also found that DCs transfected with fungal RNA restored antifungal resistance in haematopoietic transplantation.

APPLICATION OF EMERGING TECHNOLOGIES TO IMMUNOLOGIC CONTROL OF OPPORTUNISTIC INFECTIONS

Current vaccine candidates in advanced development for opportunistic pathogens are relatively immunogenic, but new developments in immunomodulation deserve consideration for future use. For example, adjuvants such CpG ODN and delivery systems such as poly glycolide poly lactide microspheres or attenuated bacterial vectors such as *Listeria monocytogenes* now on the horizon may be able to reduce the number of doses (i.e. 3 doses to one) of vaccine needed or accelerate or enhance the development of protective titres or other immune responses. These types of techniques could be useful in certain groups of immunocompromised individuals or could help overcome possible interference among multiple components of a vaccine for opportunistic infections. The recognition, for example, of the possible benefit of increased expression of FcγR and the importance of Toll like receptors and dendritic cells in determining immune responses should lead to research which will enable better immune regulation through vaccination. The potential options for better, more directed, immunomodulation may make the current term “adjuvant” obsolete. Already the observation that subcutaneous immunisation is better than the intramuscular route for inducing immune responses may be an example of exploitation of the DC in the skin. Future manipulation involving DCs may involve direct loading of antigens into the cells in vitro or targeting them in vivo. The possibility that non-specific modulators of the innate immune system could be used in combination with vaccination regimens is yet to be explored. Further, antigens themselves may be modified to modulate different immune responses.

THE CHALLENGE OF CLINICAL TRIAL DESIGN

It is possible that paradigms appropriate for paediatric and adult vaccines to be administered to healthy persons may need re-evaluation for immunisation against opportunistic infections. The duration of efficacy may need to be considered carefully in the evaluation of vaccines for people in various compromised states. Further, thought must be given to determination of whether effi-

cacy in one population can serve as proof of concept for the approach toward similar infections in other patient groups at risk, which are immunologically and physiologically equivalent or better than the indicated population. This question may become particularly important for patient populations, which are rare. A question, which may apply to all vaccines, is what clinical studies would be necessary to support the addition of an antigen component to a vaccine after licensure? For example, if a third polysaccharide element is added to a bivalent vaccine to increase coverage by the vaccine, would a second phase III efficacy trial be required? Considerable discussion will be necessary to determine what data are required for licensure of such a second generation vaccine. Another complication of trial design involves the need to maintain antibiotic treatment in all trial groups, yet show a statistically significant in groups given the vaccine also. The meeting faculty observed that improved discussion of preclinical and clinical testing approaches among government, academic and industrial entities would be essential to address practical vaccine development issues such as these.

INTERACTIONS OF COMMENSAL GUT MICROBES WITH SUBSETS OF B- AND T-CELLS IN THE MURINE HOST*

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SUMMARY

Although mechanisms operative in the induction and maintenance of specific, adaptive immunity, including 'cognate' B/T interactions, have been extensively studied and defined, we still know little about the mechanisms operative in developing and maintaining B- and T-cell dependent 'natural' immunity. Particularly, we are still rather ignorant concerning gut microbial/gut or systemic APC, T cell and B cell interactions that lead to lymphoid cell mediated 'natural' immunity: Specific or broadly reactive, activation via TCR and BCR and/or via other receptors such as the TLR series, and whether T/B interactions are operative at this level? Here we will address: 1) the general role of gut microbes in the development and maintenance of the intestinal, humoral immune system; 2) the general role of gut microbes in the development of B1 cell mediated, 'natural' gut IgA and the dependence of these B1 cells on bystander T cell help; 3) the relative contributions of B1 vs. B2 cells to gut 'natural' and specific IgA responses; 4) the role for particular 'normal' gut microbes in the initiation of inflammatory bowel diseases (IBD) in mice with a dysregulated immune system; and 5) the possible roles of gut microbes in facilitating oral tolerance, a mechanism likely operative in forestalling or ameliorating IBD. A central theme of this paper is to attempt to define the specificities of activated, functional $CD4^+$ T cells in the gut for Ags of particular, usually benign gut microbes. We will also consider the still-unresolved issue of whether the contributions of B1-derived IgA in the gut to the 'natural' Ab pool are Ag-selected and driven to proliferation/differentiation or whether the main stimuli are not via BCRs but rather other receptors (TLRs, etc.). The main experimental approach has been to use antigen-free, germ-free, or gnotobiotic (mono- or oligo-associated with precisely known bacterial species) mice.

INTRODUCTION

This overview aims to address the interactions of normally benign members of the gut microbiota with B-cells and T-cells of the mammalian host. We

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will mostly consider those host cells located in inductive sites for development of gut mucosal immunity — Peyer's patches (PP), solitary follicles (SF) — and effector sites in the gut-lamina propria (LP), inter-epithelial leukocyte (IEL) spaces. Although mechanisms operative in the induction and maintenance of specific, adaptive immunity, including 'cognate' B/T interactions, have been extensively studied and defined, we still know little about the mechanisms operative in developing and maintaining 'systemic' or mucosal lymphoid cell-dependent 'natural' immunity. Our main experimental approach has been to use antigen-free, germ-free, and gnotobiotic (mono- or oligo-associated with precisely known bacterial species) mice. For a comprehensive review of this field see *Cebra* et al. (1999). The intent of this overview is not to offer specific, practical suggestions for developing effective mucosal vaccines for particular nosocomial or opportunistic pathogenic bacteria that infect via mucosal surface. Rather, we aim to inform vaccinologists concerning how the gut mucosal immune system generally responds to enteric microbes and raise appreciation for the role of innocuous gut colonisers in possibly ameliorating particular infections via stimulation of the 'natural' gut mucosal immune system, i.e. the possible use of probiotic microbes as a complement to specific immunisation.

FINDINGS AND CONCLUSIONS

The role of benign gut microbiota in the development and maintenance of the humoral immune system; effects of gut IgA on the colonising bacteria

The main basis for implicating the 'normal' gut microbiota in the development and maintenance of gut IgA production comes from comparing antigen-free or germ-free mice with conventionally reared mice. Adult conventional mice have a plethora of IgA plasmablasts in the gut LP. A majority of all the productive plasmablasts in the entire body is found in the gut. Neonates show a lag in the outgrowth of these cells until weaning. There is a paucity of IgA plasmablasts in gut LP of adult antigen-free or germ-free mice. Thus, normal colonisation of the gut with benign, commensal microbes is accompanied by the rapid rise in IgA plasmablasts in the gut LP.

Since PP have been implicated as inductive sites for the generation of IgA-committed B cells (*Craig* and *Cebra*, 1971), we decided to assess the effects of mono-associating germ-free mice with a benign mouse commensal, *Morganella morganii* (Gram-negative rod) on activating germinal centre reactions (GCRs) in PP. Such GCRs generate IgA committed, specific B cells in immunocompetent mice. While conventionally reared mice contain PP exhibiting chronic GCRs, the PPs of germ-free mice are quiescent and lack B-blasts. Our findings (*Shroff* et al., 1995) were: (a) that GCRs in PP waxed and waned over 10-28 days post-colonisation and remained quiescent up to 314 days, even though the gut bacterial load was $>10^8$ CFU/g; (b) in order to relate the GCRs stimulated by *M. morganii* colonisation to both 'natural' and specific IgA production in the gut, we used a tissue fragment culture assay that permits quantisation of IgA produced in PPs and in each segment of gut (duodenum, jejunum, ileum, caecum, large intestine) following microbial colonisation (see Figure 1). This assay avoids enzymatic degradation of IgA in the intact gut,

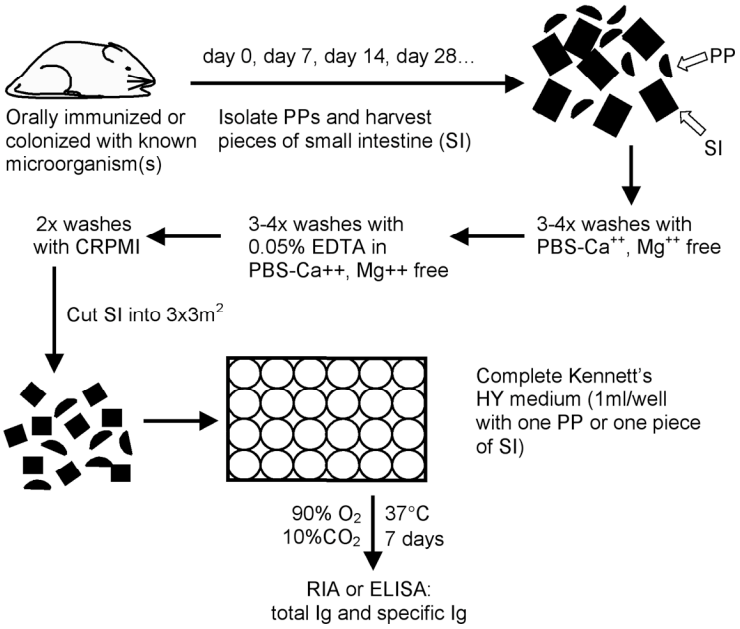

Figure 1: Assessment of humoral immune responses in GALT (gut associated lymphoid tissues) by organ fragment cultures.

variable dilution of IgA by gut fluids, and aggregation of different IgA molecules with mucin (see *Logan* et al., 1991). Our findings were that total ('natural') IgA production in the gut and PPs rose from very low levels in tissue fragment cultures from germ-free mice to about 20-25% of that from conventionally-reared mice within 14 days and remained at that level for >54 days, although GCRs in PPs had disappeared. Further, the microbial specific IgA Abs also rose to about 5% of the total IgA being produced over the same time period. As expected, plasma cells giving ELISPOTS vs. bacterial Ags also rose and these persisted for >314 days despite the cessation of GCRs in PPs; (c) the chronically colonising *M. morganii* became 'coated' with IgA, as detected by FACS analysis, by day 14 and this coating persisted >314 days without any apparent effect on the persistence of the microbe in the gut; (d) *M. morganii* that had translocated were cleared from both the spleen (58 days) and MLN (208 days), although they continued to persist in gut lumen in high numbers (>10^8 CFU/g). Thus, the chronically present GCRs in conventionally reared mammals cannot simply be accounted for by persistence of the bacteria. Likely, as will be supported below, the specific IgA Abs provide a shield to exclude bacterial products of a particular coloniser and consecutive exposure to 'novel' colonising bacteria is required to maintain the chronic GCRs in PP. Further, coating of gut bacteria by IgA did not seem to compromise their retention in the gut, at least when they were the sole colonisers. Of course, specific IgA Abs vs. microbial antigens responsible for attachment to and translocation across the gut epithelium, especially of frank and opportunistic enteric pathogens, may interfere with these processes. Likely most 'natural' and specific IgA Abs reactive with surface Ags of all gut bacteria may have little qualitative effects

on persistence of bacteria in the gut. Quantitative effects on bacterial persistence *vis a vis* other microbial competitor have yet to be accurately evaluated in gnotobiotic hosts.

Another benign gut colonising bacteria is 'segmented filamentous bacteria' (SFB). SFB colonisers of mammals were first comprehensively studied by *Davis* and *Savage* (1974) and their relationship to members of the genus Clostridia was shown using comparative 16S RNA analyses by *Snel* et al. (1995). These SFB are obligate anaerobes, Gram-positive, not-cultivatable, spore forming, segmented, gut bacteria which are major colonisers of the mammalian gut from weaning to puberty. Their ability to persist in the terminal ileum depends on use of a 'holdfast' segment to attach to the brush border of epithelial cells (ECs). Colonisation of formerly germ-free mice with SFB results in a rise in 'natural' IgA to about 2/3 of the level found in conventionally reared mice. This is the greatest rise induced by mono-association observed using any one of six other gut-colonising bacteria (*Talham* et al., 1999). However, only about 1% of this total IgA can be shown to be specifically reactive with the Ags in SFB sonicates (*Talham* et al., 1999). The GCRs in PP also wax and wane after SFB colonisation, however, the GCRs in PPs can be re-activated by super-colonisation with another gut commensal, *M. morganii*, at day 113. Subsequently, within 10 days specific IgA Abs can be detected reactive with *M. morganii* and these persists >80 days after secondary colonisation. Thus, the chronic presence of GCRs in the PPs of conventionally reared mice is likely due to continuous exposure to novel and ever changing members of the gut microbiota.

Each of seven different gut colonising bacteria, when used to mono-associate germ-free mice, resulted in its own characteristic level of total IgA production at steady state and its own characteristic proportions of demonstrably specific IgA Abs (1-15%) (*Bos* et al., 2001).

Several other principles have emerged from using SFB to mono-associate mice: (a) the immune responsiveness of both nursing dam and pups can determine the numbers and sites of colonisation of SFB (*Jiang* et al., 2001). By crossing immunocompetent mice with severe-combined immunodeficient (SCID) mice, and then back-crossing the F_1 mice to male or female parental SCID mice, we obtained four groups of germ-free offspring: Immunocompetent (+/-) pups with either immunocompetent (+/-) or SCID (-/-) mothers and SCID pups with either immunocompetent (+/-) or SCID (-/-) mothers. Pups with immunocompetent mothers showed a delay in gut colonisation with SFB, compared with pups from SCID mothers. If the pups were SCID (-/-), the eventual SFB colonisers persisted in the small intestine for the length of the experiment (70 days post partum). But if the pups were themselves immunocompetent (+/-), they cleared the SFB from small intestine 30-35 days post partum. The immunocompetent dams also prolonged the time to activate gut production of IgA by pups (if +/-, immunocompetent) compared with immunocompetent pups of SCID mothers. We suspect that suckled maternal and actively produced neonatal IgA Abs can forestall or prevent colonisation of small intestine by blocking the essential 'holdfast'/EC brush border interactions; (b) the diet can dramatically influence the level of gut colonisation by a single species of bacteria. SFB was used to mono-associate immunocompetent germ-free mice. After 20-30 days of colonisation and ingestion of conventional chow, groups of mice were split and half of each group was switched to a

chemically defined, elemental diet (*Pleasants* et al., 1986). SFB colonisation of the gut was monitored as well as IgA output in the various parts of gut. Within 5-13 days after switching to a chemically defined diet, almost all SFB disappeared from all sections of gut. Within 13 days, IgA output, relative to that of litter mates continuing on a conventional diet, dropped to about 1/3 and remained at that relative level for 55 days, when the experiment was terminated (*Thurnheer* et al., unpublished). Thus, some gut colonising bacteria are unable to survive alone in the presence of a chemically defined diet. The IgA levels rapidly dropped after switching to a chemically defined diet, either as a result of removal of SFB or because of the change in diet. Nevertheless, these observations suggest at least two subsets of IgA plasmablasts in the gut, differing in turnover time.

These latter findings, made using mono-associated hosts, may be extrapolated to attempts to orally vaccinate premature and full term human neonates vs. nosocomial or opportunistic pathogens. Neonates born of mothers effectively exposed to such pathogens may offer passive protection to their offspring via specific IgA Abs in milk but also forestall effective active immunisation with mucosal vaccines. Artificial diet formulae may be inadequate for the outgrowth of a potential probiotic gut microbe that may ameliorate such nosocomial or opportunistic infection.

The role of B1- vs. B2- cell subsets in gut mucosal IgA production; the dependence of IgA production by B1 cells on bystander T cell 'help' and the specific reactivity of $CD4^+$ T cells locally with gut microbial Ags

In a number of mammals, B cells can be divided into B1 and B2 cell subsets: B1 cells generally are surface IgM^{high}/IgD^{low} and many are $CD5^+$ and $Mac1^+$; B2 cells are surface IgM^{low}/IgD^{high} and are negative for CD5 and Mac1. In the adult mouse most B1 cells are localised in peritoneal and pleural cavities. These cells exhibit local self-renewal and do not depend upon replenishment from bone marrow stem cells. The B1 cells exhibit multi-reactivity with a variety of bacterial and autoantigens and both B1-derived IgM and IgA express germ-line encoded V-genes with few point mutations and no evidence of 'affinity maturation' (*Bos* et al., 1996). B1 cells are not found in PPs or peripheral lymph nodes (PLN). They account for the majority of 'natural' IgM in the circulation but their contribution to gut IgA, although demonstrated, has until recently not been quantitatively defined in physiologically 'normal' mice.

Our first indication that B1 cells might benefit from or require bystander T cell 'help' came from efforts to determine whether B1-derived gut IgA could be stimulated vs. intestinal murine rotavirus (EDIM strain), and neutralise/clear this multi-determinant Ag without T cells (*Kushnir* et al., 2001). SCID mice become chronically infected with rotavirus in intestinal ECs, and shed viral Ag in their faeces. We found that transfer of unfractionated peritoneal cavity cells, B1 cells plus $CD4^+$ T cells, or $CD4^+$ T cells alone would result in cessation of viral shedding, but B1 cells alone did not. Transfer of peritoneal cavity cells to infected SCID mice resulted in viral specific IgA production in the gut but transfer of B1 cells alone did not. However, transfer of both types of cells resulted in appreciable expression of 'natural' IgA in the gut. Examination of cells from recipient mice 8-10 weeks after cell transfer to infected SCID mice showed, surprisingly, that transferred, FACS-purified B1 cells also resulted in the appearance of appreciable $CD4^+$ T cells in peritoneal cavity and gut LP. Fi-

nally, experiments using congenic IgA allotype-different donors and exchange of B1 cells added to B1-depleted peritoneal cavity cells, showed that the allotype of specific, anti-viral IgA Abs was almost exclusively that of the non-B1 cell donor (*i.e.*, likely the product of B2 cells). Thus, we concluded that B1 cells could not produce effective IgA anti-rotavirus Abs, and suspected that their production of 'natural' IgA might depend on the outgrowth of $CD4^+$ T cells, contaminating the FACS-purified B1 cells. Since we transferred only 2 x 10^5 FACS-sorted cells, we reckoned that the purification could only have left about one to two thousand contaminating T cells, but that these expanded extensively over 8-10 weeks *in vivo*. Since we suspected that our cell inoculum size was 'borderline' with respect to contaminating T cell content, we transferred this dose of FACS-purified T cells into a large number of conventionally reared SCID recipients (*Jiang* et al., submitted). We found expression of gut IgA in some but not all recipients, and the level of IgA production after 8 weeks was nicely correlated with the occurrence and extent of $CD4^+$ T cell outgrowth in peritoneal cavity and spleen. Although B1 cells have been found not to require or benefit from Ag-specific, cognate, $CD4^+$ T cell 'help', we suspected that they might benefit from bystander T cell 'help', possibly developed locally in gut LP in response to microbial Ags.

To address these issues we prepared B1 cells at greater purity by treatment of donor peritoneal cavity cells with anti-Thy1 plus complement, and then sorting for B1 cells and against $CD4^+$ T cells. Such further purified B1 cells, upon transfer to conventionally reared SCID mouse hosts, showed repopulation of the recipient mice with B1 cells but not T cells. Addition of graded doses of FACS-purified $CD4^+$ T cells (0-2 × 10^5) to these B1 cells (2 × 10^5) resulted in increasing expression of 'natural' gut IgA in conventionally reared SCID mice, 8-10 weeks after transfer. A role for gut microbial Ags in this phenomenon was shown by cell transfer into conventionally reared and germ-free SCID mice: Neither B1 cells nor unfractionated peritoneal cavity cells gave rise to appreciable gut IgA production in germ-free mice, although the peritoneal cavity cells resulted in a robust (app. 80% of intact, conventionally reared BALB/c mice) gut IgA production in conventionally reared mice (*Jiang* et al., submitted for publication).

To test whether $CD4^+$ T cells of a specificity irrelevant to that of B1 cells could provide the bystander 'help' necessary for gut IgA production and whether activation/stimulation of these T cells was necessary, we used monoclonal DO11.10 T cells (ovalbumin peptide/class II^d specific transgenic mice crossed onto a RAG-2 -/- background). If the DO11.10 T cells were activated *in vivo* in the donors by giving OVA in drinking water, they facilitated gut IgA production upon co-transfer with B1 cells in conventionally reared but not in germ-free SCID mouse recipients. This observation suggested that 'activated' $CD4^+$ T cells could provide bystander 'help' but that B1 cells also needed some sort of gut microbial stimulation. If the DO11.10 T cells were taken from quiescent donors (no OVA given to donors), they would facilitate gut 'natural' IgA expression by B1 cells in conventionally reared SCID mice only if OVA was administered via drinking water to recipients (*Jiang* et al., submitted).

To determine whether mono-associated SCID mice could provide the necessary $CD4^+$ and/or B1 cell stimulation for gut IgA production, we transferred $CD4^+$ T cells plus B1 cells from conventionally reared donors into *Bacteriodes distasonis* colonised recipients. Neither 2 × 10^5 B1 cells alone nor B1

cells with either 2×10^3 or 2×10^5 $CD4^+$ T cells resulted in appreciable gut 'natural' IgA production, but transfer of unfractionated peritoneal cavity cells did. We think it likely that B2 cells in peritoneal cavity, benefiting from the 'help' of accompanying T cells, accounted for the IgA production. However, if the co-transferred $CD4^+$ T cells were from SFB-mono-associated donors, they did provide the 'help' required by B1 cells from conventionally reared donors to produce 'natural' IgA in the guts of SFB mono-associated SCID recipients (*Jiang* et al., submitted).

We conclude that: (a) B1 cells benefit from 'bystander' $CD4^+$ T cells in the gut in order to develop into IgA plasma cells (IL-5, IL-6, IL-10, TGFβ, etc., LKs); (b) the $CD4^+$ T cells must be locally activated by specific Ag in their gut locale — these Ags normally appear to be microbial Ags. The specificities of the T cells are likely unrelated to those of the B1 cells benefiting from 'bystander' help; (c) although the presence of colonising gut microbes seems to be required for the B1 to plasmablast transition in the gut, we have no evidence of Ag-specific selection and stimulation of these B1 cells. Possible 'activating' molecules include microbial Ags, microbial mitogens (LPS, peptidoglycan, CpG, etc.) and the activation may be via BCRs, toll-like receptors, or both.

Finally, the strong effect of gut microbial stimulation on T cell outgrowth can be shown by comparing fluorochrome (CFSE)-labelled $CD4^+$ or CD8+ T cells into conventionally reared vs. germ-free SCID mice. 'Homeostatic proliferation' generally leads to the rapid expansion and populating of lymphoid tissues in conventionally reared SCID mice but transfer of T cells into germ-free SCID mice dramatically diminishes their outgrowth, as judged by only minimal decrease in fluorescence intensity of the transferred cells (*Surh* et al., unpublished).

In an effort to estimate the relative contributions of B1 vs. B2 cells to 'natural' IgA and to specific anti-microbial IgA Abs we designed a nearly physiologically normal model (*Thurnheer* et al., 2003): Using germ-free newborn pups of the Igh^a allotype, we suppressed the dissemination of B cells from the bone marrow by giving bi-weekly injections (10 total injections) of anti-IgM^a beginning at day 1 post partum. On day 3 we injected 2×10^6 peritoneal cavity cells, containing about 0.8×10^6 B1 cells, from an adult C.B17 Igh^b congenic donor. After 8 weeks, we tested for 'balanced' chimerism and then mono-associated such mice with SFB, *M. morganii*, or *B. distasonis*. Examination of peritoneal cavity cells at 8 weeks showed that most germ-free mice were balanced chimeras, with almost all B1 cells from the donor and almost all (80-85%) B2 cells from the recipient. These mice were also balanced chimeras functionally, as about half of the circulating IgM was donor derived and half from host cells over a period of 70 days after colonisation with either of the three gut microbes. Following bacterial colonisation, the GCRs waxed and waned in PPs, indicating a local B2 cell response. Periodic analysis of gut tissues over 70 days following colonisation indicated that <10% of the 'natural' or bacteria-specific IgA was derived from the B1 cell donor. Thus, in these neonatal, germ-free, chimeric mice, with no known impairment of their T cell system, most of the intestinal IgA seems to be of B2 cell origin. As shown above, at least some of the gut IgA is reactive with normal members of the gut microbiota. *Macpherson* et al. (2000) have shown that anti-microbial IgA can be stimulated in TCR (-/-) mice, with no functional T cells. Since we have shown that B1 cells

seem to require 'bystander' $CD4^+$ T cell 'help', what may be the origin of the anti-microbial gut IgA Ab that arises in TCR (-/-) mice? We have shown that oral infection with reovirus, an Ag with repeating determinants, of TCR (-/-) mice results in some anti-viral IgA Ab (*Zuercher* et al., unpublished). Unlike the response in W/T mice, this Ab is insufficient to clear the virus. However, the infection does result in a vestigial GCR in PPs, likely due to limited proliferation of B2 cells. We suggest that this modest B2 cell expansion, in the absence of a cognate T cell interaction, does not result in affinity maturation of B2 cells that develop few if any progeny with mutated, expressed Ig V-genes except that B2 responses may occur in GCRs while B1 responses may not. Both responses require bystander 'help', provided by otherwise activated $CD4^+$ T cells or dendritic cells (either interdigitating or follicular). Consideration of these possibilities may be relevant to raising protective Abs vs. many microbial Ags expressed by nosocomial and opportunistic mucosal pathogens. Possibly, combination of subunit vaccine and polyclonal stimuli-particularly those that interact with both B- and T cells via toll-like receptors-may provide an effective combination of 'natural' and specific immune responses.

The role of particular members of the gut microbiota and host T cells in the initiation, development, and control of inflammatory bowel disease (IBD): The possible relationship of controlling (Trl) T cells to those mediating oral tolerance

In the past 10 years, a number of animal models for human IBD have been developed. Most of these utilise conventionally reared rats or mice, which exhibit some sort of dysregulation or imbalance of their immune systems. One well-studied mouse model involves the transfer of $CD4^+$, $CD45RB^{high}$ T cells into conventionally reared SCID mice. The recipients usually exhibit a wasting disease and develop classic symptoms of ulcerative colitis (UC) in 10-14 weeks (*Morrissey* et al., 1993; *Powrie* et al., 1993). We have found that such pathologic consequences do not develop in germ-free mice, which exhibit no wasting or symptoms of UC (*Jiang* et al., 2002). In an effort to identify a microbial provocateur, we mono-associated germ-free SCID mice with one of five gut colonising, benign bacterial species, before transfer of 'naive' $CD4^+$ T cells. $CD4^+CD45RB^{high}$ T cells have been defined as naïve or inexperienced T cells, also expressing high level of CD62L, and lack expression of other activation markers, such as CD69 and CD44, which are prevalent in the PP of germ-free mice (*Morrissey* et al., 1993; *Powrie* et al., 1993; *Talham* et al., 1999). No wasting disease or IBD symptoms developed in these mice. However, a sixth mono-associate, *Helicobacter muridarum*, did result in an accelerated development of wasting, and UC (at 4-5 weeks) upon colonisation of SCID mice prior to transfer of 'naive' T cells (*Jiang* et al., 2002). *H. muridarum* was first described as a benign commensal, living in colonic crypts of healthy, conventional, immunocompetent mice (*Phillips* and *Lee*, 1983). The developing disease was accompanied by extensive outgrowth of *H. muridarum* in colonic crypts and caecum, a severe inflammatory response in the colon with trans-mural cell infiltration, and severe loss of weight. All mono-associating, gut colonising bacteria we tried stimulate outgrowth of transferred $CD4^+$, $CD45RB^{high}$ T cells in formerly germ-free SCID mice. However, *H. muridarum* results in the development of an imbalance of activated macrophage and of INF-γ producing T cells in the gut

lamina propria of colonised, recipient SCID mice. Thus far, the specificities of $CD4^+$ T cells that initiate experimental IBD have not been defined in any animal model for IBD.

Generally, co-transfer of $CD4^+$, $CD45RB^{low}$, $CD25^+$ T cells (subset including Tr1 cells) from conventionally reared donors, along with $CD4^+$, $CD45RB^{high}$ T cells ('naïve' T cells) from conventionally reared donors into conventionally-reared SCID mice forestalls or ameliorates the development of IBD initiated by the latter (*Morrissey* et al., 1993; *Powrie* et al., 1993). The specificities of these regulatory Tr1 cells, has long been an issue. We find that Tr1 cells derived from conventionally reared donors, have no effect on the development of IBD when given along with the initiator ($CD4^+$, $CD45RB^{high}$) T cells from germ-free or conventionally reared donors. However, if the Tr1 cells are taken from immunocompetent, *H. muridarum* mono-associated mice and transferred to germ-free SCID recipients prior to colonisation with *H. muridarum*, they effectively block development of IBD initiated by 'naïve' T cells. This observation suggests a specificity of the effective Tr1 cells for microbial Ags.

These findings suggest that normally innocuous members of the gut microbiota, such as *H. muridarum*, can act as an initiator of IBD in mice with a dysregulated immune system, since immunocompetent mice with functional regulation elements, do not develop IBD upon mono-association with *H. muridarum*. It is not surprising that $CD4^+$,$CD45RB^{low}$, $CD25^+$ T cells from such mice can control the development of IBD in dysregulated SCID mice.

Possible roles of gut microbes in facilitating oral tolerance

Finally, we believe that the cellular mechanisms for mediating the forestalling or amelioration of IBD are similar to those mediating acquired oral tolerance. A typical scheme for the experimental initiation of oral tolerance to a dietary, protein Ag is to give the Ag (ovalbumin, conalbumin) orally and then the fed mice, along with unfed controls, are primed parenterally with the same Ag in adjuvant.

We find that germ-free mice are refractory to the development of oral tolerance, as judged by their failure to show a diminished response of their peripheral T cells *in vitro* upon feeding a dietary Ag, relative to unfed control mice. Mono-association of germ-free mice with the normal murine *E. coli* (Schaedler's *E. coli*), results in susceptibility to the expression of oral tolerance. Thus, gut colonisation with at least some benign, commensal microbes somehow facilitates the development of oral tolerance (*Boiko* et al., unpublished).

Thus our above observations in 3 and 4 above support the stimulation of gut $CD4^+$ T cells to either initiate IBD or down regulate development of IBD and systemic immune responses to orally administered protein Ags. It is still unclear whether microbial products act via TCR and/or the toll-like receptors of T cells. Clearly, development of effective oral vaccine formulation will require selective stimulation of protective T cells and Abs and circumvent the down regulatory effects of Tr1 cells.

GENERAL SUMMARY

Microbial colonisers of the mammalian gut play a role in the development and maintenance of the intestinal mucosal immune system. The host makes a humoral mucosal response to each benign gut bacterial species used to

colonise adult GF mice. This response is characterised by transient GCR in PPs and the increased production of IgA in the gut. Each bacterial species used for mono-association has a characteristic stimulation of steady state levels of gut IgA production and ratio of specific IgA Abs to 'natural' IgA. In many cases, the level of 'natural' IgA is appreciably higher than demonstrably specific IgA.

Both B1- and B2-cells contribute to 'natural' IgA production in the gut. The B1 cells require 'bystander' $CD4^+$ T cell 'help' to develop IgA production in intestine. At least some of these gut T cells seem to be specific for Ags associated with particular gut microbes and require local colonisation to be functionally 'activated'. It is still unclear whether B1 cells can be specifically selected and activated by particular Ags or whether they are stimulated by microbial products via TLRs rather than BCRs. Under near physiologically normal conditions, B2 rather than B1 cells appear to account for most of the 'natural' and anti-microbial specific IgA produced in the gut. B2 cells may produce anti-microbial IgA Abs without a requirement for T cell help via vestigial GCRs and possibly without the accumulation of cells with V-gene mutations that often lead to affinity maturation.

We describe a mouse model for human IBD that has a dysregulated immune system and an identifiable bacterial provocateur, the normally benign *H. muridarum*. Naïve $CD4^+$ T cells, introduced into formerly germ-free SCID mice, mono-associated with *H. muridarum*, result in development of a wasting disease and ulcerative colitis. This disease appears to be initiated by activated $CD4^+$ T cells, which infiltrate the large intestine, produce IFN-γ and activate terminal effector macrophage. The specificities of the initiator T cells are unknown. However, regulatory $CD4^+$, $CD25^+$, $CD45RB^{low}$ T cells (Tr1 cells) can forestall or ameliorate the wasting and progression of the IBD symptoms. These seem to require specific, microbial Ag stimulation and may act in a 'bystander' fashion to down-regulate the development of the initiator $CD4^+$ T cells. Such Tr1-type T cells also appear to play a role in oral tolerance and also be stimulated by colonisation of the gut with enteric microbes. Oral tolerance — systemic hypo-responsiveness of T cells following ingestion of protein Ags — is absent or diminished in germ-free mice, but can be facilitated by colonisation with *E. coli*. Since the tolerance is elicited by feeding a dietary Ag (ovalbumin, conalbumin), it is likely that any Tr1-cells elicited by colonisation with *E. coli* act in a 'bystander' fashion.

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LITERATURE

Bos, N.A., Bun, J.C.A.M., Popma, S.H., Cebra, E.R., Deenen, G.J., van der Cammen, M.J., Kroese, F.G., and Cebra, J.J.: Monoclonal immunoglobulin A derived from peritoneal B cells is encoded by both germ line and somatically mutated V_H genes and

is reactive with commensal bacteria. Infect. Immun. 64, 616-623 (1996).

Bos, N.A., Jiang, H.Q., and Cebra, J.J.: T cell control of the gut IgA response against commensal bacteria. Gut 48, 762-764 (2001).

Cebra, J.J., Jiang, H.Q., Sterzl, J., and Tlaskalova-Hogenova, H.: The role of mucosal microbiota in the development and maintenance of the mucosal immune system. In: Mucosal Immunology, 2nd Ed. (Eds.: Ogra, P.L., Mestecky, J., Lamm, M.E., Strober, W., Bienenstock, J., and McGhee, J.R.). Academic Press, New York, 267-280 (1999).

Craig, S.W. and Cebra, J.J.: Peyer's patches: An enriched source of precursors for IgA producing immunocytes in the rabbit. J. Exp. Med. 134, 188-200 (1971).

Davis, C.P. and Savage, D.C.: Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract. Infect. Immun. 10, 948-956 (1974).

Jiang, H.Q., Bos, N.A., and Cebra, J.J.: Timing, localization, and persistence of colonization by segmented filamentous bacteria in the neonatal mouse gut depend on immune status of mothers and pups. Infect. Immun. 69, 3611-3617 (2001).

Jiang, H.Q., Kushnir, N., Thurnheer, M.C., Bos, N.A., and Cebra, J.J.: Monoassociation of SCID mice with *Helicobacter muridarum*, but not four other enterics provokes IBD upon receipt of T cells. Gastroenterology 122, 1346-1354 (2002).

Kushnir, N., Bos, N.A., Zuercher, A.W., Coffin, S.E., Moser, C.A., Offit, P.A., and Cebra, J.J.: B2 but not B1 cells can contribute to $CD4^+$ T-cell-mediated clearance of rotavirus in SCID mice. J. Virol. 75, 5482-5490 (2001).

Logan, A.C., Chow, K.-P.N., George, A., Weinstein, P.D., and Cebra, J.J.: Use of Peyer's patch and lymph node fragment cultures to compare local immune responses to *Morganella morganii*. Infect. Immun. 59, 1024-1031 (1991).

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M.: A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science 288, 2222-2226 (2000).

Morrissey, P.J., Charrier, K., Braddy, S., Liggitt, D., and Watson, J.D.: $CD4^+$ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified $CD4^+$ T cells. J. Exp. Med. 178, 237-244 (1993).

Phillips, M.W. and Lee, A.: Isolation and characterization of a spiral bacterium from the crypts of rodent gastrointestinal tracts. Appl. Environ. Microbiol. 45, 675-683 (1983).

Pleasants, J.R., Johnson, M.H., and Wostmann, B.S.: Adequacy of chemically defined, water-soluble diet for germfree BALB/c mice through successive generations and litters. J. Nutr. 116, 1949-1964 (1986).

Powrie, F., Leach, M.W., Mauze, S., Baddle, L.B., and Coffman, R.L.: Phenotypically distinct subsets of $CD4^+$ T cells induce or protect from chronic intestinal inflammation in C.B-17 *scid* mice. Int. Immunol. 5, 1461-1471 (1993).

Shroff, K.E., Meslin, K., and Cebra, J.J.: Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. Infect. Immun. 63, 3904-3913 (1995).

Snel, J., Heinen, P.P., Blok, H.J., Carman, R.J., Duncan, A.J., Allen, P.C., and Collins, M.D.: Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus arthromitus*". Int. J. Systematic Bacteriol. 45, 780-782 (1995).

Talham, G.L., Jiang, H.Q., Bos, N.A., and Cebra, J.J.: Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect. Immun. 67, 1992-2000 (1999).

Thurnheer, M.C., Zuercher, A.W., Cebra, J.J., and Bos, N.A.: B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. J. Immunol. 170, 4564-4571 (2003).

DEVELOPMENT OF AN ANTI-CORE LIPOPOLYSACCHARIDE VACCINE FOR THE PREVENTION AND TREATMENT OF SEPSIS*

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SUMMARY

Sepsis continues to be a leading cause of death among hospitalised patients. Despite advances in supportive care and the availability of potent antimicrobials, the mortality exceeds 20%. The passive infusion of antibodies directed against a conserved region of the lipopolysaccharide (LPS) of Gram-negative bacteria was highly protective in an early study (*Ziegler* et al., 1982). When this and similar preparations were unable to show consistent efficacy, efforts were directed towards other strategies, including cytokine modulation. Our group found that a whole bacterial vaccine made from the *E. coli* O111:B4, J5 (Rc chemotype) mutant induced protective antibodies when given passively as treatment for sepsis in a neutropenic rat model. A subunit vaccine, composed of detoxified J5 LPS complexed to group B meningococcal outer membrane protein (OMP), provided similar protection when antibodies were given passively, or induced actively in both the neutropenic and caecal ligation/puncture models of sepsis. A phase I study in 24 subjects (at 5, 10 and 25 μg doses [based on LPS] for each group of 8) revealed the vaccine to be well-tolerated with no systemic endotoxin-like effects. Although a 2-3 fold increase in antibody levels over baseline (by ELISA assay) was observed at the 10 and 25 μg doses, the plasma from both high and low responders reduced LPS-induced cytokine generation in whole blood. Re-immunisation of 6 subjects at 12 months did not convert low responders to high responders or boost the still elevated anti-J5 LPS levels of high responders. If functional assays of anti-LPS antibodies are better predictors of vaccine efficacy than ELISA antibody levels, then it will be necessary to determine which of many potential assays best correlates with protection in animal models. We are currently comparing a panel of functional assays with protective efficacy in animal models of sepsis, as well as the ability of adjuvants to enhance vaccine efficacy. The availability of an effective anti-endotoxin vaccine will provide additional therapeutic options for the prevention and/or treatment of sepsis.

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INTRODUCTION

Sepsis, a leading cause of death in intensive care units, has increased in frequency over the last two decades (*Martin* et al., 2003). Between 1979 and 2000 there was a four-fold increase in the number of cases of sepsis (from 164,000 to nearly 660,000). The mortality remains nearly 20% despite advances in supportive care and the introduction of potent antimicrobial agents (*Martin* et al., 2003). Consequently, additional therapeutic measures have been sought. The important role of Gram-negative bacterial lipopolysaccharide (LPS) in the pathogeneses of sepsis was recognised in the 1960's and 70's (*Braude* et al., 1960); therefore, it is not surprising that initial attention to adjunctive treatment measures focused on this molecule. Elucidation of the structure of LPS revealed that the lipid-A portion was highly conserved among species of *Enterobacteriacea*e and that the core regions also had considerable conservation. As a result, it was hypothesised that antibodies against these conserved LPS structures might provide protection against a broad range of Gram-negative bacteria. Investigators developed bacterial strains in which the core region of LPS was available to the immune system (i.e. not shielded by O antigen, for example *S. minnesota* Re595 [Re chemotype] and *E. coli* O111:B4, J5 mutant [Rc chemotype]) (*McCabe*, 1972; *Ziegler* et al., 1973). Pre-clinical work with anti-core LPS antibodies induced by these killed bacterial strains was effective in animal models of sepsis (*Ziegler* et al., 1975; *Johns* et al., 1983). In this manuscript we shall briefly review earlier studies with anti-endotoxin antibodies, and then describe our own studies with a detoxified J5 LPS (dLPS)/group B meningococcal outer membrane complex (OMP) vaccine that progressed to a phase I study in human subjects.

Early studies with anti-endotoxin antibodies

Based on these studies, *Braude* and colleagues prepared a whole bacterial vaccine by boiling *E. coli* 0111: B4, Rc chemotype (hereafter, J5 mutant) and raised immune sera in healthy volunteers. In a multi-centre clinical trial, patients with suspected Gram-negative bacterial sepsis were given either pre or post-immune sera in addition to standard therapy (*Ziegler* et al., 1982). Patients with Gram-negative bacteraemia who received post-immune sera had a better survival rate (22/91 [24%]) than those receiving pre-immune sera (30/100 [38%] p=0.041). Among those with either hypotension or in profound shock, there were even more significant differences in favour of the post-immune sera. Despite the therapeutic benefit, there was no evidence that the antisera prevented infection. In this trial investigators were unable to determine whether the antibody fraction of sera was responsible for the improved survival. Further, the antigen in the whole bacterial vaccine responsible for inducing the protection was not clearly identified. Finally, since the "therapeutic product" was material from an individual volunteer and not a reproducibly made reagent, this clinical study must be viewed as a proof of principle rather than the testing of a potential therapeutic product.

Subsequent investigators were unable to confirm the findings of *Ziegler* and colleagues; however, none of these studies were similar in design to the original study and none clearly documented the maintenance of anti-endotoxin antibodies (Table 1). In one study

Table 1: Passive administration of anti-core LPS antibodies for sepsis: Previous clinical studies

Study	Product	Number of patients	Ab levels	Outcome
Ziegler (1982)	J5 serum	91	Increased	Reduced mortality, especially if shock
Baumgartner (1985)	J5 Plasma	126	Not done	9/136 controls vs. 2/126 patients died
J5 Study Group (1992)	J5 plasma	40	No increase	No protection in meningococcemia
Commetta (1992)	Screened IVIG	108	Consumption	No protection
Calandra (1988)	J5 IVIG	30	Not done	No effect
Schedel (1991)	"Enriched" IVIG	27	Consumption	Titre-related protection 1/27 vs. 9/28 survival
Fomsgaard (1989)	Screened IVIG	9	Consumption	Anti-LPS IgG reduced TNF

children with meningococcal purpura fulminans were given J5 plasma at the onset of illness (*J5 Group*, 1992). There was no evidence of benefit; however, there was no increase in anti-J5 LPS antibody when measured at 6 hours after infusion. In another study, use of J5 plasma was ineffective when given as prophylaxis to surgical patients. This study confirmed the earlier finding of *Ziegler* et al. (1982) that J5 serum did not prevent the development of Gram-negative infection (*Baumgartner* et al., 1985). Similarly, in another clinical trial IgG was prepared from the plasma of volunteers who were immunised with the whole bacterial J5 vaccine (*Calandra* et al., 1988). A single infusion of IVIG was ineffective in a clinical trial of patients with sepsis; however, there appeared to be only a two-fold response in anti-J5 LPS antibody in the starting material before fractionation into IVIG. Thus, although the level of anti-core LPS antibodies after infusion was not measured in these patients, it is unlikely that adequate levels of anti-J5 IgG were administered. In yet another study, plasma from blood donors was screened for high levels of naturally occurring anti-core LPS (*S. minnesota,* Re 595*)* antibody and high titred material was pooled and made into an IVIG (*The Intravenous Immunoglobulin Collaborative Study Group*, 1992). This preparation was compared to standard IVIG in its ability to prevent the onset of sepsis when given as prophylaxis to patients who underwent surgery. In the absence of documented infection, the levels of antibody at 2 days was <50% that of levels obtained at 2 hr post infusion (*The Intravenous Immunoglobulin Collaborative Study Group*, 1992). This enriched anti-core LPS IVIG was unable to prevent infection, sepsis or death. Thus, in all of these studies it is likely that inadequate amounts of antibodies were given or inadequate levels of antibody were maintained to test the hypothesis that anti-endotoxin antibodies were effective in the treatment of sepsis.

A number of studies (*Pollack* et al., 1983; *Goldie* et al., 1995; *Zinner* and *McCabe*, 1976) have clearly established a relationship between the level of anti-core LPS antibody at the onset of sepsis and outcome. More importantly, a decrease in anti-core LPS antibody during a septic episode forebodes a poor out-

come (*Fomsgaard* et al., 1989; *Schedel* et al., 1991; *Nys* et al., 1993; *Goldie* et al., 1995). Consequently, in the absence of documentation that there was an adequate level of circulating anti-endotoxin antibodies, it is difficult to exclude the hypothesis that anti-endotoxin antibodies might be an effective adjunctive therapy for sepsis. Indeed, in small studies, both *Schedel* et al. (1991) and *Fomsgaard* et al. (1989) each demonstrated that maintenance of "adequate levels" of anti-CGL antibody with multiple infusions corresponded to a decrease in circulating endotoxin levels and increased survival.

Despite the fact that early studies with antisera to lipid-A were unsuccessful in treating sepsis in animal models (*Bruins* et al., 1977), nevertheless, monoclonal antibodies to lipid-A were developed and tested in clinical trials without success (*Greenman* et al., 1991; *Ziegler* et al., 1991). Given the repeated failures of anti-core LPS and anti-lipid-A antibodies to affect the outcome of sepsis in clinical trials, subsequent efforts were directed towards the rapidly developing field of cytokine modulation.

Additional therapeutic strategies

Recognition of the important role of TNF-α and IL-1 in the development sepsis resulted in multiple clinical trials in which inhibitors of TNF and IL-1 activity were tested for therapeutic efficacy in sepsis. After many trials with these and other endogenous mediators of sepsis, no convincing therapeutic effect was detected (*Zeni* et al., 1997). In contrast to studies with anti-endotoxin antibodies that target an invading pathogen, however, administration of active cytokine antagonist often was associated with increases in lethal infections. These unforeseen adverse events illustrate the difficulty in trying to "fine-tune" the levels of endogenous mediators of sepsis in the host as opposed to efforts to target microbial initiators of sepsis. In view of the difficulties in trying to monitor the effect of therapy on host-defences as well as the success of the initial clinical trial with J5 antiserum, we decided to re-examine the potential utility of anti-core endotoxin antibodies, such as the J5 antibody. This effort was facilitated by the development of a neutropenic rat model of sepsis in which animals developed a lethal bacterial infection following the administration of relatively low doses of opportunistic pathogens (*Collins* et al., 1989).

CURRENT STUDIES WITH ANTI-J5 ANTIBODY

We obtained the *E. coli* 0111:J5 strain from Dr. Ziegler and prepared a heat-killed whole bacterial vaccine according to the original method. Antisera raised in rabbits with this vaccine were highly protective in a neutropenic rat model of sepsis, when given at the onset of fever (*Bhattacharjee* et al., 1994) (i.e. as therapy). The effect was clearly dose-related (*Bhattacharjee* et al., 1994), which lent credence to the argument that previous clinical trials with anti-endotoxin antibodies may not have been successful because of inadequate levels of serum administered. We further showed that IgG was the protective fraction in serum and was directed against the core J5 LPS in the whole bacterial vaccine (*Bhattacharjee* et al., 1994). Six of 8 animals that received affinity purified J5 LPS-specific IgG were protected against lethal *Pseudomonas* sepsis vs. none of 25 animals receiving pre-immune IgG. Importantly, the protection was clearly dose-related with animals receiving 9 ml/kg IgG protected versus none re-

Table 2: Local and systemic reactions following immunisation with dJ5 LPS/OMP vaccine

Reactions	Dose (based on dLPS)		
	5 μg	10 μg	25 μg
Local			
Erythema	2[a]	1	3
Induration	2	0	4
Swelling	2	8	6
Pain			
Severe	0	0	0
Moderate	8/1[b]	7/0	12/0
Mild	10/5	12/5	9/8
None	6/18	5/19	3/16
Analgesia	2	2	2
Systemic			
Fever	1	1	1
Headache	2	1	0
Fatigue	0	0	0
Haematologic			
Anaemia	0	1	0
Leukopenia	0	0	0

Volunteers were immunised at day 0, day 28 and day 56 with the indicated dose.
[a]number of reactions per 24 total immunisations (8 subjects, 3 doses).
[b]number of reactions at day 1/day2 after immunisation.

ceiving <6 ml/kg (*Bhattacharjee* et al., 1994).

Based on these findings we made a J5 LPS vaccine, which was detoxified by removing the ester-linked fatty acids through alkaline treatment (*Bhattacharjee* et al., 1996). The LPS was not immunogenic when given alone, with alum, with QS21 or when conjugated to tetanus toxoid. When complexed non-covalently with the outer membrane protein of group B-meningococcus, however, the formulation was highly immunogenic in mice, rabbits and rats. Antisera raised with this vaccine was highly protective in a neutropenic rat model after challenge with either *Klebsiella* or *Pseudomonas* when the antibody was given either as passive therapy at the time of sepsis, or when antibodies were actively induced by immunisation before the start of sepsis. In the latter instance, immunisation with this vaccine did not prevent bacteraemia, but did reduce mortality. Receipt of anti-J5 antibody reduced circulating levels of endotoxin at 24 hr after infusion and reduced the circulating TNF levels compared to the effect with pre-immune sera (*Bhattacharjee* et al., 1996). Active immunisation with the J5dLPS/OMP vaccine promoted the uptake of bacteria from the circulation and killing (i.e. decreased organ bacterial load). Immunisation both actively and passively was also protective in another animal model of sepsis, caecal ligation/puncture in mice. This model differs from the neutropenic rat model in that the sepsis is polymicrobial. With these findings we prepared a vaccine for human use.

Phase I clinical study

A Phase I study (*Cross* et al., 2003) was conducted in 24 healthy subjects. Subjects received either 5, 10, or 25 μg

Table 3: Anti-J5 LPS ELISA titres of sera from volunteers in the phase I trial

	IgG			IgA		
Group[a]	Pre	Post	Fold rise	Pre	Post	Fold rise
5μg	1.7[b] ± 0.28	3.6 ± 0.71	2.0 ± 0.18	1.3 ± 0.14	2.6 ± 0.3	2.1 ± 0.3
10μg	2.8 ± 0.5	5.8 ± 1.9	3.3 ± 0.4	4.4 ± 0.6	9.1 ± 2.0	2.0 ± 0.3
25μg	2.1 ± 0.18	4.9 ± 0.6	2.3 ± 0.3	1.8 ± 0.3	3.9 ± 0.9	2.2 ± 0.5

	IgM		
Group	Pre	Post	Fold rise
5μg	11.2 ± 0.9	16.9 ± 1.3	1.5 ± 0.1
10μg	18.9 ± 4.8	66.2 ± 24.0	3.2 ± 1.0
25μg	6.5 ± 1.1	18.2 ± 5.4	2.9 ± 0.6

[a] 8 volunteers in each group received J5 dLPS/OMP vaccine at time 0, days 28 and 56.

[b] Serum antibody levels were measured according to our previously described methods (*Cross* et al., 2003). Data represent mean ± SEM optical density units (ODU). ODU are defined as the product of the optical density and reciprocal titre for the serum dilution that gives an optical density closest to but still below 1.00 (e.g. OD 0.400 at 1:100 dilution = 40 ODU). Post levels are from the peak antibody level measured on specimens obtained up to 3 months after immunisation. Fold-rises were calculated for each subject and a geometric mean-fold rise for each group then determined.

of vaccine (based on LPS content) at time 0, 1 and 2-months (i.e. 3 total doses). There were few systemic responses (headaches/fever/fatigue) (Table 2). No temperatures >99.9°F were recorded. Most individuals had a mild-to-moderate degree of tenderness at the injection site, which usually resolved by 48-hours. For comparison, the only study to report the incidence of adverse effects with the heat-killed J5 vaccine observed 7/16 incidences of systemic reactions to the initial vaccine, and 3/9 subjects who returned for a second dose (*Schwartzer* et al., 1988). No abnormalities were seen in renal (creatinine, urinalysis), liver (serum alkaline phosphatase, transaminases, bilirubin) or haematologic (leukopenia, anaemia) studies compared to baseline studies (data not shown).

Antibody responses were measured by ELISA (Table 3). Compared to pre-immunisation levels there was a mean 3-fold increase in IgG and IgM levels in the 10 μg group. The 5 μg and 25 μg dosage groups had slightly lower responses. Subjects in all groups had higher baseline levels of IgM antibody to core LPS. We did not assess the affinity of the pre- vs. post-immune anti-core LPS antibodies. Six subjects (3 high and 3 low responders) received a single booster dose of 25μg of vaccine at 12-months to see if it were possible to convert non-responders and to boost the level of responders. High responders were defined as having >2.5 fold increase in serum IgG over baseline, while low responders had <2 fold increase. At 12 months, among responders, pre-boost levels of antibody were still elevated but had decreased by approximately 50%. There was no increase in antibody levels among the high responders following the booster dose. Subjects who did not respond after the primary series did not convert with the

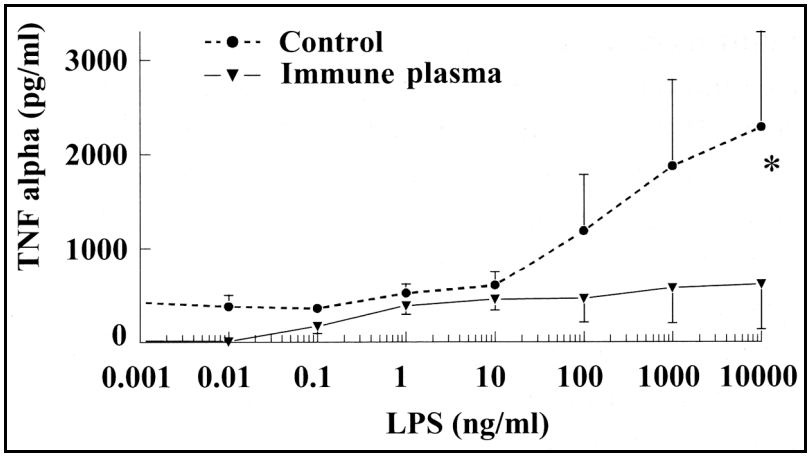

Figure 1: Effect of pre-incubation of LPS with either post-immunisation plasma or control pooled plasma from non-immunised individuals. Different doses of *E. coli* LPS were added to plasma from either one subject with >3-fold increase in anti-J5 dLPS antibody levels or to control plasma. Then the mixture was added to heparinised whole blood from a J5 LPS-naïve donor. The blood was incubated for 24 hr and the supernatant analysed for TNFα.

booster dose. Plasma from all six subjects was obtained one week after the booster dose. These were evaluated in functional assays.

Functional studies of anti-J5 LPS antibody

With most other vaccines there is usually one functional assay recognised as corresponding to vaccine efficacy. For example, opsonic antibody assays for pneumococcal immunisation are thought to better reflect vaccine efficacy than ELISA (*Johnson* et al., 1999; *Kim* and *Seoh*, 1999). Viral neutralisation assays or serum bactericidal tests have also been thought to correspond to efficacy for other vaccines. In the case of an anti-endotoxin vaccine, however, it is not readily apparent what functional assay would best reflect vaccine efficacy. Many functional activities are initiated by LPS, including induction of cytokines, fever, and coagulation as well as the initiation of complement cascades, among a great many other activities. We tested the plasma of the six volunteers in the Phase I study (three high and 3 low responders) who received a booster dose (25 mg) of vaccine at one year in an *ex vivo* cytokine assay. In this assay, LPS is added to heparinised whole blood and incubated at 37°C for 24-hours (*Kovach* et al., 1990). Cytokine generation was then measured in the supernatant. When LPS was pre-mixed with post-immune plasma before addition to the blood, there was a highly significant decrease in TNF (Figure 1) and in IL-6 generation (data not shown) compared to LPS that was exposed to control plasma (*Cross* et al., 2003). This was observed for both low and high responders. When plasma was diluted, however, the higher titred plasma had more activity. Consequently, although the ELISA antibody level did not correlate with functional activity, those with higher antibody levels did appear to have a higher LPS neutralising capacity. In a preliminary study, the plasma from a high responder enhanced the clearance of bacteria and endotoxin from the circulation of rats (*Cross* et al., 2003).

In yet another functional assay of LPS activity, pre-incubation of human

Table 4: Post-immune rabbit sera block LPS-primed superoxide response of human neutrophils

Pre-treatment	Change in OD_{560}	%Control	Anti-J5 LPS IgG (ng/ml)
None	0.023 ± 0.001		not applicable
LPS/HBSS	0.199 ± 0.033		not applicable
LPS/NRS	0.212 ± 0.005	100	109
LPS/anti-J5-1	0.143 ± 0.042	67	727
LPS/anti-J5-2	0.161 ± 0.012	76	1528
LPS/anti-J5-3	0.144 ± 0.020	68	473

Human PMNs were suspended in HBSS/2% human serum and incubated for 60 min at 37°C in medium, medium and LPS or rabbit serum with LPS. The serum from 3 different rabbits (anti-J5-1; anti-J5-2 and anti-J5-3) immunised, or from non-immunised rabbits (NRS) were used (Anti-J5 LPS antibody levels for each rabbit are indicated in the last column). After washing, the PMNs were stimulated with FMLP (10^{-7} M) for 10 min in the presence and absence of superoxide dismutase and the change in ferricytochrome C reduction between 0 and 10 min samples determined by absorption at 550 nm. NRS=normal rabbit serum; HBSS=Hank's Balanced Salt Solution. Each condition performed in triplicate. Representative experiment shown of 3 with similar results.

neutrophils with LPS primes the ability to generate superoxide in response to a neutrophil agonist, formyl-methionyl-leucyl-phenylalanine (fMLP) (*Guthrie* et al., 1984). Pre-incubation of LPS with post-immune sera from three different rabbits (anti-J5-1through 3) immunised with the J5dLPS/OMP vaccine reduced the ability of LPS to prime this response (Table 4). Although there did not appear to be an antibody dose-related inhibition of LPS priming based on ELISA antibody levels, we did not dilute out the antisera. When this was done in the *ex vivo* cytokine induction assay, differences were observed (*Cross* et al., 2003). Based on these initial studies we plan to compare the ability of high and low responder plasma to protect in the caecal ligation puncture and neutropenic rat models of sepsis, to recognise heterologous LPS in other binding assays (fluid phase, and binding to whole bacteria by flow cytometry) and to neutralise the ability of LPS to induce cytokines by THP1 and RAW cells *in vitro*. These studies may provide data as to which functional assay may correlate best with protection in animal model of sepsis. This becomes an even more important consideration since there has been considerable and ongoing debate on the methodology for measuring anti-LPS antibodies by ELISA (*Warren* et al., 1993).

PROPOSED USE OF ANTI-ENDOTOXIN VACCINE

If an effective anti-endotoxin vaccine were available for the prevention and/or treatment of sepsis, then it might be used in several different conditions. Several populations are at higher risk of sepsis and might be considered for immunisation: soldiers, police, fire fighters, as well as patients undergoing complicated abdominal or genitourinary surgery. Routine immunisation of the first three groups would require that the antibody response be long-lived. In our phase I study, subjects with elevated anti-J5 LPS antibody responses after initial immunisation still had elevated antibodies at 12 months (*Cross* et al.,

2003). In the case of patients undergoing elective surgery, an effective anti-endotoxin vaccine would need to induce antibodies after one or two doses of vaccine. Co-administration of the vaccine with an adjuvant might accelerate the antibody response in a manner similar to that of the oligonucleotide, CpG, given with hepatitis B (a vaccine also given in 3 doses) (*Davis* et al., 2000). Since after acute injury there is a Th2 polarisation, patients admitted with burns or trauma might respond to active immunisation (*Lyons* et al., 1997; *Ginnoudis* et al., 1998). We administered experimental *Klebsiella* and *Pseudomonas* vaccines to patients admitted following severe trauma and found that they responded well to both vaccines (*Campbell* et al., 1996).

Alternatively, anti-core LPS antibodies could be given passively to septic patients. In this instance, it would be essential to monitor the circulating levels of anti-core LPS antibodies. In our own pre-clinical studies in neutropenic rats there was a clear dose-related protection (*Bhattacharjee* et al., 1994), and previous clinical trials did not pay adequate attention to the maintenance of antibody levels. Additional doses of antibody may be required during a septic episode. In patients who become septic despite active immunisation with an anti-endotoxin vaccine, supplementation with passive administration of antibodies may be required to counter any consumption of antibody, as was documented in previous trials.

CONCLUSIONS

Our own bias is that many of these previous studies that investigated the efficacy of anti-endotoxin antibody therapy did not adequately measure the amount of antibody administered and did not insure adequate levels of antibody after initial infusions. Consequently, the potential role of anti-core endotoxin antibody therapy has not been sufficiently tested to discard the hypotheses. In monitoring the adequacy of therapy, the discrepancy between the ELISA antibody levels in human subjects and their activity in functional studies needs to be confirmed in a more rigorous fashion. Given the number of functional assays with which one might measure anti-endotoxin activity, this may become a daunting task. The conflicting data with previous studies of anti-endotoxin antibody therapy demands, however, that this effort be pursued in order to better evaluate the response to vaccine such as the one under present study. The current studies suggest that monitoring responses with functionally relevant assays may be an important component of clinical trials with anti-endotoxin antibodies. Moreover, our earlier studies in a neutropenic rat model of sepsis demonstrated the importance of giving adequate levels of anti-endotoxin antibodies (*Bhattacharjee* et al., 1994). The more recent study in human subjects found that even though the plasma from both high and low responders neutralised the cytokine-inducing activity of LPS, nevertheless, the activity was greater for the high responders (*Cross* et al., 2003). Consequently, it may be desirable to devise strategies to improve the antibody response with this J5 dLPS/OMP complex vaccine.

Future studies will be directed toward administration of this vaccine with adjuvants that may boost the level of anti-endotoxin antibodies and enhance the functional activity of the preparation. These strategies are currently being investigated.

LITERATURE

Baumgartner, J.D., Glauser, M.P., McCutchan, J.A., Ziegler, E.J., Van Melle, G., Klauber, M.R., Vogt, M., Muehlen, E., Luethy, R., Chiolero. R., and Geroulanos, S.: Prevention of Gram-negative shock and death in surgical patients by antibody to endotoxin core glycolipid. Lancet 2, 59-63 (1985).

Bhattacharjee, A.K., Opal, S.M., Palardy, J.E., Drabick, J.J., Collins, H., Taylor, R., Cotton, A., and Cross, A.S.: Affinity-purified *Escherichia coli* J5 lipopolysaccharide-specific IgG protects neutropenic rats against Gram-negative bacterial sepsis. J. Infect. Dis. 170, 622-629 (1994).

Bhattacharjee, A.K., Opal, S.M., Taylor, R., Naso, R., Semenuk, M., Zollinger, W.D., Moran, E.E., Young, L., Hammack, C., Sadoff, J.C., and Cross, A.S.: A noncovalent complex vaccine prepared with detoxified *Escherichia coli*J5 (Rc Chemotype) lipopolysaccharide and *Neisseria meningitidis* Group B outer membrane protein produces protective antibodies against Gram-negative bacteremia. J. Infect. Dis. 173, 1157-1163 (1996).

Braude, A.E., Jones, J.L., and Douglas, H.: The behavior *Escherichia coli* endotoxin (somatic antigen) during infectious arthritis. J. Immunol. 90, 297-312 (1960).

Bruins, S.C., Stumacher, R., Johns, M.A., McCabe, W.R.: Immunization with R mutants of *Salmonella minnesota*. III. Comparison of the protective effect of immunization with lipid A and the Re mutant. Infect. Immun. 17, 16-20 (1977).

Calandra, T., Glauser, M.P., Schellekens, J., Verhoef, J.: Treatment of gram-negative septic shock human IgG antibody to *Escherichia coli* J5: a prospective, double-blind, randomized trial. J. Infect. Dis. 158, 312-319 (1988).

Campbell, W.N., Hendrix, E., Cryz, S.J., and Cross, A.S.: Immunogenicity of a 24-valent *Klebsiella* capsular polysaccharide vaccine and an 8-valent *Pseudomnas* O-polysaccharide conjugate vaccine administered to acute trauma victims. Clin. Infect. Dis. 23, 179-181 (1996).

Collins, H.H., Cross, A.S., Dobek, A., Opal, S.M., McClain, J.B., and Sadoff, J.C.: Oral ciprofloxacin and anti-lipopolysaccharide monoclonal antibody protect leukopenic rats from lethal infection. J. Infect. Dis. 159, 1073-1082 (1989).

Cross, A.S., Opal, S.M., Palardy, J.E., Drabick, J.J., Warren, H.S., Huber, C., Cook, P., and Bhattacharjee, A.K.: Phase I study of detoxified *Escherichia coli* J5 lipopolysaccharide (J5dLPS)/group B meningococcal outer membrane protein (OMP) complex vaccine in human subjects. Vaccine 21, 4576-4587 (2003).

Davis, H.L., Cooper, C.L., Morris, M.L., Elfer, S.M., Cameron, D.W., and Heathcote, J.: CpG ODN is safe and highly effective in humans as an adjuvant in hepatitis B vaccines. Preliminary results of phase I trial with CpG 7909. In: Proceedings of the Third Annual Conference on Vacines, Abstract S25 (2000).

Fomsgaard, A., Baek, L., Fomsgaard, J.S., and Engquist, A.: Preliminary study on treatment of septic shock patients with anti-lipopolysaccharide IgG from blood donors. Scand. J. Infect. Dis. 21, 697-708 (1989).

Ginnoudis, P.V., Smith, R.M., Banks, R.E., Windsor, A.C.J., Dickson, R.A., and Guillou, P.J.: Stimulation of inflammatory markers after blunt trauma. Brit. J. Surg. 85, 986-990 (1998).

Goldie, A.S., Fearon, K.C.H., Ross, J.A., Barclay, R., Jackson, R.E., Grant, I.S., Ramsay, G., Blyth, A.S., and Howie, J.C.: Natural cytokine antagonists and endogenous anti-endotoxin core antibodies in sepsis syndrome. JAMA 274, 172-177 (1995).

Greenman, R.L., Schein, R.M.H., Martin, M.A., Wenzel, R.P., MacIntyre, N.R., Emmanuel, G., Chmel, H., Kohler, R.B., McCarthy, M., Plouffe, J., and Russell, J.A.: A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of Gram-negative sepsis. JAMA 266, 1097-1102 (1991).

Guthrie, L.A., McPhail, L.C., Henson, P.M., and Johnston, R.B. Jr.: Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme. J. Exp. Med. 160, 1656-1671 (1984).

Johns, M., Skehill, A., and McCabe, W.R.: Immunization with rough mutants of *Sal-*

monella minnesota. IV. Protection by antisera to O and rough antigens against endotoxin. J. Infect. Dis. 147, 57-62 (1983).

Johnson, S.E., Rubin, L., Romero-Steiner, S., Dykes, J.K., Pais, L.B., Rizvi, A., Ades, E., and Carlone, G.M.: Correlation of opsonophagocytosis and passive protection assay using human anticapsular antibodies in an infant mouse model of bacteremia for *Streptococcus pneumoniae*. J. Infect. Dis. 180, 133-140 (1999).

J5 Group: Treatment of severe infectious purpura in children with human plasma from donors immunized with *Escherichia coli* J5: a prospective double-blind study. J. Infect. Dis. 165, 695-701 (1992).

Kim, K.H. and Seoh, J.U.: Evaluation of antibody responses to pneumococcal vaccines with ELISA and opsonophagocytosis assay. J. Korean Med. Sci. 14, 475-479 (1999).

Kovach, N.L., Yee, E., Munford, R.S., Raetz, C.R., and Harlan, J.M.: Lipid IVA inhibits synthesis and release of tumor necrosis factor induced by lipopolysaccharide in human whole blood *ex vivo*. J. Exp. Med. 172, 77-84 (1990).

Lyons, A., Kelly, J.L., Rodrick, M.L., Mannick, J.A., and Lederer, J.A.: Major injury induces increased production of interleukin-10 by cells of the immune system with a negative impact on resistance to infection. Ann. Surg. 226, 450-460 (1997).

Martin, G.S., Mannino, D.M., Eaton, S., and Moss, M.: The epidemiology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 348,1546-1554 (2003).

McCabe, W.R.: Immunization with R mutants of *S. minnesota*. Part I: Protection against challenge with heterologous Gram-negative bacilli. J. Immunol. 108, 601-610 (1972).

Nys, M., Damas, P., Joassin, L., and Lamy, M.: Sequential anti-core glycolipid immunoglobulin antibody activities in patients with and without septic shock and their relation to outcome. Ann. Surg. 217, 300-306 (1993).

Pollack, M., Huang, A.I., Prescott, R.K., Young, L.S., Hunter, K.W., Cruess, D.F., and Tsai, C.M.: Enhanced survival in *Pseudomonas aeruginosa* septicemia associated with high levels of circulating antibody to *Escherichia coli* endotoxin core. J. Clin. Invest. 72, 1874-1881 (1983).

Schedel, I., Dreikhausen, U., Nentwig, B., Hockenschneider, M., Rauthmann, D.; Balikcioglu, S., Coldeway, R., and Deicher, H.: Treatment of Gram-negative septic shock with an immunoglobulin preparation: a prospective, randomized clinical trial. Crit. Care Med. 9, 1104-1113 (1991).

Schwartzer, T.A., Alcid, D.V., Numsuwan, V., and Gocke, D.J.: Characterization of the human anti-body response to an *Escherichia coli* O111:B4 (J5) vaccine. J. Infect. Dis. 158, 1135-1136 (1988).

The Intravenous Immunoglobulin Collaborative Study Group: Prophlactic intravenous immunoglobulin administration of standard immunoglobulin as compared with core-lipopolysaccharide immune globulin in patients at high risk of postsurgical infection. N. Engl. J. Med. 327, 234-240 (1992).

Warren, H.S., Amato, S.F., Fitting, C., Black, K.M., Loiselle, P.M., Pasternack, M.S., and Cavaillon, J.M.: Assessment of ability of murine and human anti-lipid A monoclonal antibodies to bind and neutralize lipopolysaccharide. J. Exp. Med. 177, 89-97 (1993).

Zeni, F., Freeman, B., and Natanson, C.: Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. Crit. Care Med. 25, 1095-1100 (1997).

Ziegler, E.J., Douglas, H., Sherman, J.E., Davis, C.E., and Braude, A.I.: Treatment of *E. coli* and *Klebsiella* bacteremia in agranulocytic animals with antiserum to a UDP-GAL epimerase-deficient mutant. J. Immunol. 111, 433-438 (1973).

Ziegler, E.J., McCutchan, J.A., Douglas, H., and Braude, A.I.: Prevention of lethal *Pseudomonas* bacteremia with epimerase-deficient *E.coli* antiserum. Trans. Assoc. Am. Physicians 88, 101-108 (1975).

Ziegler, E.J., McCutchan, J.A., Fierer, J., Glauser, M.P., Sadoff, J.C., Douglas, H., and Braude, A.I.: Treatment of Gram negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. N. Engl. J. Med. 307, 1225-1230 (1982).

Ziegler, E.J., Fisher, C.J. Jr., Sprung, C.L., Straube, R.C., Sadoff, J.C., Foulke, G.E., Wortel, C.H., Fink, M.P., Dellinger, R.P., Teng, N.N., et al.: Treatment of Gram-negative bacteremia in septic shock with HA-1A human monoclonal antibody against endotoxin: a randomized, double-blind, placebo-controlled trial. N. Engl. J.

Med. 325, 429-436 (1991).
Zinner, S.H. and McCabe, W.R.: Effects of IgM and IgG antibody in patients with bacteremia due to Gram-negative bacilli. J. Infect. Dis. 133, 37-45 (1976).

O ANTIGEN SEROEPIDEMIOLOGY OF *KLEBSIELLA* CLINICAL ISOLATES AND IMPLICATIONS FOR IMMUNOPROPHYLAXIS OF *KLEBSIELLA* INFECTIONS*

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SUMMARY

Prevention of *Klebsiella* infections by passive immunotherapy has received more attention during the last decade. Both K antigen- and O antigen-specific antisera and monoclonal antibodies (mAbs) have been studied with respect to phagocytosis-enhancing and *in vivo* protective capacities. Our own work has focussed on the generation of O serogroup-specific rabbit antisera and O antigen specific murine antibodies. O-specific rabbit sera were absorbed extensively with heterologous O antigen strains in order to obtain highly specific typing reagents. Using these for typing a collection of 378 clinical strains, we found that 82% of them belonged to one of the 4 serogroups O1, O2ab, O3 and O5. Phagocytosis experiments using antisera and mAbs showed that O antigen specific antibodies were able to opsonise non-encapsulated strains, while fully encapsulated bacteria were rather resistant against the opsonising effect. Nevertheless, *in vivo* experiments demonstrated a prophylactic effect on both *Klebsiella* septicaemia and pneumonia in a mouse model of lethal infection. Given the limited number of O serogroups, O antigen-specific antibodies may be suited to supplement K antigen-specific hyperimmune globulins for passive immunoprophylaxis of *Klebsiella* infections.

