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EFFECTIVE AND INEFFECTIVE DEFENCE MECHANISMS IN THE GASTROINTESTINAL TRACT

EDITORS:

HANS COTTIER
PETER J. HEIDT
VOLKER C. RUSCH
DIRK VAN DER WAAIJ

Institute for Microbiology and Biochemistry
D-35745 Herborn-Dill
Germany
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EDITORS:

Hans Cottier, M.D.
Central Laboratory
Swiss Red Cross Blood Transfusion Service
Bern
Switzerland

Peter J. Heidt, Ph.D., B.M.
Department of Microbiology and Gnotobiology
Biomedical Primate Research Centre - TNO
Rijswijk
The Netherlands

Volker C. Rusch, Dr. rer. nat.
Institute for Microbiology and Biochemistry
Herborn-Dill
Germany

Dirk van der Waaij, M.D., Ph.D.
Department of Medical Microbiology
University of Groningen
The Netherlands

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INSTITUTE FOR MICROBIOLOGY AND BIOCHEMISTRY

Kornmarkt 34
D-35745 Herborn-Dill
Germany
Telephone: +49 - (0)2772 - 41033
Telefax: +49 - (0)2772 - 41039

Contents

Contents (continued)

Contents (continued)

Contents (continued)

Participating authors

Herma Z. Apperloo-Renkema, Laboratory for Medical Microbiology, University of Groningen, Groningen, The Netherlands

Geoffrey L. Asherson, Division of Immunological Medicine, Clinical Research Centre, Northwick Park Hospital, Harrow, UK

Hans Cottier, Central Laboratory, Swiss Red Cross Blood Transfusion Service, Bern, Switzerland

Sander J.H. van Deventer, Centre for Haemostasis, Thrombosis, Atherosclerosis and Inflammation Research, Academical Medical Centre, Amsterdam, The Netherlands

A.S.M. Dofferhoff, Department of Internal Medicine, University Hospital Groningen, Groningen, The Netherlands

Jan Eva Doran, Central Laboratory, Swiss Red Cross Blood Transfusion Service, Bern, Switzerland

Peter Erb, Institute for Medical Microbiology, University of Basel, Basel, Switzerland

Martin E. Evans, Division of Infectious Diseases, Department of Medicine, Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, Bethesda, Maryland, USA

Helge Scott, Laboratory for Immunohistochemistry and Immunopathology, Institute of Pathology, University of Oslo, The National Hospital, Oslo, Norway

Dick Veenendaal, Laboratory for Medical Microbiology, University of Groningen, Groningen, The Netherlands

Torkel Wadström, Department of Medical Microbiology, University of Lund, Lund, Sweden

Richard I. Walker, Enteric Diseases Program, Naval Medical Research Institute, Bethesda, MD, USA

Gayle Delmonte Wetzel, Cellular and Molecular Biology Research, Cutter Biological, Miles Inc., Berkeley, CA, USA

EFFECTIVE AND INEFFECTIVE DEFENCE MECHANISMS IN THE GASTROINTESTINAL TRACT

HANS COTTIER, ALFRED HÄSSIG, and KURT STAMPFLI

University of Bern, and Central Laboratory,
Swiss Red Cross Blood Transfusion Service, Bern, Switzerland.

SUMMARY

Evolutionary processes have led to a mutual relationship among the hundreds of microbial species in the lower intestinal tract and between this microflora and the host. Under physiological conditions, the macro-organism benefits from this situation, however, a number of defence mechanisms is needed to contain this mass of bacteria. Success or failure of the highly complex machinery which exerts these protective functions depends not only on the force of potentially aggressive/damaging agents/events but also on the - in part genetically determined - ability of the individual to confront them. The present short review focuses on some aspects of host defence against dangerous complications that might arise from the bacterial mass in the gut. Particular topics discussed are: help and danger from the intestinal microflora; the intestinal barrier and its breakdown; effective and ineffective immune functions of the gut-associated lymphoid tissue (GALT); harmonious and protective versus excessive and hazardous inflammatory reactions; success and failure of antibacterial defence; and containment and release of bacterial endotoxin, the triggering substance that may elicit uncontrolled pro-inflammatory cascades. The latter are the hallmark in the pathogenesis of septic shock and multiple organ failure caused by severe infections with Gram-negative bacteria, a condition which often defies conventional therapy.

INTRODUCTION

The preceding Herborn Seminar Monographs have centred around three main topics:

1) microbial ecology of the human digestive tract;

2) interactions between the indigenous microflora and the host immune system; and

3) consequences of antimicrobial therapy for the composition of the intestinal microflora.

In this monograph, we shall shift our interest more to the host's side and place the emphasis on certain aspects of effective and ineffective defence mechanisms in the gastrointestinal tract.

The term "defence", in a biomedical sense, encompasses all cell or tissue structures and processes that serve to maintain, or in case of injury to restore, the integrity of the organism. Success or failure of the highly complex machinery which exerts these protective functions depends not only on the force of the aggressive/damaging agents/events but also on the ability of the host to confront them. Beside age, the nutritional status and the presence or absence of disease,

the capacity of the organism to cope with untoward influences is largely determined by genetic factors. This may best be exemplified by the numerous hereditary disorders, including those affecting the immune apparatus, which may interfere in one way or another with defensive capabilities. In view of the innumerable peptidic molecules involved in protective mechanisms and the existence of allelic polymorphism, it should also come as no surprise that each individual has its own way of reacting to potentially hazardous influences, be they exogenous or endogenous. This may manifest itself in many situations, for instance in some degree of selective ineffective immune responsiveness (*Hässig* and *Cottier*, 1986) or the reverse of it.

In the dynamic interactions between the organism and its environment, the gastrointestinal tract, with - in the adult human - an inner surface of about 250 m^2 , takes a leading position. This is not only the site where water and a vast variety of dietary substances enter the body, it also represents by far the most important area of contact between the organism and a multitude of micro-organisms, not to speak of potentially toxic chemicals. It has once been said that "the gut contains sufficient bacteria and toxins to kill the host millions of times over" (*Border*, 1989). Although this forceful sentence, expressed by a glorious surgeon, defies logic, it helps to focus our attention on the intestinal microflora and its medical importance. It has become increasingly clear that this enormous mass of microbes, if not contained, can endanger the host's life within a short time. It should be recalled at this point that septic shock, and in particular its gut-derived infectious-toxic variant (*Cottier* and *Kraft*, 1991), remains the most feared complication of major surgery, traumata and burns. It is estimated that alone in the United States more than 130,000 deaths per year are associated with bacteraemia, and a large proportion of these results from infection by micro-organisms that normally reside in the gut (*McCabe*, 1974; *Parillo*, 1985).

HELP AND DANGER FROM THE INTESTINAL MICROFLORA

Since this topic has been discussed extensively in previous Herborn Seminar Monographs, this chapter is restricted to a few remarks that relate to the general theme of this monograph. The mutual relationships among the hundreds of enteric microbial species, and between the intestinal microflora and the host, are the result of an evolutionary process of long duration. Under physiological conditions, the macro-organism benefits from the presence of this mass of micro-organisms in various ways. This may best be exemplified by axenic ("germ-free") animals who have a poorly developed immune apparatus and in addition to other deficiencies - are highly susceptible to severe infections when exposed to pathogenic microbes (*Luckey*, 1963). "Protective colonisation" by specialised micro-organisms (*Savage*, 1984) seems to contribute markedly to host defence in small rodents and probably also plays an important role in humans. Bacterial endotoxin in small amounts is able to enhance host resistance (*Urbascheck* et al., 1984), however, this effect of LPS in humans remains to be studied. Furthermore, the intestinal microflora is well known to participate in enzymatic degradation of gut content, delivering, e.g., substrates for enterocyte metabolism and other use; to be instrumental in the transformation

of bile components; to contribute to supplement of vitamins; to catabolise certain exogenous toxins; and to help maintaining the host's homeostasis in many other ways. In all these processes, the gut microflora exhibits a high degree of flexibility, for instance in the sense of adaptive enzyme induction (*Schlegel*, 1985).

Conversely, the enormous mass of bacteria in the lumen of the lower intestinal tract constitutes a permanent threat to the macro-organism. If the more or less exponential growth of these micro-organisms is not counterbalanced by continuous propulsion through the digestive tract and ultimate elimination with the faeces, a dangerous situation can develop within a short time. In immunocompromised individuals, opportunistic infections may originate from the gut content. Enteric bacteria also play a predominant role in hospitalisation; represent a hazard in catheterism; may settle on anomalous cardiac valves or protheses; are apt to damage the host by a variety of metabolic products; and enter the tissue in case of intestinal barrier failure (see below). Furthermore, they may collaborate in the metabolic transformation of various chemicals into carcinogens. The interspecies equilibrium in the intestinal microflora, which is based, among others, on mutual tolerance and optimal antagonisms and in which obligate anaerobes appear to play an important role, can be disrupted by oral antibiotic treatment. As a result, certain species, such as *Clostridium difficile*, with its natural multiple resistance, may overgrow and damage the host via toxin production (*Bartlett* and *Laughon*, 1984). It should be recalled in the present context that pathogenic properties can be transferred, even across different species, from one micro-organism to another with the help of bacterial viruses, in particular plasmids and phages, and via transduction (*Taylor*, 1983; *Luria* and *Suit*, 1987; *Finlay* and *Fulkow*, 1989). In other words, previously non-pathogenic microbes can acquire pathogenicity, and commence, for instance, to produce exotoxins, and this process is apt to expand among the microbial population within a rather short period of time. Conversely, bacterial endotoxin, chemically a lipopolysaccharide (LPS), is a constitutive component of the outer membrane of Gram-negative bacteria. It may be released as a consequence of microbial death, to some extent also during rapid proliferation of bacteria and/or by the action of activated complement (*Doran*, this fascicle).

THE INTESTINAL BARRIER AND ITS BREAKDOWN

Under physiological conditions, the mass of intestinal micro-organisms is contained within the lumen by an intricate system of structural and functional obstacles, commonly known as "intestinal barrier" or "mucosal block" (*Gebbers* and *Laissue*, 1990). In brief, it comprises - among other from inside to outside: secretory IgA and lysozyme, which are in part associated with mucus (*Clamp*, 1980); the enterocyte layer, covered by the glycocalyx, tightened by the intercellular junction complex, primarily by the *zonulae occludentes*, and equipped with protective molecules such as interferon and enzymes; and the mucosal stroma which harbours, e.g., antibodies, complement, granulocytes, macrophages, lymphocytes, plasma cells and natural killer cells as well as lymphatics and small blood vessels. Lymphoid follicles in the intestinal wall

function primarily as immunological contact structures in as much as they are covered by a specialised epithelium with so-called "M" cells that are permeable for macromolecules and small particles (*Owen*, 1977). Taken together, these structures provide for both a rather tight seclusion of, and a constant but restricted contact with, the intestinal microflora and/or its antigens. The integrity of the intestinal barrier, in particular of the epithelial layer, depends on a sufficient supply of oxygen and nutrients (*Page*, 1989), especially also of glutamine (*Souba* et al., 1990).

Intestinal barrier failure can occur in various ways. Trivial causes are, for instance, physical disruption, ulceration/perforation and suture insufficiency in the gut wall. More complex causative mechanisms include severe disturbances of the ecological balance in the intestinal microflora and endotoxin effects. Cytotoxic chemicals and ionising radiation represent well established noxious agents attacking, among other, the intestinal epithelium. Of predominant medical importance, however, are splanchnic ischaemia (*Fiddian-Green*, 1988) and consecutive reperfusion damage, involving oxygen-derived radicals (*Deitch* et al., l990a). The lesions produced by this type of injury may range from a break-up of tight junctions (*Deitch* et al., l990b) to bland epithelial necrosis and mucosal denudation. Regeneration of the enterocyte layer seems to require 4 or more days (*Bragg* and *Thompson*, 1989).

EFFECTIVE AND INEFFECTIVE IMMUNE FUNCTIONS OF THE GUT ASSOCIATED LYMPHOID TISSUE (GALT)

Since it was shown that the follicle associated intestinal epithelium is preferentially permeable to macromolecules and microparticles (*Joel* et al., 1970), it became increasingly clear that the gut associated lymphoid tissue acts also as an immunological contact organ. The outstanding magnitude of the antigenic stimulation originating from the intestinal microflora may, for example, be exemplified by the postnatal development, in mice, of the proliferative activity in regional, i.e. mesenteric, lymph nodes, which is manifold greater than in lymph nodes of other locations (*Schwander* et al., 1980). In this species, a massive influx of thymus derived lymphocytes sets in shortly after birth (*Joel* et al., 1971). Primary humoral immune responses result in the production mainly of IgM antibodies, and germinal centres form to generate great numbers of memory B cells (*Grobler* et al., 1974). In the GALT, and under physiological conditions, the newly produced memory B cells belong predominantly to the IgA class. They enter the circulation and tend to "home" back to the intestinal or other mucosal layers (*Hall*, 1979). The mechanisms responsible for this type of homing are still disputed, however, they seem to involve adhesion molecules on the cell surfaces (for references, see *Möller* et al., 1991). Dimeric IgA is coupled by epithelial cells with the secretory component and released into the lumen as secretory IgA, which is quite resistant to proteolysis and represents the most important humoral defence instrument in the gut of the healthy adult mammalian organism (*Bienenstock* and *Befus*, 1980). In humans, it takes - after an appropriate antigenic challenge - about one week until, e.g., anti-O antibody titres rise in the circulating blood (*Stuttmann* et al., 1989). In chronic ulcerative colitis, IgG antibodies seem to take the lead over IgA antibodies

Table 1: Selective list of risk factors for the development of gut derived infectious-toxic complications, e.g., following major surgery*

Age:	Less than 1 year, more than 65 years
Nutritional status:	Malnutrition Obesity
Abuse:	Alcoholism Possibly smoking Drugs
Disease:	Metabolic disorders, e.g., diabetes mellitus Arterial atherosclerosis, with regional hypoperfusion, in particular also coronary heart disease Other cardiovascular disorders Chronic obstructive bronchitis Severe infections (e.g., HIV infection /AIDS, measles, chronic tuberculosis, parasitoses and others) Malignant neoplasia
Severe trauma	
Severe burns	
Therapy:	Cytostatic chemotherapy, including immunosuppression Glucocorticosteroids Catecholamines Possibly antibiotics Catheterism (intravascular, intraluminal) Ionising radiation, especially in the abdominal region Preceding major surgery
Preceding hospitalisation	

*Excluding primary, i.e. genetically determined, immunodeficiency syndromes.

(*Brandtzaeg*, 1985). The role of cell-mediated immunity in gastrointestinal defence systems is still not adequately understood, so are the functions of intra-epithelial T cells, which usually carry a γ/δ type T cell receptor (*Goodman* and *Lefrançois*, 1988). Quite obviously, however, and despite some uncertainties about mechanisms involved, GALT is functioning effectively most of the time and in the vast majority of individuals.

Conversely, there are many possible causes of ineffective defence by the immune apparatus associated with the digestive tract. More and more genetically determined, so-called "primary" immunodeficiency syndromes are being identified and many of these - mostly hereditary - defects manifest themselves also in gastrointestinal disorders (*Cottier* et al., 1991). If one considers the innumerable peptidic molecules engaged in immune reactions, each of which may be subject to a genetic defect, this field of research and knowledge is certain to expand in the years to come. Of even greater medical importance is the multitude of acquired conditions that interfere with successful immune reactions in the gastrointestinal tract. The individual's general condition plays an essential role since it has been shown that the risk of developing severe infectious complications following major abdominal surgery is a function of age, cell-mediated

immune reactivity, examined by skin tests, and albumin concentration in the circulating blood (*Christou*, 1989). Furthermore, a great number of diseases/disorders of infectious, toxic, metabolic and/or iatrogenic nature can also cause acquired immunodeficiency (Table 1). AIDS is just one example, albeit the most dramatic, of such conditions. The defensive capabilities of GALT may also be overcome, even in otherwise healthy individuals, by an excessive microbial attack, the causative micro-organisms being pathogenic or - as often occurs in the gut derived infectious-toxic shock (GITS) - normal constituents of the intestinal microflora: we may regard this type of events as the consequence of a "relative" immunodeficiency. In particular, the primary - or even anamnestic - immune response may come too late to be able to cope with a sudden massive bacterial attack, as it can occur, e.g., after acute intestinal barrier failure. In addition, major trauma, surgery and burns are known to be followed by marked immunosuppression. The mechanisms responsible for this "Post-TSB" immunodeficiency syndrome (*Grob* et al., 1987) are still incompletely understood. They are complex and seem to involve, among other, phagocyte dysfunction, enhanced suppressor activity of cells, release of suppressor peptides, lymphocyte sequestration, and hormonal effects (*Goodwin* and *Behrens*, 1990).

INFLAMMATORY REACTIONS: HARMONIOUS AND PROTECTIVE VERSUS EXCESSIVE AND HAZARDOUS

Inflammation can be regarded as the sum of reactions originating from soluble blood plasma constituents, blood cells, the microvascular system, mast cells, and mesenchymal elements to injury (*Iversen*, 1989). This highly complex process involves complement (*Mollnes* and *Lachmann*, 1988); the coagulation, fibrinolytic (*Kaplan* and *Silverberg*, 1987) and anticoagulant (*High*, 1988) systems; immunoglobulins (see below); granulocytes, especially neutrophils (*Benestad* and *Laerum*, 1989); monocytes/macrophages (*van Furth*, 1989); mast cells (*Enerbäck* and *Norrby*, 1989); and small vessels which may interact, e.g., via adhesion molecules, with blood cells (*Möller* et al., 1991), and soluble plasma components. Most often, inflammatory reactions fulfil their *protective* function in as much as they succeed in overcoming invading microbes and/or in restoring - at least to the possible extent - tissue integrity, and then calm down.

If, however, the causative agent (e.g., bacteria, endotoxin and others) persists or increases in amount, inflammation may build up and reach a level where it gets out of antagonistic control. In such situations, the powerful forces of defence are apt to direct themselves against the host and put its life in acute *danger*, mainly via the action of pro-inflammatory mediators. Among the latter, certain cytokines, especially tumour necrosis factor (*Fong* and *Lowry*, 1990), interferon-γ (*Billiau*, 1988), interleukin-1 (*Offner* et al., 1990) and others, play a predominant role. But platelet-activating factor (*Braquet* et al., 1987), certain eicosanoids (*Hechtman* et al., 1990), oxygen derived free radicals (*Taylor* et al., 1986), proteinases liberated from phagocytes (*Neuhof*, 1990), and others also participate in this deleterious cascade of events. This complex, progressive process is the hallmark of septic shock, which in medicine has remained an unresolved crux.

ANTIBACTERIAL DEFENCE: SUCCESS AND FAILURE

Phagocytes, in particular neutrophils, are the host's most powerful weapons against bacteria and fungi. These cells kill micro-organisms in various ways, the most important being the formation of highly reactive oxygen derived radicals in the course of a respiratory burst and their secondary products (*Taylor* et al., 1986); the release of myeloperoxidase with consecutive chlorination of endogenous amines (*Grisham* et al., 1984); and oxygen independent mechanisms. The latter encompass the elaboration of cytotoxic peptides, some of which appear to be specific for Gram-negative bacteria (*Elsbach* and *Weiss*, 1985). One prerequisite for neutrophils to enter into action in time is their rapid accumulation on site, in which complement components, especially C5a, certain cytokines, endotoxin, and adhesion molecules play their part. Another important condition for successful killing of microbes is their adequate opsonisation, in preparation of endocytosis by phagocytes. Micro-organism specific antibodies are the best promoters of this process because they can, with the constant region of the molecule, also interact with Fc receptors on cells and with complement components, in particular with C3b. Depending on the bacterial species/strains involved, C3 cleavage products bind with complement receptors CRl and/or CR3 on the phagocyte surface (*Späth*, 1991). It has been shown that C3b-IgG-heterodimers are especially good opsonisers for micro-organisms to be taken up by neutrophils (*Malbran* et al., 1987). Conversely, certain microbes, such as mycobacteria, are preferentially killed by macrophages activated by T cells.

Antibacterial defence may fail for a number of reasons. Neutropenia is, in rare instances, hereditary, but most often this type of insufficient cell production occurs in the course of an acquired disorder. The same pertains to phagocyte dysfunction (*van der Valk* and *Herman*, 1987), although primary defects of this category are well known, e.g., chronic granulomatous disease of childhood (*Curnutte* et al., 1989), the Chédiak-Higashi syndrome (*White* and *Gallin*, 1986), myeloperoxidase deficiency, and others. It is not surprising that novel types of deficient phagocyte functions are reported in increasing numbers (*Cottier* et al., 1991). It must be emphasised, however, that genetically determined disorders of this sort are far less frequently encountered than acquired forms of phagocyte dysfunction. They occur in a vast variety of infectious, toxic, metabolic, and other disorders. One important cause of ineffective microbicidal activity of phagocytes seems to be inadequate opsonisation due, for instance, to local antibody consumption. Understandably, immunosuppression (see above) can lead to similar insufficiencies of the microbicidal machinery.

CONTAINMENT AND RELEASE OF BACTERIAL ENDOTOXIN

In considering the threat posed by the presence of enormous amounts of endotoxin (a constituent of the outer membrane of Gram-negative bacteria) in the gut, a few remarks seem appropriate. *In vitro* it is difficult - if at all possible - to demonstrate a direct toxicity of LPS. Rather, this substance triggers cells, especially also macrophages, to release a multitude of pro-inflammatory media-

tors which, when produced in excess, endanger the host's life. Thus, endotoxin threatens the integrity of the macro-organism as soon as it comes - in critical amounts - into contact with responsive cells, i.e. in the tissues. The question, therefore, arises as to how this may happen. It is generally assumed that the gut mucosa is "impervious" and "resistant" to this material (*Bayston* and *Cohen*, 1990). However, information on the amount of free endotoxin in the intestinal lumen of healthy adults is scarce. We also know little about intraluminal death rates for Gram-negative micro-organisms under physiological conditions. Conversely, substantial release of endotoxin has been observed following the action of certain antibiotics (*Rokke* et al., 1987) and - probably of great importance after engulfment of Gram-negative bacteria by phagocytes. These may liberate fragments of this material that are many times more active than commercially available LPS (*Duncan* et al., 1986). Killing of micro-organisms solely by complement and/or specific antibodies does not seem to be a major source of free endotoxin (*Roantree* and *Rantz*, 1960). Disregarding antibiotic treatment, which is not without hazards, we may thus theorise that endotoxin is predominantly released from Gram-negative bacteria within the tissues, via the action of phagocytes. Quite possibly, this release may be particularly important if neutrophils and macrophages die before endocytosed micro-organisms are fully degraded. The fate of liberated endotoxin in the tissues remains debatable. It may be neutralised by naturally occurring anti-LPS antibodies, although these could rapidly be consumed (*Barclay* et al., 1989). Or it may bind to LPS-binding protein (LBP), lipoproteins, anti-thrombin III, α-2-macroglobulin and/or other, poorly characterised plasma proteins (*Bayston* and *Cohen*, 1990). Elimination of such endotoxin-protein compounds seems to be achieved primarily by the liver.

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PROPERTIES, FUNCTIONS AND BIOLOGICAL SIGNIFICANCE OF T LYMPHOCYTE SUBSETS

PETER ERB

Institute for Medical Microbiology, University of Basel, Basel, Switzerland

SUMMARY

Since the discovery of the separation of killer T cells from helper T cells according to the cell-surface molecules CD8 and CD4, evidence has accumulated that further subdivision exist among the CD4+ T cell population. Mouse CD4+ T cells can be separated on the basis of different patterns of lymphokine secretion into Th1-type cells, which secrete IL-2 and IFN-γ, but not IL-4 and IL-5, and Th2-type cells which express the reverse lymphokine profile. Th1 and Th2 phenotypes are considered to represent mature stages of helper T cells. Their precursors are thought to express a mixed lymphokine profile not allowing to make the Th1/Th2 distinction. Human CD4+ T cells do not demonstrate a lymphokine profile analogous to the mouse Th1 or Th2 cells. Thus, a distinction of human CD4+ T subsets according to the lymphokine secretion pattern is not possible. Functionally, helpers and non-helpers for B cells, killers of MHC class-II antigen presenting cells and suppressors of antibody responses can be identified among human as well as mouse CD4+ T cells. However, the functions only partially correlate with the lymphokine expression in the mouse and not in the human system. Beside functional criteria, CD4+ T subsets can be defined according to surface markers, especially on the expression of CD45 isoforms. In man, CD45 isoforms do not define different lineages of CD4 T cells but rather represent maturational stages of a lineage. Thus, naive CD4+ T cells express the high molecular weight isoforms which are lost upon T cell activation.

Lamina propria lymphocytes and intraepithelial lymphocytes not only represent distinct subsets among gut mucosal T cells, they also seem to differ from T cells in other compartments of the immune system including peripheral T cells. Due to their location, both lymphocyte populations are of outstanding importance as barrier against invading agents.