INTRODUCTION

Klebsiella spp., in particular *Klebsiella* (*K.*) *pneumoniae* and *Klebsiella oxytoca*, are important pathogens causing a variety of nosocomial infections (*Hansen* et al., 1997). In particular, *Klebsiella* ventilator-associated pneumonia in the Intensive Care Unit (ICU) setting carries a high mortality of up to 50% (*Carpenter*, 1990). Given the frequent occurrence of highly antibiotic-resistant *Klebsiella* strains, passive immunoprophylaxis of *Klebsiella* infections has received increasing attention in recent years. It has been shown by us and other groups, that capsule (K antigen) -specific antibodies are opsonic for *Klebsiella* organisms and protect against disseminating *Klebsiella* infection in

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Table 1: *Klebsiella* O antigen serogroup reference strains

Strain designation	Antigen formula	Comment
Friedländer 201	O1:K-	contains O2a
7380	O2ab:K-	-
5053	O2ac:K-	-
390	O3:K11	-
1702	O4:K42	-
4425/51	O5:K57	-
NCTC 8172	O6:K64	serologically identical with O1
264 (1)	O7:K67	-
889	O8:K69	serologically identical with O1*
1205	O9:K72	contains O2ab
337	O10:K73	no *Klebsiella***
378	O11:K78	contains O4
708	O12:K80	-

* The O8 antigen was shown chemically to be distinguished from O1 by partial O-acetylation of the polysaccharide backbone structure (*Kelly* et al., 1993). However, the two antigens are not separable by means of conventional serology.

** This strain, originally described as a novel *Klebsiella* serotype, was later shown to belong to the genus *Enterobacter* because it was motile. Both the O10 and K73 antigens were removed from the list of recognised *Klebsiella* antigens. Adapted from *Trautmann* et al., 1997.

animal models. In humans, a large clinical trial performed by the group of *Donta* and colleagues (1996) has shown that pre-treatment of ICU patients with high-titred human immunoglobulins containing antibodies specific for various *Pseudomonas* O serotypes and *Klebsiella* K serotypes prevented a significant proportion of ICU acquired infections compared to a control group treated with non-specific IgG. However, for effective prophylaxis of *Klebsiella* infections, a total of 77 recognised K antigen serotypes must be taken into account. The currently available *Klebsiella* K antigen vaccine contains no more than 24 capsular types, and the immune response against individual antigens in this vaccine is variable (*Cross* and *Cryz*, 1990). Therefore, a vaccine consisting of fewer components might be desirable.

Like other Gram-negative bacteria, *Klebsiella* also possesses a somatic or O antigen. However, except for the recent decade, little work had been done on the *Klebsiella* O antigens. Several obstacles prevented the generation of highly specific O antigen typing sera and the elucidation of the sero-epidemiology of the O antigens. Firstly, O antigen-specific sera, even when produced against less encapsulated mutants, are in reality OK sera because they always contain significant amounts of K specific antibodies. Consequently, the presence of K specific antibodies in these sera can significantly confound the results of O serotyping. Secondly, simple and quick typing methods such as agglutination cannot be used because O antibodies get "buried" within the large capsule layer of most *Klebsiella* strains.

In our own work, we focussed on the development of a reliable typing method to elucidate the sero-epidemiology of the O antigens in clinical material. Also, we produced monoclonal antibodies (mAbs) specific for different epitopes of the O1 antigen in order to test their opsonising and protective effects.

MATERIALS AND METHODS

O antigen reference strains

These strains, which are listed in Table 1, were obtained from the Statens Serum Institute, Copenhagen, Denmark.

Production of rabbit antisera and mAbs against Klebsiella O antigens

Immune sera were produced by repeated intramuscular immunisations of rabbits with boiled *Klebsiella* organisms as described (*Trautmann* et al., 1996). Antisera were raised preferably against capsule-less mutants or O antigen-identical but K antigen-heterologous strains in order to avoid a confounding effect of anti-capsular antibodies on typing results. Murine mAbs were produced by conventional immunisation schedules as described (*Trautmann* et al., 1994).

Preparation of O antigens (lipopolysaccharides)

We used the hot phenol-water method as described (*Trautmann* et al., 1996).

Clinical *Klebsiella* isolates

During a 10-year period, clinical *Klebsiella* isolates from two University hospitals (Charité Virchow Klinikum, Berlin, Germany, and University Hospital of Ulm University, Ulm, Germany) were collected and frozen. Species identification was performed by determination of the biochemical reaction profile (API 20E). Isolates recovered from any body site during routine clinical diagnostics were accepted, but only primary isolates from each patient were retained. The origin of each isolate and whether it was associated with colonisation, non-invasive or invasive infection was documented.

O antigen typing

A competitive enzyme-linked immunosorbent assay (ELISA) method was using for typing. In short, the strain to be tested was grown freshly on agar plates, harvested, and boiled to release the O antigen from the outer cell layer. Bacterial cellular debris was removed by centrifugation, and the clear supernatant was added in a 1:1 ratio to an O antigen-specific rabbit antiserum. After repeated vortexing, the mixture was transferred to ELISA wells coated with the homologous O antigen lipopolysaccharide. After incubation, the mixture was washed off, and any remaining O antigen specific rabbit antibodies that had bound to the plates were detected by anti-rabbit-IgG alkaline phosphatase conjugate. In case of a positive reaction, it could be concluded that the O antigen of the test strain did not correspond to the antibody in the typing serum. Conversely, if the reaction remained negative, O antigen identity between test strain and the typing serum could be assumed. All isolates were typed with all O specific antibodies and mAbs available (*Trautmann* et al., 1996).

Opsonophagocytic assay

This was performed by means of a microtitre plate phagocytosis assay, using Ficoll-Paque-purified human neutrophils and fresh human serum as a source of complement (*Held* et al., 2000).

In vivo protection studies

Groups of 10 mice were pre-treated intraperitoneally with mAb Ru-O1 in ascending doses. Four hours later, the animals were challenged i.p. with an estimated dose of 50 organisms of *Klebsiella pneumoniae* Caroli, a fully encapsulated and highly virulent strain (serotype O1:K2). This dose corresponded to approximately 50x the lethal dose 50% (LD50) as determined previously. Mortality was recorded for 4 subsequent days (*Rukavina* et al., 1997).

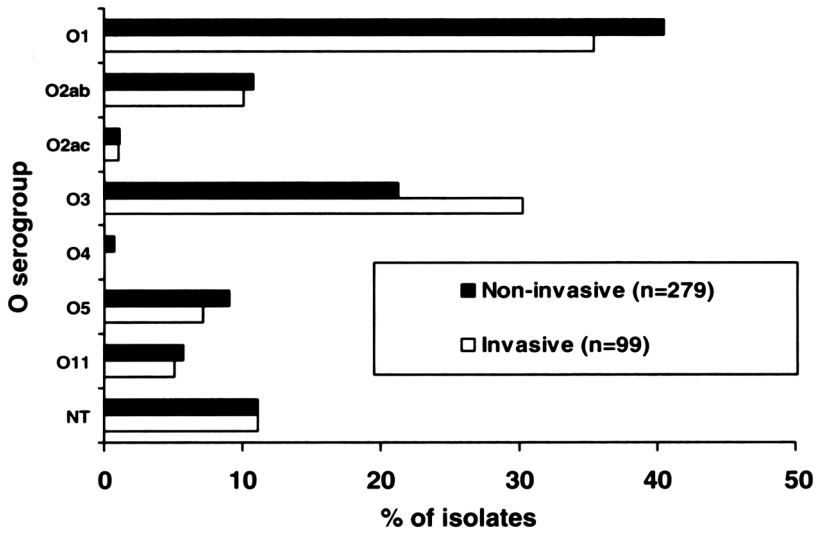

Figure 1: Relative distribution of isolates from invasive versus non-invasive infections.

RESULTS

Re-examination of O antigen reference strains

We found that the O8 antigen reference strain contained an O antigen indistinguishable by polyclonal and monoclonal serology from the O1 antigen. Therefore, we proposed to define this serogroup as a common O serogroup, although chemically, and additional O acetylation has been detected in the O8 type strain (*Kelly* et al., 1993). We also found that both of these strains contained the O2a partial antigen defined by strain 7380 (Table 1). Furthermore, we detected the O2a partial antigen in strain 1205 (O9), and the O4 antigen as a partial antigen in strain 378 (O11) (Table 1).

O antigen sero-epidemiology

A total of 378 clinical isolates were collected, 290 of which belonged to the species *K. pneumoniae* spp. *pneumoniae*, and 86 to the species *K. oxytoca*. Two isolates were identified as *K. ornithinolytica*. Ninety-nine strains were judged to have caused invasive infections because they were recovered from blood cultures (n=79), from open lung biopsies (n=7), or from the abdominal cavity during septic surgery (n=13). Typing results of invasive versus non-invasive isolates are summarised in Figure 1. Only 4 O antigens (O1, O2ab, O3, and O5) accounted for 82% of all clinical isolates, with no relevant differences between invasive and non-invasive strains (*Trautmann* et al., 1997).

O antigen specific mAbs

We raised 3 mAbs which we designated Ru-O1, IV/4-5 and V/9-5. MAb Ru-O1 reacted specifically with a high-molecular weight component of the lipopolysaccharide of serogroup O1 (and O8) strains, mAb IV/4-5 reacted with the O2a antigen, which represents a medium-weight component present in the O2ab, O2ac, and other O2a-containing strains such as O1 and O8. The broadly cross-reactive mAb V/9-5 recognises both low and high-molecular weight LPS components of all *Klebsiella* O serogroup reference strains ex-

Table 2: Opsonophagocytic effect of *Klebsiella* O antigen-specific mAbs for encapsulated and non-encapsulated *Klebsiella* strains

Strain designation	% Phagocytosis in the presence of mAb			
	Ru-O1	IV/4-5	V/9-5	K antibody
Klebsiella Caroli (O1:K2)	0	0	0	97.3 ± 2.2
Klebsiella Caroli decapsulated mutant (O1:K-)	94.4 ± 0.9	3.5 ± 1.8	21.7 ± 5.0	n.d.
strain 37 (O1:K7)	48.9 ± 4.8	38.6 ± 13.7	12.8 ± 6.4	97.0 ± 1.8
strain 37 decapsulated mutant (O1:K-)	93.5 ± 3.0	11.6 ± 6.6	59.9 ± 4.8	n.d.
strain 151 (O1:K21)	31.0 ± 8.03	3.4 ± 0.3	12.4 ± 3.7	94.3 ± 3.0
strain 151 decapsulated mutant (O1:K-)	93.9 ±1.5	54.1 ± 4.4	69.1 ± 4.8	n.d.

Values are % phagocytosis ± 1 standard deviation (3-4 separate experiments). Human neutrophils were used at a predetermined optimum ratio of bacteria to cells. The concentration of complement was 10%, and the final concentration of the mAbs was 5 μg/ml. Specific K antibody for K2 was mAb III/5-1, and polyclonal anticapsular rabbit sera raised against K7 and K21 were used in the respective experiments.

cept O7, and most of the clinical strains tested. In opsonophagocytosis experiments, a K2 antigen-specific anti-capsular mAb, III/5-1 (mouse IgM), was used as a positive control (*Trautmann* et al., 1988).

Opsonophagocytic tests

A variety of *Klebsiella* strains expressing and not expressing capsular antigens were tested in these experiments. We found that mAb Ru-O1 was the most active antibody in terms of a promotion of phagocytosis, however, even this antibody did not opsonise a fully encapsulated *Klebsiella* O1 strain, *K. pneumoniae* Caroli (Table 2). Thus, it was concluded that the capsule significantly hampered the access and functional activity of O antigen-specific antibodies (*Held* et al., 2000). Capsule-specific antibodies were highly opsonic for their homologous strains in these experiments (Table 2).

In vivo protection experiments

Although not opsonic for encapsulated *K. pneumoniae* strain Caroli, mAb Ru-O1 exerted significant protection against lethal infection with this strain. Doses necessary to provide protection were higher than those needed for a K antigen specific antibody tested for comparison (Table 3).

DISCUSSION

Our work on the *Klebsiella* O antigen serogroups has shown that the O antigen epidemiology of this genus is by far less diverse than that of other *Enterobacteriaceae*. For instance, in *E. coli*, more than 150 O antigen serogroups have been described, a significant proportion of which are found in clinical material. We found that only nine O antigen serogroups in *Klebsiella* can be accepted as truly separable groups, namely O1, O2, O3, O4, O5, O7, O9, O11 and O12. The previously described O and O8 antigens were

Table 3: Protective effect of mAb Ru-O1 (anti-O1) and mAb III/5-1 (anti-K2) in animals challenged with *Klebsiella* Caroli (O1:K2)

	No. of animals surviving at day 7/no. challenged, after pre-treatment with mAb	
Dose of mAb (μg/g)	III/5-1	Ru-O1
0.25	5/5	n.d.
0.5	5/5	n.d.
1.0	10/10	0/10
10.0	10/10	0/10
20.0	10/10	1/10
40.0	n.d.	7/10
200.0	n.d.	6/10

Adapted from *Rukavina* et al., 1997

found to be identical with O1, and several type strains were found to contain partial antigens of other strains. For instance, the O2a partial antigen was found in the type strain of O9, and the O4 partial antigen in the type strain of O11. The O2 serogroup is heterogeneous with various partial antigens that were not fully elucidated and that await their further clarification by mAb technology. We had mAbs at hand against the O2ab and O2ac partial antigens. While the former was present in all O1 and O2ab strains (i.e., in approximately 50% of clinical strains), the latter was found in only 4 out of 378 strains (1.1%). Our data are in nearly complete accordance with those obtained by *Hansen* et al. (1999) 2 years later, using a similar ELISA inhibition technique. These authors studied a total of 638 *Klebsiella* isolates from Denmark, Spain and the United States and found a virtually identical distribution of O serotypes in their clinical material. In their study, 78.9 % of strains belonged to serogroups O1, O2, O3 and O5.

Our studies with O antigen specific mAbs showed that these mAbs were able to bind to their target epitopes on whole bacteria (data not shown), but the capsule significantly hampered their opsonising effect. Nevertheless, our *in vivo* experiments with the most active of the antibodies, mAb Ru-O1, showed that protection may be achieved in spite of the relative lack of opsonic activity for encapsulated strains. We speculate that two mechanisms may account for this protective effect: Firstly, capsular antigen may be shed from growing bacteria *in vivo*, thereby exposing the O antigen layer for specific reaction with mAb, and secondly, soluble O antigen may contribute to pathogenicity by triggering pathophysiologic reactions such as disseminated intravascular coagulation or pro-inflammatory mediator release. It is possible, though not proven, that circulating O antigen specific mAb neutralises these effects, thereby contributing to protection. This mechanism may also explain why relatively large doses of O-specific antibody were needed for protection, compared to K antigen-specific antibodies (*Held* et al., 2000). Further studies will have to be done to clarify the protective mechanisms and study a possible synergism with K antigen specific antibodies before a clinical role of such antibodies can be defined.

LITERATURE

Carpenter, L.S.: *Klebsiella* pulmonary infections: Occurrence at one medical center and review. Rev. Infect. Dis. 5, 629-638 (1990).

Cross, A.S. and Cryz, S.J.: Vaccines against *Klebsiella* and *Pseudomonas* infections. In: New generation vaccines (Eds.: Woodrow, G.C. and Levine, M.M.). Marcel Dekker, New York, N.Y., 699-713 (1990).

Donta, S.T., Peduzzi, P., Cross, A.S., Sadoof, J., Haakenson, C., Cryz, S.J., Kauffman C., Bradley, S., Gafford, G., Elliston, D., Beam, T.R., John, J.R., Ribner, B., Cantey, R., Wels, C.H., Ellison, R.T., Young, E.J., Hamill, R.J., Leaf, H., Schein, R.M., Mulligan, M., Johnson, C., Griffiss, J.M., Slagle, D. and The Federal Hyperimmune Immunoglobulin Trial Study Group: Immunoprophylaxis against *Klebsiella* and *Pseudomonas aeruginosa* infections. J. Infect. Dis. 174, 537-543 (1996).

Hansen, D.S., Gottschau, A., and Kolmos, H.J.: Epidemiology of *Klebsiella* bacteraemia: a case-control study using *Escherichia coli* bacteraemia as control. J. Hosp. Infect. 37, 199-132 (1997).

Hansen, D.S., Mestre, F., Alberti, S., Hernandez-Alles, S., Alvarez, D., Domenech-Sanchez, A., Gil, J., Merino, S., Tomas, J.M., and Benedi, V.J.: *Klebsiella pneumoniae* lipopolysaccharide O typing: revision of prototype strains and O-group distribution among clinical isolates from different sources and countries. J. Clin. Microbiol. 37, 56-62 (1999).

Held, T.K., Jendrike, N.R.M., Rukavina, T., Podschun, R., and Trautmann, M.: Binding to and opsonophagocytic activity of O-antigen-specific monoclonal antibodies against encapsulated and nonencapsulated *Klebsiella pneumoniae* serotype O1 strains. Infect. Immun. 68, 2402-2409 (2000).

Kelly, R.F., Severn, W.B., Richards, J.C., Perry, M.B., MacLean, L.L., Tomas, J.M., Merino, S., and Whitfield, C.: Structural variation in the O specific polysaccharides of *Klebsiella pneumoniae* serotype O1 and O8 lipopolysaccharide: evidence for clonal diversity of *rfb* genes. Mol. Microbiol. 10, 615-625 (1993).

Rukavina, T., Ticac, B., Susa, M., Jendrike, N., Jonjic, S., Lucin, P., Marre, R., Doric, M., and Trautmann, M.: Protective effect of antilipopolysaccharide antibody in experimental *Klebsiella* infection. Infect. Immun. 65, 1754-1760 (1997).

Trautmann, M., Vogt, K., Hammack, C., and Cross, A.S.: A murine monoclonal antibody defines a unique epitope shared by *Klebsiella* lipopolysaccharides. Infect. Immun. 62, 1282-1288 (1994).

Trautmann, M., Cross, A.S., Reich, G., Held, T.K., Podschun, R., and Marre, R.: Evaluation of a competitive ELISA method for the determination of *Klebsiella* O antigens. J. Med. Microbiol. 44, 44-51 (1996).

Trautmann, M., Ruhnke, M., Rukavina, T., Held, T.K., Cross, A.S., Marre, R., and Whitfield, C.: O-antigen seroepidemiology of *Klebsiella* clinical isolates and implications for immunoprophylaxis of *Klebsiella* infections. Clin. Diagn. Lab. Immunol. 4, 550-555 (1997).

Trautmann, M., Cryz, S.J., Sadoff, J.C., and Cross, A.S.: A murine monoclonal antibody against *Klebsiella* capsular polysaccharide is opsonic *in vitro* and protects against experimental *Klebsiella pneumoniae* infection. Microb. Pathog. 5, 177-187 (1988).

ENTEROCOCCAL INFECTIONS: HOST RESPONSE, THERAPEUTIC, AND PROPHYLACTIC POSSIBILITIES*

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SUMMARY

The emergence of resistance against multiple antibiotics and the increasing frequency with which *E. faecalis* and *E. faecium* are isolated from hospitalised patients underscore the necessity for a better understanding of the virulence mechanisms of this pathogen and the development of alternatives to current antibiotic treatments. The genetic plasticity of enterococci and their ability to rapidly acquire and/or develop resistance against many clinically important antibiotics and to transfer these resistance determinants to other more pathogenic microorganisms makes the search for alternative treatment and preventive options even more important. A capsular polysaccharide antigen has recently been characterised that is the target of opsonic antibodies. A limited number of clinically relevant serotypes exist, and the development of an enterococcal vaccine based on capsular polysaccharides may improve our ability to prevent and treat these infections. Additional enterococcal surface antigens, including ABC transporter proteins and other virulence factors, such as aggregation substance, may also be useful targets for therapeutic antibodies.

INTRODUCTION

Enterococci are physiologic commensals of the gastrointestinal and female genital tracts of humans and several mammals and birds (*Aarestrup* et al., 2002). They are extremely versatile and well suited for survival under harsh conditions (*Murray*, 2000). Under most circumstances, enterococci do not cause any harm to the host, despite living in abundance in the intestinal lumen (10^5-10^8 colony-forming units per gram of faeces) (*Huycke* et al., 1998; *Noble*, 1978). Some enterococcal strains are used as probiotic agents and are believed to have beneficial effects on a number of gastro-intestinal and systemic diseases (*Franz* et al., 1999; *Mitra* and *Rabbani*, 1990; *Benyacoub* et al., 2003). However, on some occasions, the commensal relationship with the host is

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disrupted with the consequence that enterococci cause serious diseases (*Jett* et al., 1994). Enterococci are intrinsically not as virulent as other Gram-positive organisms such as *S. aureus*, pneumococci, or group A streptococci, which makes the study of their pathogenicity more difficult. A number of putative virulence factors for enterococci have been described, although their relevance to disease development is often not as obvious as for other pathogens. Enterococci are endogenously resistant and are known to have acquired further resistance mechanisms to multiple antibiotics (*Jones* et al., 1997), allowing them to prevail in hospital and nursing home settings. The immense difficulties in treating serious enterococcal infections underscore the importance of understanding virulence factors that may be targeted by alternative therapeutics. The rapid increase in enterococcal strains resistant to vancomycin (VRE) and other antibiotics (*Huycke* et al., 1998; *Jones* et al., 1997) and their ability to pass this trait on to other pathogens, i.e., *S. aureus*, indicates an urgent and expanding clinical problem.

ENTEROCOCCAL INFECTIONS

Enterococci are the third most common pathogen isolated from bloodstream infections (*Jones* et al., 1997), the single most frequently reported type of pathogen in surgical-site infections in intensive care units (*Richards* et al., 2000), and the second most common nosocomial pathogen in the U.S. (*Richards* et al., 1999). Enterococci are responsible for three to four cases of nosocomial bloodstream infections per 10,000 hospital discharges (*Banerjee* et al., 1991). These bacteria contribute significantly to patient mortality as well as to additional hospital stay (*Landry* et al., 1989). The ability of enterococci to acquire, accumulate, and transfer genetic elements such as plasmids and transposons via conjugation is one of the major reasons for their increased importance as nosocomial pathogens (*Murray*, 2000). Transfer of resistance determinants from enterococci to other more virulent Gram-positive bacteria, like staphylococci, has been observed *in vitro* (*Murray*, 2000). The first isolation of a fully vancomycin-resistant *S. aureus* strain in a patient previously colonised with VRE suggests the possibility of an *in vivo* exchange of resistance traits (*Chang* et al., 2003).

Enterococci can cause a variety of clinical syndromes including endocarditis, bacteraemia, meningitis, intra-abdominal, wound, and urinary tract infections. There are well-defined patient populations [e.g., liver-transplant patients (*Papanicolaou* et al., 1996), neonates (*Christie* et al., 1994), and patients with haematological malignancies (*Chadwick* et al., 1996)] who would clearly benefit from improved treatment options for enterococcal infections (Table 1).

PATHOGENICITY OF ENTEROCOCCI

The mechanisms by which peaceful commensals are transformed into life-threatening pathogens are not well understood. One hypothesis is that enterococci normally colonise the intestinal tract and are held in check by host

Table 1: Predominant enterococcal infections in specific patient populations

Immunocompetent patients	Immunocompromised patients	Procedure-related infections
Urinary tract infections Endocarditis	Bacteraemia/sepsis	Urinary tract infections Intra-abdominal infections Meningitis

mechanisms, but at some point develop traits to occupy new niches or exploit a possibly weakened host immune system (*Gilmore* et al., 2002). This imbalance could lead to translocation of organisms from the intestinal lumen into the bloodstream, eventually resulting in systemic spread. Successful evasion of the host defence can eventually lead to increased pathogenicity in the host and subsequent disease (*Johnson*, 1994). Additional sources of infections include intravenous, urinary, or biliary catheters, foreign bodies, the urinary tract, surgical wounds, or the oral cavity (*Jett* et al., 1994; *Gilmore* et al., 2002). Studies have shown that enterococci can also be transmitted through the hands of healthcare workers, clinical instruments (*Porwancher* et al., 1997), or from patient to patient (*Chenoweth* and *Schaberg*, 1990).

COLONISATION

Enterococci normally colonise the gastrointestinal tract of healthy humans. A number of adhesion factors of enterococci have been identified that confer binding to mucosal and other epithelial surfaces and facilitate colonisation or the formation of vegetations. Adhesion to host tissues is considered a prerequisite for the establishment of infection by many bacteria. For example, in endocarditis, firm attachment to endocardial epithelium is a precondition of successful colonisation, considering the high flow rates inside the heart (*Karchmer*, 2001; *Hoesley* and *Cobbs*, 1999).

Aggregation substance (AS) is one enterococcal virulence factor that seems to mediate the specific binding of enterococci to intestinal epithelium (*Sartingen* et al., 2000), renal epithelial cells (*Kreft* et al., 1992), human neutrophils (*Vanek* et al., 1999), and macrophages (*Sussmuth* et al., 2000). AS is a surface-bound glycoprotein encoded on sex-pheromone plasmids that mediates aggregation between bacteria and facilitates plasmid transfer (*Dunny* et al., 1995). AS augments internalisation of enterococci (*Sartingen* et al., 2000; *Olmsted* et al., 1994; *Wells* et al., 2000) and intracellular survival (*Sussmuth* et al., 2000; *Rakita* et al., 1999) and has been associated with an increased mass in valvular vegetations in rabbit endocarditis models (*Chow* et al., 1993; *Schlievert* et al., 1998). In some studies, AS seems to be more common in clinical vs. stool isolates (*Coque* et al., 1995; *Waar* et al., 2002), while other studies found no difference (*Archimbaud* et al., 2002; *Huycke* and *Gilmore*, 1995) (Table 2).

Another cell surface protein, Ace (adhesin of collagen from *E. faecalis*), which exhibits strong similarities with the *S. aureus* collagen-binding protein Cna, has recently been identified (*Rich* et al., 1999). This *E. faecalis*–specific surface component belongs to the

Table 2: Prevalence of virulence genes of enterococcal isolates from different sources

Virulence Factors	Clinical isolates	Stool isolates from healthy volunteers
Aggregation substance (AsaI)	50-90% [34, 35, 36, 37, 60, 63]	30-60% [34, 36, 37]
Esp	5-100% [35, 36, 42, 59, 60]	3-40% [35, 36, 42]
Cytolysin/haemolysin	11-70% [34, 35, 36, 37, 59, 60, 63]	0-25% [34, 35, 36, 37]
Gelatinase	55-100% [34, 36, 59, 60, 63]	27-66% [34, 35, 36]

MSCRAMM family, mediates binding to certain collagens (*Rich* et al., 1999), and may play a role in the pathogenesis of endocarditis (*Nallapareddy* et al., 2000).

Similarly, EfaA (*E. faecalis* adhesin), a serum-inducible surface protein that shows extensive similarities with several adhesins of streptococci (*Lowe* et al., 1995), is a putative endocarditis antigen and demonstrated a potential biological role in a mouse peritonitis model (*Singh* et al., 1998a).

Another putative colonisation factor is the enterococcal surface protein Esp (*Shankar* et al., 1999), a cell-wall associated protein, that shows structural similarities with the *Streptococcus agalacticae* (GBS) Rib (*Wastfelt* et al., 1996), C alpha protein of GBS (*Michel* et al., 1992), R28 of *Streptococcus pyogenes* (GAS) (*Stalhammar-Carlemalm* et al., 1999), and the *Staphylococcus aureus* biofilm-associated protein BAP (*Cucarella* et al., 2001). Esp was found to be enriched in clinical vs. stool or food isolates in several studies (*Archimbaud* et al., 2002; *Shankar* et al., 1999; *Baldassarri* et al., 2001a; *Eaton* and *Gasson*, 2002; *Willems* et al., 2001), though this could not be confirmed by others (*Waar* et al., 2002) (Table 2). Esp has been shown to contribute to the colonisation and persistence of some *E. faecalis* strains during ascending urinary tract infection (*Shankar* et al., 2001). It also seems to play a role in mediating primary attachment of enterococci to surfaces and in biofilm formation (*Toledo-Arana* et al., 2001).

SECRETED VIRULENCE FACTORS

Enterococci also secrete molecules that are putative virulence factors. For example, cytolysin/haemolysin is a bacterial toxin that is encoded by an operon consisting of 8 genes [52-56] localised on a pheromone-responsive plasmid (*Jett* et al., 1994) or on the chromosome (*Colmar* and *Horaud*, 1987; *Ike* and *Clewell*, 1992). Cytolysin shows haemolytic (against human, horse, and rabbit erythrocytes) and bacteriocidal activity against other Gram-positive bacteria (*Coque* et al., 1995). It is thought to play an important role in human infections, in which it is produced in 11-70% of strains (*Coque* et al., 1995; *Waar* et al., 2002; *Archimbaud* et al., 2002; *Huycke* and *Gilmore*, 1995; *Vergis* et al., 2002; *Eaton* and *Gasson*, 2001; *Huycke* et al., 1991, 1995; *Elsner* et al., 2000), compared to 0-25% in stool isolates (*Coque* et al., 1995; *Waar* et al., 2002; *Archimbaud* et al., 2002; *Huycke* and *Gilmore*, 1995) (Table 2). Cytolysin also contributes to enterococcal virulence in all animal models (*Huycke* et al., 1998; *Chow* et al., 1993; *Ike* et al., 1987; *Jett* et al., 1992, 1995) and a *C.*

elegans model studied (*Garsin* et al., 2001). It has recently been shown to be regulated by a quorum-sensing mechanism involving a two-component regulatory system (*Haas* et al., 2002).

Gelatinase (GelE) is an extracellular zinc metallo-endopeptidase secreted by *E. faecalis* that shares homologies with gelatinase of *Bacillus species* and *Ps. aeruginosa* elastase (*Coque* et al., 1995). It is co-transcribed with the serine protease SprE and regulated by the quorum-sensing *fsr* locus, which shows homology to the *S. aureus agr* locus and is expressed in late exponential phase at high cell densities (*Qin* et al., 2000, 2001; *Nakayama* et al., 2001a,b). GelE can hydrolyse gelatine, casein, haemoglobin, and other bioactive peptides, which provides clues for its potential role as a virulence factor in enterococci (*Makinen* et al., 1989; *Su* et al., 1991). Gelatinase can also cleave sex pheromones, which are known to be potent chemo-attractants (*Sannomiya* et al., 1990), and might therefore modulate the host response (*Hancock* and *Gilmore*, 2000). It might also play an important role in the severity of systemic disease, as shown in several independent animal studies (*Chow* et al., 1993; *Singh* et al., 1998b; *Gutschik* et al., 1979; *Dupont* et al., 1998; *Ike* et al., 1984; *Miyazaki* et al., 1993). GelE was also shown to be enriched in clinical isolates in some studies [55-100% in clinical isolates vs. 27-66% in stool isolates from healthy volunteers (*Coque* et al., 1995; *Archimbaud* et al., 2002; *Vergis* et al., 2002; *Eaton* and *Gasson*, 2001)], but contradicting observations have also been reported (*Waar* et al., 2002) (Table 2). Further investigations are needed to explore possible therapeutic uses for the above-mentioned enterococcal virulence mechanisms.

Burnie et al. (2002) examined sera of patients with enterococcal infections to identify enterococcal antigens that might be associated with protective antibodies. They identified an immunodominant ABC transporter complex that was recognised by antibodies from patients. Antibodies raised against parts of this complex conferred protection to mice in a systemic infection model. ABC (ATP-binding cassette) transporter proteins are cell membrane–associated ex- and import systems that transport a variety of molecules, including nutrients and drugs (*Fath* and *Kolter*, 1993; *Linton* and *Higgins*, 1998; *Quentin* et al., 1999). They have also been associated with polysaccharide biosynthesis in *E. faecalis* (*Xu* et al., 1998). ABC transporters have been implicated as virulence factors in staphylococcal infections in several studies (*Coulter* et al., 1998; *Lowe* et al., 1998; *Mei* et al., 1997) and as immunodominant antigens in infections due to *E. faecalis* (*Xu* et al., 1997) and *S. aureus* (*Burnie* et al., 2000). *MsrC* from *E. faecium*, another ABC transporter, which is homologous to *MsrA* of *S. aureus,* is associated with macrolide resistance (*Portillo* et al., 2000; *Singh* et al., 2001). ABC transporters share highly conserved sequences and therefore seem to be promising targets for the development of protective antibodies.

TRANSLOCATION

Enterococci possess the ability to translocate from the intestinal lumen to mesenteric lymph nodes, the liver, and the spleen (Wells et al., 1988, 1990, 1991a,b). However, the mechanisms responsible have not been fully elucidated. Enterococci are thought to be phagocytosed by tissue macrophages or intesti-

nal epithelial cells and transported across the intestinal wall into the lymphatic system (*Hancock* et al., 2000). *Olmsted* et al. (1994) showed that internalisation of enterococci by cultured intestinal cells is significantly increased in the presence of AS, although this is most likely only one of several factors that control internalisation efficiency. No study to date has been able to suggest any therapeutic approaches to prevent infection at this level of interaction between host and enterococci.

HOST RESPONSE AGAINST ENTEROCOCCAL INFECTIONS

Surprisingly little is known about host defence mechanisms against enterococcal infections, and only a few studies have attempted to investigate this area systematically. In order to survive in the host, enterococci must successfully avoid specific and non-specific host defence mechanisms. Most Gram-positive pathogens possess factors such as anti-phagocytic polysaccharide capsules, surface proteins such as the M-protein of GAS, or toxins to ensure survival in the host. After translocation or introduction into the bloodstream, enterococci are susceptible to neutrophil-mediated killing carried out mainly by complement and opsonising antibodies (*Harvey* et al., 1992; *Gaglani* et al., 1997; *Arduino* et al., 1994a,b). Certain strains of enterococci have also been shown to be capable of surviving within phagocytic cells (*Sussmuth* et al., 2000; *Rakita* et al., 1999; *Gentry-Weeks* et al., 1999; *Baldassarri* et al., 2001b), which might serve as vehicles for enterococci to translocate across the intestinal wall and disseminate into distant organs. The failure of phagocytic cells to kill intracellular enterococci might lead to systemic spread (*Wells* et al., 1988). Whether phagocytosis of enterococci represents a successful host defence mechanism or a means of immune response evasion for enterococci remains to be demonstrated.

Arduino et al. (1994a) studied the resistance of *E. faecium* to neutrophil-mediated phagocytosis using a fluorescence microscopic ingestion assay. While all *E. faecalis* strains studied were internalised, only 50% of the *E. faecium* strains were phagocytosed. Exposure to pronase, trypsin, or phospholipase C did not affect the bacterium's resistance to phagocytosis, while treatment with periodate eliminated the resistance to phagocytosis.

The authors concluded that a carbohydrate structure was responsible for the resistance to phagocytic killing, although they did not isolate or chemically characterise a specific factor. By electron microscopy, they identified small electron-dense clumps in *E. faecium* as well as in *E. faecalis* that may be consistent with capsular material (*Arduino* et al., 1994a).

ENTEROCOCCAL POLYSACCHARIDES

Little is known about capsular polysaccharides in enterococci or their roles in colonisation or persistence. Since 1935 there have been reports on serological typing systems for enterococci (formerly group D streptococci). Initially 31 subtypes of "enterococci" were described (*Takeda*, 1935). However, the main goal of these studies was the epidemiological investigation of out-

breaks rather than the taxonomic classification of isolates. Only crude extracts of bacteria were used to prepare immunising suspensions. The streptococcal group D antigen is expressed by most enterococci. Unlike the cell-wall carbohydrates characterising the serogroup A to C antigens, the group D antigen is a glycero-phosphate polymer (*Elliott*, 1962). Lancefield recognised additional cell wall or surface carbohydrates and referred to these as type-specific antigens (*Elliott*, 1959). These antigens were considered to be the structural and chemical counterparts of the group-specific substances in streptococci groups A, B, C, E, F, and G. Type-specific enterococcal antigens contain glucosamine, rhamnose, and glucose (*Elliott*, 1960). *Bleiweis* et al. (1965) attempted an analysis of the chemical composition of the type antigen from *E. faecalis* type 1. By extraction with lysozyme, they identified material that consisted of 22.5% rhamnose, 11.9% hexosamine, 14.4% glucose, 4.2% muramic acid, 11.7% alanine, 5.5% glutamic acid, and 5.8% lysine. They suggested that the type 1 antigen contained a rhamnose polymer covalently linked to a second moiety, a ribitol phosphate (*Krause*, 1972).

In 1964, Sharpe proposed a typing system for *Streptococcus faecalis* based on cell-wall type antigens that included 11 serogroups. Her antigen preparations were unaffected by trypsin but were inactivated by periodate (*Sharpe*, 1964). However, no systematic sero-epidemiologic study reported to date has used the above-mentioned system. In 1992, Maekawa et al. proposed a new serotyping system for *E. faecalis* that included nine of Sharpe's type strains. It distinguished a total of 21 serotypes, with four types being responsible for 72% of the typable strains (*Maekawa* et al., 1992,1996). However this system used formalin-killed bacteria to immunise rabbits instead of chemically defined antigen preparations (i.e. polysaccharide antigens) to produce typing sera. This serotyping system is therefore not based on defined antigenic structures such as capsules or other cell wall antigens. In recent years a number of studies have focused on polysaccharide antigens in enterococci (*Xu* et al., 1997,1998, 2000). By expressing chromosomal DNA fragments in *E. coli*, *Xu* et al. (2000) were able to identify clones that produced an antigen detectable by convalescent human sera. However, they were not able to isolate this material from the parent strain, and thus its structure remains unknown. The fact that two of the polysaccharide genes are a putative glycosyl transferase and a putative rhamnose biosynthesis gene indicate that this locus may be responsible for the synthesis of the enterococcal type antigen described by Lancefield and others. Insertional mutants of these two genes were shown to have diminished virulence in a mouse peritonitis model (*Xu* et al., 2000). *Hancock* et al. (2002) identified a serotype-specific cell wall polysaccharide biosynthetic operon. This operon consists of 11 ORFs, and mutants with insertions into certain of these genes lacked a high-molecular-weight antigen. One of the created mutants, HG101, with insertion in the *cps*I gene, was more readily cleared from a subcutaneous infection model and was found to be more susceptible to human neutrophil-mediated killing in an opsonophagocytosis assay compared to the wild-type FA2-2. Genetic evidence and preliminary carbohydrate analysis indicated a teichoic acid-like surface molecule consisting of glycerol phosphate, glucose, and galactose. Although some phenotypic effects have been observed in the mutants described above (*Xu* et al., 2000; *Hancock* and *Gilmore*, 2002), it cannot be concluded from these studies that the antigens are indeed present on the surface of enterococci. It

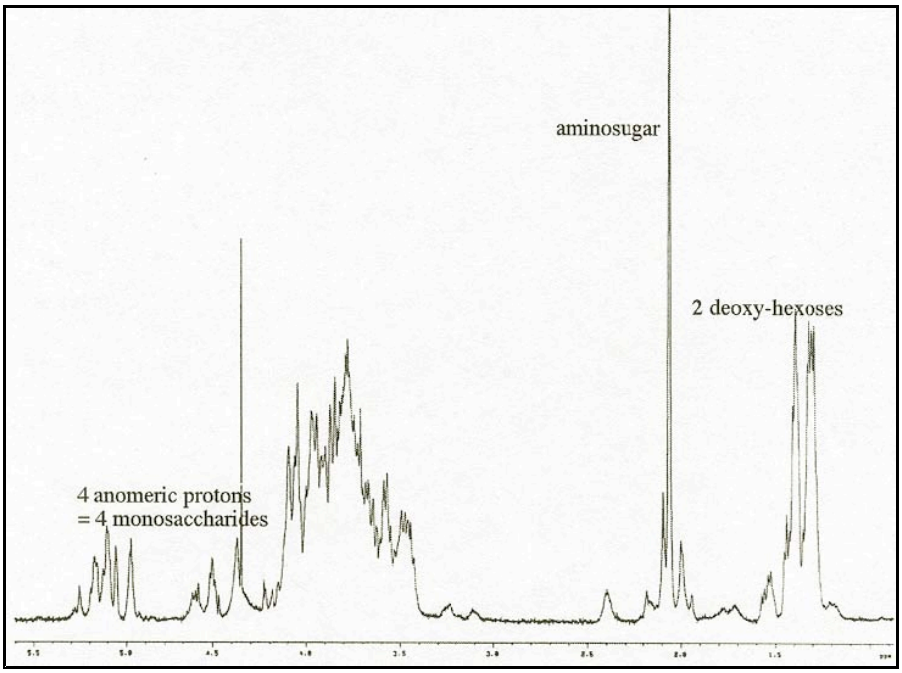

Figure 1: NMR spectroscopy of the putative type-antigen from *E. faecalis* 12030.

has not been shown for either of the polysaccharides that antibodies directed against these structures are protective.

VACCINE POTENTIAL OF ENTEROCOCCAL ANTIGENS

Data from our laboratory showed that about 57% of pathogenic enterococci (90 out of 157 strains) possess a capsule and that the capsule may be used to immunise animals as well as protect them against systemic infection (*Wang* et al., 1999; *Huebner* et al., 2000). A high-molecular-weight polysaccharide fraction isolated from strain *E. faecalis 12030* inhibited opsonic killing activity of immune rabbit sera raised against both *E. faecalis* and *E. faecium* strains. The crude antigen could be divided into two distinct polysaccharide fractions by ion-exchange chromatography, and analysis of these purified materials by NMR spectroscopy indicated that the first peak consisted of four distinct monosaccharides (see Figure 1). This first fraction most likely contained amino sugars and deoxyhexoses and is probably identical with the type-specific antigen. The second polysaccharide consisted of a glycerol-teichoic acid-like molecule with a backbone structure of –6 a-D-glucose-1-2-glycerol-3-PO_4 substituted on carbon two of the glucose molecule with an a-2-1-linked molecule of D-glucose (Figure 2) (*Wang* et al., 1999). Immunoblot and ELISA experiments indicated that the immunoreactivity of the immune rabbit sera was directed against the second polysaccharide. Rabbits immunised with the purified glycerol/glucose polymer material developed specific high-titre

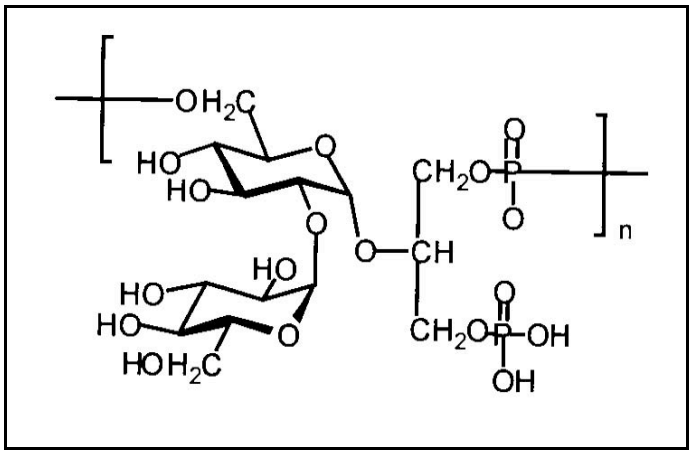

Figure 2: Chemical structure of the capsular teichoic acid from *E. faecalis* 12030.

antibodies that mediated bacterial killing in an opsonophagocytic assay. This killing activity could be abolished by absorption of the immune rabbit sera with the purified polymer. However, pretreatment of this polysaccharide with Na-periodate prior to absorption rendered the polysaccharide unable to affect killing activity. Immune-electron microscopy studies clearly indicate that those polysaccharide-specific antibodies have a capsule-like structure (see Figure 3) (*Huebner* et al., 1999). Evaluation of protective efficacy was carried out in mice that were intravenously (i.v.) challenged with live enterococci (*Huebner* et al., 2000). In non-immune mice, i.v. inoculations resulted in high bacterial levels in kidney, spleen, and liver five days after challenge. Mice immunised with four 10-µg doses of CP antigen were protected against challenge with the homologous *E. faecalis* strain. Opsonic IgGs were induced in high titres by immunising rabbits with the purified CP, and passive transfer of this antiserum to mice produced significantly lower bacterial counts in organs than did normal rabbit serum or sterile saline. Antibodies to the polysaccharide isolated from *E. faecalis* strain 12030 were protective against another *E. faecalis* strain and against two serologically related, vancomycin-resistant clinical *E. faecium* isolates. Antibodies to this CP antigen were also effective as a therapeutic reagent in mice when passive therapy was initiated up to four days after challenge with live bacteria (*Huebner* et al., 2000).

OTHER POTENTIAL VACCINE CANDIDATES

So far only the ABC transporters described above have been studied as targets of therapeutic antibodies in an appropriate animal model (*Burnie* et al., 2002). However, all of the above-mentioned putative virulence factors could theoretically be used as vaccine targets. A recombinant aggregation substance has been used to immunise rabbits, and the application of these hyperimmune sera protected mice against weight loss and kidney infections in a bacteraemia model (*Krueger*, manuscript in preparation). Protective antibodies directed

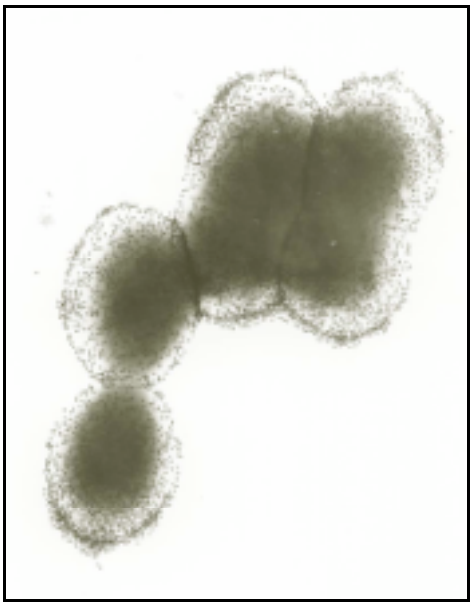

Figure 3: Immune electron microscopy of *E. faecalis* 12030 with immunogold-labelled rabbit sera raised against the purified capsular polysaccharide.

against surface proteins have been studied in a number of bacteria, and the possibility of conjugating a capsular polysaccharide to one of these proteins would provide targets against two different pathophysiologic mechanisms included in the same vaccine (*Lesinski* and *Lesinski*, 2001; *Gravekamp* et al., 1999). Further studies to evaluate these possibilities are necessary.

POSSIBLE USAGE OF AN ENTEROCOCCAL VACCINE

The development of an enterococcal vaccine to prevent and/or treat systemic infections depends on a number of factors, but must take into account the patient populations most likely to be at risk for infections due to enterococci. A number of recent studies established specific risk factors in well-defined patient populations (*Carmeli* et al., 2002; *Cetinkaya* et al., 2002; *Elizaga* et al., 2002; *Husni* et al., 2002; *Lund* et al., 2002; *Pai* et al., 2002; *Safdar* and *Maki*, 2002; *Suntharam* et al., 2002; *Timmers* et al., 2002), and the prevention of infections in high-risk patients could lead to reduced mortality and reduced hospital stay, making the cost-benefit favourable for this possibly very expensive treatment. Passive immunotherapy using hyperimmunoglobulins would be the therapy of choice, since most patients at risk are likely to need protection for only a limited period (i.e., several weeks), and in most instances there would not be sufficient time to actively immunise these patients in advance. Passive immunotherapy has been used in the prevention and treatment of a number of bacterial and viral diseases (*Keller* and *Stiehm*, 2000). The generation of antibodies with new technologies such as phage display and the genetic

manipulation of mammals that express human antibody molecules are promising techniques to explore in the future. Highly specific monoclonal antibodies (*Casadevall*, 1999) directed against enterococcal antigens could be a useful addition and/or alternative for the prevention and/or treatment of enterococcal infections in susceptible patients.

LITERATURE

Aarestrup, F.M., Butaye, P., and Witte, W.: Nonhuman reservoirs of enterococci. In: The Enterococci: Pathogenesis, molecular biology, and antibiotic resistance (Ed.: Gilmore, M.S.). ASM Press, Washington, DC, 55-99 (2002).

Archimbaud, C., Shankar, N., Forestier, C., Baghdayan, A., Gilmore, M.S., Charbonne, F., and Joly, B.: *In vitro* adhesive properties and virulence factors of *Enterococcus faecalis* strains. Res. Microbiol. 153, 75-80 (2002).

Arduino, R.C., Jacques-Palaz, K., Murray, B.E., and Rakita, R.M.: Resistance of *Enterococcus faecium* to neutrophil-mediated phagocytosis. Infect. Immun. 62, 5587-5594 (1994a).

Arduino, R.C., Murray, B.E., and Rakita, R.M.: Roles of antibodies and complement in phagocytic killing of enterococci. Infect. Immun. 62, 987-993 (1994b).

Baldassarri, L., Bertuccini, L., Ammendolia, M.G., Gherardi, G., and Creti, R.: Variant esp gene in vancomycin-sensitive *Enterococcus faecium*. Lancet 357, 1802 (2001a).

Baldassarri, L., Cecchini, R., Bertuccini, L., Ammendolia, M.G., Iosi, F., Arciola, C.R., Montanaro, L., Di Rosa, R., Gherardi, G., Dicuonzo, G., Orefici, G., and Creti, R.: *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. Med. Microbiol. Immunol. (Berl.) 190, 113-120 (2001b).

Banerjee, S.N., Emori, T.G., Culver, D.H., Gaynes, R.P., Jarvis, W.R., Horan, T., Edwards, J.R., Tolson, J., Henderson, T., and Martone, W.J.: Secular trends in nosocomial primary bloodstream infections in the United States, 1980-1989. National Nosocomial Infections Surveillance System. Am. J. Med. 91, 86S-89S (1991).

Benyacoub, J., Czarnecki-Maulden, G.L., Cavadini, C., Sauthier, T., Anderson, R.E., Schiffrin, E.J., and von der Weid, T.: Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. J. Nutr. 133, 1158-1162 (2003).

Bleiweis, A. and Krause, R.: The cell walls of group D streptococci: I. The immunochemistry of the type 1 carbohydrate. J. Exp. Med. 122, 237-249 (1965).

Burnie, J.P., Matthews, R.C., Carter, T., Beaulieu, E., Donohoe, M., Chapman, C., Williamson, P., and Hodgetts, S.J.: Identification of an immunodominant ABC transporter in methicillin-resistant *Staphylococcus aureus* infections. Infect. Immun. 68 3200-3209 (2000).

Burnie, J., Carter, T., Rigg, G., Hodgetts, S., Donohoe, M., and Matthews, R.: Identification of ABC transporters in vancomycin-resistant *Enterococcus faecium* as potential targets for antibody therapy. FEMS Immunol. Med. Microbiol. 33, 179-189 (2002).

Carmeli, Y., Eliopoulos, G.M., and Samore, M.H.: Antecedent treatment with different antibiotic agents as a risk factor for vancomycin-resistant Enterococcus. Emerg. Infect. Dis. 8, 802-807 (2002).

Casadevall, A.: Passive antibody therapies: progress and continuing challenges. Lin. Immunol. 93, 5-15 (1999).

Cetinkaya, Y., Falk, P.S., and Mayhall, C.G.: Effect of gastrointestinal bleeding and oral medications on acquisition of vancomycin-resistant *Enterococcus faecium* in hospitalized patients. Clin. Infect. Dis. 35, 935-942 (2002).

Chadwick, P.R., Oppenheim, B.A., Fox, A., Woodford, N., Morgenstern, G.R., and Scarffe, J.H.: Epidemiology of an outbreak due to glycopeptide-resistant *Enterococcus faecium* on a leukaemia unit. J. Hosp. Infect. 34, 171-182 (1996).

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R.,

Brown, W.J., Cardo, D., Fridkin, S.K.; Vancomycin-Resistant Staphylococcus aureus Investigative Team: Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. N. Engl. J. Med. 348, 1342-1347 (2003).

Chenoweth, C. and Schaberg, D.: The epidemiology of enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 9, 80-89 (1990).

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., and Zervos, M.J.: Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. 37, 2474-2477 (1993).

Christie, C., Hammond, J., Reising, S., and Evans-Patterson, J.: Clinical and molecular epidemiology of enterococcal bacteremia in a pediatric teaching hospital. J. Pediatr. 125, 392-399 (1994).

Coburn, P.S., Hancock, L.E., Booth, M.C., and Gilmore, M.S.: A novel means of self-protection, unrelated to toxin activation, confers immunity to the bactericidal effects of the *Enterococcus faecalis* cytolysin. Infect. Immun. 67, 3339-3347 (1999).

Colmar, I. and Horaud, T.: *Enterococcus faecalis* hemolysin-bacteriocin plasmids belong to the same incompatibility group. Appl. Environ. Microbiol. 53, 567-570 (1987).

Coque, T.M., Patterson, J.E., Steckelberg, J.M., and Murray, B.E.: Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. 171, 1223-1229 (1995).

Coulter, S.N., Schwan, W.R., Ng, E.Y., Langhorne, M.H., Ritchie, H.D., Westbrock-Wadman, S., Hufnagle, W.O., Folger, K.R., Bayer, A.S., and Stover, C.K.: *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. Mol. Microbiol. 30, 393-404 (1998).

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penades, J.R.: Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J. Bacteriol. 183, 2888-2896 (2001).

Dunny, G.M., Leonard, B.A., and Hedberg, P.J.: Pheromone-inducible conjugation in *Enterococcus faecalis*: Interbacterial and host-parasite chemical communication. J. Bacteriol. 177, 871-876 (1995).

Dupont, H., Montravers, P., Mohler, J., and Carbon, C.: Disparate findings on the role of virulence factors of *Enterococcus faecalis* in mouse and rat models of peritonitis. Infect. Immun. 66, 2570-2575 (1998).

Eaton, T.J. and Gasson, M.J.: Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. 67, 1628-1635 (2001).

Eaton, T.J. and Gasson, M.J.: A variant enterococcal surface protein Esp(fm) in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiol. Lett. 216, 269-275 (2002).

Elizaga, M.L., Weinstein, R.A., and Hayden, M.K.: Patients in long-term care facilities: a reservoir for vancomycin-resistant enterococci. Clin. Infect. Dis. 34, 441-446 (2002).

Elliott, S.: Group and type-specific polysaccharides of group D streptococci. Nature 184, 1342 (1959).

Elliott, S.: Type and group polysaccharides of group D streptococci. J. Exp. Med. 11, 621-630 (1960).

Elliott, S.: Teichoic acids and the group antigen of group D streptococci. Nature 193, 1105-1106 (1962).

Elsner, H.A., Sobottka, I., Mack, D., Claussen, M., Laufs, R., and Wirth, R.: Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. Eur. J. Clin. Microbiol. Infect. Dis. 19, 39-42 (2000).

Fath, M.J. and Kolter, R.: ABC transporters: bacterial exporters. Microbiol. Rev. 57, 995-1017 (1993).

Franz, C.M., Holzapfel, W.H., and Stiles, M.E.: Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47, 1-24 (1999).

Gaglani, M.J., Baker, C.J., and Edwards, M.S.: Contribution of antibody to neutrophil-mediated killing of *Enterococcus faecalis*. J. Clin. Immunol. 17, 478-484 (1997).

Garsin, D.A., Sifri, C.D., Mylonakis, E., Qin, X., Singh, K.V., Murray, B.E., Calderwood, S.B., and Ausubel, F.M.: A

simple model host for identifying Gram-positive virulence factors. Proc. Natl. Acad. Sci. USA 98, 10892-10897 (2001).

Gentry-Weeks, C.R., Karkhoff-Schweizer, R., Pikis, A., Estay, M., and Keith, J.M.: Survival of *Enterococcus faecalis* in mouse peritoneal macrophages. Infect. Immun. 67, 2160-2165 (1999).

Gilmore, M.S., Segarra, R.A., and Booth, M.C.: An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. Infect. Immun. 58, 3914-3923 (1990).

Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R., and Clewell, D.B.: Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to antibiotic determinants. J. Bacteriol. 176, 7335-7344 (1994).

Gilmore, M.S., Coburn, P.S., Nallapareddy, S.R., and Murray, B.E.: Enterococcal virulence. In The enterococci: Pathogenesis, molecular biology, and antibiotic resistance (Ed.: Gilmore, M.S.). ASM Press, Washington, D.C., 301-354 (2002).

Gravekamp, C., Kasper, D.L., Paoletti, L.C., and Madoff, L.C.: Alpha C protein as a carrier for type III capsular polysaccharide and as a protective protein in group B streptococcal vaccines. Infect. Immun. 67, 2491-2496 (1999).

Gutschik, E., Moller, S., and Christensen, N.: Experimental endocarditis in rabbits. 3. Significance of the proteolytic capacity of the infecting strains of *Streptococcus faecalis*. Acta Pathol. Microbiol. Scand. [B] 87, 353-362 (1979).

Haas, W., Shepard, B.D., and Gilmore, M.S.: Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature 415, 84-87 (2002).

Hancock, L.E. and Gilmore, M.S.: Pathogenicity of *Enterococci*. In: Gram-positive pathogens (Eds.: Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A., and Rood, J.I.). ASM Press, Washington DC, 251-258 (2000).

Hancock, L.E. and Gilmore, M.S.: The capsular polysaccharide of *Enterococcus faecalis* and its relationship to other polysaccharides in the cell wall. Proc. Natl. Acad. Sci. USA 99, 1574-1579 (2002).

Harvey, B.S., Baker, C.J., and Edwards, M.S.: Contributions of complement and immunoglobulin to neutrophil-mediated killing of enterococci. Infect. Immun. 60, 3635-3640 (1992).

Hoesley, C.J. and Cobbs, C.G.: Endocarditis at the millennium. J. Infect. Dis. 179 (Suppl 2), S360-S365 (1999).

Huebner, J., Wang, Y., Krueger, W.A., Madoff, L.C., Martirosian, G., Boisot, S., Goldmann, D.A., Kasper, D.L., Tzianabos, A.O., and Pier, G.B.: Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. Infect. Immun. 67, 1213-1219 (1999).

Huebner, J., Quaas, A., Krueger, W.A., Goldmann, D.A., and Pier, G.B.: Prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide shared among vancomycin-sensitive and -resistant enterococci. Infect. Immun. 68, 4631-4636 (2000).

Husni, R., Hachem, R., Hanna, H., and Raad, I.: Risk factors for vancomycin-resistant *Enterococcus* (VRE) infection in colonized patients with cancer. Infect. Control Hosp. Epidemiol. 23, 102-103 (2002).

Huycke, M.M., Spiegel, C.A., and Gilmore, M.S.: Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. 35, 1626-1634 (1991).

Huycke, M.M. and Gilmore, M.S.: Frequency of aggregation substance and cytolysin genes among enterococcal endocarditis isolates. Plasmid 34, 152-156 (1995).

Huycke, M.M., Joyce, W.A., and Gilmore, M.S.: *Enterococcus faecalis* cytolysin without effect on the intestinal growth of susceptible enterococci in mice. J. Infect. Dis. 172, 273-276 (1995).

Huycke, M.M., Sahm, D.F., and Gilmore, M.S.: Multiple-drug resistant enterococci: The nature of the problem and an agenda for the future. Emerg. Infect. Dis. 4, 239-249 (1998).

Ike, Y., Hashimoto, H., and Clewell, D.B.: Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. 45, 528-530 (1984).

Ike, Y., Hashimoto, H., and Clewell, D.B.: High incidence of hemolysin production by

Enterococcus (*Streptococcus*) *faecalis* strains associated with human parenteral infections. J. Clin. Microbiol. 25, 1524-1528 (1987).

Ike, Y. and Clewell, D.B.: Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. J. Bacteriol. 174 8172-8177 (1992).

Jett, B.D., Jensen, H.G., Nordquist, R.E., Gilmore, and M.S.: Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. 60, 2445-2452 (1992).

Jett, B.D., Huycke, M.M., and Gilmore, M.S.: Virulence of enterococci. Clin. Microbiol. Rev. 7, 462-478 (1994).

Jett, B.D., Jensen, H.G., Atkuri, R.V., and Gilmore, M.S.: Evaluation of therapeutic measures for treating endophthalmitis caused by isogenic toxin-producing and toxin-nonproducing *Enterococcus faecalis* strains. Invest. Ophthalmol. Vis. Sci. 36, 9-15 (1995).

Johnson, A.P.: The pathogenicity of enterococci. J. Antimicrob. Chemother. 33, 1083-1089 (1994).

Jones, R.N., Marshall, S.A., Pfaller, M.A., Wilke, W.W., Hollis, R.J., Erwin, M.E., Edmond, M.B., and Wenzel, R.P.: Nosocomial enterococcal blood stream infections in the SCOPE Program: Antimicrobial resistance, species occurrence, molecular testing results, and laboratory testing accuracy. SCOPE Hospital Study Group. Diagn. Microbiol. Infect. Dis. 29, 95-102 (1997).

Karchmer, A.W.: Infective Endocarditis. In: Heart disease: A textbook of cardiovascular medicine (Ed.: Braunwald, E.). Saunders, Philadelphia, 1723-1745 (2001).

Keller, M.A. and Stiehm, E.R.: Passive immunity in prevention and treatment of infectious diseases. Clin. Microbiol. Rev. 13, 602-614 (2000).

Krause, R.: The antigens of group D streptococci. In: Streptococci and streptococcal diseases (Eds.: Wannamaker, L. and Matsen, J.). Academic Press, New York, 67-74 (1972).

Kreft, B., Marre, R., Schramm, U., and Wirth, R.: Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. Infect. Immun. 60, 25-30 (1992).

Landry, S.L., Kaiser, D.L., and Wenzel, R.P.: Hospital stay and mortality attributed to nosocomial enterococcal bacteremia: A controlled study. Am. J. Infect. Control 17, 323-329 (1989).

Lesinski, G.B. and Westerink, M.A.: Vaccines against polysaccharide antigens. Curr. Drug Targets Infect. Disord. 1, 325-334 (2001).

Linton, K.J. and Higgins, C.F.: The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. 28, 5-13 (1998).

Lowe, A.M., Lambert, P.A., and Smith, A.W.: Cloning of an *Enterococcus faecalis* endocarditis antigen: Homology with adhesins from some oral streptococci. Infect. Immun. 63, 703-706 (1995).

Lowe, A.M., Beattie, D.T., and Deresiewicz, R.L.: Identification of novel staphylococcal virulence genes by *in vivo* expression technology. Mol. Microbiol. 27, 967-976 (1998).

Lund, B., Agvald-Ohman, C., Hultberg, A., and Edlund, C.: Frequent transmission of enterococcal strains between mechanically ventilated patients treated at an intensive care unit. J. Clin. Microbiol. 40, 2084-2088 (2002).

Maekawa, S., Yoshioka, M., and Kumamoto, Y.: Proposal of a new scheme for the serological typing of *Enterococcus faecalis* strains. Microbiol. Immunol. 36, 671-681 (1992).

Maekawa, S. and Habadera, S.: Comparative distribution of the serotypes of *Enterococcus faecalis* isolated from the urine of patients with urinary tract infections and the feces of healthy persons as determined by the slide agglutination reaction. Kansenshogaku Zasshi 70, 168-174 (1996).

Makinen, P.L., Clewell, D.B., An, F., and Makinen, K.K.: Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from *Streptococcus faecalis* (strain 0G1-10). J. Biol. Chem. 264, 3325-3334 (1989).

Mei, J.M., Nourbakhsh, F., Ford, C.W., and Holden, D.W.: Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. Mol. Microbiol. 26, 399-407 (1997).

Michel, J.L., Madoff, L.C., Olson, K., Kling,

D.E., Kasper, D.L., and Ausubel, F.M.: Large, identical, tandem repeating units in the C protein alpha antigen gene, bca, of group B streptococci. Proc. Natl. Acad. Sci. USA 89, 10060-10064 (1992).

Mitra, A.K. and Rabbani, G.H.: A double-blind, controlled trial of bioflorin (*Streptococcus faecium* SF68) in adults with acute diarrhea due to *Vibrio cholerae* and enterotoxigenic *Escherichia coli*. Gastroenterology 99, 1149-1152 (1990).

Miyazaki, S., Ohno, A., Kobayashi, I., Uji, T., Yamaguchi, K., and Goto, S.: Cytotoxic effect of hemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. Microbiol. Immunol. 37, 265-270 (1993).

Murray, B.E.: Vancomycin-resistant enterococcal infections. N. Engl. J. Med. 342, 710-721 (2000).

Nakayama, J., Cao, Y., Horii, T., Sakuda, S., Akkermans, A.D., de Vos, W.M., and Nagasawa, H.: Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in *Enterococcus faecalis*. Mol. Microbiol. 41, 145-154 (2001a).

Nakayama, J., Cao, Y., Horii, T., Sakuda, S., and Nagasawa, H.: Chemical synthesis and biological activity of the gelatinase biosynthesis-activating pheromone of *Enterococcus faecalis* and its analogs. Biosci. Biotechnol. Biochem. 65, 2322-2325 (2001b).

Nallapareddy, S.R., Qin, X., Weinstock, G.M., Hook, M., and Murray, B.E.: *Enterococcus faecalis* adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. Infect. Immun. 68, 5218-5224 (2000).

Noble, C.J.: Carriage of group D streptococci in the human bowel. J. Clin. Pathol. 31, 1182-1186 (1978).

Olmsted, S.B., Dunny, G.M., Erlandsen, S.L., and Wells, C.L.: A plasmid-encoded surface protein on *Enterococcus faecalis* augments its internalization by cultured intestinal epithelial cells. J. Infect. Dis. 170, 1549-1556 (1994).

Pai, M.P., Rodvold, K.A., Schreckenberger, P.C., Gonzales, R.D., Petrolatti, J.M., and Quinn, J.P.: Risk factors associated with the development of infection with linezolid- and vancomycin-resistant *Enterococcus faecium*. Clin. Infect. Dis. 35, 1269-1272 (2002).

Papanicolaou, G.A., Meyers, B.R., Meyers, J., Mendelson, M.H., Lou, W., Emre, S., Sheiner, P., and Miller, C.: Nosocomial infections with vancomycin-resistant *Enterococcus faecium* in liver transplant recipients: risk factors for acquisition and mortality. Clin. Infect. Dis. 23, 760-766 (1996).

Portillo, A., Ruiz-Larrea, F., Zarazaga, M., Alonso, A., Martinez, J.L., and Torres, C.: Macrolide resistance genes in *Enterococcus* spp. Antimicrob. Agents Chemother. 44, 967-971 (2000).

Porwancher, R., Sheth, A., Remphrey, S., Taylor, E., Hinkle, C., and Zervos, M.: Epidemiological study of hospital-acquired infection with vancomycin- resistant *Enterococcus faecium*: Possible transmission by an electronic ear-probe thermometer. Infect. Control Hosp. Epidemiol. 18, 771-773 (1997).

Qin, X., Singh, K.V., Weinstock, G.M., and Murray, B.E.: Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. Infect. Immun. 68, 2579-2586 (2000).

Qin, X., Singh, K.V., Weinstock, G.M., and Murray, B.E.: Characterization of fsr, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. J. Bacteriol. 183, 3372-3382 (2001).

Quentin, Y., Fichant, G., and Denizot, F.: Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. J. Mol. Biol. 287, 467-484 (1999).

Rakita, R.M., Vanek, N.N., Jacques-Palaz, K., Mee, M., Mariscalco, M.M., Dunny, G.M., Snuggs, M., Van Winkle, W.B., and Simon, S.I.: *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. Infect. Immun. 67, 6067-6075 (1999).

Rich, R.L., Kreikemeyer, B., Owens, R.T., LaBrenz, S., Narayana, S.V., Weinstock, G.M., Murray, B.E., Hook, and M.: Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. J. Biol. Chem. 274, 26939-26945 (1999).

Richards, M.J., Edwards, J.R., Culver, D.H., and Gaynes, R.P.: Nosocomial infections in medical intensive care units in the United

States. National Nosocomial Infections Surveillance System. Crit. Care Med. 27, 887-892 (1999).

Richards, M.J., Edwards, J.R., Culver, D.H., and Gaynes, R.P.: Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect. Control Hosp. Epidemiol. 21, 510-515 (2000).

Safdar, N. and Maki, D.G.: The commonality of risk factors for nosocomial colonization and infection with antimicrobial-resistant *Staphylococcus aureus, Enterococcus*, gram-negative bacilli, *Clostridium difficile*, and *Candida*. Ann. Intern. Med. 136, 834-844 (2002).

Sannomiya, P., Craig, R.A., Clewell, D.B., Suzuki, A., Fujino, M., Till, G.O., and Marasco, W.A.: Characterization of a class of nonformylated *Enterococcus faecalis*- derived neutrophil chemotactic peptides: The sex pheromones. Proc. Natl. Acad. Sci. USA 87, 66-70 (1990).

Sartingen, S., Rozdzinski, E., Muscholl-Silberhorn, A., and Marre, R.: Aggregation substance increases adherence and internalization, but not translocation, of *Enterococcus faecalis* through different intestinal epithelial cells *in vitro*. Infect. Immun. 68, 6044-6047 (2000).

Schlievert, P.M., Gahr, P.J., Assimacopoulos, A.P., Dinges, M.M., Stoehr, J.A., Harmala, J.W., Hirt, H., and Dunny, G.M.: Aggregation and binding substances enhance pathogenicity in rabbit models of *Enterococcus faecalis* endocarditis. Infect. Immun. 66, 218-223 (1998).

Segarra, R.A., Booth, M.C., Morales, D.A., Huycke, M.M., Gilmore, and M.S.: Molecular characterization of the *Enterococcus faecalis* cytolysin activator. Infect. Immun. 59, 1239-1246 (1991).

Shankar, V., Baghdayan, A.S., Huycke, M.M., Lindahl, G., and Gilmore, M.S.: Infection-derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. Infect. Immun. 67, 193-200 (1999).

Shankar, N., Lockatell, C.V., Baghdayan, A.S., Drachenberg, C., Gilmore, M.S., and Johnson, D.E.: Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. Infect. Immun. 69, 4366-4372 (2001).

Sharpe, M.: Serological types of *Streptococcus faecalis* and its varieties and their cell wall type antigen. J. Gen. Microbiol. 36, 151-160 (1964).

Singh, K.V., Coque, T.M., Weinstock, G.M., and Murray, B.E.: *In vivo* testing of an *Enterococcus faecalis* efaA mutant and use of efaA homologs for species identification. FEMS Immunol. Med. Microbiol. 21, 323-331 (1998a).

Singh, K.V., Qin, X., Weinstock, G.M., and Murray, B.E.: Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. J. Infect. Dis. 178, 1416-1420 (1998b).

Singh, K.V., Malathum, K., and Murray, B.E.: Disruption of an *Enterococcus faecium* species-specific gene, a homologue of acquired macrolide resistance genes of staphylococci, is associated with an increase in macrolide susceptibility. Antimicrob. Agents Chemother. 45, 263-266 (2001).

Stalhammar-Carlemalm, M., Areschoug, T., Larsson, C., and Lindahl, G.: The R28 protein of *Streptococcus pyogenes* is related to several group B streptococcal surface proteins, confers protective immunity and promotes binding to human epithelial cells. Mol. Microbiol. 33, 208-219 (1999).

Su, Y.A., Sulavik, M.C., He, P., Makinen, K.K., Makinen, P.L., Fiedler, S., Wirth, R., and Clewell, D.B.: Nucleotide sequence of the gelatinase gene (gelE) from *Enterococcus faecalis* subsp. *liquefaciens*. Infect. Immun. 59, 415-420 (1991).

Suntharam, N., Lankford, M.G., Trick, W.E., Peterson, L.R., Noskin, and G.A.: Risk factors for acquisition of vancomycin-resistant enterococci among hematology-oncology patients. Diagn. Microbiol. Infect. Dis. 43, 183-188 (2002).

Sussmuth, S.D., Muscholl-Silberhorn, A., Wirth, R., Susa, M., Marre, R., and Rozdzinski, E.: Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. Infect. Immun. 68, 4900-4906 (2000).

Takeda, K.: Immunisatorische Einteilung der Enterokokken. Z. Immunolog. Forschung 86, 341 (1935).

Timmers, G.J., van der Zwet, W.C., Simoons-Smit, I.M., Savelkoul, P.H., Meester, H.H., Vandenbroucke-Grauls, C.M., and

Huijgens, P.C.: Outbreak of vancomycin-resistant *Enterococcus faecium* in a haematology unit: risk factor assessment and successful control of the epidemic. Br. J. Haematol. 116, 826-833 (2002).

Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penades, J.R., and Lasa, I.: The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. Appl. Environ. Microbiol. 67, 4538-4545 (2001).

Vanek, N.N., Simon, S.I., Jacques-Palaz, K., Mariscalco, M.M., Dunny, G.M., and Rakita, R.M.: *Enterococcus faecalis* aggregation substance promotes opsonin-independent binding to human neutrophils via a complement receptor type 3mediated mechanism. FEMS Immunol. Med. Microbiol. 26, 49-60 (1999).

Vergis, E.N., Shankar, N., Chow, J.W., Hayden, M.K., Snydman, D.R., Zervos, M.J., Linden, P.K., Wagener, M.M., and Muder, R.R.: Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis*. Clin. Infect. Dis. 35, 570-575 (2002).

Waar, K., Muscholl-Silberhorn, A.B., Willems, R.J., Slooff, M.J., Harmsen, H.J., and Degener, J.E.: Genogrouping and incidence of virulence factors of *Enterococcus faecalis* in liver transplant patients differ from blood culture and fecal isolates. J. Infect. Dis. 185, 1121-1127 (2002).

Wang, Y., Huebner, J., Tzianabos, A.O., Martirosian, G., Kasper, D.L., and Pier, G.B.: Structure of an antigenic teichoic acid shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. Carbohydr. Res. 316, 155-160 (1999).

Wastfelt, M., Stalhammar-Carlemalm, M., Delisse, A.M., Cabezon, T., and Lindahl, G.: Identification of a family of streptococcal surface proteins with extremely repetitive structure. J. Biol. Chem. 271, 18892-18897 (1996).

Wells, C.L., Maddaus, M.A., and Simmons, R.L.: Proposed mechanisms for the translocation of intestinal bacteria. Rev. Infect. Dis. 10, 958-979 (1988).

Wells, C.L., Jechorek, R.P., and Erlandsen, S.L.: Evidence for the translocation of *Enterococcus faecalis* across the mouse intestinal tract. J. Infect. Dis. 162, 82-90 (1990).

Wells, C.L. and Erlandsen, S.L.: Localization of translocating *Escherichia coli, Proteus mirabilis*, and *Enterococcus faecalis* within cecal and colonic tissues of monoassociated mice. Infect. Immun. 59, 4693-4697 (1991).

Wells, C.L., Jechorek, R.P., and Gillingham, K.J.: Relative contributions of host and microbial factors in bacterial translocation. Arch. Surg. 126, 247-252 (1991).

Wells, C.L., Moore, E.A., Hoag, J.A., Hirt, H., Dunny, G.M., and Erlandsen, S.L.: Inducible expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalization by cultured enterocytes. Infect. Immun. 68, 7190-7194 (2000).

Willems, R.J., Homan, W., Top, J., van Santen-Verheuvel, M., Tribe, D., Manzioros, X., Gaillard, C., Vandenbroucke-Grauls, C.M., Mascini, E.M., van Kregten, E., van Embden, J.D., and Bonten, M.J.: Variant esp gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet 357, 853-855 (2001).

Xu, Y., Jiang, L., Murray, B.E., and Weinstock, G.M.: *Enterococcus faecalis* antigens in human infections. Infect. Immun. 65, 4207-4215 (1997).

Xu, Y., Murray, B.E., and Weinstock, G.M.: A cluster of genes involved in polysaccharide biosynthesis from *Enterococcus faecalis* OG1RF. Infect. Immun. 66, 4313-4323 (1998).

Xu, Y., Singh, K.V., Qin, X., Murray, B.E., and Weinstock, G.M.: Analysis of a gene cluster of *Enterococcus faecalis* involved in polysaccharide biosynthesis. Infect. Immun. 68, 815-823 (2000).

PSEUDOMONAS IMMUNOTHERAPY: A HISTORICAL OVERVIEW*

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SUMMARY

The historic development of vaccines to be used as immunotherapy for *Pseudomonas aeruginosa* infections, in various patient populations, is reviewed. Commentary is offered concerning the relevance of each approach in light of our current understanding of the pathological process of these infections.

INTRODUCTION

With the widespread use of penicillin and other antibiotics to control Gram-positive organisms in the 1950s, there was a shift in the type of microorganisms causing severe infections in a variety of patient populations. At this time the Gram-negative bacteria, particularly *Pseudomonas aeruginosa*, emerged as the greatest infectious threat to hospitalised patients. Although the introduction of new antibiotics with anti-pseudomonal activity showed initial promise in the control of *Pseudomonas*, the innate capacity of this organism to become resistant to newly introduced antibiotics soon became a problem in treating these infections. Thus, alternative means were sought to treat and prevent *Pseudomonas* infections. Extensive research studies on the mechanisms of

Table 1: *Pseudomonas* virulence associated and cellular factors used or suggested as antigens for immunotherapy

Lipopolysaccharide
Exotoxin A
Ribosome
Flagella
Pili
High-molecular-weight polysaccharides
Alginate/mucoid exopolysaccharide
Outer membrane proteins
Multicomponent/conjugate
DNA
Type III secretion/intoxication proteins

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pathogenesis of *P. aeruginosa* infections led to the discovery of a variety of virulence-associated factors that lent themselves to the possibility of an immunologic approach to the prevention and treatment of *Pseudomonas* infections. Some of the factors that have been tested in this regard are listed in Table 1.

Although many factors are listed, they may not all be equally effective as universal immunogens. Data have accumulated that some virulence factors may be associated with certain infection processes but not others. Proteases seem to be important virulent factors in patients with cystic fibrosis, whereas exotoxin A is not. Others have compared virulence products produced by *P. aeruginosa* isolated from patients with different site infections and suggest from their results that "(1) elastase, phospholipase C, toxin A and exo-enzyme S are produced by *P. aeruginosa* isolates from different sites of infection; (2) the production of higher levels of elastase and phospholipase C is important in all types of infections, while the production of toxin A and exo-enzyme S is important in wound infections; (3) persistent infection with *P. aeruginosa* may enhance exo-enzyme S production." Thus, neutralisation of any onespecific virulence factor by immunologic or other means may not be successful in preventing, eliminating, or improving morbidity or mortality in *P. aeruginosa* infections in all of the forms they are seen in patients. This caveat should be kept in mind when reading this review. Since the literature on immunologic approaches to control *Pseudomonas* infections is vast, this review will give only an abbreviated overview of a variety of approaches that have been attempted to prepare *Pseudomonas* vaccines over the years.

REVIEW

In the 1960s, when *P. aeruginosa* had started to replace Gram-positive cocci as the organism causing the most mortality from sepsis, a variety of immunologic approaches to the prevention and treatment of these infections was attempted. Because of the lack of knowledge of the virulence factors associated with this organism and a lack of understanding of its mechanisms of pathogenesis, most of these attempts at immunotherapy relied on cell wall components (lipopolysaccharides; LPS) as antigens. It was believed that the generation of opsonising antibody against the infecting strain would clear the bacteria from the host, thereby aborting the infection. As knowledge of the various 0 serotypes of *P. aeruginosa* became better known, multivalent LPS vaccines were developed (*Feller* et al., 1964; *Millican* et al., 1966; *Alms* and *Bass*, 1967; *Alexander* et al., 1971; *Haghbin* et al., 1973; *Young* et al., 1973; *Alexander* and *Fisher*, 1974; *Pennington* et al., 1975; *Miler* et al., 1977; *Jones* et al., 1980; *Pennington* and *Pier*, 1983). These were tested not only in animal models [1-3,9,11] and patients, especially burned patients (*Alexander* et al., 1971; *Alexander* and *Fisher*, 1974; *Jones* et al., 1980), where the incidence of lethal *P. aeruginosa* infection was very high but also in patients with various forms of cancer (*Young* et al., 1973; *Haghbin* et al., 1973; *Pennington* et al., 1975) and acute and chronic lung disease (*Pennington* et al., 1975) (Table 2). Although research and testing of these types of vaccines went on for at least two decades and in spite of positive results in animal and patient testing, especially in burns, these vaccines, because of the LPS nature and the potential

problems involved with LPS, never gained clinical acceptance.

Early investigations into virulence factors associated with *P. aeruginosa* infections described an ADP-ribosylating toxic substance that was designated exotoxin A. Some studies of *Pseudomonas* infection suggested that animals infected with *P. aeruginosa* would die a "toxic" death even in the face of treatments which reduced the infecting bacterial load to a significant degree. This suggested to some investigators that as an alternative to immunologic approaches that functioned to protect by reducing the microbial load, one might be able to enhance survival even in the face of ongoing infection by neutralisation of the toxic exoproduct, exotoxin A (*Pavlovskis* et al., 1977, 1981; *Snell* et al., 1978; *Cryz* et al., 1983) (Table 3). There were mixed results using this approach; at best, antitoxin treatment alone only increased survival time ((*Pavlovskis* et al., 1977, 1981; *Snell* et al., 1978). Only in the presence of additional treatment that simultaneously reduced microbial load did antitoxin treatment enhance long-term survival (*Snell* et al., 1978). Despite encouraging studies, mostly using burned mice, there are no clinical studies using this approach to *Pseudomonas* immunotherapy. However, exotoxin A toxoid is being used in combination with other potential protective immunogens and in multicomponent and conjugate vaccines. More about this will be described later.

For a time, ribosomes and ribosomal RNA vaccines were evaluated for their ability to enhance survival from *P. aeruginosa* infection in a variety of animal studies using unburned (*Smith* et al., 1974; *Gonggrijp* et al., 1981; *Lieberman* and *Ayala*, 1983) and burned rodents (*Lieberman* et al., 1986) (Table 4). Although some success in animal models was presented, questions about LPS contamination of ribosomal preparations cast doubt on these results, and RNA and ribosomal vaccine development fell out of favour.

When it was demonstrated that motility and attachment were associated with *P. aeruginosa* virulence, particularly in burn wound infections, many studies were directed at the study of flagella and pili as protective immunogens (*Holder* et al., 1982; *Holder* and *Naglich*, 1986; *Montie* et al., 1987; *Sato* et al., 1988; *Ochi* et al., 1991) (Table 5). Several studies showed the efficacy of flagella immunisation in a variety of burned animal studies (*Holder* et al., 1982; *Holder* and *Naglich*, 1986; *Montie* et al., 1987; *Ochi* et al., 1991). The appeal of flagella immunisation was that there are only two immunotypes of flagella in *P. aeruginosa*, thus a successful divalent vaccine could be uniformly protective (*Holder* and *Naglich*, 1986). Flagella as protective immunogens are still being investigated not only for protection against burn wound infections but also in the prevention of *P. aeruginosa* infection in cystic fibrosis patients. Pili, bacterial appendages used for attachment, have been shown to be virulence-associated factors in *P. aeruginosa* as well. Flagellated and piliated strains of *P. aeruginosa* have a 10-fold lower LD_{50} than their isogenic non-piliated mutants, and non-piliated strains lose their ability to adhere to epidermal cells *in vitro* (*Sato* et al., 1988) Antipilin serum inhibited piliated strains from adhering to these same cells, suggesting that pili, as well as flagella, were appropriate proteins to be considered as protective vaccines against *P. aeruginosa* infection. Although they have not reached points where they can be used as successful vaccines, studies on how portions of the pilin adherence binding domain and how peptides to the c-terminal receptor binding regions of four strains of *P. aeruginosa* pilin may be used as vaccines have been reported.

Table 2: Features of some early immunotherapeutic approaches to treat *Pseudomonas aeruginosa* infections using whole cells or cell wall-associated materials

Immunogen	Tested in	Active	Passive	Effect of immunisation	Reference
Heat-killed phenol preserved whole cells - one strain	Rabbits	+	+	Significant ↑ survival	*Feller* et al., 1964
Heat or formalin killed whole cells - one strain	Unburned and burned mice	+	-	Significant ↑ survival; ↓ pathologic findings in organs	*Millican* et al., 1966
Alcohol precipitate of "slime" fraction-one strain	Mice	+	+	Significant ↑ survival	*Alms* and *Bass*, 1967
LPS prepared from seven 0 serotype strains combined to make a heptovalent vaccine (Pseudogen®)†	Burned patients Burned patients	+ +	- +*	↑ survival ↑ survival	*Alexander* et al., 1971 *Alexander* and *Fisher*, 1974
Heptavalent LPS vaccine (Pseudogen®)	Cancer patients	+	-	Significant but limited ↓ in fatal infection high incidence of untoward side effects	*Young* et al., 1973
Heptavalent LPS vaccine (Pseudogen®)	Paediatric acute leukaemia patients	+	-	No control of infection observed	*Haghbin* et al. 1973
Heptavalent LPS vaccine (Pseudogen®)	Acute leukaemia and cystic fibrosis patients	+	-	Leukaemia: possibly fewer *Pseudomonas* infections; cystic fibrosis - no clinical benefit in spite of high antibody titres	*Pennington* et al., 1975
EDTA-glycine extraction of viable cells from each of 16 distinct 0 serotype strains combined to make polyvalent vaccine (later called PEV-01)	Mice Burned patients;	+ +	+ +	Significant ↑ survival ↓bacteraemia	*Miler* et al., 1977 *Jones* et al., 1980
Pseudogen and PEV-01	Acute pneumonia/ Guinea pigs			Significant protection - both vaccines	*Pennington* and *Pier*, 1983

* Given to patients who arrived or became bacteraemic within 5 days of hospital admission.
† Parke Davis and Co., Detroit, MI

Table 3: Protection studies using exotoxin A immunisation

Type of immunisation	Results	Reference
Passive	↑ Survival time (burned mice)	*Pavlovskis* et al., 1977
Passive	↑ Survival time; long term survival ↑ with additional antibiotic treatment (burned mice)	*Snell* et al., 1978
Active	↑ Survival time; lower viable bacterial counts in blood and liver (burned mice)	*Pavlovskis* et al., 1981
Passive	No effect on survival or number of bacteria found in blood, liver or skin (burned mice)	*Cryz* et al., 1983

Table 4: Protection studies using ribosomes

Immunogen (type of immunisation)	Results	Reference
Ribosomes/1 strain (active)	Significant homologous but not heterologous strain protection (mice)	*Smith* et al., 1974
Purified ribosomes prepared from crude ribosomal fractions from two strains and RNA extracted from these purified preparations (active)	Cross-protection (mice)	*Gonggrijp* et al., 1981
Ribosomal fractions one strain (active and passive)	Significant protection (C3H/HeJ and ICR mice)	*Lieberman* et al., 1983
Ribosomal fractions, two strains (active and passive	Cross protection when immunisation was prior to infection; post infection protection was time interval dependent between immunisation and infection (scald burned rats)	*Lieberman* et al., 1986

Table 5: Protection studies using flagella or pili immunisation

Immunogen (Type of Immunisation)	Results	Reference
Purified flagella (active)	Flagella antigen specific ↑ survival; uniform ↑ survival with divalent immunisation (burned mice)	*Holder* et al., 1982
Partially purified flagella (active)	Flagella antigen specific *in vitro* inhibition of motility using antiflgella	*Holder* et al., 1986
Highly purified flagella (active and passive)	Flagella antigen specific *in vitro* inhibition of motility using antiflagellar scrum; flagella antigen specific ↑ survival (burned or scalded mice)	*Montie* et al., 1987
Pili (active)	↑ Survival with challenge strain from which pili isolated (scalded rats)	*Sato* et al., 1988
Monoclonal antibody to partially purified flagella (passive)	Flagella antigen specific *in vitro* inhibition of motility; flagella antigen specific ↑ survival (burned mice)	*Ochi* et al., 1991

Table 6: Protection studies using high-molecular-weight polysaccharide (HMWP) immunisation

Valency (Type of Immunisation)	Results	Reference
Active and passive (monovalent)	↑ Protection, serotype specific (mice)	*Pier* et al., 1978
Active and passive (monovalent)	↑ Survival, homologous protection (mice)	*Pier* et al., 1981
Active and passive (divalent)	↑ Survival; some but not complete cross-protection (mice)	*Pier*, 1982
Active and passive (divalent)	Strong, serotype specific and weakly cross-reactive antibody response to active immunisation; cross protective ↑ LD_{50}; serotype specific ↑ survival with passive immunisation (mice)	*Pollack* et al., 1984
Active (monovalent)	↑ Serotype specific protection; 1000-fold more HMWP needed for protection compared to LPS (mice)	*Cryz* et al., 1984

The success or failure of these efforts remains to be seen.

In another effort to avoid some of the pitfalls of the use of LPS vaccines to prevent and treat *P. aeruginosa* infections, several investigators turned to the use of high molecular weight polysaccharides as potential vaccine candidates using both active and passive immunological procedures (Table 6). Several animal studies attest to the potential efficacy of this type of immunisation (*Pier* et al., 1978, 1981; *Pier*, 1982; *Pollack* et al., 1984; *Cryz* et al., 1984). Despite this, there does not seem to be any contemporary interest in this type of immunotherapy for the prevention and treatment of *Pseudomonas* infections.

Because of the association between mucoid *P. aeruginosa* and the pathogenesis of these infections in cystic fibrosis patients, interest was generated in using *Pseudomonas* alginate (*Woods* and *Bryan*, 1985; *Pier* et al., 1990) and mucoid exopolysaccharide (*Pier* et al., 1990, 1994; *Johansen* et al., 1994) as immunogens to prevent and treat *P. aeruginosa* infections in this patient population (Table 7). Despite some encouraging results in animal studies, the clinical application of these vaccines has not been realised.

For at least two decades there has been considerable interest in the use of a variety of outer membrane proteins as immunogens for the prevention of *P. aeruginosa* infections (*Gilleland* et al., 1984; *Hancock* et al., 1985; *Matthews-Greer* and *Gilleland*, 1987; *von Specht* et al., 1995,1996a,b; *Finke* et al., 1990; *Fox* et al., 1994; *Hughes and Gilleland*, 1995; *Mansouri* et al., 1999; *Lee* et al., 1999,2000; *Knapp* et al., 1999; *Jang* et al., 1999; *Kim* et al., 2000). Part of the reason for this is that outer membrane proteins are exposed on the *Pseudomonas* cell surface, and at least one, protein F, is conserved and antigenically related in all serotype strains. Encouraging results have been obtained in a wide variety of animal studies using either intact animal models (*Gilleland* et al., 1984; *Hancock* et al., 1985; *von Specht* et al., 1996a; *Finke* et al., 1990) or animal models that represent a number of clinically relevant circumstances: Burns (*Matthews-Greer* and *Gilleland*, 1987; *von Specht* et al., 1996a; *Jang* et al., 1999) and other immunosuppressed patient populations (*von Specht* et al., 1995; *Knapp* et al., 1999) and acute (*Fox* et al., 1994) and chronic lung disease (*Hughes and Gilleland*, 1995) (Table 8). More progress has been made in translating outer membrane protein vaccines successes in animal models to the human circumstance than any other *P. aeruginosa* virulence factor. Immunisations with outer membrane proteins have been shown to cause large, long-lived increases in antibody titre (*Lee* et al., 1999; *Mansouri* et al., 1999; *Jang* et al., 1999) and to be safe (*Kim* et al., 2000) and well tolerated (*von Specht* et al., 1996; *Mansouri* et al., 1999). Further, outer membrane proteins were shown to be generated after immunisation in burn patients (*Kim* et al., 2000). IgG prepared from these burn patients increased protection when used for passive immunisation of normal and burned mice infected with *P. aeruginosa*. Current interest in these types of vaccines continues.

From the earliest days of research into immunotherapy for *P. aeruginosa* infections, multicomponent (*Kawaharajo* and *Homa*, 1977; *Okada* et al., 1980; *Holder* and *Neely*, 1989; *Gilleland* et al., 1993; *Matsumoto* et al., 1998) or conjugate vaccines (*Tsay* and *Collins*, 1984; *Cryz* et al., 1986; *Gilleland* et al., 1993) have been developed (Table 9). The fact that development of multicomponent and conjugate vaccines continues to the present day speaks to the relevance that many investigators see in this approach. Many of the earlier

Table 7: Protection studies using alginate/mucoid exopolysaccharide immunisation

Type of Immunisation	Results	Reference
Active	↑ Antibody production; ↑ bacterial clearance associated with ↑ in alginate antibody, inconsistent results (rat agar bead, chronic lung infection model)	*Woods* and *Bryan*, 1985
Active	↑ Growth of bacteria in lung but only with immunising dose which generated opsonising antibody (rat agar bead, chronic lung infection model)	*Pier* et al., 1990
Active	Significantly fewer % of lungs infected - bacteria completely cleared; pathologic changes from acute to chronic-type inflammatory response in lungs (rat agar bead, chronic lung infection model)	*Johansen* et al., 1994
Active	Immunogenic, well tolerated; elicited long lived opsonic antibodies; mediated opsonic killing of heterologous mucoid strains (human volunteers)	*Pier* et al., 1994

Table 8: Protection studies using outer membrane protein (OMP) immunisation

Immunogen (Type of Immunisation)	Results	Reference
Protein F (active and Passive)	3-fold ↑ LD_{50} (mice)	*Gilleland* et al., 1984
Monoclonal antibody to protein F (passive [pre-infection])	2-3 fold ↑ LD_{50} (mice) 8-fold ↑ LD_{50} (burned mice)	*Hancock* et al., 1985
Proteins F and H (active)	Significant ↑ survival with protein F but not protein H immunisation (scalded mice)	*Matthews-Greer* and *Gilleland*, 1987
Proteins F, H_2 and I mixture (active)	2-26-fold ↑ LD_{50} with different challenge strains due to 30-60% variation in animals response to immunisation (mice)	*von Specht* et al., 1996a
Lipoprotein I (active)	4-5-fold ↑ LD_{50} (mice)	*Finke* et al., 1990

Table 8 (continued): Protection studies using outer membrane protein (OMP) immunisation

Immunogen (Type of Immunisation)	Results	Reference
Protein F (active)	Significant ↓ in severe pulmonary lesions - significant ↓ in lung compliance (rat agar bead, chronic lung infection model)	*Fox* et al., 1994
Recombinant F and I fusion proteins (active and passive)	Significant ↑ in LD_{50} (cyclophosphamide immunosuppressed and SCID (mice)	*von Specht* et al., 1995
Synthetic peptides of protein F (active)	Significant ↑ survival with 2/3 peptides tested - intranasal immunisation (mouse acute pneumonia model)	*Hughes* and *Gilleland*, 1995
Protein I – express in *E. coli* (active)	Immunisation well tolerated; significant ↑ in titres; long lived (human volunteers)	*von Specht* et al., 1996b
Protein F: protein I hybrid (active)	Significant ↑ in antibody; elevated antibody still measurable 6 months after last vaccination; well tolerated (human volunteers)	*Mansouri* et al., 1999
Mixed OMP (passive)	Affinity purified anti-OMP from pooled human IgG using mixed OMP; antibody purified from burn patient sera, as well. Both antisera enhanced opsonic phagocytosis of *P. aeruginosa*, *in vitro*. Passive administration of IgG ↑ LD_{50} in mice-IP challenge	*Lee* et al., 1999
Mixed OMPs (active [humans], passive [mice])	Phase I/IIa clinical trial in healthy male volunteers and significant ↑ in OMP-specific antibody; higher in IM than SC immunised; ↑ protection in normal and burned mice	*Jang* et al., 1999
Protein F: protein I hybrid expressed in *E. coli* (active and passive)	↑ Protection by both active and passive immunisation (SCID mice—IP challenge)	*Knapp* et al., 1999
Mixed OMPs (active [humans], passive [mice])	Active immunisation in burn patients; serum from immunised patients showed high opsonophagocytic activity; ↑ survival in mice - IP challenge	*Lee* et al., 2000
Mixed OMPs (burned patients)	Tested immunisation schedules found 1.0 mg doses at 3-day intervals safe and effective in conferring protection against *P. aeruginosa* bacteraemia	*Kim* et al., 2000

Table 9: Protection studies using multicomponent/conjugate preparation immunisation

Immunogen (type of Immunisation)	Results	Reference
OEP: toxoids of elastase and protease; alone and in combination (active)	↑ Survival; combined immunisation no better than single component immunisation (tail-burned mice)	*Kawaharajo* and *Homa*, 1977
OEP: elastase, protease and exotoxin A toxoids (active)	↑ Survival; challenge strain dependent (burned mice)	*Okada* et al., 1980
Low molecular weight polysaccharide: albumin (active and passive)	↑ Survival with homologous strain infection (burned mice)	*Tsay* and *Collins*, 1984
Monovalent high molecular weight: polysaccharide: exotoxin A conjugate (active)	↑ Circulating homologous 0 serotype LPS+ exotoxin A antibody; ↑ survival (burned mice)	*Cryz* et al., 1986
Hyperimmune globulin: antitoxin: protease inhibitors (passive)	↑ Survival when used together compared to individual treatments (burned mice)	*Holder* and *Neely*, 1989
Octavalent HMWP: exotoxin A conjugate (active)	↑ Circulating IgG to exotoxin A + all serotypes contained in vaccine; ↑ protection after infection with all serotypes contained in vaccine (mice)	*Cryz* et al., 1989
Elastase: exotoxin A: outer membrane protein F (active)	Reduced severe pulmonary lesions–no better than protein F vaccine alone (rat agar bead, chronic lung infection model)	*Gilleland* et al., 1993
Alginate: toxin A conjugate (acute)	Significantly fewer % of lungs infected—bacteria completely cleared; pathologic changes from acute to chronic-type inflammatory response in lungs (rat agar bead, chronic lung infection model)	*Johansen* et al., 1994
Octavalent 0 polysaccharide: toxin A conjugate (active)	High titre antibody response associated with lower incidence of infection (six year follow-up in non Pseudomonas colonised CF patients)	*Cryz* et al., 1997
Toxoids of elastase: alkaline protease: exotoxin A (active)	No protection when used separately; ↑ survival in combination (gut derived sepsis in cyclophosphamide immunosuppressed specific pathogen-free mice)	*Matsumoto* et al., 1998

Table 10: DNA vaccine approaches to *Pseudomonas aeruginosa* immunisation

DNA encoding gene (type of immunisation)	Results	Reference
Type B flagellin (active)	↑ Survival in burned *P. aeruginosa* infected mice using gene gun to immunise; no ↑ when immunisation via IM route	*Baker* et al., 1999
Outer membrane protein F (active)	↑ In opsonic activity in immune sera; ↓ in macroscopic lung lesions and bacteria in immunised mice (agar bead chronic lung infection model)	*Price* et al., 2001
Mutated exotoxin A gene producing immunologically active but non-lethal toxin (active)	Neutralisation of exotoxin A *in vitro*; significant survival in immunised (gene gun) mice challenged with 13 x LDs exotoxin A	*Denis-Mize* et al., 2000
Modified exotoxin A gene (active)	Protection of mice from intoxication with lethal dose of exotoxin A	*Shiau* et al., 2000

Table 11: Additional immunological approaches

Immunogen	Results	Reference
Pooled monoclonal antibodies directed against 0-saccharide of *Pseudomonas aeruginosa* serotype E, core saccharide of LPS from *P. aeruginosa* serotypes A, G, F, H, K, and flagellin type b (passive)	Safe and well tolerated by 8 pneumonia, 4 burns and 8 patients with both burns and pneumonia. Too few patients to assess efficacy but clinical impression of improvement	*Harrison* et al., 1997
Two epitopes from *P. aeruginosa* elastase (active)	50-70% reduction in lung histopathologic changes with one of the two peptides. Protection when lung challenged with *Burkholderia cepacia* as well as with *P. aeruginosa* (rat agar bead, chronic lung infection model)	*Sokol* et al., 2000

vaccines consisted of toxins of known *Pseudomonas* virulence factors, e.g., proteases, elastases, and exotoxin A, together with some form of cell wall-associated materials (*Kawaharajo* and *Homa*, 1977; *Johansen* et al., 1994; *Okada* et al., 1980; *Holder* and *Neely*, 1989; *Gilleland* et al., 1993; *Cryz* et al., 1997; *Matsumoto* et al., 1998). Many of these early studies were done in mice, burned mice, in particular (*Kawaharajo* and *Homa*, 1977; *Okada* et al., 1980; *Tsay* and *Collins*, 1984; *Cryz* et al., 1986; *Holder* and *Neely*, 1989). More recently, conjugate vaccines were shown to be effective in animal models of chronic lung infections (*Gilleland* et al., 1993) and gut-derived infection in immunosuppressed mice (*Matsumoto* et al., 1998). A recent clinical study follow-up has shown promising results, with high titre antibody response associated with a low incidence of infection in cystic fibrosis patients (*Cryz* et al., 1997). Current interest in this type of approach to the prevention and treatment of *P. aeruginosa* infection remains high.

In recent years a novel approach to anti-infectious agent vaccinology has been described: DNA vaccines and genetic immunisation. Genetic immunisation uses plasmids that express bacterial proteins in eucaryotic cells, and this eliminates the laborious methods of protein purification and potential for LPS contamination. In genetic immunisation, the gene for the target protein is cloned into a eucaryotic expression plasmid, usually under the control of a viral promoter such as the cytomegalovirus promoter. Cells that take up the expression plasmid produce the target protein. The predominant immune response induced depends on the target protein, the construct, the route of immunisation, and the amount of plasmid injected. Most genetic immunisation studies have been targeted toward viruses, intracellular bacterial pathogens, and pathogenic protozoa and thus have focused on stimulating strong cellular responses. However, some DNA vaccine studies have been directed to the elaboration of antibodies to protein virulence-associated factors from *P. aeruginosa* (Table 10). DNA immunisation has been shown to be effective in enhancing protection in burned mice using flagellin B as the immunogen (*Baker* et al., 1999) and in a model of chronic lung infection using outer membrane protein F as the immunogen (*Price* et al., 2001). Further, results from DNA immunisation studies using exotoxin A as the immunogen, demonstrated that antisera from the immunised mice neutralised the activity of exotoxin A, *in vitro* (*Denis-Mize* et al., 2000) and protected the animals from death by intoxication when given lethal doses of toxin (*Denis-Mize* et al., 2000; *Shiau* et al., 2000). Some difficulties in DNA vaccination derive from finding appropriate virulence factor genes to clone and choosing the best way to administer the vaccine. Results have varied according to whether DNA vaccines were delivered by gene gun or intramuscularly (*Baker* et al., 1999; *Price* et al., 2001). While this approach is intriguing and has great potential, its practical application in the clinical arena remains to be seen.

Some additional, novel immunologic approaches have been tested recently (Table 11). These include preliminary testing, in pneumonia and burn patients, of the use of combined monoclonal antibodies to a variety of *P. aeruginosa* virulence antigens (*Harrison* et al., 1997) and an animal study which showed reduced severity of experimental lung infection in animals immunised with an to epitope of *Pseudomonas* elastase (*Sokol* et al., 2000). Although the monoclonal antibodies were shown to be well tolerated and safe in a few pa-

Table 12: Type III secretion/intoxication proteins

Immunogen (Type of Immunisation)	Results	Reference
Purified translocation protein PcrV (active and passive)	Decreased lung inflammation and injury; significant ↑ survival (mouse acute lung infection model)	*Sawa* et al., 1999
Purified translocation protein PcrV (active)	Significant ↑ survival; O serotype non-specific; supplemental antitoxin treatment necessary for significant enhanced long-term survival when challenge made using very high exotoxin A generating strain (burned mice)	*Holder* et al., 2000, 2001
Anti PcrV ab (passive)	Complete survival; lethal airspace infection (mice); ↓ lung injury; bacteraemia and plasma TNF-α; significant improvement in haemodynamic parameters associated with shock (rabbit model of septic shock)	*Shime* et al., 2001
Monoclonal antibody to PcrV (passive)	Prevented sepsis and death (acute lung infection model in mice)	*Frank* et al., 2002

Table 13: Immunisation via the mucus membrane route

Immunogen used (route)	Results	Reference
Serotype specific LPS (GI tract)	↑ Survival in both burned mice and chronic lung infection models	(*Holder* et al., 1992)
Killed serotype specific whole cells (GI tract)	↑ Serotype specific survival; antibody to exotoxin A necessary for ↑ survival when challenge was with high exotoxin A producing strain (burned mice)	(*Schryvers* et al., 1987)
Live attenuated aroA deletion mutant (nasal)	↑ Protection (acute pneumonia model; mice)	(*Priebe* et al., 2003)
hybrid outer membrane protein F-1 vaccine (nasal)	Induction of IgG and IgA in sera; safe and well tolerated (human volunteers)	(*Larbig* et al., 2001)

tients with pneumonia and burns (*Harrison* et al., 1997), the full efficacy of such immunologic treatment has yet to be demonstrated in large, double-blind clinical trials.

Besides the novel immunologic approaches cited above, research in recent years has shown that the type III secretion and intoxication system is a virulence factor for *P. aeruginosa*. Type III-mediated intoxication consists of three functional sets of genes encoding secretion and chaperone proteins, proteins involved in the translocation of effectors to the cytoplasm of eucaryotic cells, and the effector toxic proteins themselves. Type III proteins have been shown to be necessary for *P. aeruginosa* virulence in mouse models, both of acute lung injury and burn wound infection. Further in both of these models, immunisation using the purified type III translocation protein, PcrV, enhanced survival when mice were challenged with lethal doses of *P. aeruginosa* (Table 12). In both the mouse-infected lung model (*Sawa* et al., 1999) and the burned mouse model (*Holder* et al., 2000, 2001), passive and active immunisation proved effective. However, in the burned mouse model, supplemental immunisation using antitoxin was necessary for full protection in PcrV immunised burned mice infected with a strain producing high amounts of exotoxin A (*Holder* et al., 2000). Additionally, passive treatment with anti-PcrV antibody improved several physiological parameters of septic shock in a study which used a *Pseudomonas* induced lung injury model in rabbits (*Shime* et al., 2001). Further, monoclonal antibody generated against PcrV protein prevented sepsis and death when used as a passive treatment for infected mice (*Frank* et al., 2002). The success of both active and passive PcrV immunisation in enhancing survival and reducing negative consequences of infection in animal models of very diverse *P. aeruginosa* infections - lung, burn wound and septic shock - suggest that further exploration of type III proteins as immunogens against *P. aeruginosa* infections in human patients is warranted.

In reviewing proposals for immunological approaches to the prevention/treatment of *P. aeruginosa* infections, one additional aspect, not related to the specific immunogens used, should be considered. Over a decade ago, it was demonstrated, and more recently substantiated, that immunological protection could be obtained by presenting *Pseudomonas* antigens to the host via mucus membranes (*Holder* et al., 1992; *Schryvers* et al., 1987; *Larbig* et al., 2001; *Priebe* et al., 2003) (Table 13). Results from the earlier studies demonstrated that feeding serotype - specific killed *P. aeruginosa* cells (*Holder* et al., 1992) or LPS (*Schryvers* et al., 1987) conferred protection in both burned mouse and chronic lung infections. Further, incorporating the protein immunogen, exotoxin A into food pellets fed to mice lead to increases in circulating anti-exotoxin IgG and IgM and the mice were protected against lethal exotoxin A challenge (*Holder* et al., 1992). Recently, nasal immunisation using an attenuated aro A deletion mutant enhanced protection in an acute pneumonia model in mice (*Priebe* et al., 2003) and the safety and immunogenicity of an outer membrane protein vaccine was demonstrated, in humans, via this route of immunisation (*Larbig* et al., 2001). Thus, it appears that *Pseudomonas* immunisation can be achieved by presenting a variety of *Pseudomonas* protective antigens to the host via its mucus membranes located in the GI tract or nasal passages. Because of that, this novel means of establishing immunological protection against various

Pseudomonas infections, apparently regardless of the immunogen used, should be investigated further.

This review gives some insight into the interest that has been engendered in immunologic approaches to the prevention and treatment of *P. aeruginosa* infections in a wide variety of clinical circumstances. The search has gone on for several decades and continues today. Some approaches have been discarded as unfruitful, e.g.: those that used cell wall components, LPS, individual exoproducts, ribosomes, high molecular muco-exopolysaccharides, alginate, etc. On the other hand, some approaches are being pursued, actively, today. For example, a flagella vaccine study has just been completed in cystic fibrosis patients and results should be available in the next several months (Gerd Doring, personal communication). Further, outer membrane protein immunisation has moved out of laboratory and animal studies into safety, efficacy and tolerance studies in human volunteers (*von Specht* et al., 1996b; *Mansouri* et al., 1999; *Jang* et al., 1999; *Kim* et al., 2000) and limited trials in some patient populations (*Lee* et al., 1999, 2000). In addition, a conjugate polysaccharide, toxin A, vaccine has shown promise in reducing *P. aeruginosa* infection in cystic fibrosis patients (*Cryz* et al., 1997). These studies should and are being pursued further.

Future development of vaccines for the prevention of *Pseudomonas* infections looks promising with clearer understanding of the role that Type III intoxication proteins play in the pathogenesis of *Pseudomonas* infections and the fact that some of these proteins may be used as immunogens for a vaccine (*Sawa* et al., 1999; *Holder* et al., 2000, 2001). Further, the new use of DNA vaccines holds great hope in the future development of immunotherapy for the prevention of *P. aeruginosa* infections in a variety of patient populations (*Baker* et al., 1999; *Denis-Mize* et al., 2000; *Shiau* et al., 2000; *Price* et al., 2001). An additional consideration for future *Pseudomonas* vaccine development would be studies on how, best, to present different immunogens to the host by the route which is the safest and provides the highest and most protective antibody titre, as well. Studies on the presentation of immunogens via the mucus membrane route should be high on this "to study" list.

In conclusion, although no *Pseudomonas* vaccine has made its way into common clinical use yet, the search continues and, currently, the goal seems more attainable.

LITERATURE

Alexander, J.W., Fisher, M.L., and MacMillan, B.G.: Immunological control of *Pseudomonas* infection in burn patients: A clinical evaluation. Arch. Surg. 102, 31-35 (1971).

Alexander, J.W. and Fisher, M.W.: Immunization against *Pseudomonas* in infection after thermal injury. J. Infect. Dis. 130, S152-S158 (1974).

Alms, T.H. and Bass, J.A.: Immunization against *Pseudomonas aeruginosa*. J. Infect. Dis. 249-256 (1967).

Baker, N.R., Conwell, J., Galloway, D.R., Kang, P.J., Neely, A.N., and Holder, I.A.: Immune response following genetic immunization with DNA encoding type B flagellin from *Pseudomonas aeruginosa*. Clin. Microbiol. Infect. 5, S40-S51 (1999).

Cryz, S.J., Furer, E., and Germanier, R.: Protection against *Pseudomonas aeruginosa* infection in a murine burn wound sepsis model by passive transfer of antitoxin A, antielastase, and antilipopolysaccharide. Infect. Immun. 39,1071-1079 (1983).

Cryz, S.J. Jr., Furer, E., and Germanier, R.: Protection against fatal *Pseudomonas aeru-*

ginosa burn wound sepsis by immunization with lipopolysaccharide and high-molecular-weight polysaccharide. Infect. Immun. 43, 795-799 (1984).

Cryz, S.J., Furer, E., Sadoff, J.C., and Germanier, R.: *Pseudomonas aeruginosa* immunotype 5 polysaccharide-toxin A conjugate vaccine. Infect. Immun. 52, 161-165 (1986).

Cryz, S.J. Jr., Sadoff, J.C., Cross, A.S., and Furer, E.: Safety and immunogenicity of a polyvalent *Pseudomonas aeruginosa* immunotype 5 0 polysaccharide-toxin A conjugate vaccine effect of a booster dose on antibody levels in humans. Infect. Immun. 56, 1829-1830 (1989).

Cryz, S.J. Jr., Lang, A., Wedgwood, J., Que, J.U., Furer, E., and Schaad, U.: Immunization of cystic fibrosis patients with a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. Behring Inst. Mitt. 98, 345-349 (1997).

Denis-Mize, K.S., Price, B.M., Baker, N.R., and Galloway, D.R.: Analysis of immunization with DNA encoding *Pseudomonas aeruginosa* exotoxin A. FEMS Immunol. Med. Microbiol. 27, 147-154 (2000).

Feller, J., Vial, A.B., Callahan, W., and Galloway, D.R.: Use of vaccine and hyperimmune serum for protection against pseudomonas septicemia. J. Trauma 4, 451-456 (1964).

Finke, M., Duchene, M., Eckhardt, A., Domdey, H., and von Specht, B.U.: Protection against experimental *Pseudomonas aeruginosa* infection by recombinant *P. aeruginosa* lipoprotein I expressed in *Escherichia coli*. Infect. Immun. 58, 2241-2244 (1990).

Fox, C.W., Campbell, G.D. Jr., Anderson, W., Zayeca, J.H., Gilleland, L.B., and Gilleland, H.E. Jr.: Preservation of pulmonary function by an outer membrane protein F vaccine. Chest 105, 1545-1550 (1994).

Frank, D.W., Vallis, A., Wiener-Kronish, J.P., Roy-Burman, A., Spack, E.G., Mullaney, B.P., Megdoud, M., Marks, J.D., Fritz, R., and Sawa, T.: Generation and characterization of a protective monoclonal antibody to *Pseudomonas aeruginosa* PcrV. J. Infect. Dis. 186, 64-73 (2002).

Gilleland, H.E., Parker, M.G., Matthews, J.M., and Berg, R.D.: Use of a purified outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine in mice. Infect. Immun. 44, 49-54 (1984).

Gilleland, H.E., Gilleland, L.B., and Fowler, M.R.: Vaccine efficacy of elastase, exotoxin A, and outer-membrane protein F in preventing chronic pulmonary infection by *Pseudomonas aeruginosa* in a rat model. J. Med. Microbiol. 38, 79-86 (1993).

Gonggrijp, R., Mullers, W.J.H.A., and van Boven, C.P.A.: Serotype non-specific protection induced by ribonucleic acid isolated from the ribosomal vaccine of *Pseudomonas aeruginosa*. Infect. Immun. 33, 178-185 (1981).

Haghbin, M., Armstrong, D., and Murphy, M.L.: Controlled prospective trial of *Pseudomonas aeruginosa* vaccine in children with acute leukemia. Cancer 32, 761-766 (1973).

Hancock, R.E.W., Muthaia, L.M., and Mouat, E.C.A.: Immunotherapeutic potential of monoclonal antibodies against *Pseudomonas aeruginosa* protein F. Eur. J. Clin. Microbiol. 4, 224-227 (1985).

Harrison, F., Rohm, D., Kohzuki, T., and Noguchi, H.: Pharmacokinetics, tolerability, and preliminary efficacy of human anti *Pseudomonas aeruginosa* monoclonal antibodies in pneumonia and burn infection patients. Hybridoma 16, 413-420 (1997).

Holder, I.A., Wheeler, R., and Montie, T.C.: Flagella preparations from *Pseudomonas aeruginosa* infections in burned mice: Experimental results and theoretic consideration. Infect. Immun. 35, 276-280 (1982).

Holder, I.A. and Naglich, J.G.: Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: Immunization using divalent flagella preparations. J. Trauma 26, 118-122 (1986).

Holder, I.A. and Neely, A.N.: Combined host and specific anti-pseudomonas directed therapy for *Pseudomonas aeruginosa* infections in burned mice: Experimental results and theoretic consideration. J. Burn Care Rehabil. 10, 131-137 (1989).

Holder, I.A., Orloff, M., and Neely, A.N.: Oral *Pseudomonas aeruginosa* immunization enhances survival in mice subsequently burned and infected with *P. aeruginosa*. Int. J. Antimicrob. Agents 1, 245-252 (1992).

Holder, I.A., Neely, A.N., and Frank, D.W.: Pcrv immunization protects burned mice from *P. aeruginosa* infection. Abstract,

100th Annual Meeting of the American Society for Microbiology, Los Angeles: page 279 (2000).

Holder, I.A., Neely, A.N., and Frank, D. Type III secretion/intoxication system important in virulence of *P. aeruginosa* infections in burns. Burns 27:129-130 (2001)

Hughes, E.E. and Gilleland, H.E. Jr.: Ability of synthetic peptides representing epitopes of outer membrane protein F of *Pseudomonas aeruginosa* to afford protection against *P. aeruginosa* infection in a murine acute pneumonia model. Vaccine 17, 158-168 (1995).

Jang, I.-J., Kim, I.-S., Park, W.J., Yoo, K.S., Yim, D.S., Kim, H.K., Shin, S.G., Chang, W.H., Lee, N.G., Jung, S.B., Ahn, D.H., Cho, Y.J., Ahn, B.Y., Lee, Y., Kim, Y.G., Nam, S.W., and Kim, H.S.: Human immune response to a *Pseudomonas aeruginosa* outer membrane protein vaccine. Vaccine 17, 158-68 (1999).

Johansen, H.K., Espersen, F., Cryz, S.J. Jr., Hougen, H.P., Fomsgaard, A., Rygaard, J., and Hoiby, N.: Immunization with *Pseudomonas aeruginosa* vaccines and adjuvant can modulate the type of inflammatory response subsequent to infection. Infect. Immun. 62, 3146-3155 (1994).

Jones, R.J., Roe, E.A., and Gupta, J.L.: Controlled trial of *Pseudomonas* immunoglobulin and vaccine in burn patients. Lancet 2, 1263-1265 (1980).

Kawaharajo, K. and Homa, J.Y.: Effects of elastase, protease and common antigen (OEP) from *Pseudomonas aeruginosa* on protection against burns in mice. Japan J. Exp. Med. 47, 495-500 (1977).

Kim, D.K., Kim, J.J., Kim, J.H., Woo, Y.M., Kim, S., Yoon, D.W., Choi, C.S., Kim, I., Park, W.J., Lee, N., Jung, S.B., Ahn, B.Y., Nam, S.W., Yoon, S.M., and Choi, W.J.: Comparison of two immunization schedules for a *Pseudomonas aeruginosa* outer membrane proteins vaccine in burn patients. Vaccine 19, 1274-1283 (2000).

Knapp, B., Hundt, E., Lenz, U., Hungerer, K.D., Gabelsberger, J., Domdey, H., Mansouri, E., Li, Y., and von Specht, B.U.: A recombinant hybrid outer membrane protein for vaccination against *Pseudomonas aeruginosa*. Vaccine 17, 1663-1666 (1999).

Larbig, M., Mansouri, E., Freihorst, J., Tummler, B., Kohler, G., Domdey, H., Knapp, B., Hungerer, K.D., Hundt, E., Gabelsberger, J., and von Specht, B.U.: Safety and immunogenicity of an internasal *Pseudomonas aeruginosa* hybrid outer membrane protein F-1 vaccine in human volunteers. Vaccine 19, 2291-2297 (2001).

Lee, N.-G., Ahn, B.-Y., Jung, S.B., Kim, Y.G., Lee, Y., Kim, H.S., and Park, W.J.: Human anti *Pseudomonas aeruginosa* outer membrane proteins Igg cross-protective against infection with heterologous immunotype strains of *P. aeruginosa.* FEMS Immunol. Med. Microbiol. 25, 330-347 (1999).

Lee, N.G., Jung, S.B., Ahn, B.Y., Kim, Y.H., Kim, J.J., Kim, D.K., Kim, I.S., Yoon, S.M., Nam, S.W., Kim, H.S., and Park, W.J.: Immunization of burn-patients with a *Pseudomonas aeruginosa* outer membrane protein vaccine elicits antibodies with protective efficacy. Vaccine 18, 1952-1961 (2000).

Lieberman, M.M. and Ayala, E.: Active and passive immunity against *Pseudomonas aeruginosa* with a ribosomal vaccine and antiserum in C3H/Hej mice. J. Immunol. 131, 1-3 (1983).

Lieberman, M.M., Walker, H.L., Ayala, E., and Chapa, L.: Active and passive immunization with *Pseudomonas aeruginosa* ribosomal vaccines and antisera in the burned rat model. J. Surg. Res. 40, 138-144 (1986).

Mansouri, E., Gabelsberger, J., Knapp, B., Hundt, E., Lenz, U., Hungerer, K.D., Gilleland, H.E. Jr., Staczek, J., Domdey, H., and von Specht, B.U.: Safety and immunogenicity of a *Pseudomonas aeruginosa* hybrid outer membrane protein F-I vaccine in human volunteers. Infect. Immun. 67, 1461-1470 (1999).

Matsumoto, T., Tateda, K., Furuya, N., Miyazaki, S., Ohno, A., Ishii, Y., Hirakata, Y., and Yamaguchi, K.: Efficacies of alkaline protease, elastase and exotoxin A toxoid vaccines against gut-derived *Pseudomonas aeruginosa* sepsis in mice. J. Med. Microbiol. 47, 303-308 (1998).

Matthews-Greer, J.M. and Gilleland, H.E. Jr.: Outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine against heterologous immunotype strains in a burned mouse model. J. Infect. Dis. 155, 1282-1291 (1987).

Miler, J.M., Spilsbury, J.F., Jones, R.J., Roe, E.A., and Lowbury, E.J.L.: A new polyvalent *Pseudomonas* vaccine. J. Med. Microbiol. 10,19-27 (1977).

Millican, R.C., Evans, G., and Markley, K.: Susceptibility of burned mice to *Pseudomonas aeruginosa* and a protection by vaccination. Annals Surg. 163, 603-610 (1966).

Montie, T.C., Drake, D., Sellin, H., Slater, O., and Edmonds, S.: Virulence and protection with a flagella vaccine against *Pseudomonas aeruginosa* infection. In: Basic Research and Clinical Aspects of *Pseudomonas aeruginosa* (Eds.: Doring, G., Holder, I.A., and Botzenhart, K.). Karger, Basel, 233-248 (1987).

Ochi, H., Ohtsuka, H., Yokota, S.L., Uezumi, I., Terashima, M., Irie, K., and Noguchi, H.: Inhibitory activity on bacterial motility and i*n vivo* protective activity of human monoclonal antibodies flagella of *Pseudomonas aeruginosa*. Infect. Immun. 59, 550-554 (1991).

Okada, K., Kawaharajo, K., Kasai, T., and Hemma, I.Y.: Effects of somatic component of *Pseudomonas aeruginosa* on protective immunity in experimental mouse burn infection. Japan J. Exp. Med. 40, 53-61 (1980).

Pavlovskis, O.R., Pollack, M., Callahan, L.T., and Iglewski, B.H.: Passive protection by antitoxin in experimental *Pseudomonas aeruginosa* burn infections. Infect. Immun. 18, 596-602 (1977).

Pavlovskis, O.R., Edman, D.C., Leppla, S.H., Wretlind, B., Lewis, L.R., and Meyer, N.A.: Protection against experimental *Pseudomonas aeruginosa* infection in mice by active immunization with exotoxin A toxoids. Infect. Immun. 32, 681-689 (1981).

Pennington, J.E., Reynolds, H., Wood, R., Robinson, R.J., and Levine, A.: Use of a *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med. 58, 629-636 (1975).

Pennington, J.E. and Pier, G.B.: Efficacy of cell wall *Pseudomonas aeruginosa* vaccines for protection against experimental pneumonia. Rev. Infect. Dis. 5 (suppl. 5), S852-S857 (1983).

Pier, G.B., Sidbery, H.F., Zolyomi, S., and Sadoff, J.C.: Isolation and characterization of a high-molecular-weight polysaccharide from the slime of *Pseudomonas aeruginosa*. Infect. Immun. 22, 908-918 (1978).

Pier, G.B., Sidberry, H..F., and Sadoff, J.C.: High-molecular-weight polysaccharide antigen from *Pseudomonas aeruginosa* immunotype 2. Infect. Immun. 34, 461-468 (1981).

Pier, G.B.: Cross-protection by *Pseudomonas aeruginosa* polysaccharides. Infect. Immun. 38, 1117-1122 (1982).

Pier, G.B., Small, G.J., and Warren, H.B.: Protection against mucoid *Pseudomonas aeruginosa* in rodent models of endobronchial infections. Science 249, 537-539 (1990).

Pier, G.B., DesJardin, D., Grout, M., Garner, C., Bennett, S.E., Pekoe, G., Fuller, S.A., Thornton, M.O., Harkonen, W.S., and Miller, H.C.: *P. aeruginosa* mucoid exopolysaccharide (alginate) vaccine. Infect. Immun. 62, 3972-3979 (1994).

Pollack, M., Pier, G.B., and Prescott, R.K.: Immunization with *Pseudomonas aeruginosa* high-molecular-weight polysaccharides prevents death from *Pseudomonas* burn infections in mice. Infect. Immun. 43, 759-760 (1984).

Price, B.M., Galloway, D.R., Baker, N.R., Gilleland, L.B., Staczek, J., and Gilleland, H.E. Jr.: Protection against *Pseudomonas aeruginosa* chronic lung infection in mice by genetic immunization against outer membrane protein F (OprF) of *P. aeruginosa*. Infect. Immun. 19, 3510-3515 (2001).

Priebe, G.P., Meluleni, G.J., Coleman, F.T., Goldberg, J.B., and Pier, G.B.: Protection against fatal *Pseudomonas aeruginosa* pneumonia in mice after nasal imminization with a live, attenuated aroA deletion mutant. Infect. Immun. 71, 1453-1461 (2003).

Sato, H., Okinaga, K., and Saito, H.: Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infections. Microbiol. Immunol. 32, 131-139 (1988).

Sawa, T., Yahr, T.L., Ohara, M., Kurahashi, K., Gropper, M.A., Wiener-Kronish, J.P., and Frank, D.W.: Active and passive immunization with the *Pseudomonas V* antigen protects against type III intoxication and lung injury. Nature Med. 5, 392-398 (1999).

Schryvers, A.B., Schollaardt, T., Woods, D.E., William, K., and Bryan, L.E.: Efficacy of oral immunization with *Pseudomonas aeruginosa* lipopolysaccharide. Serodiag. Immunother. 3, 379-392 (1987).

Shiau, J.W., Tang, T.K., Shih, Y.L., Tai, C., Sung, Y.Y., Huang, J.L., Yang, H.L.: Mice immunized with DNA encoding modified *Pseudomonas aeruginosa* exotoxin A develop protective immunity against exotoxin intoxication. Vaccine 19, 1106-1112 (2000)

Shime, N., Sawa, T., Fujimoto, J., Faure, K., Allmond, L.R., Karaca, T., Swanson, B.L., Spack, E.G., and Wiener-Kronish, J.P.: Therapeutic administration of anti-Pcrv F $(Ab')_2$ in sepsis associated with *Pseudomonas aeruginosa*. J. Immunol. 167, 5880-5886 (2001).

Smith, R.L., Wysocki, J.A., Bruun, J.N., Decourcy, S.J. Jr., Blakemore, W.S., and Mudd, S.: Efficacy of ribosomal preparations from *Pseudomonas aeruginosa* to protect against intravenous *Pseudomonas* challenge in mice. J. Reticoendothel. Soc. 15, 22-30 (1974).

Snell, K., Holder, I.A., Leppla, S.A., and Saelinger, C.D.: Role of exotoxin and protease as possible virulence factors in experimental infections with *Pseudomonas aeruginosa*. Infect. Immun. 19, 839-845 (1978).

Sokol, P.A., Kooi, C., Hodges, R.S., and Cachia, P.: Immunization with a *Pseudomonas aeruginosa* elastase peptide reduces severity of experimental lung infections due to *P. aeruginosa* or *Burkholderia cepacia*. J. Infect. Dis. 171, 1682-1692 (2000).

Tsay, G.C. and Collins, M.S.: Preparation and characterization of a nontoxic polysaccharide-protein conjugate that induces active immunity and passively protective antibody against *Pseudomonas aeruginosa* immunotype 1 in mice. Infect. Immun. 45, 217-221 (1984).

von Specht, B.U., Knapp, B., Muth, G., Broker, M., Hungerer, K.D., Diehl, K.D., Massarrat, K., Seemann, A., and Domdey, H.: Protection of immunocompromised mice against lethal infection with *Pseudomonas aeruginosa* by active or passive immunization with recombinant *P. aeruginosa* outer membrane protein F and outer membrane protein I fusion proteins. Infect. Immun. 63, 1855-1862 (1995).

von Specht, B.U., Strigl, G., Ehret, W., and Brendel, W.: Protective effect of an outer membrane vaccine *Pseudomonas aeruginosa* infection. Infection 15, 408-412 (1996a).

von Specht, B.U., Lucking, H.C., Blum, B., Schmitt, A., Hungerer, K.D., and Domdey, H.: Safety and immunogenicity of a *Pseudomonas aeruginosa* outer membrane protein I vaccine in human volunteers. Vaccine 14, 1111-1117 (1996b).

Woods, D.E. and Bryan, L.E.: Studies on the ability of alginate to act as a protective immunogen against infection with *Pseudomonas aeruginosa* infection. J. Infect. Dis. 151, 581-588 (1985).

Young, L.S., Meyer, R.D., and Armstrong, D.: *Pseudomonas aeruginosa* vaccine in cancer patients. Ann. Intern. Med. 79, 518-527 (1973).

RECOMBINANT OprF-OprI AS A VACCINE AGAINST *PSEUDOMONAS AERUGINOSA* INFECTIONS*

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SUMMARY

A vaccine against *Pseudomonas aeruginosa* based on recombinant outer membranes has been developed. After intramuscularly injection into patients with severe burns, antibodies against *P. aeruginosa* were induced. Vaccination was well tolerated. Intranasal application of the vaccine into volunteers induced specific sIgA antibodies. We conclude that the newly developed vaccine may be suitable for protection of the main risk groups *of P. aeruginosa* infections. In particular for the protection of burn patients and patients with cystic fibrosis.

INTRODUCTION

Pseudomonas aeruginosa represents a leading cause of nosocomial infections and pneumonia in hospitals (*Gallagher* and *Watanakunakorn*, 1989; *Gordon* et al., 1998; *Holder*, 1988; *Holzheimer* et al., 1990; *Pennington*, 1994) pathogen affects mainly immunocompromised patients, such as patients with large burns (*McManus* et al., 1985; *Pruitt* et al., 1984, 1998) or patients under immunosuppressive or cytostatic therapy for the prevention of organ rejection after transplantation (*Korvick* et al., 1991) or for cancer treatment (*Griffith* et al., 1989). Also compromised local defence mechanisms, such as an impaired mucociliary clearance in patients with cystic fibrosis (*Burns* et al., 2001) artificial ventilation or paraplegia can enhance the susceptibility to pulmonary *P. aeruginosa* infections. The eradication of *Pseudomonas* frequently proves difficult due to antibiotic resistance and the ability to form a biofilm in case of chronic infection (*Hanberger* et al., 1997; *Hancock*, 1986; *Hoiby* et al., 2001; *Hsueh* et al., 1998; *Srikumar* et al., 1988; *Tassios* et al., 1998).

Clearance of *P. aeruginosa* in systemic infection is mediated predominantly by antibodies of the IgG1 isotype and by complement-dependent opsonisation (*Hong* and *Ghebrehiwet*, 1992). Secretory IgA antibodies are likely to be the first line of defence (*McGhee* et al., 1999) for prevention of adherence and subsequent infection of mucosal tissues like the lung, the urogenital tract or the paranasal sinuses. A clinical vaccine against *P. aeruginosa,* therefore, should induce protective antibodies of both isotypes.

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Since the 1960s a number of experimental vaccines were developed and tested for the prevention of *P. aeruginosa* infections in burn patients. The most promising of the vaccines tested were the two vaccines based on lipopolysaccharides (LPS) as antigens (Pseudogen™, a heptavalent preparation, and PEV-01 a 16-valent vaccine). Both vaccines appeared to effectively stimulate the induction of antibodies against all O-serotype strains contained in the vaccine when tested in burn patients and lead to a reduction of mortality from *P. aeruginosa* infection in this group of patients (*Alexander* and *Fisher*, 1974; *Jones* et al., 1979; *Miller* et al., 1977). Clinical studies with Pseudogen™ vaccine were also carried out in patients with cystic fibrosis (CF), malignancies, and in intensive care patients with respiratory failure. However, both LPS vaccines did not meet the approval for routine clinical use because of their toxicity associated with their lipid A fraction. Subsequently, subunit vaccines based on purified oligosaccharides from LPS and conjugated to *P. aeruginosa* exotoxin (*Cryz* et al., 1987a,b,1988) or mucoid exopolysaccharide [alginate] (*Pier*, 1982,1985,1994,1997) or isolated flagella were shown to be less toxic and have been successfully shown to elicit antibodies in a number of volunteers and patient groups (*Cryz* et al., 1987a,b,1988; *Pier*, 1982,1985,1994, 1997). However, currently there is still no approved vaccine against *P. aeruginosa* available for routine use for which safety and efficacy have been shown in clinical trials in patients from one of the major risk groups of *P. aeruginosa* infection.

Our research over the last decade has focused on the development of a vaccine against *P. aeruginosa* based on the outer membrane proteins [OPRs] of *P. aeruginosa*. A vaccine based on OPRs may have several advantages. First, they are highly conserved and induce a cross protective immunity among all 17 known *P. aeruginosa* serotypes (*Mutharia* et al., 1982; *von Specht* et al., 1987). Second, OPRs can be produced by recombinant DNA technology free of contaminating LPS. Third, the cloned genes of OPRs would be applicable for naked DNA immunisation (*Cohen*, 1993; *Donnely* et al., 1994; *Price* et al., 2002) or could be transfected into special vectors like non-pathogenic *Salmonella* strains to induce a mucosal immune response (*Kraehenbuhl* and *Neutra*, 1992; *Toth* et al., 1994). The efficacy of OPRs as a vaccine candidate was shown by us and other research groups (*Finke* et al., 1990, 1991; *Gilleland* et al., 1984,1988; *Matthews-Greer* and *Gilleland*, 1987; *von Specht* et al., 1987,1995) in various animal models. We have cloned the major OPRs, outer membrane protein F (OprF) (*Duchêne* et al., 1988) and outer membrane protein I (OprI) (*Duchêne* et al., 1989). After identification of the protective epitopes on both proteins we have generated a recombinant hybrid protein consisting of the entire OprI molecule fused to the carboxy terminal sequence (aa 190-342) of OprF (*von Specht* et al., 1995). The hybrid protein comprised the presence of the main known protective epitopes (*Gabelsberger* et al., 1997; *Gilleland* et al., 1995; *Gilleland* and *Gilleland*, 1995; *Hancock* and *Wong*, 1997). A modified histidine tagged hybrid protein Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$, resulted in a synergistically enhancement of protection in an immune suppressed mouse model (*von Specht* et al., 2000). Subsequently two vaccine formulations for different clinical applications were developed. A systemic vaccine formulation aimed for protection by means of IgG isotype antibodies against systemic infections like in burn patients. It consists of Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ adsorbed to $Al(OH)_3$. The second for-

mulation was designed for protection by the induction of s-IgA antibodies against mucosal infections, like isolated lung infections in Cystic Fibrosis patients. The mucosal vaccine is based on a nasally applicable gel, produced by mixing the Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ protein with sodium dodecylsulfate (SDS) and aerosil. In this article, we summarise the data we recently generated with both vaccine types in phase 1 and phase 2 clinical trials in volunteers and selected patient groups.

METHODS

Expression of Met-Ala-$(His)_6$ $OprF_{190-342}$-$OprI_{21-83}$ protein in *E. coli* and purification

The expression and purification of Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ protein has been described in detail (*Mansouri* et al., 1999).

Vector

The recombinant vector pTrc-His-F-I, carrying the hybrid gene encoding parts of OprF and OprI from *P. aeruginosa*, was constructed as described previously (*Gabelsberger* et al., 1997). The vector was transfected into *E. coli* XL-1 Blue bacteria and the expression of Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ protein induced using standard procedures (*Ausubel* et al., 1997).

Purification

Forty grams cell wet mass was lysed by one passage through a Gaulin press at 1,200 psi. The cell extract was clarified at 48,000 x g for 90 min at 4°C and passed through a 0.45 μm filter. The crude extract was purified by affinity chromatography on a Ni-NTA superflow column. The specific eluate was concentrated by centrifugation in MACROSEP 10 units by a factor of 3. The pH of this eluate was lowered to 5.9 by adding 0.02 mol/L NaH_2PO_4 monohydrate, pH 3.0, incubated at 4° C overnight and than clarified for 10 min at 4°C and 5000 x g to precipitate the lipopolysaccharides. The pH was re-titrated to 7.0-7.2 by adding a 0.1 N NaOH solution drop wise. The neutralised protein solution was filter (0.22 μm) sterilised and stored at 4°C overnight. Finally, the purified protein was concentrated to about 1 mg/ml by ultra filtration using a stirred Amicon cell and a YM10 membrane and then extensively dialysed against sterile, pyrogen-free PBS at 6°C for 20 h.

Vaccine preparation 1 (Parenteral vaccine) (*Mansouri* et al., 1999)

Recombinant OprF-OprI was adsorbed to $Al(OH)_3$ (Alhydrogel™), Superfos, Vedbaek, Denmark and Thimerosal (Caesar & Lorenz, Hilden, Germany) was added as a preservative. A Thimerosal stock solution was prepared, using a sterile, pyrogen-free physiological saline solution. For the 1 mg/ml vaccine preparation, a dispersion of 3% [w/v] of $Al(OH)_3$ was mixed with the OprF-OprI solution and the Thimerosal stock solution to yield final concentrations of OprF-OprI: 1 mg/ml, $Al(OH)_3$: 3 mg/ml and Thimerosal: 0.05 mg/ml. $Al(OH)_3$ and the OprF-OprI solution were mixed and stirred for 30 min, and the Thimerosal solution was then added. This was followed by additional stirring for 10 min. For the 0.1 mg/ml OprF-OprI vaccine preparation, pyrogen-free physiological saline solution was added to yield final concentrations of 0.1 mg/ml OprF-OprI, 0.3 mg/ml $Al(OH)_3$ and 0.05 mg/ml Thimerosal. Aliquots of one ml were aseptically introduced into sterile pyrogen-

free glass vials, and the vials stoppered and sealed.

Vaccine Preparation 2 (*Mansouri* et al., 1997)

80 mg Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ protein were mixed with 0.54 g sodiumdodecylsulfate and 0.6 g aerosil. The emugel was stirred three times for one minute at 300 rpm in an UMC 5-stirring machine. Aliquots were aseptically introduced into sterile pyrogen-free cryovials and stored at 4°C.

Safety evaluations

The identity and purity of the Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ protein and the expression of the relevant epitopes was assessed by western blot analysis and epitope specific monoclonal antibodies as described in detail recently. After intramuscularly injection of vaccine preparation 1 and after intranasal application of vaccine 2 into rats no signs of histopathological changes were detectable (*Mansouri* et al., 1997,1999).

All volunteers and patients gave their informed written consent in accordance with institutional review board-approved protocols. As specified by the German regulations for vaccine studies, protocols concerning the preparation of the vaccine and the laboratory and animal safety testing of the vaccine were deposited at the Paul Ehrlich Institute, Langen, Germany.

VACCINATION STUDY 1: DOSE FINDING AND SAFETY STUDY IN HUMAN VOLUNTEERS (*Mansouri* et al., 1999)

Subjects and study plan

Thirty-two healthy volunteers (16 male; 16 female; >18 years of age) were randomly allotted to 4 groups. All volunteers received three consecutive injections of the vaccine into the deltoid muscle of the left arm with 20 μg (0.2 ml of 100 μg/ml), 50 μg (0.5 ml of 100 μg/ml), 100 μg (0.1 ml of 1 mg/ml) or 500 μg (0.5 ml of 1 mg/ml) OprF-OprI, respectively, at 4-week intervals and a fourth injection after six months at the same dose. All volunteers underwent a physical examination and had histories taken to rule out any conditions which would have necessitated exclusion from the study. Before, two and 14 days after each vaccination, blood samples were taken and sent to the clinical laboratory for a complete blood count and evaluation of the liver specific enzymes, creatinine and urea. Reactions to the vaccine were assessed for 3 consecutive days and documented by the volunteers. The local and systemic responses were graded on a subjective scale of 0 to 3, with the respective scores representing absent, mild, moderate and severe reactions. The vaccinees were instructed to take their temperature before and 12, 24, 48 and 72 hours after vaccination. In addition, each volunteer underwent a physical examination two days after vaccination. For the determination of OprF-and OprI-specific antibodies, venous blood samples were taken on day 0 (prior to immunisation), and two weeks after each vaccination.

Analysis of the immune response

Before and two weeks after each vaccination antibody titres against OprI, OprF and OprF-OprI were determined by ELISA. A significant increase in antibody titres within all the different dosage groups could be measured. The specificity of the antibodies against native *P. aeruginosa* OprF and OprI was confirmed by Western blotting (data not shown). Wild type OprI (6kD) and

Table 1: IgG antibody titres against OprF-OprI in volunteers vaccinated with 100 μg vaccine dose data are summarised from *Mansouri* et al., 1999

Day	Vaccinations given	IgG antibody titre [mean (SD)]
0	0	200
14	1	1218 (623)
42	2	1503 (448)
70	3	3645 (505)

OprF (33kD) were both recognised by the immune sera. Considerable differences were observed between the dosage groups and also between volunteers belonging to the same dosage group.

Statistical analysis showed that after only one vaccination a maximal response was observed for the groups which had received the 100 μg (table 1) or the 500 μg dose. No statistically significant increase of specific antibody titter was measured in these groups after the first and second revaccination. After vaccination with the 20μg OprF-OprI dose, a significant antibody response was measured only after revaccination. Six months after the third vaccination the antibody titres against OprF-OprI were still significantly elevated in all groups. A further booster vaccination after 6 month induced a 3-10 fold increase of the specific antibody titres (Table 1).

Systemic protection against *P. aeruginosa* is mediated in humans predominantly by specific IgG1 antibody and both antibody-mediated and complement-mediated phagocytosis (*Hong* and *Ghebrehiwet*, 1992). To address the question whether the vaccine would be protective in patients, IgG subclasses of antibodies against OprF-OprI were determined. In all groups a significant increase in IgG1 antibodies was observed. In addition binding of serum C1q on *P. aeruginosa* coated plates was tested by ELISA (*Eckhardt* et al., 2003) before immunisation and after the third vaccination. A significant increase of C1q binding to antibodies was detected after the third vaccination in all 26 sera tested.

The ability of the OprF-OprI vaccine to boost the opsono-phagocytic efficacy of the sera of the volunteers was measured by incubation of viable *P. aeruginosa* bacteria (ATCC strain 27313) with the sera of the volunteers before and after the third vaccination. The OprF-OprI hybrid protein vaccine demonstrated the ability to boost the opsono-phagocytic activity of the antisera obtained from 73% of the volunteers tested by this assay (*Mansouri* et al., 1999).

CLINICAL TRIAL IN BURN PATIENTS (*Larbig* et al., 2001)

Study population

Eight adult burn patients with the following inclusion/exclusion criteria:
Inclusion criteria: Age between 18-60 years, II° or III° burn, burns covering between 35% and 55% of the total body surface, ABSI score (*Tobiasen* et al., 1982) between 6 and 10.

Exclusion criteria: Patients with any concomitant diseases, patients with electrical burns, adults whose case records include a former confirmed infection with *P. aeruginosa,* suspected or documented hypersensitivity against one

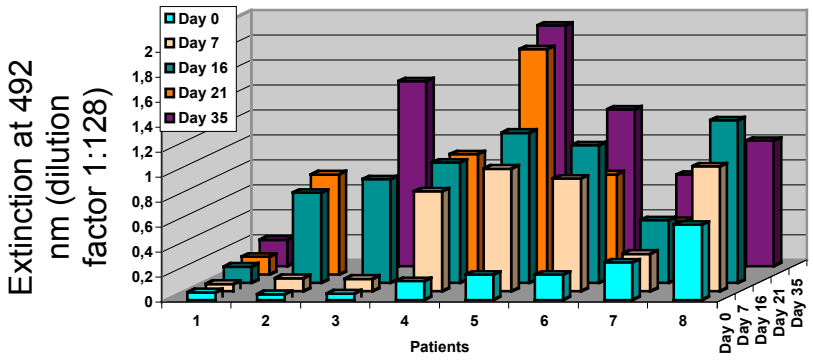

Figure 1: Anti OprF-OprI specific antibody in sera of burn patients detected by ELISA. Patients were vaccinated intramuscularly with 100 μg OprF-I three times at days 0, 7 and 21.

of the substances used in the vaccine or any chemically related substances or pregnancy.

Dose and administration

OprF-OprI was given as an intramuscularly injection into the left upper arm, if not possible due to a burn injury the right side was taken. If both arms were burned we applied the vaccines into the gluteal muscle. As far as possible we sticked to the administration schedule: 1 ml OprF-I (=100 μg) into the left arm, 0,5 ml Tetanol (Tetanus-Toxoid, at least 40 I.E per ml, Chiron Behring, Germany) injection into the right arm, 1 ml Tetagam injection (100-170 mg Immunglobulin, Chiron Behring, Germany) into the left gluteal muscle. The 100 μg dose was chosen because it has been demonstrated to be effective and adequate to induce sufficient antibodies against OprF-I in our volunteer study (*Mansouri* et al., 1999).

Local and systemic responses were graded with a scale from 0 to 3, with scores representing absent, mild, moderate, and severe reactions, respectively. Body temperature, blood pressure and heart rate were measured before vaccination and 1, 2, 4 and 24 h after each vaccination. In addition, each patient underwent a physical examination 2 days after vaccination.

Response to vaccination in burn patients

Antibody titres against OprF-OprI and tetanus were determined by ELISA before each vaccination and at days 7, 16, 21 and 35. Eleven patients were enrolled and received at least 2 vaccinations. The patients were 21 to 60 years of age (2 females and 9 males). The mean age of all patients was 39 years. Three out of the 11 patients died during the study time because of cardiovascular complications. Eight patients received the three scheduled doses of the vaccine and completed all the post vaccination follow-up visits, and the data on these patients are presented in this study. The burned skin areas covered between 35 and 48% (mean 38%) of the body sur-

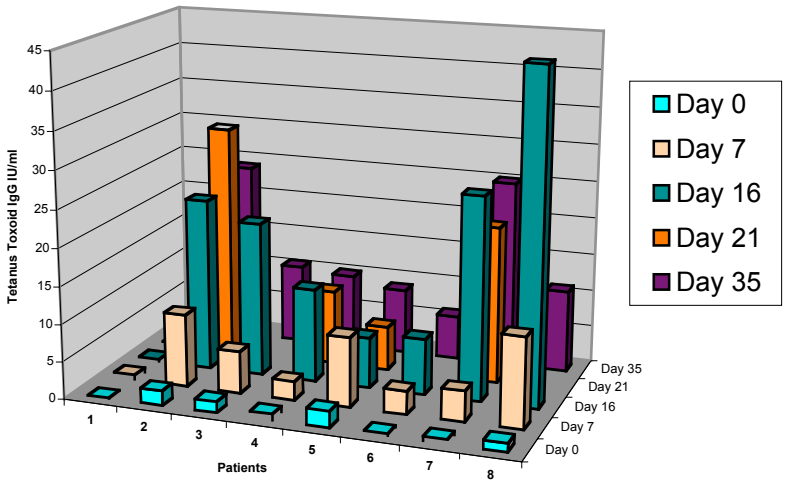

Figure 2: Antibody response against Tetanus toxoid. Patients were vaccinated at the day of admission to the hospital. Antibody response was measured at days 7, 16, 21 and 35.

face. The ABSI score was between 7 and 10 (mean 8). Those patients, who died during the study suffered from the largest burn surfaces (46% or above) and had the highest ABSI scores (9 or higher). The serological tests (ELISA) for detection of antibodies against *P. aeruginosa* and tetanus toxoid showed for 7 patients seroconversion (= at least 3 times higher titre than the pre-vaccination value). The kinetics of the serum antibody against OprF-I and the tetanus toxoid responses are given in Figures 1 and 2.