INTRODUCTION

The importance of the immune system for the protection of the host against infectious agents is well recognised. The immune system is equipped in a very sophisticated way to recognise foreign antigens and to distinguish them from self molecules. The underlying mechanism, the specific immune response, is the result of a complex co-operation between T cells, B cells and antigen presenting cells (APC), as well as of various cytokines secreted by these cells. To initiate an immune response antigen is taken up by MHC class-II-positive (also called Ia+) APC and processed, e.g. proteins are proteolytically degraded to peptides. Such peptides are associated with MHC products, re-ex-

Table 1: CD4+ T subsets defined according to the LK-profile[1]

LK secreted[2]	LK not secreted	Mouse	Man
IL-2, IFN-γ	IL-4, IL-5, IL-6	Th1	not found
IL-4, IL-5, IL-6	IL-2, IFN-γ	Th2	Th2
IL-2, IL-4, IL-5, IFN-γ		Th0	Th0

[1] Only selected lymphokines are listed in the Table
[2] Cloned CD4+ T cells are stimulated with either mitogens or with antigens and APC. 24h later, SN are collected and tested for the presence of selected lymphokines.

pressed on the surface of the APC and presented to CD4+ T cells with the appropriate receptors. Thus, T cells recognise with their heterodimeric α/β receptors (TcR) combinations of peptide antigen (foreign) and MHC class-II molecules (self) (reviewed by *Grey* et al., 1990). Once activated, CD4+ T cells evoke various functions, e.g. they help B cells for antibody production or CD8+ T cells to become killer cells. CD8+ T cells - in order to perform their effector function - must recognise foreign antigen in context of MHC class-I determinants expressed on the surface of target cells. Recently, the existence of a second T cell lineage was revealed which expresses a γ/δ TcR instead of an α/β TcR (reviewed by *Raulet*, 1989; *Kaufmann* and *Kabelitz*, 1991). Both the α/β and the γ/δ TcR are noncovalently linked with the CD3 complex which transduces the activation signal upon contact with the antigen. The majority of the γ/δ T cells lack the CD4 and CD8 markers, although some have been shown to express the CD8 and to lesser extent the CD4 molecules. While the

Table 2: Representative CD4+ T cell subsets

Properties of T cell clones:	9A/B	9F/D	8/25-1	8/25-2	10H/A	9/6
LK-secretion:						
- IL-2 secretion	-	+	+	+	+	+
- IL-3 secretion	+	+	+	+	+	+
- IL-4 secretion	+	+	-	-	-	-
- IFN-γ secretion	-	+	+	-	+	+
Effect on B cells:						
- cognate help	+	+	+	-	-	-
- non-cognate help	+	n.t.	n.t.	+	-	-
- suppression of help	-	-	-	-	-	+
Effect on MHC class-II+ APC:						
- antigen-specific killing	-	-	-	-	+	+
Major characteristic:	helper	helper	helper	helper	killer	suppressor and killer
Th-phenotype:	Th2	Th0	Th1	Th1	Th1	Th1

n.t. : not tested
\+ : strong
\- : none

functions of the α/β T cells are well defined, the functional properties and the biological significance of the γ/δ T cells are not yet clearly revealed. Mycobacterial components and heat shock proteins have been found as frequent ligands for γ/δ T cells (*Kaufmann* and *Kabelitz*, 1991; *Janis* et al., 1989; *O'Brien* et al., 1989; *Haas* et al., 1990), implying that this population of T cells might be important non-MHC-restricted effectors in the antimicrobial immunity. The CD4 and CD8 determinants are so-called adhesion molecules, i.e. they bind to the non-polymorphic portions of either the MHC class-II (in the case of CD4) or class-I (CD8) products and stabilise cell interactions by this connection (*Parnes*, 1989; *Eichmann* et al., 1989). Thus, two classes of T cells can be defined according to the cell surface molecules CD4 and CD8, and both differ in their functional properties. The CD4+ T cells are commonly classified as helper/inducer and the CD8+ T cells as killer/ suppressor cells. However, already ten years ago provisional evidence emerged that the CD4+ T cells represent a rather heterogeneous population, a fact, which was confirmed when T cell cloning became available. In this review, we will discuss peripheral CD4+ T cell subsets in the two best investigated systems, mouse and man, with special emphasis on their characteristics, functional properties and biological significance. In addition, gut mucosal T cell subsets, which demonstrate some unique features will also be briefly reviewed.

PERIPHERAL CD4+ T CELL SUBSETS

T cell subsets can be defined according to either (A) their lymphokine secretion pattern, (B) their functional properties or (C) surface markers which they express. Preferentially, the concordance of all three criteria would be best for a unequivocal subset definition. However, this is only partially the case.

CD4+ T subsets defined according to the lymphokine profile:

Mosmann and colleagues (1986; 1989) were the first to describe two mutually exclusive subsets among mouse CD4+ T cell clones, termed Th1 and Th2, based on their ability to secrete different lymphokines (LK). Th1 essentially produce IL-2 and IFN-γ but not IL-4 and IL-5, while Th2 secrete IL-4, IL-5 and IL-6, but neither IL-2 nor IFN-γ (Table 1). However, the distinct LK-secretion pattern of Th1 and Th2-clones did not turn out to be exclusive. Recently, clones intermediate between Th1 and Th2 have been described (*Firestein* et al., 1989; *Gajewski* and *Fitch*, 1988; *Yokoyama* et al., 1989), termed Th0, which secrete IL-2, IL-4 and IFN-γ (Table 1).

In man, the clear-cut Th1/Th2 distinction cannot be made (*Paliard* et al., 1988; *Umetsu* et al., 1988; *Rotteveel* et al., 1988). The majority of human CD4+ T clones described demonstrate the characteristics of Th0, i.e. secret IL-2 and IFN-γ in addition to IL-4. Some human T cell clones have the phenotype of Th2, secreting IL-4, but not IL-2 and IFN-γ (Table 1). However, T cell clones similar to Th1 have not been identified so far. Thus, the Th1 and Th2 dichotomy may not hold true as far as human CD4+ T cells are concerned.

Characterising a number of CD4+ T clones generated by directly cloning splenic T cells from primed BALB/c mice we identified stable subsets which clearly fitted into the Th1/Th2/Th0 scheme in terms of the LK-secretion profile (Table 2) (*Erb* et al., 1991).

Table 3: Th2-type clones can switch into the Th0 phenotype.

Conditions of stimulation of clone 9A/B[1]	days between assay and last stimulation	mRNA expression[2] IL-2	IL-3	IL-4	IL-5	IL-6	IFN-γ
A20.2J + KLH	9	-	+	+	+	+	-
	28	+	+	+	+	+	+
Con-A	9	-	+	+	+	+	-
	28	-	+	+	+	+	-

[1] 9A/B T cells (10^6/ml) were activated with either con-A (5 μg/ml) or with the B lymphoma A20.2J (10^6/ml) as APC and KLH (50 μg/ml). After 6h, the cells were harvested and mRNA was determined for selected lymphokines by Northern blot analysis.

[2] +: mRNA expression.
-: no mRNA expression.

However, clones with an unusual LK-secretion pattern were also found, such as clone 8/25-2, which expressed a LK-profile similar to Th1, but failed to secrete IFN-γ (Table 2). It is noteworthy that these clones kept their original phenotypes stable over years with an exception discussed below. Thus, it is likely that the CD4+ T cells are even more heterogeneous as originally thought.

In vivo, evidence for the existence of CD4+ subsets is also available, but the LK-secretion pattern is much less distinct than in cloned T cells (*Hayakawa* and *Hardy*, 1988; *Powers* et al., 1988; *Mohler* and *Butler*, 1990; *Schoenbeck* et al., 1989; *Heinzel* et al., 1989; *Bass* et al., 1989). Indeed, when CD4+ T cells from normal mice are stimulated with mitogens or antigen and APC, the LK-pattern that is produced does not match the Th1/Th2 pattern. In short-term cultured T cell lines LK-secretion phenotypes intermediate between Th1 and Th2 patterns are also frequently found (*Kelso* and *Gough*, 1988; *Carding* et al., 1989; *Street* et al., 1990; *Swain* et al., 1990; *Weinberg* et al., 1990). These observations lead to the suggestion that Th1 and Th2 subsets represent final stages in Th differentiation and thus, express mutually exclusive LK-secretion precursors, e.g. Th0, express a mixed LK-secretion pattern (*Firestein* et al., 1989; *Street* et al., 1990; *Mosmann* and *Moore*, 1991). Indeed, it was reported that Th0 obtained from unprimed mice converted into Th2 cells after prolonged culture *in vitro* (*Torbett* et al., 1990). A conversion of Th0 to Th1 or of Th1 to Th2 and the reverse was not found.

Recently, we found that a classical Th2 clone (9A/B, see Table 2) is able to transcribe and secrete IL-2 and IFN-γ in addition to IL-4 and IL-5 depending on the resting state of the Th2 cells as well as on the mode of activation (manuscript submitted). The resting state is defined as the time between the last stimulation of the T clone with antigen and irradiated feeder cells and the assay. Thus, short-term (<2 weeks) rested 9A/B cells demonstrated the classical Th2 LK-profile independent of the mode of activation, while long-term (>3 weeks) rested 9A/B cells showed the Th0 profile, provided they had been activated with antigen and APC, but not with mitogens (Table 3). Our results question the proposed unidirectional differentiation pathway of Th0 to Th2 and suggest that certain Th0 clones may represent an additional independent subset rather than a stage along the development from precursors to Th2 or Th1.

The differential LK-secretion pattern of CD4+ subsets could also stem from differences in their triggering require-

Table 4: CD4+ T cell subsets defined according to functions

Function	Mouse	Man
Help to B cells for antibody production	Th2, Th0, (Th1)	Th0, Th2
Killing of MHC class-II + APC	Th1	Th0, (Th2)
Suppression of antibody responses	Th1	Th0, Th2
Activation of macrophages	Th1	Th0
Delayed type hypersensitivity (DTH)	Th1	?

( ): Function has been demonstrated by some, but not all investigators

ments. *In vivo*, it is possible to induce either Th1-type or Th2-type responses depending on the immunogens used. Antigens that preferentially stimulate Th1-mediated responses include *Brucella abortus* and many viruses (*Finkelman* et al., 1988; *Coutelier* et al., 1987). Certain parasites and helminths induce a strong, predominant Th2 response, as manifest by a significant production of IgE and weak or absent DTH (*Heinzel* et al., 1989; *Liew* et al., 1990; *Pearce* et al., 1991). *In vitro*, Th2 cells, but not Th1 cells are reported to proliferate in response to IL-1 when costimulated with anti-TcR antibodies or mitogens (*Fernandez-Botran* et al., 1988; *Greenbaum* et al., 1988). In addition, the IL-2-induced proliferation of Th1, but not of Th2-clones is strongly inhibited by immobilised anti-CD3 antibodies (*Williams* et al., 1990). Finally, differences between Th1 and Th2-clones have also been found in their response to a tolerogenic signal (*Gilbert* et al., 1990).

CD4+ T cell subsets defined according to functions:

Functionally, both murine T cell subsets were originally described to help B cells for antibody production (Table 4). Indeed, Th2 cells, which produce IL-4, IL-5 and IL-6 efficiently provide noncognate or cognate help to B cells for antibody responses (*Erb* et al., 1991, see also Table 2). However, the capacity of Th1 cells to provide cognate MHC-restricted help for Ag-specific antibody responses is controversial. Some investigators found efficient B cell help provided by Th1-clones (*Mosmann* et al., 1986; *Dekruyff* et al., 1989; *Erb* et al., 1991), while others did not (*Killar* et al., 1987; *Boom* et al., 1988). We found very few real helper clones among many Th1-clones tested (e.g. clone 8/25-1, Table 2) which provided helper activity by cognate interaction. The majority of our Th1-clones did not help at all. Irrespective whether Th1 cells help B cells or not, it is clear that Th2 are much more efficient helper cells. In any case, it is of important biological significance that both Th2 and Th1 cells markedly influence isotype secretion of B cells (*Stevens* et al., 1988). Thus, Th2 preferentially help B cells for IgG1 and IgE production, due to the secretion of IL-4, while Th1 promote IgG2a and suppress IgE production due to IFN-γ secretion. Thus, IL-4 is the switch factor for IgE and IFN-γ for IgG2a. As already mentioned, infection of certain inbred strains of mice with protozoan or helminth parasites, e.g. *Leishmania*, evokes a strong IgE response mediated by Th2 cells which predominate in these mice (*Boom* et al., 1988; *Locksley* and *Scott*, 1991). Other functions have been more or less only found among the Th1 subset (Table 4). Thus, Th1-clones mediate strong antigen specific delayed type hypersensitivity (DTH) and they are, therefore, also called inflammatory Th cells (*Bottomly*,

1988). Some of the Th1 are cytotoxic, activate macrophages (due to IFN-γ secretion) or mediate suppression of antibody responses (*Killar* et al., 1987; *Erb* et al., 1990; *Erb* et al., 1991; *Yokoyama* et al., 1989).

In man, CD4+ T cells can be divided into helper and non-helper for B cells, but there is little correlation to the LK-secretion phenotype (Table 4). Thus, both Th0 as well as Th2 cells help or do not help, kill or suppress (*Umetsu* et al., 1988; *Rotteveel* et al., 1988).

It is of interest, that beside the well-known CD8+ CTL, cytotoxic T cells also exist among the CD4+ T cell population. CD4+ CTL lyse target cells in an antigen-specific and MHC class-II restricted way (*Fleischer*, 1984; *Tite* and *Janeway*, 1984; *Braakman* et al., 1987; *Ju* et al., 1990; *Erb* et al., 1990). In other words, CD4+ cytotoxic T cells eliminate their own MHC class-II+ APC. CD4+ CTL have originally been claimed to be an 'artefact' of *in vitro* cultured T cell clones (*Fleischer*, 1984). However, this possibility can now be ruled out as it has been recently shown that CD4+ CTL are present in the spleen of *in vivo* primed mice (*Erb* et al., 1990). Different types of APC express a differential sensitivity to killing. MHC class-II+ tumour cells or non-transformed, but activated APC (e.g. B cell blasts) are highly sensitive to killing, while non-activated APC (e.g. normal B cells) are resistant to killing (*Erb* et al., 1990). The differential susceptibility of MHC class-II+ APC to lysis suggests an important role of CD4+ CTL in immunoregulation, i.e. down-regulation of antigen presentation by eliminating stimulated APC. In addition, the preferential killing of MHC class-II+ tumour cells as well as the lysis of certain virus-infected cells (*Meurer* et al., 1991; *Jacobson* et al., 1984; *Schmid*, 1988; *Bourgault* et al., 1989; *Wing* et al., 1990) strongly supports the participation of CD4+ CTL in immune surveillance. Moreover, CD4+ CTL may also be involved in certain autoimmune diseases. Thus, they may be responsible for the elimination of cells which were accidentally stimulated and became Ia+, but whose primary function is not antigen presentation. Examples for such a role may be the CD4-mediated lysis of MHC class-II+ astrocytes of the brain (*Sun* and *Wekerle*, 1986) or of MHC class-II+ β-cells of the pancreas (*DeBerardinis* et al., 1988; *Wang* et al., 1991), a mechanism which could be responsible for the pathogenesis of the experimental autoimmune encephalomyelitis or of type I diabetes.

CD4+ T cell subsets defined according to surface markers:

Beside the lymphokine profile or functional properties, surface markers also represent a good tool for defining CD4+ T cell subsets. Functional dichotomy in normal CD4+ T cells was found in human, rat and recently in the mouse, using mAb that react with various isoforms of T200 leukocyte common antigen, also termed CD45 (*Morimoto* et al., 1985b; *Smith* et al., 1986; *Pulido* et al., 1988; *Spickett* et al., 1983; *Bottomly* et al., 1989; *Luqman* et al., 1991). CD45 is a major cell surface glycoprotein expressed on haematopoietic cells except mature erythrocytes. This cell surface antigen has been characterised in human, rat, mouse and has similar properties in all species (reviewed by *Thomas*, 1989). A unique feature of CD45 is its heterogeneity in molecular weight (Figure 1). The structural variation is cell type specific in that antibodies to CD45 distinguish between T cells and B cells, between CD4+ and CD8+ T cells and even between subsets of CD4+ T cells. Part of the heterogeneity is due to the presence of three exons which are differentially spliced in the mRNA. This gives theo-

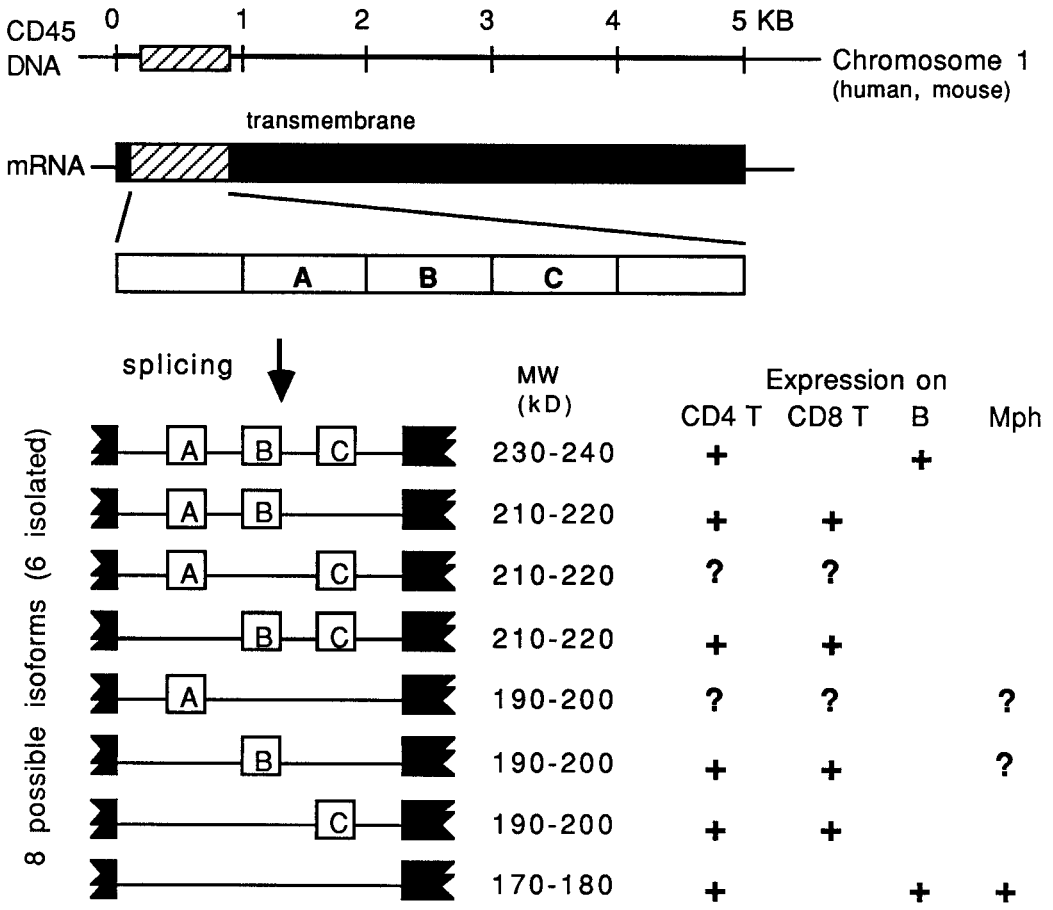

Figure 1: Schematic diagram of the CD45 complex.
The CD45 cDNA is 4991 base pairs (bp) long and contains 34 exons encoding 1291 amino acids. Exons 3-15 encode amino acids 1-537 of the external domain. The striped bar contains the three variably expressed exons A, B, and C. Splicing leads to 8 possible isoforms of which 6 have been identified. MW indicates the approximate molecular weights of the glycoproteins for each type of isoform. The expressed column indicates the possible expression patterns for different isoforms. Adapted according to *Thomas* (1989) and *Luqman* et al. (1991).

retically rise to eight different mRNA and hence eight isoforms (Figure 1) from which six have been identified. The differential splicing results in differences in the primary sequence of the extracellular domain. Monoclonal antibodies exist which have known specificity for particular CD45 isoforms referred to as CD45R (Table 5). Some mAb require the expression of exon A (anti-CD45RA) or exon B (anti-CD45RB) for binding, while others recognise a null isoform with all variable exons spliced out (anti-CD45R0). In human, the mAb 2H4 reacts with a high molecular weight form of CD45 (=CD45RA) (*Morimoto* et al., 1985b). This CD45 high molecular weight marker is lost upon T cell activation. The 2H4-positive subset which proliferates upon mitogen stimulation, but much less to antigen stimulation, provides poor help for specific immunoglobulin synthesis (*Morimoto* et

Table 5: List of mAb with known specificity for CD45 isoforms

Species	mAb	Reference	CD45RA	CD45RB	CD45RO
Man	2H4	Morimoto, 1985	+	-	-
	UCHL-1	Smith, 1986	-	-	+
	PD-7/26	Pulido, 1988	-	+	-
Mouse	C363.16A	Bottomly, 1989	-	+	-
	14.8	ATCC TIB 164	+	-	-
	M1/9.3.4HL2	ATCC TIB 122	+	+	-
Rat	MRC OX22	Spickett, 1983	_	+	-

al., 1985b). In contrast, the 2H4-low T subset poorly proliferates upon mitogen stimulation, but highly to soluble antigen and provides good help to B cells (*Morimoto* et al., 1985a). As mentioned, human T cells loose the 2H4 marker upon activation, whereas activated T cells are now reactive with an other mAb termed UCHL-1, which recognises a low molecular weight form of CD45R (=CD45R0) (*Smith* et al., 1986). These observations led to the assumption that 2H4+ T cells are naive, while UCHL-1+ T cells are of the memory type (*Sanders* et al., 1988). More recently, it has been reported, that memory T cells for IFN-γ secretion express the CD45RB isoform on the surface (*Mason* and *Powrie*, 1990). A summary of the human CD4+ T subsets characterised according to CD45 isoforms has been given in Table 6.

In the rat, mAb OX22 has also been found to bind to the high molecular weight form of CD45 (CD45RB) (*Spickett* et al., 1983). OX22+ T cells are mainly producing IL-2 and mediate DTH, while OX22- T cells provide help to B cells (*Powrie* and *Mason*, 1990).

In the mouse, anti-CD45 mAb (Table 5) raised against the high molecular weight isoform of CD45 preferentially bind to Th2-type cells (*Luqman* et al., 1991) Indeed, Th2-clones express the

Table 6: Expression of CD45 isoforms on CD4+ human T cells

Properties / Functions	CD45RA	CD45RO	CD45RB
Naive T	++	+/-	?
Inducer of suppression	++	+/-	?
Memory T for B help	+/-	++	?
Proliferation to recall antigen	+/-	++	?
Proliferation to mitogens	++	+	?
IL-2 secretion	++	++	?
IL-4 secretion	+	++	?
IFN-γ secretion	+/-	++	++
TNF secretion	++	++	?
CD29, CD2, LFA-1, LFA-3 expression	+	++	?
Phenotype change upon activation	yes	no	no

++ : high expression
+ : low expression
+/- : very low expression
? : not yet defined

Table 7: CD45 isoforms expressed on murine CD4+ T subsets

Isoform	Th1	Th2	Th0
CD45RA	-	++	?
CD45RB	-	++	?
CD45R0	++	-	?

++ : high expression
+ : low expression
+/- : very low expression
? : not yet defined

high molecular weight isoforms of CD45 containing two or three of the alternatively spliced exons, while the two-exon and three-exon forms are absent in Th1-clones, in which the CD45R0 dominates (Table 7) (*Luqman* et al., 1991). Nothing is known about the CD45 isoform expression on Th0 cells.

Taken together, CD45 isoforms do not define different lineages of cells but correspond to maturational stages of a lineage. Thus, the high molecular weight isoforms are lost from naive T cells upon activation. Murine Th2-clones seem to represent the exception of the rule in that the expression of the high molecular weight isoforms is still maintained, despite these cells are considered to be end stages of Th differentiation.

CD44, an extensively glycosylated one-chain molecule which is widely distributed on a diverse range of cell types, is also expressed on subsets of CD4+ and CD8+ T cells. In both cases, CD44-cells appear to correspond to

Table 8: Comparison of intraepithelial (IEL) and lamina propria lymphocytes (LPL)

Properties		IEL	LPL
Surface marker expression	CD3, CD2	>90%	>90%
	CD4	5-10%	65-80%
	CD8	75-90%	20-35%
	α/β TcR	90%	100%
	γ/δ TcR	10%	<1%
	CD45RA	high	low
	CD45R0 (UCHL-1)	high	high
	IL-2R (TAC)	none	high
	MHC class II	very low	high
LK secretion	IL-2	no	yes
	IL-3	yes	yes
	IFN-γ	yes	?
	GM-CSF	yes	?
Functions	Cytotoxicity	yes	yes(?)
	Help to B cells	uncertain	yes
	DTH	yes	?
	Proliferation to recall antigen	low	low

virgin, unstimulated T cells, while the memory cells that have been previously exposed to antigen are found in the CD44+ population (*Haynes* et al., 1989).

Another immunoglobulin-like surface glycoprotein, CD28 is found on about 95% of human peripheral CD4+ T cells and approximately 50% of CD8+ T cells (*June* et al., 1990). The density of CD28 expression divides human CD4+ T clones into two subsets (*Rotteveel* et al., 1988). The clones with low CD28 expression produce IL-2, IFN-γ and TNF-α and display anti-CD3-mediated cytotoxicity. The clones with high CD28 expression produce minimal amounts of LK and are not cytotoxic. Thus, both CD44 and CD28 seem to be activation markers rather than lineage markers.

Finally, *Takada* et al. (1989) discriminated human cytotoxic from non-cytotoxic CD4+ T cells by the cell surface marker Leu 8. Cytotoxic CD4+ T cells lack Leu 8 antigen, but express the CD2 marker in high density in contrast to non-cytotoxic CD4+ T cells.

In summary, murine CD4+ T cell subsets have been defined mainly on the basis of different lymphokine secretion patterns and functions, but both, LK-profiles and functions of the subsets correlate only partially. The classification of human CD4+ T cell subsets is primarily based on their functional properties and on certain surface markers, especially on the expression of CD45 isoforms. Lymphokine secretion profiles neither correlate with function nor surface marker expression.

INTESTINAL T CELL SUBSETS

T cells in the gut are either localised in organised lymphoid organs, such as the Peyer's patches and the lymphoid follicles in the colonic mucosa, or are disseminated in the intestinal lamina propria (lamina propria lymphocytes: LPL) and above the basement membrane between epithelial cells (intraepithelial lymphocytes: IEL). While the T cells in the gut organised lymphoid organs are more or less analogous to the peripheral T cells described above, gut mucosal lymphocytes, especially LPL and IEL seem to differ from T cells in other compartments of the immune system in various aspects (reviewed by *Jalkanen*, 1990; *MacDonald* and *Spencer*, 1990; *Mowat*, 1990; *Zeitz* et al., 1990). Indeed, evidence is accumulating that the gut mucosal T cells may form a discrete compartment of the immune system on its own. Despite their apparent importance, surprisingly little is known about their nature and their function is incompletely understood, especially in man.

A comparison - by no way comprehensive - of some major properties and functions of the IEL and LPL are given in Table 8. It is quite obvious that both, IEL and LPL, represent distinct subsets. While the majority of the intraepithelial T cells are CD8-positive, T cells from the lamina propria have almost similar CD4/CD8 expression to that of peripheral blood lymphocytes (2:1). Moreover, in contrast to LPL, IEL do not express conventional T cell activation markers, such as the IL-2 receptor (TAC) or MHC class-II antigens. However, the expression of UCHL-1 which is a marker for differentiated memory T cells is not consistent with IEL being entirely a resting population. It is, therefore, likely that IEL have already interacted with antigen before migrating into the epithelium (*Jalkanen*, 1990).

Functionally, IEL and presumably

Table 9: Mouse CD8+ IEL consist of two ontogenetically distinct populations

90% CD8+ IEL comprise 2 populations	
Thy-1 positive	Thy-1 negative
α/β TcR	γ/δ TcR or α/β TcR
heterodimeric α/β CD8+	homodemeric α/α CD8+
Thymus-dependent	Thymus-independent
Ag stimulation required	no Ag stimulation required
Peyer's patch ➔ blood ➔ TD ➔ mucosa of gut	directly bone marrow derived?

also LPL evoke cytotoxic activity. Whether the cytotoxicity of LPL is also due to CD4+ T cells is not known, but might be possible due to the fact that LPL lack the Leu-8 marker. Lamina propria T cells, but not IEL have been convincingly shown to provide high helper activity for Ig-synthesis. Finally, both IEL and LPL do not well proliferate to recall antigen, which in the case of LPL is again a difference to classical memory T cells. Lamina propria T cells can, therefore, be characterised as differentiated effector cells which respond to antigen challenge with the production of certain lymphokines and cells for antibody production (*Zeitz* et al., 1990).

CD8+ IEL have been recently characterised in more detail in the mouse model (*Guy-Grand* et al., 1991). Table 9 summarises the results. Two ontogenetically different subsets have been identified. One subset bears the α/β TcR, the α/β CD8 chains and expresses the Thy-1 marker. The other subset has either the γ/δ or α/β TcR, bears the homodimeric α/α CD8 chains and does not express the Thy-1 marker. The Thy-1+ subset, which is thymus-dependent, represents most likely the progeny of precursors cells arising in Peyer's patches under antigenic stimulation, which then by way of the blood and the thoracic duct colonise the mucosa of the gut due to their gut-homing property. The Thy-1 negative subset which does not require a thymus for differentiation, presumably derives directly from the bone marrow. Although the functional role of the Thy-1 negative subset is not known, it is highly likely that it recognises an antigenic repertoire different from the Thy-1+ subset.

Taken together, IEL are one of the least understood populations of lymphocytes with many unique features. According to their properties it has been suggested that IEL represent a population of committed end-stage effector cells similar to plasma cells, which have been fully differentiated before entering the epithelium. Both, IEL and LPL are of outstanding importance for the host' integrity due to the fact that they by virtue of their position have the closest direct connection to foreign antigen including many potential pathogenic organisms. Thus, they represent an important barrier against invading antigens.

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ABBREVIATIONS USED

APC : antigen-presenting cells
CTL : cytotoxic T lymphocytes
DTH : delayed type hypersensitivity
Ia : I region associated
IEL : intraepithelial lymphocytes
IFN-γ : interferon-gamma
LK : lymphokine(s)
LPL : lamina propria lymphocytes
MHC : major histocompatibility complex
TcR : T cell receptor
Th : T helper cell(s)
TNF : tumour necrosis factor

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ANTIGEN SPECIFIC T SUPPRESSOR FACTORS, A PROPER SUBJECT FOR STUDY: THEIR RELATION TO THE T CELL RECEPTOR, THEIR CLASS AND THEIR MODE OF ACTION

GEOFFREY L. ASHERSON

Division of Immunological Medicine, Clinical Research Centre,
Northwick Park Hospital, Harrow, UK

INTRODUCTION

The object of this review is to draw attention to recent work on antigen-specific T suppressor factors (TsF) and the evidence that many of them share epitopes with the α and β chains of the T cell receptor (TCR) and that their production makes use of the standard α/β TCR. It also comments on the mode of action of certain TsF, which are of particular interest to the author. It is not comprehensive and focuses on work based on T cell hybridomas. It does not cover much of the earlier literature, where basic facts about the genetic restriction and mode of action of antigen-specific TsF are summarised (*Dorf* and *Benacerraf*, 1984; *Asherson* et al., 1986).

The introduction shows the way in which the broad concept of suppressor cells has been refined as it became clear that many cells have pleomorphic effects and upregulate certain immune responses while downregulating others. The concluding remarks deal with the biological significance of antigen-specific T suppressor factors and certain unsolved problems, such as the possible selective use of I-E restriction by suppressor cells and the continuing puzzle of I-J.

Historically, the concept of downregulation in the immune response goes back to work in the sixties on specific antibody mediated depression of antibody production and cellular immunity. In the early seventies, two groups described downregulation of the immune response by T cells and the term suppressor cell arose (*Gershon* et al., 1971; *Asherson* et al., 1971). The use of a single term to describe several different types of cells with different modes of action caused confusion. The term was often taken to imply that T suppressor (Ts) cells were "professional", i.e. they were committed to downregulation and had no other function. The use of T cell lines and hybridomas has shown that some suppressor cells have other activities; in other cases a suppressor cell acts (directly or indirectly) though a known cytokine. Some examples are given below.

Downregulation due to cytokines with negative effects:

Certain cytokines have negative effects and are made *inter alia* by T cells. The best example is TGF-β, which depresses T cell proliferation and NK cytotoxicity (*Lotz* et al., 1990). It is also required for the action of a T suppressor cell which downregulates allergic encephalomyelitis (*Karpus* and *Swanborg*, 1991).

The role of lymphokines in the polarisation of the immune response:

The apparent polarisation of the immune response between antibody production and delayed hypersensitivity was noted a quarter of a century ago (*Asherson* and *Stone*, 1965). The recent

work on Th1 and Th2 cells gives an approach to the underlying mechanism. To a first approximation, the Th1 subset makes IFN and IL-2, while the Th2 subset makes IL-4. The former affects cellular immunity, while the later controls antibody production. The two subsets have a reciprocal relationship to each other. In particular, the IFN made by Th1 cells limits the proliferation of Th2 cells and antagonises the effect of IL-4, while IL-10 made by Th2 cells limits cytokine production by Th1 cells (*Fiorentino* et al., 1989, 1991; *Mossmann* et al., 1990). Because the cells and lymphokines involved augment certain responses while downregulating others, they cannot be usefully described as suppressor cells and factors.

Ts-1 and Ts-3 cells which make antigen-specific factors

By definition, Ts-1 (Ts-inducer or Ts-afferent) cells act at the induction stage of a particular immune response, while Ts-3 cells act at the effector stage. They have different modes of action and are made by cells with different properties. In particular, the antigen-specific TsF-1 made by Ts-1 cells induces idiotope directed cells by a process of immunisation. In contrast, the antigen-specific TsF-3 made by Ts-3 has indirect mode of action through the macrophage and the T acceptor cell. Moreover certain Ts-1 cells resemble T helper cells and make IL-2 and IL-4 (*Kuchroo* et al., 1990). In contrast, Ts-3 cells behave like professional suppressor cells, i.e. they produce an antigen-specific TsF and *pro tem* have no other known action.

These antigen-specific T suppressor factors are distinct from cytokines, in being antigen-specific and genetically restricted in their action. In general, they are disulphide bonded heterodimers with an antigen binding and a non antigen binding chain. Evidence is now available that in some cases the antigen binding chain conveys antigen-specificity, while the non antigen binding chain is responsible for the genetic restriction in the action of the factor. It would be rational to designate these two chains as α and β.

Much of the confusion with suppressor cells has arisen from mistaken view that the term implies a single class of suppressor cell. Similarly, study of immunoglobulins was chaotic before classes of immunoglobulin were recognised. Antigen-specific T cell factors in general have many different effects and an important object of current research is to define distinct classes. In fact, TsF-3 has many different actions which are listed in Table I. By analogy with immunoglobulin these different actions of TsF have structural implications for the constant region of the two chains. Hence study of mode of action of TsF

Table 1: Inhibitory effects of T suppressor factor which acts at the efferent stage (TsF-3)

Contact and delayed hypersensitivity in immune mice
Passive transfer of contact sensitivity
*via macrophage which releases macrophage suppressor factor
*via T acceptor cell which releases antigen non-specific mediator
Granuloma formation
Tumour rejection
Phagocytosis by subset of macrophages
Antibody production

*The cell binds TsF, which acts like a mobile receptor for antigen (cf. IgE). Contact with antigen then causes the release of an antigen non-specific inhibitory mediator of the passive transfer of contact sensitivity.

is complementary to structural studies.

The finding of a disulphide bonded heterodimer produced by a T cell, whose function is antigen and MHC restricted invites comparison with the TCR.

Consider the following analogy:

- The Ig receptor for antibody production and the secreted antibody use the same H and L chain gene rearrangements.
- The TCR for TsF-3 production and TsF-3 use the same α and β chain gene rearrangements.

Current evidence is that TsF-3 studied share epitopes with the α and β chains of the TCR. Moreover, studies of the blocking of TsF secretion with antibody to the α and β chain would indicate that the analogy with surface and secreted immunoglobulin is valid. However, not all the data are in yet and there is little molecular biology.

In due course more information will be needed about the control of the production and secretion of these factors. A beginning has been made through studies of the effect of IL-2 on TsF-3 production and on T suppressor cells (*Perrin* et al., 1989; *Madar* et al., 1987).

STRUCTURE

Antigen-specific TsF-3 is a disulphide bonded heterodimer with antigen binding and non antigen binding chains

Most antigen-specific TsF's are disulphide bonded heterodimers, possessing an antigen binding and a non antigen binding chain. In particular this is true for all the TsF-3 that have been examined. Chain structure of TsF is analysed by reduction with or without alkylation. Usually the chains are partially purified by affinity chromatography on antigen or immobilised antibody to give separate antigen binding and non antigen binding chains. The chains are then tested by biological assay using complementation. This is based on the fact that they have no biological activity when tested singly, but cause suppression, e.g. of contact sensitivity or of an *in vitro* model of granuloma formation when tested together.

Complementation between the two chains occurs, using the assay of inhibition of contact sensitivity in immune mice with monoclonal picryl-TsF (unpublished observations). It also occurs when biological activity is assayed by inhibition of the passive transfer of contact sensitivity by chains from picryl-specific and DNP-specific monoclonal TsF (*Fairchild* et al, 1990). The antigen binding and non antigen binding chains also complement each other when they are used to coat the T accep-

Table 2: Properties of T acceptor cell

- Lyt-2^+ I-J^+ T cells found after immunisation with contact sensitiser, but not in unimmunised mice.
- Production prevented by cyclophosphamide and adult thymectomy
- Binds TsF to its surface and can then be panned on antigen
- Releases nsTsF-1 when activated by antigen (haptenised spleen cells) corresponding to TsF. This interaction is I-J restricted.
- Cross-linking of molecules of TsF required for activation.
- The haptenised spleen cell can be replaced by antigen together with a KCl extract of spleen cells of the appropriate genotype.

See *Zembala* et al. (1982a,b)

tor cell (see Table 2), which then release an antigen non-specific inhibitory mediator (nsTsF-1) when activated by antigen. This is seen with chains from conventional TNP-specific and monoclonal cryptococcal-specific TsF. In contrast, separate chains do not complement each other in the inhibition of phagocytosis by a subset of macrophages (*Blackstock* et al., 1991a,b). It is not known whether separate chains complement each other when used to coat the macrophage. [This cell, after coating with intact TsF, releases an antigen non-specific inhibitory mediator when activated by antigen (*Ptak* et al., 1978)].

Sometimes the antigen binding and non antigen binding chains occur separately (*Asherson* et al., 1984a) or only one of the chains is produced. *Taniguchi* and colleagues (1980, 1981) described a hybridoma in which the two separate chains were released by freezing and thawing, but appeared in the supernatant as a disulphide bonded heterodimer. In a more physiological system, the spleen cells of mice, injected with water soluble chemically reactive haptene [e.g. picrylsulphonic acid (trinitrobenzenesulphonic acid), or "oxazolone-thioglycollic acid"] release the antigen binding chain of TsF without further activation. This is biologically inactive alone but can be assayed by complementation with the non antigen binding chain (*Zembala* et al., 1984). However, constitutive liberation of the antigen binding chain alone is not shown by a inducible picryl-specific hybridoma (unpublished observations)

In fact, after activation with the antigen, the spleen cells from mice injected with chemically reactive, water soluble haptene produce "complete" TsF which does not require complementation. This activation can be achieved *in vivo* by applying contact sensitiser to the skin, or by injecting haptenised cells, and *in vitro* by culturing with haptenised cells (*Blackstock* et al., 1991a,b; *Colizzi* et al, 1983). The non antigen binding chain occurs in KCl extracts of normal cells (*Asherson* 1984a). This might be due to the β chains of the TCR present in the extract as it is known that different non antigen binding chains can complement a particular antigen binding chain (*Perrin* et al., 1989b). See next section on "Antigen-specific factors bear α and β chain TCR determinants".

There is some confusion in the literature as to whether the two chains of TsF covalently link, by reformation of disulphide bonds in complementation assays. There may be a real difference between different TsF's and different assay systems (*Taniguchi* et al., 1981; unpublished observations). However technical factors may be important. For instance, the use of strictly oxygen free conditions renders the reduced intra-chain disulphide bonds more susceptible to alkylation. Another factor is the use of acylation by succinic anhydride instead of alkylation by iodoacetamide to prevent reformation of disulphide bonds. Succinylation increases the negative charge on molecules as it effectively replaces positively charged amino groups by negatively charged carboxylic groups (*Perrin* et al., 1989b). Iodoacetamide is more selective for sulphydral groups and does not cause charge reversal. In our studies on monoclonal TNP- and cryptococcal-specific TsF, iodoacetamide was used and the chains complemented each other without reformation of interchain disulphide bonds. However, if alkylation or acylation is omitted, complementation may be due to reformation of the original molecule through oxidation of the interchain reduced disulphide bonds. This might be the case in studies on DNP-specific TsF in which alkylation was not undertaken (*Fairchild* et al, 1990).

There are other antigen-specific T cell factors which are disulphide bonded

heterodimers. Antigen-specific T helper factor, which specifically increases the contact sensitivity reaction to picryl induced by haptenised cells, is an example and its two chains can be assayed by complementation (*Little* et al., 1985).

In contrast to TsF-3, the chain structure of TsF-1 varies. Monoclonal TMA (phenyltrimethylamino)- and GAT-specific monoclonal TsF-1 have a single chain structure (*Jayaraman* and *Bellone*, 1985), while other GAT-, ABA-, NP- and sheep red blood cell-specific TsF have a two chain structure (*Jendriska* et al., 1986).

In summary, antigen-specific TsF which acts at the expression stage of the immune response, has a two chain disulphide-bonded structure, with one antigen binding and one non antigen binding chain, which can be detected in a complementation assay.

Antigen-specific T cell factors bear α and β chain TCR determinants

Analogy with immunoglobulin suggests that antigen-specific T cell factors are made by a cell with the standard TCR-T3 complex using α/β or possibly γ/δ chains and the soluble factors should closely resemble the TCR. However, the finding that only a small minority of T cell hybridomas had evidence of β chain gene rearrangement, while other hybridomas only expressed the V_β2.5

Table 3: α and β chain determinants of the T cell receptor (TCR) on antigen-specific T cell factors and on the cell that makes them

		Determinants on factor					Determinants on hybridoma			
Type of factor		TCR		I-J	Class markers		TCR		Others	
Specificity	Source	α	β		eff.	aff.	α	β		Reference
TsF-3 (efferent)										
TNP	hyb.	α	V_β8	I-J			α	V_β8	I-J	Zembala (unpubl.)
DNP	hyb.	α	V_β8					V_β8		Fairchild (1990)
NP	hyb.	α	-				α	β*	CD3	Dorf (1989) Collins (1990)
OA	hyb.	α	β		+	-		β	CD3	Iwata (1989)
Schisto.	conv.		V_β8	I-J	+					Perrin (1989)
TsF-1 (afferent)										
NP	hyb.	α	β		-	+	α	β		Kuchroo (1990)
ABA	hyb.								CD3	Weiner (1988)
HGG	line	α	-					β		Takata (1990)
KLH	hyb.						α			Koseki (1989)
Poly-18	hyb.	α	-			+		β		Bissonette (1991)
ThF (T helper factor)										
TNP	hyb.		V_β8							Dieli (unpubl.)
FGG	line		V_β8							Guy (1989)
KLH	line		V_β8							Guy (1989)
OA	hyb.	α	β		-	+		β	CD3	Iwata (1989a,b)
H-2D	hyb.		V_β8							Kwong (1987)

*β chain from BW 5147 (*Collins* et al., 1990)

Table 4: Anti-$V_\beta 8$ and anti-I-J monoclonal antibodies inhibit activation of T suppressor hybridoma

Pre-treatment of hybridoma before activation by antigen	Inhibition of contact sensitivity by TsF
None	100%*
anti-$V_\beta 8$ (F23.1)	26%**
anti-I-J^k (Ig8)	31%**
anti-I-E^k (HB32)	100%
anti-I-A^b	100%

P2.2.B4 picryl specific hybridoma was pre-treated for 1 hour with purified Ig, prepared from ascites with Protein A. After washing, antigen was added (picrylated spleen cells). The supernatant at 24 hours contained TsF which was assayed by its ability to inhibit contact sensitivity in actively immunised mice (unpublished observations).

*The data was normalised by setting the suppression caused by the hybridoma untreated with antibody at 100%.

**Highly significant as compared with hybridoma not treated with antibody ($P<0.005$)

chain of the BW 5147 thymoma (*Imai* et al., 1986; *Lee* and *Davis*, 1988) confused the issue (*Kronenberg* et al., 1985; *Hedrick* et al 1985; *Möller*, 1988). This was despite evidence that a virus transformed Ts-1 specific for lysozyme and producing soluble TsF-1 possessed mRNA for both α and β chains (*De Santis* et al., 1985, 1987).

The current weight of evidence suggests that most and perhaps all TsF-3 and those TsF-1 which possess two chains are coded for by the α and β chains of the TCR (Table 3). What is the evidence? First, in the few cases studied, the antigen binding chain has α chain determinants, while the non antigen binding chain has β chain determinants. In some cases the β chain is coded for by the BW 5147 genes. However, this does not pose a conceptual problem as the non antigen binding chain of one hybridoma can complement the α chain of another hybridoma and indeed convey genetic restriction (*Fairchild* et al, 1990; *Perrin* et al., 1989b). In particular, the TCR and soluble TsF can be assembled with the α chain (which conveys antigen-specificity) from the Ts and the β chain from the BW 5147 thymoma line (*Kuchroo* et al., 1990). The recent development of BW 1100, which lacks the genes for the α and β chains, will allow investigators to study hybridoma TsF, which only has chains derived from T suppressor cell (*White* et al., 1989).

There is a further experimental point. A "good" hybridoma yields supernatant active at dilutions of $>10^4$ (unpublished observations). Hence, as emphasised by *Dorf*, a minority of cells may be responsible for producing TsF. For this reason reselection, by adherence to antigen or to anti-CD3, followed by cloning is an important preliminary to critical experiments (*Kuchroo* et al., 1988).

Evidence that the T cell uses the TCR in the activation that leads to liberation of TsF is provided by studies in which antibody against the β chain ($V_\beta 8$) (*Staerz* et al., 1985) is used to inhibit TsF production. Our recent studies, using inducible I-E restricted, TNP-specific hybridoma, illustrate this point. One chain bound to antigen, while the non antigen binding chain was absorbed by and could be eluted from monoclonal antibody to $V_\beta 8$ and I-J determinants. The same antibodies blocked the induction of TsF production when used to pretreat the hybridoma. See Table 4

(unpublished observations). [Note in passing that the antibody to I-J may block TsF production by combining with a molecule distinct from the TCR (*Nakayama* et al., 1989)]. It may be deduced that determinants on the TsF molecule also occur on the T cell and are involved in activation. The implication is that there is important similarity between the non antigen binding chain of the TsF and the β chain of the T cell receptor.

Dorf and his group pinpointed the role of CD3, by showing that hybridomas, reselected by panning for CD3 positivity, had increased TsF-3 production. Using these hybridomas, it was then possible to demonstrate the α chain of TCR on the surface of the hybridoma by immunoprecipitation (*Kuchroo*, 1988).

Similarly, *Weiner* and colleagues (1988) studied an azobenzenearsonate (ABA)-specific TsF1 which inhibited delayed hypersensitivity and antigen-induced production of IL-2. They established $CD3^+$ and $CD3^-$ lines and showed that many but not all $CD3^+$ lines were unstable over 6 weeks. Only the $CD3^+$ cell lines constitutively released TsF. The implication is that the cells which made TsF possessed CD3 and presumably the TCR/CD3 complex.

Some of the data on antigen-specific T helper factors also bear on the similarity between antigen-specific T cell factors and the T cell receptor. *Guy* and colleagues (1989) developed a cloned antigen-specific and MHC restricted Th2 cell line which secreted IL-4 when stimulated with antigen and an antigen-specific factor which augmented the IgG antibody response *in vitro*. The factor had $V_\beta 8$ determinants and biosynthetic labelling showed that the cell and the antigen-specific factor were heterodimeric 85 kDa molecules with components of 40-45 kDa.

Role of antigen binding chain of TsF in determining antigen-specificity and of non antigen binding chain with $V_\beta 8$ determinants in determining genetic restriction

Moorhead's group studied a DNP-specific TsF whose action was class I (K or D) restricted. The antigen binding chain possessed an epitope of the TCR α chain constant region, while the non antigen binding chain expressed an epitope of variable region of the β chain ($V_\beta 8$). Moreover activation of the hybridoma by antigen to produce TsF was blocked by antibody (F23.1) to $V_\beta 8$. Complementation studies, using chains from hybridomas with different class I genetic restrictions, showed that the restriction (K or D) was controlled by the non antigen binding chain. (This was measured by the ability to bind to DNP-immune lymph node cells with accessible K^k and D^k determinants.) The ability of the antigen binding chain to bind to antigen suggested that it conveyed antigenic specificity. However, not all combinations of antigen binding and non antigen binding chains complemented each other (*Fairchild* et al., 1990).

Some of the observations of antigen-specific T helper factor also suggest that the non antigen binding chain determines genetic restriction. Our group studied an antigen-specific T helper factor which augments the induction stage of the contact sensitivity reaction when used to coat the haptenised spleen cells used for immunisation. The ThF was a disulphide bonded heterodimer and absorption with monoclonal antibody showed I-A α and β chain determinants on the nonantigen binding chain. These determinants controlled the antigen-specificity of the factor as shown by studies using T helper factor from F1 mice (*Little* et al., 1987, 1988). Recently, *Dieli* (personal com-

munication) showed that the non antigen binding chain had $V_\beta 8$ determinants but it is not clear whether the I-A determinants were an integral part of the chain or whether the non antigen binding chain formed an non covalent complex with I-A.

Perrin et al. (1988, 1989b) studied schistosomal and PPD specific "conventional" TsF's, which they assayed by their ability to inhibit an *in vitro* model of granuloma formation. The TsF possessed $V_\beta 8$ determinants of the TCR. Complementation studies, using the antigen binding and non binding chains of schistosomal specific TsF of two different genetic specificities, confirmed that the non antigen binding chain conveyed the genetic restriction. Studies using antigen binding chains of schistosomal- and PPD-specificity showed that this chain conveyed antigen-specificity and complemented the non antigen binding chain from TsF of different specificity. This role of the β chain in determining MHC genetic restriction is in keeping with the finding of *Kappler* and colleagues (1987) that a certain V_β gene is strongly associated with reactivity to allogeneic I-E.

Does TsF always have a non antigen binding chain similar to the β chain of the TCR?

It is clear that some TsF-1 lack a separate non antigen binding chain. However, all relevant studies have shown that the antigen binding chain of TsF bears TCR α chain determinants. In contrast, three studies have raised doubts about the involvement of TCR β chain determinants or of the TCR β chain.

For instance, *Imai* and colleagues (1986) described KLH-specific hyridomas. cDNA studies showed an α chain derived from the suppressor cell, but the β chain was of the thymoma line (BW 5147) on Southern blot analysis. As cell surface labelling showed a disulphide bonded heterodimer, they concluded that the α chain might be coupled with a hitherto unknown chain. However, these findings would now be interpreted as indicating that functional molecules of TCR and of TsF could be assembled using the α chain of the Ts cell and the β chain of BW 5147.

Takata and colleagues (1990) studied a class I restricted clone which suppressed antibody production to ovalbumin *in vitro*. The clone was maintained in crude rat Con A supernatant and periodically stimulated with antigen. The TsF was weakly absorbed by antibody to the α chain but not to an antibody specific for the α/β chain or $V_\beta 8$. It is possible that the line only produced

Table 5: Classification of soluble antigen-specific T cell factors

I Antigen-specific T cell factors with negative effects
TsF-1 which only acts when given at the induction stage
TsF-2 (characteristically anti-idiotypic) which activates Ts-3
TsF-3 which only acts when given at the expression stage
TsF which modulates IgE response
II Antigen-specific T cell factors with positive effects
Antigen-specific T helper factor which augments induction of contact sensitivity*
Antigen-specific ThF which augments antibody production*
Antigen-specific ThF which augments tumour rejection*
Antigen-specific T cell factor which causes local oedema in the contact sensitivity reaction*

*It is not clear whether these belong to different classes.

the antigen binding chain of the TsF and that the non antigen binding chain was provided by the other cells in the culture or (in view of the weakness of the absorption by antibody) that technical factors were important.

Finally, *Zheng* and colleagues (1989) studied a peptide-specific Ts-1 hybridoma. Antisense oligodeoxynucleotide of part of the α chain blocked TsF production. However, antisense constructs to part of the β chain had no effect. It is possible that technical factors were important e.g. limited action of the antisense oligodeoxynucleotide, which depends upon the amount of mRNA, or provision of the non antigen binding chain by factors added during the assay.

In summary, most antigen-specific T suppressor factors behave as a soluble form of the TCR, and the apparent exceptions may have special, technical explanations. The liberation of antigen-specific factors similar but not identical to the TCR may be based on differential splicing, as mRNA alternative splicing with the addition of base pairs between Vβ and Cβ has been described (*Behlke* and *Loh*, 1988). However, a study of a cDNA library of the DNP-specific hybridoma showed no evidence of this (*Fairchild* et al., 1990). Alternatively the soluble antigen-specific product may be a post-translational modification or proteolysed form of the α/β TcR.

CLASSES OF SOLUBLE ANTIGEN-SPECIFIC T CELL FACTORS

The classes of immunoglobulins were originally suspected on the basis of different biological properties, such as the ability of some antibodies to fix complement or coat mast cells. It was then confirmed by raising class-specific antibody to epitopes on the constant region and finally explored at a DNA level. At present only the first two have been undertaken for antigen-specific T cell products. Table 5 gives a classification of the main soluble antigen-specific T cell factors.