The vaccine was well tolerated. No serious side effects were observed. The vaccination did not appear to enhance an inflammatory response in the burn patients. None of the subjects acquired systemic *P. aeruginosa* infections during or after the treatment of their burns.

MUCOSAL VACCINATION TRIAL IN VOLUNTEERS (*Göcke* et al., 2003)

Study subjects and study plan

Twelve healthy male volunteers (mean age 24.3, range 21.8 to 26.7 years) were included in this study. Exclusion criteria were, beside current or chronic conditions, a previous *P. aeruginosa* infection. Serum and saliva were assayed for total IgG and IgA to exclude undiscovered humoral immune defects. 100 μl of the emulgel, containing 1 mg OprF-I were applied to the concha nasalis for the mucosal vaccination, while the systemic vaccination was performed by injection of 1 ml, containing 100 μg OprF-I, into the deltoid muscle.

We compared two vaccination schedules, one with three consecutive nasal vaccinations at three weeks intervals, the other a combined mucosal/systemic schedule with two nasal vaccinations followed by a systemic booster, also at

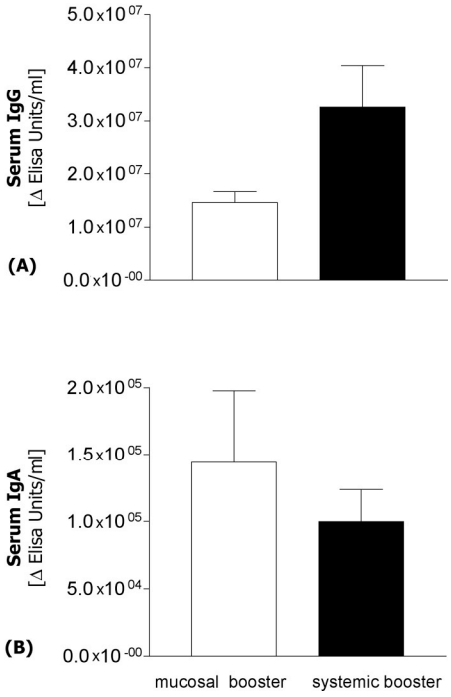

Figure 3: Increase in serum antibody levels against OprF-I of *P. aeruginosa* of IgG and IgA isotype (A and B, respectively) after 3 consecutive nasal ("mucosal booster") or two consecutive nasal vaccinations followed by a systemic booster ("systemic booster"). For further details of the study see " Mucosal vaccination trial in volunteers" in the text and reference (*Göcke* et al., 2003).

three weeks intervals. The participants were randomly assigned to the two schedules (n=6 per group).

The volunteers were monitored for adverse effects for 5 days after each vaccination by physical examination, blood samples, and body temperature. The induction of OprF-I-specific antibodies was analysed by comparing blood and saliva samples obtained prior to the primary and 4 weeks after the second booster vaccination. OprF-I-specific antibodies were determined as described previously (*Mansouri* et al., 1999).

Results of the mucosal vaccination study

Apart from a brief local discomfort (burning, tickling in the nose and tension in the muscle) and an occasional episode of fever of less than 24 hrs (n=3 in 36 vaccinations), no adverse effects were observed. All vaccinees showed a seroconversion irrespective of the vaccination schedule. The systemic booster elicited OprF-I-specific IgG antibody titres in serum twice as high as the nasal booster, while specific IgA antibodies did not differ between both vaccination groups (Figure 3). The different booster schedules appeared not to affect the levels of mucosal antibodies as obtained in saliva (*Göcke* et al., 2003).

DISCUSSION

As already pointed out in the introduction, *P. aeruginosa is* a leading cause of morbidity and mortality in immunocompromised patients. The pathogen can cause severe and often fatal sepsis in burn patients, cancer patients receiving chemotherapy or transplant patients treated with immunosuppressive drugs. Beside causing septicaemia *P. aeruginosa* infections occur frequently in organs suffering from a local impairment of immune barriers. *P. aeruginosa* is the main cause of nosocomial pneumonia in the United States (*Pennington*, 1994). Chronic lung infection by mucoid strains of *P. aeruginosa* is the leading cause of death in CF-patients (*Koch* and *Hoiby*, 1993). Other risk groups are patients with artificial ventilation or paraplegia for lung infection and carriers of contact lenses for eye infection with *P. aeruginosa.*

Systemic vaccination with LPS based vaccines against *P. aeruginosa* was shown in the 1960s to reduce the incidence of *P. aeruginosa* sepsis and to reduce the mortality from this organism (*Alexander* and *Fisher*, 1974; *Jones* et al., 1979; *Miller* et al., 1977). However, due to endotoxic complications these vaccines were not approved for routine clinical use. We developed a vaccine based on recombinant outer membrane proteins. The protective efficacy of native outer membrane proteins of *P. aeruginosa* against *P. aeruginosa* infection has been shown in animal models by us and various other research groups, and recently in burn patients by researchers of the Cheil Jedang Corp. (Korea) (*Finke* et al., 1991,1990; *Gilleland* et al., 1984,1988; *Matthews-Greer* and *Gilleland*, 1987; *Jung* et al., 2000; *Kim* et al., 2000). However trace contaminations with *P. aeruginosa* LPS in this native outer membrane preparations induced LPS specific antibodies and resulted in systemic and local side effects by the vaccination (*Kim* et al., 2000).

We therefore choose the recombinant approach. A hybrid protein carrying the known protective epitopes of the main outer membrane proteins F and I was expressed in *E. coli.* Systemic vaccination of volunteers and burn patients was well tolerated. Even after the 500μg dose none of the volunteers reported any adverse effects like fever or local oedema. In burn patients the 100μg vaccine dose was able to induce an antibody response against the vaccine in 7 of the 8 treated patients. Seroconversion against Tetanus toxoid was observed in the same 7 patients and did also not occur the patient not responding to OprF-OprI vaccination. All patients had a continuous haemodynamic monitoring during the observation period. No signs for an activation of the mediator cascade, like fever or increase of heart rate was observed. Beside the primary vaccination of patients at the time point of the delivery to the hospital, risk groups like soldiers, or patients waiting for an organ transplantation could be prophylactically vaccinated.

In chronically infected CF-patients serum IgG antibodies against outer membrane proteins and LPS can be detected at very high titres. At this stage the patients are obviously unable to clear the pathogen from the airways despite the presence of high levels of antibodies. Since colonisation of the upper respiratory tract seems to precede the pulmonary infection in CF (*Johanson* et al., 1979; *Burns* et al., 2001) and IgA is the predominant isotype on the mucosal surface of the upper airways (*Pilette* et al., 2001) induction of secretory IgA on the airway mucosa may play a critical role for the prevention of adherence and subsequent colonisation of the patho-

genic microorganism (*Johanson* et al., 1979). Induction of s-IgA antibodies is particularly enhanced by presentation of the antigen at local inductive sites of the mucosa, like the Peyer's patches in the gut or the lymphatic tissue in the nose. Antibody secreting B-cells (ASC) preferentially migrate to effector tissues corresponding to the inductive site (*Butcher* and *Picke*, 1996). The expression of mucosal homing receptors is a prerequisite for the induction of mucosal immunity. It should be noted that IgG predominates in the lower airways (*Quiding-Jabrink* et al., 1997; *Kim* and *Malik*, 2003). An ideal vaccine, therefore, would induce high titres of both, local IgA in the upper airways, and IgG in the lower airways. While an oral or intestinal immunisation almost exclusively induces antibody-secreting ASC with a mucosal homing pattern, nasal immunisation was shown to induce a more promiscuous pattern of the ASC with IgA secreting mucosal ASC, and IgG secreting systemic ASC (*Kim* and *Malik*, 2003). Nasal vaccination has been shown to induce specific antibodies in the lower respiratory tract (*Rudin* et al., 1999) and to be protective in mice against pneumococcal infection (*Hvalbye* et al., 1999). In our mucosal vaccination study we investigated if intranasal application of the OprF-I vaccine would induce the desired s-IgA antibodies in the mucosal of the upper airways secretions together with a systemic IgG response in humans. Supporting the potential of a nasal vaccine, the nasal OprF-I immunisations induced high levels of systemic and mucosal antibodies of IgA and IgG isotype as obtained in serum and saliva. Since a systemic booster following a mucosal primary was shown to enhance both the systemic and the mucosal immune response, we investigated a further schedule with a systemic booster replacing the second mucosal booster (*Muszkat* et al., 2000). In our volunteer trial, the systemic booster further enhanced the serum IgG response without compromising the induction of specific s-IgA antibodies in saliva. Moreover, according to preliminary data obtained from induced sputum samples, the systemic booster appears to enhance even the specific IgA levels at the pulmonary airway surface (*Baumann* et al., 2002).

A mucosal vaccine augmenting the sIgA protection in the respiratory tract may be beneficial also for other patient groups. Patients undergoing major surgery or other severe stress show a compromised oropharyngeal barrier function, a frequent upper airway colonisation with *P. aeruginosa* and an increased risk of a *P. aeruginosa* pneumonia (*Johanson* et al., 1972,1979).

This patient group has increased considerably during the last decades.

In summary we believe that the results from our phase 1 and 2 studies summarised in this report are in support of a further development of the Met-Ala-$(His)_6OprF_{190-342}$-$OprI_{21-83}$ *Pseudomonas* vaccine to a clinical use in the major risk groups like burn patients and CF patients.

LITERATURE

Alexander, J.W. and Fisher, M.W.: Immunization against *Pseudomonas* infection after thermal injury. J. Infect. Dis. 130, 152-158 (1974).

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., and Struhl, K. (eds.).: Current protocols in molecular biology 1994-1997 - High efficiencytransformation by electroporation. John Wiley and Sons, New York, 1.8.4-1.8.6. (1997).

Baumann, U., Göcke, K., Hagemann, H.,

Freihorst, J., and von Specht, B.U.: Mucosal immune response after nasal and systemic vaccination with a recombinant outer membrane protein F and I of *Pseudomonas aeruginosa* in healthy volunteers. Pediatr. Pulmonol. 24 (Suppl.), 275 (2002).

Burns, J.L., Gibson, R.L., McNamara, S., Yim, D., Emerson, J., Rosenfeld, M., Hiatt, P., McCoy, K., Castile, R., Smith, A.L., and Ramsey, B.W. : Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J. Infect. Dis. 183, 44-452 (2001).

Butcher, E.C. and Picke, L.J.: Lymphocyte homing and homeostasis. Science 272, 60-66 (1996).

Cohen, J.: Naked DNA points way to vaccines. Science 259, 1691-1692 (1993).

Cryz, S.J. Jr., Fürer, E., Cross, A.S., Wegmann, A., Germanier, R., and Sadoff, J.C.: Safety and immunogenicity of *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine in humans. J. Clin. Invest. 80, 51-56 (1987a).

Cryz, S.J. Jr., Sadoff, J.C., and Fürer, E. : Octavalent *Pseudomonas aeruginosa*-O-polysaccharide-toxin A conjugate vaccine. Microb. Pathogen. 6, 75-80 (1987b).

Cryz, S.J. Jr., Sadoff, C., Ohmann, D., and Fürer, E.: Characterization of the human immune response to a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. J. Lab. Clin. Med. 111, 701-707 (1988).

Donnelly, J.J., Ulmer, J.B., and Liu, M.A. : Immunization with polynucleotides. Immunologist 2, 20-26 (1994).

Duchêne, M., Schweizer, A., Lottspeich, F., Krauss, G., Marget, M., Vogel, K., von Specht, B.U., and Domdey, H.: Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. J. Bacteriol. 170, 155-162 (1988).

Duchêne, M., Barron, C., Schweizer, A., von Specht, B.U., and Domdey, H.: *Pseudomonas aeruginosa* outer membrane lipoprotein I gene: molecular cloning sequence, and expression in *Escherichia coli*. J. Bacteriol. 171, 4130-4137 (1989).

Eckhardt, A., Heiss, M.M., Ehret, E., Permanetter, M., Duchêne, M., Domdey, H., and von Specht, B.U.: Evaluation of protective mAbs against *Pseudomonas aeruginosa* outer membrane protein I by C1q binding assay. Zbl. Bakt. 1991; 275:100-111.

Finke, M., Duchêne, M., Eckhardt, A., Domdey, H., and von Specht, B.U.: Protection against experimental *Pseudomonas aeruginosa* infection by recombinant *P. aeruginosa* lipoprotein I expressed in *Escherichia coli*. Infect. Immun. 58, 2241-2244 (1990).

Finke, M., Muth, G., Reichhelm, T., Thoma, M., Duchêne, M., Hungerer, K.D., Domdey, H., and von Specht, B.U.: Protection of immunosuppressed mice against infection with *Pseudomonas aeruginosa* by recombinant *P. aeruginosa* lipoprotein I and lipoprotein I-specific monoclonal antibodies. Infect. Immun. 59, 1251-1254 (1991).

Gabelsberger, J., Knapp, B., Bauernsachs, S., Lenz, U., von Specht, B.U., and Domdey, H.: A hybrid outer membrane protein antigen for vaccination against *Pseudomonas aeruginosa*. Behring Inst. Mitt. 98, 302-314 (1997).

Gallagher, P.G. and Watanakunakorn, Ch.: *Pseudomonas* bacteremia in a community teaching hospital, 1980-1984. Rev. Infect. Dis. 11, 846-852 (1989).

Gilleland, H.E. Jr., Parker, M.G., Matthew, J.M., and Berg, R.D.: Use of purified outer membrane protein F (porin) of *Pseudomonas aeruginosa* as a protective vaccine in mice. Infect. Immun. 44, 49-54 (1984).

Gilleland, H.E. Jr., Gilleland, L.B., and Matthews-Greer, J.M.: Outer membrane protein F preparation of *Pseudomonas aeruginosa* as a vaccine against chronic pulmonary infection with heterologous immunotype strains in rats. Infect. Immun. 56, 1017-1022 (1988).

Gilleland, H.E. Jr., Hughes, E.E., Gilleland, L.B., Matthews-Greer, J.M., and Staczek, J.: Use of synthetic peptides to identify surface-exposed, linear B-cell epitopes within outer membrane protein F of *Pseudomonas aeruginosa*. Curr. Microbiol. 31, 279-286 (1995).

Gilleland, L.B. and Gilleland, H.E. Jr.: Synthetic peptides representing two protective, linear B cell epitopes of outer membrane protein F of *Pseudomonas aeruginosa* elicit whole-cell-reactive antibodies that are functionally pseudomonad specific. Infect. Immun. 632347-2351 (1995).

Göcke, K., Baumann, U., Hagemann, H., Gabelsberger, J., Hahn, H., Freihorst, J.,

and von Specht, B.U.: Mucosal vaccination with a recombinant OprF-I vaccine of *Pseudomonas aeruginosa* in healthy volunteers: Comparison of a systemic vs. a mucosal booster schedule. FEMS Immunol. Med. Microbiol. 37, 167-171 (2003).

Gordon, S.M., Serke, J.M., Keys, T.F., Ryan, T., Fatica, C.A., Schmitt, S.K., Borsh, J.A., Cosgrove, D.M., and Yared, J.P.: Secular trends in nosocomial bloodstream infections in a 55-bed cardiothoracic intensive care unit. Ann. Thorac. Surg. 65, 95-100 (1998).

Griffith, S.J., Nathan, C., Selander, R.K., Chamberlin, W., Gordon, S.T., Kabins, S., and Weinstein, R.A.: The epidemiology of *Pseudomonas aeruginosa* in oncology patients in a general hospital. J. Infect. Dis. 160, 1030-1036 (1989).

Hanberger, H., Hoffmann, M., Lindgren, S., and Nilson, L.E. High incidence of antibiotic resistance among bacteria in 4 intensive care units at a university hospital in Sweden. Scand. J. Infect. Dis. 29, 602-614 (1997).

Hancock, R.E.W.: Intrinsic antibiotic resistance of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 18, 653-656 (1986).

Hancock, R.E.W. and Wong, R.: Potential of protein OprF of *Pseudomonas* in bivalent vaccines. Behring Inst. Mitt. 98, 283-290 (1997).

Hoiby, N., Krogh, J.H., Moser, C., Song, Z., Ciofu, O., and Kharazmi, A.: *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. Microbes Infect. 3, 23-35 (2001).

Holder, I.A.: *Pseudomonas* immunotherapy. Serodiagn. Immunother. 2, 7-16 (1988).

Holzheimer, R.G., Quoika, P., Pätzmann, D., and Füssle, R.: Nosocomial infections in general surgery: Surveillance report from a German University. Clin. Infect. 18, 219-225 (1990).

Hong, Y.Q. and Ghebrehiwet, B.: Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin. Immun. Immunopath. 62, 1333-1338 (1992).

Hsueh, P.R., Teng, L.J., Chen, Y.C., Ho, S.W., and Lu, K.T.: Persistence of a multidrug-resistance *Pseudomonas aeruginosa* clone in an intensive care burn unit. J. Clin. Microbiol. 2, 1347-1351 (1998).

Hvalbye, B.K., Aaberge, I.S., Lovik, M., and Haneberg, B.: Intranasal immunization with heat-inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection. Infect. Immun. 67, 4320-4325 (1999).

Johanson, W.G. Jr., Pierce, A.K., Sanford, J.P., and Thomas, G.D. Nosocomial respiratory infections with gram-negative bacilli. The significance of colonization of the respiratory tract. Ann. Intern. Med. 77, 701-706 (1972).

Johanson, W.G. Jr., Woods, D.E., and Chaudhuri, T.: Association of respiratory tract colonization with adherence of gram-negative bacilli to epithelial cells. J. Infect. Dis. 139, 667-673 (1979).

Jones, R.J., Roe, E.A., and Gupta, J.L.: Controlled trials of a polyvalent *Pseudomonas* vaccine in burns. Lancet 2, 977-983 (1979).

Jung, S.B., Ahn, B.Y., Kim, Y.H., Kim, J.J., Kim, D.K., Kim, I.S., Yoon, S.M., Nam, S.W., Kim, H.S., and Park, W.J.: Immunization of burn-patients with a *Pseudomonas aeruginosa* outer membrane protein vaccine elicits antibodies with protective efficacy. Vaccine 18, 1952-1961 (2000).

Kim, D.K., Kim, J.J., Kim, J.H., Woo, Y.M., Kim, S., Yoon, D.W., Choi, C.S., Kim, I., Park, W.J., Lee, N., Jung, S.B., Ahn, B.Y., Nam, S.W., Yoon, S.M., and Choi, W.J.: Comparison of two immunization schedules for a *Pseudomonas aeruginosa* outer membrane proteins vaccine in burn patients. Vaccine 19, 1274-1283 (2000).

Kim, K.J. and Malik, A.B.: Protein transport across the lung epithelial barrier. Am. J. Physiol. Lung Cell. Mol. Physiol. 284, L247-259 (2003).

Koch, C. and Hoiby, N.: Pathogenesis of cystic fibrosis. Lancet 341, 1065-1069 (1993).

Korvick, J.A., Marsh, J.W., Starzl, T.E., and Yu, V.L.: *Pseudomonas aeruginosa* bacteremia in patients undergoing liver transplantation: An emerging problem. Surgery 109, 62-68 (1991).

Kraehenbuhl, J.P. and Neutra, M.R.: Molecular and cellular basis of immune protection of mucosal surfaces. Physiol. Rev. 72, 853-879 (1992).

Larbig, M., Mansouri, E., Freihorst, J., Tümmler, B., Kohler, G., Domdey, H., Knapp, B., Hungerer, K.D., Hundt, E.,

Gabelsberger, J., and von Specht, B.U.: Safety and immunogenicity of an intranasal *Pseudomonas aeruginosa* hybrid outer membrane protein F-I vaccine in human volunteers. Vaccine 19, 2291-2297 (2001).

Mansouri, E., Blome-Eberwein, S., Knapp, B., Hungerer, K.D., Germann, G., and von Specht, B.U.: Clinical study to assess the immunogenicity and tolerability of a recombinant *Pseudomonas aeruginosa* high efficiency transformation by electroporation. Curr. Protocols Mol. Biol. 1994-1997 (1997).

Mansouri, E., Gabelsberger, J., Knapp, B., Hundt, E., Lenz, U., Hungerer, K.D., Gilleland, H.E. Jr., Staczek, J., Domdey, H., and von Specht, B.U.: Safety and immunogenicity of a *Pseudomonas aeruginosa* hybrid outer membrane protein F-I vaccine in human volunteers. Infect. Immun. 67, 1461-1470 (1999).

Matthews-Greer, J.M. and Gilleland, H.E. Jr.: Outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine against heterologous immunotype strains in a burned mouse model. J. Infect. Dis. 155, 1282-1291 (1987).

McGhee, J.R., Czerkinsky, C., and Mestecky, J.: Mucosal vaccines: An overview. In: Mucosal immunology (Eds.: Ogra, P.L., Mestecky, J., Lamm, M.E., Strober, W., Bienenstock, J., and McGhee, J.R.). Academic Press, San Diego, California, 844-850 (1999).

McManus, A.T., Mason, A.D. Jr., McManus, W.F., and Pruitt, B.A. Jr.: Twenty-five year review of *Pseudomonas aeruginosa* bacteremia in a burn center. Eur. J. Clin. Microbiol. 4, 219-223 (1985).

Miller, J.M., Spilsbury, J.F., Jones, R.J., Roe, E.A., and Lowbury, E.J.L.: A new polyvalent *Pseudomonas* vaccine. J. Med. Microbiol. 10, 19-27 (1977).

Muszkat, M., Yehuda, A.B., Schein, M.H., Friedlander, Y., Naveh, P., Greenbaum, E., Schlesinger, M., Levy, R., Zakay-Rones, Z., and Friedman, G.: Local and systemic immune response in community-dwelling elderly after intranasal or intramuscular immunization with inactivated influenza vaccine. J. Med. Virol. 61, 100-106 (2000).

Mutharia, L.M., Nicas, T.L., and Hancock, R.E.W.: Outer membrane proteins of *P. aeruginosa* serotype strains. J. Infect. Dis. 146, 770-779 (1982).

Pennington, J.E.: *Pseudomonas aeruginosa* pneumonia and other respiratory tract infections. In: *Pseudomonas aeruginosa* infections and treatment (Ed.: Baltch, A.). Marcel Dekker Inc., New York, 159-181 (1994).

Pier, G.B.: Safety and immunogenicity of high-molecular weight polysaccharide vaccine from immunotype 1 *Pseudomonas aeruginosa*. J. Clin. Invest. 69, 303-308 (1982).

Pier, G.B.: *Pseudomonas aeruginosa* surface polysaccharide vaccines. In: *Pseudomonas aeruginosa*: New therapeutic approaches from basic research, Vol. 36 (Eds.: Speert, D.P. and Hancock, R.E.W.). S. Karger, Basel, 157-167 (1985).

Pier, G.B., Des Jardin, D., Grout, M., Garner, C., Bennet, S.E., and Pekoe, G.: Human immune response to *Pseudomonas aeruginosa* mucoid exopolysaccharide (alginate) vaccine. Infect. Immun. 62, 3972-3979 (1994).

Pier, G.B.: Rational for development of immunotherapies that target mucoid *Pseudomonas aeruginosa* infection in cystic fibrosis patients. Behring Inst. Mitt. 98, 350-360 (1997).

Pilette, C., Ouadriri, Y., Godding, V., Vaerman, J.P., and Sibille, Y.: Lung mucosal immunity: Immunoglobulin-A revisited. Eur. Respir. J. 18, 571-588 (2001).

Price, B.M., Barten Legutki, J., Galloway, D.R., von Specht, B.U., Gilleland, L.B., Gilleland, H.E. Jr., and Staczek, J.: Enhancement of the protective efficacy of an oprF DNA vaccine against *Pseudomonas aeruginosa*. FEMS Immunol. Med. Microbiol. 33, 89-99 (2002).

Pruitt, B.A. Jr., Colonel, M.C., and McManus, A.T.: Opportunistic infections in severely burned patients. Am. J. Med. 30, 146-154 (1984).

Pruitt, B.A. Jr., McManus, A.T., Kim, S.H., and Goodwin, C.W.: Burn wound infections: Current status. World J. Surg. 22, 135-145 (1998).

Quiding-Jabrink, M., Nordstrom, I., and Granstrom, G.: Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations. A molecular basis for the compartmentaliza-

tion of effector B cell responses. J. Clin. Invest. 99, 1281-1286 (1997).

Rudin, A., Riise, G.C., and Holmgren, J.: Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infect. Immun. 67, 2884-2890 (1999).

Srikumar, R., Kon, T., Gotoh, N., and Poole, K.: Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. Antimicrob. Agents Chemother. 42, 65-71 (1988).

Tassios, P.T, Gennimata, V., Maniatis, A.N., Fock, C., and Legakis, N.J.: Emergence of multidrug resistance in ubiquitous and dominant *Pseudomonas aeruginosa* serogroup O:11. The Greek *Pseudomonas aeruginosa* Study Group. J. Clin. Microbiol. 36, 897-901 (1998).

Tobiasen, J., Hiebert, J., and Edlich, R.: Prediction of burn mortality. Surg. Gynecol. Obstet. 154, 711-714 (1982).

Toth, A., Schödel, F., Duchêne, M., Massarrat, K., Blum, B., Schmitt, A., Domdey, H., and von Specht, B.U.: Protection of immunosuppressed mice against translocation of *Pseudomonas aeruginosa* from the gut by oral immunization with recombinant *Pseudomonas aeruginosa* outer membrane protein I expressing *Salmonella dublin*. Vaccine 12, 1215-1221 (1994).

von Specht, B.U., Strigl, G., Ehret, W., and Brendel, W.: Protective effect of an outer membrane vaccine against *Pseudomonas aeruginosa* infection. Infection 15, 408-412 (1987).

von Specht, B.U., Knapp, B., Muth, G., Bröker, M., Hungerer, K.D., Diehl, K.D., Massarrat, K., Seemann, A., and Domdey, H. : Protection of immunocompromised mice against lethal infection with *P. aeruginosa* by active or passive immunization with recombinant *P. aeruginosa* outer membrane protein F and outer membrane protein I fusion proteins. Infect. Immun. 63, 1855-1861 (1995).

von Specht, B.U., Gabelsberger, J., Knapp, B., Hundt, E., Schmidt-Pilger, H., Bauernsachs, S., Lenz, U., and Domdey, H.: Immunogenic efficacy of differently produced recombinant vaccines candidates against *Pseudomonas aeruginosa* infections. J. Biotechnol. 83, 3-12 (2000).

ACTIVE AND PASSIVE IMMUNISATION AGAINST *CLOSTRIDIUM DIFFICILE* DIARRHOEA AND COLITIS*

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SUMMARY

Clostridium difficile, a Gram-positive bacterium, is the major cause of hospital-acquired infectious diarrhoea and colitis in industrialised nations. *C. difficile* colonisation results from antibiotic administration and subsequent loss of protection provided by intestinal flora. *C. difficile*-induced colitis is caused by the release of two exotoxins, toxin A and B. Host factors including advanced age, pre-existing severe illness and weakened immune defences predispose individuals to symptomatic infection. The generation of antibody responses to toxin A through natural exposure is associated with protection from disease. In addition, an inability to acquire immunity to toxin A puts individuals at risk for recurrent and/or severe disease. Immunological approaches for the management of this disease are being developed which could reduce the reliance on antibiotics for treatment and allow for re-establishment of the natural barrier provided by an intact commensal flora. An active vaccine and various immunotherapeutic strategies under evaluation may prove to be effective against severe or relapsing *C. difficile* infection.

INTRODUCTION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium that is commonly found in the environment. The organism is transmitted by the faecal-oral route through the ingestion of resistant spores that survive passage through the stomach, ultimately residing in the colon. Antimicrobial therapy creates an ecological niche, which allows *C. difficile* spores to germinate in the colon. The bacterium colonises the luminal surfaces of colonic epithelial cells and produces two large exotoxins (toxins A and B), which are principally responsible for the disease manifestations associated with this infection. *C. difficile* is currently the most frequent cause of nosocomial infectious diarrhoea (*McFarland* et al., 1989; *Kelly* et al., 1994) and is responsible for an estimated $1 billion in health care costs annually in the US alone (*Kyne* et al., 2002).

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INCIDENCE, RISK FACTORS AND MODES OF TRANSMISSION

The incidence of *C. difficile* carriage in the healthy adult population is ~1-3%. The rate of colonisation increases substantially to ~20% with antibiotic usage (*McFarland* et al., 1989), due to the alteration of the protective commensal flora. Up to 60% of healthy neonates and infants are colonised with *C. difficile* without clinical symptoms (*Larson* et al., 1982; *Viscidi* et al., 1981). The total number of *C. difficile*-associated diarrhoea (CDAD) cases is estimated to be at least 300,000 annually in the US. The incidence can be higher in hospitals, nursing homes and other long-term care facilities where CDAD outbreaks occur. In such settings, diarrhoea helps disseminate the spores that can be found on environmental surfaces, equipment and staff clothing and can be difficult to eradicate. Viable spores have been cultured from various surfaces years after being deposited (*Fekety* et al., 1981; *Kim* et al., 1981). Such contamination constitutes a continuous source of infection for those at risk for CDAD. A variety of factors increase the risk of acquiring CDAD. The most important factor is antibiotic therapy with certain agents, but advanced age (>65), serious underlying illness, an institutional setting and immunodeficiency due to AIDS or chemotherapy, also increase the risk of developing disease. Despite the fact that up to 31% of high-risk hospitalised patients are colonised with *C. difficile*, only a subset develops disease symptoms (*McFarland* et al., 1989; *Samore* et al., 1994).

CLINICAL SYMPTOMS, DIAGNOSIS AND TREATMENT

C. difficile colonisation of adults produces a spectrum of clinical symptoms ranging from asymptomatic carriage to life threatening pseudomembranous colitis. Diarrhoea often appears 1-2 weeks after initiation of antibiotic therapy, which can be accompanied by modest fever and abdominal cramping. Moderate or severe colitis develops in a subset of patients and the most serious form of the infection, pseudomembranous colitis, carries the risk of intestinal perforation and death.

The diagnosis of *C. difficile* infection most commonly relies on the detection of toxin in stool filtrates. A toxin-specific enzyme-linked immunosorbent assay (ELISA) is often employed due to the quick turn-around time and ease of use. The most sensitive and specific test measures the cytotoxic activity in stool supernatants but this method takes up to 3 days for results and requires cell culture capabilities. The anaerobic culture of *C. difficile* from stool does not necessarily aid in diagnosis since non-toxigenic strains exist and these are not associated with disease. However, this method is useful for identifying isolates that are toxin assay negative but are toxigenic under appropriate growth conditions and also for defining the asymptomatic carrier state.

The treatment of CDAD typically involves the cessation of the offending antibiotic, initiation of oral metronidazole or vancomycin therapy and fluid replacement. Metronidazole is generally considered a first-line therapeutic for CDAD due to cost and the concern that oral vancomycin might induce the emergence of vancomycin-resistant enteropathogens. The response rate to initial therapy is ~95% but up to 20% of

patients relapse within 1-2 weeks of completing a course of antibiotics (*Teasley* et al., 1983). The risk of relapsing disease increases markedly with each additional relapse such that individuals who have experienced two or more relapses have a 65% risk of further recurrence (*McFarland* et al., 1994). In one study, it was found that over 50% of the relapse incidents are due to reinfection with a different *C. difficile* strain rather than recrudescence of the primary infection (*Wilcox* et al., 1998). *C. difficile* organisms isolated following a recurrent episode are sensitive to antimicrobial therapy, indicating that relapsing disease is not due to the acquisition of antibiotic resistance. Further exposure to *C. difficile* and/or the imbalance in the normal intestinal flora perpetuated by metronidazole or vancomycin therapy likely contribute to the development of relapsing disease. Host factors associated with protection from primary and recurrent CDAD will be discussed below.

TOXIN STRUCTURE AND MECHANISMS OF ACTIONS

C. difficile is a non-invasive organism which possesses multiple virulence factors that aid in colonisation and may promote disease. These include various adherence factors such as flagellar proteins (*Tasteyre* et al., 2001), surface layer proteins (*Calabi* et al., 2002), and a surface-exposed adhesin (*Waligora* et al., 2001). In addition, all pathogenic strains of *C. difficile* express one or two large exotoxins (A and B) and the majority expresses both toxins. Toxin A (308 kDa) and toxin B (270 kDa) exhibit 49% amino acid identity. These toxins belong to the large clostridial cytotoxin (LCT) family (*von Eichel-Streiber* et al., 1996; *Warny* and *Kelly*, 2003). This family includes *Clostridium sordellii* haemorrhagic and lethal toxins and *Clostridium novyi* alpha toxin. LCT family members are structurally and functionally related proteins with the following properties: 1) high molecular weight; 2) an amino-terminal enzymatic domain; 3) a central hydrophobic region; and 4) a carboxy-terminal domain carrying carbohydrate recognition sequence repeats. The C-terminal region functions as a multivalent lectin which recognises host cell surface carbohydrate receptors. This high avidity binding allows for internalisation by target cells via receptor-mediated endocytosis in clathrin-coated pits (*von Eichel-Streiber* et al., 1996). Certain cultured cell lines do not possess toxin receptors yet these cells effectively internalise toxin A and B (presumably by fluid-phase endocytosis) and become intoxicated, but this requires higher toxin concentrations than for receptor-bearing cells (*Tucker* et al., 1990). Oligosaccharide receptors for toxin A are expressed on the apical membranes on intestinal epithelia of small animals (hamsters, rabbits and certain mouse strains) and in humans. A toxin B receptor has not been identified, which is consistent with the insensitivity of animals to toxin B incubated administered orally (see below). The central hydrophobic region is believed to be necessary for the translocation of the toxins from endocytic vesicles into the cytoplasm, where the toxins interact with their GTPase substrates. Following endosomal acidification, the toxins undergo structural changes that expose the hydrophobic region (*Qa'Dan* et al., 2000), forming potassium permeable channels which facilitate translocation to the cytosol (*Barth* et al., 2001). The enzymatic domain catalyses the transfer of glucose from UDP-glucose as donor molecule to threonine 35/37 of members

of the Rho family of small GTP-binding proteins (including Rho, Rac, cdc42) (*Just* et al., 1995a,b). This covalent addition irreversibly inactivates these proteins, which regulate the actin cytoskeleton, among other functions. The loss of actin cytoskeletal network is lethal to cells and causes a distinctive rounded cell phenotype, which is exploited for the diagnosis of toxigenic *C. difficile* in stool filtrates.

ROLE OF TOXINS IN DISEASE

Studies in animals have contributed greatly to our understanding of the pathogenicity of toxins A and B. When administered orally, the purified toxins are capable of inducing the full spectrum of disease manifestations typical of *C. difficile* infection. Purified toxin A possesses potent enterotoxic and pro-inflammatory activity, as determined in ligated loop studies in mice, rats, hamsters and rabbits (*Kurtz* et al., 2001; *Lyerly* et al, 1982) Toxin A is also cytotoxic to cultured cells in low nanogram quantities. By contrast, toxin B does not exhibit enterotoxic activity in animals but is a more potent cytotoxin than toxin A (*von Eichel-Streiber* et al., 1996). When administered intragastrically, toxin A is lethal to mice and hamsters but toxin B is not (*Lyerly* et al., 1985). The toxins appear to act synergistically when co-administered by the intragastric route, suggesting that toxin A may initially affect epithelial integrity allowing entry of the more potent cytotoxin, toxin B. Indeed, after mechanically compromising the epithelial barrier, toxin B can cause systemic toxicity and death (*Lyerly* et al., 1985). The hamster is a natural model of *C. difficile* diarrhoea and colitis and some laboratory colonies experience outbreaks of *C. difficile* infection (*Chang* and *Rohwer*, 1991). A single dose of oral clindamycin to hamsters followed by intragastric inoculation with toxigenic *C. difficile* organisms produces fulminant disease symptoms (diarrhoea, ruffled fur, lethargy, etc) leading to death within 2-3 days. Necropsy reveals severe haemorrhagic caecitis. This model of *C. difficile* diarrhoea and colitis is a stringent test for vaccines and immunotherapies.

By contrast to the observations with toxin-producing strains, intragastric administration of culture filtrates from non-toxigenic strains does not result in disease (*Lyerly* et al., 1985), confirming the principal role of the toxins in the pathogenicity of *C. difficile*. Recently, certain related strains (serogroup F) have been shown to possess a toxin A-B+ phenotype. Examination at the genetic level revealed that these strains do not have an intact toxin A gene but do express an unusual variant toxin B (*Chaves-Olarte* et al., 1999). Strains of this phenotype have been associated with clinical disease, suggesting that toxin B alone can cause intestinal symptoms in humans (*Alfa* et al., 2000; *Johnson* et al., 2001; *Limaye* et al., 2000). *Ex vivo* studies using human colonic explants indicated that toxin B can induce a loss in transepithelial resistance and pro-inflammatory cytokine signalling consistent with enterotoxic activity (*Riegler* et al., 1995). In addition, toxin B was recently found to possess enterotoxic and pro-inflammatory activity in human intestinal xenografts in immunodeficient (*scid*) mice (*Savidge* et al., 2003). The enterotoxic potential of toxin B in humans is an important consideration for the design of vaccines and immunotherapies, as discussed below.

VACCINATION WITH *C. DIFFICILE* TOXOIDS IN ANIMAL MODELS

One approach to defining the roles of toxins A and B in the pathogenesis of *C. difficile* infection has been to examine the protective capacity of toxin-specific immunity in animals.

Active vaccination

Animals have been vaccinated with various forms of *C. difficile* toxoids ranging from crude culture filtrates to partially purified preparations. Hamsters vaccinated parenterally with formalin-inactivated toxins (toxoids) A and B in culture filtrate, but not individual toxoids in culture filtrate, were protected from lethal ileocaecitis induced with clindamycin and toxigenic *C. difficile* (*Fernie* et al., 1983; *Kim* et al., 1987; *Libby* et al., 1982). *Kim* et al. (1987) found that toxoid A plus B and toxoid A alone (but not toxoid B) similarly protected hamsters from fatal *C. difficile* challenge, while culture filtrate from non-toxigenic *C. difficile* strains did not confer protection. Although the different immunisation schemes, antigen dose levels and adjuvant formulations employed in these studies make direct comparisons of these findings difficult, the protection afforded by toxin-specific immunity was clear.

The rapid onset of fulminant, lethal disease in hamsters represents a rigorous test of vaccine-induced immunity because of the requirement to neutralise the enterotoxicity, mucosal damage and inflammation mediated by the toxins as well as the systemic toxicity due to toxins entering the circulation. While initial vaccine studies used protection from lethal disease as the primary efficacy measure, more detailed assessments of protection from enterotoxicity and diarrhoea were carried out which provided proof-of-principle in support of the development of an effective vaccine against CDAD. An evaluation of the routes of delivery of an inactivated culture filtrate vaccine in hamsters assessed protection from both lethal disease and diarrhoea and found that a sequential combination of intranasal and intraperitoneal immunisation with vaccine plus cholera toxin and Ribi adjuvants, respectively, provided complete protection from death and diarrhoea, suggesting that induction of both systemic and mucosal immunity was necessary for optimal protection (*Torres* et al., 1995).

Using a more purified toxoid preparation, various clinically compatible vaccination regimens were tested in hamsters to determine the routes of administration capable of eliciting protection from death and diarrhoea (*Giannasca* et al., 1999). The combination of rectal immunisation with *E. coli* heat-labile toxin adjuvant and intramuscular (i.m.) administration with alum provided full protection from *C. difficile* challenge, irrespective of the sequence employed. Intranasal or intragastric vaccination in combination with i.m. administration was partially protective against diarrhoea, as was i.m. vaccination with alum. While assessing the requirement of alum adjuvant during i.m. administration, it was unexpectedly found that the toxoid preparation without adjuvant was best able to consistently protect hamsters from diarrhoea and death of all the regimens tested, and it elicited high levels of serum toxin A and B neutralising activity, as determined in the cell culture assay. No detectable anti-toxin antibodies were found in saliva or faeces, suggesting that serum antibodies were the principal effector molecules.

In order to define the domains of the large toxins that contain protective epitopes, recombinant peptides have been generated and evaluated in small animal models. A large portion of the cell-binding domain of toxin A was cloned

and expressed in *E. coli* (*Price* et al., 1987). This 104 kDa polypeptide retained its ability to agglutinate rabbit erythrocytes. Antibodies raised against this peptide neutralised the enterotoxic activity of native toxin A in the rabbit intestinal loop assay (*Lyerly* et al., 1990) and a mAb (PCG-4) generated with this antigen had similar neutralising activity (*Lyerly* et al., 1986). When hamsters were vaccinated with this peptide, partial protection from death and diarrhoea due to *C. difficile* challenge was observed, thereby establishing a role for this toxin A domain in protective immunity. Another polypeptide from this cell-binding domain of toxin A was generated and used to explore the intranasal route of immunisation in mice (*Ward* et al., 1999a). This antigen induced specific antibodies in both serum and lung lavage fluid but not in small intestinal secretions.

Because *C. difficile* disease manifestations in humans are largely confined to the intestinal mucosa, delivery systems capable of presenting non-toxic domains of toxin A to intestinal immune induction sites have been explored. Various length polypeptides spanning the cell-binding domain of toxin A were expressed as fusions with tetanus toxin C fragment in an attenuated *Salmonella typhimurium* vaccine strain known to be effective against murine typhoid disease (*Ward* et al., 1997). Following intragastric (i.g.) immunisation of mice, it was found that one construct containing 14 toxin A carbohydrate recognition domain (CRD) repeats generated serum anti-toxin A responses. Using a less attenuated *aro A, aroD S. typhimurium* host strain given to mice by the i.n. or i.g. routes, these 14 CRD repeats generated toxin A-binding and -neutralising antibodies in serum (*Ward* et al., 1999b). Analysis of pulmonary and intestinal lavage samples subsequent to i.n. or i.g. vaccination, respectively, revealed that toxin A-specific IgA was induced. Using another live vector delivery system, 720 amino acids comprising most of the toxin A cell-binding domain was expressed as a fusion protein with the signal sequence of *E. coli* haemolysin A in an attenuated *Vibrio cholerae* vaccine strain and used to orally immunise rabbits (*Ryan* et al., 1997). It was found that anti-toxin A serum IgG antibodies were induced and protection from enterotoxicity was demonstrated using the ligated ileal loop assay. The host range specificity of these live vectors limits their evaluation to susceptible species, making it difficult to compare with findings generated in the hamster model.

Passive immunisation

Because active vaccination elicits both cellular and humoral immune responses, passive vaccination with immune sera has been employed to define the relative roles of the two branches of the immune system in protection from *C. difficile* diarrhoea and colitis.

Oral administration

Toxin-mediated diseases typically require the production of toxin-specific antibodies for protection. Because *C. difficile* intoxication begins with release of toxin molecules at the luminal surfaces of the caecum and colon, investigators have examined whether anti-toxin preparations administered orally could neutralise enterotoxicity. Bovine antibodies have been tested as a means to provide protection against various enteric pathogens following oral delivery (*Korhonen* et al., 2000). A *C. difficile* bovine IgG concentrate was prepared by immunising gestating cows with culture filtrate toxoid and processing the resulting colostrum. This antibody formulation contained toxin binding and neutralising activity and was able to prevent diarrhoea and death in hamsters

when administered before and during clindamycin/*C. difficile* challenge (*Kelly* et al., 1996; *Lyerly* et al., 1991).

In order to define the toxin polypeptide domains which could elicit antibodies with protective activity when delivered orally, *Kink* and *Williams* (*Kink* and *Williams*, 1998) created multiple recombinant peptides that together spanned the entire toxin proteins. These peptides were used to immunise hens for the production of egg IgY antibodies, which were orally administered to hamsters to assess passive protection from CDAD in a prophylactic and therapeutic setting. They observed that antibodies to the cell-binding domains of both toxins were most effective in eliciting toxin-neutralising antibodies. Administration of anti-toxin A neutralising antibodies alone prior to challenge was sufficient to prevent disease, while neutralising antibodies to both toxins was required for complete therapeutic protection from death and diarrhoea. Furthermore, hamsters effectively treated with antibodies did not develop relapsing disease months after treatment was halted.

Parenteral administration

Early *C. difficile* studies established a principal, if not exclusive, role for humoral immunity in protection from disease. Prior to the availability of *C. difficile* toxin-specific antisera, *C. sordellii* anti-toxin was tested for cross-reactivity and passive immune protection in hamsters (*Allo* et al., 1979). This anti-toxin preparation neutralised the cytotoxicity of *C. difficile* toxins and was able to fully protect animals from death while significantly preventing diarrhoea when administered by the i.m. route on three consecutive days surrounding clindamycin challenge. These observations suggested that circulating antitoxin could indeed confer protection from enterotoxicity. However, the very high antitoxin doses administered perhaps clouded the physiological relevance of these results.

The ability of circulating anti-toxin IgG to mediate intestinal protection was further established by subsequent studies in mice and hamsters. The intravenous administration of IgG monoclonal antibodies directed against the cell-binding domain of toxin A to gnotobiotic mice and subsequent oral challenge with *C. difficile* resulted in complete protection from death and diarrhoea (*Corthier* et al., 1991). Polyclonal antibodies with toxin neutralising activity induced with toxoid vaccine were administered to hamsters by the i.p. route and were able to protect animals from oral challenge in a dose-dependent manner (*Giannasca* et al., 1999).

MECHANISMS OF PROTECTION IN ANIMAL MODELS

The ability of an antitoxin antibody preparation to convey full protection from oral *C. difficile* challenge in mice and hamsters indicates that antibodies are the essential effector molecules in these animal models. The oral administration of toxin-specific antibodies is capable of neutralising the enterotoxicity and mucosal inflammation caused by the toxins presumably by intercepting the toxins in the intestinal lumen rendering them inactive. This "immune exclusion" likely models the action of secretory antibodies elicited via mucosal vaccination with toxoid or natural exposure to *C. difficile* toxins. Indeed, colonic aspirates from *C. difficile* patients were shown to possess toxin A-specific secretory IgA capable of inhibiting toxin A binding to receptors (*Kelly* et al., 1992).

The ability of serum antibodies to

prevent enterotoxicity and mucosal damage is mechanistically less obvious. The protective role for circulating antibodies in *C. difficile* animal models suggested that toxin-specific IgG was the critical effector molecule because of the elevated IgG titres relative to IgA and IgM levels, but the contribution of IgA or IgM antibodies could not be dismissed. Furthermore, rodents including mice and hamsters possess an efficient hepatobiliary transport system for serum IgA and IgM, which directs substantial amounts of polymeric immunoglobulins into the intestinal tract (*Delacroix* et al., 1985; *Vaerman* and *Langendries*, 1997). Accordingly, the most conclusive evidence for the role of anti-toxin IgG in protection from enterotoxicity was provided by the i.v. administration of monoclonal IgG to gnotobiotic mice (*Corthier* et al., 1991). While the precise mechanism by which anti-toxin IgG neutralises enterotoxicity has not been established, the direct effects of the toxins on epithelial cells probably play a role. Toxins A and B have been shown to increase the permeability of polarised intestinal epithelial cells (*Hecht* et al., 1988,1992) through the specific inactivation of Rho proteins which regulate tight junctions and their interaction with the actin cytoskeleton. Thus, the barrier function of intestinal epithelium appears to be highly sensitive to the action of the toxins and the increase in epithelial permeability may lead to enhanced paracellular transport of soluble molecules including antibodies. In support of this hypothesis, intravenously-administered anti-toxin A monoclonal was detected in the caecal contents of gnotobiotic mice following oral challenge with toxigenic *C. difficile* but not in unchallenged mice (*Corthier* et al., 1989). If this model is correct, the "leakage" of serum proteins into the intestinal lumen can occur in the absence of fluid loss (diarrhoea) or gross changes in the epithelium, as described for hamsters protected by parenteral immunisation with different toxoid vaccine preparations (*Giannasca* et al., 1999; *Kim* and *Rolfe*, 1989).

ANTIBODY RESPONSES TO TOXINS IN HUMANS

Many healthy adults (~60%) have detectable serum IgG and IgA to toxins A and B (*Viscidi* et al., 1983) despite only a small population (2-3%) being colonised (*Kelly* and *Lamont*, 1998), as determined by culturing stool on selective media. It is not known if the prevailing responses in adults are a reflection of childhood exposure or sub-clinical infection(s) as adults. The ability to mount an effective immune response following exposure to *C. difficile* appears to impact the course of disease expression. Indeed, only a small proportion of high-risk hospitalised patients develop symptomatic infection while up to 31% are colonised with *C. difficile* (*McFarland* et al., 1994; *Samore* et al., 1994). Following symptomatic infection, many individuals develop anti-toxin A and B antibodies in serum (*Viscidi* et al., 1983; *Aronsson* et al., 1985), including toxin neutralising IgA (*Johnson* et al., 1995), as well as in stool and this response appears to be associated with protection from subsequent infection. The important role of acquired immunity to this disease is supported by the observations that individuals with recurrent *C. difficile* diarrhoea were found to mount poor anti-toxin responses despite repeated exposure to these antigens (*Aronsson* et al., 1985; *Leung* et al., 1991; *Warny* et al., 1994). A comprehensive prospective analysis of hospitalised patients receiving antibiotics

revealed that the development of anti-toxin A IgG in the serum of colonised patients was associated with asymptomatic carriage of *C. difficile* (*Kyne* et al., 2001). Patients who developed elevated serum anti-toxin A IgG titres in response to colonisation were 48 times less likely to suffer from diarrhoea than those who did not. Furthermore, patients who developed circulating antitoxin A IgG antibodies soon after a primary episode of *C. difficile* diarrhoea were much less likely to experience recurrent diarrhoea (*Kyne* et al., 2000). Thus, two recent prospective studies strongly suggest that the magnitude and kinetics of the IgG response to toxin A play an important role in determining the clinical outcome of *C. difficile* infection.

These data also raise the intriguing possibility that circulating anti-toxin A IgG antibodies may act as effector molecules in immune protection from *C. difficile* diarrhoea in humans. The observation that total stool IgG levels are elevated in patients with *C. difficile* diarrhoea (*Warny* et al., 1994), consistent with the results previously described in experimentally-infected mice (*Corthier* et al., 1989), allows one to speculate that serum exudation may facilitate access of circulating antibodies to the intestinal lumen where antibody neutralisation of enterotoxicity and protection of the intestinal mucosa may occur.

IMMUNOLOGICAL APPROACHES TO CLINICAL MANAGEMENT

Because antimicrobial therapy is the principal inciting agent for CDAD, the need for non-antibiotic approaches for the clinical management of this disease is apparent. Interventions that allow for the restoration of the commensal flora and exploit its protective effect hold the greatest promise for primary prevention and secondary prophylaxis. Active and passive immunisation strategies are being developed which may yield effective alternatives to anti-microbial therapy for use in certain clinical settings.

Active vaccination

The development of an investigational vaccine comprised of a partially purified preparation containing inactivated toxins A and B is in progress. This parenteral toxoid vaccine was recently tested in young, healthy volunteers for safety and immunogenicity (*Kotloff* et al., 2001). The *C. difficile* vaccine was administered by intra-muscular injection to volunteers at one of three dose levels (6.25 mg, 25 mg, and 100 mg) with or without aluminum hydroxide adjuvant on days 1, 8, 30 and 60. The vaccine was generally well tolerated with some local injection site soreness, which was mainly associated with the aluminum hydroxide adjuvant. Analysis of toxin A-specific IgG responses in serum by ELISA showed that all subjects seroconverted, and exhibited a range of 42- to 92-fold increases over baseline across all doses and formulations. Toxin A-neutralising titres, as determined in the cell culture cytotoxicity assay, were elevated 32- to 43-fold over baseline. Positive anti-toxin B IgG responses were seen in 90% of volunteers. Anti-toxin faecal IgA was stimulated less frequently than in serum, as might have been expected following parenteral vaccination. The potent immune responses elicited by the vaccine suggested that a vaccine might prove useful as an immunological alternative to anti microbial therapy.

Although the antibody titres stimulated through vaccination were substantial, the magnitude of these responses relative to those associated with protection from symptomatic infection was unknown. In order to bridge these data, the sera from clinical study described

above were tested in the standardised ELISA use to demonstrate the relationship between anti-toxin IgG levels and resistance to hospital-acquired *C. difficile* diarrhoea and recurrent illness. The kinetics of toxin A-specific IgG induction during the course of vaccination was assessed relative to the "threshold" level associated with protection (*Aboudola* et al., 2003). It was found that, across all dose levels and formulations, 57% of subjects reached or surpassed the threshold by day 15 of the vaccine course. Furthermore, by day 90, all subjects exceeded this level and were found to have a median titre 50-fold higher than the threshold. Because the toxoid vaccine elicited substantial toxin A-neutralising titres, this activity was also measured in sera from asymptomatic *C. difficile* carriers as well as those with *C. difficile*-associated diarrhoea. It was found that none of the patients with diarrhoea developed neutralising antibodies while only 1 of 18 carriers demonstrated a detectable titre, suggesting that this functional activity does not correlate with protection from symptomatic infection. In summary, parenteral immunisation with *C. difficile* toxoid vaccine elicits toxin A-binding antibody titres which greatly exceed the levels associated with protection from disease symptoms.

The inability to mount substantial *C. difficile* toxin-binding antibody responses despite repeated exposure is a hallmark of recurrent *C. difficile* diarrhoea. Because this syndrome is particularly difficult to treat, new approaches which may prove beneficial to these sufferers are needed. The positive findings with the toxoid vaccine in healthy volunteers prompted an initial pilot test of the vaccine in three patients with chronic, relapsing *C. difficile* diarrhoea to assess safety and immunogenicity (*Sougioultzis* et al., 2004). In this open label study, volunteers undergoing vancomycin therapy were administered 50 mg doses of the vaccine without adjuvant on days 0, 7, 28 and 56. The patients continued vancomycin therapy until the fourth vaccine dose on day 56.

All three subjects remained free of recurrent CDAD for the two month follow-up period in the absence of vancomycin. These preliminary observations suggest that active vaccination may be an effective strategy for treatment of recurrent CDAD. Larger controlled clinical studies will be needed to establish this approach as an immunological alternative to long-term antimicrobial therapy.

Passive vaccination

The prevalence of serum antibodies against *C. difficile* toxins A and B in healthy populations has prompted investigators to test the therapeutic activity of intravenous immune globulin (IVIG) preparations derived from plasma donors in individuals experiencing severe or recurrent *C. difficile* infection. In the first application of IVIG treatment for *C. difficile* infection, five children with relapsing *C. difficile* colitis who were found to have lower anti-toxin A IgG titres than healthy children were administered 400 mg/kg IVIG containing toxin A- and B-specific IgG antibodies (*Leung* et al., 1991). All treated children responded favourably to therapy, with resolution of colitis symptoms and diarrhoea and clearance of toxin B from stool samples. IVIG therapy has also shown promise in the treatment of adults with severe and/or recurrent *C. difficile* diarrhoea and colitis (*Beales*, 2002; *Salcedo* et al., 1997; *Warny* et al., 1995).

These case reports provide proof-of-principle that intravenously administered antibodies can confer rapid protection from the enterotoxic and inflammatory actions of *C. difficile* toxins. The limited availability of IVIG precludes its general

use as a therapeutic for severe or recurrent *C. difficile* diarrhoea. An intriguing alternative to standard IVIG preparations, which rely on anti-toxin antibodies raised in response to natural exposure to the organism, is the production of hyper-immune globulin derived from volunteers immunised with *C. difficile* toxoid vaccine. This strategy is being employed to produce an immune globulin preparation which a higher specific activity than IVIG developed from source plasma. The dose requirements for therapeutic activity would need to be determined empirically in clinical trials.

The oral administration of anti-*C. difficile* antibodies has also been explored for the treatment of severe or recurrent CDAD. The bovine IgG preparation found to be effective in animal models, as described above, was evaluated in a clinical study aimed at determining the survival of bovine IgG following passage through the GI tract (*Kelly* et al., 1997). The preparation was administered in liquid form or within enteric capsules and the effect of antacid treatment or therapy with a proton pump inhibitor was also assessed. The degradation of bovine IgG during transit by intestinal proteases was found to substantially reduce the activity of anti-*C. difficile* antibodies recovered in stool. Further development of this approach has not been reported.

The passive therapies described above rely on the activity of polyclonal anti-toxin antibodies. Although monoclonal antibodies (mAbs) against toxins A or B have been produced for many years, only recently have they entered development as therapeutics intended for clinical evaluation. However, the selection of antibody clones with the best chance of demonstrating clinical activity is complicated by several factors. Firstly, both toxins A and B will likely need to be neutralised for optimal efficacy and because no cross-neutralising mAbs have been reported, it is likely that at least two antibodies will be required. Secondly, because the toxins possess distinct functional domains for which their respective roles in human disease have not been precisely defined, choosing the critical epitopes within the large toxin molecules may be difficult. Thirdly, since the cell-binding domains of toxins A and B are comprised of 30 and 19 carbohydrate binding sites, respectively, the adherence to target cells may be difficult to block with a single mAb. In addition, sequence variation amongst toxin types may reduce the activity of mAbs against certain *C. difficile* strains. In total, the use of monoclonal antibodies as therapeutics represents a novel strategy against CDAD that may require more than one mAb component for optimal clinical efficacy.

Nevertheless, monoclonal therapeutics are being developed as alternative strategies. Mice expressing human immunoglobulin gene repertoires are being employed to generate human IgG antibodies against *C. difficile* toxin A and possibly toxin B. In addition, recombinant human antibodies specific for toxins A and B have been engineered into corn as a cost-effective production system. These interesting approaches may be evaluated in the clinic in the near future. The mounting interest in developing immune-based strategies for combating *C. difficile* disease validates the belief that symptomatic infections which arise due to insufficient host responses can be managed through active or passive immunisation. Furthermore, these immunological interventions also allow for the restoration of the natural protective barrier of an intact commensal flora, which together should reduce the reliance on antibiotics for treatment of this iatrogenic infection.

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LITERATURE

Aboudola, S., Kotloff, K.L., Kyne, L., Warny, M., Kelly, E.C., Sougioultzis, S., Giannasca, P.J., Monath, T.P., and Kelly, C.P.: *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. Infect. Immun. 71, 1608-1610 (2003).

Alfa, M.J., Kabani, A., Lyerly, D., Moncrief, S., Neville, L.M., Al-Barrak, A., Harding, G.K., Dyck, B., Olekson, K., and Embil, J.M.: Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. 38, 2706-14 (2000).

Allo, M., Silva, J. Jr., Fekety, R., Rifkin, G.D., and Waskin, H.: Prevention of clindamycin-induced colitis in hamsters by *Clostridium sordellii* antitoxin. Gastroenterology 76, 351-355 (1979).

Aronsson, B., Grantsrom, M., Mollby, R., and Nord, C.E.: Serum antibody response to *Clostridium difficile* toxins in patients with *Clostridium difficile* diarrhoea. Infection 13, 97-101 (1985).

Barth, H., Pfeifer, G., Hofmann, F., Maier, E., Benz, R., and Aktories, K.: Low pH-induced formation of ion channels by *Clostridium difficile* toxin B in target cells. J. Biol. Chem. 276, 10670-10676 (2001).

Beales, I.L.: Intravenous immunoglobulin for recurrent *Clostridium difficile* diarrhoea. Gut 51, 456 (2002).

Calabi, E., Calabi, F., Phillips, A.D., and Fairweather, N.F.: Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. Infect. Immun. 70, 5770-5778 (2002).

Chang, J. and Rohwer, R.G.: *Clostridium difficile* infection in adult hamsters. Lab. Anim. Sci. 41, 548-552 (1991).

Chaves-Olarte, E., Low, P., Freer, E., Norlin, T., Weidmann, M., von Eichel-Streiber, C. and Thelestam, M.: A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. J. Biol. Chem. 274, 11046-11052 (1999).

Corthier, G., Muller, M.C., Elmer, G.W., Lucas, F., and Dubos-Ramare, F.: Interrelationships between digestive proteolytic activities and production and quantitation of toxins in pseudomembranous colitis induced by *Clostridium difficile* in gnotobiotic mice. Infect. Immun. 57, 3922-3927 (1989).

Corthier, G., Muller, M.C., Wilkins, T.D., Lyerly, D., and L'Haridon, R.: Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against *Clostridium difficile* toxin A. Infect. Immun. 59, 1192-1195 (1991).

Delacroix, D.L., Malburny, G.N., and Vaerman, J.P.: Hepatobiliary transport of plasma IgA in the mouse: contribution to clearance of intravascular IgA. Eur. J. Immunol. 15, 893-899 (1985).

Fekety, R., Kim, K.H., Brown, D., Batts, D.H., Cudmore, M., and Silva, J. Jr.: Epidemiology of antibiotic associated colitis; isolation of *Clostridium difficile* from the hospital environment. Am. J. Med. 70, 906-908 (1981).

Fernie, D.S., Thompson, R.O., Batty, I., and Walker, P.D.: Active and passive immunization to protect against antibiotic associated caecitis in hamsters. Dev. Biol. Stand. 53, 325-323 (1983).

Giannasca, P.J., Zhang, Z.X., Lei, W., Boden, J.A., Giel, M.A., Monath, T.P., and Thomas, W.D. Jr.: Serum antitoxin antibodies mediate systemic and mucosal protection from *Clostridium difficile* disease in hamsters. Infect. Immun. 67, 527-538 (1999).

Hecht, G., Pothoulakis, C., LaMont, J.T., and Madara, J.L.: *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. 82, 1516-24 (1988).

Hecht, G., Koutsouris, A., Pothoulakis, C., LaMont, J.T., and Madara, J.L.: *Clostridium difficile* toxin B disrupts the barrier function of T84 monolayers. Gastroenterology 102, 416-423 (1992).

Johnson, S., Sypura, W.D., Gerding, D.N., Ewing, S.L., and Janoff, E.N.: Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. Infect. Immun. 63, 3166-3173 (1995).

Johnson, S., Kent, S.A., O'Leary, K.J., Merrigan, M.M., Sambol, S.P., Peterson, L.R., and Gerding, D.N.: Fatal pseudomembranous colitis associated with a variant *Clostridium difficile* strain not detected by toxin A immunoassay. Ann. Intern. Med. 135, 434-438 (2001).

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K.: Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature 375, 500-503 (1995a).

Just, I., Wilm, M., Selzer, .J, Rex, G., von Eichel-Streiber, C., Mann, M., and Aktories, K.: The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. J. Biol. Chem. 270, 13932-13936 (1995b).

Kelly, C.P., Pothoulakis, C., Orellana, J., and LaMont, J.T.: Human colonic aspirates containing immunoglobulin A antibody to *Clostridium difficile* toxin A inhibit toxin A-receptor binding. Gastroenterology 102, 35-40 (1992).

Kelly, C.P., Pothoulakis, C., and LaMont, J.T.: *Clostridium difficile* colitis. N. Engl. J. Med. 330, 257-262 (1994).

Kelly, C.P., Pothoulakis, C., Vavva, F., Castagliuolo, I., Bostwick, E.F., O'Keane, J.C., Keates, S., and LaMont, J.T.: Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of *C. difficile* toxins. Antimicrob. Agents Chemother. 40, 373-379 (1996).

Kelly, C.P., Chetham, S., Keates, S., Bostwick, E.F., Roush, A.M., Castagliuolo, I., LaMont, J.T., and Pothoulakis, C.: Survival of anti-*Clostridium difficile* bovine immunoglobulin concentrate in the human gastrointestinal tract. Antimicrob. Agents Chemother. 41, 236-241 (1997).

Kelly, C.P. and Lamont, J.T.: *Clostridium difficile* infection. Annu. Rev. Med. 49, 375-390 (1998).

Kim, K.H., Fekety, R., Batts, D.H., Brown, D., Cudmore, M., Silva, J. Jr., and Waters, D.: Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J. Infect. Dis. 143, 42-50 (1981).

Kim, P.H., Iaconis, J.P., and Rolfe, R.D.: Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect. Immun. 55, 2984-2992 (1987).

Kim, P.H. and Rolfe, R.D.: Characterization of protective antibodies in hamsters immunized against *Clostridium difficile* toxins A and B. Microb. Ecol. Health Dis. 2, 47-59 (1989).

Kink, J.A. and Williams, J.A.: Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. Infect. Immun. 66, 2018-2025 (1998).

Korhonen, H., Marnila, P., and Gill, H.S.: Bovine milk antibodies for health. Br. J. Nutr. 84, S135-S146 (2000).

Kotloff, K.L., Wasserman, S.S., Losonsky, G.A., Thomas, W. Jr., Nichols, R., Edelman, R., Bridwell, M., and Monath, T.P.: Safety and immunogenicity of increasing doses of a *Clostridium difficile* toxoid vaccine administered to healthy adults. Infect. Immun. 69, 988-995 (2001).

Kurtz, C.B., Cannon, E.P., Brezzani, A., Pitruzzello, M., Dinardo, C., Rinard, E., Acheson, D.W., Fitzpatrick, R., Kelly, P., Shackett, K., Papoulis, A.T., Goddard, P.J., Barker, R.H. Jr., Palace, G.P., and Klinger, J.D.: GT160-246, a toxin binding polymer for treatment of *Clostridium difficile* colitis. J. Antimicrob. Chemother. 45, 2340-2347 (2001).

Kyne, L., Warny, M., Qamar, A., and Kelly, C.P.: Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N. Engl. J. Med. 342, 390-397 (2000).

Kyne, L., Warny, M., Qamar, A., and Kelly, C.P.: Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. Lancet 357, 189-193 (2001).

Kyne, L., Hamel, M.B., Polavaram, R., and

Kelly, C.P.: Health Care Costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. Clin. Infect. Dis. 34, 346-353 (2002).

Larson, H.E., Barclay, F.E., Honour, P., and Hill, I.D.: Epidemiology of *Clostridium difficile* in infants. J. Infect. Dis. 146, 727-733 (1982).

Leung, D.Y., Kelly, C.P., Boguniewicz, M., Pothoulakis, C., LaMont, J.T., and Flores, A.: Treatment with intravenously administered gamma globulin of chronic relapsing colitis induced by *Clostridium difficile* toxin. J. Pediatr. 118, 633-637 (1991).

Libby, J.M., Jortner, B.S., and Wilkins, T.D.: Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. 36, 822-829 (1982).

Limaye, A.P., Turgeon, D.K., Cookson, B.T., and Fritsche, T.R.: Pseudomembranous colitis caused by a toxin A (-) B(+) strain of *Clostridium difficile*. J. Clin. Microbiol. 38, 1696-1697 (2000).

Lyerly, D.M., Lockwood, D.E., Richardson, S.H., and Wilkins, T.D.: Biological effects of toxins A and B of *Clostridium difficile*. Infect. Immun. 35, 1147-1150 (1982).

Lyerly, D.M., Saum, K.E., MacDonald, D.K., and Wilkins, T.D.: Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. 47, 349-352 (1985).

Lyerly, D.M., Phelps, C.J., Toth, J., and Wilkins, T.D.: Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect. Immun. 54, 70-76 (1986).

Lyerly, D.M., Johnson, J.L., Frey, S.M., and Wilkins, T.D.: Vaccination against lethal *Clostridium difficile* enterocolitis with a nontoxic recombinant peptide of toxin A. Curr. Microbiol. 21, 29-32 (1990).

Lyerly, D.M., Bostwick, E.F., Binion, S.B., and Wilkins, T.D.: Passive immunization of hamsters against disease caused by *Clostridium difficile* by use of bovine immunoglobulin G concentrate. Infect. Immun. 59, 2215-2218 (1991).

McFarland, L.V., Mulligan, M.E., Kwok, R.Y., and Stamm, W.E.: Nocosomial acquisition of *Clostridium difficile* infection. N. Engl. J. Med. 320, 204-210 (1989).

McFarland, L.V., Surawicz, C.M., Greenberg, R.N., Fekety, R., Elmer, G.W., Moyer, K.A., Melcher, S.A., Bowen, K.E., Cox, J.L., Noorani, Z., et al.: A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. JAMA 271, 1913-1918 (1994).

Price, S.B., Phelps, C.J., Wilkins, T.D., and Johnson, J.L.: Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. 16, 55-60 (1987).

Qa'Dan, M., Spyres, L.M., and Ballard, J.D.: pH-induced conformational changes in *Clostridium difficile* toxin B. Infect. Immun. 68, 2470-2474 (2000).

Riegler, M., Sedivy, R., Pothoulakis, C., Hamilton, G., Zacherl, J., Bischof, G., Cosentini, E., Feil, W., Schiessel, R., LaMont, J.T., et al.: *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium *in vitro*. J. Clin. Invest. 95, 2004-2011 (1995).

Ryan, E.T., Butterton, J.R., Smith, R.N., Carroll, P.A., Crean, T.I., and Calderwood, S.B.: Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect. Immun. 65, 2941-2949 (1997).

Salcedo, J., Keates, S., Pothoulakis, C., Warny, M., Castagliuolo, I., LaMont, J.T., and Kelly, C.P.: Intravenous immunoglobulin therapy for severe *Clostridium difficile* colitis. Gut 41, 366-70 (1997).

Samore, M.H., Degirolami, P.C., Tlucko, A., Lichtenberg, D.A., Melvin, Z.A., and Karchmer, A.W.: *Clostridium difficile* colonization and diarrhea at a tertiary care hospital. Clin. Infect. Dis. 18, 181-187 (1994).

Savidge, T.C., Pan, W.H., Newman, P., O'Brien, M., Anton, P.M., and Pothoulakis, C.: *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. Gastroenterology 125, 413-420 (2003).

Sougioultzis, S., Kyne, L., Drudy, D., Keates, S., et al.: *Clostridium difficile* toxoid vaccine in recurrent *C. difficile* associated diarrhea. Submitted for publication (2004).

Tasteyre, A., Barc, M.-C., Collignon, A., Boureau, H., and Karjalainen, T.: Role of FliC and FliD flagellar proteins of *Clostrid-*

ium difficile in adherence and gut colonization. Infect. Immun. 69, 7937-7940 (2001).

Teasley, D.G., Gerding, D.N., Olson, M.M., Peterson, L.R., Gebhard, R.L., Schwartz, M.J., and Lee, J.T. Jr.: Prospective randomized trial of metronidazole versus vancomycin for *Clostridium difficile*-associated diarrhea and colitis. Lancet 2, 1043-1046 (1983).

Torres, J.F., Lyerly, D.M., Hill, J.E., and Monath, T.P.: Evaluation of formalin-inactivated *Clostridium difficile* vaccine administered by parenteral and mucosal routes of immunization in hamsters. Infect. Immun. 63, 4619-4627 (1995).

Tucker, K.D., Carring, P.F., and Wilkins, T.D.: Toxin A *of Clostridium difficile* is a potent cytotoxin. J. Clin. Microbiol. 28, 869-871 (1990).

Vaerman, J.P. and Langendries, A.: Hepatobiliary transport of IgA in the golden Syrian hamster (*Mesocricetus auratus*). Immunol. Lett. 55, 19-26 (1997).

Viscidi, R., Willey, S., and Bartlett, J.G.: Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. Gastroenterology 81, 5-9 (1981).

Viscidi, R., Laughon, B.E., Yolken, R., Bo-Linn, P., Moench, T., Ryder, R.W., and Bartlett, J.G.: Serum antibody response to toxins A and B of *Clostridium difficile*. J. Infect. Dis. 148, 93-100 (1983).

von Eichel-Streiber, C., Warfolomeow, I., Knautz, D., Sauerborn, M., and Hadding, U.: Morphological changes in adherent cells induced by *Clostridium difficile* toxins. Biochem. Soc. Trans. 19, 1154-1160 (1991).

von Eichel-Streiber, C., Boquet, P., Sauerborn, M., and Thelestam, M.: Large clostridial cytotoxins - a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol. 4, 375-382 (1996).

Waligora, A.-J., Hennequin, C., Mullany, P., Bourlioux, P., Collignon, A., and Karjalainen, T.: Characterization of a cell surface protein of *Clostridium difficile* with adhesive properties. Infect. Immun. 69, 2144-2153 (2001).

Ward, S.J., Douce, G., Dougan, G., Wren, B.W.: Delivery of non-toxic fragments of *Clostridium difficile* toxin A to the mucosal immune system. Rev. Med. Microbiol. 8, S34-S36 (1997).

Ward, S.J., Douce, G., Dougan, G., and Wren, B.W.: Local and systemic antibody responses induced by intranasal immunization with the nontoxic binding domain of toxin A from *Clostridium difficile*. Infect. Immun. 67, 5124-5132 (1999a).

Ward, S.J, Douce, G., Figueiredo, D., Dougan, G., and Wren, B.W.: Immunogenicity of a *Salmonella typhimurium* aroA aroD vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. Infect. Immun. 67, 2145-2152 (1999b).

Warny, M., Vaerman, J.P., Avesani, V., and Delmée, M.: Human antibody response to *Clostridium difficile* toxin A in relation to clinical course of infection. Infect. Immun. 62, 384-389 (1994).

Warny, M., Denie, C., Delmee, M., and Lefebvre, C.: Gamma globulin administration in relapsing *Clostridium difficile*-induced pseudomembranous colitis with a defective antibody response to toxin A. Acta Clin. Belg. 50, 36-39 (1995).

Warny, M. and Kelly, C.P.: Pathogenicity of *Clostridium difficile* toxins. In: Microbial pathogenesis and the intestinal epithelial cell (Ed. Hecht, G.). ASM Press, Washington, D.C., 503-524 (2003).

Wilcox, M.H., Fawley, W.N., Settle, C.D., and Davidson, A.: Recurrence of symptoms in *Clostridium difficile* infection - relapse or reinfection? J. Hosp. Infect. 38, 93-100 (1998).

DENDRITIC CELL-BASED VACCINATION AGAINST OPPORTUNISTIC FUNGI*

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SUMMARY

Efficient responses to the different forms of fungi require different mechanisms of immunity. Dendritic cells (DCs) are uniquely able to decode the fungus-associated information and translate it in qualitatively different T helper (Th) immune responses, *in vitro* and *in vivo*. DCs sense fungi in a morphotype-specific manner, through the engagement of distinct recognition receptors ultimately affecting cytokine production and co-stimulation. Adoptive transfer of different types of DCs activates protective and non-protective Th cells as well as regulatory T cells and affects the outcome of the infections. DCs transfected with fungal RNA also restore antifungal resistance in haematopoietic transplantation. Thus, the remarkable functional plasticity of DCs in response to fungi can be exploited for the deliberate targeting of cells and pathways of cell-mediated immunity in response to fungal vaccines.

INTRODUCTION

Infections caused by systemic fungal pathogens are a significant health problem in immunocompetent and immunocompromised host. Opportunistic fungal pathogens, which more typically require immunosuppression to infect the host, include *Candida albicans*, which is a normal inhabitant of the human gut, and *Aspergillus fumigatus*, which is ubiquitous in the environment. As a pathogen *C. albicans* is associated with a wide spectrum of diseases in humans, ranging from allergy, severe intractable muco-cutaneous diseases to life-threatening bloodstream infections (*Calderone*, 2002). Aspergilli are respiratory pathogens, and pulmonary infections are usually acquired through the inhalation of conidia able to reach small airways and the alveolar space, where the impaired host defence mechanisms allow hyphal germination and subsequent tissue invasion. *A. fumigatus* is associated with a wide spectrum of diseases ranging from benign colonisation of the lung and allergy to life-threatening diseases such as invasive pulmonary aspergillosis or allergic broncho-pulmonary aspergillosis (*Latgé*, 2001). The delicate balance between the host and these otherwise harmless fungi may turn into a parasitic relationship, resulting in the

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development of severe infections. However, fungi are not mere passive participants in the infectious process and a hypothetical set of virulence factors has been attributed to them (*Denning*, 2000; *Rooney* and *Klein*, 2002). Among these, the ability to form hyphae from budding yeasts or from swelling conidia and the subsequent filamentous growth are thought to be important for virulence (*Hogan* et al., 1996).

Host defence mechanisms against fungi are numerous and range from relatively primitive and constitutively expressed non-specific defences to sophisticated adaptive mechanisms that are specifically induced during infection (*Romani* and *Kaufmann*, 1998). Although the role of innate immunity was originally considered to be a process for defence of the host early in infection, it is now clear that there is an important reciprocal relationship between innate and adaptive immune responses. Through the involvement of a set of germline-encoded pattern recognition receptors (PRRs) and Toll-like receptors (TLRs) that recognise and are triggered by evolutionarily conserved molecules essential to pathogen function (PAMPs, pathogen -associated molecular patterns), cells of the innate immune system not only discriminate between different pathogens, but also contribute to discrimination between self and pathogens at the level of the adaptive T helper (Th) immunity (*Medzhitov* and *Janeway*, 1997; *Schnare* et al., 2001). Cytokines and other mediators play an essential role in the process and, indeed, may ultimately determine the type of effector response that is generated towards the pathogens (*Romani*, 1996). The recognition of fungi at sites of infection leads to the production of chemokines and cytokines that not only activate the innate cell population but also drive the adaptive immune response down different pathways of differentiation. As the different Th cell subsets are endowed with the ability to release a distinct panel of cytokines, capable of delivering the activating and deactivating feedback signals to effector phagocytes, the activation of an appropriate Th subset may be instrumental in the generation of a successful immune response to the fungal pathogens (*Puccetti* et al., 1995; *Romani*, 1997). To limit the pathologic consequences of excessive inflammatory cell-mediated immune reactions, the immune system resorts to a number of protective mechanisms including the reciprocal cross-regulatory effects of Th1 and Th2-type effector cytokines, such as interferon (IFN)-γ and interleukin (IL)-4, and the generation of regulatory T cells (Treg). Thus, innate and adaptive immune responses are intimately linked and controlled by sets of molecules and receptors that act to generate the most effective form of immunity for protection against fungal pathogens.

It has became apparent that understanding how immune responses are activated will enable the construction of better vaccines and vaccine strategies that are effective at eliciting acquired protective immunity to pathogens. The model has brought DCs to centre stage as promising targets for intervention for immunotherapy and vaccine development (*Steinman* and *Pope*, 2002) and has shifted the emphasis from the "antigen" towards the "adjuvant" (*Gallucci* et al., 1999). Thus, the promise of a fungal vaccine will demand for an adjuvant capable of both stimulating the appropriate type of response best tailored to combating the infection and being effective in conditions of immunosuppression.

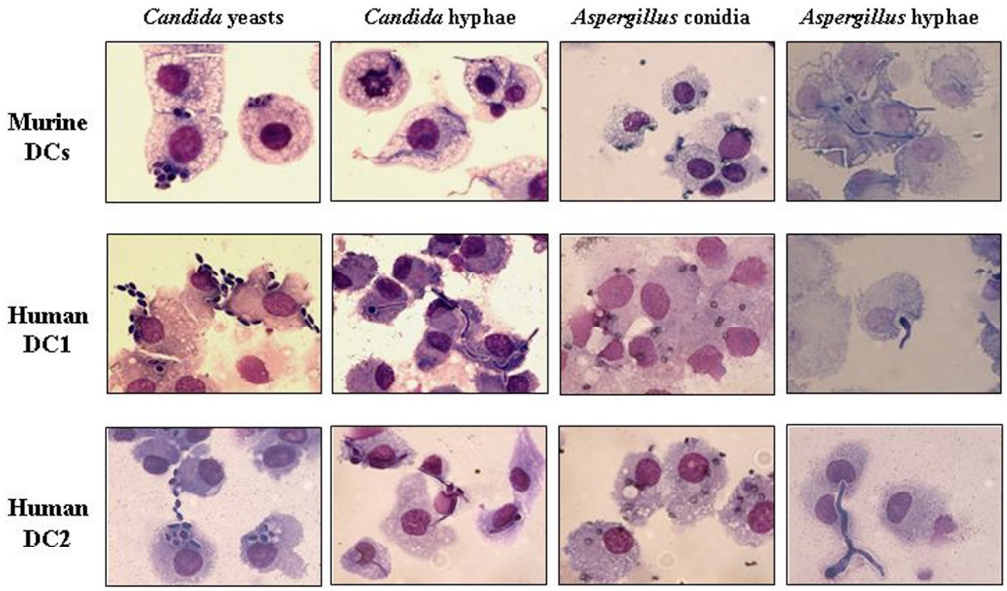

Figure 1: Dendritic cells internalise yeasts and hyphae of *Candida albicans* and conidia and hyphae of *Aspergillus fumigatus*. Murine DC1 were purified from spleens and immature human DC1 and DC2 from $CD11c^+$ blood mononuclear cells, as described (*Bozza* et al., 2003). DCs were exposed to un-opsonised yeasts for 30 min or un-opsonised conidia and hyphae for 60 min before the assessment of phagocytosis, as described (*Bozza* et al., 2003). After a Diff Quik staining, aliquots of cells were spun down on slides on a cytocentrifuge and mounted in buffered glycerol to be examined for conidia internalisation by light microscopy. For each experiment, at least 5 fields in each slide were counted, and at least 200 DCs were analyzed in each well. All conditions were tested in triplicates.

DCs AS NATURAL ADJUVANTS

Since their original discovery in 1973, DCs have assumed centre stage as the key initiator of adaptive immunity (*Lanzavecchia* and *Sallusto*, 2001). In infections, they are central in the balancing act between immunopathology and protective immunity generated by host-microbe interactions. DCs are strategically located at the interface of potential pathogen entry sites and take up antigen, move into secondary lymphoid tissues and activate both helper and cytotoxic T cells. Pathogen-mediated activation induces DCs to undergo maturation consisting in antigen acquisition down-regulation, increased expression of the Major Histocompatibility Antigen Complex (MHC) and co-stimulatory molecules, IL-12 production, and altered expression of chemokine receptors (*Lanzavecchia* and *Sallusto*, 2001). As they mature, DCs migrate to the T cell areas of lymphoid organs, where they translate the tissue-derived information into the language of Th cells, providing them with an antigen-specific "signal 1", a co-stimulatory "signal 2" and a "signal 3" which determines the polarisation of naive Th cells into Th1 or Th2 cells. In addition to DCs initiating immunity, certain subpopulations of DCs are able to down-regulate immune responses (*Shortman* and *Heath*, 2001). The ability of DCs to influence the pattern of cytokine secreted by T cells represents a critical

function, which can profoundly influence the final outcome of the immune response to pathogens. Several factors appear to influence the ability of DCs to polarise T-cell cytokine responses, including the DC subsets, the nature of the maturation stimuli and the host microenvironment (*Shortman* and *Liu*, 2002). At the end, DCs represent the critical link between innate and adaptive immunity, upon which, appropriate concerted action is required for a successful host defence against an invading pathogen. Progress in our understanding of DC biology and their critical function in immunity have prompted investigations to explore their potential use in immunotherapy and prophylaxis.

INTERACTIONS BETWEEN FUNGI AND DCs

In vitro

Efficient responses to the different forms of fungi require different mechanisms of immunity (*Romani*, 1997; *Romani* and *Kaufmann*, 1998). DCs showed a remarkable functional plasticity in response to the different forms of fungi, being able to discriminate between the different forms in terms of maturation, cytokine production and induction of Th cell reactivity, *in vitro* and *in vivo* (*Fè d'Ostiani* et al., 2000; *Huang* et al., 2001; *Bacci* et al., 2002; *Bozza* et al., 2002a,2003; *Claudia* et al., 2002; *Garlanda* et al., 2002). Both murine and human DCs were able to phagocytose *Candida* yeasts, *Aspergillus* conidia, and hyphae from both (Figure 1). The uptake of the different fungal elements occurred through different forms of phagocytosis. Transmission electronic microscopy indicated that internalisation of yeasts and conidia occurred predominantly by coiling phagocytosis, characterised by the presence of overlapping bilateral pseudopods that led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional zipper-type phagocytosis, characterised by the presence of symmetrical pseudopods which strictly followed the contour of the hyphae before fusion. However, the fate of the different forms of the fungi inside cells appeared to be quite different. Two and four hours later, numerous yeast cells were found partially degraded inside phagosomes. In contrast, as early as one hour after infection, *Candida* hyphae appeared to escape the phagosome and were found lying free in the cytoplasm of cells. For *Aspergillus*, two hours after the exposure, numerous conidia were found inside DCs with no evidence of conidia destruction, as opposed to hyphae, that were rapidly degraded once inside cells. As killing of conidia would seem to be a necessary prerequisite to obtain efficient antigen presentation, it can be postulated that either a small number of conidia are actually degraded by mature DCs thus allowing their antigen processing and presentation or, alternatively, antigens could be processed and regurgitated by other infected phagocytes and then transferred to DCs for presentation.

Multiple receptors on phagocytes and DCs participate in the microbial recognition event either independently or through receptor cooperativity (*Mosser* and *Karp*, 1999). Receptors that have been identified on immature DCs include PRRs, lectins such as the mannose receptors (MR), DEC-205 and DC-SIGN as well as Fc receptors (FcεRI and FcγR) and receptors for a number of components of the complement system (CR). Work on innate recognition of pathogens has defined a number of PAMPs and their cognate

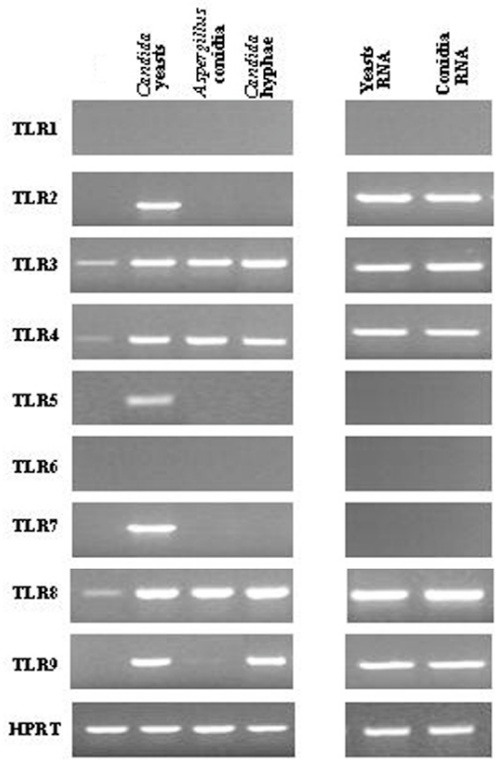

Figure 2: Fungi activate Toll-like receptor expression on dendritic cells. Murine CD11c$^+$ DCs were purified from spleens (for *Candida*) or from lungs (for *Aspergillus*) and exposed to un-opsonised *Candida* yeasts, *Candida* hyphae and *Aspergillus* conidia or fungal RNA, for 60 or 120 min, respectively, as described (*Bacci* et al., 2002; *Bozza* et al., 2003). TLR expression was assessed by RT-PCR. cDNA levels were normalised against the HPRT gene. None, cells exposed to the diluent alone.

PRRs on phagocytes (*Medzhitov* and *Janeway*, 1997; *Romani*, 1996). For fungi, PAMPs include cell-wall components such as glucans, mannans, mannoproteins and phospholipomannan (*Calderone*, 2002) capable of mediating phagocytosis and activation of pro-inflammatory pathways upon recognition by MR and β-glucan receptors, mannose-binding lectins (MBL) and CR3 through the involvement of TLRs 2 and 4 (*Ezekowitz* et al., 1990; *Brown* et al., 2003; *Cambi* et al., 2003; *Wang* et al., 2001; *Mambula* et al., 2002; *Netea* et al., 2002).

Internalisation of yeasts, conidia or hyphae involved different receptors (*Claudia* et al., 2002; *Romani* et al., 2002). Live un-opsonised yeasts, conidia or hyphae were mainly internalised through a phagocytic process. Internalisation of yeasts and conidia occurred through the lectin-like receptors, including MR, DC-SIGN and dectin-1. For hyphae, the internalisation by DCs mainly occurred through CR3 and FcgR II and III. The results are consistent with the view that fungi have exploited common pathways for entry into DCs, which may include a lectin-like pathway for unicellular forms and opsono-dependent pathways for filamentous forms. In terms of sugar specificity, this may vary among fungi, as DCs recognise *Candida* yeasts through a mannose-fucose receptor (*Newman* and

Holly, 2001) and *Aspergillus* conidia through a lectin receptor of galactomannan specificity (*Bozza* et al., 2002a). It also appears that unicellular fungal forms may exploit the CR3 receptor on DCs as a niche to avoid degradation through the multi-lectin pathway while allowing their own persistence. In doing so, fungi share with pathogenic bacteria the ability to avert activation of phagocytes by entry through complement receptors that are not accompanied by phagocyte activation (*Ehlers* and *Daffè*, 1998). Interestingly, the entry of heat-inactivated fungi may occur through different pathways, as inactivated *Candida* yeasts were mainly internalised through CR3 (*Claudia* et al., 2002), a finding that may have important implications in terms of vaccination strategies against fungi.

TLR2 and 4 have been implicated in the activation of phagocytes by fungi (*Wang* et al., ,2001; *Mambula* et al., 2002; *Netea* et al., 2002). It is believed that microbial detection by DCs trough TLRs is responsible for pathogen discrimination and the initiation of the appropriate effector response accordingly (*Schnare* et al., 2001). Distinct patterns of TLR expression were observed on splenic and pulmonary DCs upon exposure to *Candida* and *Aspergillus*, respectively. Both yeasts and conidia up-regulated the expression of TLR3, TLR4 and TLR8, but only yeasts up-regulated the expression of TLR2, TLR5, TLR7 and TLR9. The exposure to *Candida* hyphae was followed by the up-regulated expression of TLR3, TLR4, TLR8 and TLR9 (Figure 2). Similar results were obtained upon exposure to *Aspergillus* hyphae (data not shown). The extent to which TLR expression on DCs implicates the functional activity of TLRs in response to fungi is far from being understood. Nevertheless, it is intriguing that the TLR9 agonist CpG-ODN could convert an *Aspergillus* allergen to a potential protective antigen, suggesting the potential for TLR agonists to act upon the degree of flexibility of the immune recognition pathways to antigens and allergens (*Bozza* et al., 2002b).

It has recently been shown that fungal RNA acts as potent DC activator (*Bacci* et al., 2002; *Claudia* et al., 2002; *Bozza* et al., 2003). Others have shown that pulsing DCs with antigen-encoded mRNA resulted in the loading of both MHC class I and II antigen presentation pathways and the delivery of an activation signal (*Ni* et al., 2002). Although extracellular mRNA induced DC activation by signalling through a nucleotide receptor (*Ni* et al., 2002), fungal RNA also activated TLR expression on DCs (Figure 2). The expression of TLR2, TLR3, TLR4, TLR8 and TLR9 was upregulated upon exposure to fungal RNA from both yeasts and conidia. As DCs efficiently took up extracellular fungal RNA (Figure 3), this indicates that DCs are allowed to orchestrate the immune response against both intracellular and extracellular fungi.

Upon exposure to fungi or fungal RNA, DCs underwent functional maturation, as indicated by the upregulated expression of co-stimulatory molecules and MHC class II antigens and cytokine production (*Bacci* et al., 2002; *Claudia* et al., 2002; *Bozza* et al., 2003). The production of cytokines occurred differently in response to un-opsonised yeasts, conidia and hyphae and the pattern of cytokine production correlated with the pattern of receptor entry and/or the levels of opsonisation. Upon phagocytosis of yeasts or conidia, high and sustained levels of IL-12 were observed. However, DCs produced IL-4 and IL-10 in response to hyphae. It was also found that the receptor exploitation on DCs and fungal opsonisation dramatically affected the pattern of cytokine production.

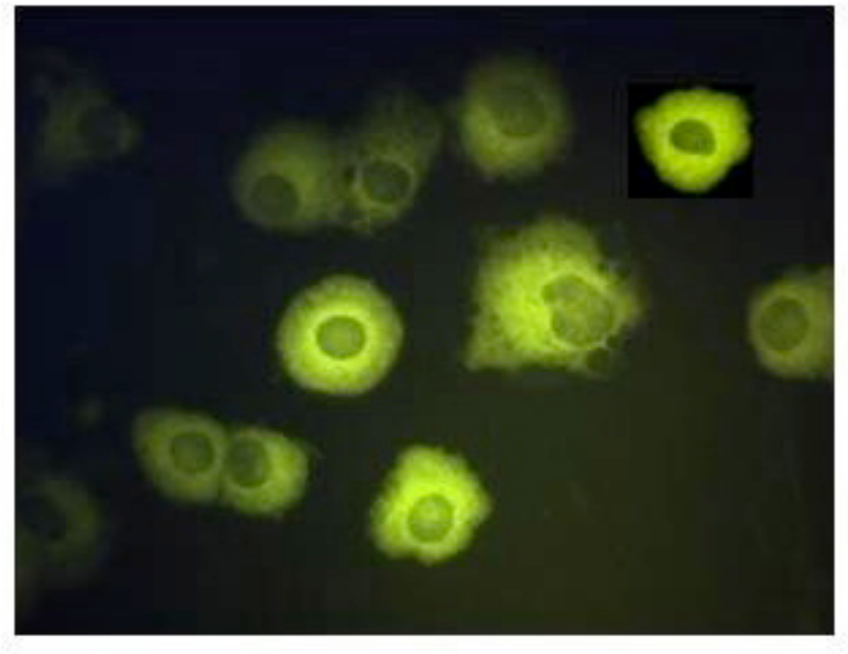

Figure 3: Uptake of fungal RNA by dendritic cells. Fluorescent probe syto17 was added to fungal total RNA (25μg) at the concentration 100μM and incubated for 2 h in dark. After removal of unbound dye, cells were transfected with labelled RNA and the cationic lipid N-[1-(2,3-dioleoyloxypropyl]-*N,N,N*,-trimethylammonium methylsulfate (DOTAP) for 120 min at 37°C (*Bacci* et al., 2002; *Bozza* et al., 2003). Cells were washed with PBS and fixed with paraformaldehyde 4% for 10 min. Photographs were taken using a high Resolution Microscopy Colour Camera AxioCam Colour, using the AuxioVision Software Rel. 3.0.

In vivo

DCs have a primary role in pathogens surveillance at the mucosal surfaces (*Huang* et al., 2001). Studies *in vivo* suggested that DCs had the ability to internalise *Aspergillus* conidia and *Candida* yeasts at the sites of the infection (*Bozza* et al., 2002a; *Montagnoli* et al., 2003). Soon after the infection, *Candida* yeasts were found inside DCs from the gut and *Aspergillus* conidia inside pulmonary DCs. In the case of *Candida*, the fungus appeared to translocate across the epithelial layers and to be subsequently phagocytosed by DCs (unpublished observations). In the case of *Aspergillus*, it should be considered that, in normal circumstances, a state of tolerance to inhaled antigens is achieved through several mechanisms, including IL-10 production by local DCs (*Akbari* et al., 2001). It is known that DCs of the respiratory tract are specialised for uptake/processing but not for antigen presentation, the latter requiring cytokine maturation signals that are encountered after migration to regional lymph nodes. We found that DCs present in the alveolar spaces phagocytosed conidia, translocated to the space below, within the alveolar septal wall, and reached the draining lymph nodes where fungus-pulsed DCs instructed local development of antifungal Th reactivity (see below).

DCs TRANSLATE FUNGUS-ASSOCIATED INFORMATION TO Th LYMPHOCYTES

Upon exposure to *Candida* or *Aspergillus*, DCs activated different types of naive $CD4^+$ Th cells *in vitro* and *in vivo* (*Fè d'Ostiani* et al., 2000; *Bacci* et al., 2002; *Bozza* et al., 2002a,2003; *Claudia* et al., 2002; *Romani* et al., 2002; *Garlanda* et al., 2002). *In vitro*, $CD4^+$ T splenocytes co-cultured with yeast-pulsed DCs produced high levels of IFN-γ, but not IL-4 or IL-10. In contrast, DCs exposed to *Candida* hyphae induced low levels of IFN-γ, but high levels of IL-4 and IL-10. *Candida*-pulsed DCs were also capable of priming antigen-specific $CD4^+$ Th responses *in vivo*. Adoptive transfer of purified DCs, pulsed with yeasts or hyphae, resulted in priming of $CD4^+$ T cells for Th1 or Th2 cytokine production, respectively (see below). *In vivo* studies confirmed that the opsonic phagocytosis of fungi is responsible for type 2 cytokine production and Th2 cell activation, an effect counteracted by the Th1-promoting activity of the non-opsonic entry through MR (*Claudia* et al., 2002). In the case of *Aspergillus*, the migration and maturation of pulmonary DCs in mice with aspergillosis correlated with their ability to induce T cell priming in the lymph nodes and spleens. The number of IFN-γ-producing $CD4^+$ T cells greatly increased in both the lymph nodes and spleens of mice injected with *Aspergillus* conidia, while IL-4-producing cells were increased in mice exposed to hyphae (*Romani* et al., 2002).

There is compelling evidence that Treg specialised in the attenuation of immune responses play a critical role in immune regulation (*Read* and *Powrie*, 2001). Immune responses driven by Th1 and Th2 cells are also influenced by Treg whose main function is counter-regulation or suppression of immune responses mediated by Th1 and Th2. Different types of Treg have been found to be implicated in the control of organ-specific autoimmunity, transplantation tolerance and inflammatory responses evoked by enteric organisms. Pathogen-specific Treg, with immunosuppressive activity, have also been described (*McGuirk* et al., 2002). Although protective immunity to *C. albicans* is mediated by antigen-specific Th1 cells, paradoxically, some Th2 cytokines are required for the maintenance of the antifungal immune resistance (*Romani*, 1997). Therefore, in addition to the Th1/Th2 balance, other mechanisms seem to be involved in the regulation of Th1 immunity to the fungus. A role for DCs in the induction of Treg has been described (*Roncarolo* et al., 2001). DCs from Payer's patches induced the activation of $CD4^+CD25^+$ T cells negatively regulating antifungal Th1 reactivity in mice with gastrointestinal candidiasis (*Montagnoli* et al., 2002). Activation of Treg required DCs expressing co-stimulatory molecules and producing IL-10, the last activity being strictly dependent on local levels of opsonising antibodies (*Montagnoli* eet al., 2003). Adoptive transfer of IL-10-producing *Candida*-pulsed DCs induced the activation of $CD4^+CD25^+$ T cells in the mesenteric lymph nodes, decreased the inflammatory response at sites of infection and contributed to the occurrence of memory protective immunity to the fungus. As hyphae, more than yeasts, are endowed with the ability to activate IL-10-producing DCs, at least *in vitro* (*Montagnoli* et al., 2002), it appears that DCs orchestrate the overall immune response to *C. albicans*, including active priming to the yeasts and tolerance to the hyphae. Whether these apparently contradictory roles could be attributed to distinct DC lineages or to a

single DC type, which are instructed by environmental stimuli to perform different functions is still a matter of debate (*Shortman* and *Heath*, 2001). Nevertheless, our data point out an extreme functional plasticity of DCs in response to the different forms of fungi.

All together, these data indicate that DCs fulfil the requirement of a cell uniquely capable of discriminating between the different forms of fungi in terms of the type of immune response elicited. The emerging paradigm calls for the exploitation of distinct receptors on DCs by the different forms of unopsonised or opsonised fungi and the dependency of the DC activation program and ensuing Th cell response on the receptor choice and mode of entry. Indeed, i) the non-opsonic phagocytosis through MR results in the production of pro-inflammatory cytokines, including IL-12, and expression of co-stimulatory molecules and MHC class II antigens; ii) up-regulation of co-stimulatory molecules also occurs along with the production of IL-4/IL-10 upon the opsonic entry through CR3 and FcgR; iii) both the expression of co-stimulatory molecules and class II antigens and the production of IL-12 are inhibited by entry through CR3. *In vivo* studies confirmed that the opsonic phagocytosis of fungi is responsible for type 2 cytokine production and Th2 cell activation, an effect counteracted by the Th1-promoting activity of the non-opsonic entry through MR. It is conceivable that the balance between the two types of phagocytosis at different body sites very likely determines the type of immune response elicited, which may help to explain the longstanding notion of compartmentalisation in antifungal Th immunity (*Romani* and *Kaufmann*, 1998). The results are also in line with evidences in humans showing an increased susceptibility to fungal infection in patients with defective MBL but not antibody or complement deficiency (*Calderone*, 2002; *Latgé*, 2001).

EXPLOITING DCs AS FUNGAL VACCINES

Fungus-pulsed DCs activated $CD4^+$ Th cell responses upon adoptive transfer into immunocompetent mice (*Bacci* et al., 2002; *Bozza* et al., 2002a,2003). The analysis of antigen specific proliferation and cytokine production by $CD4^+$ T cells from draining lymph nodes and spleens revealed that levels of IFN-γ were higher, and those of IL-4 lower, in mice immunised with yeast- or conidia-pulsed DCs as compared to mice receiving unpulsed or hypha-pulsed DCs. The ability of fungus-pulsed DCs to prime for Th1 and Th2 cell activation upon adoptive transfer *in vivo* correlated with the occurrence of resistance and susceptibility to the infections. Resistance to either *C. albicans* or *A. fumigatus* infection was greatly increased upon transfer of yeast-pulsed or conidia-pulsed DCs, respectively, as indicated by the decreased fungal burden in the target organs. The fungal burden was not reduced upon transfer of unpulsed or hypha-pulsed DCs, being actually increased upon transfer of the latter. Therefore, adoptively transferred fungus-pulsed DCs are able to prime specific antifungal Th responses *in vivo*, the quality of which depends on forms of the fungus and nature of cytokines. Indeed, the ability to induce anti-candidal protective Th1 immunity *in vivo* was impaired upon transfer of DCs exposed to the yeasts in the absence of IL-12, and potentiated upon transfer of DCs exposed to the hyphae in the absence of IL-4 (*Fè d'Ostiani* et al., 2000). These

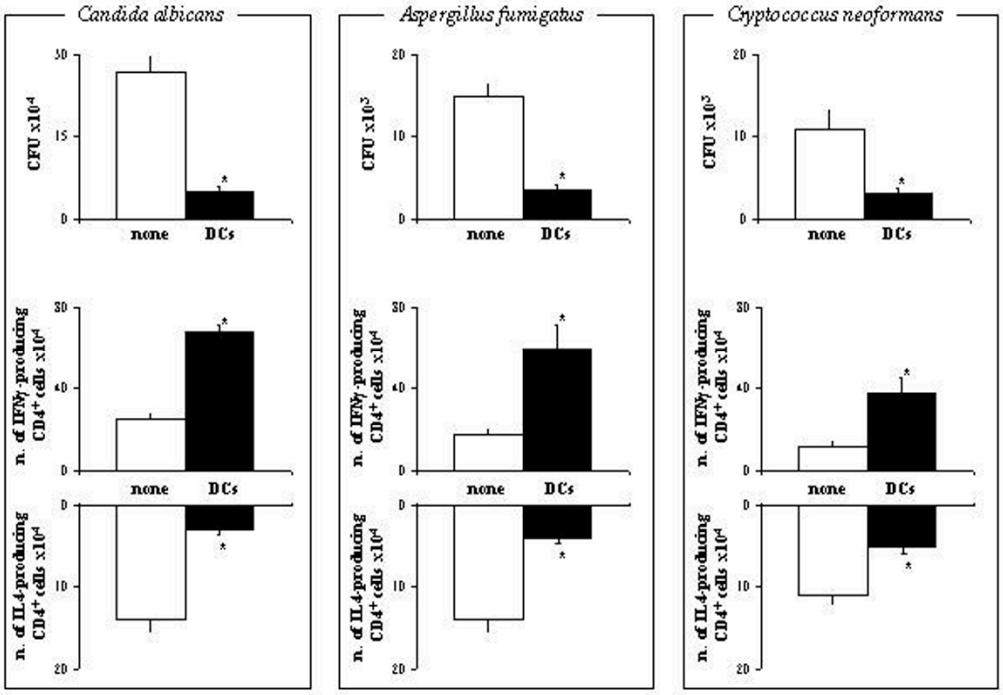

Figure 4: Adoptively transferred fungus RNA-transfected-dendritic cells induce Th1-mediated resistance to fungal infections. Splenic DCs were transfected with RNA from *Candida* or *Cryptococcus* yeasts, or *Aspergillus* conidia as described (*Bacci* et al., 2002; *Bozza* et al., 2003). DCs (5 x 10^5) were administered into recipient mice subcutaneously, 2 and 1 week before the intravenous injection of 5 x 10^5 *C. albicans* or *A. fumigatus* conidia or the intranasal injection of 10^4 *C. neoformans*. Resistance to infection was assessed in terms of colony forming units (CFU, mean ± SE) and number of cytokine producing $CD4^+$ T cells (ELISPOT assay) a week after the infection in the kidneys (candidiasis and aspergillosis) or in the lungs (cryptococcosis) (*Bacci* et al., 2002; *Bozza* et al., 2003). *Indicates $p<0.05$ (mice receiving pulsed DCs versus mice not receiving DCs).

results suggest that production of IL-12 or IL-4 by DCs may crucially contribute to the induction of protective and non-protective immune responses in fungal infection. Interestingly, inactivated yeasts failed to induce DCs maturation *in vitro* and DCs pulsed with inactivated yeasts failed to promote Th1 immunity upon adoptive transfer *in vivo* (data not shown). Therefore, these data may account for the long-standing observation of the inability of inactivated *Candida* to induce memory anti-candidal protective immune responses (*Romani* and *Kaufmann*, 1998).

Antifungal protective immunity *in vivo* was also observed upon adoptive transfer of DCs transfected with fungal RNA (*Bacci* et al., 2002; *Bozza* et al., 2003). The efficacy was restricted to DCs transfected with RNA from yeasts or conidia but not with hyphal RNA. *Ex vivo* DCs, transfected with yeast RNA or conidial RNA, adoptively transferred into otherwise susceptible recipients, conferred protection against *C. albicans* or *A. fumigatus* infection, respectively (Figure 4). The effect was fungus-specific, as no cross-protection was observed upon adoptive transfer of DCs

pulsed with either fungal species (*Bozza* et al., 2003). It is of interest that DCs transfected with RNA from *Cryptococcus neoformans*, an opportunistic fungus on occasion, also induced protection in a murine model of pulmonary cryptococcosis (Figure 4), a finding expanding upon the vaccinating potential of DCs in fungal infections. The frequency of IFN-γ-producing Th1 cells was increased and that of IL-4-producing cells decreased in protected mice (Figure 4), a finding suggesting the occurrence of a Th1-dependent antifungal resistance.

The infusion of fungus-pulsed or RNA-transfected DCs accelerated the recovery of functional antifungal Th1 responses in mice with allogeneic haematopoietic stem cell transplantation (HSCT), an experimental model in which autologous reconstitution of host stem cells is greatly reduced to the benefit of a long-term, donor type chimerism in more than 95% of the mice and low incidence of graft-versus-host disease (*Mencacci* ete al., 2001). Patients receiving T cell-depleted HSCT are unable to develop antigen-specific T cell responses soon after transplantation (*Velardi* et al., 1988). However, functional recovery of the T cell system after T cell-depleted allogeneic HSCT has been demonstrated (*Verfuerth* et al., 2000) and both donor and recipient DCs may participate to the reconstitution of the T cell repertoire in transplantation through distinct pathways of antigen presentation (*Lechler* et al., 2001). We have demonstrated that an imbalanced production of Th1 and Th2 cytokines was responsible for the susceptibility to fungal infections in our HSCT model. However, readdressing the balance between Th1 and Th2 subsets, as by treatment with Th2 cytokine antagonists, accelerated the recovery of Th1-mediated antifungal resistance (*Mencacci* et al., 2001). The recovery of functional Th1 cells producing IFN-γ was accelerated by the infusion of fungus-pulsed or RNA-transfected DCs, a finding suggesting that DCs may contribute to the educational program of T cells in HSCT during reconstitution, as already suggested (*Lechler* et al., 2001).

All together, our studies will suggest that DCs could act as effective vaccines against fungal infections and that RNA-transfected DCs could be of vaccinating potential in conditions that negate the use of attenuated microorganisms, such as immunosuppression, or in the case of poor availability of protective antigens.

CONCLUSIONS AND PERSPECTIVES

DCs have a unique role in infections, as they are regarded as both sentinel for innate recognition and initiator of Th cell differentiation and functional commitment. Through the use of distinct recognition receptors, murine DCs showed a remarkable functional plasticity in the recognition of fungi. It appears that the DC/fungi interaction dynamics, more than fungal dimorphism, could be responsible for fungal virulence. The implications of these findings are manifold. First, as cytokines are known to modulate the expression of opsonic and non-opsonic receptors (*Raveh* et al., 1998), and antibodies differently opsonise fungi (*Casadevall*, 1995), it is likely that the levels of cytokines may influence the DC/fungi interaction *in vivo* and that the different ability of antibodies to opsonise fungi may contribute to the protective and non-protective activity of antibodies in fungal infections (*Casadevall*, 1995). Second, as clinical

resistance represents a significant component of the overall drug resistance of the anti-fungals (*Alexander* and *Perfect*, 1997), one major strategy to prevent antifungal drug resistance is to improve the immune functions of the immunocompromised host. A variety of cytokines, including chemokines and growth factors proved to be beneficial in experimental and clinical fungal infections (*Romani* and *Kaufmann*, 1998). However, establishing the clinical utility of cytokines as therapy for fungal infections in patients has been difficult. The Th1/Th2 balance itself was also found to be the target of immunotherapy. Thus, the deliberate targeting of cells and pathways of cell-mediated immunity to the fungus may represent a useful strategy in developing effective strategies of vaccination to fungi. The ultimate challenge will be to design fungal vaccines capable of inducing optimally effective immunities by targeting specific receptors on DCs *in vivo*. This implicates that we have to learn from pathogens how to manipulate DCs for immunotherapy.

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LITERATURE

Akbari, O., DeKruyff, R.H., and Umetsu, D.T.: Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat. Immunol. 2, 725-731 (2001).

Alexander, B.D. and Perfect, J.R.: Antifungal resistance trends towards the year 2000. Implications for therapy and new approaches. Drugs 54, 657-678 (1997).

Bacci, A., Montagnoli, C., Perruccio, K., Bozza, S., Gaziano, R., Pitzurra, L., Velardi, A., d'Ostiani, C.F., Cutler, J.E., and Romani, L.: Dendritic cells pulsed with fungal RNA induce protective immunity to *Candida albicans* in hematopoietic transplantation. J. Immunol. 168, 2904-2913 (2002).

Bozza, S., Gaziano, R., Spreca, A., Bacci, A., Montagnoli, C., di Francesco, P., and Romani, L.: Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. J. Immunol. 168, 1362-1371 (2002a).

Bozza, S., Gaziano, R., Lipford, G.B., Montagnoli, C., Bacci, A., Di Francesco, P., Kurup, V.P., Wagner, H., and Romani, L.. Vaccination of mice against invasive aspergillosis with recombinant *Aspergillus* proteins and CpG oligodeoxynucleotides as adjuvants. Microb. Infect. 4, 1281-1290 (2002b).

Bozza, S., Perruccio, K., Montagnoli, C., Gaziano, R., Bellocchio, S., Burchielli, E., Nkwanyuo, G., Pitzurra, L., Velardi, A., and Romani, L.: A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation. Blood 102, 3807-3814 (2003).

Brown, G.D., Herre, B., William, D.L., Willment, J.A., Marshall, A.S.J., and Gordon, S.: Dectin-1 mediates the biological effects of β-glucans. J. Exp. Med. 197, 1119-1124 (2003).

Calderone, R.: *Candida* and candidiasis. ASM Press, Washington, DC, 2002.

Cambi, A., Gijzen, K., de Vries, J.M., Torensma, R., Joosten, B., Adema, G.J., Netea, M.G., Kullberg, B.J., Romani, L., and Figdor, C.G.: The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. Eur. J.

Immunol. 33, 532-538 (2003).
Casadevall, A.: Antibody immunity and invasive fungal infections. Infect. Immun. 63, 4211-4218 (1995).
Claudia, M., Bacci, A., Silvia, B., Gaziano, R., Spreca, A., and Romani, L.: The interaction of fungi with dendritic cells: Implications for Th immunity and vaccination. Curr. Mol. Med. 2, 507-524 (2002).
Denning, D.W.: *Aspergillus*. In: Principles and practice of infectious diseases, fifth ed. (Eds.: Mandel, G.L., Bennett, J.E., and Dolin, R.). W.B. Saunders, Philadelphia, 2674-2682 (2000).
d'Ostiani, C.F., Del Sero, G., Bacci, A., Montagnoli, C., Spreca, A., Mencacci, A., Ricciardi-Castagnoli, P., and Romani, L.: Dendritic cells discriminate between yeast and hyphae of the fungus *Candida albicans*: Implications for initiation of T helper cell immunity *in vitro* and *in vivo*. J. Exp. Med. 15, 1661-1674 (2000).
Ehlers, M.R.W. and Daffè, M.: Interactions between *Mycobacterium tuberculosis* and host cells: are mycobacterial sugars the key? Trends Microbiol. 6, 328-335 (1998).
Ezekowitz, R.A., Sastry, K., Bailly, P., and Warner, A.: Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. J. Exp. Med. 172, 1785-1794 (1990).
Gallucci, S., Lolkema, M., and Matzinger, P.: Natural adjuvants: Endogenous activators of dendritic cells. Nature Med. 5, 1249-1255 (1999).
Garlanda, C., Hirsch, E., Bozza, S., Salustri, A., De Acetis, M., Nota, R., Maccagno, A., Riva, F., Bottazzi, B., Peri, G., Doni, A., Vago, L., Botto, M., De Santis, R., Carminati, P., Siracusa, G., Altruda, F., Vecchi, A., Romani, L., and Mantovani, A.: Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. Nature 420, 182-186 (2002).
Hogan, L.H., Klein, B.S., and Levitz, S.M.: Virulence factors of medically important fungi. Clin. Microbiol. Rev. 9, 469-488 (1996).
Huang, Q., Liu, D., Majewski, P., Schulte, L.C., Korn, J.M., Young, R.A., Lander, E.S., and Hacohen, N.: The plasticity of dendritic cell responses to pathogens and their components. Science 294, 870-875 (2001).
Lanzavecchia, A. and Sallusto, F.: Regulation of T cell immunity by dendritic cells. Cell 106, 263-266 (2001).
Latgé, J.P.: The pathobiology of *Aspergillus fumigatus*. Trends Microbiol. 9, 382-389 (2001).
Lechler, R., Ng, W.F., and Steinman, R.M.: Dendritic cells in transplantation - Friend or foe? Immunity 14, 357-368 (2001).
Mambula, S.S., Sau, K., Henneke, P., Golenbock, D.T., and Levitz, S.M.: Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J. Biol. Chem. 277, 39320-39326 (2002).
McGuirk, P., McCann, C., and Mills, K.H.: Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: A novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J. Exp. Med. 195, 221-231 (2002).
Medzhitov, R. and Janeway, C.A. Jr.: Innate immunity: The virtues of a nonclonal system of recognition. Cell 91, 295-298 (1997).
Mencacci, A., Perruccio, K., Bacci, A., Cenci, E., Benedetti, R., Martelli, M.F., Bistoni, F., Coffman, R., Velardi, A., and Romani, L.: Defective antifungal T-helper 1 (TH1) immunity in a murine model of allogeneic T-cell-depleted bone marrow transplantation and its restoration by treatment with TH2 cytokine antagonists. Blood 97, 1483-1490 (2001).
Montagnoli, C., Bacci, A., Bozza, S., Gaziano, R., Mosci, P., Sharpe, A.H., and Romani, L.: B7/CD28-dependent $CD4^{+}CD25^{+}$ regulatory T cells are essential components of the memory protective immunity to *Candida albicans*. J. Immunol. 169, 6298-6308 (2002).
Montagnoli, C., Bozza, S., Bacci, A., Gaziano, R., Mosci, P., Morschhauser, J., Pitzurra, L., Kopf, M., Cutler, J., and Romani, L.: A role for antibody in the generation of memory antifungal immunity. Eur. J. Immunol. 33, 1193-1204 (2003).
Mosser, D.M. and Karp, C.L.: Receptor mediated subversion of macrophage cytokine production by intracellular pathogens. Curr. Opin. Immunol. 11, 406-411 (1999).

Netea, M.G., van der Graaf, C.A., Vonk, A.G., Verschueren, I. van der Meer, J.W., and Kullberg, B.J.: The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. J. Infect. Dis. 185, 1483-1489 (2002).

Newman, S.L. and Holly, A.: *Candida albicans* is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. Infect. Immun. 69, 6813-6822 (2001).

Ni, H., Capodici, J., Cannon, G., Communi, D., Boeynaems, J.M., Kariko, K., and Weissman, D.: Extracellular mRNA induces dendritic cell activation by stimulating tumor necrosis factor-a secretion and signaling through a nucleotide receptor. J. Biochem. Chem. 277, 12689-12696 (2002).

Puccetti, P., Romani, L., and Bistoni, F.: A TH1/TH2-like switch in candidiasis: New perspectives for therapy. Trends Microbiol. 3, 237-244 (1995).

Raveh, D., Kruskal, B.A., Farland, J., and Ezekowitz, R.A.: Th1 and Th2 cytokines cooperate to stimulate mannose-receptor-mediated phagocytosis. J. Leuk. Biol. 64, 108-113 (1998).

Read, S. and Powrie, F.: $CD4^+$ regulatory T cells. Curr. Opin. Immunol. 13, 644-649 (2001).

Romani, L.: Cytokines in the innate and adaptive immunity to *Candida albicans*. In: Cytokines and chemokines in infectious diseases handbook (Eds.: Kotb, M., and Calandra, T.). Humana Press, Totowa, 227-241 (1996).

Romani, L.: The T cell responses against fungal infections. Curr. Opin. Microbiol. 9, 484-490 (1997).

Romani, .L and Kaufmann, S.H.E.: Immunity to fungi. Res. Immunol. 149, 277-281 (1998).

Romani, L., Bistoni, F., and Puccetti, P.: Fungi, dendritic cells and receptors: a host perspective of fungal virulence. Trends Microbiol. 10, 508-514 (2002).

Roncarolo, M.G., Levings, M.K., and Traversari, C.: Differentiation of T regulatory cells by immature dendritic cells. J. Exp. Med. 193, F5-F9 (2001).

Rooney, P.J. and Klein, B.S.: Linking fungal morphogenesis with virulence. Cell Microbiol. 4, 127-137 (2002).

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S., and Medzhitov, R.: Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. 2, 947-950 (2001).

Shortman, K. and Heath, W.R.: Immunity or tolerance? That is the question for dendritic cells. Nat. Immunol. 2, 988-989 (2001).

Shortman, K. and Liu, Y.J.: Mouse and human dendritic cells subtypes. Nat. Immunol. 2, 151-161 (2002).

Steinman, R.M. and Pope, M.: Exploiting dendritic cells to improve vaccine efficacy. J. Clin. Invest. 109, 1519-1526 (2002).

Velardi, A., Terenzi, A., Cucciaioni, S., Millo, R., Grossi, C.E., Grignani, F., and Martelli, M.F.: Imbalances within peripheral blood T-helper (CD4+) and T-suppressor (CD8+) cell population in the reconstitution phase after human bone marrow transplantation. Blood 71, 1196-1200 (1988).

Verfuerth, S.K., Peggs, P.V., Barnett, L., O'Reilly, R.J., and Mackinnon, S.: Longitudinal monitoring of immune reconstitution by CDR3 size spectratyping after T-cell-depleted allogeneic bone marrow transplant and the effect of donor lymphocyte infusion on T-cell repertoire. Blood 95, 3990-3995 (2000).

Wang, J.E., Warris, A., Ellingsen, E.A., Jorgensen, P.F., Flo, T.H., Espevik, T., Solberg, R., Verweij, P.E., and Aasen, A.O.: Involvement of CD14 and Toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae. Infect. Immun. 69, 2402-2406 (2001). Immun. 69, 2402-2406 (2001).

RECOMBINANT ANTIBODIES: A NATURAL PARTNER IN COMBINATORIAL ANTIFUNGAL THERAPY*

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SUMMARY

Monotherapy, in the form of amphotericin B or one of its liposomal derivatives, is the usual treatment for invasive fungal infections, due to lack of a safe, effective combination of antifungal drugs. Combination therapy is not necessarily beneficial – there may be mutual antagonism or indifference, increased toxicity or interference with concomitant medication. But the benefits of a well-tolerated, synergistic combination would be great – the enhanced efficacy would improve clinical outcome, reduce the need for prolonged courses of treatment and prevent the emergence of antifungal drug resistance. Antifungal antibodies would be a natural partner in a combinatorial approach to antifungal therapy. Analysis of the antibody response which occurs in patients with invasive candidiasis, being treated with amphotericin B, showed a close correlation between recovery and antibody to the immunodominant heat shock protein 90 (hsp90). The molecular chaperone hsp90 is essential for yeast viability. Mycograb® is a human recombinant antibody to hsp90 which shows intrinsic antifungal activity and synergy with amphotericin B both *in vitro* and *in vivo*. It is now the subject of a multinational, double-blind, placebo-controlled trial, in patients with culture-confirmed invasive candidiasis on liposomal amphotericin B.

INTRODUCTION

Invasive candidiasis is the most prevalent of the systemic fungal infections. It is a deep-seated, life-threatening form of the infection due to yeasts of the genus Candida. The commonest species is *Candida albicans,* but non-albicans species account for an increasing proportion of the infections. Almost any organ can become infected and the infection frequently disseminates, via the bloodstream, to multiple organs. At risk groups include immunosuppressed and neutropenic patients, organ transplant recipients, individuals receiving total parental nutrition or peritoneal dialysis, those with cerebrospinal fluid shunts and drug abusers. Today one of the most commonly affected patient groups is intensive care unit patients. These patients are debilitated, but not neutropenic, and are particularly at risk if the gastrointestinal tract is damaged, by disease, trauma or surgery, because the gut is a major harbinger of *Candida*.

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Reviewing the literature, *Fridkin* and *Jarvis* (1996) estimated that the mortality attributable to *Candida* was 38%, with crude mortality rates of 50% to 60%.

Treatment of candidiasis is dependent on three main classes of chemotherapeutic agents. The first of these is the polyenes, of which conventional amphotericin B (Fungizone®) is the parent compound. Lipid-based formulations of amphotericin B (Abelcet®, Ambisome® and Amphocil®) were developed to reduce the toxicity of amphotericin B. These are well-established, mainline therapeutics for the treatment of life-threatening, culture-confirmed, deep-seated infections. The second class is the onazoles, such as fluconazole and voriconazole. Fluconazole is the most commonly used antifungal drug for the treatment of superficial candidiasis (thrush). It is sometimes given to high-risk patients as prophylaxis against invasive candidiasis or as empiric treatment in suspected cases. It is not recommended for non-albicans species, many of which are intrinsically resistant to fluconazole (*Martins* and *Rex*, 1996) nor for neutropenic patients because it fungistatic, not fungicidal. Acquired resistance to fluconazole can occur among strains of *C. albicans,* and is particularly seen in patients with AIDS receiving long-term treatment for candidal oesophagitis. The echinocandins, such as caspofungin, are a new class of antifungals. In a recently published study in patients with invasive candidiasis (*Mora-Duarte* et al., 2002), caspofungin was found to be at least as effective as conventional amphotericin B, though differences in efficacy between the two groups were mainly a reflection of failures due to Fungizone toxicity. *Cryptococcus neoformans* is intrinsically resistant (because it lacks the target enzyme) and *Candida parapsilosis* shows relative resistance compared to other candidal species. Caspofungin recently received market authorisation, in the USA and Europe, for the treatment of invasive aspergillosis refractory to amphotericin B.

There are increasing reports of clinically significant antifungal drug resistance (*Espinel-Ingroff*, 1997; *Hope* et al., 2002; *Krcmery* and *Barnes*, 2002; *Kontoyiannis* and *Lewis*, 2002). Combination therapy is therefore being suggested as a means of combating resistance and improving clinical outcome, just as it is for serious bacterial infections (*Kontoyiannis* and *Lewis*, 2002). Potential problems with this approach include: Antagonism between static and cidal drugs, as occurs between fluconazole and amphotericin B (*Arganoza* et al., 1997); indifference between drugs that have the same target; increased risk of side effects when several potentially toxic antifungal drugs are used in combination and greater risk of undesirable interactions with other drugs such as immunosuppressive agents. Ideally an antifungal partner should be synergistic (enhancing the efficacy of the other antifungal drug), broad spectrum (against all clinically significant *Candida* species) and safe (both in terms of intrinsic toxicity and lack of interactions with other drugs). Passive immunotherapy, in the form of a naturally occurring antifungal antibody, has the potential to be an ideal partner for combination therapy.

RATIONALE FOR ANTIBODY THERAPY

Candida species commonly inhabit the mucosal surfaces of the gut and oropharynx, without producing symptoms, being held in check by a wide va-

riety of innate and specific defence systems. But if *Candida* does gain access to the bloodstream, it can spread to set up foci of infection in one or more systemic organs, and thereby become a life-threatening infection. Moreover, *Candida* itself is immunosuppressive, predisposing the patient to additional infections by bacteria, such as *Staphylococcus aureus* (*Carlson*, 1982; *Vartivarian* and *Smith*, 1993). In the past there has been considerable debate over the relative importance of antibody-mediated versus innate and cell-mediated immunity (CMI) in defence against candidiasis. The importance of phagocytic cells such as neutrophils can be demonstrated both experimentally and clinically (candidiasis being associated with deficiencies in the number and function of neutrophils) (*Vartivarian* and *Smith*, 1993). Evidence for the importance of CMI comes from animal studies and the well-documented association between specific defects in CMI and chronic mucocutaneous candidiasis (*Vartivarian* and *Smith*, 1993). Similarly HIV infection is associated with oral and oesophageal candidiasis. However these defects in CMI predispose to superficial, mucocutaneous candidiasis not deep-seated invasive candidiasis (*Matthews* et al., 1988a). Numerous studies have shown immune sera to be protective in animal models of candidiasis involving systemic forms of the infection resulting from intravenous injection with *Candida* (*Casadevall*, 1995; *Matthews* et al., 1996). Many of these early experiments were conducted with immune sera in which the nature of the protective antibody was uncharacterised. Without knowing the titre or specificity of the antibody such experiments were difficult to reproduce. It is now possible not only to characterise an antibody and its target, defining its sequence and mapping epitopes reactivity, but also to bulk produce it to current Good Manufacturing Standards (camp) at an economically viable cost. Antibody-based therapeutics can now be significant contenders in the development of novel antifungal drugs. Unlike vaccines, they avoid the need for the recipient to be immunocompetent and provide an immediate benefit to the patient – as required for a life-threatening infection.

Analysis of the antibody response produced by patients, being treated with amphotericin B, who survived invasive candidiasis, showed that such patients produced a strong, sustained antibody response to the 47 kilodalton antigen (*Matthews* et al., 1984,1987). On sequencing this was identified as the carboxy end of the stress protein heat shock protein 90 (hsp90) (*Matthews* and *Burnie*, 1989; *Panaretou* et al., 1999; *Swoboda* et al., 1995). Fatal cases produced no or falling antibody titres to this antigen. Antibody to this antigen is significantly ($p<0.05$) commoner in patients with systemic candidiasis than those colonised with *Candida* (*Porsius* et al., 1990), and common in patients with AIDS and chronic mucocutaneous candidiasis (*Matthews* et al., 1988a). Epitope mapping (*Matthews* et al., 1991a) defined the immunodominant antibody binding site of hsp90 and this epitope was used to raise both mouse monoclonal and human recombinant antibodies, which were protective in mouse models of invasive candidiasis (*Matthews* et al., 1991b,1995). Homologous epitopes have been identified in both yeasts and filamentous fungi, namely *C. albicans, C. parapsilosis, Torulopsis glabrata, Candida tropicalis, Candida krusei* and *Aspergillus fumigatus* (*Matthews*, 1991; *Santhanam* and *Burnie*, 2000; *Burnie* and *Matthews*, 1991; *Kumar* et al., 1993). Mycograb® is a human genetically recombinant antibody ("grAb") against the immunodominant epitope of the *Candida* hsp90 antigen, which has been produced to

cGMP standards and is currently being assessed in clinical trials in patients with culture-confirmed invasive candidiasis.

HEAT SHOCK PROTEIN 90: AN ANTIGEN TARGET

Heat shock proteins (also known as stress proteins) are ubiquitous families of proteins, produced both constitutively and inducibly, in response to a wide variety of stressful stimuli. The hsp90 family plays an essential role in cell physiology (*Csermely* et al., 1998; *Matthews* et al., 1998) acting as molecular chaperones for a variety of cellular proteins, including steroid receptors, protein kinases involved in signal transduction and endothelial nitric-oxide synthase. Their induction in response to stressful stimuli is a means of helping the cell to protect its components from the degradative effects of stress. When an organism invades the host, its environment becomes highly stressful - temperature, pH, ionic strength and nutritional composition all abruptly change - and it comes under attack from the host's immune system. In response, hsp levels rise in the invading pathogen which, by chaperoning key cellular proteins, helps to counter-balance the degradative effects of this adverse environment. In turn, the hsps themselves have become abundant targets against which the host directs its immune response. There are many bacterial, parasitic and fungal infections in which hsps have been identified as immunodominant antigens, and in some cases it has now been established that this immunity is protective. For example, in animal models of tuberculosis and histoplasmosis, vaccination with hsp65 and hsp60, respectively, induced protective cell-mediated immunity (*Bonnefoy* et al., 1994; *Matthews* et al., 1998). Vaccine-induced antibody to hsp90 has been correlated with protection against malaria in a squirrel monkey model (*Bonnefoy* et al., 1994).

Hsp90 is essential for yeast viability. In the relatively non-virulent yeast *Saccharomyces cerevisiae*, deletion of the two genes encoding hsp82 (the homologue of *C. albicans* hsp90) leads to cell death, while deletion of one gene leaves the yeast viable but unable to grow at higher temperatures (*Borkovich* et al., 1989). Higher concentrations of hsp82 are required for growth at temperatures above the optimal growth temperature. The hsp90 of *C. albicans* can confer hsp90 functions in *S. cerevisiae* (*Panaretou* et al., 1999). Overexpression of hsp90 by a transformant of *S. cerevisiae* was associated with a significant increase in virulence in mice compared to the parent strain, producing an infection more like that seen with *C. albicans* (*Hodgetts* et al., 1996). Therefore, hsp90 appears to be a virulence factor and overexpressed hsp90 may play a key part in helping the yeast adapt to its new stressful environment at higher body temperatures.

Protein extracts from exponentially growing *C. albicans* or *S. cerevisiae* yield not only full length hsp90 but also subfragments of 72-76 kDa and 47 kDa, which are the result of partial degradation within viable yeast cells (*Panaretou* et al., 1999). Mice infected with candidal protoplasts failed to produce an antibody response to hsp90 or its subfragments (*Pitarch* et al., 2001), compatible with loss of this antigen family during removal of the yeast cell wall. Immuno-electronmicroscopy studies suggested partial localisation of the 47 kilodalton antigen in the cell wall (*Matthews* et al., 1988b), and immunohistochemical staining of infected mice kid-

ney sections suggests binding of Mycograb® around yeast cells. Likewise, surface-expressed hsp90 serves as an antigen in Chagas' disease, ascariasis, leishmaniasis, toxoplasmosis and infection due to *Schistosoma mansoni* (*Johnson* et al., 1989; *Kumari* et al., 1994; *Dragon* et al., 1987; *Rojas* et al., 2000; *Streit* et al., 1996).

These features make an antibody-based hsp90 inhibitor, replicating a naturally occurring antibody response to candidal hsp90, an obvious candidate for combination antifungal therapy.

MYCOGRAB®: AN ANTIFUNGAL ANTIBODY AGAINST HSP90

Mycograb® was derived from the anti-hsp90 antibody cDNA of patients recently recovered from invasive candidiasis (*Matthews* and *Burnie*, 2001; *Matthews* et al., 2003). It consists of the antigen-binding variable domains of antibody heavy and light chains linked together to create a recombinant protein which is expressed in *Escherichia coli*. It does not have an Fc component and therefore its activity is not dependent on recruitment of white blood cells or complement. It is simply dependent on its ability to bind to and inhibit hsp90. Its antifungal activity *in vitro* can be demonstrated using assays designed to assess conventional antifungal drugs (*Matthews* et al., 2003). It has shown a broad range of activity against all yeasts tested – compatible with the conserved nature of the target antigen in different yeast species. Using these same assays it is possible to demonstrate synergy with amphotericin B (*Matthews* et al., 2003). In contrast, for all strains examined to date, it has usually shown indifference when used in combination with fluconazole - the exception being a fluconazole-sensitive strain of *C. albicans* with which it showed synergy. This mutual enhancement of activity when combined with amphotericin may simply reflect the effect of combining two drugs directed against two different targets within the fungus or it may have as its basis the increased leakiness of yeast cells in the presence sub-lethal doses of amphotericin, thereby giving Mycograb® greater access to intracellular hsp90 (*Matthews* et al., 2003).

Serum levels of amphotericin B in patients are 1 to 2 μg/ml (*Bekersky* et al., 2002; *Bindschadler* et al., 1969; *Groll* et al., 1998), consistent with a therapeutic response occurring when the minimum inhibitory concentration (MIC) of the *Candida* isolate is equal or less than 0.5 μg/ml (*Rex* et al., 2001), but therapeutic failure when the MIC is greater than to 1 μg/ml (*Nguyen* et al., 1998). Mycograb®, at levels readily achievable in the serum, is able to significantly reduce the MIC of amphotericin B to 0.5 μg/ml or less, even for strains which previously had an MIC > 1 μg/ml (*Matthews* et al., 2003).

In a mouse model of systemic candidiasis, intravenous administration of Mycograb® alone produced a statistically significant improvement in the infections caused by each species examined (*Matthews* et al., 2003). Amphotericin B alone cleared the *C. tropicalis* infection, but failed to clear infections caused by *C. albicans, C. krusei, C. glabrata* or *C. parapsilosis* from one or more organs. By combining Mycograb® with amphotericin B, complete resolution of infection was achieved for *C. albicans, C. krusei* and *C. glabrata*; for *C. parapsilosis* the liver and spleen were cleared, but renal counts were unaltered by either drug alone or in combination (*Matthews* et al., 2003).

The immunological reactivity of Mycograb® with candidal hsp90 can be

demonstrated by immunoblot, immunohistochemistry and by ELISA (presenting the target epitope as a synthetic peptide). Following two dimensional gel electrophoresis of candidal extracts, the Mycograb® preferentially binds to the two truncated forms of hsp90, represented by the 40 and 47 kDa spots – indicating that the epitope is more accessible to antibody binding here than in the full-length hsp90 protein (*Matthews* et al., 2003). This is compatible with the observation that patients recovering from invasive candidiasis much more commonly had antibody against the 47 kDa antigen band on immunoblots of a one-dimensional gel of *Candida* than antibody to the full-length hsp90 protein (*Matthews* et al., 1984, 1987; *Matthews* and *Burnie*, 1989).

At the primary structure level, hsp90 is composed of three domains: The N-terminal region (Met^{1}-Arg^{400}), the middle region (Glu^{401}-Lys^{615}) and the C-terminal region (Asp^{621}-Asp^{732}) (*Matsumoto* et al., 2002). The assembly of the hsp90-substrate protein complex requires ATP and involves a conformational change in the hsp90. Hsp90 has two ATP binding sites, one in the C-terminal domain and one in the N-terminal domain. The C-terminal ATP binding site is the first example of a cryptic chaperone nucleotide-binding site, which is opened by occupancy of the N-terminal site (*Soti* et al., 2002). This process requires communication between these two sites through the middle domain, which has a γ-phosphate-binding motif similar to other GHKL family members involving QQSKILKVI, which overlaps the N-terminal end of the peptide recognised by Mycograb® (*Matthews* et al., 1991a; *Dutta* and *Inouye*, 2000). The importance of the middle region in yeast hsp90 is illustrated by the finding that point mutations in this domain caused deficient binding to the N-terminal region which in turn was associated with the yeast cells being unable to grow higher temperatures (37°C) (*Matsumoto* et al., 2002). Since the interaction between the N-terminal and middle regions is essential for the *in vivo* function of hsp90 in yeasts (*Matsumoto* et al., 2002), this could explain the antifungal activity of both Mycograb® (which binds the middle region) and radicicol (which binds the N-terminal region) (*Schulte* et al., 1999).

Hsp90 is part of the steroid hormone receptor superfamily of proteins (*Pratt*, 1993). In the water mold *Achlya ambisexualis*, sexual reproduction involves branching in the opposite mating type, which is induced by the binding of a steroid hormone to a steroid hormone receptor complexed with hsp90 (*Brunt* and *Silver*, 1986). Induction of hsp90 by steroids may be responsible for the upregulation of the stress response in *C. albicans* observed following treatment with 17-β-oestradiol (*O'Connor* et al., 1998). The frequency of *Candida* infections in pregnant women and the oestrogen-dependence of *Candida* colonisation in the rat model, could be linked to this steroid-induced enhanced protection, which results in the treated yeasts becoming resistant to an otherwise lethally high temperature (48.5°C) and oxidative stress (menadione exposure). Exposure to the steroid also induced yeast-to-hyphal transformation (which is thought to be linked to virulence) and increased colony size.

There may be additional means by which Mycograb® achieves benefits in the infected human host, since the epitope recognised by this antibody is highly conserved and present in human hsp90 (*Hickey* et al., 1986; *Matthews* et al., 1991a). The binding of hsp90 to endothelial nitric oxide synthase leads to the release of nitric oxide (*Garcia-Gardena* et al., 1998) which in turn regulates cardiovascular haemodynam-

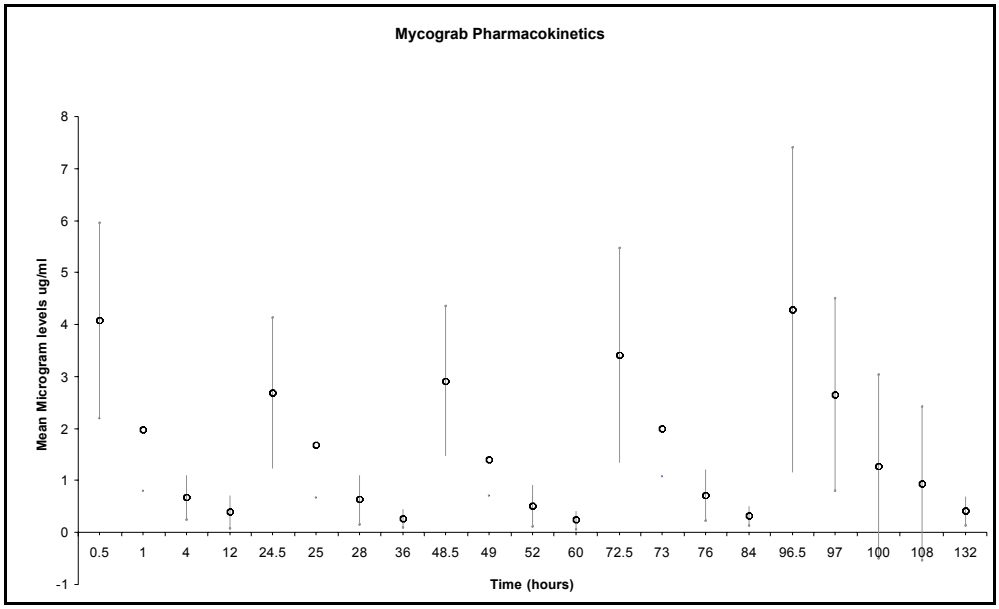

Figure 1: Mean serum levels (with standard deviation) of Mycograb® in 10 patients given Mycograb® at a dose of 1 mg/kg body weight b.d. for 5 days.

ics and causes large vessel vasodilatation. Other pathways catalysed by hsp90 include activation of the prekallikrein-kininogen complex, leading to release of bradykinin, another active biological – responsible, for example, for the angio-oedema seen with ACE inhibitors (*Kusukam* et al., 2002). Inhibition of such pathways, which could be activated by release of endogenous human hsp90 from damaged tissues or candidal hsp90 from lysed yeasts, may be of benefit in counteracting many of the signs of septic shock.

CLINICAL TRIALS

Mycograb® was first assessed in an open-labelled, tolerance and pharmocokinetic study, carried out in the UK, involving five patients with invasive candidiasis receiving liposomal amphotericin B (Abelcet®). After a test dose of Mycograb® (0.1 mg/kg), a single dose of Mycograb® was given (1 mg/kg) followed, at least 24 hours later, by two doses (1 mg/kg) given 8 or12 hours apart. All patients were closely monitored both clinically and by a wide range of laboratory parameters (blood chemistry, haematology, coagulation profile). No treatment-related adverse effects were observed. Mycograb® was undetectable at the 0.1 mg/kg dose. Blood samples taken 30 minutes after receiving a single i.v. bolus of 1 mg/kg, gave serum levels ranging from 1.5 to 4.0 mg/l. Serum levels become undetectable by 8 hours. When two doses were given, with an interval of 8 hours or 12 hours, a slight increase in the levels occurred following the second dose indicating some tissue accumulation. This first study was designed to obtain preliminary data on safety and pharmacokinetics, and not for the assessment of efficacy, but among the three patients who

Table 1: The serum pharmacokinetic data from the same 10 patients receiving Mycograb® over a 5-day period. The data was interpreted by non-compartmental pharmacokinetic analysis PK Solutions 2.0 for C_{max} (μg/ml), AUC_{0-t}, $AUC_{0-\infty}$, (μg (min/ml), $t_{1/2\alpha}$ (min), $t_{1/2\beta}$ (hours) and Mean Residence Time (MRT) (hours)

	Day number (n = 10)				
	1	2	3	4	5
C_{max} (μg/ml)	9.9	4.9	7.4	6.6	8.3
AUC_{0-t} (μg (min/ml)	13.2	10.0	9.5	12.1	27.7
$AUC_{0-\infty}$ (μg (min/ml)	19.2	12.6	12.1	15.3	34.8
$t_{1/2\alpha}$ (min)	18	24	18	24	24
$t_{1/2\beta}$ (hours)	10.7	6.5	7.4	6.8	12.0
MRT (hours)	10.8	6.8	6.9	6.9	14.4

received the full dose of 1mg/kg b.d., albeit only for 24 hours, there was an association with cultures becoming negative and improvement in one or more clinical parameters.

Mycograb® is now the subject of a double-blind, placebo-controlled efficacy and safety study involving over 30 centres in 10 countries. All patients have culture-confirmed invasive candidiasis and are being treated with liposomal amphotericin B (Abelcet® or Ambisome®), in combination with a 5 day course of Mycograb® (1 mg/kg b.d.) or placebo (saline). Patients are carefully monitored both clinically and by laboratory parameters, including fungal cultures. Assessment of efficacy is based on clinical response, mycological response, overall mortality and *Candida*-associated mortality. In addition, the test and control arm will be compared to determine whether the need for prolonged courses of amphotericin B (> 10 days) is less in the Mycograb®-treated group – the aim being to develop a shorter, more effective course of treatment, using this combination of antifungals, in place of a prolonged course of monotherapy with its associated increased cost and risk of toxicity.

So far, blood samples taken for pharmacokinetics (Figure 1) have suggested serum levels are not affected by varying degrees of renal insufficiency, liver failure or the patient receiving haemodialysis. Mycograb® was not detectable in urine samples The data was interpreted by non-compartmental pharmacokinetic analysis PK Solutions 2.0 for C_{max} (μg/ml), AUC_{0-t}, $AUC_{0-\infty}$, (μg (min/ml), $t_{1/2\alpha}$ (min), $t_{1/2\beta}$ (hours) and Mean Residence Time (MRT) (hours). This showed (Table 1) that the C_{max} levels obtained were generally in the range required to achieve demonstrable synergy with amphotericin B *in vitro* for the strains of *C. albicans, C. krusei* and *C. tropicalis* (4 μg/ml) and *C. glabrata and C. parapsilosis* (8 μg/ml). Mouse pharmacokinetic studies suggest that tissue levels may be sustained for longer periods than serum levels (*Matthews* et al., 2003).

OTHER DISEASE TARGETS

Other infectious diseases in which hsp90 plays a key role in the physiology of the organism and its ability to meet the challenge of survival in the human

host, could benefit from treatment with an hsp90 inhibitor such as Mycograb®. Hsp90 is an immunodominant antigen in *Aspergillus fumigatus* (*Burnie* and *Matthews*, 1991; *Kumar* et al., 1993). The role of humoral immunity in host defence against aspergillosis is uncertain, but Mycograb®, given in combination with a cell-wall active antifungal such as amphotericin B or an echinocandin, may be able to reach the target hsp90 and inhibit it, and thereby be of benefit in the treatment of invasive aspergillosis. Since invasive aspergillosis is relatively refractory to treatment, it is likely that a more prolonged course, and possibly higher doses, of Mycograb® would be required in such cases.

Several different families of hsps play important roles in parasitic infections, being involved in differentiation, protection from the host cell's killing mechanisms and virulence (*Polla*, 1991). The importance of humoral immunity to malaria was demonstrated by *Cohen* et al. (1961), who showed clinical improvement in African children suffering from severe malaria following passive transfer of immunoglobulin from immune adults. *Bonnefoy* et al. (1994), in a squirrel monkey vaccination trial found a close correlation between antibody response to hsp90 and resistance to heavy challenge from highly virulent *Plasmodium falciparum*. Analysis of the antibody responses to hsp90, hsp70 and hsp65 in Thai patients with malaria showed that antibody titres to hsp90 were particularly high (*Zhang* et al., 2001). Recently it has been shown that hsp90 is essential for *P. falciparum* growth in human erythrocytes, suggesting hsp90 as a potential drug target for antimalarials (*Banumathy* et al., 2003).