Serological differences

Monoclonal antibodies exist which distinguish between TsF-1 and TsF-3 even when the antigen-specificities of the factors are identical, and some of these antibodies have been used in ELISA assays. The monoclonal mouse antibody B16G was raised against a TsF-1 which limits rejection of the tumour P815. It also absorbs NP-specific TsF-1, but not TsF-2 (idiotype specific) or NP-specific TsF-3 (*Steele* et al., 1987; *Gallina* et al., 1990). Monoclonal rat antibody 14-30 has similar properties (*Ferguson* and *Iverson*, 1986). *Sorensen* and *Pierce* (1985) also produced monoclonal rat antibodies against TsF-1. In contrast, another rat monoclonal antibody, 14-12, only reacts with TsF-3 (*Ferguson* et al. 1985). In the few cases studied, these determinants are on the non antigen binding chain of the TsF, the same chain as is responsible for the genetic restriction in the action of TsF (*Perrin* et al., 1989b).

Other serological determinants may be indicators of MHC restriction and not class-specific. For instance, many of the TsF-1 and TsF-3 bear I-J determinants. This has been regarded provisionally as a marker of the TCR with I-E genetic restriction, but see *Nakayama* et al. (1989). In keeping with this, TsF-3 of DNP specificity is class I restricted and is I-J negative. However, no correlative study exists using hybridomas against the same determinant with different genetic restrictions.

T helper factor of picryl specificity carries α and β chain I-A determinants

on its non antigen binding chain (*Little* et al., 1985, 1987, 1988). As preliminary data indicate that this also carries $V_\beta 8$ determinants, it is possible that the I-A is bound non covalently by the non antigen binding chain and is not an integral part of the molecule (*Dieli*, personal communication). The I-A serology probably defines a distinctive class of factor, but it is possible that it simply reflects the genetic restriction in the action of the factor.

Some, but not all, TsF carry $V_\beta 8$ determinants. This is also shown by certain TsF-1, TsF-3 and antigen-specific T helper factor (see Table 3). This is clearly an indication of Vβ gene usage and not a class marker.

T suppressor inducer factor (TsF-1)

The Ts-1 is now usually called T suppressor inducer cell and the factor TsF-1 or TsiF. The defining feature of TsF-1 is that it acts only when given at the induction stage of the immune response and not at the effector stage (*Jendrisak* et al., 1986; *Kuchroo* et al., 1990; *Gallina* et al., 1990). TsF-1 form a distinct class which differ biologically in their time and mode of action, serologically and sometimes structurally from TsF-3. The key difference is their time of action. NP- and TMA-specific TsF-1 give rise to an Ts2 which is idiotype specific and makes a corresponding TsF-2. This in turn activates antigen-specific Ts-3 to produce TsF-3. Some of the TsF-1 are disulphide bonded heterodimers like TsF-3, while others are single chain molecules with antigen binding capacity and I-J determinants on a single chain (*Gallina* et al., 1990; *Jendrisak* et al., 1986). They are sometimes IgH restricted in their action. It presumably reflects their role in inducing anti-idiotypic Ts-2 and is related to the effect of IgH allotypes on the idiotopes of antibodies and hence on the idiotopes of the TCR.

The relation of the Ts-1 to helper cells was investigated in a recent study. Two of three Ts-1 made IL-2 or IL-2 and IL-4. Their distinctive feature was the production of antigen-specific TsF. It is unclear whether this is a potential of many helper cells when stimulated appropriately or represents a distinct subset of cells (*Kuchroo* et al., 1990).

T suppressor factor 3 (TsF-3)

There are several lines of evidence suggesting that TNP-, oxazolone-, NP- and cryptococcal-specific TsF-3 belong to the same class. The serological evidence has been summarised above. In addition, all these TsF-3 have a mode of action through the macrophage and the T acceptor cell (unpublished observations; *Blackstock* et al, 1991b). Additional evidence comes from studies on the activation of the T acceptor cell (Table 2). This cell, when coated with TsF, is activated to release its antigen non specific inhibitory mediator (nsTsF-1) by bivalent low molecular weight haptene. This activation, *inter alia*, requires the haptene to crosslink separate molecules of TsF on the surface of the T acceptor cell. Thus lysine with two TNP groups attached causes activation, while lysine with only one TNP group is inactive. "Mixed haptene", i.e. lysine with one TNP and one oxazolone group attached, is also inactive. However when the T acceptor cells are coated with a mixture of TNP- and oxazolone-specific TsF, the mixed haptene causes activation. In other words, crosslinking of TNP-specific to oxazolone-specific TsF on the surface of the T acceptor cell leads to activation. Similar studies show that monoclonal NP- and conventional oxazolone-specific TsF cause activation when crosslinked by appropriate mixed haptene (*Asherson* et al., 1984b,c).

Multiple effects of TsF-3:

These are listed in Table 1. It is likely that all the effects ascribed to these TsF-3 are due to the same molecular species. In particular, the same cryptococcal-specific TsF inhibits phagocytosis by macrophages and coats them for the production of macrophage suppressor factor, as judged by their common monoclonal origin, similar molecular weight (ca. 70-80 kDa), and similar structure (antigen binding site and I-J determinants on the same molecule). In the case of monoclonal TNP-, cryptococcal- and NP-specific TsF, the same hybridoma supernatant acts through the macrophage and the T acceptor cell. Moreover, the same NP-specific hybridoma supernatant (and presumably the same TsF) affects both antibody production and the effector stage of delayed hypersensitivity (*Hausmann* et al, 1985)

Antigen-specific TsF which depresses IgE response:

The monoclonal ovalbumin-specific TsF described by *Iwata* et al (1989a,b, 1990) probably belongs to a separate class. Its distinctive feature is modulation of the IgE antibody response and its glycosylation inhibiting activity (GIF). The unstimulated hybridomas liberated a glycosylation inhibition factor, which lacks an antigen combining site. However activation by antigen leads to the release of an ovalbumin specific molecule which also has glycosylation inhibition factor activity. This antigen-specific factor has α chain determinants of the TCR and was associated with the non specific GIF chain which bears I-J determinants.

Antigen-specific T helper factor (ThF)

The ThF, which augments the induction of contact sensitivity, is I-A restricted in its action and is an afferent acting factor. (*Colizzi* et al, 1985; *Little* et al., 1987, 1988). In contrast the T cell factor which causes local oedema in the contact sensitivity reaction is genetically unrestricted and may be regarded as an efferent acting factor (*Van Loveran* et al, 1984, 1986). However, there is no formal study indicating whether these two factors are different.

In summary, there is good evidence that TsF-1 and TsF-3 belong to different families on serological and biological grounds. The TsF-3 may divide further as some are selective for the IgE response. The antigen-specific helper factors also belong to a different family, but for the moment the evidence is mainly biological. Finally the TsF-3 have several different actions which are due in all probability to the same molecule.

MODES OF ACTION OF TSF-3

Introduction

The availability of hybridomas making TsF allows structural and molecular biological studies and renews interest in the mode of action of TsF. In fact, studies with "conventional" TNP- and oxazolone-specific TsF showed that it had two distinct modes of action: one through the macrophage and the other through the T acceptor cell. See Table 2. In both cases the TsF behaves like a mobile receptor and coats these cells. This mode of action is formally analogous to that of IgE. This is a class of antibody which acts as a mobile receptor and coats the mast cell. The mast cell then releases histamine and other mediators when exposed to antigen. Both systems show the need for crosslinking of the IgE or TsF on the surface of the

cell. However, with TsF, there is an additional need for genetic matching between the TsF and the haptene on the surface of the antigen presenting population.

Biological role:

There are several reasons why evolutionary pressure may have driven the selection of these complexities. The macrophage and the T acceptor cell probably provide amplification so that a limited amount of antigen-specific TsF has a greater effect. Moreover, the production of antigen non specific inhibitory mediators allows antigen-specific TsF to limit the inflammation to the other antigens liberated by an invading micro-organism or parasite. Finally, the complexity of the system provides multiple levels of control by antigen and presumably cytokines so that the inhibitory response is under tight control. An additional feature may be that the genetic restriction in the production and action of TsF causes a variation of the immune response between related animals and hence reduces the likelihood that infection will kill many members of a species. Similar considerations are presumably responsible for the complexity of the complement system.

Inhibition of contact sensitivity skin reaction in immune mice

Dorf's group was the first to describe the inhibition of the delayed sensitivity skin reaction by injecting monoclonal Ts-3 into immunised mice. The assay is sensitive and in our hands cloned TNP-specific hybridoma supernatant (after stimulation with antigen) causes 50% inhibition of contact sensitivity at a dilution of 1/1,000 , when injected shortly before challenge (unpublished observations). The TsF inhibits the early, 2 hour phase of the contact sensitivity reaction. On current views, this early phase is due to an antigen-specific T cell factor which coats mast cells and perhaps other cells. These cells, when exposed to antigen, liberate serotonin and perhaps other mediators (*van Loveren* et al., 1984; *Kops* et al., 1984).

TsF also limits the 24 and 48 hour skin reaction and diminishes the 48 hour reaction even when given at 24 hours - a time at which the reaction is well developed. This indicates that the TsF (directly or indirectly) limits the cytokine production needed for the persistence of the reaction perhaps by limiting the influx of cells. Paradoxically TsF has no effect on local passive transfer. This suggests that TsF is unable to act, if a large number of antigen-specific and other cells are injected into the skin test site. Perhaps its key mode of action is in preventing the entry of cells. Unfortunately, it is difficult to understand the details of the action of TsF in inhibiting contact sensitivity, because of our ignorance of the basis of the inflammation seen in contact sensitivity [see *Piguet* et al. (1991) on the role of TNF-α].

Inhibition of the passive transfer of contact sensitivity

The inhibition of contact sensitivity in immunised mice provides a convenient assay for monoclonal TsF-3. However, a more analytical approach is to study the inhibition of the passive transfer of contact sensitivity. In this system, cells from immune mice are incubated *in vitro* with the factor under study. They are then injected into naive recipients and contact sensitivity assessed. This system has been used to show that TsF-3 has no direct effect on the passive transfer of contact sensitivity, but two indirect effects: one via the macrophage and the other via the T acceptor cell.

Action of TsF through the macrophage:

In its action through the macrophage, TsF-3 coats this cell (Thy-1 negative, adherent peritoneal exudate cells). Exposure to antigen then leads to the re-

lease of an antigen non specific inhibitory mediator which is called macrophage suppressor factor (MSF). It is detected by its ability to inhibit the passive transfer of contact sensitivity (*Ptak* et al., 1978). The activation of the macrophage to release TsF is antigen-specific, and the haptene needs to be on cells which match the TsF at I-J (*Dieli* et al., 1991). This was assessed using B10.A(3R) and B10.A(5R) mice and it is possible that the TsF uses I-E as the restriction element and that the I-J gene modulates this interaction, by influencing the amount of I-E or the cell type which expresses it. MSF has a molecular weight around 10-20 kDa on gel filtration. Its production is not affected by indomethacin which suggests that MSF is not a carrier protein for prostaglandin. It differs from the non specific inhibitory mediator produced by the T acceptor cell in lacking I-J determinants (*Blackstock* et al., 1991b).

The original findings, using "conventional" TNP- and oxazolone-specific TsF-3, have now been extended to monoclonal TNP- and cryptococcal-specific TsF using TNP-modified spleen cells or soluble cryptococcal polysaccharide and spleen cells as a source of antigen presenting cells (unpublished observations; *Blackstock* et al., 1991b). It is interesting that inhibition of antibody production by TsF-3 may be mediated through macrophages but detailed analysis is not available (*Hausman* et al., 1985).

Action of TsF through the T acceptor cell:

Briefly, the T acceptor cell arises following immunisation with contact sensitiser and is not found in unimmunised mice (Table 2). Its antigen-specificity is unimportant in its interaction with TsF. It binds TsF to its surface (*Zembala* et al, 1982a,b,c). The T acceptor cell coated with TsF is activated by antigen and liberates an antigen non specific mediator called the first non specific T suppressor factor or nsTsF-1. Like MSF, it is detected by its ability to inhibit the passive transfer of contact sensitivity. The activation of the T acceptor cell to release its antigen non specific inhibitory mediator, nsTsF-1, is antigen-specific, and the haptene must correspond to that of the TsF and be on cells which match the TsF at I-J. There is no requirement for matching to the antigen used to generate the T acceptor cell or to its genotype. NsTsF-1 has a molecular weight around 50 kDa on gel filtration. It bears I-J determinants in contrast to macrophage suppressor factor (*Zembala* et al., 1982; *Asherson* et al., 1984; *Blackstock* et al., 1991b). In this, it resembles the antigen non specific inhibitor liberated by staphylococcal enterotoxin B (*Taub* et al., 1989).

The finding that "conventional" TNP- and oxazolone- and monoclonal NP-specific TsF act through the T acceptor cell has now been extended to monoclonal TNP- and cryptococcal-specific TsF using TNP-modified spleen cells and soluble cryptococcal polysaccharide (unpublished observations; *Blackstock* et al., 1991a).

It was originally thought that the non specific mediator, nsTsF-1, acted directly on the cell that transfers contact sensitivity. However, experiment showed that it was unable to affect passive transfer by a population depleted of I-A bearing T cells. This suggested that nsTsF-1 inhibited contact sensitivity by a mechanism involving I-A^+ T cells. Further analysis showed that nsTsF-1 is a "permissive factor" which allows a specifically immunised cell to release a second antigen non specific inhibitory mediator, nsTsF-2, when activated by specific antigen (*Zembala* et al., 1986).

The nsTsF-2 bears I-A determinants and is I-A restricted in its production and action. Further experiments indicate that the genetic restriction in the action

of nsTsF-2 is related to the haplotype of the I-A determinants that it carries (*Asherson* et al., 1989). This suggests that there is an interaction between an I-A determinant on the factor and a cell which has a receptor for I-A which may be the TCR.

TsF inhibits phagocytosis by a subset of macrophages

In its action via the macrophage and the T acceptor cell, TsF acts indirectly and antigen is required to release the inhibitory mediators. However, it also has a direct mode of action on a subset of macrophages, which does not appear to require antigen. Moreover there is no evidence that the factor coats the macrophages which then liberate a factor into the medium which suppresses phagocytosis.

Blackstock and colleagues (1989a,b; 1991a,b) showed that conventional and monoclonal cryptococcal-specific TsF inhibited phagocytosis by a subset of macrophages. This subset was I-A^+ and comprised 8-27% of macrophages in the peritoneal exudate. This inhibition of phagocytosis appears to be a general property of antigen-specific TsF-3 which depress contact sensitivity and is shown by TNP- and oxazolone-specific TsF. However there is an important difference between the inhibition of phagocytosis, and the coating of the macrophage which then releases MSF when exposed to antigen. The inhibition of phagocytosis does not require the addition of antigen. Thus TNP-specific conventional TsF still inhibits phagocytosis after careful purification to remove antigen. Similarly, monoclonal cryptococcal-specific TsF inhibits phagocytosis after purification on antigen and subsequent elution. In contrast, antigen is required in order for the macrophage and T acceptor cell coated with TsF-3 to release antigen non specific inhibitory mediators.

Inhibition of the granuloma formation

The pathology in schistosomiasis is mainly due to the granuloma reaction and fibrosis around the eggs. *Perrin* and colleagues (1989b) studied conventional schistosomal-specific TsF-3. The Ts were activated by antigen *in vitro* to release TsF, and the TsF diminished granuloma formation *in vivo* and in an *in vitro* model. IL-2 blocked the production of TsF both *in vivo* and *in vitro*. However it had no effect on the action of TsF-3. The target of the TsF *in vitro* was the immune population used to generate the granuloma; incubating the antigen coated beads (around which the granuloma formed) in TsF had no effect.

Inhibition of antibody production

Monoclonal NP-specific TsF-3 inhibits antibody production. It may act late in the response, by inhibiting the production or response to key lymphokines, as TsF given 4 hours before measuring plaques reduces the response. The TsF acts in the first instance on an adherent cell. However it is not known whether this cell the acts directly on a B cell or a T cell, or via other cells (*Hausman* et al., 1985).

CONCLUDING REMARKS

Biological significance

The biological role of TsF-3 may be to alter the balance between immunopathology and handling of the microorganism. It is relevant that the action of TsF-3 is tightly controlled by antigen and MHC and that the final mediators are antigen non specific (*Zembala* et al.,

1986) Hence, TsF specific for a particular antigen or epitope of the pathogen will limit inflammation caused by other antigens.

I-E is sometimes involved in activating Ts-3, while I-A is involved in the production and action of the second antigen non specific inhibitory mediator (nsTsF-2). Hence alteration of the I-A:I-E ratio may affect the magnitude of the negative control in a complex fashion. In fact, there is polymorphism in the control regions for the human equivalent of I-A and I-E (*Andersen* et al., 1991) and differential expression of these may influence the balance between immunopathology and the handling of the micro-organism.

Ts-1 and Ts-3 cells bind to antigen, and of TsF-3 coats macrophages and T acceptor cells. This may have implications for antigen presentation. *Lanzavecchia* et al. (1990) has shown that B cells present antigen to T cells. They bind intact antigen by their Ig receptor, internalise and process it, and then present it to T cells. The T cells are activated and *inter alia* liberate lymphokines which provide T cell help for antibody production.

By analogy Ts cells, and cells (macrophages and T acceptor) coated with antigen-specific TsF may bind antigen (*Zembala* et al., 1982c) and then process it and present it to T cells. In the case of B cell presentation, the combination of antibody with antigen alters which epitopes are presented (due to selective protection by the antibody, during intravacuolar proteolysis) and serves to change the epitope to which the animal responds. This may also be true for this T cell presentation. This hypothetical mechanism involving Ts and/or TsF would have the effect of turning off the inflammatory response to one epitope, while changing the dominant epitope presented to T cells. This would provide a mechanism for directing the immune response towards relevant epitopes for handling infection.

Unanswered biological problems

I-E genetic restriction of T suppressor cells:

Oliveira and *Mitchison* (1988) postulated that T suppressor cells are characteristically, but not always I-E restricted. This view, if correct, raises the question of the mechanistic basis of the association. For instance, do suppressor cells use the TcR to selectively recognise I-E and is this linked to a particular constant region associated with suppressor function? Alternatively is there a molecule, which resembles CD4 in binding class II but which is selective for I-E?

It may be relevant that the certain superantigens - bacterial and viral products which activate the TCR of certain V_β genes - are often I-E restricted (*Marrack* and *Kappler*, 1990). A good example is Staphylococcal enterotoxin-B, which is a common cause of food poisoning. This activates T cells to liberate an $I\text{-}J^+$ antigen non specific inhibitory mediator from T cells (Taub et al., 1988). Has evolutionary accommodation between host and pathogen led to a reduction of immunopathology through the selective use of I-E by superantigens and hence to a selective activation of suppressor cells?

The continuing puzzle of I-J:

The basis of I-J genetic restriction in the production and action of certain Ts-F's and the epitopes recognised by sera raised across I-J differences are a continuing puzzle (*Murphy*, 1987).

The common feature between the molecules recognised by anti-I-J monoclonal antibodies is unclear. On the one hand, they react with the non antigen binding chain of TsF. Their ability to block the production of TsF by a hybridoma (unpublished observations) might suggest, but does not prove, that

they react with the T cell receptor. In some experiments the I-J phenotype depended on the I-E environment in which the T cells developed, which led to the suggestion that I-J is an idiotype determinant on the TCR influenced by the MHC specificity of the receptor. In particular, the introduction of an $I\text{-}E^k$ transgene into an $I\text{-}J^b$ mouse, changes the phenotype to $I\text{-}J^k$ (*Flood* et al., 1986). However, this evidence is indirect and *Nakayama* and colleagues (1989) have indicated that the I-J determinant may be on a molecule distinct from the TCR.

On the other hand, I-J monoclonal antibody reacts with antigen non specific mediators including the first non specific inhibitory mediator (nsTsF-1) of contact sensitivity, an inhibitor of antibody production induced by superantigen (*Taub* et al., 1989) and glycosylation inhibition factor - a molecule which inhibits phospholipase A2 activity (*Jardieu* et al., 1986). These molecules may be truncated or modified forms of the β chain of the TCR.

Similarly, the nature of I-J genetic restriction is unclear. There is a technical point. The difference between B10.A(3R) and B10.A(5R) used to define I-J genetic restrictions can be interpreted as showing that the I-J direct gene product is the restricting element or I-E is the restricting element which the I-J gene modifies. For instance, I-J may lead to pre- or post-translational modification of I-E or alter its amount or location. Alternatively the I-J gene may affect the T cell receptor and this might select between different post-translational I-E variants. In any event the description of an adaptive molecule which may be distinct from the TCR is clearly important. *Nakayama* and colleagues (1989) provide a helpful summary.

Burnet drew attention to the tension between the biological and the biochemical approaches to immunology and saw the biochemical approach as negative. In fact, both are needed. In the study of antigen-specific T cell factors, the biological approach was needed to discover factors and their mode of action, while the molecular biological approach is critical to clarifying the precise relation between the TCR and these soluble products and defining classes of antigen-specific T cell factors. It should soon be possible to outline the amino acid sequences on T suppressor factors which renders them cytophilic for other cells and enables them to act as mobile antigen-specific receptors.

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NOTE ADDED IN PRESS

Dorf and colleagues (1992) have summarised recent work on T suppressor factor and I-J, while *Asherson* and colleagues (1994) have offered a possible explanation of the I-J phenomenon in terms of an endogenous superantigen.

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A REASSESSMENT OF THE T-DEPENDENCE/INDEPENDENCE OF POLYSACCHARIDE AND PROTEIN ANTIGENS

GAYLE DELMONTE WETZEL

Cellular and Molecular Biology Research, Cutter Biological, Miles Inc., Berkeley, CA, USA

SUMMARY

This article reviews the literature concerning thymus independent antigens as well as presenting experimental results concerning immune responses to these antigens and their analogues. The review focuses on the nature of thymus independent antigens, the sites or tissues responsible for responses to these antigens, subpopulations of B cells responding to these and other antigens, memory to these antigens and the roles of T cell and macrophage derived factors in responses to these antigens. In the literature, there is evidence that T cells, macrophages and products from these cell types can regulate responses to so-called thymus independent antigens. It is even possible that such factors may be required for responses to these antigens.

Experiments with murine peritoneal and splenic B cells revealed that interleukin-5 can increase antibody responses to thymus independent type 1 antigens. Proliferation by murine splenic B cells stimulated with the thymus independent type 2 antigen analogue system, anti-IgD-dextran was increased by interleukin-5 as well as by IL-1. Proliferation induced by a different thymus independent type 2 analogue system using anti-IgM + dextran sulphate was increased by interleukins 4 and 5. Proliferation by mouse splenic B cells was also increased by interleukins 4 and 5 when the thymus independent antigen analogue system anti-IgM + lipopolysaccharide was used. Hence, different interleukins appear to be important for responses to different types of thymus independent antigens.

Human peripheral blood B lymphocytes proliferated in response to two thymus independent type 2 analogues, anti-IgD-dextran and anti-IgM-dextran. Proliferation by these cells was increased by interleukin-2 but not by the other interleukins tested. No evidence of B cell responses was obtained from cells prepared from peritoneal dialysis fluids of patients undergoing continuous ambulatory peritoneal dialysis. This was due to a lack of B cells in these fluids and not to immunoincompetence since a vigorous T cell response to a bacterial superantigen was observed by cells from these fluids.

Hence, various strategies for antibacterial immunity have been developed in different tissues of different species and interleukins can influence the antibody responses to some of these bacterial components.

INTRODUCTION

Protection against pathogenic bacteria is certainly one of the most important functions of an immune system and therefore it is not surprising that many

anti-bacterial strategies have been developed. Antibodies to bacteria are one of the immune systems' central defence mechanisms and they are effective in several ways including opsonisation and complement mediated lysis. Many germline antibody genes encode specificities directed against polysaccharide components among which are those found on the outer surfaces and capsules of bacteria. Interestingly, and probably not fortuitously, many of these outer components of bacteria, the polysaccharides, lipoproteins and lipopolysaccharides, can activate B lymphocytes in the apparent absence of T cells. Hence these types of antigens have been called thymus independent (TI).

TI antigens have several interesting features as do the responses to these antigens. The antigens themselves are generally polymeric in nature and some act as polyclonal activators at high concentrations. Immunological responses to these TI antigens differ from those to thymus dependent antigens in several important respects. One difference lies in the relative amounts of isotypes and subclasses of immunoglobulins produced. The secondary, or memory, responses are also somewhat different. It is now becoming clear that certain specific sites and cell types are very important in responses to TI antigens. There is also some controversy as to whether the B cells which respond to TI antigens are from the same population as those which respond to thymus dependent antigens. Finally, although responses to some of these antigens are termed thymus independent, recent findings indicate a role for T cells as well as macrophages in both generating and regulating antibody responses to these agents.

This report will deal with these issues first by reviewing some of what is available in the literature. We then present experimental findings of our own which bear on some of these issues and extend our understanding of some aspects of responses to TI antigens by peritoneal lymphoid elements and other peripheral tissues.

The nature of TI antigens and responses to them

The observation that athymic nude mice could mount an antibody response to certain antigens probably gave rise to the name thymus independent. This observation was clarified by demonstrations that purified B cells from mice (*Snow* et al., 1983), humans (*Golding* et al., 1981) and other species can make antibodies *in vitro* to so-called TI antigens. Responses to these antigens are predominantly of the IgM isotype, although IgG subclasses (*Hurme*, 1976; *Klaus* et al., 1976; *Slack* et al., 1980) as well as IgA (*Taubman* et al., 1986) can sometimes be seen. The IgM response to these antigens can be long-lived, presumably due to antigen persistence or to polyclonal activation or both.

CBA/N mice, and some other *xid* (X chromosome linked immunodeficient) mice, can respond to some TI antigens and not to others (*Mosier* et al., 1976; *Cohen* et al., 1976). This led to a classification of TI antigens into those to which CBA/N mice could respond, called TI-1 antigens, and those to which CBA/N mice could not respond, dubbed TI-2 antigens. Using the responsiveness of CBA/N mice as a criterion for TI-1 or TI-2 classification is perhaps not the best, however, since *xid* B cells can not only bind and present what have been called TI-2 antigens (*Kirkland* et al., 1980) but they can also respond to TI-2 antigens if IL-2 (*Delovitch* et al., 1983), a helper T cell population (*Braley-Mullen*, 1982; *Lindsten and Andersson*, 1979; *Klaus* et al., 1976), or a TI-1 antigen (*Couderc* et al., 1984) is concomitantly present.

Several other parameters distinguish the responses to different TI antigens, and they seem to follow a pattern. Besides the physical differences between so-called TI-1 and TI-2 antigens, considered below, the IgG subclasses evoked by these classes of antigens tend to be different. Thus while IgG_1 predominates over IgG_2 and IgG_3 in thymus dependent responses, little thymus independent IgG_1 is seen. Responses to TI-1 antigens tend to evoke comparable levels of IgG_2 and IgG_3 whereas TI-1 antigens evoke far more IgG_3 than IgG_2 (*Slack* et al., 1980).

Some TI-2 antigens tend to persist for long periods *in vivo* since macrophages apparently are ineffective at degrading them. Hence, responses to TI-2 antigens can persist for extended periods.

Another difference between responses to TI-1 and TI-2 antigens is that responses to TI-2 antigens tend to arise later in ontogeny than those to TI-1 antigens. This is true for both mice and humans (*McKearn* and *Quintans*, 1979; *Golding* and *Rittenberg*, 1984; *van Rees* et al., 1987).

Both TI-1 and TI-2 antigens cause B cell membrane depolarisation but only TI-1 antigens lead to the G_0 to G_1 transition (*Monroe* and *Cambier*, 1988). Moreover, TI-1 antigens can initiate proliferation in antigen specific B cells while TI-2 antigens appear to require interleukins, as well, for induction of proliferation (*Stein* et al., 1986).

Many of the differences in responses to TI-1 and TI-2 antigens probably result from differences in their structures. As previously stated, TI antigens tend to be polymeric. Some examples are polysaccharides like levan, dextran or ficoll and polymerised flagellin. These are considered TI-2 type antigens. Lipopolysaccharides and lipoproteins are other TI antigen examples, although these are considered TI-1 type. A controversy early in the study of TI responses centred around a putative requirement for a mitogenic carrier moiety. Early studies suggested that both TI-1 and TI-2 antigens induced B cell proliferation and the carriers needed only to be polymeric, not mitogenic or immunogenic (*Klaus* et al., 1975). The epitope density was shown to affect the immunogenicity of certain TI-2 antigens (*Desaymard* and *Howard*, 1975a; 1975b). In addition, the chain length has been implicated in the immunogenicity of TI antigens (*Peacock* et al., 1983). Several studies state that a mitogenic principle is required for responses to TI-1 antigens (*Coutinho* and *Gronowicz*, 1975; *Hare* et al., 1980; *Alarcon-Riquelme* and *Moller*, 1990). There is some confusion in the literature concerning the relative importance of each of these structural parameters contributing to the thymic independence of these antigens.

A clarifying series of experiments showed that if a polyclonal activator was present on an antigen, then a polymeric structure was not required for thymic independence of the antibody response (*Ishizaka* et al., 1979). Along these lines, TNP-liposomes have been classified as TI-2 antigens whereas insertion of lipid A (a polyclonal activator) into TNP-liposomes converts them to TI-1 type antigens (*Tadakuma* et al., 1982). This is probably a useful unifying concept: polyclonally activating moieties can render oligovalent antigens thymic independent; otherwise extensive B cell surface Ig crosslinking provided by high epitope density on repeating structures appears necessary to render responses to antigens independent of certain T cell requirements. The absence of a polyclonal activator on (TI-2) antigens probably makes them less immunogenic regardless of their polymeric nature and the extent of epitope density. This, in turn, probably explains why

responses to TI-2 antigens are more difficult to elicit and seem to have more requirements than TI-1 antigen responses.

Sites of Responses to TI Antigens

Antibody responses to different TI antigens *in vivo* reveal some differences in their induction requirements as well as differences in the characteristics of the response patterns. The locations of responses to different antigens can be different as can the cells required for, or contributing to, these antibody responses.

The spleen is a very important site of antibody formation especially in response to TI antigens. Splenectomy in rats (*Gray* et al., 1985; *Amlot* et al., 1985) and humans (*Amlot* and *Hayes*, 1985) abolishes primary *in vivo* responses to TI-2 antigens confirming that the spleen, almost exclusively, accounts for TI-2 antibody responses (*Veerman* and *Vries*, 1976; *van Rees* et al., 1987). Antibody Forming Cells (AFC's) elicited by both thymus dependent (TD) and TI antigens are first seen in the outer part of the peri-arteriolar lymphoid sheath (*Veerman* and *Vries*, 1976; *Eikelenboom* et al., 1982). Both types of antigen cause AFC localisation in the marginal zone (MZ) while there is some subsequent migration to the follicle (*Veerman* and *Vries*, 1976). Interestingly, spleens respond faster to TI antigens although most antigens must first transit through lymph nodes (*Delemarre* et al., 1989). In fact, two days after immunisation with a TI-2 antigen, AFC's can be seen in the spleen (*Claassen* et al., 1986).

Some TI-2 antigens are taken up exclusively by splenic marginal zone macrophages (*Chao* and *MacPherson*, 1990, *Humphrey* and *Grennan*, 1981). This would appear to account for the importance of the splenic marginal zone in TI-2 antigen responses. Others have shown, however, that MZ macrophages and AFC's can distribute differentially suggesting that MZ macrophages are not absolutely required for TI-2 responses. Although the requirement for MZ macrophages may not be absolute, recent reports indicated that the MZ is essential for TI-2 responses (*Claassen* et al., 1989). These observations are consistent with the report that recirculating B cells go to the splenic MZ to make TI-2 responses (*Lane* et al., 1986) whereas some other TI antigens can generate AFC's in the bone marrow (*Koch* et al., 1982) or some lymph nodes (*Goud* et al., 1990) as well as the spleen.

These and several other pieces of evidence support the concept that macrophages are required for TI-2 antigen responses. *In vivo* depletion of macrophages abolishes TI-2 antibody responses (*Delemarre* et al., 1990). The beige mouse, in addition to other immune defects, has a macrophage defect as well as impaired TI-2 responses (*Pflumio* et al., 1990). IL-1, a macrophage product, is reported to be absolutely required for TI-2 responses (*Sinha* et al., 1987). Furthermore, *in vitro* proliferation induced by fluoresceinated ficoll conjugates has been reported to require macrophages (*Pillai* and *Scott*, 1981). Marginal zone macrophages have been described as exclusively responsible for TI-2 antigen uptake (*Chao* and *MacPherson*, 1990). Together, these observations suggest that macrophages are required for TI-2 responses and a population of marginal zone macrophages alone may be competent to provide this helper function.

Subpopulations of B Cells Responding to TD, TI-1 and TI-2 Antigens

The subject of B cell subpopulations is a controversial one and this contro-

versy includes the possibility of different subpopulations responding to different antigenic forms. One early datum contributing to the idea of different subpopulations for different antigens concerns the *xid* B cell defect. Since *xid* B cells respond to TD and TI-1 antigens, it appeared that a TI-2 antigen responsive population bearing the lyb5 surface antigen was missing (*Mosier* et al., 1976, *Cohen* et al., 1976). The previously mentioned reports that *xid* B cells could respond to TI-2 antigens when a T helper cell (*Braley-Mullen*, 1982, *Lindsten* and *Andersson*, 1979, *Klaus* et al., 1976), IL-2 (*Delovitch* et al., 1983) or a TI-1 antigen (*Couderc* et al., 1984) was present confuse the issue of differentially responsive B cells subpopulations. Furthermore, neonatal CBA/N B cells respond to TI-2 antigens in the presence of T helper cells exactly as do normal neonatal CBA/J B cells in the presence of T helper cells do (*Lindsten* et al., 1979). Hence, the defect in these B cells has been described as one of development, rather than as one of a missing population. Moreover, CBA/N B cells can bind and present TI-2 antigens (*Kirkland* et al., 1980). Therefore, it is unclear how the *xid* B cell defect defines separate subpopulations of TI responsive B cells.

One report showed that anti-Lyb7 blocked TI-2 responses and not those to TI-1 antigens (*Subbarao* et al., 1979). Similarly, anti-IgM or anti-IgD will both block TD responses but anti-IgM alone blocks TI responses (*Cambier* et al., 1978). Others have shown that TI-2 responsive B cells are more radiosensitive than TI-1 responsive B cells (*Lee* and *Woodland*, 1985). These differences may simply reflect differing triggering requirements for TI-1 antigens compared to TI-2 antigens rather than the existence of separately responsive subpopulations. Indeed, by elimination experiments, it has been found that the populations responding to TD and TI antigens overlap appreciably (*Hurwitz* et al., 1982). This is probably the strongest finding and before one accepts the existence of separately responsive populations more support is necessary.

Some studies have shown that TI-2 responsive B cells are long-lived and migrate into the bone marrow (*Koch* et al., 1982). More recent studies using adoptive transfers have shown that B cells responding to TI-2 antigens are long-lived whereas TD antigen responsive B cells are both long-lived and short-lived (*Udhayakumar* et al., 1988).

It has also been shown that once B cells have been primed by a TD antigen, they can be activated by either TD or TI forms of antigen (*Rennick* et al., 1983). Furthermore, one report states that memory to both TD and TI antigens resides in the surface Complement Receptor (presumably the CR2) negative B cell subpopulation (*Lindsten* et al., 1985). Another group, however, opposes this view claiming that primed B cells fall into two distinct populations responding differentially to either TI or TD antigens (*Rittenberg* and *Tittle*, 1978).

It appears that there is no consensus yet as to whether truly distinct B cell populations responding in a primary or secondary response to TD, TI-1 or TI-2 antigens exist. Until some clarifying experiments unite all these data, caution in accepting separately responding subpopulations is probably well advised.

Generation of Memory by TI-Antigens

Although a classical anamnestic response is not usually elicited by a secondary challenge with TI antigens, there is evidence that a secondary response does occur. Some have reported a secondary response to TI-2 antigens (*Hurme*, 1976) and shown TI antigen-mediated induction of T helper cells

(*Bretscher*, 1984). Indeed, immunisation with a TI antigen yields protection against a fatal secondary challenge with *Trichinella spiralis* (*Lim* and *Choy*, 1990). Others, however, failed to observe a memory response and showed that the failure was due to suppressive anti-carrier (*Fernandez* and *Moller*, 1978) or anti-hapten (*Brodeur* and *Wortis*, 1980) antibodies. Furthermore, it has been reported that TI-2 antigens may induce suppressor T cells under some conditions (*Fraser* and *Braley-Mullen*, 1981). Indeed, nude mice, which should lack T suppressor cells, can give rise to secondary TI-2 antibody responses (*Schott* and *Merchant*, 1979).

A major factor contributing to these apparently contradictory findings concerning the induction of TI memory may be genetic. There are several reports of strain differences in the ability of TI antigens to generate memory and a secondary response (*Fernandez* and *Moller*, 1979, *Motta* and *Truffa-Bachi*, 1980, *Motta* et al., 1981, *Colle* et al., 1983, *Shidani* et al., 1983). The genetic basis for this difference is poorly understood at present, but it may relate to some triggering mechanisms. Two groups suggest that both TI-1 and TI-2 antigens can generate memory B cells but only TI-1 antigens can activate these memory cells (*Colle* et al., 1983b, *Truffa-Bachi* et al., 1983). Others claim that both types of antigen can generate memory but that lipopolysaccharide (LPS) is concomitantly required for TI-2 antigen memory generation (*Zhang* et al., 1988).

There is also some controversy concerning the cells responsible for these anamnestic responses. One group identified surface Complement Receptor-negative B cells as the carriers of memory for both TD and TI antigens (*Lindsten* et al., 1985). However, others report that the precursors for a TI-2 response are long-lived (*Udhayakumar* et al., 1988) whereas memory to TI-1 antigens declines and its maintenance requires antigen persistence and B cell renewal from the bone marrow (*Colle* et al., 1988, *Burlen* et al., 1988). These apparently differing conclusions may, in fact, not be mutually exclusive. Rather, they may simply reflect different apparent phenotypes of B cells at different stages in their response to antigen.

Role of T cell and Macrophage Derived Factors in TI Responses

Although responses to the antigens we have been considering are called thymus independent, there is much evidence to show that T cells and their products can influence these responses. For this reason, it has been proposed that these antigens be renamed thymus regulated instead of thymus independent (*Mond* and *Brunswick*, 1987). The term thymus independent originated from the observation of responses in athymic nude mice. However, we now appreciate that nude mice are seldom devoid of all T cells. Furthermore, some mediators secreted by T cells are also secreted by mast cells and large granular lymphocytes which are also present in nude mice. Hence, responses to some of these antigens may indeed be dependent on influences from T cells. Nevertheless, the term thymus independent is historical, and as such, will probably remain. It is this T cell regulation which is considered here.

Quite early it was observed that supernatants from concanavalin A (Con A) stimulated cultures (*Chen* and *Leon*, 1976) could increase responses to TI-2 antigens and that gamma interferon (IFN-γ) could suppress these responses (*Johnson* et al., 1975). These observations are consistent with the findings that optimal TI-2 induced IgG is seen in the presence of T helper cells (*Klaus* et al., 1976) and that TI-2 antigens can in-

Table 1: Interleukin-5 enhances antibody secretion by peritoneal mouse B lymphocytes induced with bacterial products

Addition	Anti-IL-5R mAb	Anti-TNP PFC/culture		
		None	DXS	LPS + DXS
Medium	-	1 ± 1	13 ± 6	373 ± 50
	+	1 ± 1	0 ± 0	400 ± 25
IL-5	-	9 ± 2	96 ± 19	1211 ± 84
	+	2 ± 2	5 ± 3	389 ± 54

5,000 Ly^+ peritoneal B cells were cultured 5 days with the indicated stimuli. DXS was used at 5 μg/ml and the monoclonal anti-IL-5R antibody R52.120 at 1 μg/ml. Standard errors for triplicate determinations are shown.

duce helper T cells (*Bretscher*, 1984). Non specific helper T cells have been shown to increase TI-2 antigen responses (*Wood* et al., 1982). In fact, T cells generally have been found to increase and suppress TI-1 antigen responses (*Tanay* and *Strober*, 1985). Furthermore, addition of Con A activated T cells can regulate TI responses (*Primi* et al., 1982) and addition of Con A itself can increase responses to TI-1 antigens (*Golding* et al., 1982). More precisely, addition of Con A to human B cells responding to the TI-1 antigen TNP-*Brucella abortus* results in recruitment of more B cell precursors and in larger B cell burst sizes (*Golding* and *Rittenberg*, 1984).

Further support for a role of T cell derived factors influencing TI antigen responses exists. Surface IgD positive neonatal B cells can respond to the TI-1 antigen TNP-*Brucella abortus* but a different set of interleukins is required to support sIgD negative B cell responses to this antigen (*McFadden* and *Vitetta*, 1984, *Waldschmidt* et al., 1985). Allogeneic amplifier T cells reportedly increase responses to TI-2 antigens but not to TI-1 antigens (*Braley-Mullen*, 1982). Cyclosporin A blocks interleukin production and secretion by T cells and also blocks secondary TI-1 antigen responses (*Shidani* et al., 1983). Similarly, *xid* B cells can respond to TI-2 antigens in the presence of IL-2 (*Delovitch* et al., 1983). These observations suggest that T cell derived interleukins can indeed be very important in responses to TI antigens.

Table 2: Interleukin-5 enhancement of antibody secretion by murine peritoneal B lymphocytes induced with the TI-1 antigen TNP-LPS

Stimulus	Anti-TNP PFC/culture		
	Medium	IL-4	IL-5
Medium	10 ± 4	56 ± 4	768 ± 60
TNP-LPS	108 ± 16	114 ± 8	1448 ± 122

50,000 murine 1.075 g/cc Percoll separated peritoneal B cells were cultured 5 days and PFC determinations were performed. Interleukins were used at 10 U/ml and TNP-LPS at 0.1 μg/ml. Standard deviations are given for triplicate determinations.

Table 3: Interleukin-5 enhances murine peritoneal B cell proliferation induced by bacterial products

Addition	cpm/culture x 10^{-3}			% of input B cells yielding clones
	Medium	DXS	LPS	LPS + DXS
none	0.5	4.9	20.4	16.8 ± 5.1
IL-2	1.2	7.5	21.9	21.1 ± 6.0
IL-3	1.3	11.4	23.7	ND
IL-4	1.8	9.3	25.1	18.3 ± 5.5
IL-5	5.2	25.9	49.8	45.7 ± 10.2

For thymidine incorporation (cpm/culture), 5 x 10^4 peritoneal B cells were cultured 2 days and pulsed during the terminal 6 hours of culture. LPS was used at 50 μg/ml, DXS at 5 μg/ml and the interleukins at 10 U/ml. For determination of the percentages of responding cells, Poisson analyses were performed on peritoneal B cells cultured at about 1, 2, 4 and 8 cells/well for four days. Each input contained 60 microcultures. Frequencies are presented with associated 95% confidence intervals.

More precise studies, using purified and cloned interleukins have successfully demonstrated their roles in TI antibody responses. Several groups have shown that IL-1 helps TI responses (*Pike* et al., 1987; *Stein* et al., 1986; *Wetzel*, 1989; 1990) and some even suggest that IL-1 is absolutely required for responses to both TI-1 and TI-2 antigens (*Sinha* et al., 1987). In contradistinction to some (*Pike* et al., 1987), a role for IL-4 in TI-2 antigen responses has been proposed (*Stein* et al., 1986). These same investigators, however, assert no relevant role for IL-2 or IL-5 (*Stein* et al., 1986). This is in contrast to other studies which do demonstrate IL-2 (*Pike* et al., 1987, *Mond* and *Brunswick*, 1987, see below) and IL-5 (*Pike* et al., 1987, *Wetzel*, 1989, 1990, 1991a, see below) involvement in responses to TI antigens.

The weight of the evidence suggests that interleukins can not only regulate TI antibody responses, but may in fact be required in some cases. The experimental sections which follow address the specific contributions of some interleukins, notably IL-1, IL-2, IL-4 and IL-5 in responses to TI antigens and analogues to try to extend our understanding in this area.

MATERIALS AND METHODS

Murine Studies

All the materials and methods for the experiments with peritoneal and splenic mouse B lymphocytes have been described previously (*Wetzel*, 1991a,b, 1990, 1989).

Human Studies

Cells:

Human peripheral blood lymphocytes were obtained by separating buffy coats from normal donors on Lymphocyte Separation Medium followed by treatment with 1 mM L-leucyl methyl ester for 30 minutes at room temperature. T cells were then depleted by rosette formation with AET treated SRBC and subsequent centrifugation on Lymphocyte Separation Medium. In some cases T cells were further depleted by treatment with anti-CD8 monoclonal antibody plus complement. Peritoneal dialysates were obtained by informed consenting patients undergoing continuous ambulatory peritoneal dialysis as outpatients at the George Washington

Table 4: Comparison of the ability of different interleukins to enhance antibody secretion by splenic murine B cells induced with the TI-1 type antigen TNP-LPS

Additions	anti-TNP PCF/10^6 cells (x 10^{-3})
none	0.8 ± 0.1
rIL-2	0.6 ± 0.1
rIL-4	0.8 ± 0.1
rIL-5	2.5 ± 0.5

100,000 resting murine splenic B cells were cultured 5 days with 0.1 μg/ml TNP-LPS. The indicated interleukins were used at 10 U/ml. Standard deviations are given. Shown are the results of three experiments.

Medical Center at the George Washington University in Washington DC and were obtained and provided kindly by Dr. Susan Lu. Cells were obtained by centrifugation and were treated with L-leucyl methyl ester as described above.

Reagents:

The monoclonal anti-IgD-dextran and anti-IgM-dextran conjugates were prepared by Dr. Andrew Lees and were provided as a kind gift. PHA-P and staphylococcal enterotoxin B were purchased from Sigma, St. Louis, MO. The human interleukins, with the exception of recombinant human IL-5 which was prepared by Dr. Tavernier from Hoffmann La Roche and was provided as a gift, were purchased as recombinant materials from Boehringer Mannheim and Genzyme.

RESULTS

The peritoneum of mice is a location where one might expect encounter with bacterial products. Hence we examined the antibody response of purified murine peritoneal B lymphocytes to two polyclonal activators which are, or mimic, bacterial products: lipopolysaccharide (LPS) and dextran sulphate (DXS). It can be seen in Table 1 that DXS by itself induced a weak Plaque Forming Cell (PFC) response which was increased by interleukin-5 (IL-5). LPS+DXS induced a much larger response which could be increased about threefold by IL-5. This IL-5 mediated increase was blocked by monoclonal antibody to the IL-5 receptor.

Next, the response of these peritoneal B cells to a prototype TI-1 antigen, TNP-LPS, was examined. The influence of two different interleukins on the antibody response was observed and these are shown in Table 2. TNP-LPS by itself was able to stimulate a vigorous PFC response at both antigenic and polyclonally activating doses. Among the interleukins tested, IL-5 alone increased the observed levels of PFCs.

The ability of these bacterial products to stimulate proliferation in these peritoneal cells was then examined. Results in Table 3 measure both thymidine incorporation and the fraction (%) of responding B cells. These measurements showed that, of the tested interleukins, IL-5 was the most effective at enhancing B cell proliferation stimulated by LPS+DXS. In the presence of IL-5, two- to three- fold more B cells were recruited to proliferate than observed with the combination of LPS+DXS alone.

The peritoneal B cells examined in

Table 5: The effect of different interleukins on resting murine splenic B cell proliferation induced by the thymus independent antigen type 2 analogue anti-∂-dextran

	cpm/culture x 10^{-3}							
	Addition							
Anti-∂-dextran (ng/ml)	None	IL-1	IL-2	IL-3	IL-4	IL-5	IL-6	IFN-γ
0.0	2.8	3.8	3.2	1.9	3.0	6.0	2.0	2.3
0.01	2.9	4.9	2.7	2.5	4.6	9.1	2.7	4.0
0.1	20.6	27.7	14.3	12.8	23.5	34.3	15.9	15.7
1.0	135.6	140.7	95.3	102.6	94.2	161.4	71.2	58.2

10^5 resting murine splenic B cells were cultured 2 days and pulsed with tritiated thymidine during the terminal 6 hours of culture. 100 U/ml IL-1, IL-6 and IFN-γ were used whereas 10 U/ml of the other interleukins were used.

the first three tables were larger and more metabolically active that their resting B cell counterparts from the spleen or other sites. Since there is some traffic between the spleen and peritoneum, and since splenic precursors can participate in antibody responses at other locations, we decided to investigate the response characteristics of resting splenic B cells. TI-1 antibody responses of these splenic B cells stimulated by TNP-LPS are shown in Table 4. Like the responses of their peritoneal counterparts, these cells demonstrated increased antibody secretion when IL-5 was present.

Responses to TI-2 antigens are not easily demonstrated with pure B cells due to the requirements for accessory cells. Hence, we have chosen a model system developed by others where anti-IgD coupled to high molecular weight dextran (anti-∂-dextran) is thought to mimic TI-2 antigens (*Brunswick* and *Mond*, 1988). This system allows the study of proliferation induced by this TI-2 analogue. Table 5 shows the proliferative responses of purified resting, splenic B cells stimulated by this anti-∂-dextran conjugate. Like the antibody and proliferative responses of peritoneal and splenic B cells previously shown, IL-5 was the interleukin most active at increasing the response induced by anti-∂-dextran. IL-1 showed some increase as well, but not to the same extent as that seen with IL-5.

Attempts to stimulate clonal B cell responses with the anti-∂-dextran conjugate were unsuccessful. We then tested whether DXS could provide additional signalling to anti-∂-dextran stimulated B cells. The results are presented in Table 6. Proliferation induced by different doses of anti-∂-dextran was increased by IL-5, as previously shown. However, these responses were also increased by DXS itself. Even further stimulation was seen when both DXS and IL-5 were added to anti-∂-dextran. This triple combination appeared to provide optimal signalling since responses were comparable to those seen with the anti-∂-dextran conjugate and LPS.

We attempted to quantitate resting splenic B cell precursor frequencies for responses to TI-1 and TI-2 antigens using two analogue model systems. The combination of anti-IgM+DXS was used to model TI-2 antigens and anti-IgM+LPS to model TI-1 antigens. Since these combinations are not covalently linked, their analogy to strict TI-1 and TI-2 antigens is only partial. How-

Table 6: Mitogen and Interleukin-5 enhancement of anti-∂-dextran induced proliferation of small, resting murine B cells

	Addition to culture				
Anti-∂-dextran (ng/ml)	none	IL-5	DXS	DXS + IL-5	LPS
0.0	0.5	2.0	0.9	7.7	73.4
0.01	0.5	2.9	0.8	8.3	79.7
0.1	4.0	15.3	5.5	32.3	130.1
1.0	36.0	81.8	44.5	110.2	124.5
10.0	37.0	73.0	75.4	111.1	112.9

5 x 10^4 resting, Percoll separated splenic murine B lymphocytes were cultured 2 days and pulsed with ^{3}HTdR during the terminal 6 hours of culture. Data presented are cpm x 10^{-3}. IL-5 was used at 3 U/ml, DXS at 5 μg/ml and LPS at 50 μg/ml.

ever, they do allow quantitation to be made which would otherwise not be possible without selecting antigen specific B cells and thereby perturbing the surface antigen receptors uncontrollably. Table 7 gives the summary of data from several experiments determining the frequencies of B cells proliferating in response to the two model antigen-analogue systems. Interestingly both IL-4 and IL-5 were able to increase proliferation in both systems. The IL-4 mediated increase seen with anti-IgM+DXS contrasts to that seen with the anti-∂-dextran plus IL-4.

Table 8 shows a comparison of the interleukin-mediated enhancement of proliferation induced by LPS versus that induced by anti-IgM+LPS. Whereas IL-4 increased both types of proliferative responses, IL-5 was active only when the combination of stimuli were used. Neither IL-2 nor IL-6 enhanced either of the proliferative response.

The data in the previous Tables were obtained with B cells from murine spleen and peritoneum. One is often reminded that humans differ from mice. Hence, several experiments with human B lymphocytes have been performed. Table 9 shows the responses of human peripheral blood B lymphocytes to two TI-2 analogues: anti-human IgD-dextran (anti-h∂-dex) and anti-human IgM-dextran (anti-hIgM-dex). With both TI-2 analogues, good proliferation was observed. IL-2 enhanced this proliferation whereas IL-1, IL-4, IL-5 or IL-6, did not.

The previous sections showed some differences in the responses of murine splenic and peritoneal B cells. To see if human peritoneal B cells responded differently from their peripheral blood counterparts, peritoneal fluids from patients on chronic ambulatory peritoneal dialysis (CAPD) were collected. While cells from these fluids come from patients, they represent one of the few sources of such cells and so, although not derived from normal volunteers, were used as a first estimate of human peritoneal lymphocyte responsiveness.