Other candidal antigens which could be used as targets for the development of therapeutic antifungal antibodies include cell-surface adhesins, antibodies to which can prevent binding of the yeast to host cell receptors (*Lee* et al., 1996), heat shock mannoproteins (*Polonelli* et al., 1994a) and yeast killer-toxin-like anti-idiotype antibodies (*Polonelli* et al., 1994b). The close association between recovery from cryptococcosis and the host's antibody response to the polysaccharide capsule of this yeast makes antibody therapy an attractive goal, which is being explored by *Casadevall* and co-workers. Passive administration of monoclonal antibodies against the capsular polysaccharide of *C. neoformans* can prolong the survival of lethally infected mice (*Shapiro* et al., 2002), provided the murine antigen-binding V regions are paired with human C regions of the correct isotype, in these mouse-human chimeric antibodies (*McLean* et al., 2002).

CONCLUSION

Antifungal antibodies offer a new approach to the treatment of these important, life-threatening infections. They provide a means of directly applying our growing knowledge of the immunology and pathogenesis of candidiasis to the development of completely novel therapeutics. By using passive antibody therapy rather than vaccines, they avoid the need for the recipient to be immunocompetent and provide an immediate benefit to the patient. They are a natural partner for combination therapy. Mycograb® has been primarily designed for use in combination with existing cell-wall active antifungal drugs, which facilitate its penetration to the target hsp90 antigen. It is believed that the

synergy between Mycograb® and amphotericin B will provide a much more effective therapeutic combination, which treats the infection relatively quickly, thereby reducing cost and risk of toxicity. Future potential applications lie in the treatment of other infectious diseases in which hsp90 plays a key role in the survival of the pathogen in the host, and may include invasive aspergillosis and malaria.

LITERATURE

Arganoza, M.T., Vazquez, J.A., Yoon, S., and Akins, R.A.: Differential susceptibilities and azole antagonisms of *Candida* species to amphotericin B. ICAAC, Abstract D-133 (1997).

Banumathy, G., Singh, V., Pavithra, S.R., and Tatu, U.: Heat shock protein 90 function is essential for *Plasmodium falciparum* growth in human erythrocytes. J. Biol. Chem. 278, 18336-18345 (2003).

Bekersky, I., Fielding, R.M., Dressler, D.E., Lee, J.W.E., Buell, D.N., and Walsh, T.J.: Plasma protein binding of amphotericin B and pharmocokinetics of bound versus unbound amphotericin B after administration of intravenous liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate. Antimicrob. Agents Chemother. 46, 834-840 (2002).

Bindschadler, D.D., Bennet, J.E., and Abernathy, R.S.: A pharmacologic guide to the clinical use of amphotericin B. J. Infect. Dis. 120, 427-436 (1969).

Bonnefoy, S., Gysin, J., Blisnick, T., Guillotte, M., Carcy, B., Pereira da Silva, L., and Mercereau-Puijalon, O.: Immunogenicity and antigenicity of a *Plasmodium falciparum* protein fraction (90-110 kDa) able to protect squirrel monkeys against asexual blood stages. Vaccine 12, 32-40 (1994).

Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J., and Lindquist, S.: Hsp82 is an essential protein that is required at higher temperatures. Mol. Cell. Biol. 9, 3919-3930 (1989).

Brunt, S.A. and Silver, J.C.: Cellular localization of steroid hormone-regulated proteins during sexual development in *Achlya*. Exp. Cell Res. 165, 306-319 (1986).

Burnie, J.P. and Matthews, R.C.: Heat Shock Protein 88 and *Aspergillus* infection. J. Clin. Microbiol. 29, 2099-2106 (1991).

Carlson, E.: Synergistic effect of *Candida albicans* and *Staphylococcus aureus* on mouse mortality. Infect. Immun. 38, 921-924 (1982).

Casadevall, A.: Antibody immunity and invasive fungal infections. Infect. Immun. 63, 4211-4218 (1995).

Cohen, S., McGregor, I.A., and Carrington, S.: Gamma-globulin and acquired immunity to human malaria. Nature 192, 733-737 (1961).

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., and Nardai, G.: The 90 kDa molecular chaperone family: Structure, function and clinical applications. A comprehensive review. Pharmacol. Ther. 79, 129-168 (1998).

Dragon, E.A., Sias, S.R., Kato, E.A., and Gabe, J.D.: The genome of *Trypanosoma cruzi* contains a constitutively expressed, tandemly arranged multicopy gene homologous to a major heat shock protein. Mol. Cell. Biol. 7, 1271-1275 (1987).

Dutta, R. and Inouye, M.: GHKL, an emergent ATPase/kinase superfamily. Trends Biochem. Sci. 25, 24-28 (2000).

Espinel-Ingroff, A.: Clinical relevance of antifungal resistance. Infect. Dis. Clin. North Am. 11, 929-944 (1997).

Fridkin, S.K. and Jarvis, W.R.: Epidemiology of nosocomial fungal infection. Clin. Microbiol. Rev. 9, 499-511 (1996).

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W.C.: Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature 392, 821-824 (1998).

Groll, A.H., Piscitelli, S.C., and Walsh, T.J.: Clinical pharmacology of systemic antifungal agents: A comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. Adv. Pharmacol. 44, 343-499 (1998).

Hickey, E., Brandon, S.E., Sadis, S., Smale, G., and Weber, L.A.: Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia. Gene 43, 147-154 (1986).

Hodgetts, S., Matthews, R.C., Morrissey, G., Mitsutake, K., Piper, P., and Burnie, J.P.: Over-expression of *Saccharomyces cerevisiae* Hsp90 enhances virulence of this yeast in mice. FEMS Immunol. Med. Microbiol. 16, 229-234 (1996).

Hope, W., Morton, A., and Eisen, D.P.: Increase in prevalence of nosocomial non-*Candida albicans* candidaemia and the association of *Candida krusei* with fluconazole use. J. Hosp. Infect. 50, 56-65 (2002).

Johnson, K.S., Wells, K., Bock, J.V., Nene, V., Taylor, D.W., and Cordingley, J.S.: The 86-kilodalton antigen from *Schistosoma mansoni* is a heat-shock protein homologous to yeast HSP-90. Mol. Biochem. Parasitol. 36, 19-28 (1989).

Kontoyiannis, D.P. and Lewis, R.E.: Antifungal drug resistance of pathogenic fungi. Lancet 359, 1135-1144 (2002).

Krcmery, V. and Barnes, A.J.: Non-albicans *Candida* spp. causing fungaemia: Pathogenicity and antifungal resistance. J. Hosp. Infect. 50, 243-260 (2002).

Kumar, A., Reddy, L.V., Sochanik, A., and Kurup, V.P.: Isolation and characterization of a recombinant heat shock protein of *Aspergillus fumigatus*. J. Allergy Clin. Immunol. 91, 1024-1030 (1993).

Kumari, S., Lillibridge, C.D., Bakeer, M., Lowrie, R.C., Jayaraman, K., and Philipp, M.T.: *Brugia malayi*: The diagnostic potential of recombinant excretory/secretory antigens. Exp. Parasitol. 79, 489-505 (1994).

Kusumam, J., Tholanikunnel, B.G., and Kaplan, A.P.: Heat shock protein 90 catalyzes activation of the prekallikrein-kininogen complex in the absence of factor X11. Proc. Natl. Acad. Sci. USA. 99, 896-900 (2002).

Lee, K.K., Yu, L., Macdonald, D.L., Paranchych, W., Hodges, R.S., and Irvin, R.T.: Anti-adhesin antibodies that recognize a receptor-binding motif (adhesintope) inhibit pilus/fimbrial-mediated adherence of *Pseudomonas aeruginosa* and *Candida albicans* to asialo-GM1 receptors and human buccal epithelial cell surface recptors. Can J. Microbiol. 42, 479-486 (1996).

Martins, M.D. and Rex, J.H.: Resistance to antifungal agents in the critical care setting: Problems and perspectives. New Horizons 4, 338-344 (1996).

Matsumoto, S., Tanaka, E., Nemoto, T.K., Ono, T., Takagi, T., Imai, J., Kimura, Y., Yahara, I., Kobayakawa, T., Ayuse, T., Oi, K., and Mizuno, A.: Interaction between the N-terminal and middle regions is essential for the *in vivo* function of hsp90 molecular chaperone. J. Biol. Chem. 277, 34959-34966 (2002).

Matthews, R.C., Burnie, J.P., and Tabaqchali, S.: Immunoblot analysis of the serological response in systemic candidosis. Lancet ii, 1415-1418 (1984).

Matthews, R.C., Burnie, J.P., and Tabaqchali, S.: Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to *Candida albicans*. J. Clin. Microbiol. 25, 230-237 (1987).

Matthews, R., Burnie, J., Smith, D., Clark, I., Midgley, J., Conolly, M., and Gazzard, B.: *Candida* and AIDS: Evidence for protective antibody. Lancet 101, 263-266 (1988a).

Matthews, R., Wells, C., and Burnie, J.: Characterisation and cellular localisation of the immunodominant 47-KDa antigen of *Candida albicans*. J. Med. Microbiol. 27, 227-232 (1988b).

Matthews, R.C. and Burnie, J.P.: Cloning of a DNA sequence encoding a major fragment of the 47 kilodalton stress protein homologue of *Candida albicans*. FEMS Microbiol. Lett. 60, 25-30 (1989).

Matthews, R.C.: HSP 90, yeasts and *Corynebacterium jeikeium*. Epidemiol. Infect. 107, 273-283 (1991).

Matthews, R.C., Burnie, J.P., and Lee, W.: The application of epitope mapping in the development of a new serological test for systemic candidosis. J. Immunol. Methods 143, 73-79 (1991a).

Matthews, R.C., Burnie, J.P., Howat, D., Rowland, T., and Walton, F.: Autoantibody to heat-shock protein 90 can mediate protection against systemic candidosis. Immunology 74, 20-24 (1991b).

Matthews, R., Hodgetts, S., and Burnie, J.: Preliminary assessment of a human recombinant antibody fragement to hsp90 in murine invasive candidiasis. J. Infect. Dis. 171, 1668-1671 (1995).

Matthews, R. and Burnie, J.: Antibodies

against *Candida*: Potential therapeutics? Trends Microbiol. 4, 354-358 (1996).

Matthews, R.C., Maresca, B., Burnie, J.P., Cardona, A., Carratu, L., Conti, S., Deepe, G.S., Florez, A.M., Franceschelli, S., Garcia, E., Gargano, L.S., Kobayashi, G.S., McEwen, J.G., Ortiz, B.L., Oviedo, A.M., Polonelli, L., Ponton, J., Restrepos, A., and Storlazzi, A.: Stress proteins in fungal diseases. Med. Mycol. 36 (Suppl. 1), 45-51 (1998).

Matthews, R. and Burnie, J.: Antifungal antibodies: A new approach to the treatment of systemic candidiasis. Curr. Opin. Invest. Drugs 2, 472-476 (2001).

Matthews, R.C., Rigg, G., Hodgetts, S., Carter, T., Chapman, C., Gregory, C., Illidge, C., and Burnie, J.P.: Preclinical assessment of the efficacy of mycograb, a human recombinant antibody against fungal hsp90. Antimicrob. Agents Chemother. 47, 2208-2216 (2003).

McLean, G.R., Torres, M., Elguezabal, N., Nakouzi, A., and Casadevall, A.: Isotype can affect the fine specificity of an antibody for a polysaccahride antigen. J. Immunol. 169, 1379-1386 (2002).

Mora-Duarte, J., Betts, R., Rotstein, C., Colombo, A.L., Thompson-Moya, L., Smietana, J., Lupinacci, R., Sable, C., Kartsonis, N., Perfect, J.; Caspofungin Invasive Candidiasis Study Group: Comparison of caspofungin and amphotericin B for invasive candidiasis. New. Engl. J. Med. 347, 2020-2029 (2002).

Nguyen, M.H., Clancy, C.J., Yu, V.L., Yu, Y.C., Morris, A.J., Snydman, D.R., Sutton, D.A., and Rinaldi, M.G.: Do *in vitro* susceptibility data predict the microbiologic response to Amphotericin B? Results of a prospective study of patients with *Candida* fungemia. J. Infect. Dis. 177, 425-430 (1998).

O'Connor, C., Essman, M., and Larsen, B.: 17-β-estradiol upregulates the stress response in *Candida albicans*: Implications for microbial virulence. Infect. Dis. Obstetrics Gynecol. 6, 176-181 (1998).

Panaretou, B., Sinclair, K., Prodromou, C., Johal, J., Pearl, L., and Piper, P.W.: The Hsp90 of *Candida albicans* can confer Hsp90 functions in *Saccharomyces cerevisiae*: A potential model for the processes that generate immunogenic fragments of this molecular chaperone in *C. albicans* infections. Microbiology 145, 3455-3463 (1999).

Pitarch, A., Diez-Orejas, R., Molero, G., Pardo, M., Sanchez, M., Gil, C., and Nombela, C.: Analysis of the serologic response to systemic *Candida albicans* infection in a murine model. Proteomics 1, 550-559 (2001).

Polla, B.S.: Heat shock proteins in host-parasite interactions. Parasitol. Today 7, 38-41 (1991).

Polonelli, L., Gerloni, M., Conti, S., Fisicaro, P., Cantelli, C., Portincasa, P., Almondo, F., Barea, P.L., Hernando, F.L., and Ponton, J.: Heat shock mannoproteins as targets for secretory IgA in *Candida albicans*. J. Infect. Dis. 169, 1401-1405 (1994a).

Polonelli, L., De Bernardis, F., Conti, S., Boccanera, M., Gerloni, M., Morace, G., Magliani, W., Chezzi, C., and Cassone, A.: Idiotypic intravaginal vaccination to protect against candidal vaginitis by secretory yeast killer toxin-like anti-idiotype antibodies. J. Immunol. 152, 3175-3182 (1994b).

Porsius, J.C., van Vliet, H.J.A., van Zeijl, J.H., Goessens, W.H.F., and Michel, M.F.: Detection of an antibody response in immunocompetent patients with systemic candidiasis or *Candida albicans* colonisation. Eur. J. Clin. Microbiol. Infect. Dis. 9, 352-355 (1990).

Pratt, W.: The role of heat shock proteins in regulating the function, folding and trafficking of the glucocorticoid receptor. J. Biol. Chem. 268, 21455-21459 (1993).

Rex, J.H., Pfaller, M.A., Walsh, T.J., Chaturvedi, V., Espinel-Ingroff, A., Ghannoum, M.A., Gosey, L.L., Odds, F.C., Rinaldi, M.G., Sheehan, D.J., and Warnock, D.W.: Antifungal usceptibility testing: Practical aspects and current challenges. Clin. Microbiol. Rev. 14, 643-658 (2001).

Rojas, P.A., Martin, V., Nigro, M., Echeverria, P.C., Guarnera, E.A., Pszenny, V., and Angel, S.O.: Expression of cDNA encoding a *Toxoplasma gondii* protein belonging to the heat- shock 90 family and analysis of its antigenicity. FEMS Microbiol. Lett. 190, 209-213 (2000).

Santhanam, J. and Burnie, J.P.: A PCR-based approach to sequence the *Candida tropicalis*

HSP90 gene. FEMS Immunol. Med. Microbiol. 29, 35-38 (2000).

Schulte, T., Akinaga, S., Murakata, T., Agatsuma, T., Sugimoto, S., Nakano, H., Lee, Y.S., Simen, B.B., Argon, Y., Felts, S., Toft, D.O., Neckers, L.M., and Sharma, S.V.: Interaction of radicicol with members of the heat shock protein 90 family of molecular chaperones. Mol. Endocrinol. 13, 1435-1448 (1999).

Shapiro, S., Beenhouwer, D.O., Feldmesser, M., Taborda, C., Carroll, M.C., Casadevall, A., and Scharff, M.D.: Immunoglobulin G monoclonal antibodies to *Cryptococcus neoformans* protect mice deficient in complement component C3. Infect. Immun. 70, 2598-2604 (2002).

Soti, C., Racz, A., and Csermely, P.: A nucleotide-dependent molecular switch controls ATP binding at the C-terminal domain of hsp90. J. Biol. Chem. 277, 7066-7075 (2002).

Streit, J.A., Donelson, J.E., Agey, M.W., and Wilson, M.E.: Developmental changes in the expression of *Leishmania chagasi* pg63 and heat shock protein in a human macrophage cell line. Infect. Immun. 64, 1810-1818 (1996).

Swoboda, R.K., Bertram, G., Budge, S., Gooday, G.W., Gow, N.A.R., and Brown, A.J.P.: Structure and regulation of the HSP90 gene from the pathogenic fungus *Candida albicans*. Infect. Immun. 63, 4506-4514 (1995).

Vartivarian, S. and Smith, C.B.: Pathogenesis, host resistance and predisposing factors. In: Candidiasis (Ed.: Bodey, G.P.). 2nd edition. Raven Press Ltd., New York, 59-84 (1993).

Zhang, M., Hisaeda, H., Kano, S., Matsumoto, Y., Hao, Y.P., Looaresuwan, S., Aikawa, M., and Himeno, K.: Antibodies specific for heat shock proteins in human and murine malaria. Microbes Infect. 3, 363-367 (2001).

BIOLOGIC PROPERTIES AND VACCINE POTENTIAL OF THE STAPHYLOCOCCAL POLY-N-ACETYL GLUCOSAMINE SURFACE POLYSACCHARIDE*

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SUMMARY

Staphylococci have become the most common causes of nosocomial bacterial infections, and this fact, along with increasing problems associated with antimicrobial resistance, spurs the need for finding immunotherapeutic alternatives to prevent and possibly treat these infections. Most virulent, clinical isolates of both coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* carry the *ica* locus which encodes proteins that synthesise a polymer of ß-1-6 linked N-acetyl glucosamine residues (PNAG). Animal studies have shown purified PNAG can elicit protective immunity against both CoNS and *S. aureus*, suggesting its potential as a broadly protective vaccine for many clinically important strains of staphylococci.

INTRODUCTION

In the past 25 years Gram-positive cocci in general, and staphylococci in particular, have become the primary bacterial organisms isolated from nosocomial infections (*Richards* et al., 1999; *Sohn* et al., 2001). Associated with this increase in occurrence is the increase in antimicrobial resistance (*Lowly*, 2003; *DeLisle* and *Perl*, 2003) which has lead to intense interest in alternative strategies to prevent and control infection. One obvious approach is the development of immunotherapeutics that could be used prophylactically for prevention of infection in high-risk patients and possibly therapeutically as an adjunct for standard antibiotic therapy. The challenge of developing such reagents lies principally in identification of antigenic targets for vaccines and definition of immune effectors that mediate resistance to infection.

For extant vaccines that prevent bacterial infections by targeting the killing of the microbial cell, surface polysaccharides have been the most effective. Usually these are referred to as capsular polysaccharides, and immunogenic polysaccharides or protein-polysaccharide conjugates from *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* have proven

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highly successful in controlling infections due to these pathogens (*Lakshman* and *Finn*, 2002; *Pelton*, 2002; *Obaro*, 2002; *Pozsgay*, 2000; *Barbour* et al., 1995; *Ward*, 1991). Many comparable vaccines based on surface polysaccharides are being developed for bacterial pathogens such as group B streptococcus (*Baker* et al., 1999), *Klebsiella pneumoniae* (*Campbell* et al., 1996), Enterococci (*Huebner* et al., 1999,2000) and *Pseudomonas aeruginosa* (*Theilacker* et al., 2003; *Hatano* and *Pier*, 1998). This is predicated upon the strong consensus that when it is feasible to induce immunity to bacterial surface polysaccharides this usually results in the most effective vaccine.

For *Staphylococcus aureus*, two major groups of surface polysaccharides have been identified and targeted for vaccine development. Work by Karakawa and colleagues (*Fournier* et al., 1984; *Sompolinsky* et al., 1985; *Moreau* et al., 1990) established a capsule typing system for *S. aureus* composed on 11 different serologic types. Two of these, types 1 and 2, appear to be expressed by only individual clones of *S. aureus* and are not found among clinical isolates (*Murthy* et al., 1983; *West* et al., 1987). However, for the remaining 9 serotypes, only two of these, types 5 and 8, have actually been shown to be antigens that represent serologically distinct capsules (*Fournier* et al., 1984; *Moreau* et al., 1990). There is no antigenic or definitive serologic characterisation for any of the other capsule types that indicates they are distinct surface polysaccharides. However, the vast majority of isolates of *S. aureus* express either the type 5 or type 8 capsule, making these reasonable targets for vaccine development. Indeed, intense interest has been focused on such development (*Shinefield* et al., 2002; *Lee* et al., 1997; *Naso* and *Fattom*, 1996; *Welch* et al., 1996; *Fattom* and *Naso*, 1996; *Fattom* et al., 1996) and a recent clinical trial of a bivalent type5/type 8 conjugate vaccine given to haemodialysis patients showed a reduction in rates of bacteraemia during the early phases of the study, but this reduction was not maintained at the conclusion of the study after 54 weeks (*Shinefield* et al., 2002).

A second surface polysaccharide, found on both *S. aureus* and *S. epidermidis*, is a poly-N-acetyl glucosamine (PNAG) antigen associated with a number of important biologic and pathologic properties of these organisms (*Tojo* et al., 1988; *Kojima* et al., 1990; *Takeda* et al., 1991; *Muller* et al., 1993a; *Mack* et al., 1994,1996; *McKenney* et al., 1998). The antigen was first described by *Tojo* et al. (1988) as the capsular polysaccharide/adhesin (PS/A) of *S. epidermidis* although a definitive chemical composition and structure was not given. The first report on the chemical properties of this antigen came from *Mack* et al. (1996) who had previously attributed to this antigen the property of mediating intercellular adherence of coagulase-negative staphylococci (CoNS) and named the factor the polysaccharide intercellular adhesin (PIA). Later on *McKenney* et al. (1999) found the same material expressed in *S. aureus*, although they mistakenly identified N-acetyl succinate as a major component of the vaccine. Recent studies have corrected this misidentification (*Maira-Litran* et al., 2002a) and attributed it to the generation of a degradation product of the PNAG molecule that was produced during acid hydrolysis in order to perform NMR determinations of the structure of PS/A (*Joyce* et al., 2003). Another variant of the PNAG polymer was described as the slime-associated antigen (SAA) (*Baldassarri* et al., 1996) which was reported to contain about 70% glucosamine. Likely the rest of the material was contaminants. Finally, *Rupp* and colleagues (1992) described a

haemagglutinin of *S. epidermidis*, which was later shown to be PIA (*Mack* et al., 1999) There is now clear consensus that PS/A, PIA and SAA are all chemically PNAG.

PS/A, PIA AND PNAG-RELATEDNESS OF THEIR CHEMICAL AND BIOLOGICAL PROPERTIES

PS/A was identified by immunologic means as a capsule of many important clinical isolates of CoNS that had the property of forming a biofilm or producing "slime" *in vitro* when grown on plastic or glass (*Tojo* et al., 1988). Christensen and colleagues (*Christensen* et al., 1982,1983a; *Younger* et al., 1987; *Baddour* et al., 1988) were instrumental in identifying slime-producing CoNS as major causes of biomedical device infections starting in the early 1980s. Isolation of PS/A identified a major factor in the slime whose properties appeared to promote adherence of bacteria to plastic and formation of a biofilm (*Tojo* et al., 1988). Transposon mutants were identified that lost production of PS/A (*Muller* et al., 1993b), but the exact genes that were interrupted were never identified. The PS/A mutants were found to have reduced virulence in models of endocarditis (*Shiro* et al., 1994,1995) and expression of PS/A antigen was needed to promote resistance of CoNS to innate opsonic factors.

PIA was first described in 1992 by *Mack* et al. (1992) as a factor whose expression was induced by glucose leading to increased intercellular adhesion among CoNS. A genetic locus in CoNS involved in production of a hexosamine polysaccharide involved in intercellular accumulation was then identified in a strain of *S. epidermidis*. PIA was next isolated and purified and then reported to be a small molecular weight (<28 kDa) linear polymer of ß-1-6-linked N-acetyl glucosamine residues (*Mack* et al., 1996) with some O-linked substituents of succinate and phosphate. *Heilmann* et al. (1996a) followed this up by identifying the biosynthetic locus for PIA, termed the *ica* locus for intercellular adhesin and initially reported the presence of 3 open reading frames (ORFs), *ica*, *ica*B, and *ica*C and a divergently transcribed apparent regulator, the *ica*R gene, separated from *ica*A by an approximately 200 base-pair promoter region. A fourth ORF, *ica*D, was then identified (*Gerke* et al., 1998) whose coding sequence started in the 5' end of the *ica* gene and finished in the 3' beginning of the *ica*B gene. Expression of the *ica*A and *ica*D proteins in membranes resulted in the synthesis of an oligomer of ß-1-6-linked N-acetyl glucosamine about 20 residues in length using UDP-N-acetyl glucosamine as a starting substrate, and addition of the *ica*C protein further increased the oligomer's size. The role for *ica*B remains undefined. Clearly these genes and their protein products are responsible for synthesis of the PIA.

The distinction between PIA and PS/A was founded on the reported inability to show that the *ica* locus was needed for initial adherence of CoNS to plastic tissue culture wells that were manufactured in Europe (*Heilmann* et al., 1996b). However, in the same report, loss of *ica* genes resulted in a loss of adherence of *S. epidermidis* to glass (*Heilmann* et al., 1996b). Thus it was suggested that PS/A mediated the initial adherence of CoNS to plastic and similar surfaces while PIA mediated accumulation of the cells into a biofilm i.e., intercellular accumulation. When *McKenney* et al. studied (1998) the

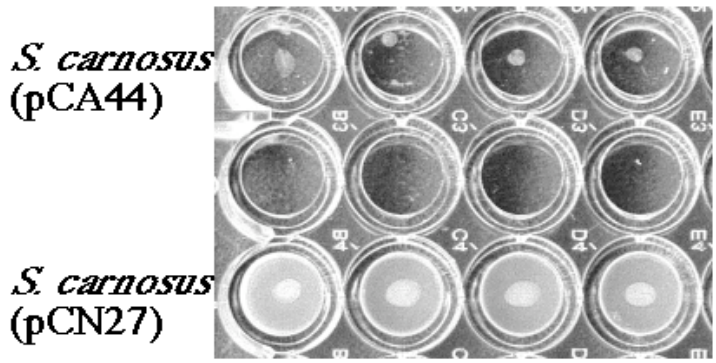

Figure 1: Biofilm formation in tissue culture wells (Corning) by *S. carnosus* carrying a plasmid with the *ica* genes from *S. epidermidis* (pCN27) and expressing PNAG or carrying the plasmid without additional DNA (pCA44). In contrast to the initial report that *S. carnosus* (pCN27) did not make a biofilm on plastic (*Heilmann* et al., 1996a) this experiment showed that with the Corning brand of tissue culture plate a biofilm is formed. The inability of *S. carnosus* (pCN27) to form a biofilm on one brand of tissue culture plate was the basis for distinguishing PIA and PS/A, which are now clearly known to be the same molecule.

cloned *ica* genes expressed in *S. carnosus* provided by *Heilmann* et al. (1996b) they found in fact they could isolate the PS/A material and indicated it was a high molecular weight glucosamine polymer containing N-linked succinate. However, the succinate was subsequently found to have been misidentified (*Maira-Litran* et al., 2002a; *Joyce* et al., 2003) and, in fact, they had isolated a high molecular weight ß-1-6-linked N-acetyl glucosamine with evidence of small amounts of O-linked succinate and acetate. Thus, both PS/A and PIA were found to be chemically identical, with some differences reported in the molecular size and larger differences in the biologic functions of these molecules.

As it turned out, the claim that PS/A mediated initial adherence of CoNS to plastic and PIA the accumulation of cells into biofilms was largely predicated on the results of studies with *S. carnosus* carrying the cloned *ica* genes and its interaction with tissue culture wells. When Heilmann performed the biofilm assay on tissue culture plates from the United States (Corning Brand) using the identical methods she used in Germany to characterise the *ica* locus, she found that in fact *S. carnosus* carrying the *ica* genes readily formed a biofilm on the plastic plates manufactured in the United States (Figure 1). Thus, even this distinction between PS/A and PIA was found to be due to some trivial differences in manufacture of tissue culture wells and it is now accepted that PIA and PS/A are the same chemical entity-PNAG.

OCCURRENCE OF *ICA* GENES AND PNAG-EXPRESSION IN *S. AUREUS*

In 1999 *McKenney* et al. (1999) reported that the *ica* genes were present in clinical isolates of *S. aureus* and expression of the PNAG antigen (incorrectly identified as poly-N-succinyl glucosamine or PNSG) was mostly associ-

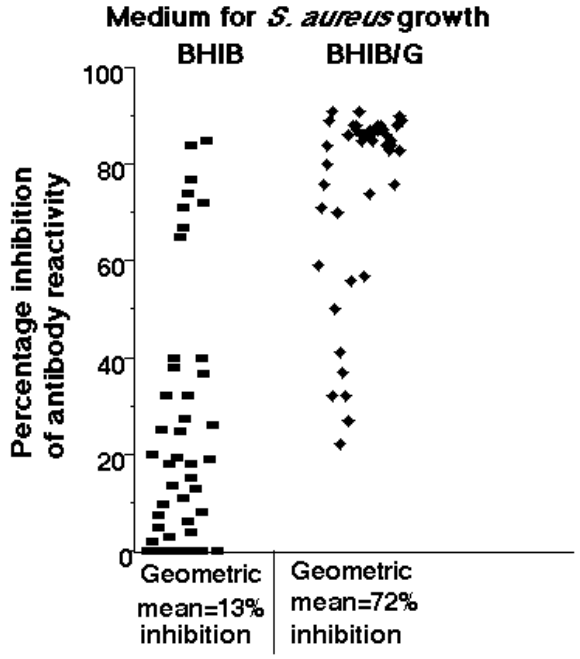

Figure 2: Induction of expression of PNAG in clinical isolates of *S. aureus* following growth in glucose-supplemented media. Strains were grown in either brain-heart infusion broth (BHIB) or BHIB supplemented with 0.25% glucose (BHIB/G) overnight, cells recovered by centrifugation and used to adsorb out a standard dilution of rabbit antibody to purified PNAG (*McKenney* et al., 1999). The antiserum was added to an ELISA plate coated with purified PNAG and the percentage inhibition of antibody binding measured. The geometric mean percentage inhibition of antibody binding, indicative of PNAG-expression, was significantly lower ($p<0.01$, t-test) in strains grown in BHIB compared to those grown in BHIB/G.

ated with *in vivo* growing organisms. However, when grown *in vitro* in rich medium (brain heart infusion broth) supplemented with glucose, there was increased expression of the PNAG antigen (Figure 2). They also showed expression of PNAG by *S. aureus* in lung sections from 2 cystic fibrosis patients and in 6 of 9 sputum samples also from cystic fibrosis patients (*McKenney* et al., 1999). Strains of *S. aureus* isolated from infected mice had increased PNAG expression *in vitro*, but after 5 passages the expression returned to a low state (*McKenney* et al., 1999). *Cramton* et al. (1999) rapidly followed this up with a similar report that the *ica* locus was present in *S. aureus* and was needed for biofilm formation by this organism. This report made no distinction between initial adherence and accumulation of cells into biofilms. As both properties in *S. aureus* were affected by deleting most of the *ica* locus. Several subsequent reports confirmed that the *ica* genes were found in most clinical isolates of *S. aureus* (*Fowler* et al., 2001; *Arciola* et al., 2001) and those reports that did not find *ica* genes in the majority of isolates (*Arciola* et al., 2001) were criticised for using primers designed for the *S. epidermidis ica* genes for investigating *S. aureus* (*Rohde* et al., 2001). There is about 70-80% identity at the nucleotide level of the *ica* genes in these two species (*McKenney* et al.,

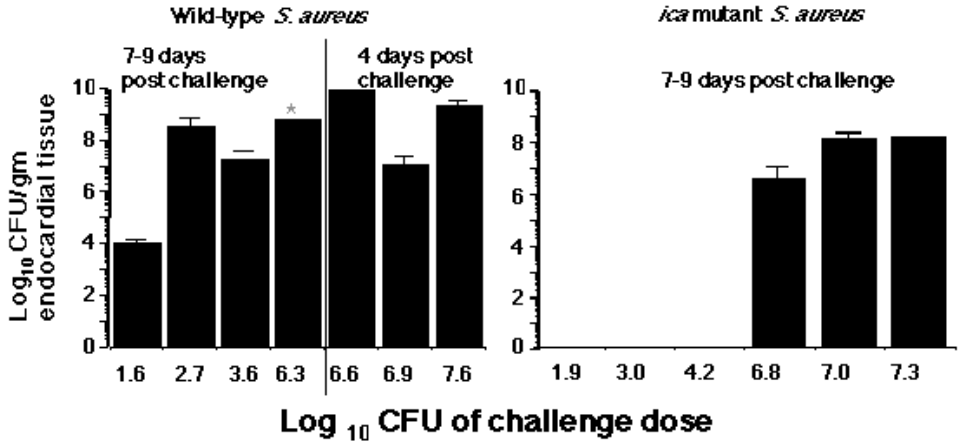

Figure 3: Virulence of a wild-type and isogenic mutant of *S. aureus* strain 10833 deleted for the *ica* locus in a rat model of endocarditis (*Lee* et al., 1997). Rats with intra-aortic catheters were infected with the dose of the wild type or mutant strain indicated on the X-axis and sacrificed at the time shown above the data bars, endocardial vegetations identified, excised, weighed, homogenised and serial dilutions plated for bacterial enumeration. The lower limit of detection (10 CFU/vegetation) is indicated and rats challenged with the indicated doses had no detectable vegetations or bacteria in their hearts. Bars represent means and error bars the SEM. Rats challenged with the higher doses of the wild-type strain had to be sacrificed early as they would not survive a longer period, further illustrating the enhanced virulence of the wild type strain compared to the *ica*-mutant in this model of infection. By comparative analysis of the overall CFU/gm of vegetation achieved, regardless of the day of sacrifice, it took approximately 4 logs more of the *ica*-deleted strain to reach comparable vegetation levels as did the wild type parental strain.

1999; *Cramton* et al., 1999), so primers based more on *S. aureus* sequences would be optimal for finding these genes in *S. aureus*. Among bovine isolates of *S. aureus* causing mastitis, 100% of 35 strains were found to carry the *ica* genes (*Vasudevan* et al., 2003). *Peacock* et al. (2002) identified 7 *S. aureus* genes encoding putative virulence factors out of 33 studied that were strongly associated with invasive strains when compared with strains of *S. aureus* carried by healthy blood donors and the *ica* genes were one of these 7. Thus, the presence of *ica* and the expression of PNAG is strongly associated with virulent strains of *S. aureus* and *S. epidermidis* (*Muller* et al., 1993a; *Ziebuhr* et al., 1997; *O'Gara* and *Humphreys*, 2001).

ROLE OF THE PNAG SURFACE POLYSACCHARIDE IN VIRULENCE OF STAPHYLOCOCCAL INFECTIONS

Accepting that PS/A, PIA and SAA are all basically PNAG polymers synthesised by proteins encoded by the *ica* locus, there is a fair amount of data that this polymer plays an important role in the virulence of infections due to CoNS. However, outside of epidemiologic associations of the occurrence of the *ica* locus in invasive isolates of *S. aureus* (*Peacock* et al., 2002), there is surpris-

ingly little information available about the role of PNAG in virulence of this species. Data reported in abstract form (*McKenney* et al., 2001) indicate a reduced level of virulence of *S. aureus* strains deleted for the *ica* locus when tested in a model of endocarditis in rats (Figure 3). In this model it was found the infectious dose for 50% (ID_{50}) of the animals infected with the wild type strain was <43 CFU, as all five animals infected with this dose had evidence of endocarditis, while for the *ica*-deleted strain the ID_{50} was 6.9 x 10^6 CFU ($p<0.001$, logit analysis). Ten of 24 animals infected with the wild-type strain at doses $\leq 10^{6.3}$ died 7-9 days after infection while none of 16 infected with the mutant strain died ($p<0.001$, Fisher's exact test). Thus, in endocarditis it appears from this one study that PNAG is a virulence factor for *S. aureus*. In contrast, *Francois* et al. (2003) reported no difference in virulence between wild-type and *ica* deletent *S. aureus* strains in a model of foreign-body infection using tissue cages implanted into guinea pigs. However, in this model the cages are first implanted in the animals and left for three weeks before infection, allowing the cages to become coated with host proteins. Given the ability of *S. aureus* to bind to numerous host proteins including fibrinogen, fibronectin, collagen and others (*Patti* et al., 1994; *Foster* and *Hook*, 1998; *Wann* et al., 2000) it is not surprising that when confronted with a foreign body coated with host proteins the surface PNAG is not required for adherence and biofilm formation and thus a role in virulence may not be manifest in this setting.

Early studies on biofilm-producing phenotypic variants of *S. epidermidis* (*Christensen* et al., 1983b,1987) indicated that the variants unable to make a strong biofilm were less virulent in a mouse model of foreign body infection. In contrast, *Patrick* et al. (1992) suggested *in vitro* slime production was not necessarily associated with pathogenesis of CoNS, particularly in the absence of a foreign body. A later study in mice showed wide heterogeneity in the ability of strains of CoNS with different biofilm phenotypes to produce infections (*Patrick* et al., 1995) but concluded there was some association between biofilm elaboration and virulence. *Deighton* et al. (1996) compared the virulence of 5 biofilm-positive and 5 biofilm-negative strains in a mouse abscess model without a foreign body implanted and found the biofilm-positive strains caused more abscesses that persisted longer with higher bacterial counts compared with the 5 biofilm-negative strains. However, these studies were conducted without knowledge as to the biochemical or genetic basis for biofilm production and classifying strains as biofilm positive or negative was based on *in vitro* measurements, which are known to vary widely based on conditions used to assess biofilm formation.

Subsequent studies with genetically manipulated strains of *S. epidermidis* gave more conclusive data that the biofilm-positive phenotype was associated with virulence. Transposon mutants of *S. aureus* strain M187 that lead to a biofilm-negative phenotype (*Muller* et al., 1993b) were found to be avirulent in a rabbit model of endocarditis (*Shiro* et al., 1994) following high-dose inoculation, and similarly were poorly virulent in a model of endocarditis following haematogenous spread from a contaminated intravascular catheter (*Shiro* et al., 1995). These studies focused on the role of the PNAG-polymer as an anti-phagocytic bacterial capsule, that in addition to promoting adherence of Staphylococci to biomaterials also prevented opsonic killing due to endogenous complement and phagocytic activ-

ity. However, *Perdreau-Remington* et al. (1998) did not find any difference in virulence in a rabbit model of endocarditis when comparing the strong biofilm-producing *S. epidermidis* strain RP62A with a chemical mutant deficient in production of biofilm. In a rat model of intravenous catheter associated infection (*Ulphani* and *Rupp*, 1999), *Rupp* et al. (1999) showed that there was less infection with a mutant of *S. epidermidis* strain 1457 unable to make the PNAG polymer compared with the parental strain. Another study showed the same effect with an *ica* mutant in strain O-47 (*Rupp* et al., 2001). In a related model of foreign body infections in mice, the same strain of *S. epidermidis* deficient in production of biofilms caused fewer abscesses and adhered to the implanted foreign body less well than did the parental strain. Overall, the general consensus from these studies is that elaboration of the PNAG polymer by CoNS, particularly *S. epidermidis*, is not only epidemiologically associated with pathogenic strains (*Gelosia* et al., 2001) but plays an important role in virulence as determined by animal studies.

ROLE OF THE PNAG SURFACE POLYSACCHARIDE IN VACCINATION

As *ica* genes and PNAG-expression are found commonly among clinical isolates of both CoNS and *S. aureus*, it is obviously an attractive vaccine candidate with the potential to elicit immunity to both of these common causes of nosocomial infection. As this polymer was first identified as PS/A in CoNS, the first studies on the vaccine potential of the PNAG polymer were performed with the PS/A material, although the level of purity of the vaccine could not be ascertained as its chemical nature was not known at the time. Nonetheless, from subsequent studies it is highly likely that the major component of the vaccine was PNAG. This immunogen was shown to reduce the number of days that rabbits had positive blood cultures, in comparison to non-immune controls, in a model of catheter-related bacteraemia (*Kojima* et al., 1990). Passive therapy using polyclonal and monoclonal antibody to the polymer also conferred protection. In a rabbit model of endocarditis, immunisation with the PS/A/PNAG polymer also markedly reduced the rate of occurrence of positive blood cultures and protected against the development of infected vegetations (*Takeda* et al., 1991). When it was discovered that the *ica* locus was present in most isolates of *S. aureus* and PNAG was expressed, it was also found that active or passive immunisation protected mice against infection with 8 different clinical isolates in a kidney infection model (*McKenney* et al., 1999). Additionally, rabbit antisera raised to purified PNAG has shown passive protective efficacy against infection in a rat model of endocarditis using a wild-type strain of *S. aureus* but not an isogenic strain deleted for the *ica* locus (Figure 4). In this experiment, rats with intra-aortic catheters were challenged with *S. aureus* strain 10833 with either an intact or deleted *ica* locus; the challenge dose for the wild-type strain was 2×10^4 CFU/rat whereas for the less virulent mutant strain the challenge dose had to be 9×10^6 CFU/rat in order to achieve comparable levels of infected vegetations with these two strains. Four days after infection animals were sacrificed and vegetations identified, excised, weighed and homogenised for bacterial levels. Immune serum to PNAG signifi-

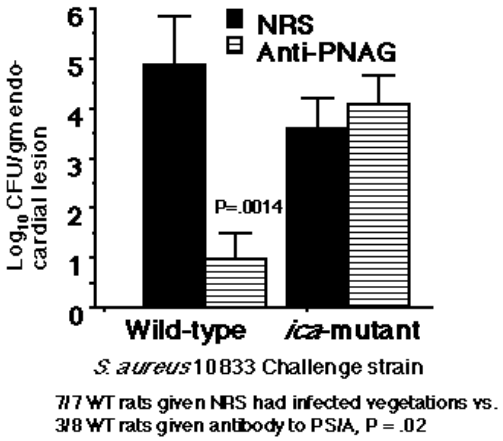

Figure 4: Passive protection mediated by rabbit antibody to purified PNAG in a rat model of endocarditis. Animals with intra-aortic catheters were treated with 0.5 ml of either normal (NRS) or immune serum to PNAG and then infected with either 2 x 10^4 CFU/rat of the wild-type, parental strain or 9 x 10^6 CFU/rat for the less virulent *ica*-mutant strain. This higher challenge dose for the mutant strain was needed in order to achieve comparable levels of infection in the aortic valve vegetations. Four days later animals were sacrificed and levels of bacteria in the vegetations determined. Bars represent means and error bars the SEM.

cantly (p=0.0014, t test) reduced the bacterial levels in vegetations in rats infected with the wild type strain but had no effect in animals infected with the *ica* deletent (Figure 4). All 7 of the animals infected with the wild-type strain and treated passively with normal rabbit serum had infected vegetations compared with only 3 of 8 animals treated with immune serum (p=0.02, Fisher's exact test). This experiment provided additional data indicating the potential of antibody to PNAG to protect against *S. aureus* infection and also showed the specificity of the protection in regard to the inability to protect against infection with the strain lacking an intact *ica* locus.

Although to date there are the only 3 published studies in the peer-reviewed literature on the vaccine potential of PNAG, there is continued on-going work on the immunochemical properties of the antigen to enhance immunogenicity and protective efficacy. A recent abstract (*Maira-Litran* et al., 2002b) indicated that conjugating PNAG to diphtheria toxoid enhanced its immunogenicity in mice and rabbits compared with antibody levels obtained using unconjugated PNAG (*Maira-Litran* et al., 2002a). The antisera had opsonic killing activity against a variety of *S. aureus* strains and one *S. epidermidis* strain. Another abstract (*Kropec* et al., 2002) showed that antibodies to PNAG were produced by cystic fibrosis patients with staphylococcal colonisation or infection, indicating that the antigen was expressed *in vivo* at a sufficient level to induce antibody. Overall, continued work on a PNAG vaccine is progressing, with both direct animal studies and correlative studies on responses of infected humans on-going, with the ultimate goal of a clinical assessment of active and passive immunotherapies directed at this antigen.

CONCLUSION

It is now clear that the various forms of staphylococcal surface polysaccharides identified as PS/A, PIA and SAA are the same chemical entity-PNAG. The structure was first identified by W. Fischer as reported by *Mack* et al. (1996) although the material isolated in this case was of a small molecular weight. Papers describing an N-linked succinate component (*McKenney* et al., 1998,1999) were incorrect in this identification (*Maira-Litran* et al., 2002a; *Joyce* et al., 2003). The biosynthetic proteins for PNAG are encoded by the *ica* locus first identified by *Heilmann* et al. (1996a) in *S. epidermidis* and subsequently by *McKenney* et al. (1999) in *S. aureus* followed shortly thereafter by *Cramton* et al. (1999). Studies in *S. epidermidis* and other CoNS show a clear association of PNAG production and virulence based on both epidemiologic studies of clinical isolates and animal studies of phenotypic variants and genetic mutants. Immunisation with PNAG protected against infection in rabbits due to catheter-associated bacteraemia (*Kojima* et al., 1990) and endocarditis (*Takeda* et al., 1991). In *S. aureus,* PNAG production is found in virtually all clinical isolates and immunisation has been reported to protect mice against infection caused by up to 8 different clinical isolates (*McKenney* et al., 1999). PNAG purified from an overproducing mutant of *S. aureus* strain MN8 (*Jefferson* et al., 2003) is immunogenic in laboratory animals (*Maira-Litran* et al., 2002a) and work reported in abstract form indicates conjugation of PNAG to carrier proteins enhances immunogenicity. Further studies in different animal models and identification of the optimal form of PNAG for testing in animal, and eventually human, immunogenicity studies is clearly warranted and if the proper types of immune effectors mediating resistance can be identified then there is a potential for PNAG to mediate protective immunity against the majority of virulent strains of CoNS and *S. aureus*.

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LITERATURE

Arciola, C.R., Baldassarri, L., and Montanaro, L.: Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter-associated infections. J. Clin. Microbiol. 39, 2151-2156 (2001).

Baddour, L.M., Smalley, D.L., Hill, M.M., and Christensen, G.D.: Proposed virulence factors among coagulase-negative staphylococci isolated from two healthy populations. Can. J. Microbiol. 34, 901-905 (1988).

Baker, C.J., Paoletti, L.C., Wessels, M.R., Guttormsen, H.K., Rench, M.A., Hickman, M.E., and Kasper, D.L.: Safety and immunogenicity of capsular polysaccharide-tetanus toxoid conjugate vaccines for group B streptococcal types Ia and Ib. J. Infect. Dis. 179, 142-150 (1999).

Baldassarri, L., Donelli, G., Gelosia, A., Vo-

glino, M.C., Simpson, A.W., and Christensen, G.D.: Purification and characterization of the staphylococcal slime-associated antigen and its occurrence among *Staphylococcus epidermidis* clinical isolates. Infect. Immun. 64, 3410-3415 (1996).

Barbour, M.L., Mayonwhite, R.T., Coles, C., Crook, D.W.M., and Moxon, E.R. The impact of conjugate vaccine on carriage of *Haemophilus influenzae* type b. J. Infect. Dis. 171, 93-98 (1995).

Campbell, W.N., Hendrix, E., Cryz, S., and Cross, A.S.: Immunogenicity of a 24-valent *Klebsiella* capsular polysaccharide vaccine and an eight-valent *Pseudomonas* O-polysaccharide conjugate vaccine administered to victims of acute trauma. Clin. Infect. Dis. 23, 179-181 (1996).

Christensen, G.D., Simpson, W.A., Bisno, A.L., and Beachey, E.H.: Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect. Immun. 37, 318-326 (1982).

Christensen, G.D., Parisi, J.T., Bisno, A.L., Simpson, W.A., and Beachey, E.H.: Characterization of clinically significant strains of coagulase-negative staphylococci. J. Clin. Microbiol. 18, 258-269 (1983a).

Christensen, G.D., Simpson, W.A., Bisno, A.L., and Beachey, E.H.: Experimental foreign body infections in mice challenged with slime-producing *Staphylococcus epidermidis*. Infect. Immun. 40, 407-410 (1983b).

Christensen, G.D., Baddour, L.M., and Simpson, W.A.: Phenotypic variation of *Staphylococcus epidermidis* slime production *in vitro* and *in vivo*. Infect. Immun. 55, 2870-2877 (1987).

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., and Gotz, F.: The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. 67, 5427-5433 (1999).

Deighton, M.A., Borland, R., and Capstick, J.A.: Virulence of *Staphylococcus epidermidis* in a mouse model: significance of extracellular slime. Epidemiol. Infect. 117, 267-280 (1996).

DeLisle, S. and Perl, T.M.: Vancomycin-resistant enterococci: A road map on how to prevent the emergence and transmission of antimicrobial resistance. Chest 123 (Suppl. 5), 504S-518S (2003).

Fattom, A.I. and Naso, R. Staphylococcal vaccines: A realistic dream. Ann. Med. 28, 43-46 (1996).

Fattom, A.I., Sarwar, J., Ortiz, A., and Naso, R.: A *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP-specific antibodies protect mice against bacterial challenge. Infect. Immun. 64, 1659-1665 (1996).

Foster, T.J. and Hook, M.: Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol. 6, 484-488 (1998).

Fournier, J.M., Vann, W.F., and Karakawa, W.W.: Purification and characterization of *Staphylococcus aureus* type 8 capsular polysaccharide. Infect. Immun. 45, 87-93 (1984).

Fowler, V.G., Jr., Fey, P.D., Reller, L.B., Chamis, A.L., Corey, G.R., and Rupp, M.E.: The intercellular adhesin locus ica is present in clinical isolates of *Staphylococcus aureus* from bacteremic patients with infected and uninfected prosthetic joints. Med. Microbiol. Immunol. (Berl.) 189, 127-131 (2001).

Francois, P., Tu Quoc, P.H., Bisognano, C., Kelley, W.L., Lew, D.P., Schrenzel, J., Cramton, S.E., Gotz, F., and Vaudaux, P.: Lack of biofilm contribution to bacterial colonisation in an experimental model of foreign body infection by *Staphylococcus aureus* and *Staphylococcus epidermidis*. FEMS Immunol. Med. Microbiol. 35, 135-140 (2003).

Gelosia, A., Baldassarri, L., Deighton, M., and van Nguyen, T.: Phenotypic and genotypic markers of *Staphylococcus epidermidis* virulence. Clin. Microbiol. Infect. 7, 193-199 (2001).

Gerke, C., Kraft, A., Sussmuth, R., Schweitzer, O., and Gotz, F.: Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. J. Biol. Chem. 273, 18586-18593 (1998).

Hatano, K. and Pier, G.B.: Complex serology and immune response of mice to variant high-molecular- weight O polysaccharides isolated from *Pseudomonas aeruginosa* serogroup O2 strains. Infect. Immun. 66, 3719-3726 (1998).

Heilmann, C., Schweitzer, O., Gerke, C.,

Vanittanakom, N., Mack, D., and Gotz, F.: Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol. Microbiol. 20, 1083-1091 (1996a).

Heilmann, C., Gerke, C., Perdreau-Remington, F., and Gotz, F.: Characterization of tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. Infect. Immun. 64, 277-282 (1996b).

Huebner, J., Wang, Y., Krueger, W.A., Madoff, L.C., Martirosian, G., Boisot, S., Goldmann, D.A., Kasper, D.L., Tzianabos, A.O., and Pier, G.B.: Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. Infect. Immun. 67, 1213-1219 (1999).

Huebner, J., Quaas, A., Krueger, W.A., Goldmann, D.A., and Pier, G.B.: Prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide shared among vancomycin-sensitive and -resistant enterococci. Infect. Immun. 68, 4631-4636 (2000).

Jefferson, K.K., Cramton, S.E., Gotz, F., and Pier, G.B.: Identification of a 5-nucleotide sequence that controls expression of the ica locus in *Staphylococcus aureus* and characterization of the DNA-binding properties of IcaR. Mol. Microbiol. 48, 889-899 (2003).

Joyce, J.G., Abeygunawardana, C., Xu, Q., Cook, J.C., Hepler, R., Przysiecki, C.T., Grimm, K.M., Roper, K., Ip, C.C., Cope, L., Montgomery, D., Chang, M., Campie, S., Brown, M., McNeely, T.B., Zorman, J., Maira-Litran, T., Pier, G.B., Keller, P.M., Jansen, K.U., and Mark, G.E.: Isolation, structural characterization, and immunological evaluation of a high-molecular-weight exopolysaccharide from *Staphylococcus aureus*. Carbohydr. Res. 338, 903-922 (2003).

Kojima, Y., Tojo, M., Goldmann, D.A., Tosteson, T.D., and Pier, G.B.: Antibody to the capsular polysaccharide/adhesin protects rabbits against catheter related bacteremia due to coagulase-negative staphylococci. J. Infect. Dis. 162, 435-441 (1990).

Kropec, A.P., Briggs, S., and Pier, G.B.: Abstracts of the 103rd General Meeting of the American Society for Microbiology, Abstract E-117 (2002).

Lakshman, R. and Finn, A.: Meningococcal serogroup C conjugate vaccine. Expert. Opin. Biol. Ther. 2, 87-96 (2002).

Lee, J.C., Park, J.S., Shepherd, S.E., Carey, V., and Fattom, A.: Protective efficacy of antibodies to the *Staphylococcus aureus* type 5 capsular polysaccharide in a modified model of endocarditis in rats. Infect. Immun. 65, 4146-4151 (1997).

Lowy, F.D.: Antimicrobial resistance: The example of *Staphylococcus aureus*. J. Clin. Invest. 111, 1265-1273 (2003).

Mack, D., Siemssen, N., and Laufs, R.: Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic adherent *Staphylococcus epidermidis* - Evidence for functional relation to intercellular adhesion. Infect. Immun 60, 2048-2057 (1992).

Mack, D., Nedelmann, M., Krokotsch, A., Schwarzkopf, A., Heesemann, J., and Laufs, R.: Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: Genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect. Immun. 62, 3244-3253 (1994).

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., Laufs, R.: The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: Purification and structural analysis. J. Bacteriol. 178, 175-183 (1996).

Mack, D., Riedewald, J., Rohde, H., Magnus, T., Feucht, H.H., Elsner, H.A., Laufs, R., and Rupp, M.E.: Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. Infect. Immun. 67, 1004-1008 (1999).

Maira-Litran, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G. 3rd., Goldmann, D.A., and Pier, G.B.: Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. Infect. Immun. 70, 4433-4440 (2002a).

Maira-Litran, T., Kropec, A., Pier, G.B.: Abstracts of the 103rd General Meeting of the American Society for Microbiology, Abstract E-121 (2002b).

McKenney, D., Hubner, J., Muller, E., Wang,

Y., Goldmann, D.A., and Pier, G.B.: The ica locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide/adhesin. Infect. Immun. 66, 4711-4720 (1998).

McKenney, D., Pouliot, K.L., Wang, Y., Murthy, V., Ulrich, M., Doring, G., Lee, J.C., Goldmann, D.A., and Pier, G.B.: Broadly protective vaccine for *Staphylococcus aureus* based on an *in vivo*-expressed antigen. Science 284, 1523-1527 (1999).

McKenney, D., Pouliot, K.L., Maira-Litran, T., et al.: Abstracts of the 101st General Meeting of the American Society for Microbiology, Abstract D-44 (2001).

Moreau, M., Richards, J.C., Fournier, J.M., Byrd, R.A., Karakawa, W.W., and Vann, W.F.: Structure of the type-5 capsular polysaccharide of *Staphylococcus aureus*. Carbohydr. Res. 201, 285-297 (1990).

Muller, E., Takeda, S., Shiro, H., Goldmann, D., and Pier, G.B.: Occurrence of capsular polysaccharide adhesin among clinical isolates of coagulase-negative staphylococci. J. Infect. Dis. 168, 1211-1218 (1993a).

Muller, E., Huebner, J., Gutierrez, N., Takeda, S., Goldmann, D.A., and Pier, G.B.: Isolation and characterization of transposon mutants of *Staphylococcus epidermidis* deficient in capsular polysaccharide/adhesin and slime. Infect. Immun. 61, 551-558 (1993b).

Murthy, S.V., Melly, M.A., Harris, T.M., Hellerqvist, C.G., and Hash, J.H.: The repeating equence of the capsular polysaccharide of *Staphylococcus aureus* M. Carbohydr. Res. 117, 113-123 (1983).

Naso, R. and Fattom, A.: Polysaccharide conjugate vaccines for the prevention of gram-positive bacterial infections. Adv. Exp. Med. Biol. 397, 133-140 (1996).

Obaro, S.K.: The new pneumococcal vaccine. Clin. Microbiol. Infect. 8, 623-633 (2002).

O'Gara, J.P. and Humphreys, H.: *Staphylococcus epidermidis* biofilms: Importance and implications. J. Med. Microbiol. 50, 582-587 (2001).

Patrick, C.C., Plaunt, M.R., Hetherington, S.V., and May, S.M.: Role of the *Staphylococcus epidermidis* slime layer in experimental tunnel tract infections. Infect. Immun. 60, 1363-1367 (1992).

Patrick, C.C., Hetherington, S.V., Roberson, P.K., Henwick, S., and Sloas, M.M.: Comparative virulence of *Staphylococcus epidermidis* isolates in a murine catheter model. Pediatr. Res. 37, 70-74 (1995).

Patti, J.M., Allen, B.L., McGavin, M.J., and Hook, M.: MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. 48, 585-617 (1994).

Peacock, S.J., Moore, C.E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G., and Day, N.P.: Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect. Immun. 70, 4987-4996 (2002).

Pelton, S.I.: Acute otitis media in an era of increasing antimicrobial resistance and universal administration of pneumococcal conjugate vaccine. Pediatr. Infect. Dis. J. 21, 599-604 (2002).

Perdreau-Remington, F., Sande, M.A., Peters, G., and Chambers, H.F.: The abilities of a *Staphylococcus epidermidis* wild-type strain and its slime-negative mutant to induce endocarditis in rabbits are comparable. Infect. Immun. 66, 2778-2781 (1998).

Pozsgay, V.: Oligosaccharide-protein conjugates as vaccine candidates against bacteria. Adv. Carbohydr. Chem. Biochem. 56, 153-199 (2000).

Richards, M.J., Edwards, J.R., Culver, D.H., and Gaynes, R.P.: Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. Crit. Care Med. 27, 887-892 (1999).

Rohde, H., Knobloch, J.K., Horstkotte, M.A., and Mack, D.: Correlation of *Staphylococcus aureus* icaADBC genotype and biofilm expression phenotype. J. Clin. Microbiol. 39, 4595-4596 (2001).

Rupp, M.E. and Archer, G.L.: Hemagglutination and adherence to plastic by *Staphylococcus epidermidis*. Infect. Immun. 60, 4322-4327 (1992).

Rupp, M.E., Ulphani, J.S., Fey, P.D., and Mack, D.: Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. Infect. Immun. 67, 2656-2659 (1999).

Rupp, M.E., Fey, P.D., Heilmann, C., and Gotz, F.: Characterization of the importance of *Staphylococcus epidermidis* auto-

lysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. J. Infect. Dis. 183, 1038-1042 (2001).

Shinefield, H., Black, S., Fattom, A., Horwith, G., Rasgon, S., Ordonez, J., Yeoh, H., Law, D., Robbins, J.B., Schneerson, R., Muenz, L., Fuller, S., Johnson, J., Fireman, B., Alcorn, H., and Naso, R.: Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis. N. Engl. J. Med. 346, 491-496 (2002).

Shiro, H., Muller, E., Gutierrez, N., Boisot, S., Grout, M., Tosteson, T.D., Goldmann, D., and Pier, G.B.: Transposon mutants of *Staphylococcus epidermidis* deficient in elaboration of capsular polysaccharide/ adhesin and slime are avirulent in a rabbit model of endocarditis. J. Infect. Dis. 169, 1042-1049 (1994).

Shiro, H., Meluleni, G., Groll, A., Muller, E., Tosteson, T.D., Goldmann, D.A., and Pier, G.B.: The pathogenic role of *Staphylococcus epidermidis* capsular polysaccharide/ adhesin in a low-inoculum rabbit model of prosthetic valve endocarditis. Circulation 92, 2715-2722 (1995).

Sohn, A.H., Garrett, D.O., Sinkowitz-Cochran, R.L., Grohskopf, L.A., Levine, G.L., Stover, B.H., Siegel, J.D., and Jarvis, W.R., Pediatric Prevention Network: Prevalence of nosocomial infections in neonatal intensive care unit patients: Results from the first national point-prevalence survey. J. Pediatr. 139, 821-827 (2001).

Sompolinsky, D., Samra, Z., Karakawa, W.W., Vann, W.F., Schneerson, R., and Malik, Z.: Encapsulation and capsular types in isolates of *Staphylococcus aureus* from different sources and relationship to phage types. J. Clin. Microbiol. 22, 828-834 (1985).

Takeda, S., Pier, G.B., Kojima, Y., Tojo, M., Muller, E., Tosteson, T., and Goldmann, D.A.: Protection against endocarditis due to *Staphylococcus epidermidis* by immunization with capsular polysaccharide/adhesin. Circulation 84, 2539-2546 (1991).

Theilacker, C., Coleman, F., Mueschenborn, S., Grout, M., and Pier, G.B.: Construction and characterization of a *Pseudomonas aeruginosa* mucoid exopolysaccharide/alginate conjugate vaccine. Infect. Immun. 71, 3875-3884 (2003).

Tojo, M., Yamashita, N., Goldmann, D.A., and Pier, G.B.: Isolation and characterization of a capsular polysaccharide/adhesin from *Staphylococcus epidermidis*. J. Infect. Dis. 157, 713-722 (1988).

Ulphani, J.S. and Rupp, M.E.: Model of *Staphylococcus aureus* central venous catheter-associated infection in rats. Lab. Anim. Sci. 49, 283-287 (1999).

Vasudevan, P., Nair, M.K., Annamalai, T., and Venkitanarayanan, K.S.: Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92, 179-185 (2003).

Wann, E.R., Gurusiddappa, S., and Hook, M.: The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. J. Biol. Chem. 275, 13863-13871 (2000).

Ward, J.: Prevention of invasive *Haemophilus influenzae* type-B disease - Lessons from vaccine efficacy trials. Vaccine 9 Suppl., S17-S24 (1991).

Welch, P.G., Fattom, A., Moore, J., Jr., Schneerson, R., Shiloach, J., Bryla, D.A., Li, X., and Robbins, J.B.: Safety and immunogenicity of *Staphylococcus aureus* type 5 capsular polysaccharide-*Pseudomonas aeruginosa* recombinant exoprotein A conjugate vaccine in patients on hemodialysis. J. Am. Soc. Nephrol. 7, 247-253 (1996).

West, T.E., Lewis, B.A., and Apicella, M.A.: Immunological characterization of an exopolysaccharide from the *Staphylococcus aureus* strain Smith diffuse. J. Gen. Microbiol. 133, 431-438 (1987).

Younger, J.J., Christensen, G.D., Bartley, D.L., Simmons, J.C., and Barrett, F.F.: Coagulase-negative staphylococci isolated from cerebrospinal fluid shunts: Importance of slime production, species identification, and shunt removal to clinical outcome. J. Infect. Dis. 156, 548-554 (1987).

Ziebuhr, W., Heilmann, C., Gotz, F., Meyer, P., Wilms, K., Straube, E., and Hacker, J.: Detection of the intercellular adhesion gene cluster (ica) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. Infect. Immun. 65, 890-896 (1997).

DEVELOPMENT OF STAPHVAX™, A POLYSACCHARIDE CONJUGATE VACCINE AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION: FROM THE LAB BENCH TO PHASE III CLINICAL TRIALS*

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SUMMARY

Staphylococcus aureus is the most common nosocomial pathogen and is responsible for approximately one-third of hospital-acquired bacteraemias. The emergence of strains with multidrug resistance, including resistance to vancomycin, the antibiotic of last resort, presents the medical community with a major public health problem. Alternative therapies, including immunotherapy, have been in development for several decades. The discovery of *S. aureus* capsular polysaccharides from clinical isolates, and their importance to pathogenicity via anti-phagocytic activity, opened a new window of opportunity for development of vaccines and immunotherapy against this pathogen. A conjugate vaccine, StaphVAX™ that includes the two most prevalent capsular polysaccharides, types 5 and 8, coupled to a carrier protein efficient in promoting a Th2 response, was developed. In a recent Phase 3 clinical study in haemodialysis patients, StaphVAX™ was shown to prevent *S. aureus* bacteraemia for up to 10 months following a single immunisation. The history, epidemiology, serology, and development of StaphVAX™, including preclinical and clinical studies demonstrating efficacy are described in this review.

INTRODUCTION

S. aureus is the number one cause of infection in hospitalised patients, accounting for 20-25% of all nosocomial infections (*Pfaller* et al., 1998). Contrary to the general belief, bacteraemia is the most prevalent type of *S. aureus* infection in hospitalised patients, followed by lower respiratory tract infections and skin/soft tissue infections. In a recent and comprehensive survey that included clinical sites in the USA, Canada, Europe, it was found that *S. aureus* accounted for 22% of all blood infections (8,929 of 40,497 infections), 23.2% of all lower respiratory tract (3,371/14,552 infections) and 39.2% (2,928/7,474 infections) of all skin and soft tissue infections (*Diekema* et al., 2001, 2002).

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The ability of *S. aureus* to acquire antibiotic resistance and to adapt to new antibiotics is well established (*Lowy*, 2003). It is well recognised that the extensive use of antibiotics has resulted in increased resistance among. *S. aureus* clinical isolates. In some areas, more than 95% of *S. aureus* isolates are now resistant to penicillin or ampicillin and more than 50% have developed resistance to methicillin (*Brumfitt* and *Hamilton-Miller*, 1990; *Boyce*, 1990; *Begley*, 1994). Methicillin resistant *S. aureus* (MRSA) infections are observed primarily in hospital settings but there have been alarming reports recently of community acquired MRSA infections (*Naimi* et al., 2001). There are numerous examples demonstrating that vancomycin, presently the antibiotic of last resort against multidrug resistant *S. aureus* infections has been unable to clear *S. aureus* infections (*Moore* et al., 2003; *Grabs* and *Lord*, 2002; *Gopal* et al., 1976). The ability of *S. aureus* to become vancomycin resistant was long believed to be limited only to laboratory setting (*Noble* et al., 1992). However, the first clinical isolate of *S. aureus* with intermediate sensitivity to vancomycin (8-16 μg/ml) was identified in Japan (*Hiramatsu*, 1997,1998). Soon after this report, more isolates with intermediate resistance to vancomycin (VISA) were reported in the USA and elsewhere (*CDC*, 1996). VISA strains were found to adapt and develop intermediate resistance by thickening of their cell walls (*Lowy*, 2003). More recently the first truly vancomycin resistant *S. aureus* (VRSA) was isolated and reported (*CDC*, 2002). The newly isolated strain was found to have acquired vancomycin resistance by acquiring the van A gene identical to that found in vancomycin resistant enterococci (*Lowy*, 2003).

RATIONALE, IDENTIFICATION AND DEVELOPMENT OF VACCINE CANDIDATES

With the advent of antibiotics, development of immunological approaches to management of staphylococcal infections has languished. Despite the large body of work in support of such approaches, the prospect of an immune-based solution to staphylococcal infections has been clouded with uncertainty (*Foster*, 1991). *Wright* and *Douglas* (1989) noted that phagocytosis was already in 1903 considered a major line of defence against *S. aureus* infections. Another significant clinical finding was reported by *Quie* (1972), who discovered that immune compromised children with “chronic granulomatous disease” had frequent *S. aureus* infections and that these occurrences were directly related to the dysfunction of the phagocytic cells. In spite of these leads, attempts to identify and isolate *S. aureus* antigens that stimulate opsonic antibodies against clinically significant "conventional" isolates were unsuccessful. Eventually, most investigators abandoned the search for immunological strategies to protect against *S. aureus* infection. As Dr. David Rogers, a prominent investigator in the field stated at the New York Academy of Science "The Staphylococci: Ecologic Perspective" meeting in 1965, protective immunity and human antibody response to staphylococci "... have gone about as fur as they kin go" (*Rogers* and *Melly*, 1965; *Fattom* and *Naso*, 1996a).

In spite of its ability to produce a large variety of toxins and extracellular products (*Foster*, 1991), *S. aureus* cannot be generally equated with other organisms, such as *Clostridium tetani*, *Corynebacterium diphtheriae*, or *Bor-*

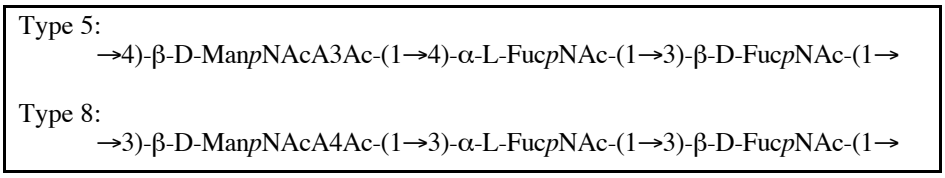

Figure 1: The structures of *S. aureus* types 5 and type 8 CP.

detella pertusis, which produce human illness primarily through elaboration of extracellular products and toxins. The hallmarks of *S. aureus* infection are dissemination of *S. aureus* through the blood and multiplication of the organism at the nidus of infection. *S. aureus* is part of the normal human flora and exists in the nasopharyngeal cavity of ~25% of healthy adults (*Essawi* et al., 1998a). Healthy people are not at risk for *S. aureus* infections and in fact can readily clear infections by this organism. Once hospitalised, however, *S. aureus* becomes the most common infectious agents in the hospital setting (*Diekema* et al., 2002). Systemic *S. aureus* infections such as endocarditis, osteomyelitis, meningitis, etc. often result from haematogenous seeding from bacteraemia due to the ability of *S. aureus* to evade immunological clearance mechanisms, especially opsonophagocytosis. Thus, staphylococcal pathobiology appears to be more like that of the pneumococci and meningococci rather than diphtheria, tetanus, or pertusis. For this reason, eliminating the organism from the host is of primary concern in preventing and treating staphylococcal infections.

In 1983 the field was advanced significantly when typing sera against *S. aureus* clinical isolates were developed (*Karakawa* and *Vann*, 1982). It was quickly shown that *S. aureus* clinical isolates possess capsular polysaccharides (CP) that contribute to the ability of the bacteria to evade opsono-phagocytosis. Subsequently, CP-specific antibodies were shown to mediate type-specific opsono-phagocytosis and bacterial killing by polymorphonuclear cells (PMNs) (*Karakawa* et al., 1988). Of the 13 known capsular types, two, types 5 and 8, were shown to comprise the majority of the clinical isolates (*Arbeit* et al., 1984; *Sompolinsky* et al., 1985). Recent studies using isolates from different countries showed that 93% *S. aureus* isolates were of either type 5 or type 8 (33% and 60%, respectively) (*Fattom* et al., 1995; *Essawi* et al., 1998b). These two capsular types also comprise >80% of *S. aureus* isolated from sheep, goats, cows with mastitis, and chickens with osteomyelitis (*Daum* et al., 1994; *Poutrel* et al., 1988). Ultimately, *S. aureus* types 5 and 8 CP were isolated, purified, and their chemical structures elucidated (*Fournier* et la., 1987; *Moreau* et al., 1990) (Figure 1).

Types 5 and 8 CP were found to be of small molecular size compared to CP of several other pathogenic bacteria. Furthermore, immunogenicity studies of the purified *S. aureus* CP showed them to be non-immunogenic in mice (*Fattom* et al., 1990). This property has been predictive for poor immunogenicity in infants and immunocompromised patients, two populations at high risk for *S. aureus* infections. Linking CP to carrier proteins to produce conjugate vaccines was shown to be effective in increasing the immunogenicity of bacterial polysaccharides and to confer T-cell dependent properties on their immune response (*Robbins* and *Schneerson*, 1990; *Chu* et al., 1983). Two conjugate vac-

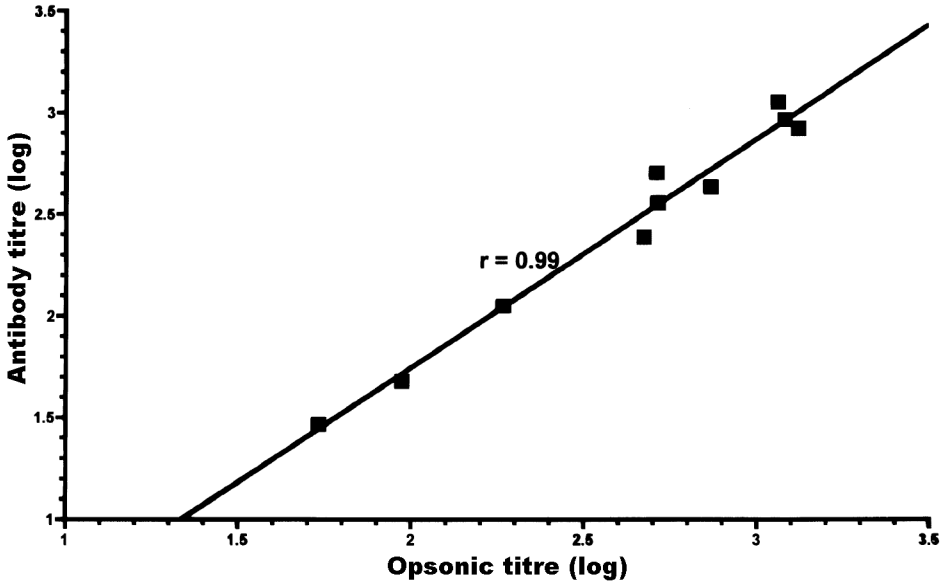

Figure 2: Opsono-phagocytosis of type 5 *S. aureus* by CP 5-rEPA conjugate induced antibodies in mice; correlation with ELISA antibodies
Opsono-phagocytosis assays were performed as described by *Karakawa* et. al (1988) except an HL60 cell line was used instead of freshly isolated human PMNs. The dilution that produced 50% kill was determined and the opsono-phagocytic titre was determined as $1/\text{dilution}_{50\%}$ after subtracting the background kill determined by the addition of non-immune sera.

cines, in which CP type 5 and type 8 were linked to carrier proteins, have been prepared using *Pseudomonas aeruginosa* exotoxin A as a carrier protein, and were evaluated in animals. Data showed that these conjugates elicited high antibody titres in mice and in rabbits. Moreover, the conjugation conferred T-cell dependent properties on the CP as evidenced by a booster response following a second injection or as shown upon carrier priming (*Fattom* et al., 1990).

Antibodies generated in vaccinated mice in response to monovalent conjugates (i.e. type 5 or type 8 CP conjugate alone) or a bivalent vaccine containing both type 5 and type 8 conjugates, exhibited both high affinity and type specificity. It was also found that antibodies generated by the vaccine(s) were functional in that there is a high degree of correlation between the amount of antibody by ELISA compared to opsonic activity. Figure 2 shows *in vitro* opsono-phagocytosis data generated by using murine sera from vaccinated mice.

EFFICACY IN ANIMAL MODELS

Active immunisation with StaphVAX™ was evaluated in a lethal mouse challenge model and was shown to protect mice from *S. aureus* challenge. It was also observed that there was a correlation between antibody titres and protection in the surviving mice (*Fattom* et al., 1996a). To further evaluate the mechanism of protection demonstrated by active immunisation, immunoglobu-

Table 1: Longevity of S. *aureus* CP5 and CP8 immune response in healthy adult volunteers following administration of CP5 or CP8 conjugate vaccines

Vaccine (Lot #)	N[1]	Antibody concentration Geometric mean IgG levels and range (μg/ml)				%[Ab][2]
		Pre-immune	6 weeks	6months	47 months	
S. aureus T5-rEPA (Lot # 50179)	8/23	10 (7-13)	367 (246-479)	292 (255-289)	122 (68-128)	42
S. aureus T5-rEPA (Lot # 4907)	11/25	8 (6-13)	241 (177-350)	175 (51-115)	100 (73-141)	57
S. aureus T8-rEPA (Lot # 51008)	9/22	11 (9-25)	81 (60-116)	71 (51-115)	52[3] (41-68)	73

[1]Number of volunteers available for evaluation at 47 months/number of original participants.
[2]Antibody levels remaining at 47 months compared to 6 months (%).
[3]Type 8 levels were measured at 33 months post last immunisation.

lin G (IgG) was purified from plasma obtained from human volunteers who received a dose of the bivalent type 5 and type 8 conjugate vaccine, designated as StaphVAX™. The IgG, called Altastaph™, was used to passively immunise animals, which were subsequently challenge with *S. aureus* lethal challenge (*Fattom* et al., 1996b). The geometric mean CP-5 specific antibody level in animals administered Altastaph™ was 111 μg/ml on the day of challenge with a half-life of 6 days. All animals that received Altastaph™ were protected against the challenge. Moreover, compared to animals administered non-specific IgG, animals passively immunised with Altastaph™ and challenged with a sublethal dose of S. *aureus* showed a faster clearance of the bacteraemia. Examination of the passively immunised animals revealed that while kidneys and livers from immunised animals were free of infection, *S. aureus* abscesses developed in kidneys and livers of animals receiving control IgG (*Fattom* et al., 1996b). The efficacy of the StaphVAX™ specific antibodies was also shown in a rat endocarditis challenge model (*Lee* et al., 1997). These data confirmed that protection against *S. aureus* infection is an antibody-mediated mechanism and that the CP-specific antibodies could serve as a surrogate marker for *in vivo* protection. Moreover, these data may suggest also that *in vitro* opsono-phagocytosis is a reasonable predictor for *in vivo* protection.

IMMUNOGENICITY OF STAPHVAX™ IN HUMANS

The type 8 CP and type 5 CP conjugate vaccines were initially evaluated in healthy adult human volunteers (*Fattom* et al., 1993). A total of 76 vaccinees received two injections of either type 5 or type 8 conjugates in saline at 25 μg CP/dose. The vaccines were well tolerated. No significant systemic or serious local reactions were reported. Minor tenderness and erythema was observed in few volunteers, however, these reactions were transient and generally disappeared within 48 hrs.

An interesting observation from this study was that nearly all individuals, presumably due to repeated exposure to

Table 2: Evaluation of IgG subclasses at 6 weeks and 33-47 month post vaccination in adult volunteers receiving type 5 CP conjugate vaccine

CP	Lot #	Subclass	N	Antibody titres (GM-μg/ml)			reduction (%)
				Pre-immune	6 weeks	33-47 months	
T5	49704	IgG1	6	1.3	47.17[a]	17.64[b]	61
		IgG2	6	1.83	60.6[c]	26.51[d]	54
		IgG3	2	<0.1	1.47	0.69	53
		IgG4	0	<0.1	<0.1	<0.1	n.a.
T5	50179	IgG1	6	0.22	11.27[e]	4.57[f]	53
		IgG2	6	2.27	173.83[g]	43.2[h]	67
		IgG3	1	1.4	100	78	22
		IgG4	2	0.89	8.39	6.32	22

Unpaired t-test: *a vs. b*: p=0.008; *e vs. f*: p=0.041.
Mann-Whitney Rank Sum Test: *c vs. d*: p=0.004, *g vs. h*: p=0.047.

S. aureus not leading to clinical disease, have low levels of pre-existing antibody to *S. aureus* CP, and are therefore immunologically primed to the CP. In these early studies, background low levels of antibodies (approximately 10-15 μg/ml) to each of the CP were measured. Following a single dose of conjugate vaccines, there was a 10-20-fold increase in CP-specific antibody levels. Both IgG and IgM classes were induced after the first injection. A second injection of conjugate vaccine 6 weeks later did not stimulate a further increase in antibody levels, indicating that the first dose resulted in a near maximum booster response in these subjects. Sera were obtained from several subjects available for blood drawing at 47 months after type 5 vaccination and 33months after type 8 vaccination. The antibody levels were 42% to 57% of the levels measured 6 months after vaccination. Antibody levels to type 8 CP were approximately 73% of the values measured at 6 months after vaccination (Table 1). Evaluation of the different subclasses at the two time points revealed a similar decline in titre in all four IgG subclasses (Table 2). These data show that CP-conjugate vaccines elicit a long-term immune response with a slow decline over time and that there is no selective decline in titres among the different IgG subclasses.

The functionality of *S. aureus* antibodies in sera from healthy volunteers participating in subsequent StaphVAX™ clinical trials were tested in an *in vitro* opsono-phagocytic assay and compared to sera obtained prior to vaccination. Post-vaccination sera demonstrated significantly higher levels of type specific opsono-phagocytic activity. In addition, there was an excellent correlation between type specific antibody levels measured by ELISA and opsono-phagocytic (Figure 3).

TARGET POPULATIONS

Hospitalised patients in general and especially those undergoing invasive medical procedures including surgery are at risk for *S. aureus* infections.

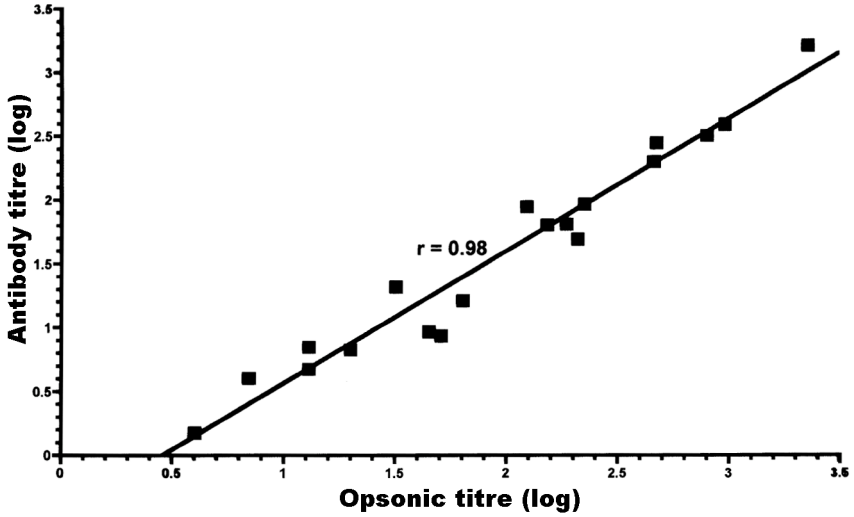

Figure 3: Opsono-phagocytosis of *S. aureus* type 5 by human sera from healthy volunteers immunised with StaphVAX™; correlation with ELISA antibodies
Opsono-phagocytosis assays were performed as described by *Karakawa* et. al (1988) except an HL60 cell line was used instead of freshly isolated human PMNs. Individual human sera were added to the reaction mixture and the 50% kill was determined. Opsono-phagocytic titres was determined as $1/\text{dilution}_{50\%}$

Other populations such as end stage renal disease patients (ESRD) on haemodialysis and other patients with chronic diseases such as residents of nursing homes are also at relatively high risk for *S. aureus* infections (*Fattom* and *Naso*, 1996b). While surgery patients are at high risk for *S. aureus* infections for a limited short period of time, patients with chronic disease, such as ESRD patients, are at continuous, long-term risk because of their underlying disease and routine medical procedures used to treat them (e.g. dialysis procedures). ESRD patients were chosen for the clinical development of StaphVAX™ (*Fattom* and *Naso*, 1996b) due to their relatively high incidence of *S. aureus* disease and their good response to StaphVAX™.

Other Early Clinical Trials

Initial clinical studies of type 5 and type 8 CP conjugates in healthy volunteers showed the vaccine components to be safe and immunogenic (*Fattom* et al., 1993). Subsequent trials evaluated a *S. aureus* monovalent type 5-rEPA conjugate in haemodialysis patients with ESRD (*Welch* et al., 1996). No serious local or systemic reactions or liver enzyme abnormalities were observed following the first or the second immunisation. Although a 18-fold increase in IgG antibodies to type 5 CP was observed, the geometric mean IgG level was 56% of that achieved in normal healthy volunteers immunised with same lot of vaccine. Furthermore, although all subjects responded with higher type 5 CP antibodies, only 13/16 responded with > 5- fold increase in titre, compared to 23/23 responders in normal healthy volunteers. Moreover, a faster decline in antibody level was observed in ESRD patients compared to normal healthy adults receiving the same vaccine six months after vaccination, 39% and 14%, respectively (*Welch* et al., 1996). These data indicated that while the *S. aureus* type 5 CP conjugate vaccine is immu-

Table 3: StaphVAX™ dose evaluation in haemodialysis patients[1]

Dose (μg) CP T5/T8	N	Type 5 IgG (μg/ml)			Type 8 (IgG μg/ml)		
		Day 0	Day 42	%[2]	Day 0	Day 42	%[2]
25/25	15	6	62	80	10	31	47
75/55	16	4	82	75	3	50	75
118/83	17	4	172	88	6	143	88

[1]Results are expressed as µg/ml IgG specific antibodies.
[2]Percent responders (>4fold increase and >25µg/ml IgG).

nogenic and can be used for active immunisation in some populations, other patient populations might require either higher doses of the vaccine or the use of the vaccine with an adjuvant.

The type 5 CP and type 8 CP conjugates were combined into one injection (StaphVAX™) and evaluated for immunogenicity in healthy volunteers and in ESRD patients. Results showed that the combining of the two conjugates did not affect the immunogenicity of each individual CP (Unpublished data). Furthermore, it was observed that CP-specific antibodies appear to peak in concentration within 10-14 days after immunisation confirming that the immune systems of most people are already primed to *S. aureus* CP. These results suggest that patients at short-term risk of *S. aureus* infection (e.g., elective surgery patients) might also benefit from vaccination.

StaphVAX™ was also evaluated in ESRD patients at higher doses than previously used in healthy volunteers. The antibody levels achieved were shown to be dose dependent however antibody levels were generally lower in ESRD patients than in healthy volunteers and they declined more rapidly (Table 3). Moreover, the percent of ESRD patients responding increased to nearly 90% at higher vaccine doses, a significant improvement over that achieved with lower doses in this population.

PHASE III EFFICACY TRIAL

StaphVAX™ was formulated to contain 100 μg each CP conjugated to rEPA for evaluation of its efficacy against *S. aureus* bacteraemia in a Phase III, double blinded, randomised, stratified, and placebo controlled clinical trial. Eighteen hundred ESRD patients on haemodialysis were enrolled to receive either one injection of StaphVAX™ or phosphate buffered saline (PBS). Patients were stratified by their nasopharingeal carriage of *S. aureus* and dialysis access. The primary endpoint of the trial was prospectively defined as significant reduction in *S. aureus* bacteraemia for one year. The safety and the immunogenicity of StaphVAX™ were secondary endpoints in this study. The vaccine was shown to be safe and elicited high levels of antibodies to both type 5 and type 8 CP components with 88% responding to type 5 and 84% responding to type 8. At peak CP-specific geometric mean antibody titres were approximately 230 μg/ml and 206 μg/ml for type 5 and type 8, respectively. At 54

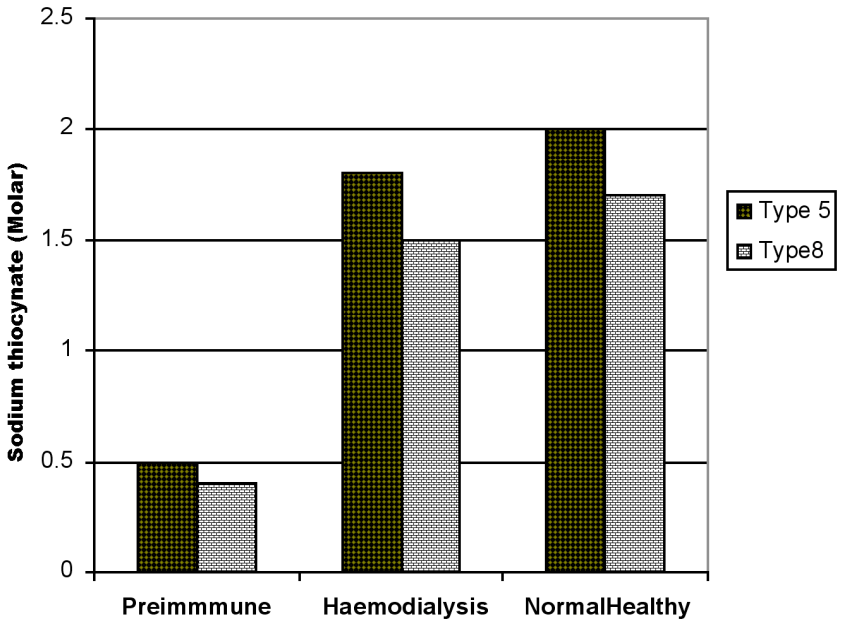

Figure 4: Comparison of the affinity of StaphVAX™ induced antibodies from healthy volunteers and haemodialysis patients.
Human sera were diluted to yield an OD of 2.0 in ELISA plates coated with the appropriate polysaccharide. The amount of thiocyanate added to result in 50% reduction in OD was determined. Results are expressed as geometric mean sodium thiocyanate concentrations.

weeks post vaccination, the antibody levels declined to approximately 74 μg/ml for type 5 and 65.5 μg/ml for type 8. The efficacy of StaphVAX™ at one year, the primary end point for this study, was 26% (reduction in bacteraemia) compared to placebo and was not statistically significant (p=0.228). In a *post-hoc* analysis evaluating the performance of the vaccine through various earlier time points, however, StaphVAX™ was shown to reduce *S. aureus* bacteraemia by 64% through 32 weeks follow-up (p=0.02) and by 57% through 40 weeks (p=0.02). When the antibody levels were matched with the efficacy, it appeared that protection fell off when geometric mean antibody levels in the population fell below approximately 80 μg/ml (*Shinefield* et al., 2002).

Opsono-phagocytosis is the principal mechanism for clearance of infections caused by Gram-positive bacteria including *S. aureus*. Circulating antibodies to CP recognise invading *S. aureus* cells and opsonise them. Complement is deposited on opsonised cells and binds to polymorphonuclear cells (PMNs) through complement receptors that induces the phagocytosis of the opsonised cells by PMNs. Examining the calculated protective antibody levels for S. aureus from our study reveals that they are far higher than those observed with other bacterial infections such as pneumococcal and meningococcal infections. The requirement for high antibody levels to protect against *S. aureus* bacteraemia may be related to health condition of ESRD patients. These patients often suffer from uncontrolled diabetes, hyper-uraemia, impaired complement, and low complement receptor density on their neutrophils, in addition to other defects or impairments in the perform-

ance of their lymphocytes. These conditions may cause an inefficiency and impairment of the opsono-phagocytosis mechanism (*Pirofski* and *Casadevall*, 1998; *Haag-Weber* et al., 1989; *Nolan* et al., 1978). Further, an optimum performance of these immune functions for protection against invading bacteria would require a high quality and functionality of the elicited antibodies. The affinity of antibodies generated by StaphVAX™ in haemodialysis patients was evaluated and compared to that exhibited by antibodies generated in immunocompetent healthy volunteers (Figure 4). The amount of thiocyanate needed to prevent CP-specific antibodies from binding to immobilised antigen is proportional to the affinity of the antibodies. Data presented above show that the affinity of anti-CP antibodies produced by StaphVAX™ in haemodialysis was equivalent to that of antibodies induced in healthy volunteers. Moreover, when tested in an *in vitro* opsono-phagocytosis assays, the antibodies to type 5 and type 8 CP generated in the ESRD patients performed equally well to the antibodies formed in healthy volunteers. (*Fattom* et al., 2004).

EXTENDING THE EFFICACY

Haemodialysis patients are at continuous and long-term risk patients for *S. aureus* infection and could benefit from the presence of protective levels of antibodies at all times. A periodic booster immunisation may be needed to rebuild trough concentrations of antibodies and restore or prolong the efficacy of the vaccine beyond the 10 months of significant protection seen in the phase 3 trial. Previous experiences with conjugate vaccines in adults showed that a booster immunisation at six weeks after the first immunisation did not result in boosting the antibody levels (*Fattom* et al., 1993; *Chu* et al., 1983; *Schneerson* et al., 1986). To see the effects of booster immunisation on specific antibody levels and vaccine safety when the booster is given longer periods of time after the initial vaccination, seventy-nine ESRD patients, previously immunised with StaphVAX™ in the phase 3 trial, were recruited for a booster study. These subjects had received their initial vaccination with StaphVAX™ 2-3 years previous to the booster. Results from the booster study showed that the CP-specific antibodies levels increased to about 60% of the peak levels achieved with the first immunisation resulting in >80% of the participants achieving or exceeding the calculated protective levels i.e. ~80µg/ml. In addition, the decline of specific antibody levels after the booster was slower than that observed after the initial immunisation.

PLANNED CONFIRMATORY EFFICACY STUDY

In a currently planned confirmatory Phase III clinical trial of StaphVAX™ in ESRD patients on haemodialysis, a booster immunisation will be administered at 8 months and its impact on the levels of CP-antibodies and on extension of efficacy will be evaluated.

CONCLUSION

StaphVAX™, an experimental *S. aureus* polysaccharide conjugate vaccine, was shown to be safe, immunogenic, and efficacious, as determined by reduction in *S. aureus* bacteraemia through up to 10 months post-immunisation, in ESRD patients. Preliminary booster studies strongly suggest that ESRD patients can respond to booster immunisations with StaphVAX™ with increased levels of vaccine-specific antibodies. Studies are planned to further evaluate the value of booster doses to prolong efficacy in patients who may be at long-term risk for infection. Since StaphVAX™ induces high levels of CP-specific antibodies within 10-14 days post-immunisation, the vaccine may also have potential in preventing *S. aureus* infections in individuals at short-term risk for infection. For patients such as surgery patients, one immunisation with may be sufficient to achieve protective levels of antibody throughout the risk (e.g., hospitalisation) period. Additional safety and immunogenicity clinical trials of StaphVAX™ in several patient populations at short-term risk of *S. aureus* infections are being planned.

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LITERATURE

Arbeit, R.D., Karakawa, W.W., Vann, W.F., and Robbins, J.B.: Predominance of two newly described capsular polysaccharide types among clinical isolates of *Staphylococcus aureus*. Diagn. Microbiol. Infect. Dis. 2, 85-91 (1984).

Begley, S.: Antibiotics, the end of miracle drugs? Newsweek 46-51 (1994).

Boyce, J.M.: Increasing prevalence of methicillin-resistant *Staphylococcus aureus* in the United States. Infect. Control Hosp. Epidemol. 11, 639-642 (1990).

Brumfitt, W. and Hamilton-Miller, J.: The worldwide probem of methicillin-resistant *Staphylococcus aureus*. Drugs Exp. Clin. Res. 16, 205-214 (1990).

CDC: Reduced susceptibility of *Staphylococcus aureus* to vancomycin - Japan, 1996. MMWR 46, 624-635 (1997).

CDC: *Staphylococcus aureus* resistant to vancomycin - United States, 2002. MMWR 51, 565-567 (2002).

Chu, C., Schneerson, R., Robbins, J.B., and Rastogi, S.C.: Further studies on the immunogenicity of *Haemophilus influenzae* type b and pneumococcal type 6A polysaccharide-protein conjugates. Infect. Immun. 40, 245-256 (1983).

Daum, R.S., Fattom, A., Freese, S., and Karakawa, W.: Capsular polysaccharide serotypes of coagulase-positive staphylococci associated with tenosynovitis, osteomyelitis, and other invasive infections in chickens and turkeys: Evidence for new capsular types. Avian Dis. 38, 762-771 (1994).

Diekema, D.J., Pfaller, M.A., Schmitz, F.J., Smayevsky, J., Bell, J., Jones, R.N., and Beach, M.; SENTRY Partcipants Group: Survey of infections due to *Staphylococcus* species: Frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997- 1999. Clin. Infect. Dis. 32 (Suppl. 2), S114-S132 (2001).

Diekema, D.J., Pfaller, M.A., and Jones, R.N.: Age-related trends in pathogen frequency and antimicrobial susceptibility of bloodstream isolates in North America. SENTRY

Antimicrobial Surveillance Program, 1997-2000. Int. J. Antimicrob. Agents 20, 412-418 (2002).

Essawi, T., Na'was, T., Hawwari, A., Wadi, S., Doudin, A., and Fattom, A.I.: Molecular, antibiogram and serological typing of *Staphylococcus aureus* isolates recovered from Al-Makased Hospital in East Jerusalem. Trop. Med. Int. Health 3, 576-583 (1998a).

Essawi, T., Na'was, T., Hawwari, A., Wadi, S., Doudin, A., and Fattom, A.I.: Molecular, antibiogram and serological typing of *Staphylococcus aureus* isolates recovered from Al-Makased Hospital in East Jerusalem. Trop. Med. Int. Health 3, 576-583 (1998b).

Fattom, A., Schneerson, R., Szu, S.C., Vann, W.F., Shiloach, J., Karakawa, W.W., and Robbins, J.B.: Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. 58, 2367-2374 (1990).

Fattom, A., Schneerson, R., Watson, D.C., Karakawa, W.W., Fitzgerald, D., Pastan, I., Li, X., Shiloach, J., Bryla, D.A., and Robbins, J.B.: Laboratory and clinical evaluation of conjugate vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides bound to *Pseudomonas aeruginosa* recombinant exoprotein A. Infect. Immun. 61, 1023-1032 (1993).

Fattom, A., Naawas, T., Hawwari , A., et al.: *S. aureus* nasal carriage as a risk factor for pneumonia but not bacteremia in shock trauma patients. In: Proceedings of the 95th General Meeting of the American Society of Microbiology (1995)

Fattom, A.I. and Naso, R.: Staphylococcal vaccines: A realistic dream. Ann. Med. 28, 43-46 (1996a).

Fattom, A.I. and Naso, R.: *Staphylococcus aureus* vaccination for dialysis patients -an update. Adv. Ren. Replace Ther. 3, 302-308 (1996b).

Fattom, A.I., Sarwar, J., Ortiz, A., and Naso, R.: A *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP-specific antibodies protect mice against bacterial challenge. Infect. Immun. 64, 1659-1665 (1996b).

Fattom, A., Fuller, S., Propst, M., Winston, S., Muenz, L., He, D, Naso, R., and Horwith, G.: Safety and immunogenicity of a booster dose of *Staphylococcus aureus* types 5 and 8 capsular polysaccharide conjugate vaccine (StaphVAX((R))) in hemodialysis patients. Vaccine 23, 656-663 (2004).

Foster, T.J.: Potential for vaccination against infections caused by *S. aureus*. Vaccine 9, 221-227 (1991).

Fournier, J.M., Hannon, K., Moreau, M., Karakawa, W.W., and Vann, W.F.: Isolation of type 5 capsular polysaccharide from *Staphylococcus aureus*. Ann. Inst. Pasteur Microbiol. 138, 561-567 (1987).

Gopal, V., Bisno, A.L., and Silverblatt, F.J.: Failure of vancomycin treatment in *Staphylococcus aureus* endocarditis. *In vivo* and *in vitro* observations. JAMA 236, 1604-1606 (1976).

Grabs, A.J. and Lord, R.S.: Treatment failure due to methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to vancomycin. Med. J. Aust. 176, 563 (2002).

Haag-Weber, M., Hable, M., Schollmeyer, P., Hort, W., and Urth, S.L.: Metabolic response of neutophils to uremia nad dialysis. Kidney Int. 36 (Suppl. 27), S293-S298 (1989).

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y., and Kobayashi, I.: Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 350, 1670-1673 (1997).

Hiramatsu, K.: The emergence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Japan. Am. J. Med. 104, 7S-10S (1998).

Karakawa, W.W. and Vann, W.F.: Capsular polysaccharides of *Staphylococcus aureus*. In: Seminars in infectious disease (Eds.: Weinstein, L. and Fields, B.N.). Stratton Intercontinental Medical Book Corp., New York, 285-293 (1982).

Karakawa, W.W., Sutton, A., Schneerson, R., Karpas, A., and Vann, W.F.: Capsular atibodies induce type-specific phagocytosis of capsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. Infect. Immun. 56, 1090-1095 (1988).

Lee, J.C., Park, J.S., Shepherd, S.E., Carey,

V., and Fattom, A.: Protective efficacy of antibodies to the *Staphylococcus aureus* type 5 capsular polysaccharide in a modified model of endocarditis in rats. Infect. Immun. 65, 4146-4151 (1997).

Lowy, F.D.: Antimicrobial resistance: The example of *Staphylococcus aureus*. J. Clin. Invest. 111, 1265-1273 (2003).

Moore, M.R., Perdreau-Remington, F., and Chambers, H.F.: Vancomycin treatment failure associated with heterogeneous vancomycin-intermediate *Staphylococcus aureus* in a patient with endocarditis and in the rabbit model of endocarditis. Antimicrob. Agents Chemother. 47, 1262-1266 (2003).

Moreau, M., Richards, J.C., Fournier, J.M., Byrd, R.A., Karakawa, W.W., and Vann, W.F.: Structure of the type 5 capsular polysaccharide of *Staphylococcus aureus*. Carbohydr. Res. 201, 285-297 (1990).

Naimi, T.S., LeDell, K.H., Boxrud, D.J., Groom, A.V., Steward, C.D., Johnson, S.K., Besser, J.M., O'Boyle, C., Danila, R.N., Cheek, J.E., Osterholm, M.T., Moore, K.A., and Smith, K.E.: Epidemiology and clonality of community-acquired methicillin-resistant *Staphylococcus aureus* in Minnesota, 1996-1998. Clin. Infect. Dis. 33 990-996 (2001).

Noble, W.C., Virani, Z., and Cree, R.G.: Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol. Lett. 72 195-198 (1992).

Nolan, C., Beaty, H., and Bagdade, J.: Impaired granulocyte bactericidal function in patients with poorly controlled diabetes. Diabetes 127, 889-894 (1978).

Pfaller, M.A., Jones, R.N., Doern, G.V., and Kugler, K.: Bacterial pathogens isolated from patients with bloodstream infection: Frequencies of occurrence and antimicrobial susceptibility patterns from the SENTRY antimicrobial surveillance program (United States and Canada, 1997). Antimicrob. Agents Chemother. 42, 1762-1770 (1998).

Pirofski, L.A. and Casadevall, A.: Use of licensed vaccines dor active immunization of immunocompromised host. Clin. Microbiol. Rev. 11, 1-26 (1998).

Poutrel, B., Boutonnier, A., Sutra, L., and Fournier, J.M.: Prevalence of capsular polysaccharide types 5 and 8 among *Staphylococcus aureus* isolates from cow, goat, and ewe milk. J. Clin. Microbiol. 26, 38-40 (1988).

Quie, P.G.: Bactericidal function of human polymorphonuclear leukocytes. Pediatrics 50, 264-270 (1972).

Robbins, J.B. and Schneerson, R.: Polysaccharide-protein conjugates. A new generation of vaccines. J. Infect. Dis. 161, 821-832 (1990).

Rogers, D.E. and Melly, M.A.: Speculation on the immunology of staphylococcal infection. Ann. NY Acad. Sci. 128, 274-284 (1965).

Schneerson, R., Robbins, J.B., Parke, J.C. Jr., Bell, C., Schlesselman, J.J., Sutton, A., Wang, Z., Schiffman, G., Karpas, A., and Shiloach, J.: Quantitative and qualitative analysis of serum antibodies elicited in adults by *Haemophilus influenzae* type B and pneumococcus type 6A capsular polysaccharide-tetanus toxoid conjugates. Infect. Immun. 52, 519-528 (1986).

Shinefield, H., Black, S., Fattom, A., Horwith, G., Rasgon, S., Ordonez, J., Yeoh, H., Law, D., Robbins, J.B., Schneerson, R., Muenz, L., Fuller, S., Johnson, J., Fireman, B., Alcorn, H., and Naso, R.: Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis. N. Engl. J. Med. 346, 491-496 (2002).

Sompolinsky, D., Samra, D., Karakawa, W.W., Vann, W.F., Schneerson, R., and Malik, Z.: Encapsulation and capsular types in isolates of *Staphylococcus aureus* from different sources and relationship to phage types. Infect. Immun. 22, 828-834 (1985).

Welch, P.G., Fattom, A., Moore, .J, Jr., Schneerson, R., Shiloach, J., Bryla, D.A., Li, X., and Robbins, J.B.: Safety and immunogenicity of *Staphylococcus aureus* type 5 capsular polysaccharide-*Pseudomonas aeruginosa* recombinant exoprotein A conjugate vaccine in patients on hemodialysis. J. Am. Soc. Nephrol. 7, 247-253 (1996).

Wright, A.E. and Douglas, S.R.: An experimental investigation of the role of the blood fluids in connection with phagocytosis. 1903. Rev. Infect. Dis. 11, 827-834 (1989).

CLEARANCE OF *HELICOBACTER PYLORI* INFECTION THROUGH IMMUNISATION: THE SITE OF T CELL ACTIVATION CONTRIBUTES TO VACCINE EFFICACY*

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SUMMARY

H. pylori vaccine development has progressed rapidly in animal models. Both *H. pylori*-associated pathogenesis and protective immunity are $CD4^+$ T cell dependent, with no discernable phenotypic difference to distinguish pathogenic T cells from protective T cells. Functionally however, protective T cells promote enhanced inflammation upon *H. pylori* challenge. Additionally, only mouse models such as phagocyte oxidase- or IL-10-deficient mice that respond to *H. pylori* infection with intense gastritis are capable of demonstrating spontaneous eradication of the bacteria. These data, combined with recent descriptions of down-regulatory T cells in infected humans and mice, support an emerging model of *H. pylori* pathogenesis in which *H. pylori* induces inflammation that is limited by regulatory T cells in the stomach. Immunisation therefore may succeed by activating T cells in peripheral lymph nodes that are capable of promoting qualitatively or quantitatively different inflammation when recruited to the stomach. Evidence in support of this model will be discussed.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the world's most successful pathogens, infecting greater than 50% of the earth's population (*Marshall*, 1995). Prevalence of infection ranges from 20% in some developed nations to greater than 90% in some developing nations. *H. pylori* is a Gram-negative bacterium whose primary niche is the human gastric mucosa, where it resides in the mucus and on the surface of gastric epithelial cells. A direct role for *H. pylori* in gastritis and peptic ulcer disease has now been established through the successful culture of *H. pylori* from gastric biopsies (*Marshall* and *Warren*, 1984), the fulfilment of Koch's postulates in human volunteers (*Marshall* et al., 1985; *Morris* and *Nicholson*, 1987), and numerous studies documenting the complete and permanent remission of ulcers following antimicrobial therapy (*NIH Consensus Conference*, 1994). *H. pylori* is also recognised as a risk factor for the development of gastric adenocarcinoma and has been categorised by the

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World Health Organisation as a Class I human carcinogen (*World Health Organization*, 1994).

A number of antimicrobial therapies have been developed for treatment of *H. pylori* infection, with eradication rates ranging from 60% to over 90%. These therapies typically include at least two antibiotics and a proton pump inhibitor, and must be taken several times per day for up to 14 days. The complexity of therapy however, often results in poor patient compliance, and the cost of these drugs is prohibitive in nations where *H. pylori* is endemic. Additionally, significant resistance to antibiotics such as clarithromycin and metronidazole are already being reported. Finally, from an immunologic perspective, even successful eradication therapy does not protect the host from potential re-infection, nor protect asymptomatic hosts at risk for developing gastric cancer. Therefore, interest in a *H. pylori* vaccine is quite high.

HOST RESPONSE

H. pylori infection induces histologic gastritis in all infected individuals (*Dooley* et al., 1989), with subgroups progressing to symptomatic gastritis and peptic ulcer disease. The inflammation has both an acute and chronic character, with a monocytic and polymorphonuclear component remaining prevalent after lymphocytes are recruited to the mucosa. *H. pylori* infection is typically associated with focal neutrophil infiltration of the gastric epithelium, most often in the gland necks (*Warren*, 2000). The lamina propria becomes infiltrated with lymphocytes, normally absent from the stomach, which may then form a moderately diffuse pattern extending the full thickness of the mucosa. Lymphocytes will also occasionally form focal patterns, and the development of lymphoid follicles with germinal centres has been noted. Long-term manifestations of infection involve changes in the architecture of the epithelial cell monolayer, including disorganisation of the epithelial cells, atrophy, and metaplasia.

In addition to the persistent inflammation that accompanies *H. pylori* infection, a strong adaptive immune response also develops. The presence of *H. pylori*-specific serum IgG antibodies remains one of the quickest and simplest methods for detecting *H. pylori* infection. Studies performed on gastric biopsies and washings have also demonstrated the presence of *H. pylori*-specific IgA at the gastric mucosa (*Rathbone* et al., 1986; *Wyatt* et al., 1986; *Blanchard* et al., 1999a,b). Numerous studies have also documented strong *H. pylori*-specific T cell responses using lymphocytes isolated from infected individuals (*Karttunen* et al., 1990,1995; *Karttunen*, 1991; *Sharma* et al., 1994; *Fan* et al., 1994; *Di Tommaso* et al., 1995; *D'Elios* et al., 1997; *Lindholm* et al., 1998; *Sommer* et al., 1998; *Bamford* et al., 1998) (Table 1). Both peripheral blood mononuclear cells (PBMC) and lamina propria mononuclear cells (LPMC) from gastric explants respond to *H. pylori* stimulation *in vitro* by secretion of cytokines or by proliferation. These studies routinely result in a predominance of interferon-γ-producing T cells, consistent with *H. pylori* inducing a Th1 mediated, pro-inflammatory response.

Table 1: T cell cytokine and proliferation response following *in vitro* stimulation with *H. pylori* antigen is characterised by IFN-γ production

Cells	Assay	*H. pylori* positive patient		*H. pylori* negative patient	Reference
PBMC	ELISA	↑IFN-γ[a]	<	↑IFN-γ	*Karttunen* et al, 1990
	^{3}H-thymidine	Proliferation	<	Proliferation	
PBMC	ELISA	↑TNFα	<	↑TNFα	*Karttunen*, 1991
		↑IL-2	=	↑IL-2	
	^{3}H-thymidine	Proliferation	<	Proliferation	
PBMC	^{3}H-thymidine	Proliferation		Proliferation	*Sharma* et al., 1994
PBMC, LPMC	ELISA	↑IFN-γ	<	↑IFN-γ	*Fan* et al., 1994
	^{3}H-thymidine	Proliferation	<	Proliferation	
LPMC	ELISPOT	↑IFN-γ	<	↑IFN-γ	*Karttunen* et al., 1995
PBMC and LPMC (T cell clones)	^{3}H-thymidine	↑Proliferation		n.d.[b]	*Di Tommaso* et al., 1995
LPMC (T cell clones)	RT-PCR + ELISA	↑IFN-γ		-IFN-γ[c]	*D'Elios* et al., 1997
		↑TNFα		-TNFα	
		↑IL-4		-IL-4	
LPMC (T cell clones)	Immunohisto-chemistry	↑IFN-γ		-IFN-γ	*Lindholm* et al., 1998
		↑TNFα		-TNFα	
		↑IL-4	=	↑IL-4	
LPMC	Flow cytometry	↑IFN-γ		n.d.	*Sommer* et al., 1998
		↑IL-4			
LPMC	Flow cytometry	↑IFN-γ		n.d.	*Bamford* et al., 1998
		↑IL-2			

[a] ↑ indicates in increase following *in vitro* stimulation.
[b] n.d. indicates not determined.
[c] - indicates little or no cytokine was detected.

VACCINE PROTOTYPES IN ANIMAL MODELS

In the early stages of *H. pylori* vaccine research, immunologists and microbiologists had at least two reasons to doubt the potential success of such a vaccine. First, because *H. pylori* is a non-invasive mucosal pathogen, successful vaccination would most likely require oral delivery. Previous vaccine research had established that to stimulate efficacious immunity in gastrointestinal tissue, direct immunisation of mucosal tissue was required, optimally through oral immunisation. This complicated vaccine design, as ingested proteins are poor immunogens, and the acid environment of the stomach must be traversed to gain access to the lymph tissue-rich intestines. This problem had hindered the development of oral vaccines in humans for years, and had yet to be successfully overcome. Second, the *H. pylori*-induced adaptive immune response is ineffective following natural infection. Since *H. pylori* is able to persist in the face of an active immune response, it seemed unlikely that stimulation of a similar immune response through immunisation would be effective.

Oral vaccine research in animals

The development of a *Helicobacter* mouse model with the cat pathogen, *H. felis* (*Lee* et al., 1990), allowed researchers to test the efficacy of vaccination in mice (*Czinn* et al., 1993; *Chen* et al., 1992). The vaccination protocol was based upon an experimental Sendai virus model in which the mucosal adjuvant, cholera toxin, was combined with viral antigen to stimulate immunity in the upper respiratory tract of mice (*Nedrud* et al., 1987). A similar protocol effectively stimulated an anti-Helicobacter humoral response when cholera toxin was combined with Helicobacter proteins and delivered orally to mice (*Czinn* and *Nedrud*, 1991). When applied to the *H. felis* challenge model, nearly 80% of the mice were found to be protected from chronic infection (*Czinn* et al., 1993; *Chen* et al., 1992). Although these experiments were performed with crude bacterial lysate, several other laboratories soon expanded these studies to include successful immunisations with purified Helicobacter proteins such as the *Helicobacter* urease enzyme (*Michetti* et al., 1994; *Ferrero* et al., 1994) and heat shock protein (*Ferrero* et al., 1995).

Several laboratories also demonstrated that infected mice could be therapeutically immunised to accomplish eradication of the bacteria (*Corthesy-Theulaz* et al., 1995; *Doidge* et al., 1994). This concept was strengthened when a similar study was performed on ferrets infected with endogenous *H. mustelae* (*Cuenca* et al., 1996). The therapeutic immunisation experiments were of profound importance because they demonstrated that vaccination succeeds not because it induces an immune response prior to infection, but because immunisation must induce a quantitatively or qualitatively different immune response than normally induced by chronic infection.

Despite the excitement generated by these and most other *H. pylori* vaccine experiments, enthusiasm has always been tempered by two observations. First, when immunised mice are challenged with *Helicobacter* bacteria they respond with gastric inflammation that is histologically indistinguishable from the inflammation that accompanies natural infection. This response is termed "post-immunisation gastritis" and it can persist for months after the challenge organisms have been eradicated, although it eventually does dissipate (*Garhart* et al., 2002). Second, protec-

tion is often incomplete. In many experiments protective immunity has been defined as a significant reduction in bacterial load. In fact, in one experiment, where antibiotic therapy was applied to protected mice, there was a rapid remission of post-immunisation gastritis, suggesting the presence of *Helicobacter* organisms that went undetected by enzyme indicators and culture techniques (*Ermak* et al., 1997). Both of these observations illustrate the need to develop a better understanding of *H. pylori* pathogenesis and immunity.

By the mid-1990s, clinical isolates of *H. pylori* had been successfully adapted to several animal models including mice and some nonhuman primates. All early observations previously made in the *H. felis* model were confirmed and expanded with *H. pylori* (*Marchetti* et al., 1995; *Ghiara* et al., 1997). As a general rule, all of these immunisations have relied upon some variation of the original protocol, a purified or crude protein antigen combined with either cholera toxin or *E. coli* heat labile toxin (LT), given in multiple doses to the recipient animal prior to or subsequent to challenge.

Alternatives routes of mucosal immunisation

Cholera and *E. coli* LT enterotoxins are potent adjuvants for protein antigens delivered orally in animal models. Both increase the immunogenicity of protein antigens without having to form covalent linkages or emulsions, and less than 10 μg is required to retain adjuvanticity. Small doses of enterotoxin however are sufficient for toxicity when given to humans, as demonstrated in a recent clinical trial testing a therapeutic *H. pylori* vaccine (see clinical trials) (*Michetti* et al., 1999). Side effects such as diarrhoea and cramping may occur. Therefore, efforts at developing a safe and efficacious vaccine for *H. pylori* in humans have moved towards avoiding the inherent risk involved in taking oral enterotoxin. One strategy has been to develop *E. coli* LT with point mutations that reduce or eliminate toxicity without reducing adjuvanticity (*Marchetti* et al., 1998). This strategy has met with some success and is currently under further development.

A second strategy has been the search for alternative routes of immunisation. Both rectal and intranasal immunisations have been tested to induce mucosal immunity that disseminates to the stomach upon challenge with *H. pylori* in mice (*Kleanthous* et al., 1998). There is evidence in the mouse model that intranasal immunisation is more efficacious than the oral immunisation (*Garhart* et al., 2003a). The rectal and intranasal immunisation protocols are similar to oral immunisation in that multiple doses are required and a bacterial toxin adjuvant is necessary. However, the success of these alternative routes of mucosal immunisation is actually a major advance in vaccine development, since they require less antigen in mice (100 μg for intranasal versus 2 to 4 mg for oral) and the risks associated with the toxin adjuvant are significantly reduced.

Systemic immunisation against H. pylori infection

Intranasal immunisation, although successful in mice, remain experimental and controversial in humans. A mucosal adjuvant is still required and intranasal application does not preclude ingestion of some part of the vaccine, consequently still exposing the patient to risk for toxicity. Additionally, recent reports indicate that CT and LT enterotoxins can target the central nervous system via the olfactory epithelium and nerves, and can induce histologic inflammation within the olfactory bulb (*Fujihashi* et al., 2002). Therefore, we and others have pursued the possibility of employing

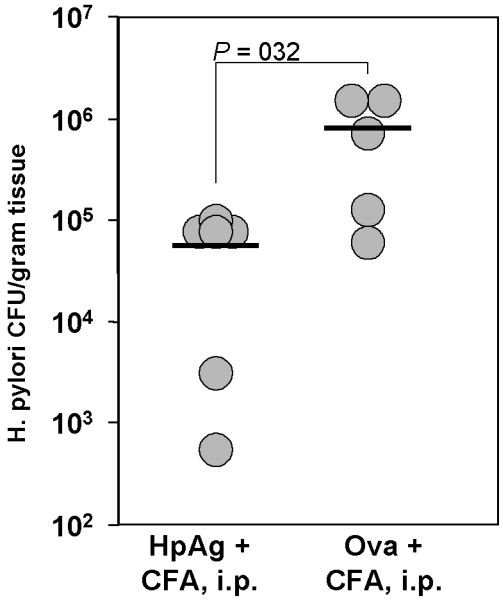

Figure 1. Systemic immunisation of mice against *H. pylori* reduces the bacterial load. Mice were immunised i.p. with a single dose of 100 µg of either *H. pylori* lysate or ovalbumin emulsified in complete Freund's adjuvant. Mice were challenged with 10^7 *H. pylori* 28 days post-immunisation and the number of colony forming units in gastric biopsies was determined 28 days post-challenge. Statistical analysis was performed by ANOVA.

traditional systemic vaccination to induce protective immunity against *H. pylori*. We have found that both intraperitoneal and subcutaneous prophylactic immunisations can result in significant reduction in bacterial load by four weeks after challenge of mice with infectious *H. pylori* (*Gottwein* et al., 2001; *Eisenberg* et al., 2003). Similar levels of protection can be induced by either Th1 (Freund's complete adjuvant) or Th2 (aluminium hydroxide or Freund's incomplete adjuvant) polarising vaccine regimens. An example of this immunity is shown in Figure 1 where mice were immunised with either *H. pylori* lysate or ovalbumin protein emulsified in complete Freund's adjuvant and given a single injection of 100 µg protein by intra-peritoneal injection. Mice were challenged with $1x10^7$ CFU *H. pylori* 28 days after immunisation and then assessed 28 days after challenge. Although immunisation did not provide sterilising immunity, there was a significant reduction in bacterial load (p= 0.032). We have achieved similar reductions when immunising neonatal mice within 24 hours of birth (*Eisenberg* et al., 2003), thus demonstrating the potential application for young children prior to contracting *H. pylori*. Several additional laboratories have demonstrated success with other adjuvants (*Guy* et al., 1998; *Weltzin* et al., 2000).

The results of these systemic immunisation experiments provide valuable insight into *H. pylori* immunity. Whereas systemic immunisation typically fails when applied against other mucosal pathogens, they can be efficacious against *H. pylori*. Thus it appears that immunisation by almost any route, including oral (which targets the Peyer's Patches of the small intestine), intranasal, rectal, and systemic can gen-

erate some degree of protective immunity when applied to mice. The relevant feature of a successful *H. pylori* vaccine therefore might not be stimulation of the mucosal immune response, but rather stimulation of an immune response in a tissue or lymph node designed to optimise immune responsiveness. This concept will be discussed further below.

CLINICAL TRIALS

The early success of oral vaccination against *H. felis* and *H. pylori* in mice led to the rapid development of a prototype oral vaccine for use in humans. Doses of either 180, 60, or 20 mg of recombinant *H. pylori* urease was administered with 5 μg *E. coli* LT and given to infected volunteers as an oral therapeutic vaccine (*Michetti* et al., 1999). Vaccination was delivered in four doses similar to the protocol used for mice. The vaccine significantly enhanced the number of circulating *H. pylori*-specific IgA-secreting cells over those in placebo immunised control volunteers demonstrating immunogenicity. Most encouraging was the significant reduction in bacterial load of urease LT-immunised subjects compared to control volunteers ($p=0.032$). Enthusiasm was somewhat dampened by the prevalence of diarrhoeal episodes induced by the *E. coli* LT adjuvant. Sixty six percent of the volunteers who completed the study experienced some level of diarrhoea, but the study confirmed the possibility of positive influence on gastric immunity in humans through oral vaccination. Several additional clinical trials have now been performed by other laboratories in which vaccine formulations were shown to be immunogenic as well. However, none have achieved the efficacy of the original study. Buoyed by the promise of this initial study, a new generation of trial vaccines is now being developed and tested. A more thorough understanding of *H. pylori* immunity will aid in the development of a better human vaccine.

IMMUNE EFFECTOR MECHANISMS IN *H. PYLORI* IMMUNITY

One means of optimising a vaccine for *H. pylori* would be to specifically design a vaccine to enhance that aspect of the immune system that mediates the protective immune response. Many studies have now been performed to elucidate how the immune system actually eradicates *H. pylori* once stimulated by immunisation. The focus has been to identify effector mechanisms or cells that are essential for protection, and to differentiate those factors from their counterparts that are also present during the chronic inflammation that accompanies natural infection.

The role of antibodies in the protective immune response

Since *H. pylori* predominantly resides at the apical surface of the gastric epithelium, the types of known immune effector mechanisms that might actually come into contact with *H. pylori* seem limited. The existence of tight junctions between epithelial cells severely limits the ability of leukocytes to cross the epithelium. Polymeric IgA however, is transported across the epithelium via the polymeric immunoglobulin receptor and released into the lumen. Although no correlation had been established between IgA levels and protective anti-*H. pylori*

immunity, IgA seemed the most likely immune effector molecule for interacting with *H. pylori* to mediate protection. However, in our studies with IgA-deficient mice, protective immunity was achieved at a level similar to that in wild type mice (*Blanchard* et al., 1999c). Because secretory IgM levels were found to compensate for the lack of IgA, we subsequently repeated these experiments with total antibody knock-out mice. Our results were consistent with those of others using the same model in that lack of antibody production in mice did not compromise the ability of an oral vaccine to induce protective immunity (*Ermak* et al., 1998; *Sutton* et al., 2000). Therefore, although secreted antibody may contribute to *H. pylori* immunity, it is not required.

The role of T cells in the protective immune response

The cellular requirements for protective immunity have been difficult to identify. Two studies using MHC I knockout mice and MHC II knockout mice have suggested the requirement for $CD4^+$ cells but not for $CD8^+$ cells in generating protective immunity (*Pappo* et al., 1999; *Ermak* et al., 1998). We found that injection of Helicobacter-primed $CD4^+$ T cells was sufficient to transfer protective immunity to otherwise immunodeficient $rag1^{-/-}$ mice (*Gottwein* et al., 2001). These studies demonstrate that T cell help is required to generate an adaptive immune response but do not advance our insight into the mechanism of protection. To further refine our understanding, many groups have used mice deficient in specific cytokines or cytokine receptors to elucidate which T cells may be most important in providing protective immunity. The most widely studied of the T cell cytokines have been IL-4 and IFN-γ, but it is now apparent that neither of these cytokines is essential to induce the protective immune response (*Lucas* et al., 2001; *Akhiani* et al., 2002; *Sawai* et al., 1999; Garhart, 2003a,b).

The role of innate host factors in the protective immune response

The importance of innate factors in *H. pylori* immunity has only recently been addressed. However, as discussed below, it may be that immunity is accomplished through the enhancement of inflammation by appropriately activated T cells. It is important therefore, to determine how innate factors may be contributing to *H. pylori* immunity. Two recent studies have demonstrated that although inducible nitric oxide synthase (iNOS) is upregulated in inflamed gastric tissue following challenge, iNOS deficient mice can be effectively immunised against *H. pylori* (*Garhart* et al., 2003a; *Blanchard* et al., 2003). This was true even when mice were deficient in both iNOS and phagocyte oxidase, the two primary host innate anti-bacterial defence mechanisms (*Blanchard* et al., 2003). In a separate study, mast cells have been shown to be unnecessary to achieve protection in mice from *H. pylori* through vaccination (John Nedrud and Steve Czinn, personal communication).

One non-T cell, pro-inflammatory factor that does seem to be necessary for protection is IL-12. Two separate laboratories have now demonstrated that mucosal immunisation of IL-12-deficient mice fails to induce significant protection as compared to non-immunised control mice (*Garhart* et al., 2003a; *Akhiani* et al., 2002). Both groups employed the IL-12 p40 subunit knockout to eliminate the formation of biologically active heterodimeric p70. Elimination of p40 also prevents formation of IL-23. Whether IL-12, IL-23, or both are required for the induction of protection remains to be determined. Regardless, whereas both IFN-γ and

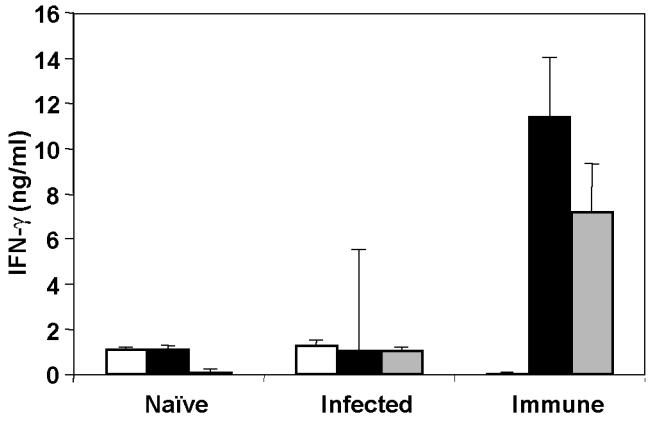

Figure 2. Memory T cells from immune mice produce IFN-γ in response to antigen presentation by mucosal epithelial cells. MODE-K epithelial cells ($1x10^4$) were combined with 1 x 10^6 $CD4^+$ spleen cells from naïve, infected, or immune mice and pulsed with either PBS (white bars) or *H. pylori* lysate (black bars). To demonstrated class II-restricted antigen presentation anti-MHC-II blocking antibody was also tested (gray bars). Supernatants were assessed after 48 hours for IFN-γ by ELISA.

IL-12 p40 knockout mice are capable of generating inflammation in response to *H. pylori* challenge, only IL-12 p40 is required to induce a protective state. These findings indicate that the character of the inflammatory response in IFN-γ knockout mice is qualitatively different than that in IL-12 p40 knockout mice.

H. PYLORI-ASSOCIATED INFLAMMATION AND IMMUNOREGULATION

Most efforts at defining *H. pylori* immunity have focused on identifying a specific effector mechanism. Another interesting possibility is the ability of *H. pylori* to down-regulate the inflammatory or immune response. This concept may seem counter-intuitive since studies in both mice and humans routinely report that infection with *H. pylori* results in *H. pylori*-specific IFN-γ producing T cells, and infection induces both inflammation and adaptive immune mechanisms. However, close inspection of the data suggests that *H. pylori* may in fact suppress the immune response, or at least the aspect of the immune response required for eradication of the bacteria. This was evident in several early studies in which it was demonstrated that T cells from infected patients responded no better than T cells from seronegative patients with regard to *H. pylori*-induced cytokine production and proliferation (Table 1). In several studies, cells from control donors actually responded as well as, or significantly stronger than cells from infected donors with more IFN-γ production or proliferation in recall assays against *H. pylori* antigen (*Karttunen* et al., 1990; *Karttunen*, 1991; *Karttunen* et al., 1995; *Fan* et al., 1994; *Sharma* et al., 1994). This observation perhaps did not garner the attention it deserved and latter studies have focused exclusively on T cells or T cell clones from infected individuals.

In mice, the data has tended to establish *Helicobacter* infection results in

strong T cell reactivity *in vitro* compared to T cells from naïve mice. Several of those studies were performed with the *H. felis* mouse model (*Mohammadi* et al., 1996; *Fox* et al., 2000) but one laboratory reported *H. pylori*-infected mice had a significant increase in IFN-γ production in recall assays compared to naïve control mice (*Smythies* et al., 2000). Our own studies in the *H. pylori* mouse model demonstrate only weak induction of IFN-γ production by T cells from infected animals. Whereas we have been able to detect cytokines such as IFN-γ and IL-2 in response to *H. pylori* infection, these responses are significantly weaker than those induced by our immunisation strategies (*Eisenberg* et al., 2003). Others have also noted increased IFN-γ production in immunised mice compared to infected control mice (*Garhart* et al., 2003a; *Goto* et al., 1999)**.** We have noted these differences regardless of the type of antigen presenting cell used to activate T cells. Figure 2 illustrates that antigen presentation by a mouse gastrointestinal epithelial cell line, to mimic what may be occurring in the gastric mucosa, induced low levels of IFN-γ by $CD4^+$ T cells from *H. pylori*-infected mice while immunised mice responded with significantly greater levels of cytokine. This IFN-γ production was partially diminished by anti-MHC class II antibody. As discussed above, IFN-γ is not required for induction of protective immunity. Nevertheless, it remains a good marker for a pro-inflammatory response when present.

$CD25^+$ Immunoregulatory T cells

In support of an immunoregulatory capacity for *H. pylori,* there is new evidence in both mice and humans that *H. pylori*-specific T regulatory cells are present in the infected host and actually work to limit the T cell or inflammatory response to *H. pylori*. Thus, when peripheral blood mononuclear cells (PBMC) from infected patients were examined *in vitro* and compared to non-infected donor PBMC, proliferation and IFN-γ production were equivocal between the two groups (*Lundgren* et al., 2003). However, when PBMC were depleted of $CD25^+$ cells (a cell phenotype implicated as a suppressive regulatory T cell), the remaining cells responded in a significantly stronger manner than non-infected controls in recall assays for proliferation and IFN-γ production. These studies were taken a step further in mice where lymph node cell populations were transferred to nude mice recipients prior to challenge with *H. pylori* (*Raghavan* et al., 2003). If $CD25^+$ cells were removed from the lymph node cells prior to transfer, the mice developed significantly more inflammation and ultimately had significantly fewer bacteria in the gastric mucosa following challenge. Therefore, in the absence of immunisation there are cells present that are capable of reducing the bacterial load in the gastric mucosa.

IL-10 producing regulatory T cells

A second type of suspected immunoregulatory cell is the IL-10 producing T cell. Intestinal colonisation of $IL\text{-}10^{-/-}$ mice with normal bacterial flora results in pronounced colitis suggesting that under normal circumstances a population of IL-10 producing T cells must prevent this inflammation. T cells that produce high amounts of IL-10 have been termed Tr1 cells and have been isolated from both mice and humans (*Groux* et al., 1997; *Muminova* et al., 1999). We have recently shown that IL-10 producing regulatory T cells may also be present in the stomach in response to *H. pylori* infection. Infection of the mouse stomach with *H. pylori* results in persistent infection, but only mild inflammation. Figure 3 illustrates that infection of $IL\text{-}10^{-/-}$ mice, however,

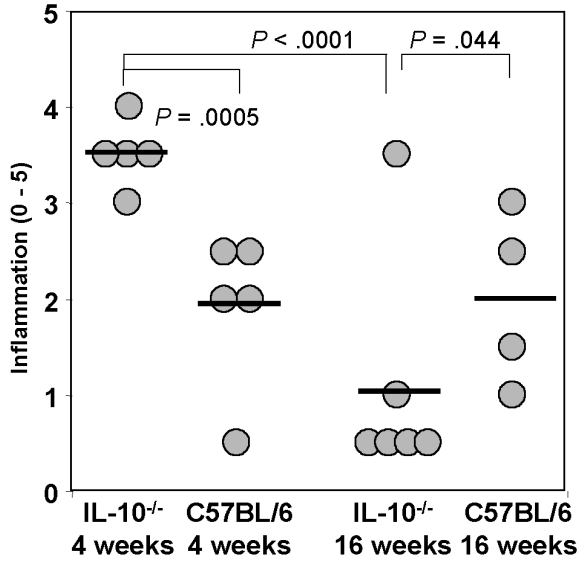

Figure 3. Il-$10^{-/-}$ mice develop severe inflammation relative to C57BL/6 mice in response to *H. pylori* infection. Mice were inoculated on two consecutive days with $1x10^7$ CFU *H. pylori* SS1. Subsets of each group were sacrificed and examined at either 4 weeks or 16 weeks post-inoculation and assessed for inflammation by examination of H&E stained sections. Statistical analysis was performed by ANOVA.

results in significantly greater inflammation by 4 weeks post inoculation (p=0.0005). Additionally, the *H. pylori* are spontaneously eradicated from these mice, but not from wild-type mice (data not shown). Spleen cells from the IL-$10^{-/-}$ mice also produce significantly greater levels of IFN-γ than wild type counterparts. By 16 weeks post-inoculation, in the absence of *H. pylori*, the inflammation in the IL-$10^{-/-}$ mice is significantly reduced (p<0.0001). Wild type mice, which remain infected, maintain a constant level of gastritis, significantly greater than the IL-$10^{-/-}$ mice at 16 weeks. Similar results with regard to bacterial load and inflammation in the IL-$10^{-/-}$ model have been reported by others (*Chen* et al., 2001).

Inflammation and immunoregulation

As stated above, challenge of immunised mice results in post-immune gastritis, which can be significantly greater than the gastritis induced by natural infection, at least within the first several weeks of challenge (*Garhart* et al., 2002). While some consider this a detriment to vaccination, the gastritis does dissipate over time. It may be that since the gastric mucosa lacks any organised or diffuse lymphoid structures, inflammation is essential to recruit the appropriate T cells to the stomach. Also, as previously mentioned, transfer of CD25-deficient lymph node cells to nude mice increases the inflammatory response following *H. pylori* challenge, as well as reducing the bacterial load, providing further evidence that inflammation may hold the key to *H. pylori* eradication (*Raghavan* et al., 2003). This concept is strengthened by our IL-$10^{-/-}$ studies in which eradication of the *H. pylori* was again accompanied by significant increases in gastritis.

We have recently described another model in which mice are able to spontaneously eradicate *H. pylori* from the

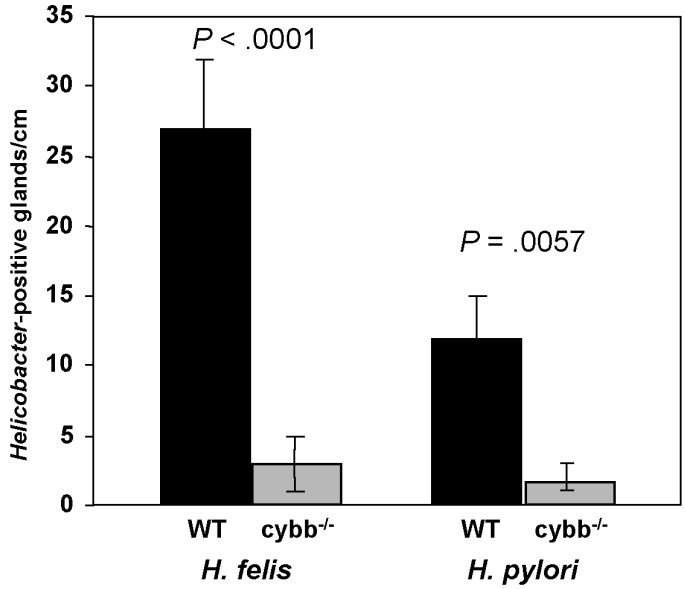

Figure 4. Phagocyte oxidase-deficient mice (cybb$^{-/-}$) respond to Helicobacter infection with severe inflammation and a reduced bacterial load relative to C57BL/6 mice. Mice were inoculated with 1x10^{7} CFU *H. pylori* SS1 or *H. felis* CS1 and sacrificed at 21 days post-inoculation for inflammation and bacterial load. Bacterial load was determined by direct enumeration of infected glands by examination of silver-stained histologic sections. Statistical analysis was performed by ANOVA.

gastric mucosa (*Blanchard* et al., 2003). Neutrophils and macrophages from NADPH phagocyte oxidase deficient mice (cybb$^{-/-}$) lack the ability to generate superoxide anions, a primary innate cellular antimicrobial defence mechanism (*Pollack* et al., 1995). This mouse line serves as an experimental model for human chronic granulomatous disease. Typically these mice have increased susceptibility to bacterial infection and delayed bacterial clearance when experimentally infected with bacteria (*Pollack* et al., 1995). When these mice are infected with either *H. pylori* or *H. felis* however, the inflammatory response is significantly greater than in wild-type controls. The bacterial load in these mice drops significantly, and in some cases the *Helicobacter* organisms are eradicated from the gastric mucosa within three weeks of infection (Figure 4). Although iNOS expression in the gastric tissue of mice with gastritis is elevated, mice deficient in iNOS, resembled wild type mice and similarly failed to eradicate *H. pylori*. Thus, the cybb$^{-/-}$ mouse is only the second mouse model described to date (in addition to the IL-10$^{-/-}$ mouse) capable of spontaneously eradicating *H. pylori*. Both models develop pronounced gastritis in response to infection.

A NEW MODEL OF *H. PYLORI* PATHOGENESIS AND IMMUNITY

Recent reports indicate that a reduction in *H. pylori* numbers in the gastric mucosa requires pro-inflammatory events. These have included the pres-

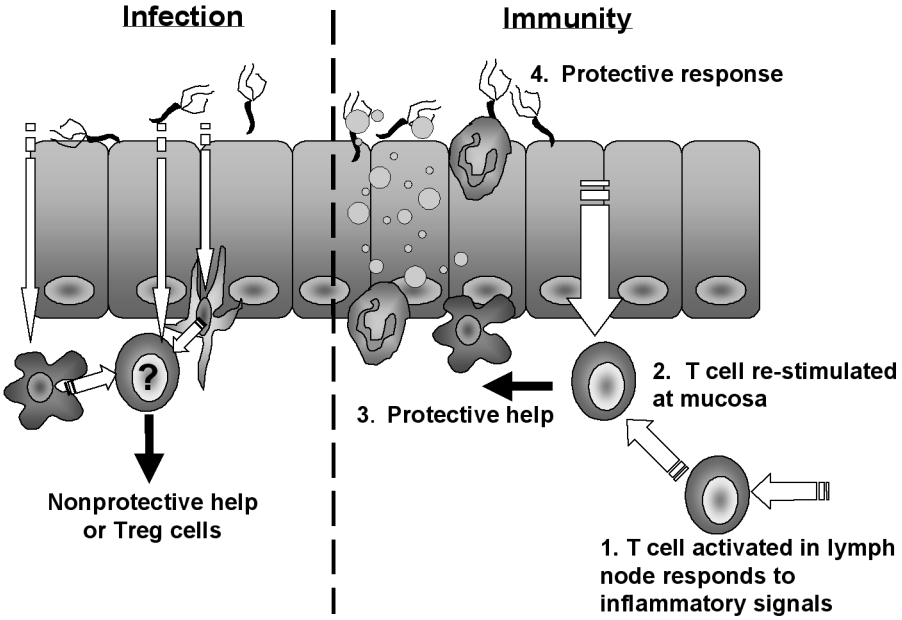

Figure 5. Model for *H. pylori* pathogenesis and immunity. *H. pylori* infection of the gastric mucosa results in activation of T cells recruited to the lamina propria (left side of figure). Antigen presentation may occur via MHC II-expressing epithelial cells, dendritic cells that bridge the tight junctions, or by macropohages that scavenge for bacteria and bacterial products that breech the epithelial monolayer. The activated T cells fail to elicit an effective immune response. Immunisation activates T cells in lymph nodes or other peripheral tissues resulting in fully active helper cells (right side of figure). Challenge of the gastric mucosa recruits these T cells to the site of inflammation where effective help results in a protective inflammatory response.

ence of post-immunisation gastritis when immunised mice are challenged (*Michetti* et al., 1994; *Pappo* et al., 1995; *Garhart* et al., 2002; *Goto* et al., 1999), a requirement for IL-12 in developing protective immunity (*Garhart* et al., 2003a; *Akhiani* et al., 2002), increased IFN-γ production upon challenge of immunised mice (*Goto* et al., 1999; *Garhart* et al., 2003a; *Eisenberg* et al., 2003; *Gottwein* et al., 2001), and spontaneous eradication only in mice that develop robust gastritis in response to infection (*Blanchard* et al., 2003; *Chen* et al., 2001). Therefore, previous theories that the induction of protective immunity requires a shift in immune character from a Th1 to a Th2 response, or even a mixed Th1/Th2 response, no longer accommodate the accumulating data. Additionally, when one considers that *H. pylori* infection does in fact stimulate *H. pylori*-specific T cells but fails to eradicate the infection, while immunisation by a number of different routes results in significant reduction in the *H. pylori* burden, a new model for *H. pylori* pathogenesis and immunity begins to emerge.

Whereas previous theories have promoted a Th1/Th2 dichotomy for pathogenesis and immunity, it is possible that *H. pylori*, while inducing a Th1 dominated response, survives in the stomach because it actually limits the inflammatory and immune response through the induction of *H. pylori*-specific immunoregulatory T cells. The studies mentioned above using IL-$10^{-/-}$ mice and describing $CD25^{+}$ regulatory T

cells in both mice and humans support this hypothesis. We propose that activation of T cells in the gastric mucosa results in a population of down-regulatory cells that limits both the inflammatory and immune response (Figure 5). When immunisations are applied however, activation of the T cells occurs in peripheral lymph nodes where activation of these T regulatory cells is not favoured. When the T cells initially activated in lymph nodes are recruited to the gastric mucosa as a result of *H. pylori* challenge, they are capable of promoting either a more severe inflammatory response or a qualitatively different immune response than is induced by natural infection.

This theory is consistent with what we know about immunoregulation of the intestinal mucosa. To prevent detrimental immunity and inflammation from occurring in response to normal indigenous bacterial flora, specific T cells down-regulate the response to those antigens resulting in maintenance of immunologic quiescence (*Khoo* et al., 1997; *Groux* et al., 1997; *Chen* et al., 1994; *Powrie*, 1995; *Powrie* et al., 1993). It is believed that conditions in the lamina propria such as antigen presentation by epithelial cells, the presence of IL-10 and TGF-ß, and immunoregulatory dendritic cells favour the induction of the regulatory T cells. Similar events may occur in the gastric mucosa. In fact, the increased incidence of gastro-oesophageal reflux disease following *H. pylori* eradication has led to speculation that *H. pylori* may have formed a symbiotic relationship with humans, and could be seen by the host as normal flora (*Blaser*, 1999). In this respect, the fraction of individuals that develop symptomatic gastritis and peptic ulcer disease may represent those individuals that have an aberrant response to *H. pylori,* in the same way that patients who suffer from inflammatory bowel disease (IBD) are believed to react inappropriately to their own intestinal flora. Further studies regarding the immunoregulation of the gastric mucosa should continue to improve our understanding of how protective immunity is accomplished against *H. pylori,* and will most likely be essential for the development of an efficacious vaccine for use in humans.

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LITERATURE

Akhiani, A.A., Pappo, J., Kabok, Z., Schon, K., Gao, W., Franzen, L.E., Lycke, N.: Protection against *Helicobacter pylori* infection following immunization is IL-12-dependent and mediated by Th1 cells. J. Immunol. 169, 6977-6984 (2002).

Bamford, K.B., Fan, X., Crowe, S.E., Leary, J.F., Gourley, W.K., Luthra, G.K., Brooks, E.G., Graham, D.Y., Reyes, V.E., and Ernst, P.B.: Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype. Gastroenterology 114, 482-492 (1998).

Blanchard, T.G., Nedrud, J.G., and Czinn, S.J.: Local and systemic antibody responses in humans with *Helicobacter pylori* infection. Can. J. Gastroenterol. 13, 591-594 (1999a).

Blanchard, T.G., Nedrud, J.G., Reardon, E.S.,

and Czinn, S.J.: Qualitative and quantitative analysis of the local and systemic antibody response in mice and humans with *Helicobacter* immunity and infection. J. Infect. Dis. 179, 725-728 (1999b).

Blanchard, T.G., Czinn, S.J., Redline, R.W., Sigmund, N., Harriman, G., and Nedrud, J.G.: Antibody-independent protective mucosal immunity to gastric *Helicobacter* infection in mice. Cell. Immunol. 191, 74-80 (1999c).

Blanchard, T.G., Yu, F., Hsieh, C.L., and Redline, R.W.: Severe inflammation and reduced bacteria load in murine helicobacter infection caused by lack of phagocyte oxidase activity. J. Infect. Dis. 187, 1609-1615 (2003).

Blaser, M.J.: Hypothesis: The changing relationships of *Helicobacter pylori* and humans: Implications for health and disease. J. Infect. Dis. 179, 1523-1530 (1999).

Chen, M., Lee, A., and Hazell, S.: Immunisation against gastric *Helicobacter* infection in a mouse/*Helicobacter felis* model. Lancet 339, 1120-1121 (1992).

Chen, Y., Kuchroo, V.K., Inobe, J.-I., Hafler, D.A., and Weiner, H.L.: Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. Science 265, 1237-1240 (1994).

Chen, W., Shu, D., and Chadwick, V.S.: *Helicobacter pylori* infection: Mechanism of colonization and functional dyspepsia. Reduced colonization of gastric mucosa by *Helicobacter pylori* in mice deficient in interleukin-10. J. Gastroenterol. Hepatol. 16, 377-383 (2001).