Preliminary experiments revealed no responsiveness to B cell stimulating agents (data not shown). This is probably explained by the paucity of B cells in these peritoneal dialysate preparations, as revealed in Table 10. This table shows the prevalence of cells exhibiting B and T lymphocyte markers in 5 CAPD patients. As can be seen, only one patient showed significant B cell levels and this patient, although showing no overt signs of inflammation, was clearly different from the other patients in the population. Nevertheless, T cell reactivity, especially to the bacterially

Table 7: Interleukins increase clonal B cell growth initiation by anti-IgM plus dextran sulphate and by anti-IgM plus LPS

Stimuli	Addition	Average frequency (%) of B cells yielding clones	s.e.
anti-IgM + DXS	none	14.5	5.9
	IL-1	24.4	7.0
	IL-4	29.4	7.9
	IL-5	21.1	6.8
anti-IgM + LPS	none	32.1	6.8
	IL-4	70.6	10.7
	IL-5	51.0	9.0

Resting murine splenic B cells were cultured with the indicated stimuli and Poisson analyses were performed to determine the fraction of input B cells stimulated to proliferate. These are presented as percentages of the input B cells which responded. Shown also are the associated standard errors. Four experiments were performed for anti-IgM + DXS and ten experiments for anti-IgM + LPS. LPS was used at 50 μg/ml, DXS at 5 μg/ml and anti-IgM was 1 μg/ml of the b-7-6 monoclonal rat anti-mouse IgM antibody.

derived superantigen staphylococcal enterotoxin B (SEB), could be reproducibly detected in peritoneal cell preparations from these patients. This is shown in Table 11 where proliferation responses induced by a T cell mitogen and SEB can be observed.

DISCUSSION

The experiments presented in the previous sections provide clear evidence that T cell products can regulate B cell responses to thymus independent activators and antigens. Local immune responses to TI-1 antigens by murine peritoneal B cells were increased by IL-5. Similarly, murine splenic B cell responses to TNP-LPS were also increased by IL-5. These data suggest a role for IL-5 in murine responses to TI-1 antigens. Interestingly, the data in Table 8 suggest that resting B cells must perceive surface antigen receptor cross-linking as well as a second polyclonally activating signal to induce IL-5 responsiveness. The data in Tables 5, 6 and 7, using anti-∂-dextran and anti-μ + DXS as models of TI-2 antigens, and with anti-μ + LPS as a TI-1 analogue model support this interpretation. We (*Wetzel*, 1991a, 1991b) and others (*Pike* et. al., 1987) have also found a role for IL-5 and IL-1 in supporting murine B cell responses driven by TI-2 antigens.

The data in Table 6 suggest that there may be several forms of TI-2 antigens. Addition of DXS to anti-∂-dextran increased the responses observed although not obviously changing the quality of these responses, i.e. IL-5 increases responses to both agents. Furthermore, DXS alters the response to anti-IgM by allowing clonal B cell proliferation in the presence of the combination of anti-IgM+DXS, as shown in Table 7. Again, IL-5 is active in this system but IL-4 is now also active. DXS by itself is a weak activator, as shown in Table 1, and hence, although apparently polyclonally activating, may best be classified as TI-2 type in its action. These properties of DXS are inter-

Table 8: Determination of the proportion of small, resting B cells proliferating in response to thymus regulated antigen type 1 analogues plus interleukins

Added interleukin	Primary stimuli	
	PLS	LPS + anti-IgM
none	2.7 ± 2.1	20.1 ± 5.4
IL-2	3.2 ± 2.2	11.6 ± 4.0
IL-4	29.0 ± 6.9*	60.2 ± 11.5*
IL-5	5.4 ± 2.7	37.5 ± 8.2*
IL-6	1.5 ± 1.7	21.9 ± 5.7

Percoll separated small, resting, murine splenic B cells were cultured in Terasaki plates at four different cell inputs (from 1 to about 8 cells/well, with no filter cells) with 60 wells/input dose for each determination. After 4 days, wells were examined for cell growth and Poisson analysis was used to calculate the frequency of input B cells stimulated to proliferate. These frequencies are presented as percentages of the input B cells (i.e. a frequency of 0.1 = 10%) and are given with the associated 95% confidence intervals.
*Asterisks mark those frequencies which are statistically different from the appropriate controls at less than the 5% level.
LPS was used at 50 μg/ml and the b-7-6 monoclonal anti-IgM used at 10 μg/ml. Interleukins were used at 10 U/ml except for IL-6 which was used at 1000 U/ml.

esting but it remains difficult to interpret them before a more complete understanding of how DXS interacts with B cells is available. In any case, the data are suggestive that more than one form of TI-2 antigen may exist. Furthermore, the data suggest that different interleukins can be important in responses to different forms of TI antigens.

The responses by human B cells revealed different interleukin receptivities from those seen with mouse B cells. Proliferation by human peripheral blood B lymphocytes induced with anti-hδ-dextran and anti-hμ-dextran was increased by IL-2 but not the other interleukins tested, as seen in Table 9. As described above, mouse B cells show different reactivities. The reason for this difference is not immediately obvious. Nevertheless, B cells from both species reveal interleukin receptiveness when stimulated by TI-2 analogues and therefore provide evidence that T cells can regulate and may even be required for TI-2 responses in some situations.
The difference between species in interleukin responsiveness may parallel the difference seen between B cells from different tissues in the mouse. Murine peritoneal B cells respond to IL-5 by itself, as seen in Tables 1 and 2 and elsewhere (*Wetzel*, 1989, 1990, 1991c), although these responses are small. Induction of resting splenic B cell IL-5 responsiveness requires surface receptor engagement and perception of a polyclonally activating stimulus. Hence, it may be possible to reveal human B cell IL-5 responsiveness with the appropriate stimulus. Our initial attempts to reveal human B cell IL-5 responsiveness using peritoneal cell populations were unsuccessful due to the lack of B cells in these populations. Nevertheless, human B cell IL-5 responses have been observed by others using some appropriate bacterial activators (*Mond* and *Harriman*, personal communications).

The finding of T cell responsiveness to SEB in lymphocyte preparations from the peritonea of CAPD patients, seen in Table 11, shows at least some functional and responsive anti-bacterial immune mechanisms in this human or-

Table 9: Stimulation of human peripheral blood B lymphocytes by the thymus independent type 2 antigen analogues anti-IgD-dextran and anti-IgM-dextran

		cpm/culture x 10^{-3}					
		Addition					
Exp.	Stimulus	none	IL-1	IL-2	IL-4	IL-5	IL-6
1	medium	0.5	0.4	1.2	0.3	ND	ND
	anti-∂-dextran	36.6	51.1	64.9	28.1	ND	ND
2	medium	5.9	7.3	12.6	ND	6.6	ND
	anti-∂-dextran	65.8	59.4	90.1	ND	55.2	ND
3	medium	4.3	4.6	12.7	ND	ND	1.8
	anti-∂-dextran	14.1	10.8	37.0	ND	ND	7.1

5 x 10^4 human peripheral blood B lymphocytes were cultured 2 days (experiment 1) or 3 days (experiments 2 and 3) with 0.1 μg/ml of the indicated anti-immunoglobulin-dextran conjugate and pulsed for the terminal 16 hours of culture with tritiated thymidine. IL-1 was used at 100 U/ml, IL-2 at 30 U/ml, IL-4 at 20 U/ml, rIL-5 at 200 pg/ml and IL-6 at 1000 U/ml.

gan. These results, coupled with those from the previous tables, show the importance of bacterial immunity. Both B cells and T cells have developed mechanisms to deal with this type of insult. It appears that each of these lymphocyte populations can respond independently as well as communicate with each other. Thus, T cells are capable of regulating B cell responses to TI antigens. The specific mediators developed for these regulatory interactions can vary according to the type of stimulation encountered by the B cell and by the tissue or species of B cell origin. It is probable that the complexity of these regulatory networks and responding populations is only beginning to be appreciated.

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Brunswick, M., Finkelman, F.D., Highet,

Table 10: Phenotypic analysis of lymphoid cells from peritoneal fluids of patients on continuous ambulatory peritoneal dialysis

	% of cells analysed expressing the indicated marker					
	Patient number					Monocyte depleted
Marker	1	2	3	4	5	blood
CD3	38.7	51.2	34.5	9.7	9.8	59.1
CD4	23.7	34.8	12.4	ND	ND	21.6
CD19	2.0	0.1	0.1	0.0	0.7	20.1
CD20	0.6	1.3	ND	ND	ND	7.9
LeuM3	35.6	12.4	9.1	12.1	5.2	0.3

Cells from peritoneal fluids were recovered by centrifugation and then stained with the appropriated fluorescently labelled monoclonal antibodies. Cells were then analysed on a FACSCAN and the numbers shown are the percentages of either small cells or of total cells positive for binding the indicated fluorescent antibodies. Anti-CD3 and anti-CD4 monoclonals detect all and helper T cells, respectively. Anti-CD19 and anti-CD20 monoclonal antibodies detect human B cells. Anti-LeuM3 detects human monocytes and macrophages.

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Table 11: Proliferative responses of cells recovered from peritoneal fluids from patients on continuous ambulatory peritoneal dialysis

		cpm x 10^{-3}				
		Interleukin added to culture				
Exp.	Stimulus	None	IL-1	IL-2	IL-5	IL-6
1	medium	0.7	1.5	2.7	1.7	1.5
	SAC	1.5	1.7	1.9	1.9	0.9
	SEB	4.1	4.5	6.7	4.5	4.8
2	medium	0.3		0.8		
	SEB	3.6		6.2		
	PHA-P	1.2		1.8		
3	medium	0.4		1.3		
	SEB	4.0		7.1		
	PHA-P	1.5		2.9		

All experiments were performed with cultures of L-Leucil Methyl Ester treated with peritoneal dialysis fluid cells. Experiment 1 used 1000 cells per well and experiments 2 and 3 used 500 cells per well. Experiment 1 was assayed on day 3 of culture whereas experiments 2 and 3 were assayed at day 5. Cultures were pulsed with 1 μCi of ^{3}HTdR for 16 hours prior to harvest and incorporated radioactivity was measured by liquid scintillation counting. *Staphylococcus aureus* protein A (SAC) was used at 10 μg/ml. Staphylococcal enterotoxin B (SEB) was used at 10 ng/ml and Phytohaemagglutinin-P (PHA-P) was used at 2 μg/ml. Interleukins 1, 2, 5 and 6 were used at 100 U/ml, 30 U/ml, 200 pg/ml, and 1000 U/ml, respectively.

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BACTERIAL TOXINS: NEW ASPECTS ON THEIR ROLE IN GASTROINTESTINAL INFECTIONS

TORKEL WADSTRÖM, MARIA HELD, AND ÅSA LJUNGH

Department of Medical Microbiology, University of Lund, Lund, Sweden

INTRODUCTION

Already Theodor Escherich at the end of the century suspected that certain strains of the indigenous intestinal microflora of young children produced soluble toxins causing diarrhoea and other gastrointestinal diseases. Only about a decade later the major symptoms of both cholera and shigellosis were proposed by other investigators to be caused by extracellular protein toxins (*Guerrant*, 1985; *Wadström* et al., 1986; *Wadström*, 1988).

Despite these early observations it was not until the early seventies, after the characterisation of cholera toxin and cholera-like *Escherichia coli* heat-labile (LT) enterotoxins, that it was demonstrated that many gastrointestinal pathogens produce enterotoxins.

The toxins produced by gastrointestinal pathogens can be divided into three major classes (Table 1):

1: Cytotonic enterotoxins not inducing cell damage of target enterocytes but stimulating cell secretory mechanisms.
2: Cytotoxic enterotoxins taken up into cells and causing cell damage.
3: Cytolytic toxins / membrane damaging toxins.

Toxins of class 1 activate the adenylate and guanylate cyclase systems of enterocytes. *E. coli* STa, belongs to this class while the mode of action of the STb toxin is still not known. However STb producing strains are rarely isolated from human infections but are common in enterovirulent strains of young pigs and calves (*Flock*, personal communication). These cytotonic enterotoxins induce cholera-like watery diarrhoea commonly defined as enterotoxic enteropaties to discriminate these infections from infections with class 2 and 3 pathogens causing epithelial cell damage and inflammation (*Guerrant*, 1985; *Cane* and *Guerrant*, 1989). Interestingly *C. difficile* and *C. jejuni* infections as well as salmonellosis and *Aeromonas* induced diarrhoea are also characterised by an initial phase with watery cholera-like stools probably via yet undefined class 1 toxins.

However *Keusch* and colleagues (1991) described that also the cytotoxic Shiga toxin may act on small bowel epithelium in an early stage of intestinal colonisation to induce watery stools typical of class 1 toxins. Later stages are characterised by epithelial cell invasion and cell death, after intracellular multiplication and synthesis of cytotoxic Shiga toxin of class 2.

We now want to compare and discuss the possible roles of toxins produced by various enteric microbes as additional virulence factors to induce mucosal barrier damage in various stages of acute and chronic intestinal infections and inflammatory bowel diseases such as ulcerative colitis. However, first some aspects on toxigenic *Clostridium botulinum* and other toxin producing microbes in the gastrointestinal tract of young infants will be discussed.

Certain strains of *Clostridium botulinum* in infant food, such as bee honey, can colonise the intestinal tract of young infants with a poorly developed indigenous microflora (*Arnon*, 1990; *Popoff*, 1990) and cause flaccid paralysis of certain muscles and sudden infant death syndrome (SIDS) (*Akotories* and *Just*, 1990).

More recent studies in Japan also suggest that haemagglutinating strains of *C. botulinum* are able to colonise the human intestinal tract while non-haemagglutinating strains seem to lack adhesins to colonise the human gut and induce SIDS (*Tabita* et al., 1991).
Bettelheim and colleagues (1990) have recently reported that Shiga-like toxin (Syn. Verotoxin) producing strains of *E. coli* are commonly isolated from children with SIDS in Australia but not from age-matched control children. Studies in England have shown that strains of coagulase-negative staphylococci (CNS) producing a delta-haemolysin-like cytolytic toxin (class 3 toxin) are commonly isolated from children with SIDS and also from children with certain forms of nicrotising colitis (*Scheifele* and *Bjornson*, 1988). These findings are interesting in the perspective that it has not been ruled out whether potent staphylococcal immunomodulating toxins such as toxic shock toxin 1 (TSS 1) (*Newbould* et al., 1989) and the related enterotoxins within the same toxin superfamily may under certain conditions be associated with SIDS and similar fulminating toxicoses later in life. These toxins are classified today into a new class of toxins often called immunomodulating toxins or superantigens (*Alouf*, 1986; 1991).

It is yet too early to speculate on the possible role of toxins produced by other enteric organisms than haemagglutinating *C. botulinum* to induce SIDS and maybe also similar fulminant toxicoses in older children and adults. However it is most likely that certain strains of potent toxigenic staphylococci

Table 1: Classification of toxins produced by gastrointestinal pathogens

Class 1	Cytotonic enterotoxin	Prototype toxins Cholera toxin *Escherichia coli* LT and ST[1]
Class 2	Cytotoxic toxins	Shiga toxin Shiga-like toxins (Syn. Vero-like toxins) *H. pylori* vacuolising toxin *Clostridium difficile* toxin A[2]
Class 3	Cytolytic toxins (Cell membrane damaging toxins, haemolysins)	Aeromonas alpha and beta toxins *E. coli a*nd Shigella haemolysin *H. pylori* haemolysin[3] *C. difficile* toxin B

[1]Other members of this class are *Salmonella* LT like toxins (*Prasad* et al., 1990; *Stephen*, 1991), and *Campylobacter jejuni* enterotoxin(s). See also: *Cane* and Guerrant, 1989.
[2]Shiga-like toxins or Vero-like cytotoxins is a family of toxins produced by 0157:H 7 and certain other specific serotypes of *E. coli* (*Karmali*, 1989; *Wadström* and *Ljungh*, 1990; *Keusch* et al., 1991)
[3]*Helicobacter pylori* may produce a number of toxins not yet defined, as well as cytotoxic phospholipase A2 and C (*Raedsh* et al., 1989; *Slomiany* et al., 1987; *Slomiany* et al., 1989).

and other "abnormal" organisms in the gastrointestinal tract may be able to colonise when the gut flora has been disturbed by e.g. antibiotic therapy. This field is certainly now open for more investigations.

CLOSTRIDIUM DIFFICILE INFECTIONS

Dubos-Ramare and *Corthier* (1990) reported on the influence of a "low protein protective diet" on toxin production by *Clostridium difficile* in gnotobiotic mice. These studies confirm previous observations that the diet can influence the intestinal microflora and that proteolytic digestion by certain microflora members may be necessary for toxigenic *C. difficile* strains to colonise the gut and produce toxins.

Studies by *Borriello* (1989) indicate that specific surface fimbriae may be the intestinal colonisation factors and necessary for a successful colonisation of the colon to allow toxin delivery at the epithelium level similar to how fimbrial colonisation factors (CFAI, CFAII etc.) allow enterotoxigenic *E. coli* (ETEC) to colonise the small bowel.

It has been speculated that the low sensitivity of children under 2 to 4 years of age to *C. difficile* toxin induced diarrhoea may be due to lack of toxin receptors of immature enterocytes while intestinal colonisation is possible due to specific receptors in the colonised mucosa for *C. difficile* surface adhesins (*Borriello*, 1989). Further research in this area may reveal a new strategy to prevent *C. difficile* infections by oral feeding with nontoxigenic strains with good ability to colonise the colon mucosa.

STAPHYLOCOCCAL ENTEROCOLITIS

Before the discovery of *C. difficile* as a common cause of antibiotic associated enterocolitis, certain strains of *S. aureus* were suggested as the major cause of nosocomial enterocolitis (*Kapral*, 1986). However, studies in recent years have not confirmed the role of enterotoxigenic *S. aureus* strains as a common cause of enterocolitis but recent observations that certain strains of coagulase negative staphylococci producing not yet defined cytolytic toxins (*Scheifele* and *Bjornson*, 1988) suggest that further research in this area is needed.

OTHER AETIOLOGIES FOR ACUTE AND CHRONIC ENTEROCOLITIS

C. difficile as well as other enteric pathogens such as *C. jejuni* and *Aeromonas hydrophila* have been reported to cause both acute and chronic infections of the small and large bowel of children and adults. Both *C. jejuni* and *A. hydrophila* infections are commonly associated with consumption of certain foods. Acute *Aeromonas* cholera-like infantile diarrhoea is common in many developing countries (*Ljungh*, 1987; *Wadström* and *Ljungh*, 1990) associated with high bacterial counts in certain waters, especially during the warm seasons. We know very little about the environmental reservoirs of enterotoxin and cytotoxin producing *Aeromonas* (*A. hydrophila, A. sobria, A. caviae* and a few more species) in water and food products in countries

with temperate climates and low hygienic standards (*Wadström* and *Ljungh*, 1991). Moreover, certain cytotoxin producing as well as non-cytotoxin producing *Aeromonas* belonging to the indigenous gut microflora of pigs may also colonise humans. However few gut microflora studies of humans involving selective search for these oxidase positive "haemolytic *E. coli*-like organisms" have yet been carried out. Interestingly, a recent report from England suggest that certain strains of *Aeromonas hydrophila* are associated with chronic colitis (*Grimminger*, 1990; *Willoughby* et al. 1989). Recent observations that certain *Bacteroides* strains in both animal and man can produce enterotoxins indicate that more studies have to be performed to diagnose possible new pathogens in the aetiology of acute, sub-acute as well as relapsing forms of enterocolitis, especially after travelling to countries with warm climates. We have initiated a study in Lund to define the possible role of toxigenic aerobes in patients with intestinal symptoms that remain for more than a week after returning home from travels in southern Europe or other continents.

TOXIGENIC ENTERIC BACTERIA AND ULCERATIVE COLITIS

Studies in the sixties showed that haemolytic *E. coli* were commonly isolated from young pigs with diarrhoea (*Thayer*, 1987). Such strains were later shown to produce also heat-labile and heat-stable (LT and ST) enterotoxins (*Järnerot*, 1986; *Wadström* and *Ljungh*, 1990).

Early studies on the human faecal flora showed that haemolytic *E. coli* strains were more commonly isolated from patients with chronic colitis and patients with subacute phase of ulcerative colitis (*Fiocchi*, 1986). More recently, tests for cytotoxins in stools of such patients and isolated *E. coli* strains revealed that strains produced also the non-haemolytic Shiga-like toxin (*Ljungh* and *Wadström*, 1988; *Ljungh* et al., 1991). Apart from direct toxic effects on enteric cells, this group of toxins may induce e.g. platelet aggregation which can be important in the pathogenesis of ulcerative colitis (*Rose* et al., 1985). Interestingly, further studies on surface properties of these toxigenic strains revealed specific heat and protease sensitive structures binding to various subepithelial extracellular matrix (ECM) components such as fibronectin, vitronectin and various collagens (*Ljungh* and *Wadström*, 1988; *Ljungh* et al., l991). Certain strains of *E. coli* and maybe also other enteric micro-organisms may thus be able to colonise in mucosal lesions of ulcerative colitis. However, despite the fact that antibiotics seem not to have effects on relaps-

Table 2: Putative effects of *Helicobacter pylori* on the human gastric epithelium

Phospholipases:	- Destruction of the hydrophobic lining on the mucus layer (*Raedsch* et al., 1989; *Slomiany* et al., 1987, 1989)
	- cytotoxic effects on the epithelium
Ammonia produced close to the cell surface	
Cytotoxins	
Vacuolising toxin(s)	
Cytolytic toxin(s)[1]	

[1] No isolation procedure for cell associated cytolytic toxin(s) has yet been published; neither it has been published whether certain strains produce extracellular as well as cell associated toxins.

ing ulcerative colitis (*McLaren* and *Gutnick*, 1982) it seems tempting to speculate that certain toxigenic *E. coli* and other organisms may precipitate relapses by causing damage of the mucosal barrier.

POSSIBLE ROLE OF THE *LACTOBACILLI* AND OTHER MEMBERS OF THE GUT FLORA TO COMBAT INTESTINAL DAMAGE BY TOXIGENIC MICROBES

Studies in recent years in Uppsala and Copenhagen (*Tvede* and *Rask-Madsen*, 1990) showed that faecal enemas have a dramatic effect on intestinal symptoms in patients with acute and relapsing *C. difficile* enterocolitis. *Streptomyces boulardii* may also combat toxigenic *C difficile* and aid in restoring a normal colonic microflora. It is also likely that more recent research on antibiotic-like bacteriocins of various lactobacilli will make it possible to design one or two strains for successful therapy of *C. difficile* infections.

It is yet too early to speculate about the possibility to use such organisms in the treatment of ulcerative colitis.

HELICOBACTER PYLORI - A TOXIGENIC GASTRIC PATHOGEN

Marshall and *Warren* rediscovered spiral gastric pathogens in 1983 (*Rathbone* and *Hartley*, 1989; *Peterson*, 1991) with the first successful culture of a micro-aerophilic pathogen initially called *Campylobacter pylori*, now known as *Helicobacter pylori*. Organisms closely related to *H. pylori* have been isolated more recently also from primates, ferrets *(H. mustelae)* and cats (*H. felis)*. High motility and high urease production are necessary for the microbe to colonise the gastric mucosa and to induce so called "type B gastritis". The formation of ammonia from urea at the gastric epithelium (*Smoot* et al., 1990; *Turbett* et al., 1991), which the organism colonises by specific surface adhesins, is very toxic for the cells. Close cell adherent *H. pylori* can probably deliver ammonia directly on the epithelial cells but may also damage the intercellular tight junction during invasion down to subepithelial tissues and survive as coccoidal forms (*Jones* and *Curry*, 1990).

Several investigators have described the following toxins produced by strains of *H. pylori* isolated from gastric and duodenal ulcer lesions (Table 2):

1. Haemolysins also cytolytic for tissue culture cells (*Gregor* et al., 1990, *Wadström* and *Ljungh*, unpublished observations)
2. Vacuolising toxins (*Blaser*, 1990; *Cover* et al., 1990; *Leunk* et al., 1990).
   It is not clear whether the toxins described by these investigators are identical.
3. Phospholipase A2 and C: The possible cytolytic effects of these cell membrane active cell toxic enzymes (*Möllby*, 1978; *Raedsch* et al., 1989) have not yet been studied while they have been proposed to destroy the normal hydrophobic mucosal cell lining of the gastric epithelium (*Wadström* and *Aleljung*, 1990).
   H. pylori toxins have also been proposed as important virulence factors in the development of stomach and duodenal ulcer diseases (*Rathbone*, 1989) and for development of the

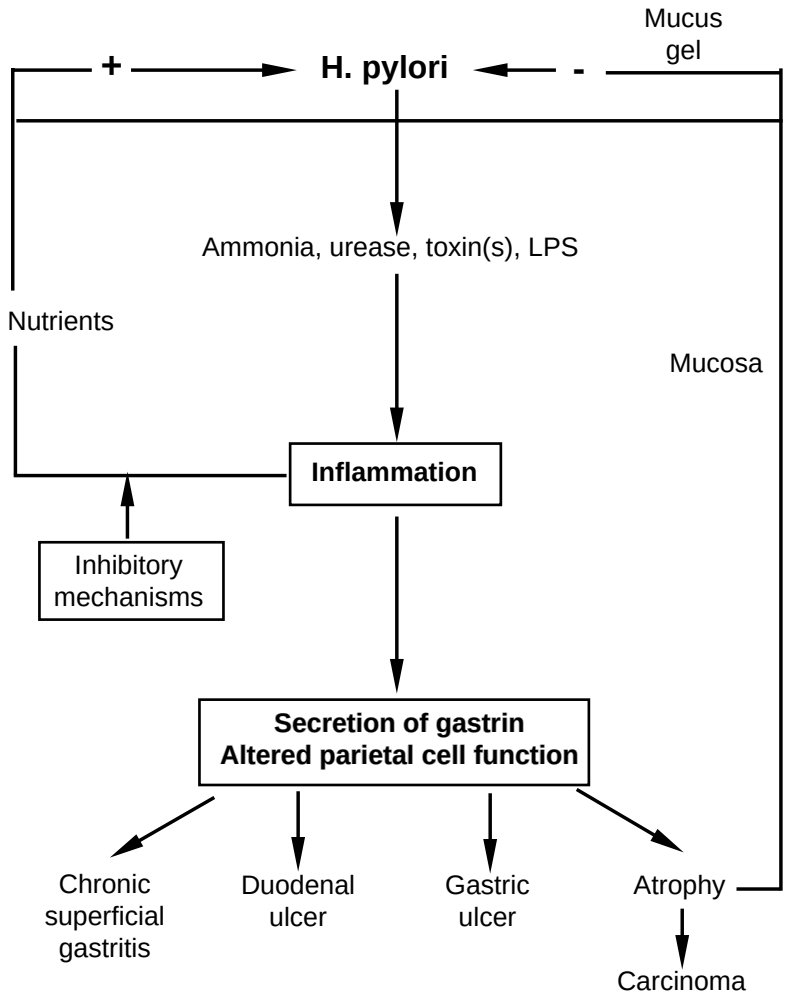

Figure 1: Model for the relationship between *Helicobacter pylori* products, inflammation and gastroduodenal pathology (modified after *Blaser*, 1990).

lesions in oesophagitis.