Corthesy-Theulaz, I., Porta, N., Glauser, M., Saraga, E., Vaney, A.C., Haas, R., Kraehenbuhl, J.P., Blum, A.L., and Michetti, P.: Oral immunization with *Helicobacter pylori* urease B subunit as a treatment against *Helicobacter* infection in mice. Gastroenterology 109, 115-121 (1995).

Cuenca, R., Blanchard, T.G., Czinn, S.J., Nedrud, J.G., Monath, T.P., Lee, C.K., and Redline, R.W.: Therapeutic immunization against *Helicobacter mustelae* in naturally infected ferrets. Gastroenterology 110, 1770-1775 (1996).

Czinn, S.J. and Nedrud, J.G.: Oral immunization against *Helicobacter pylori*. Infect. Immun. 59, 2359-2363 (1991).

Czinn, S.J., Cai, A., and Nedrud, J.G.: Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. Vaccine 11, 637-642 (1993).

D'Elios, M.M., Manghetti, M., De Carli, M., Costa, F., Baldari, C.T., Burroni, D., Telford, J.L., Romagnani, S., and Del Prete, G.: T helper 1 effector cells specific for *Helicobacter pylori* in the gastric antrum of patients with peptic ulcer disease. J. Immunol. 158, 962-967 (1997).

Di Tommaso, A., Xiang, Z., Bugnoli, M., Pileri, P., Figura, N., Bayeli, P.F., Rappuoli, R., Abrignani, S., and De Magistris, M.T.: *Helicobacter pylori*-specific CD4+ T-cell clones from peripheral blood and gastric biopsies. Infect. Immun. 63, 1102-1106 (1995).

Doidge, C., Crust, I., Lee, A., Buck, F., Hazell, S., and Manne, U.: Therapeutic immunisation against *Helicobacter* infection. Lancet 343, 914-915 (1994).

Dooley, C.P., Cohen, H., Fitzgibbons, P.L., Bauer, M., Appleman, M.D., Perez-Perez, G.I., and Blaser, M.J.: Prevalence of *Helicobacter pylori* infection and histologic gastritis in asymptomatic persons. N. Engl. J. Med. 321, 1562-1566 (1989).

Eisenberg, J.C., Czinn, S.J., Garhart, C.A., Redline, R.W., Bartholomae, W.C., Gottwein, J.M., Nedrud, J.G., Emancipator, S.E., Boehm, B.B., Lehmann, P.V., and Blanchard, T.G.: Protective efficacy of anti-*Helicobacter pylori* immunity following systemic immunization of neonatal mice. Infect. Immun. 71, 1820-1827 (2003).

Ermak, T.H., Ding, R., Ekstein, B., Hill, J., Myers, G.A., Lee, C.K., Pappo, J., Kleanthous, H.K., and Monath, T.P.: Gastritis in urease-immunized mice after *Helicobacter felis* challenge may be due to residual bacteria. Gastroenterology 113, 1118-1128 (1997).

Ermak, T.H., Giannasca, P.J., Nichols, R., Myers, G.A., Nedrud, J., Weltzin, R., Lee, C.K., Kleanthous, H., and Monath, T.P.: Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. J. Exp. Med. 188, 2277-2288 (1998).

Fan, X.J., Chua, A., Shahi, C.N., McDevitt, J., Keeling, P.W., and Kelleher, D.: Gastric

T lymphocyte responses to *Helicobacter pylori* in patients with *H. pylori* colonisation. Gut 35, 1379-1384 (1994).

Ferrero, R.L., Thiberge, J.-M., Huerre, M., and Labigne, A.: Recombinant antigens prepared from the urease subunits of *Helicobacter* spp: Evidence of protection in a mouse model of gastric infection. Infect. Immun. 62, 4981-4989 (1994).

Ferrero, R.L., Thiberge, J.M., Kansau, I., Wuscher, N., Huerre, M., and Labigne, A.: The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proc. Natl. Acad. Sci. USA 92, 6499-6503 (1995).Karttunen, R., Andersson, G., Poikonen, K., Kosunen, T.U., Karttunen, T., Juutinen, K., and Niemela, S.: *Helicobacter pylori* induces lymphocyte activation in peripheral blood cultures. Clin. Exp. Immunol. 82, 485-488 (1990).

Fox, J.G., Beck, P., Dangler, C.A., Whary, M.T., Wang, T.C., Shi, H.N., and Nagler-Anderson, C.: Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces *Helicobacter*-induced gastric atrophy. Nat. Med. 6, 536-542 (2000).

Fujihashi, K., Koga, T., van Ginkel, F.W., Hagiwara, Y., and McGhee, J.R.: A dilemma for mucosal vaccination: Efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine 20, 2431-2438 (2002).

Garhart, C.A., Redline, R.W., Nedrud, J.G., and Czinn, S.J.: Clearance of *Helicobacter pylori* infection and resolution of postimmunization gastritis in a kinetic study of prophylactically immunized mice. Infect. Immun. 70, 3529-3538 (2002).

Garhart, C.A., Heinzel, F.P., Czinn, S.J., and Nedrud, J.G.: Vaccine-induced reduction of *Helicobacter pylori* colonization in mice is interleukin-12 dependent but gamma interferon and inducible nitric oxide synthase independent. Infect. Immun. 71, 910-921 (2003a).

Garhart, C.A., Nedrud, J.G., Heinzel, F.P., Sigmund, N.E., and Czinn, S.J.: Vaccine-induced protection against *Helicobacter pylori* in mice lacking both antibodies and interleukin-4. Infect. Immun. 71, 3628-3633 (2003b).

Ghiara, P., Rossi, M., Marchetti, M., Di Tommaso, A., Vindigni, C., Ciampolini, F., Covacci, A., Telford, J.L., De Magistris, M.T., Pizza, M., Rappuoli, R., and Del Giudice, G.: Therapeutic intragastric vaccination against *Helicobacter pylori* in mice eradicates an otherwise chronic infection and confers protection against reinfection. Infect. Immun. 65, 4996-5002 (1997).

Goto, T., Nishizono, A., Fujioka, T., Ikewaki, J., Mifune, K., and Nasu, M.: Local secretory immunoglobulin A and postimmunization gastritis correlate with protection against *Helicobacter pylori* infection after oral vaccination of mice. Infect. Immun. 67, 2531-2539 (1999).

Gottwein, J.M., Blanchard, T.G., Targoni, O.S., Eisenberg, J.C., Zagorski, B.M., Redline, R.W., Nedrud, J.G., Tary-Lehmann, M., Lehmann, P.V., and Czinn, S.J.: Protective anti-*Helicobacter* immunity is induced with aluminum hydroxide or complete Freund's adjuvant by systemic immunization. J. Inf. Dis. 184, 308-314 (2001).

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G.: A CD4+ T-cell subset inhibits antigen-specific T cell respones and prevents colitis. Nature 389, 737-742 (1997).

Guy, B., Hessler, C., Fourage, S., Haensler, J., Vialon-Lafay, E., Rokbi, B., and Millet, M.J.: Systemic immunization with urease protects mice against *Helicobacter pylori* infection. Vaccine 16, 850-856 (1998).

Karttunen, R.: Blood lymphocyte proliferation, cytokine secretion and appearance of T cells with activation surface markers in cultures with *Helicobacter pylori*. Comparison of the responses of subjects with and without antibodies to *H. pylori*. Clin. Exp. Immunol. 83, 396-400 (1991).

Karttunen, R., Karttunen, T., Ekre, H.P., and MacDonald, T.T.: Interferon gamma and interleukin 4 secreting cells in the gastric antrum in *Helicobacter pylori* positive and negative gastritis. Gut 36, 341-345 (1995).

Khoo, U.Y., Proctor, I.E., and Macpherson, A.J.S.: CD4+ T cell down-regulation in human intestinal mucosa: Evidence for intestinal tolerance to lumenal bacterial antigens. J. Immunol. 158, 3626-3634 (1997).

Kleanthous, H., Myers, G.A., Georgakopoulos, K.M., Tibbitts, T.J., Ingrassia, J.W.,

Gray, H.L., Ding, R., Zhang, Z.Z., Lei, W., Nichols, R., Lee, C.K., Ermak, T.H., and Monath, T.P.: Rectal and intranasal immunizations with recombinant urease induce distinct local and serum immune responses in mice and protect against *Helicobacter pylori* infection. Infect. Immun. 66, 2879-2886 (1998).

Lee, A., Fox, J.G., Otto, G., and Murphy, J.: A small animal model of human *Helicobacter pylori* active chronic gastritis. Gastroenterology 99, 1315-1323 (1990).

Lindholm, C., Quiding-Jarbrink, M., Lonroth, H., Hamlet, A., and Svennerholm, A.M.: Local cytokine response in *Helicobacter pylori*-infected subjects. Infect. Immun. 66, 5964-5971 (1998).

Lucas, B., Bumann, D., Walduck, A., Koesling, J., Develioglu, L., Meyer, T.F., Aebischer, T.: Adoptive transfer of CD4+ T cells specific for subunit A of *Helicobacter pylori* urease reduces *H. pylori* stomach colonization in mice in the absence of interleukin-4 (IL-4)/IL-13 receptor signaling. Infect. Immun. 69, 1714-1721 (2001).

Lundgren, A., Suri-Payer, E., Enarsson, K., Svennerholm, A.M., and Lundin, B.S.: *Helicobacter pylori*-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to *H. pylori* in infected individuals. Infect. Immun. 71, 1755-1762 (2003).

Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P.: Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science 267, 1655-1658 (1995).

Marchetti, M., Rossi, M., Giannelli, V., Giuliani, M.M., Pizza, M., Censini, S., Covacci, A., Massari, P., Pagliaccia, C., Manetti, R., Telford, J.L., Douce, G., Dougan, G., Rappuoli, R., and Ghiara, P.: Protection against *Helicobacter pylori* infection in mice by intragastric vaccination with *H. pylori* antigens is achieved using a non-toxic mutant of *E. coli* heat-labile enterotoxin (LT) as adjuvant. Vaccine 16, 33-37 (1998).

Marshall, B.J. and Warren, J.R.: Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1, 1311-1315 (1984).

Marshall, B.J., Armstrong, J.A., McGechie, D.B., and Glancy, R.J.: Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. Med. J. Aust. 142, 436-439 (1985).

Marshall, B.J.: The 1995 Albert Lasker Medical Research Award. *Helicobacter pylori*. The etiologic agent for peptic ulcer. JAMA 274, 1064-1066 (1995).

Michetti, P., Corthesy-Theulaz, I., Davin, C., Haas, R., Vaney, A.C., Heitz, M., Bille, J., Kraehenbuhl, J.P., Saraga, E., and Blum, A.L.: Immunization of BALB/c mice against *Helicobacter felis* infection with *Helicobacter pylori* urease. Gastroenterology 107, 1002-1011 (1994).

Michetti, P., Kreiss, C., Kotloff, K.L., Porta, N., Blanco, J.L., Bachmann, D., Herranz, M., Saldinger, P.F., Corthesy-Theulaz, I., Losonsky, G., Nichols, R., Simon, J., Stolte, M., Ackerman, S., Monath, T.P., and Blum, A.L.: Oral immunization with urease and *Escherichia coli* heat-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. Gastroenterology 116, 804-812 (1999).

Mohammadi, M., Czinn, S., Redline, R., and Nedrud, J.: *Helicobacter*-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. J. Immunol. 156, 4729-4738 (1996).

Morris, A. and Nicholson, G.: Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. Am. J. Gastroenterol. 82, 192-199 (1987).

Muminova, Z., Saparov, A., Oliver, J.R., Thomas, J.S., and Weaver, C.T.: Antigen-specific T cell clones derived from the lamina propria of TCR transgenic mice have a Tr1-like cytokine phenotype. FASEB J. 13, A942 (1999).

Nedrud, J.G., Liang, X.P., Hague, N., and Lamm, M.E.: Combined oral/nasal immunization protects mice from Sendai virus infection. J. Immunol. 139, 3484-3492 (1987).

NIH Consensus Conference: *Helicobacter pylori* in peptic ulcer disease. JAMA 272, 65-69 (1994).

Pappo, J., Thomas, W.D., Jr., Kabok, Z., Taylor, N.S., Murphy, J.C., and Fox, J.G.: Effect of oral immunization with recombinant urease on murine *Helicobacter felis* gastritis. Infect. Immun. 63, 1246-1252 (1995).

Pappo, J., Torrey, D., Castriotta, L., Savinainen, A., Kabok, Z., and Ibraghimov, A.: *Helicobacter pylori* infection in immunized mice lacking major histocompatibility complex class I and class II functions. Infect. Immun. 67, 337-341 (1999).

Pollack, J.D., Williams, D.A., Gifford, M.A.C., Li, L.L., Du, X., Fisherman, J., Orkin, S.H., Doerschuk, C.M., and Dinauer, M.C.: Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. Nature Genet. 9, 202-209 (1995).

Powrie, F., Leach, M.W., Mauze, S., Caddle, L.B., and Coffman, R.L.: Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C.B-17 scid mice. Int. Immunol. 5, 1461-1471 (1993).

Powrie, F.: T cells in inflammatory bowel disease: Protective and pathogenic roles. Immunity 3, 171-174 (1995).

Raghavan, S., Fredriksson, M., Svennerholm, A.M., Holmgren, J., and Suri-Payer, E.: Absence of CD4+CD25+ regulatory T cells is associated with a loss of regulation leading to increased pathology in *Helicobacter pylori*-infected mice. Clin. Exp. Immunol. 132, 393-400 (2003).

Rathbone, B.J., Wyatt, J.I., Worsley, B.W., Shire,s S.E., Trejdosiewicz, L.K., Heatley, R.V., and Losowsky, M.S.: Systemic and local antibody responses to gastric *Campylobacter pyloridis* in non-ulcer dyspepsia. Gut 27, 642-647 (1986).

Sawai, N., Kita, M., Kodama, T., Tanahashi, T., Yamaoka, Y., Tagawa, Y., Iwakura, Y., and Imanishi, J.: Role of gamma interferon in *Helicobacter pylori*-induced gastric inflammatory responses in a mouse model. Infect. Immun. 67, 279-285 (1999).

Sharma, S.A., Miller, G.G., Perez-Perez, G.I., Gupta, R.S., and Blaser, M.J.: Humoral and cellular immune recognition of *Helicobacter pylori* proteins are not concordant. Clin. Exp. Immunol. 97, 126-132 (1994).

Smythies, L.E., Waites, K.B., Lindsey, J.R., Harris, P.R., Ghiara, P., and Smith, P.D.: *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. J. Immunol. 165, 1022-1029 (2000).

Sommer, F., Faller, G., Konturek, P., Kirchner, T., Hahn, E.G., Zeus, J., Rollinghoff, M., and Lohoff, M.: Antrum- and corpus mucosa-infiltrating CD4(+) lymphocytes in *Helicobacter pylori* gastritis display a Th1 phenotype. Infect. Immun. 66, 5543-5546 (1998).

Sutton, P., Wilson, J., Kosaka, T., Wolowczuk, I., and Lee, A.: Therapeutic immunization against *Helicobacter pylori* infection in the absence of antibodies. Immunol. Cell. Biol. 78, 28-30 (2000).

Warren, J.R.: Gastric pathology associated with *Helicobacter pylori*. Gastroenterol. Clin. North Am. 29, 705-751 (2000).

Weltzin, R., Guy, B., Thomas, W.D., Jr., Giannasca, P.J., and Monath, T.P.: Parenteral adjuvant activities of *Escherichia coli* heat-labile toxin and its B subunit for immunization of mice against gastric *Helicobacter pylori* infection. Infect. Immun. 68, 2775-2782 (2000).

World Health Organization: Schistosomes, Liver Flukes and *Helicobacter pylori*. International Agency for Research on Cancer, Lyon, 177-241 (1994).

Wyatt, J.I., Rathbone, B.J., and Heatley, R.V.: Local immune response to gastric *Campylobacter* in non-ulcer dyspepsia. J. Clin. Pathol. 39, 863-870 (1986).

NEISSERIA MENINGITIDIS, *NEISSERIA LACTAMICA* AND *MORAXELLA CATARRHALIS* SHARE CROSS-REACTIVE CARBOHYDRATE ANTIGENS*

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SUMMARY

Carriage of commensal bacteria species is associated with the development of natural immunity to meningococcal disease, with lipo-oligosaccharides (LOS) of meningococci being one of the main virulence factors associated with severity of meningococcal disease. Meningococcal reference strains and isolates from the commensal species *Neisseria lactamica* and *Moraxella catarrhalis* were assessed for the presence of cross-reactive glycoconjugate antigens. Binding of human blood group antibodies of the P and Ii system to meningococcal immunotype reference strains were in accordance with the presence of known LOS carbohydrate structures. Binding studies with meningococcal immunotyping antibodies and blood group phenotyping antibodies to *N. lactamica* strains from different European countries showed, that a greater number of isolates obtained from native Greek and Scottish adults and children bound anti-meningococcal L(3,7,9) immunotyping ($p<0.001$), pK ($p=0.035$) and paragloboside ($p<0.001$) blood group typing antibodies compared to isolates obtained from children of Russian immigrants in Greece. A greater number of *M. catarrhalis* strains isolated from children in Scotland bound anti-L(3,7,9) antibodies (38.2%) compared to strains isolated from adults (22.2%) ($p=0.017$). These findings provide evidence that blood group like glycoconjugate antigens found on the commensal species *Neisseria lactamica* and *Moraxella catarrhalis* might be involved in the development of natural immunity to meningococcal endotoxins during childhood, and might be exploited as anti-meningococcal vaccine candidates.

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INTRODUCTION

Evidence of common oligosaccharide structures for meningococci and *N. lactamica* (NL) or meningococci and *Moraxella catarrhalis* (MC) has been reported to share oligosaccharide antigens with some carbohydrate structures found on human tissues including paragloboside, P, P1, p^K, and Ii blood group antigens (*Mandrell* et al., 1988). The expression of blood group related LOS on meningococcal carrier strains and outbreak strains differs greatly. While disease is mainly associated with meningococcal immunotype L(3,7,9) showing homology with the paragloboside antigen (a precursor of the human nP1 blood group antigen), carrier strains isolated in Britain were found to express LOS immunotypes similar to the p^K and ceramide-dihexocide blood group antigens L1 and L8, respectively (*Jones* et al., 1992). There has been no systematic screening of commensal NL or MC isolates from different regions of Europe with the immunotype antibodies used to classify NM immunotypes or antibodies to human blood group antigens.

Thirteen major LOS types were identified for *N. meningitidis* using polyclonal and monoclonal antibodies by passive haemagglutination inhibition techniques and whole cell ELISA (*Abdillahi* and *Poolman*, 1988; *Scholten* et al., 1994). The majority of meningococcal isolates express one or more of the immunotypes L1-L12, while nontypable and L13 immunotypes are rare. The twelve major LOS types have a relative molecular weight ranging from 3.15 to 7.1 kDa. The oligosaccharide chain, also referred to as the α-chain or variable LOS region one (R1), is composed of the saccharides glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc). Sialylated forms contain the terminal saccharide *N*-acetylneuramic acid (NeuNAc) is added to terminal galactose residues by endogenous or exogenous sialyl transferases. Abbreviations for core moieties are used as follows: glycero-D-manno-heptopyranoside (Hep or heptose); phospho-ethanolamine (PEA); 2-keto-3-deoxyoctulosonic (KDO). The complete structures of immunotypes L10 – L13 are not elucidated, but there is evidence that L10 contains the paragloboside residue and L11 shows some homology with L1. The PEA residue of immunotype L2 can be expressed in two forms that undergo phase variation: The PEA on the G3 region can be linked in (1→6) or (1→7) conformation. The PEA can be replaced by a hydrogen (H) atom. The PEA residue of immunotypes L4 and L6 express both PEA (1→6) and (1→7) linkages. The expression of meningococcal immunotypes is associated with serogroups. Immunotypes L8, L9, L10, L11 and L12 are found on group A strains, while serogroup B and C meningococci express immunotypes L1 – L8 (*Scholten* et al., 1994; *Dell* et al., 1990; *Gu* et al., 1992; *Jennings* et al., 1983; *Kim* et al., 1994; *Kogan* et al., 1997; *Michon* et al., 1990; *Pavliak* et al., 1993; *Plested* et al., 1999; *Rahman* et al., 1998a; *Wakarchuk* et al., 1998)

Immunotypes and pathogenicity

LOS immunotype expression is thought to be linked to the pathogenicity of the organism. Immunotypes L(3,7,9) are isolated predominantly from patients with invasive meningococcal disease. Other immunotypes are found predominantly among carrier strains. Immunotypes L3, L7 and L9 are thought to be similar in their immunochemical structures with immunotype L3 being sialylated by endogenous sialyl transferases. Immunotypes L3 and L7 are found on

serogroup B and C meningococci and they have similar G2 core components, PEA (1→3) HepII. Immunotype L9 is expressed on group A strains (*Jones* et al., 1992; *Plested* et al., 1999).

The presence of the sialylated phenotype on invasive meningococci is associated with resistance to complement-mediated killing by masking the terminal galactose with NeuNAc. This mechanism is thought to reduce the recognition of the epitope by anti-LOS antibodies directed against the non-sialylated epitopes. Free or membrane bound sialyl-L(3,7,9) also upregulates neutrophil activation markers and results in increased injury of epithelial cell lines. Sialyl L(3,7,9) phenotypes can evade the complement mediated bacteriolysis cascade and reduces complement and anti-LOS antibody mediated phagocytosis by professional phagocytes (*Mandrell* et al., 1991,1993; *Mandrell* and *Apicella*, 1993; *Hammerschmidt* et al., 1994; *McLeod Griffiss* et al., 2000).

Expression of major and minor immunotypes by *N. meningitidis*

Meningococci are able to express more than one immunotype. Isolates from patients with meningococcal disease in the Netherlands (1989-1990) showed different immunotype combinations (*Scholten* et al., 1994).

1. Group A meningococci L9 (54%), L9,8 (8%), L10 (24%), L10,11 (8%) and non-typable (NT) (8%).
2. Group B meningococci L1 (1%), L1,8 (11%), L2 (10%), L3,7 (36%), L3,7,1 (4%), L3,7,1,8 (2%), L3,7,8 (28%), L4 (4%), and L8 (5%).
3. Group C meningococci L1,8 (2%), L2 (30%), L3,7 (37%), L3,7,1 (1%), L3,7,1,8 (3%), L3,7,8 (7%), L4 (15%), L8 (3%), and NT (3%).

The expression of multiple immunotypes within a meningococcal population is thought to allow the organism to diversify its antigenic structure, evading selective pressure of the host's immune system. Sialylation and the expression of paragloboside gene cluster IgtABE are the main phase variable phenotypes known (*Jennings* et al., 1999).

The expression of meningococcal immunotypes undergoes phase variation due to *in vitro* growth conditions. The variability of meningococcal phenotypes and LOS expression depends on the growth rate and phase, as well as the presence of exogenous sialyl transferases (*Berrington* et al., 2002).

LOS based vaccines

The most common LOS immunotype associated with disease is L(3,7,9) found in both group B and C outbreak strains of meningococci in Europe and America, and group A in sub-Sahara-Africa (*Varaine* et al., 1997; *Booy* and *Kroll*, 1998; *Fonkoua*, 2002). Meningococcal LOS is closely associated with the severity and fatality of disease. This is mainly due to its involvement in inducing large amounts of pro-inflammatory cytokines in a CD14 dependent mechanism. Anti-meningococcal LOS antibodies are not only bactericidal, but also opsonising in nature, resulting in the phagocytosis of invading bacteria and LOS containing blebs by human monocytes. Normal human serum of adults usually contains antibodies against meningococcal LOS, suggesting its important role in development of natural immunity to meningococcal disease (*Braun* et al., 2002). Several anti-meningococcal LOS vaccine candidates are currently under investigation (*Katial* et al., 2002; *Saunders* et al., 1999).

P-related blood group system

Carbohydrate antigens are widely distributed on human blood cells and tissues. Their expression is facilitated through glycosyl-transferases during the post-translational modification of

Table 1: Blood group antigens of the P and Ii system

Blood group	α chain moiety
CDH	Galβ (1→4) Glcβ
P globoside	GalNAcβ (1→3) Galα (1→4) Galβ (1→4) Glcβ
p^k, CD77	Galα (1→4) Galβ (1→4) Glcβ
P1	Galα (1→4) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
Paragloboside	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i a	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
i b	*S* - Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ
I a αβ, I b β	Galβ (1→4) GlcNAcβ (1→3; 1→6) Galβ (1→4) GlcNAcβ (1→4) Galβ (1→4) Glcβ
I c α	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→6) GalNAcβ
I c β	Galβ (1→4) GlcNAcβ (1→6) GalNAcβ
I d α,β	Galβ (1→4) GlcNAcβ (1→6) GalNAcβ

CDH, ceramide-di-hexocide; I, adult; i, foetal; S, sialyc acid; the glucose is linked to the membrane anchored ceramide (Glcβ (1→1) Ceramide)

proteins (glycoproteins) or linkage to ceramide (N-linked fatty acyl sphingosine). The ABO blood group antigens can be expressed either as glycoproteins or as ceramide glycolipids. The P-blood group system is thought to be expressed in glycolipid form on red blood cells and other tissues (*Prokop* and *Uhlenbruck*, 1965; *Race* and *Sanger*, 1975; *Hakomori* and *Kannagi*, .1986; *Bailly* et al., 1992; *Brown* et al., 1993) It consists of a single oligosaccharide chain linked to a membrane-anchored ceramide (Table 1). One member of the P-system, the globotriaosylceramide (p^K or CD77) is associated with the differentiation and maturation of human B cells (*Butch* and *Nahm*, 1992) and B-cell Burkitt lymphomas (*Wiels* et al., 1981). While other members of the P-system are readily expressed on human red blood cells, the expression of p^K is relatively rare (*Marcus* et al., 1976). It is thought that oligosaccharides with a terminal galactose residue can be found in sialylated or non-sialylated forms due to the sialyl-transferases found in human serum, an enzyme that is also associated with the sialylation of meningococcal LOS (*Wakarchuk* et al., 1998; *Mandrell* et al., 1991,1993; *Mandrell* and *Apicella*, 1993; *Hammerschmidt* et al., 1994; *McLeod Griffiss* et al., 2000).

Ii-blood group system

Similar to the p^K antigen, Ii determinants are associated with developmental maturation in humans. Although, p^K might be expressed in children and adults, i-antigens are found in foetal tissue but rarely in children or adults. I-antigen expression coincides with the loss of i-blood group moieties (*Marsh* and *Jenkins*, 1960; *Marsh*, 1961; *Wiener*, 1973). The i-determinant has a single chain oligosaccharide structure linked to a membrane anchored ceramide, while the I-blood group antigens consist of a branched structure at the third terminal saccharide. Ia and Ib are glycolipids linked to ceramide, while the carbohydrate antigens Ic and Id form the glycosyl structure of glycoproteins linked to the amino acids serine and threonine.

Structural homology of *N. meningitidis* LOS with blood group antigens

The oligosaccharide moiety of the α-chain of NM LOS shares structural homology with some human blood group antigens (Table 2), and these

Table 2: Oligosaccharide and core structures of meningococcal LOS immunotypes

LOS type	Terminal oligosaccharide α chain oligomer of the G1 region	Core		
		[A]	G2	G3
L1	Galα (1→4) Galβ (1→4) Glcβ	-	PEA (1→3)	H
L2	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	Glcα (1→3)	PEA H, (1→6), (1→7)
L3	S (2→3) Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	PEA (1→3)	H
L4	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	H (→3)	PEA (1→6), (1→7)
L5	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	Glcβ (1→4)	Glcα (1→3)	H
L6	GalNAcβ (1→3) Galα (1→4) Glcβ	-	H(→3)	PEA (1→6), (1→7)
L7	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	PEA (1→3)	H
L8	Galβ (1→4) Glcβ	-	PEA (1→3)	H
L9	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	-	PEA (n.e.)	n.e.
L10	Galβ (1→4) GlcNAcβ (1→3) Galβ (1→4) Glcβ	n.e.	PEA (n.e.)	n.e.
L11	Galα (1→4) Galβ (1→4) Glcβ	n.e.	PEA (n.e.)	n.e.
L12	n.e.	n.e.	PEA (n.e.)	n.e.
L13	n.e.	n.e.	n.e.	n.e.

Abbreviations: Gal, galactose; GlcNAc, N-acetylglucosamine; Glc, glucose; S, sialyc acid; Hep, heptose (glycero-D-manno-heptopyranoside); PEA, phospho-ethanolamine; H, hydrogen; [A], Glcβ (1→4) insertion of the α chain; n.e., not elucidated.

Table 3: Primary structure of α and β chains of MC LOS of MC immunotypes A, B and C

LOS type	Chain	Terminal oligosaccharides (variable regions)	Homology with human oligosaccharides	Homology with meningococcal immunotypes
A	α	Galα (1→4) Galβ (1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	GlcNAcα(1→2) Glcβ		
B6	α	Glcα (1→2) Glcβ		
	β	Glcβ		
B7	α	Glcα (1→2) Glcβ		
	β	Glcα (1→2) Glcβ		
B8	α	Glcα (1→2) Glcβ		
	β	Galβ(1→4) Glcα (1→2) Glcβ	Dihexoceramide	L8
B9	α	Galβ(1→4) Glcα (1→2) Glcβ	Dihexoceramide	L8
	β	Galβ(1→4) Glcα (1→2) Glcβ	Dihexoceramide	L8
B10	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galβ(1→4) Glcα (1→2) Glcβ	Dihexoceramide	L8
B11	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
C8	α	Glcα (1→2) Glcβ		
	β	Galβ(1→4) GlcNAcα(1→2) Glcβ	Paragloboside	L2, L(3,7,9), L5
C10	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galβ(1→4) GlcNAcα(1→2) Glcβ	Paragloboside	L2, L(3,7,9), L5
C11	α	Galα (1→4) Galβ(1→4) Glcα (1→2) Glcβ	p^K blood group	L1, L11
	β	Galα (1→4) Galβ(1→4) GlcNAcα(1→2) Glcβ	P1 blood group	

Abbreviations: Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine ; Glc, glucose.

structures have been identified in the LOS of several isolates of NM and *N. gonorrhoea* (*Mandrell* et al., 1988; *Kim* et al., 1989). The G1 region of L1 and L11 meningococcal LOS immunotypes show identical terminal oligosaccharide residues of ceramide trihexocide, Galα (1→4) Galβ (1→4) Glcβ, identical to the human p^k blood group antigen (CD77) (*Griffiss* et al., 1987a,b).

The lacto-N-neotetranose residue, Galβ (1→4) GlcNAcβ (1→3) Galβ, a 4.5 kilodalton LOS component of immunotypes L2, L(3,7,9), L4 and L5 is identical to paragloboside with different distributions in NM, *N. gonorrhoea* and NL (*Kim* et al., 1989). Additionally, paragloboside, a precursor of P1 blood group antigen found in 75% of Caucasians, is the terminal structure of the human I-erythrocyte antigen and the embryonic i-antigen (*Mandrell* et al., 1988; *Hakomori* and *Kannagi*, 1986; *Tsai* and *Civin*, 1991). It is also an epitope homologous to type 14 pneumococcal polysaccharide capsules (*Siddiqui* and *Hakomori*, 1973). Immunotype L6 shares its two terminal sugars, Galβ (1→4) GlcNAcβ, with the P blood group antigen, and L8 shares its terminal disaccharide with ceramide dihexocide, Galβ (1→4) Glcβ, the common precursor of the P blood group system and steroid receptors.

Structural homology of *M. catarrhalis* LOS with blood group antigens

The oligosaccharide moieties of the α-or ß-chains of *M. catarrhalis* LOS shares structural homology with some human blood (Table 3). Serological typing of MC is based upon its LOS. All serotypes of MC have LOS of a similar molecular weight, about 5.5 kDa (*Holme* et al., 1999). This differs greatly from immunotypes found in meningococci and *N. gonorrhoeae, which* have variable sizes of LOS, 4.1-5.0 kDa and 3-5 kDa, respectively (*Schneider* et al., 1984).

There are three major LOS types (A, B, and C) found in approximately 95% of all MC isolates identified in 61%, 29%, and 5% of isolates, respectively (*Rahman* et al., 1997,1998a,b; *Rahman* and *Holme*, 1996; *Vaneechoutte* et al., 1990a,b; *Edebrink* et al., 1994,1995, 1996; *Masoud* et al., 1994). Lipid A is anchored in the outer membrane of the bacterial envelope linked to KDO-I that is linked to KDO-II. A Glcβ (3→1) Glc moiety is linked to (1→5) KDO-I forming the backbone of all known MC immunotypes. The LOS β-chain is linked to the 4^{th} carbon, the α-chain to the 6^{th} carbon of the glucose (1→5) KDO-1 residue. Both chains are variable in length and oligosaccharide composition that determines the MC immunotypes. In contrast to the LOS of meningococci, heptose is not present in the LOS of MC (*Holme* et al., 1999). The major difference between the immunotypes of MC is that groups A and C contain GlcNAcα within its β-chain, while group B contains Glcα in its place. Several authors have reported some structural and antigenetic homology or similarity between commensal bacteria, or *Neisseria* and *Moraxella* species (*Rahman* et al., 1998b; *Jonsson* et al., 1992,1993,1994).

OBJECTIVES

The aim of this study was to test the hypothesis that LOS found on the commensal species *N. lactamica* and *M. catarrhalis* share cross-reactive antigens with meningococcal immunotypes and human blood group antigens that might

induce protective immunity against meningococcal disease. The objectives of this study were: 1) to assess *N. lactamica* and *M. catarrhalis* isolates for binding of antibodies to blood group antigens and NM immunotype antigens and 2) to compare binding of these antibodies by commensal isolates from different geographical regions of Europe and the age groups.

MATERIAL AND METHODS

Bacterial strains

Standard meningococcal immunotype strains L1-L12 were obtained from Dr. W. D. Zollinger, Washington D.C. *N. lactamica* isolates were obtained from our culture collection or colleagues in different regions of Europe: Dr. P. Krizova, National Reference Laboratory for Meningococcal Disease, Prague, Czech Republic; Dr. K. Jónsdóttir, University Hospital, Reykjavik, Iceland; Dr. G. Tzanakaki, National Meningococcal Reference Laboratory, National School of Public Health, Athens, Greece; Dr. S. Clarke, Scottish Meningococcal and Pneumococcal Reference Laboratory (SMPRL), Glasgow, Scotland. *M. catarrhalis* clinical isolates from our culture collection were kindly provided by Dr. El-Ahmer. None of the NL or MC isolates were agglutinated by standard meningococcal capsular serogroup reagents.

Bacterial growth conditions

Cultures were grown overnight at 37ºC on human blood agar (HBA) containing: lysed erythrocytes concentrates (100 ml) obtained from the blood transfusion services, University of Cologne; special peptone (23 g) (Difco); corn starch (1 g) (Sigma); NaCl (4.5 g) (Sigma); D-glucose (1 g) (Sigma); technical grade agar (10 g) (OXOID); K_2HPO_4 (4 g) and KH_2PO_4 (1 g); 900 ml of distilled water.

Antibodies

Primary antibodies used to detect expression of meningococcal immunotype antigens were as follows: L1 (17-1-L1), L(3,7,9) (12C10), L8 (6E7-10) and L10 (14-1-L10) (all mouse IgG) were provided by W. Zollinger, Walter Reid Army Institute for Research, Washington D.C. Antibodies directed against human blood group antigens were used as follows: paragloboside (mouse IgM) (1B12-1B7, ATCC); P1 (mouse IgM) (Z202) and Ii (human IgG) (Z248) (both Diagnostic Scotland, Edinburgh, UK); P (rabbit IgG) (A0302 118, DAKO); pK (rat IgM) (MCA579, Serotec). The following horseraddish peroxidase (HRP) conjugated secondary antibodies were used: protein A (*S. aureus*) (Sigma); anti mouse IgM μ chain (goat) (Sigma); anti-rabbit IgG γ chain (rat) (Biosource); and anti rat IgM μ chain (mouse) (Biosource).

Whole cell ELISA (WCE)

WCE was used to screen for binding of antibodies to blood group and immunotype antigens on bacteria based on previously published methods for the detection of antigens on meningococci (*Abdillahi* and *Poolman*, 1988; *Scholten* et al., 1994). Bacteria were grown overnight on HBA, harvested in sterile filtered 0.5% (v/v) buffered formalin and washed twice in sterile filtered phosphate buffered saline (PBS). The cell suspension was adjusted to a final concentration of 10^{10} bacteria ml^{-1} which correlated to an absorption of OD_{546}=0.600. The cell suspension (100 μl) was distributed into sterile flat bottom 96 well PVC microtitre plates (Greiner) and allowed to dry overnight

at 56°C. The coated plates were kept at room temperature (RT) for up to three months.

Assay

The coated plates were washed 3 times with washing solution containing Tween 80 (0.25 ml) (Sigma) in 1 litre of tap water, aspirated, and blocked for 15 min with 50 µl CT buffer containing casein (3 g) (Sigma) and Tween 80 (0.11 ml) dissolved in 1 litre of distilled water at 37°C. The CT buffer was removed and 50 µl of primary antibodies diluted in CT buffer were added to the appropriate wells. The plates were incubated at 37°C for 30 min in a moist chamber. The samples were washed three times in washing solution. The peroxidase-conjugated secondary antibody diluted in CT buffer (100 µl) was added to each appropriate well and incubated for 30 min at 37°C in a moist chamber. The wells were washed three times with washing solution (100 µl), and 100 µl of freshly prepared 3,3',5,5' tetramethyl-benzidine (TMB) (Sigma) substrate diluted 1 in 5 in distilled water was added to the appropriate wells. The plates were incubated at room temperature in the dark for 15 min. The peroxidase activity was stopped by adding 50 µl of sulphuric acid (2N) to each well. The absorption of each well was measured at an optical density of 450 nm with a reference filter at 630 nm using a 96 well plate reader (Dynex MRX II) and analysed with the Dynex Revelation software for PCs .Two separate batches of bacteria were assessed in duplicate in three independent experiments. Each ELISA plate contained a negative control for non-specific binding of the HRP-conjugated secondary antibody tested for each individual strain. This sample was used to set the negative value against which the samples incubated with the primary and secondary antibody was judged as negative or positive. An increase in the absorbance of more than 0.5 in the reading for the test compared to the negative control was considered to be positive for binding of the antibody (+). Values below 0.5 were scored as negative (-) (*Scholten* et al., 1994).

Every ELISA plate contained a positive control, reported to express the immunotype antigens, immunotype reference strains L1, L3, L7, L8 and L10. Quality control between plates was assessed by adding 5 µl of the HRP-conjugated secondary antibody and TMB substrate (100µl) to an empty well. Variability in absorbance between plates was less than 0.100.

Statistical analysis

Binding of meningococcal immunotype or blood group antibodies were expressed as positive (1) or negative (0) scores. Statistical analysis was performed with the software package SPSS version 10.0.7a for Macintosh (SPSS Inc.). Differences in WCE scores across groups with two factor models were examined using Chi-Square tests (Fisher's exact test, $F\chi^2$) and symmetric interval-by-interval measures (Pearson's regression, *PR*), and groups with more than two factor models were examined using Chi-Square tests (Pearson's two sided Chi-Square test, $P\chi^2$) and symmetric interval by interval measures (Pearson's regression one-sided test, *PR*). P values of <0.05 were considered statistically significant.

Table 4: Whole cell ELISA to detect binding of antibodies to blood group antigens by meningococcal immunotype reference strains

	Blood group				Immunotype				
Reference strain	P	P1	p^K	Paragloboside	I	L1	L(3,7,9)	L8	L10
L1	+	-	+	-	-	+	-	+	-
L2	+	-	-	+	+	-	+	-	-
L3	+	-	+	-	+	-	+	+	-
L4	-	-	-	+	-	-	-	-	-
L5	-	-	-	+	+	-	+	-	-
L6	+	+	-	-	-	-	-	-	-
L7	+	+	+	+	+	-	+	+	-
L8	+	+	+	+	+	-	+	+	-
L9	-	-	+	+	+	-	+	+	-
L10	-	-	+	+	+	-	-	+	+
L11	+	-	+	-	+	-	-	+	-
L12	-	-	-	+	-	-	-	-	+

Data: detection of antigens; + binding of typing antibodies; -, absence of antibody binding.

RESULTS

Binding of blood group and immunotype antibodies to meningococcal immunotype strains

Blood group antibodies

The immunotype reference strains bound the following blood group antibodies: L1 reacted with P and p^K; L2 with P, paragloboside and Ii; L3 with P, p^K, and Ii; L4 with paragloboside; L5 with paragloboside and Ii; L6 with P1; L7 and L8 reacted with all blood group antibodies used; L9 with P1, p^K, paragloboside and Ii; L10 with p^K, paragloboside and Ii; L11 P, p^K and Ii; L12 reacted only with paragloboside (Table 4). The detection of anti-I antibodies to meningococcal reference strains using either HRP-conjugated protein A or HRP-conjugated anti-human IgG was identical.

Meningococcal immunotype antibodies

The immunotype reference strains bound the following anti-LOS antibodies: L1 reacted with L1 and L8; L2 with L(3,7,9); L3 with L(3,7,9) and L8; L4 with none; L5 with L(3,7,9); L6 did not bind any of the antibodies used; L7 reacted with L(3,7,9) and L8; L8 with L8 and L(3,7,9); L9 with L(3,7,9) and L8; L10 with L10 and L8; L11 with L8; and L12 reacted with L10 (Table 4).

Detection of blood group or immunotype antigens on NL from different sources

The binding of antibodies to blood group antigens and LOS immunotypes to NL isolates from the Czech Republic (n=4), from children (n=2) and adults (n=10) in Scotland, from Russian immigrant children in Greece (n=27), and from native Greek children (n=34), juveniles and adults (n=38) (*Kremastinou* et al., 1999a,b) were assessed by WCE in three independent experiments. The binding of blood group antibodies (Table 5) and meningococcal immunotype antibodies (Tables 6, 7, and 8) are summarised by country and age group.

Table 5: Binding of antibodies to human blood group antigens by commensal *N. lactamica* isolates obtained from Czech, Icelandic and Scottisch childrenand adults, and Russian immigrant children in Greece

Origin	Age(years)	n	P	P1	pK	Paragloboside	Ii	no binding
Czech Rep	≤13	4	1 (25.0)	2 (50.0)	2 (50.0)	4 (100)	4 (100)	0 (0.0)
Scotland	>0	12	1 (8.3)	2 (16.7)	8 (66.7)	8 (66.6)	7 (58.3)	6 (50.0)
Russia	≤13	27	10 (37)	4 (14.8)	8 (29.6)	1 (3.7)	8 (29.6)	0 (0.0)
Iceland	≤13	1	1 (100)	1 (100)	0 (0.0)	1 (100)	1 (100)	0 (0.0)

Data: number and percentage (%) of isolates binding the human blood group phenotyping antibodies; n.t.: not tested; n.s., not significant.

Significantly fewer NL isolates from Russian immigrant children in Greece expressed p^K (p=0.035) and paragloboside (p<0.001) antigens compared to isolates from Scotland. There was no significant difference in the expression of P, P1 or Ii antigens between the samples from different countries tested. Meningococcal immunotypes L(3,7,9) (p<0.001) and the L(3,7,9) positive L8 negative phenotype (L379+L8-) (p<0.001) were expressed by fewer NL isolates obtained from Russian immigrant children in Greece compared to isolates obtained from Scottish, Czech or from native Greek adults or children (Table 7).

The majority of NL isolates obtained from children (60.6%) and adult (82.2%) carriers expressed antigens cross-reactive with meningococcal immunotype L(3,7,9). There was no significant difference in the binding of meningococcal immunotyping antibodies between strains isolated from juveniles (age 14 to 19) compared to isolates obtained from adult carriers (age ≥20 years). These two populations were consequently grouped together as adults (age ≥14 years). Significantly more strains isolated from adults bound antibodies against meningococcal immunotype L(3,7,9) (p=0.014) and phenotype L(3,7,9) positive L8 negative (p=0.009) compared to isolates obtained from children carriers.

Binding of antibodies to blood group and L(3,7,9) antigens by *M. catarrhalis* isolates from Scotland

The binding of blood group antibodies against P, P1, p^K, paragloboside, I (n=126) to isolates obtained from children (n=27) and adults (n=99) was measured by WCE. The binding of meningococcal immunotyping antibodies L1, L(3,7,9) and L10 to isolates of children (n=87) and adults (n=99) was measured.

Most clinical isolates of MC bound one or more antibodies to the blood group antigens tested (Table 9). Significantly more strains isolated from adults bound antibodies to P1 (p=0.024), paragloboside (p=0.034) and Ii (p=0.022) compared to isolates obtained from children. Binding of antibodies to P, p^K or strains that did not bind any of the blood groups tested did not differ significantly between isolates obtained from adults or children.

Most clinical isolates of MC obtained from children (n=89) bound one or more meningococcal immunotyping antibodies. The majority of *M. catarrhalis* strains isolated from children (71.9%) bound immunotyping L1 antibodies, while L8 antigens were detected on a small number of isolates (5.6%). Significantly more strains isolated from children (38.2%) bound antibodies to L(3,7,9) compared to isolates obtained from adults (n=99) (22.2%) (P=0.017).

Table 6: Binding of meningococcal immunotyping antibodies by commensal *N. lactamica* isolates obtained from Czech, Icelandic, Greek and Scottisch children and adults, Russian immigrant children in Greece

Origin	Age (years)	n	L1	L(3,7,9)	L8	L(3,7,9)+ L8+	L(3,7,9)+ L8-	L(3,7,9)- L8+	L(3,7,9)- L8-
Czech Rep	≤13	4	1 (25.0)	4 (100)	2 (25.0)	2 (50.0)	2 (50.0)	0 (0.0)	0 (0.0)
Scotland	≤13	2	0 (0.0)	2 (100.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)
Scotland	≥14	10	1 (10.0)	7 (70.0)	2 (20.0)	2 (20.0)	5 (50.0)	0 (0.0)	3 (30.0)
Iceland	≤13	1	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
Russia	≤13	27	4 (14.8)	9 (33.3)	4 (14.8)	4 (14.8)	5 (18.5)	0 (0.0)	18 (66.7)
Greece	≤13	34	12 (35.3)	25 (73.5)	3 (0.8)	3 (0.8)	22 (64.7)	0 (0.0)	9 (26.5)
Greece	≥14	38	10 (26.3)	32 (84.2)	4 (10.5)	4 (10.5)	28 (73.7)	0 (0.0)	6 (15.8)

Data: number and percentage (%) of isolates binding the human blood group phenotyping antibodies.

Table 7: Binding of meningococcal immunotyping antibodies by commensal *N. lactamica* isolates by region

Origin	n	L1	L(3,7,9)	L8	L(3,7,9)+ L8+	L(3,7,9)+ L8-	L(3,7,9)- L8+	L(3,7,9)- L8-
Czech Rep	4	1 (25.0)	4 (100.0)	2 (50.0)	2 (50.0)	2 (50.0)	0 (0.0)	0 (0.0)
Scotland	12	1 (8.3)	9 (75.0)	2 (16.7)	2 (16.7)	7 (58.3)	0 (0.0)	3 (25.0)
Russia	27	4 (14.8)	9 (33.3)	4 (14.8)	4 (14.8)	5 (18.5)	0 (0.0)	18 (66.7)
Greece	72	22 (30.6)	57 (79.2)	7 (9.7)	7 (9.7)	50 (69.4)	0 (0.0)	15 (20.8)

Data: number and percentage (%) of isolates binding the human blood group phenotyping antibodies; n.t.: not tested; n.s., not significant.

Table 8: Binding of meningococcal immunotyping antibodies by commensal *N. lactamica* isolates by age

Age (years)	n	L1	L(3,7,9)	L8	L(3,7,9)+ L8+	L(3,7,9)+ L8-	L(3,7,9)- L8+	L(3,7,9)- L8-
≤13	71	16 (22.5)	43 (60.6)	10 (14.1)	10 (14.1)	33 (46.5)	0 (0.0)	28 (39.4)
≥14	45	12 (26.7)	37 (82.2)	5 (11.1)	5 (11.1)	32 (71.1)	0 (0.0)	8 (17.8)

Data: number and percentage (%) of isolates binding the human blood group phenotyping antibodies; n.t.: not tested; n.s., not significant.

Table 9: Binding of antibodies to blood group antigens by clinical *M. catarrhalis* isolates obtained from Scottisch children and adults

Origin	Age (years)	n	P	P1	pK	Paragloboside	Ii	no binding
Scotland	≤13	27	1 (3.7)	2 (7.4)	20 (74.1)	1 (3.7)	1 (3.7)	6 (22.2)
Scotland	≥14	99	15 (15.2)	28 (28.3)	60 (60.6)	21 (21.2)	23 (23.2)	26 (26.1)

Data: number and percentage (%) of isolates binding the human blood group phenotyping antibodies; n.t.: not tested; n.s., not significant.

DISCUSSION

Binding of antibodies to blood group and meningococcal immunotype antigens by meningococci in relation to previous studies

All immunotype antibodies used bound to strains previously reported to express these antibodies (*Scholten* et al., 1994). The anti-paragloboside antibody bound to all immunotype reference strains reported to express this antigen as its terminal moiety. The anti-paragloboside antibody did not bind to the sialylated paragloboside moiety of immunotype L3. In addition, immunotype L8 that bound anti-paragloboside IgM co-expresses immunotype L(3,7,9). Immunotype L12 bound anti-paragloboside, an antigen found on immunotype L10. The antibody against immunotype L(3,7,9), an antigen that contains the paragloboside moiety, did not bind to immunotypes L4, or L10 that bound anti-paragloboside antibodies. These findings suggest that the L(3,7,9) antibody recognises an epitope that is either not accessible in these immunotypes, or that the L(3,7,9) antibody is directed against an epitope other than or in addition of the oligosaccharide moiety. This epitope might be present in the core structure of the L(3,7,9) antigen, or it might include a combination of core and paragloboside structure.

The binding of blood group antibodies corresponds to the presence of these antigens within the published structures of meningococcal LOS. These findings provide evidence that antibodies used for blood group typing can detect similar antigens on meningococci. Antibodies found in human serum directed against blood group antigens might also cross-react with meningococcal oligosaccharide moieties, for example anti-I antibodies found in human serum. Their possible biological functions in relation to complement dependent bactericidal activity, ability to neutralise the toxicity of meningococcal LOS, or ability to opsonise meningococci and commensal species has not been reported.

Binding of antibodies to blood group and meningococcal immunotype antigens by NL isolates from different countries in Europe

Several authors have investigated carriage rates of meningococci and NL within normal populations in the USA (*Gold* et al., 1978), Norway (*Holten* et al., 1978), Nigeria (*Blakebrough* et al., 1982), Spain (*Saez-Nieto* et al., 1985), England (*Cartwright* et al., 1987; *Coen* et al., 2000), Faroer Islands (*Olsen* et al., 1991), Wales (*Davies* et al., 1996), Greece (*Kremastinou* et al., 1999a,b), and New Zealand (*Simmons* et al., 2000). There has, however, been no systematic survey of antigens on NL cross-reactive with those on meningococci. All of these surveys investigating carriage of NL reported, that carriage rates of NL were higher in young children (12-65%) compared to young adults (2-5%). Carriage of NL were found to exceed those for carriage of meningococci within the younger age groups by up to 6 to 1 and the two species are not isolated from the same individual (G. Tzanakaki, personal communication). This demonstrates that NL is a commensal found world-wide in young children, and its association with the development of natural immunity to meningococcal disease appears to be of great importance in many communities.

The significant differences in the binding of antibodies to carbohydrate antigens by strains isolated from carriers from different European regions indicate that regional phenotypic differences of NL isolates might contribute to the development of different herd im-

munities. The majority of NL isolates from children and adults tested, expressed the cross-reactive L(3,7,9) meningococcal immunotype associated with virulence in pathogenic meningococci, suggesting that this phenotype on commensal species might be a major antigen involved in the development of natural immunity to meningococcal disease. This could lead to greater susceptibility to meningococcal disease in some populations, for example, those in which there is a low proportion of commensal strains expressing the L(3,7,9) or L8 epitopes. Little is known about the LOS immunotypes of meningococcal isolates from Eastern Europe, the Americas, Australia or African countries.

Binding of antibodies to blood group and L(3,7,9) antigens by *M. catarrhalis* obtained from adults and children

The majority of MC isolates (73.8%) bound one or more antibodies to blood group antigens or L(3,7,9) immunotype. Antibodies to the blood group p^K (63.5%) were bound by most of the isolates, and a large proportion of isolates obtained from children (38.2%) and adults (22.2%) bound the monoclonal meningococcal immunotyping antibody to L(3,7,9). These findings support our hypothesis, that carriage of MC might induce protective immunity against meningococcal disease. Most of these isolates were from adults with respiratory tract infections. Strains isolated from children with otitis media showed a higher proportion of binding of L(3,7,9) antibodies to those MC isolates compared to isolates causing disease in adults. Similar studies with MC isolates from children from different geographical regions and ethnic groups with respiratory or ear infections and carrier isolates need to be carried out. Little is known about natural antibodies induced by MC cross-reactive to meningococci or NL. Naturally occurring IgG2 antibodies that bound to whole cells of MC were detected in children older than 5 years (*Goldblatt* et al., 1990). The development of natural immunity to MC is thought to be facilitated by glycoconjugates (*Murphy* and *Bartos*, 1989). Carriage rates of MC are higher in early childhood compared to NL and meningococcal carriage rates combined (*Vaneechoutte* et al., 1990a,b; *Faden* et al., 1991; *Harrison* et al., 1999), and consecutive carriage of genetically and phenotypically different MC strains is common among young children (*Rahman* et al., 1998b; *Faden* et al., 1994).

The presence of antibodies to MC in the serum of older children, the frequent carriage rate of multiple strains by children, and its cross-reactivity with some of meningococcal antigens provides evidence that MC might be involved in the development of natural immunity to meningococcal disease as described by *Goldschneider*, *Gotschlich* and *Artenstein* (1969a,b). The higher frequency of carriage of MC compared to NL and meningococci further suggests that MC might play an important role in the development of antibodies that protect against meningococcal disease.

CONCLUSIONS

The commensal species *N. lactamica* and *M. catarrhalis* express antigens that bound antibodies used for meningococcal immunotyping and typing of human blood group antigens. Significant differences in the binding of antibodies to carbohydrate antigens were observed among *N. lactamica* strains isolated

from children and adult carriers from different European regions. This indicates that regional phenotypic differences of *N. lactamica* isolates might contribute to the development of different herd immunities that could lead to greater susceptibility to meningococcal disease in some of these populations. Differences in the expression of carbohydrate antigens between isolates of *M. catarrhalis* obtained from children and adults, the presence of anti-MC antibodies in older children, its frequent presence in the pharyngeal cavities of children, and apparent high levels of strains with L(3,7,9) epitope isolated from children with otitis media provide evidence that MC might be involved in the development of natural immunity to meningococcal LOS. The presence of glycoconjugate antigens on commensal bacteria might be exploited as anti-meningococcal vaccine candidates.

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LITERATURE

Abdillahi, H. and Poolman, J.T.: Typing of group-B *Neisseria meningitidis* with monoclonal antibodies in the whole-cell ELISA. J. Med. Microbiol. 26, 177-180 (1988).

Bailly, P., Piller, F., Gillard, B., Veyrieres, A., Marcus, D., and Cartron, J.P.: Biosynthesis of the blood group Pk and P1 antigens by human kidney microsomes. Carbohydr. Res. 228, 277-287 (1992).

Berrington, A.W., Tan, Y.C., Srikhanta, Y., Kuipers, B., van der Ley, P., Peak, I.R., and Jennings, M.P.: Phase variation in meningococcal lipooligosaccharide biosynthesis genes. FEMS Immunol. Med. Microbiol. 34, 267-275 (2002).

Blakebrough, I.S., Greenwood, B.M., Whittle, H.C., Bradley, A.K., and Gilles, H.M.: The epidemiology of infections due to *Neisseria meningitidis* and *Neisseria lactamica* in a northern Nigerian community. J. Infect. Dis. 146, 626-637 (1982).

Booy, R. and Kroll, J.S.: Bacterial meningitis and meningococcal infection. Curr. Opin. Pediatr. 10, 13-18 (1998).

Braun, J.M., Blackwell, C.C., Poxton, I.R., Ahmer, O.E., Gordon, A.E., Madani, O.M., Weir, D.M., Giersen, S., and Beuth, J.: Proinflammatory responses to lipooligosaccharide of *Neisseria meningitidis* immunotype strains in relation to virulence and disease. J. Infect. Dis. 185, 1431-1438 (2002).

Brown, K.E., Anderson, S.M., and Young, N.S.: Erythrocyte P antigen: Cellular receptor for B19 parvovirus. Science 262: 114-117 (1993).

Butch, A.W. and Nahm, M.H.: Functional properties of human germinal centre B cells. Cell. Immunol. 140, 331-344 (1992).

Cartwright, K.A., Stuart, J.M., Jones, D.M., and Noah, N.D.: The Stonehouse survey: Nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol. Infect. 99, 591-601 (1987).

Coen, P.G., Cartwright, K., and Stuart, J.: Mathematical modelling of infection and disease due to *Neisseria meningitidis* and *Neisseria lactamica*. Int. J. Epidemiol. 29, 180-188 (2000).

Davies, A.L., O'Flanagan, D., Salmon, R.L., and Coleman, T.J.: Risk factors for *Neisseria meningitidis* carriage in a school during a community outbreak of meningococcal infection. Epidemiol. Infect. 117, 259-266 (1996).

Dell, A., Azadi, P., Tiller, P., Thomas-Oates, J., Jennings, H.J., Beurret, M., and

Michon, F.: Analysis of oligosaccharide epitopes of meningococcal lipopolysaccharides by fast-atom-bombardment mass spectrometry. Carbohydr. Res. 200, 59-76 (1990).

Edebrink, P., Jansson, P.E., Rahman, M.M., Widmalm, G., Holme, T., Rahman, M., and Weintraub, A.: Structural studies of the O-polysaccharide from the lipopolysaccharide of *Moraxella* (*Branhamella*) *catarrhalis* serotype A (strain ATCC 25238). Carbohydr. Res. 257, 269-284 (1994).

Edebrink, P., Jansson, P.E., Rahman, M.M., Widmalm, G., Holme, T., and Rahman, M.: Structural studies of the O-antigen oligosaccharides from two strains of *Moraxella catarrhalis* serotype C. Carbohydr. Res. 266, 237-261 (1995).

Edebrink, P., Jansson, P.E., Widmalm, G., Holme, T., and Rahman, M.: The structures of oligosaccharides isolated from the lipopolysaccharide of *Moraxella catarrhalis* serotype B, strain CCUG 3292. Carbohydr. Res. 295, 127-146 (1996).

Faden, H., Waz, M.J., Bernstein, J.M., Brodsky, L., Stanievich, J., and Ogra, P.L.: Nasopharyngeal flora in the first three years of life in normal and otitis-prone children. Ann. Otol. Rhinol. Laryngol. 100, 612-615 (1991).

Faden, H., Hong, J.J., and Pahade, N.: Immune response to *Moraxella catarrhalis* in children with otitis media: Opsonophagocytosis with antigen-coated latex beads. Ann. Otol. Rhinol. Laryngol. 103, 522-524 (1994).

Fonkoua, M.C.: Meningococcal meningitis in Africa. Am. J. Nurs. 102, 17 (2002).

Gold, R., Goldschneider, I., Lepow, M.L., Draper, T.F., and Randolph, M.: Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in infants and children. J. Infect. Dis. 137, 112-121 (1978).

Goldblatt, D., Turner, M.W., and Levinsky, R.J.: *Branhamella catarrhalis*: Antigenic determinants and the development of the IgG subclass response in childhood. J. Infect. Dis. 162, 1128-1135 (1990).

Goldschneider, I., Gotschlich, E.C., and Artensen, M.S.: Human immunity to meningococcus. I. The role of humoral antibodies. J. Exp. Med. 129, 1307-1326 (1969a).

Goldschneider, I., Gotschlich, E.C., and Artensen, M.S.: Human immunity to meningococcus. II. Development of natural immunity. J. Exp. Med. 129, 1327-1346 (1969b).

Griffiss, J.M., O'Brien, J.P., Yamasaki, R., Williams, G.D., Rice, P.A., Schneider, H.: Physical heterogeneity of neisserial lipooligosaccharides reflects oligosaccharides that differ in apparent molecular weight, chemical composition, and antigenic expression. Infect. Immun. 55, 1792-1800 (1987a).

Griffiss, J.M., Schneider, H., Mandrell, R.E., Jarvis, G.A., Kim, J.J., Gibson, B., and Apicella, M.A.: The immunochemistry of neisserial LOS. Antonie van Leeuwenhoek 53, 501-7 (1987b).

Gu, X.X., Tsai, C.M., and Karpas, A.B.: Production and characterization of monoclonal antibodies to type 8 lipooligosaccharide of *Neisseria meningitidis*. J. Clin. Microbiol. 30, 2047-2053 (1992).

Hakomori, S. and Kannagi, R.: Carbohydrate antigens in higher animals. In: Handbook of experimental immunology (Ed. Weir, D.M.). Blackwell Scientific, London (1986).

Hammerschmidt, S., Birkholz, C., Zahringer, U., Robertson, B.D., van Putten, J., Ebeling, O., and Frosch, M.: Contribution of genes from the capsule gene complex (cps) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol. Microbiol. 11, 885-896 (1994).

Harrison, L.M., Morris, J.A., Telford, D.R., Brown, S.M., and Jones, K.: The nasopharyngeal bacterial flora in infancy: Effects of age, gender, season, viral upper respiratory tract infection and sleeping position. FEMS Immunol. Med. Microbiol. 25, 19-28 (1999).

Holme, T., Rahman, M., Jansson, P.E., and Widmalm, G.: The lipopolysaccharide of *Moraxella catarrhalis* structural relationships and antigenic properties. Eur. J. Biochem. 265, 524-529 (1999).

Holten, E., Bratlid, D., and Bovre, K.: Carriage of *Neisseria meningitidis* in a semi-isolated arctic community. Scand. J. Infect. Dis. 10, 36-40 (1978).

Jennings, H.J., Johnson, K.G., and Kenne, L.: The structure of an R-type oligosaccharide core obtained from some lipopolysaccharides of *Neisseria meningitidis*. Carbohydr. Res. 121, 233-241 (1983).

Jennings, M.P., Srikhanta, Y.N., Moxon, E.R., Kramer, M., Poolman, J.T., Kuipers, B., and van der Ley, P.: The genetic basis of the phase variation repertoire of lipopolysaccharide immunotypes in *Neisseria meningitidis*. Microbiology 145, 3013-3021 (1999).

Jones, D.M., Borrow, R., Fox, A.J., Gray, S., Cartwright, K.A., and Poolman, J.T.: The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. Microb. Pathog. 13, 219-224 (1992).

Jonsson, I., Holme, T., Krook, A., Rahman, M., and Thoren, M.: Variability of surface-exposed antigens of different strains of *Moraxella catarrhalis*. Eur. J. Clin. Microbiol. Infect. Dis. 11, 9199-9122 (1992).

Jonsson, I., Holme, T., Krook, A., and Thoren, M.: Serological cross-reactions between *Moraxella* (*Branhamella*) *catarrhalis* and other oropharyngeal bacteria. Eur. J. Clin. Microbiol. Infect. Dis. 12, 289-293 (1993).

Jonsson, I., Holme, T., and Krook, A.: Significance of isolation of *Moraxella catarrhalis* in routine cultures from the respiratory tract in adults: Antibody response studied in a whole cell EIA. Scand. J. Infect. Dis. 26, 553-558 (1994).

Katial, R.K., Brandt, B.L., Moran, E.E., Marks, S., Agnello, V., and Zollinger, W.D.: Immunogenicity and safety testing of a group B intranasal meningococcal native outer membrane vesicle vaccine. Infect. Immun. 70, 702-707 (2002).

Kim, J.J., Mandrell, R.E., and Griffiss, J.M.: *Neisseria lactamica* and *Neisseria meningitidis* share lipooligosaccharide epitopes but lack common capsular and class 1, 2, and 3 protein epitopes. Infect. Immun. 57, 602-608 (1989).

Kim, J.J., Phillips, N.J., Gibson, B.W., Griffiss, J.M., and Yamasaki, R.: Meningococcal group A lipooligosaccharides (LOS): Preliminary structural studies and characterization of serotype-associated and conserved LOS epitopes. Infect. Immun. 62, 1566-1575 (1994).

Kogan, G., Uhrin, D., Brisson, J.R., and Jennings, H.J.: Structural basis of the *Neisseria meningitidis* immunotypes including the L4 and L7 immunotypes. Carbohydr. Res. 298, 191-199 (1997).

Kremastinou, J., Tzanakaki, G., Velonakis, E., Voyiatzi, A., Nickolaou, A., Elton, R.A., Weir, D.M., and Blackwell, C.C.: Carriage of *Neisseria meningitidis* and *Neisseria lactamica* among ethnic Greek school children from Russian immigrant families in Athens. FEMS Immunol. Med. Microbiol. 23, 13-20 (1999a).

Kremastinou, J., Tzanakaki, G., Pagalis, A., Theodondou, M., Weir, D.M., and Blackwell, C.C.: Detection of IgG and IgM to meningococcal outer membrane proteins in relation to carriage of *Neisseria meningitidis* or *Neisseria lactamica*. FEMS Immunol. Med. Microbiol. 24, 73-78 (1999b).

Mandrell, R.E., Griffiss, J.M., and Macher, B.A.: Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. J. Exp. Med. 168, 107-126 (1988).

Mandrell, R.E., Kim, J.J., John, C.M., Gibson, B.W., Sugai, J.V., Apicella, M.A., Griffiss, J.M., and Yamasaki, R.: Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J. Bacteriol. 173, 2823-2832 (1991).

Mandrell, R.E., Griffiss, J.M., Smith, H., and Cole, J.A.: Distribution of a lipooligosaccharide-specific sialyltransferase in pathogenic and non-pathogenic *Neisseria*. Microb. Pathog. 14, 315-327 (1993).

Mandrell, R.E. and Apicella, M.A.: Lipo-oligosaccharides (LOS) of mucosal pathogens: Molecular mimicry and host-modification of LOS. Immunobiology 187, 382-402 (1993).

Marcus, D.M., Naiki, M., and Kundu, S.K.: Abnormalities in the glycosphingolipid content of human Pk and p erythrocytes. Proc. Natl. Acad. Sci. USA 73, 3263-3267 (1976).

Marsh, W.L. and Jenkins, W.J.: Anti-i: A new cold antibody. Nature 188, 753-755 (1960).

Marsh, W.L.: Anti-i, a cold antibody defining the Ii relationship in human red cells. Br. J. Haematol. 7, 200-209 (1961).

Masoud, H., Perry, M.B., and Richards, J.C.: Characterization of the lipopolysaccharide of *Moraxella catarrhalis*. Structural analysis of the lipid A from *M. catarrhalis* serotype

A lipopolysaccharide. Eur. J. Biochem. 220, 209-216 (1994).

McLeod Griffiss, J., Brandt, B.L., Saunders, N.B., and Zollinger, W.: Structural relationships and sialylation among meningococcal L1, L8, and L3,7 lipooligosaccharide serotypes. J. Biol. Chem. 275, 9716-9724 (2000).

Michon, F., Beurret, M., Gamian, A., Brisson, J.R., and Jennings, H.J.: Structure of the L5 lipopolysaccharide core oligosaccharides of *Neisseria meningitidis*. J. Biol. Chem. 265, 7243-7247 (1990).

Murphy, T.F. and Bartos, L.C.: Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarrhalis*. Infect. Immun. 57, 2938-2941 (1989).

Olsen, S.F., Djurhuus, B., Rasmussen, K., Joensen, H.D., Larsen, S.O., Zoffman, H., and Lind, I.: Pharyngeal carriage of *Neisseria meningitidis* and *Neisseria lactamica* in households with infants within areas with high and low incidences of meningococcal disease. Epidemiol. Infect. 106, 445-457 (1991).

Pavliak, V., Brisson, J.R., Michon, F., Uhrin, D., and Jennings, H.J.: Structure of the sialylated L3 lipopolysaccharide of *Neisseria meningitidis*. J. Biol. Chem. 268, 14146-14152 (1993).

Plested, J.S., Makepeace, K., Jennings, M.P., Gidney, M.A., Lacelle, S., Brisson, J., Cox, A.D., Martin, A., Bird, A.G., Tang, C.M., Mackinnon, F.M., Richards, J.C., and Moxon, E.R.: Conservation and accessibility of an inner core lipopolysaccharide epitope of *Neisseria meningitidis*. Infect. Immun. 67, 5417-5426 (1999).

Prokop, O. and Uhlenbruck, G.: Human blood and serum groups. McClaren and Sons, London (1965).

Race, R.R. and Sanger, R.: Blood groups in man (6th edition). Backwell Scientific Publications, Oxford (1975).

Rahman, M. and Holme, T.: Antibody response in rabbits to serotype-specific determinants in lipopolysaccharides from *Moraxella catarrhalis*. J. Med. Microbiol. 44, 348-354 (1996).

Rahman, M., Holme, T., Jonsson, I., and Krook, A.: Human immunoglobulin isotype and IgG subclass response to different antigens of *Moraxella catarrhalis*. APMIS 105 213-220 (1997).

Rahman, M.M., Stephens, D.S., Kahler, C.M., Glushka, J., and Carlson, R.W.: The lipooligosaccharide (LOS) of *Neisseria meningitidis* serogroup B strain NMB contains L2, L3, and novel oligosaccharides, and lacks the lipid-A 4'-phosphate substituent. Carbohydr. Res. 307, 311-324 (1998a).

Rahman, M., Jonsson, A.B., and Holme, T.: Monoclonal antibodies to the epitope alpha-Gal-(1-4)-beta-Gal-(1-3) of *Moraxella catarrhalis* LPS react with a similar epitope in type IV pili of *Neisseria meningitidis*. Microb. Pathog. 24, 299-308 (1998b).

Saez-Nieto, J.A., Dominguez, J.R., Monton, J.L., Cristobal, P., Fenoll, A., Vazquez, J., Casal, .J, and Taracena, B.: Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in a school population during an epidemic period in Spain. Hygiene (London) 94, 279-288 (1985).

Saunders, N.B., Shoemaker, D.R., Brandt, B.L., Moran, E.E., Larsen, T., and Zollinger, W.D.: Related Immunogenicity of intranasally administered meningococcal native outer membrane vesicles in mice. Infect. Immun. 67, 113-119 (1999).

Schneider, H., Hale, T.L., Zollinger, W.D., Seid, R.C. Jr., Hammack, C.A., and Griffiss, J.M.: Heterogeneity of molecular size and antigenic expression within lipooligosaccharides of individual strains of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Infect. Immun. 45, 544-549 (1984).

Scholten, R.J., Kuipers, B., Valkenburg, H.A., Dankert, J., Zollinger, W.D., and Poolman, J.T.: Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. J. Med. Microbiol. 41, 236-243 (1994).

Siddiqui, B. and Hakomori, S.: A ceramide tetrasaccharide of human erythrocyte membrane reacting with anti-type XIV pneumococcal polysaccharide antiserum. Biochim. Biophys. Acta 330, 147-155 (1973).

Simmons, G., Martin, D., Stewart, J., and Bremner, D.: Carriage of *Neisseria lactamica* in a population at high risk of meningococcal disease. Epidemiol. Infect. 125, 99-104 (2000).

Tsai, C.M. and Civin, C.I.: Eight lipooligosaccharides of *Neisseria meningitidis* react

with a monoclonal antibody which binds lacto-N-neotetraose (Gal beta 1-4 GlcNAc beta 1-3 Gal beta 1-4 Glc). Infect. Immun. 59, 3604-3609 (1991).

Vaneechoutte, M., Verschraegen, G., Claeys, G., Weise, B., and Van den Abeele A.M.: Respiratory tract carrier rates of *Moraxella* (*Branhamella*) *catarrhalis* in adults and children and interpretation of the isolation of *M. catarrhalis* from sputum. J. Clin. Microbiol. 28, 2674-2680 (1990a).

Vaneechoutte, M., Verschraegen, G., Claeys, G., and Van Den Abeele, A.M.: Serological typing of *Branhamella catarrhalis* strains on the basis of lipopolysaccharide antigens. J. Clin. Microbiol. 28, 182-187 (1990b).

Varaine, F., Caugant, D.A., Riou, J.Y., Konde, M.K., Soga, G., Nshimirimana, D., Muhirwa, G., Ott, D., Hoiby, E.A., Fermon, F., and Moren, A.: Meningitis outbreaks and vaccination strategy. Trans. R. Soc. Trop. Med. Hyg. 91, 3-7 (1997).

Wakarchuk, W., Gilbert, M., Martin, A., Wu, Y., Brisson, J.-R., Thibault, P., and Richards, J.C.: Structure of an α2,6-sialylated lipooligosaccharide from *Neisseria meningitidis* immunotype L1. Eur. J. Biochem. 254, 626-633 (1998).

Wiels, J., Fellous, M., and Tursz, T.: Monoclonal antibody against a Burkitt lymphoma-associated antigen. Proc. Natl. Acad. Sci. USA 78, 6485-6488 (1981).

Wiener, A.S.: Blood group distributions. Ann. Intern. Med. 79, 137 (1973).