4. Very low levels of extracellular toxin production seems common among *H. pylori* strains as reported by several investigators (*Blaser*, 1990; *Leunk* et al., 1990). However, it is too early to speculate if *in vivo*-like growth conditions, such as adding gastric mucins in fractions to culture medium, may enhance toxin production. It is also possible that certain cells of the human gastric epithelium are more susceptible to the toxin(s) than HeLa cells or other *in vitro* tissue culture cells used for screening for *H. pylori* toxins. Our own studies confirm a recent report by *Smoot* and co-workers (1990) that the vacuolising cytotoxin of *H. pylori* does not produce drastic effects on the cell membrane and changes in membrane permeability. The possible role of this toxin and cytolytic (haemolytic) toxins in the pathogenesis of *H. pylori* infections is thus still quite obscure. However, strains which are

associated with stomach and duodenal ulcer disease may be more potent toxin producers than strains isolated from patients with mild and often symptom free acute or chronic gastritis. Interestingly, patients also produced antibodies to cytolytic toxins described by *Figura* and *Blaser*, but a high serum antibody titre does not seem to influence the cause of the disease (*Leunk* et al., 1990; *Cover* et al., 1990; *Wadström*, in preparation). It thus seems likely that toxin formation may be important just in the first initial stages of gastritis and development of acute duodenal and stomach ulcer disease.

Studies in Brussels indicate that more than half of the patients with *H. pylori* infections respond with an antibody titre to crude *H. pylori* toxin(s) as determined in toxin neutralisation tests (NT) in tissue culture assays (*Goosens*, personal communication). Moreover, biotyping of *H. pylori* strains from various geographic regions suggests that toxin(s) are commonly produced by different biotypes of strains. However, studies in Lund on strains isolated from patients with gastritis and ulcer disease show that toxin production occurs only at low levels in Swedish strains (titre <1/8-1/16). Work is now in progress in several laboratories to explore how to enhance toxin production in laboratory cultures in order to purify toxin(s) and phospholipase C which, like other bacterial phospholipases, is probably per se cytolytic.

A model for the relationship between *H. pylori* products, inflammation and gastroduodenal pathology is presented in Figure 1.

FUTURE PROSPECTIVE

The discoveries in the last two decades of a number of new toxins and produced by "old and new" enteropathogens or putative enteropathogens such as *Aeromonas hydrophila* will stimulate further studies on how to prevent gut colonisation and diseases induced by such microbes. The great complexity of toxins and toxin families (such as Shiga-like toxins) indicate that vaccines seem less likely as prophylaxis for these infections but that new strategies to develop the probiotic concept to prevent and combat these infections by various toxigenic enteric organisms is now a fruitful area to explore for both human and animal medicine.

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ENDOTOXIN INDUCED ENDOGENOUS MEDIATORS IN THE PATHOGENESIS OF SEPTICAEMIA

DAAN W. HOMMES[1], YONGQIANG ZHAO[2], ENRICO BERNARDI[3], JAAP JANSEN[1], SANDRA A. RADEMA[1], and SANDER J.H. VAN DEVENTER[1,*]

[1]Centre for Haemostasis, Thrombosis, Atherosclerosis and Inflammation Research, Academical Medical Centre, Amsterdam, The Netherlands; [2]Department of Haematology, Peking Union Medical College Hospital, Beijing, China; and [3]Department for Thrombosis and Haemostasis, University of Padua, Italy.

SUMMARY

Endotoxin is a major causal Gram-negative bacterial factor in the development in Gram-negative septicaemia. The bowel contains a vast amount of endotoxin, that is derived by shedding of outer membrane fragments of either growing or dead bacteria. Normally, very little endotoxin is absorbed by the gut. In animal models, conditions that damage the mucosal lining, such as ischaemia/reperfusion, profound metabolic changes, or bowel inflammation, may cause transmigration of endotoxin. In humans, similar conditions, including bowel ischaemia and extensive inflammatory bowel disease, may similarly lead to "intestinal endotoxaemia". Some investigators have proposed that continuous endotoxin uptake may be an important causative factor in the development of multiple organ failure. Although it is possible that endotoxin uptake through damaged bowel mucosa has a role in the perpetuation of septicaemia in critically ill patients, there are currently no data to either substantiate or falsify this hypothesis .

Despite improvements in the management of critically ill patients, the mortality of septicaemia remains high. Immunotherapy, in particular treatment with cross-reactive anti endotoxin (glyco)lipid antibodies, may improve survival in septic patients with Gram-negative bacteraemia. Clinical studies with antibodies that neutralise an important endogenous mediator of endotoxicity, tumour necrosis factor, are underway, and may show additional benefit.

INTRODUCTION

Gram-negative septicaemia remains a major cause of death among hospitalised patients and is the commonest cause of death in intensive care units. In the United States the annual incidence of septicaemia is estimated to be 400.000, leading to 200.000 cases of septic shock and 100.000 deaths (*Parrillo* et al., 1990). Gram-negative septicaemia is caused by bacterial products, such as endotoxins, that enter the blood stream and trigger a series of reactions including complement activation (via both the classical and alternative pathways) (*Vukajlovitch* et al., 1987), platelet activation (*Doebber* et al.,

*Dr. van Deventer is a fellow of the Netherlands Academy of Arts and Sciences.

1985), activation of the coagulation and fibrinolytic system (*Harris* et al., 1987) and the induction of the synthesis and release of various cytokines from mononuclear and endothelial cells (*Tracey* et al., 1986; *Michie* et al., 1988; *van Deventer* et al., 1990; *Fong* et al., 1989a; *Girardin* et al., 1988; *Hack* et al., 1989).

Endotoxins are lipopolysaccharides (LPS) from the Gram-negative bacterial cell wall. Endotoxin is composed of three portions: the oligosaccharide side chain, the core polysaccharide and the lipid A component. Lipid A is structurally similar among many Gram-negative bacteria, and it represents the biologically active part of the endotoxin molecule, being responsible for the toxic properties of endotoxin (*Appelmelk* et al., 1988; *Rietschel* et al., 1982). Naturally occurring antibodies to lipid A are associated with increased survival in human septicaemia (*McCabe* et al., 1972), and a recent clinical trial with a human monoclonal anti-endotoxin antibody HA-1A, which binds to the lipid A component, showed a significant reduction of mortality in patients with septicaemia (*Ziegler* et al., 1991).

In the last decade, extensive research has given us considerable insight into the interplay of endotoxin with the immune system. It has become apparent that almost all toxic effects of lipid A are mediated by endogenous (glyco)proteins, called cytokines, that are released by monocytes and endothelial cells. Here we briefly review the role of endotoxin and cytokines in septicaemia, and discuss novel therapeutic approaches in Gram-negative septicaemia, with special emphasis on gut derived endotoxin as a cause of septicaemia.

CYTOKINES

The normal human immune response is regulated by cytokines, polypeptide hormones that are synthesised and release by various cell-types. Cytokines regulate the biological function of various tissues by modifying gene expression and cellular metabolism. Many cytokines have been molecularly cloned and recombinant proteins are available to investigate their role in immune responses and inflammation. Although each cytokine has a distinct sequence, structure and individual receptors, cytokines often share similar biological properties, and multiple cytokines can act synergistically or antagonistically on the same target cells. Although cytokines were first identified in monocytes and macrophages, it is now clear that many cytokines can be produced by other cells, including those that are traditionally not considered to be "immunocompetent": Fibroblasts, endothelial cells, bone marrow, epidermal keratinocytes, stromal cells and brain astrocytes are able to produce substantial amounts of cytokines in response to various stimuli.

Cytokines can be divided into two main groups: I. cytokines that predominantly function as *growth factors* (interleukin-1 [IL-2], interleukin-3 [IL-3], interleukin-4 [IL-4]) and II. cytokines that predominantly have *pro-inflammatory properties* (interleukin-1 [IL-1], interleukin-6 [IL-6], interleukin-8 [IL-8] tumour necrosis factor [TNF]). IL-2 and IL-4 stimulate the growth and functional activities of T and B lymphocytes, and IL-3, together with granulocyte-macrophage colony stimulating factor (GM-CSF), is a growth and progression factor for haematopoietic cells. The pro-inflammatory cytokines (IL-1,

IL-6, IL-8, TNF) have been extensively studied and are now known to have a pivotal role in the pathogenesis of septicaemia. In the context of a normal immune response these cytokines are produced in very small amounts (usually below the detection limits in serum), necessary for a normal host defence response. In septicaemia however, uncontrolled cytokine release may cause hypotension, organ damage, catabolism, and death.

INTERLEUKIN-1

IL-1 was one of the first endogenous pyrogens to be identified, and has a large variety of other effects including the induction of PGE2 synthesis, growth of fibroblasts, bone resorption, expression of cell adhesion molecules, sleep, anorexia, synthesis of collagenase and growth and differentiation of T and B cells (*Dinarello*, 1988). In intestinal disease states, IL-1 is also responsible for an increase of intestinal mucus production (*Han* et al., 1987), which may constitute a normal host defence response. In animal models of experimental colitis, IL-1β is predominantly produced by the enterocytes of the colonic mucosa (*Radema* et al., 1991), suggesting a central role of IL-1 in inflammatory bowel disease.

Two separate IL-1 molecules have been cloned, IL-1α and IL-1β, each one encoded by a separate gene on chromosome 2 (*Lomedico* et al, 1984; *Auron* et al., 1984). Although the two forms only share a 26% amino-acid homology, they have similar activities and bind to the same cellular receptor (*Urdal* et al., 1988). The administration of IL-1 to animals induces several systemic changes typical of inflammatory reactions, including fever, neutrophilia, synthesis of hepatic acute phase proteins, hypotension and increased corticosteroid production (*Dinarello*, 1989). In contrast, pretreatment with low doses of recombinant IL-1β (30-300 ng/kg) protects against death and tissue injury in animal models of Gram-negative infection (*van der Meer* et al., 1988), radiation (*Neta* et al., 1986), contact hypersensitivity (*Robertson* et al., 1987), arthritis (*Jacobs* et al., 1988) and hyperoxia (*White* et al., 1987). One explanation for these protective effects is an increase of endogenous prostaglandin levels (Cominelli et al., 1990).

TUMOUR NECROSIS FACTOR

A century ago, infection-induced haemorrhagic necrosis of tumours was described in man by *Coley* (1983). It took almost a century until the responsible factor, tumour necrosis factor (TNF, also known as cachectin), was independently isolated by two groups of investigators (*Aggarwal* et al., 1985; *Beutler* et al., 1985a; *Beutler* et al., 1985b). TNF is predominantly produced by monocytes and macrophages and has a still growing list of biological properties, including activation of polymorphonuclear leukocytes (PMN), increasing leukocyte/endothelial cell adhesion, cachexia, inhibition of lipoprotein lipase, tumoricidal activity, bone resorption, and procoagulant activity (*Sherry* and *Cerami*, 1988; *Tracey*, 1989; *Beutler* et al, 1985a; *Tsujimoto* et al., 1986). TNF was found to be produced in large quantities by some tumour cells, including the myelomonocytic cell line RAW 264.7 from which it was initially

isolated (*Beutler* et al., 1985a; *Mahoney* et al., 1985). TNF is encoded by a gene located on chromosome 6 and is synthesised as a 232 amino-acid peptide. Cleavage of a relatively long propeptide yields the secreted mature hormone (*Jones* et al., 1989). In volunteers, circulating TNF can be detected 30 minutes after endotoxin administration, reaching a peak at 90-120 minutes (*Jones* et al., 1989; *Michie* et al., 1988). In experimental animals, high doses of TNF cause a syndrome which is indistinguishable from septic shock, characterised by hypotension, metabolic acidosis, haemorrhagic infarction of the gastrointestinal tract, and release of catecholamines and glucocorticoids (*Tracey* et al., 1986). Sustained exposition to low and chronically administered doses of TNF causes a profound catabolic state that resembles the metabolic changes that are observed in patients with chronic inflammatory diseases, including anorexia and wasting, suppression of lipoprotein lipase (*Tracey* et al., 1987; *Oliff* et al., 1987), and a marked resorption of fat.

INTERLEUKIN-6

IL-6 was first detected in T cell supernatants as a helper T factor that was observed to induce immunoglobulin secretion by B cells (*Schimple* and *Wecker*, 1972; *Kishimoto* and *Ishizaka*, 1973). IL-6 is a potent inducer of the expression of acute phase proteins by hepatocytes (*Castell* et al., 1989), and has the capability of activating B and T lymphocytes. In addition, IL-6 causes haematopoietic stem cell growth, maturation of megakaryocytes, neural cell differentiation, mesangial cell growth and myeloid leukaemic cell differentiation. The human IL-6 gene is located on chromosome 7, and the secreted protein is glycosylated. A wide variety of cells may produce IL-6, but it is likely that in septicaemia macrophages and endothelial cells are its main source.

In summary, IL-6 has emerged as a major systemic acute signal-peptide which elicits a variety of host defence responses of the host.

INTERPLAY OF CYTOKINES

There is accumulating evidence of considerable interplay between IL-1, TNF and IL-6, and some of their functions overlap. TNF and IL-1 may reciprocally stimulate their synthesis *in vitro* as well as *in vivo* (*Le* and *Vilcek*, 1989) and both cytokines trigger the production of IL-6 (*Le* and *Vilcek*, 1989). In contrast, IL-6 impairs LPS-induced TNF and IL-1 production by mononuclear cells *in vitro* (*Aderka* et al., 1989; *Schindler* et al., 1990). Furthermore, IL-1, IL-6 and TNF all are endogenous pyrogens and trigger the liver to express genes for acute phase proteins. Both IL-1 and TNF, but not IL-6, induce the production of cycloxygenase and phospholipase A_2. Corticosteroids suppress both the transcription and translation of IL-1 and TNF as well as that of other cytokines. Both TNF and IL-1 are responsible for the recruitment of neutrophils to inflammatory sites by activation of several mechanisms. The first step in neutrophil recruitment is adhesion to endothelial cells, which is reflected by a transient precipitous fall in circulating neutrophil numbers in experimental endotoxaemia in humans (*van Deventer*, 1988). Both IL-1 and TNF

elaborate rapid expression of the adhesion molecules ELAM-1, GMP-140 and ICAM-1 on endothelial cells (*Bevilacqua* et al., 1989) that are involved in binding of neutrophils. These cytokines also cause expression of receptors, such as VCAM-1 and the VLA integrins, that mediate lymphocyte adhesion to endothelial cells (*Stoolman*, 1989).

NOVEL ENDOTOXIN-INDUCED CYTOKINES

Although many of the host-immune responses and inflammatory changes during septicaemia can be attributed to IL-1, IL-6 and TNF, it has become clear that other cytokine hormones participate in the overall host-defence response. Macrophages, when stimulated with LPS, release a multitude of induced proteins, many with yet undefined structures and functions (*Nathan*, 1987). Some biological activities that were previously ascribed to known cytokines may in fact result from the release of other mediators. For example, it has become clear that both *in vivo* neutrophil chemo-attractive potential of TNF and the neutrophil activating capability of IL-1 result from the induction of secondary protein mediators such as IL-8. IL-8 is a member of a class of small cytokines (SCY-family) that have similar cDNA sequences and genomic structure. Other small cytokines which belong to this SCY-family and which are now identified are the murine macrophage inflammatory proteins 1 and 2 (MIP1, MIP2), platelet factor 4 (PF4) and monocyte chemo-attractant protein 1 (MCP1). Most of these cytokines have only been recently identified and many of their biological properties remain unknown. It seems that IL-8 is importantly involved in the pathogenesis of septicaemia. IL-8 is generated by macrophages and endothelial cells after stimulation by LPS, TNF or IL-1, and is chemotactic for neutrophils, basophils and T cells but not for monocytes. In addition it is a potent stimulator of neutrophils. In rats, injection of recombinant IL-8 caused extensive recruitment and accelerated migration of neutrophils across high endothelial venules (*Larsen* et al, 1989).

GUT-DERIVED ENDOTOXINS IN SEPTICAEMIA

In critically ill patients endotoxins may transmigrate the gut mucosal lining, and cause additional activation of mediator systems. Surgical manipulation may further contribute to endotoxin release from the bowel. Endotoxaemia itself impairs glutamine metabolism, thereby causing a breakdown of the gut mucosal barrier (*Souba* et al., 1990), and "bowel rest" during total parenteral nutrition, results in enhanced endotoxin-induced splanchnic cytokine responses (*Fong* et al., 1989b). Thus, in various conditions in which critically ill patients are unable to tolerate enteral feeding, "intestinal endotoxaemia" may be an important pathophysiological entity (*van Deventer* et al., 1988a), and in this context the gut has been named the "motor of MOF" (multiple organ failure). We here briefly review studies on the transport of intestinal endotoxins and the mechanism and pathogenesis of endotoxin absorption.

DOES THE NORMAL BOWEL ABSORB ENDOTOXIN?

Large amounts of endotoxin are present in the gut, and even in germ-free animals the intestinal endotoxin concentration is substantial (*Rush* et al., 1989). A major point of dispute remains to what extent the normal intestine absorbs endotoxin. Early experimental studies showed an increase in radioactivity in the liver of rabbits (not in other organs) that were fed ^{32}P O111;B4 lipopolysaccharide (*Ravin* et al., 1960). However, no substantial transport of ^{51}Cr labelled bacterial lipopolysaccharide through the intact bowel could be detected (*Sanford* and *Noyes*, 1958). These results should be interpreted cautiously because radioactive label can dissociate from endotoxin, thereby causing false positive results. Using immunohistochemistry, lipopolysaccharides that were instilled into the proximal large bowel of normal rats were not detected in the bowel wall, nor in intestinal lymph nodes, peritoneal cavity, or liver sinusoids (*Schoeffel* et al., 1989) .

It has been the traditional view that endotoxins are normally present in human portal blood (*Pain* and *Bailey*, 1987; *Tachiyama* et al., 1988; *Lumsden* et al., 1988). In this view, Kupffer cells in the liver prevent systemic endotoxaemia by removal of endotoxins, which would explain the occurrence of systemic endotoxaemia in liver failure. For two reasons this hypothesis now appears to be incorrect. Firstly, although a high incidence of portal endotoxaemia in normal humans has been reported (*Prytz* et al., 1976; *Jacob* et al., 1977; *Pain* and *Bailey*, 1987; *Tachiyama* et al., 1988), in two recent prospective studies in consecutive patients without diseases of bowel or liver, no endotoxin could be detected in the portal or systemic circulation (*van Deventer* et al., 1988b; *Brearly* et al., 1985). Secondly, it is difficult to explain why Kupffer cells, that are main sources of TNF, IL-1, and other cytokines, do not release these mediators after stimulation with intestinal endotoxin, while these cells release substantial amounts of TNF following systemic administration of small amounts of endotoxin (*Fong* et al., 1989b). Presently, most investigators therefore agree that the intact bowel mucosa provides an effective defence barrier for endotoxin, and that endotoxin transmigration results from damage to the integrity of the bowel mucosa. Consequently, in liver failure, intestinal damage due to portal hypertension, rather than Kupffer cell depression, seems to induce endotoxin uptake from the gut.

INTESTINAL ENDOTOXAEMIA

Circumstantial evidence implicates many pathophysiological conditions, as potential causes of "intestinal endotoxaemia". Although the precise molecular mechanisms and kinetics of endotoxin transmigration in these circumstances remain to be elucidated, a decreased production of mucus and damage to the integrity of the bowel mucosa, resulting in disruption of tight junctions between mucosal cells, seem to be pathophysiologically important. Here, we briefly summarise clinical and experimental data that provide evidence for a pathophysiological role of gut-derived endotoxin in haemorrhagic shock, intestinal ischaemia, jaundice, bowel obstruction and other conditions.

Haemorrhagic shock and intestinal ischaemia

In rats, haemorrhagic shock rapidly results in systemic endotoxaemia and bacteraemia (87% and 50% respectively, after 2 hours) (*Rush* et al., 1988). The mucosal damage in haemorrhagic shock and its mechanism has been elegantly studied by *Deitch* and colleagues. Using horseradish peroxidase as a marker, they demonstrated an increased intestinal permeability in experimental haemorrhagic shock, that coincided with the appearance of subepithelial oedema and focal necrosis of the ileal and coecal mucosa. In addition translocation of bacteria, most commonly *Escherichia coli* and *Enterococcus* species, to mesenteric lymph nodes, liver and spleen was observed (*Baker* et al., 1988; *Deitch* et al., 1990a). Interestingly, systemic endotoxaemia itself also caused a significant bacterial translocation accompanied by disruption of mucosal barrier (*Deitch* et al., 1987; 1989a; *Navaratnam* et al., 1990; *O'Dwyer* et al., 1988). It appears that the mucosal damage in haemorrhagic shock is mediated by release of oxidants that are derived from the xanthine oxidase system. Inhibition of xanthine oxidase by oral administration of allopurinol or its inactivation by feeding a tungsten-supplemented molybdenum-free diet, resulted in significant decreases in bacterial translocation (*Deitch* et al., 1988; 1990b). One should keep in mind however that it is likely that bacterial translocation and endotoxin absorption are different processes, that may be induced by different stimuli.

Multiple studies have demonstrated that intestinal ischaemia is rapidly followed by systemic endotoxaemia (*Cuevas* and *Fine*, 1972; *Nozickova* et al., 1977; *Olofsson* et al., 1985). In rats, bowel ischaemia results in subsequent appearance of endotoxin in the thoracic duct, the portal vein, and the systemic circulation (*Olofsson* et al., 1985). Likewise, in dogs with superior mesenteric occlusion, a high endotoxin concentration may be observed (*Nozickova* et al., 1977). Thus in intestinal ischaemia, as well as in other conditions, the thoracic duct appears to be a major route of endotoxin uptake (*Daniele* et al., 1970; *Olofsson* et al., 1986; *Olofsson*, 1988). Disruption of the intestinal barrier in intestinal ischaemia may be caused by oxygen radicals that are formed during ischaemia, and released following reperfusion (*Schoenberg* and *Beger*, 1990).

Obstructive jaundice

The clinical finding that the incidence of postoperative renal impairment is particularly high in obstructive jaundice (*Bailey*, 1976; *Wilkinson* et al., 1976; *Cahill*, 1983), has prompted clinical and experimental studies on endotoxaemia as a pathogenic factor in this setting. Indeed, jaundiced patients or experimental rats have a high incidence of portal and systemic endotoxaemia (*Wilkinson* et al., 1976; *Cahill* et al., 1987; *Pain* and *Bailey*, 1987; *Blumgart*, 1988; *Thompson* et al., 1988; *van Bossuyt* et al., 1990; *Diamond* et al., 1990). It has been shown that in rats internal bile drainage, but not external drainage, protects against intestinal endotoxin uptake (*Gouma* et al., 1986). Although ligation of the common bile duct in mice leads to subepithelial oedema of the ileal villi and an increase in bacterial translocation (*Deitch* et al., 1990c) and jaundice may cause a depression of the mononuclear phagocytic system (*Drivas* et al., 1976), the absence of bile salts in the intestinal lumen therefore seems to be the pivotal factor for intestinal endotoxin uptake in jaundice. This hypothesis is supported by clinical studies as well as animal exper-

iments that demonstrated a reduced incidence of postoperative endotoxaemia and a decreased occurrence of postoperative complications after oral administration of bile salts (*Thompson* et al., 1986; *Cahill* et al., 1987; *van Bossuyt* et al., 1990). However, this hypothesis seems to be refuted by a recent report that demonstrated that both internal and external drainage reversed endotoxaemia and reduced mortality in obstructive jaundiced rats (*Diamond* et al., 1990).

Bowel obstruction

Bowel obstruction rapidly causes systemic endotoxaemia (*Roscher* et al., 1988), as well as an increase in bacterial translocation (*Deitch* et al., 1989b; 1990d). Intestinal endotoxaemia following bowel obstruction likely results from an increase in the endotoxin concentration as a consequence of bacterial overgrowth (particularly in the small intestine) (*Roscher* et al., 1988), and a decreased barrier function of the bowel mucosa (*Roscher* et al., 1988; *Deitch* et al., 1990d). The latter is possibly related to toxic effects of extremely high endotoxin concentrations on epithelial integrity (*Roscher* et al., 1988; *Walker* and *Provaznik*, 1978).

Inflammatory bowel disease

Severe inflammatory bowel disease may cause extensive ulceration of the small or large intestine, and may be complicated by systemic endotoxaemia (*Palmer* et al., 1980; *Fink* et al., 1988). Surprisingly, even in patients with severe damage to the bowel mucosa, septic shock is not frequently observed. It is possible that the continuous exposure to endotoxin in these patients causes tolerance to its biological effects. Alternatively, patients with inflammatory bowel disease may develop specific anti-endotoxin defence mechanisms. For example, patients with Crohn's disease frequently have high titres of anti-lipid A antibodies.

Other conditions

Protein malnourished mice are more susceptible to endotoxin-induced bacterial translocation than controls (*Li* et al., 1989). Endotoxaemia was present in 44% of severely malnourished Thai children, and correlated with the presence of vitamin A deficiency (*Klein* et al., 1988). Systemic endotoxaemia may also be induced in monkeys by heat stress, and the occurrence of endotoxaemia predicts mortality (*Gathiram* et al., 1987).

PHYSICAL BINDING OF ENDOTOXIN IN THE GUT

As discussed above, endotoxins are present in large quantities in the gut, derived from endogenous Gram-negative flora. In an experimental model of serotonin-induced intestinal endotoxaemia in mice, systemic endotoxaemia may be reduced by the use of kaopectate and charcoal particles that bind endotoxin in the gut. Lactulose and pectin, however, showed no effect on the endotoxaemia in the same study (*Ditter* et al., 1983). When lactulose was orally administered preoperatively to jaundiced patients, the level of postoperative portal and systemic endotoxaemia diminished (*Pain* and *Bailey*, 1986). Oral, nonresorbed antibiotics do not significantly reduce the amount of gut endotoxins, with the exception of polymyxin (*van der Waaij* et al., 1985). In fact, in mice a transient rise in the faecal endotoxin content was observed after selective bowel decontamination with streptomycin, neomycin and amphotericin B (*Rogers* et al., 1985). Prolonged administration of neomycin to humans may

however decrease the faecal endotoxin concentration. It is nevertheless difficult to explain the finding that in experimental animals kanamycin protects against endotoxaemia induced by vaso-active amines or bowel ischaemia (*Ravin* et al., 1960). Bile salts have been reported to reduce intestinal uptake of endotoxins in patients with obstructive jaundice (*Thompson* et al., 1986), and preoperative administration of sodium deoxycholate to these patients has been shown to prevent portal endotoxaemia (*Cahill*, 1983). In other conditions however, in particular septicaemia, there is yet no direct proof for the clinical usefulness of any drug that interferes with intestinal endotoxin uptake.

TREATMENT STRATEGIES FOR SEPTICAEMIA

In the last decade, numerous studies have led to different (immuno)therapeutical approaches in septicaemia. A key finding in immunotherapy for Gram-negative septicaemia was the demonstration of therapeutic efficacy of antibodies that bind epitopes on the endotoxin core or the lipid A component in animal models of endotoxaemia and Gram-negative infection. The development of this strategy began with the production of polyclonal antisera to endotoxin core by immunising rabbits with heat inactivated mutants of *S. typhimurium* TV119, *E. coli* O111:B4 and *S. minnesota*. Because these mutants lack the immunodominant O-polysaccharides, they allow antisera to be raised against the conserved inner core determinants. Antisera obtained from immunised rabbits conferred passive protection against the dermal Shwarzman reaction caused by endotoxin derived from a wide variety of Gram-negative organisms (*Braude* and *Douglas*, 1972) and protected mice and neutropenic rabbits from lethal bacteraemia due to *E. coli*, *Klebsiella*, *Pseudomonas* and *Proteus* (*Tate* et al., 1966; *Chedid* et al., 1968; *McCabe* and *Greely*, 1972; *Ziegler* et al., 1973). Subsequently, a human polyclonal antiserum to endotoxin was developed by immunising human volunteers with the heat inactivated *E. coli* J5 mutant. In one large clinical trial, treatment resulted in a marked reduction in mortality in patients with objectively documented Gram-negative bacteraemia (*Ziegler* et al., 1982), and prophylactic use of the antiserum prevented mortality due to septic shock and reduced the morbidity in surgical patients at high risk for developing Gram-negative infections (*Ziegler* et al., 1982; *Baumgartner* et al., 1985). In patients with prolonged neutropenia however, prophylactically administered polyclonal anti-J5 serum did not reduce the number of Gram-negative bacteraemic episodes (*McCutchan* et al., 1983). For several reasons, including the variability on antibody titres and the risk of transmission of infectious agents, administration of immune serum does not seem to be practical in septic patients. Monoclonal antibodies have constant and established specificities, and can be produced in large amounts. The results from recently published large multicentre trials employing monoclonal anti-endotoxin antibodies directed against the J5 mutant of *E. coli* showed a remarkable reduction in the 28 day mortality of 37-39% in Gram-negative bacteraemic patients and 39-42% in patients with septic shock (*Gorelick* et al., 1990; 1990b; *Ziegler* et al., 1991). There were no adverse effects that could be attributed to the treatment. Thus, the efficacy and safety of monoclonal antibodies to endotoxin seems established and they

therefore have the potential of becoming the immunotherapeutic drug of choice in clinical medicine in the treatment of Gram-negative septicaemia. A major problem in treating septic patients is case selection. Of all patients that are clinically diagnosed as having Gram-negative septicaemia, only one third has Gram-negative bacteraemia, and one third is endotoxaemic. It is tempting to speculate that a rapid method of bedside endotoxin determination would be of help in selecting patients for immunotherapy .

OUTLOOK FOR FUTURE TREATMENTS

The protective effects of TNF-antibodies were first established with a polyclonal rabbit antiserum against murine-TNF. This experiment showed a significant protection in a lethal endotoxaemia model in mice (*Beutler* et al., 1985c). Subsequent investigations in rabbits showed similar results (*Mathison* et al., 1988). Strong evidence was provided by a study which showed that monoclonal anti-TNF antibodies protected against the development of septic shock and lethal tissue injury in acutely bacteraemic baboons (*Tracey* et al., 1987). Large, multicentre trials using anti-TNF antibodies as the immunotherapeutic drug for septicaemia are currently underway. Clearly, TNF is not the only inflammatory mediator, and recently it has been shown that rabbits can be protected from the lethality of endotoxaemia by pretreatment with recombinant interleukin-1 receptor antagonist (*Wakabayashi* et al., 1991). All these treatment strategies are exciting, but their clinical efficacy has yet to be proved.

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THE RELEASE OF ENDOTOXIN AND INTERLEUKIN-6 DURING TREATMENT WITH DIFFERENT ANTIBIOTICS ALONE OR IN COMBINATION WITH TAUROLIDINE OF RATS WITH EXPERIMENTAL GRAM-NEGATIVE SEPSIS

A.S.M. DOFFERHOFF[1*], H.L. BARTELS[2], H.G. DE VRIES-HOSPERS[3], J.H. NIJLAND[1], J. BIJZET[1], V.J.J. BOM[1], P.O.M. MULDER[1], J. WEITS[1], D. VAN DER WAAIJ[3], and R.P. BLEICHRODT[4]

[1]Department of Internal Medicine, [2]Central Animal Laboratory, [3]Laboratory of Medical Microbiology, and [4]Department of General Surgery, University Hospital Groningen, Groningen, The Netherlands.

SUMMARY

To evaluate the role of different antibiotics in the release of endotoxin and the production of interleukin 6 (IL-6) during the treatment of experimental *Escherichia coli* septical peritonitis, we obtained serial blood samples from septic rats treated with placebo, ceftazidime, aztreonam or imipenem. We also studied the effect of taurolidine, given alone or in combination with aztreonam, on the release of endotoxin and IL-6. In rats treated with placebo or taurolidine, we demonstrated a correlation between viable *E. coli* counts and the levels of free endotoxin and IL-6. Despite decreasing levels of viable *E. coli* counts after treatment with ceftazidime, aztreonam or imipenem, levels of free endotoxin increased in all animals. We did not notice any significant differences in the extent of endotoxin release between the different treatment groups. However, we did find significant differences in the IL-6 production between the different treatment groups. After two hours of treatment, IL-6 levels had increased in all animals with the highest levels in the imipenem treated animals, whereafter IL-6 levels decreased again in the rats treated with imipenem or ceftazidime. IL-6 levels further increased in the rats treated with placebo or aztreonam. The increase in IL-6 levels was associated with poor outcome. The increase in IL-6 levels in the aztreonam treated animals is thought to be the result of the formation of long bacterial filaments in the abdominal cavity. In the present study, treatment with taurolidine could not prevent or inhibit the release of endotoxin or IL-6. Unexpectedly, treatment with taurolidine alone or in combination with aztreonam caused a dramatic increase in IL-6 levels, which was associated with an increased mortality. We conclude that antibiotics can cause the release of endotoxin in spite of decreasing levels of bacteraemia *in vivo*. We have demonstrated an antibiotic type-dependent increase in plasma IL-6 levels, and we found an association between the level of IL-6 and mortality.

*Present address: Department of Internal Medicine, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands.

INTRODUCTION

The mortality rate due to severe Gram-negative sepsis and in particular that associated with shock, is still up to 50% in spite of appropriate antimicrobial therapy and optimum supportive care (*Kreger* et al., 1980). From the beginning of the antibiotic era it has been suggested that in some circumstances shock might be precipitated by bacterial cell lysis and the sudden release of endotoxin (LPS) from Gram-negative bacteria exposed to antibiotics (*Galpine*, 1949; *Spink* et al., 1948; *Hopkin*, 1977; 1978; 1985). These findings are supported by *in vitro* studies that have shown an enhanced endotoxin release from Gram-negative bacteria after treatment with antibiotics (*Cohen* and *McConnell*, 1985; 1986; *McConnel* and *Cohen*, 1986; *Dofferhoff* et al., 1991a). In addition, animal studies have shown that antibiotic therapy for experimental Gram-negative sepsis and/or meningitis can promote endotoxin release *in vivo* (*Shenep* et al., 1985; *Tauber* et al., 1987; *Rokke* et al., 1988; *Andersen* and *Solberg*, 1984). Recently, also antibiotic-induced endotoxin liberation during the treatment of human septicaemia has been demonstrated (*Shenep* et al., 1988; *Dofferhoff* et al., 1991a). Endotoxin toxicity is considered to be largely mediated by the monocyte/macrophage derived tumour necrosis factor-α (TNF) (*Michie* et al., 1988; *Waage* et al., 1989; *Cannon* et al., 1990; *Michalek* et al., 1980; *Tracey* and *Lowry*, 1990). It is also known that endotoxin can further promote the lethal effects of TNF suggesting that both factors determine the clinical outcome (*Rothstein* and *Schreiber*, 1988). It has also been suggested that IL-6, either by itself or by interactions with TNF, interleukin-1 (IL-1) or LPS, is involved in the pathogenesis of septic shock (*Waage* et al., 1989; *Fong* et al., 1989; *Jirik* et al., 1989; *Tracey* and *Lowry*, 1990).

In a previous study (*Dofferhoff* et al., 1991a), we have demonstrated the release of endotoxin from *Escherichia coli* by several newer (β-lactam) antibiotics nowadays frequently used in the management of patients with septic shock and found an antibiotic type- and dose dependent increase in the release of endotoxin from an *in vitro* culture of *E. coli.* In a subsequent study, we have analysed the influence of antibiotic-induced release of endotoxin from *in vitro* cultured *E. coli* on TNF production by human monocytes and also found an antibiotic type- and dose dependent increase in TNF production by monocytes as the result of antibiotic-induced release of endotoxin (*Dofferhoff* et al., 1991b). To study the release of endotoxin and the cytokine IL-6 *in vivo*, we analysed the release of these mediators in rats treated for an experimental Gram-negative sepsis with different antibiotics. Also the influence of treatment with taurolidine, an endotoxin-binding agent, alone or together with antibiotic treatment, was evaluated in the present study.

MATERIALS AND METHODS

Organisms

For the endotoxin release studies *E. coli* strain ATCC 25922 was used. Organisms were stored at -70˚C and prior to use subcultured onto blood agar. Subsequently one colony was inoculated into 9 ml Brain Heart Infusion (BHI) broth (Difco laboratories) and incubated at 37˚C overnight. This overnight culture was centrifuged at 5,000

RPM for 15 minutes. The supernatant was discarded and the bacteria were resuspended in 9 ml fresh BHI broth and incubated at 37˚C for another two hours. This suspension was centrifuged at 5,000 RPM for 15 min., the supernatant was discarded and the bacteria were resuspended into 8 ml sterile NaCl 0.9%. The MIC's for this *E. coli* strain were as follows: ceftazidime, 0.25 mg/l; aztreonam, 0.125 mg/l; imipenem, 0.0675 - 0.125 mg/l.

Antibiotics

Antibiotics used were ceftazidime (Glaxo BV, Nieuwegein, The Netherlands), aztreonam (Squibb BV, Rijswijk, The Netherlands), and imipenem/cilastatin (Merck, Sharp & Dome BV, Haarlem, The Netherlands). Also taurolidine 2% (Multipharma BV, Weesp, The Netherlands), an antibacterial agent with anti-endotoxin properties was used. This solution was made isotonic through the addition of 5 ml of a 50% glucose solution.

Sepsis model and experimental protocol

Male Wistar rats (200 - 300 g), locally bred at the Central Animal Laboratory of the University of Groningen, were used throughout the study. During the experiments, the animals were allowed to drink and eat *ad libitum* and were housed at 21˚C in labelled cages. Prior to the start of the experiment, the bacterial suspension was divided into four equal aliquots. The challenge dose for the different pairs of rats ranged from 2 to 5 x 10^9 CFU of *E. coli*. One aliquot (2 ml) was administered intraperitoneally (i.p.) to each of the paired rats. Two hours after the bacterial challenge four rats were randomly assigned to receive either 1 ml sterile NaCl 0.9% i.v., 200 mg/kg ceftazidime or aztreonam in NaCl 0.9% (1 ml infusion solution) i.v., or 50 mg/kg imipenem in NaCl 0.9% (1 ml) intra-veneously (i.v.). It was not possible to administer imipenem in a dose of 200 mg/kg because precipitation occurred above a concentration of 10 mg/ml. These experiments were repeated four times and were performed on five different days. In the experiments with taurolidine, 1 ml of isotonic 2% solution of taurolidine was administered i.v. as a single agent or together with aztreonam. The experiments with taurolidine were performed in quadruplicate. The antibiotics were given as a bolus i.v. injection, while taurolidine was administered i.v. hourly. For the administration of the drugs the tail vein or the penile vein was used. It was verified that the plasma concentrations of the antibiotics two hours after the bolus injection were >10 mg/l (data not shown). Heart blood samples were taken aseptically from anaesthetised animals just before the administration of the antibiotics (t = 0) and 2, 4, and 6 hours after the administration of the drugs. From each of the rats, 1 ml of blood was transferred into pyrogen free Falcon tubes (Becton Dickinson, New Jersey, USA) containing 50 units of heparin (Leo Pharmaceutical Products BV, Weesp, The Netherlands). These tubes were immediately immersed in melting ice. Plasma was prepared at 4˚C by centrifugation at 2,000 RPM for 10 min. Plasma samples of 0.1 ml were removed for the IL-6 assay. For the endotoxin assay, 0.1 ml plasma was transferred to pyrogen free Falcon tubes containing 0.9 ml pyrogen free NaCl 0.9% and thoroughly mixed. Free endotoxin was separated from bacterial cell-bound endotoxin by filtration of an aliquot of each sample through a 0.45 μm pyrogen free filter (Millex HA, Millipore SA, Molsheim, France). All samples were stored at -80˚C. At the end of the experiment (the time of death or 6 hours after the administration of the antibiotics) the abdomi-

nal cavity was opened and fluid was aspirated for microscopical examination. During the administration of the drugs and during the heart punctures, the animals were anaesthetised with halothane.

Viable counts

Twenty-five μl aliquots of the whole blood or the dilutions were plated onto blood agar in duplicate and colony counts were done after overnight incubation. The minimal detectable number of CFU/ml was 40.

Endotoxin assay

Endotoxin was measured using the chromogenic limulus amoebocyte lysate (LAL) assay (KabiVitrum BV, Amsterdam, The Netherlands) according to the prescriptions of the manufacturer and adapted to a microtiter scale with minor modifications. In brief, 25 μl aliquots of the test sample were added in duplicate to 25 μl LAL in a pyrogen free microtiter tray. After incubating at 37˚C for 10 min., 50 μl of a prewarmed chromogenic substrate (S 2423)-buffer solution was added. After incubating at 37˚C for 4 min., colour development was terminated by addition of 100 μl 20% acetic acid. The optical density was measured at 405 nm and the endotoxin level calculated from a calibration curve using *E. coli* 0111:B4 as the standard (12 EU/ml = 1 ng/ml). Appropriate standards and controls were included within each assay. The between-assay coefficient of variation was 4.7%. Detection level was 0.05 EU/ml.

IL-6 assay

The biologic activity of IL-6 was determined using an IL-6 dependent cell line B9 as previously described (*Helle* et al., 1988) (control values < 5 U/ml).

Statistical analysis

Differences between variables were tested with the Wilcoxon signed rank test or the Mann-Whitney U test. Differences between treatment groups were tested by the Kruskal-Wallis analysis by ranks. Correlations were analysed with the Spearman rank correlation test. A p-value < 0.05 (two-tailed) was considered statistically significant. Viable counts, endotoxin levels and cytokine levels are expressed as median values.

RESULTS

Viable counts

The viable counts of control and antibiotic treated rats of E. *coli* ATCC 25299 at different time intervals are shown in Figure 1. Of the 28 rats tested, 24 rats had a positive blood culture with *E. coli* two hours after the i.p. administration of the bacteria (median level for the whole group: 3×10^3 cfu/ml, range: 0 - 5×10^4 cfu/ml). In control rats there was during the following 6 hour period (or until the time of death) a significant increase in viable counts from 4×10^3 cfu/ml (range: 0 - 14×10^3) to 2×10^5 (range: 0.4 - 12×10^5) cfu/ml. Two hours after the administration of the different antibiotics the median levels of viable organisms were significantly decreased ($p < 0.05$), except for taurolidine monotherapy. In the animals treated with ceftazidime, viable counts slightly increased after two hours of treatment from 20 cfu/ml (range: 0 - 280) to 160 cfu/ml (range: 0 - 700). In the animals treated with aztreonam alone or in combination with taurolidine, viable counts decreased persistently until the animals died (2 animals

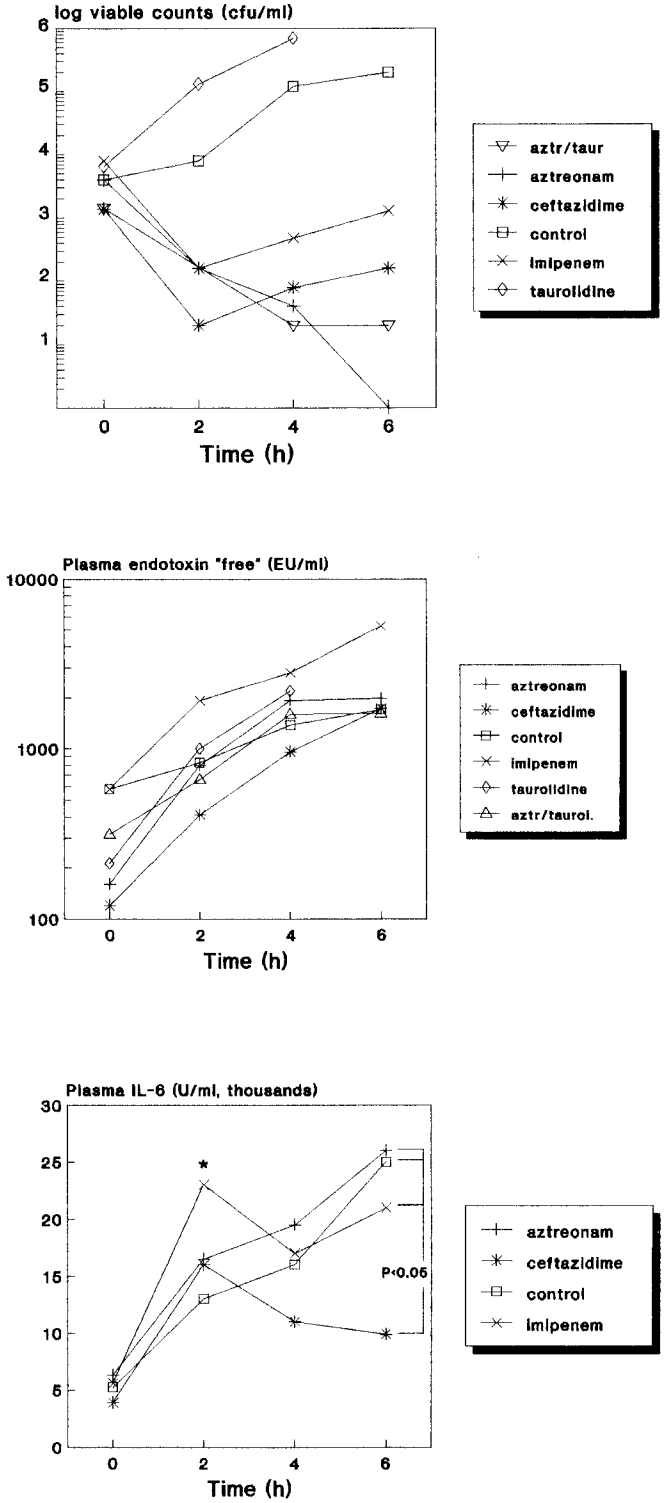

Figure 1: Median levels of viable counts (top), free endotoxin (middle) and plasma IL-6 (bottom) during the treatment of experimental Gram-negative sepsis ($^{*}p < 0.05$).

in the aztreonam group and 3 animals in the aztreonam/taurolidine group) or until the end of the study period. Median levels at the end of the study period were 0 cfu/ml (range: 0 - 40) and 20 cfu/ml (range: 0 - 40), respectively. In the imipenem treated animals, viable counts started to increase again after two hours of treatment, probably as the result of the lower initial dose and the short half-life of this drug in animals. Taurolidine monotherapy resulted in a steady and significant increase in viable counts up to 7×10^5 cfu/ml (range: 0 - 1×10^6) (Figure 1) at 4 hours of treatment. Six hours after the first injection of taurolidine only one animal was still alive. After 2 and 4 hours of treatment viable counts were significantly higher in the taurolidine treated animals than in the control rats ($p < 0.05$).

Plasma levels of free endotoxin during antibiotic treatment

Two hours after the i.p. injection of *E. coli* plasma levels of free endotoxin were detectable in all rats (median level free endotoxin: 223 EU/ml, range: 0.2 - 1,710 EU/ml) (Figure 1). Plasma levels of free endotoxin increased during the 6 hours following the administration of placebo or antibiotics in all but one animal. The maximal increases in the levels of free endotoxin in the control rats varied from four-fold to 15-fold. Despite the decreases in viable counts, in all but one rat treated with either ceftazidime, aztreonam or imipenem, plasma levels of free endotoxin increased significantly during the treatment period ($p < 0.05$) (Figure 1). During this period, there were no statistically significant differences in the levels of free endotoxin between the different treatment groups (Figure 1). In the rats treated with antibiotics, maximal increases in the levels of free endotoxin varied from two-fold to 25-fold. In one rat treated with aztreonam blood cultures were sterile during the entire study period. In this rat plasma levels of free endotoxin decreased from 8.5 EU/ml on admission to 3 EU/ml 6 hours after the administration of the antibiotic.

In the rats treated with taurolidine alone, plasma levels of free endotoxin also significantly increased during the treatment period from a median level of 212 EU/ml (range: 0.2 - 276 EU/ml) to 2175 EU/ml (range: 1 - 3,600 EU/ml). During the study period the levels of free endotoxin in the taurolidine treated rats did not significantly differ from the levels of free endotoxin in the control rats. In the rats treated with a combination of aztreonam and taurolidine, plasma levels of free endotoxin were not different from those in rats treated with taurolidine or aztreonam alone (Figure 1). In none of the different groups we found an association between the levels of free or total endotoxin and survival.

Levels of interleukin-6 during antibiotic treatment

Two hours after the bacterial challenge, plasma levels of IL-6 were increased in all animals, median level 4850 U/ml (range: 300 - 12,000 U/ml). In control animals, IL-6 levels significantly ($p < 0.05$) increased during the study period from 5,250 U/ml (range: 3,000 - 6,600 U/ml) to 25,000 U/ml (range: 2,600 - 38,000 U/ml) (Figure 1). In both control animals and antibiotic treated animals, after two hours of treatment there was an increase in median IL-6 levels (Figure 1). This initial rise in IL-6 levels between 0 and 2 hours after the administration of the drugs, was significantly higher in the imipenem treated group than the control rats ($p < 0.05$). After 2 hours of treatment with ceftazidime, median plasma levels of IL-6 decreased from 16,000 U/ml (range: 7,600 - 25,000 U/ml) at two hours to 11,000 U/ml (range:

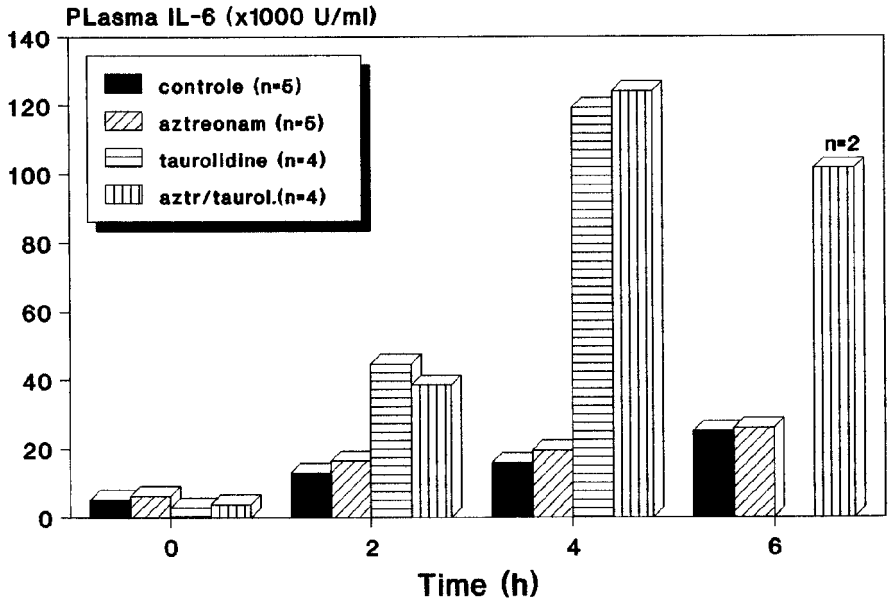

Figure 2: Median plasma levels of IL-6 during the treatment of septic rats with placebo and aztreonam, and the influence of the administration of taurolidine.

7,800 - 15,000 U/ml) at 6 hours of treatment (Figure 1). In the imipenem treated animals also a decrease was seen between 2 and 4 hours of treatment, however between 4 and 6 hours of treatment with imipenem IL-6 levels started to rise again from 17,000 U/ml (range: 9,700 - 31,000 U/ml) at 4 hours to 23,000 U/ml (range: 11,000 - 25,000 U/ml) at 6 hours (Figure 1). Treatment with aztreonam resulted in a significant increase in median plasma levels of IL-6 from 6300 U/ml (range: 5,100 - 7,100 U/ml) at the start of the treatment to 26,000 U/ml (range: 15,000 - 29,000 U/ml) at the time of death or at 6 hours of treatment (Figure 1). At the end of the study period median IL-6 plasma levels were significantly higher ($p < 0.05$) in the control animals and the aztreonam treated animals than the ceftazidime treated animals.

In the animals treated with taurolidine, alone or in combination with aztreonam, we noticed a dramatic increase in the plasma IL-6 levels from 2,970 (range: 300 - 5,260 U/ml) to 119,200 U/ml (range: 105,000 - 200,000 U/ml) in the taurolidine group and from 3,850 U/ml (range: 2,600 - 7,200 U/ml) to 101,550 U/ml (range 3,100 - 200,000 U/ml) in the rats treated with a combination of aztreonam and taurolidine (Figure 2). The increases in plasma IL-6 levels in the taurolidine group and the aztreonam/taurolidine group were significantly higher than in the control group and the aztreonam group, respectively ($p < 0.05$) (Figure 2).

The kinetics of IL-6 production was associated with survival. In the whole study group IL-6 levels increased from t=0 to 2 hours of treatment whereafter in the surviving animals IL-6 levels stabilised, while in the non-surviving animals IL-6 levels increased up to the time of death (Figure 3).

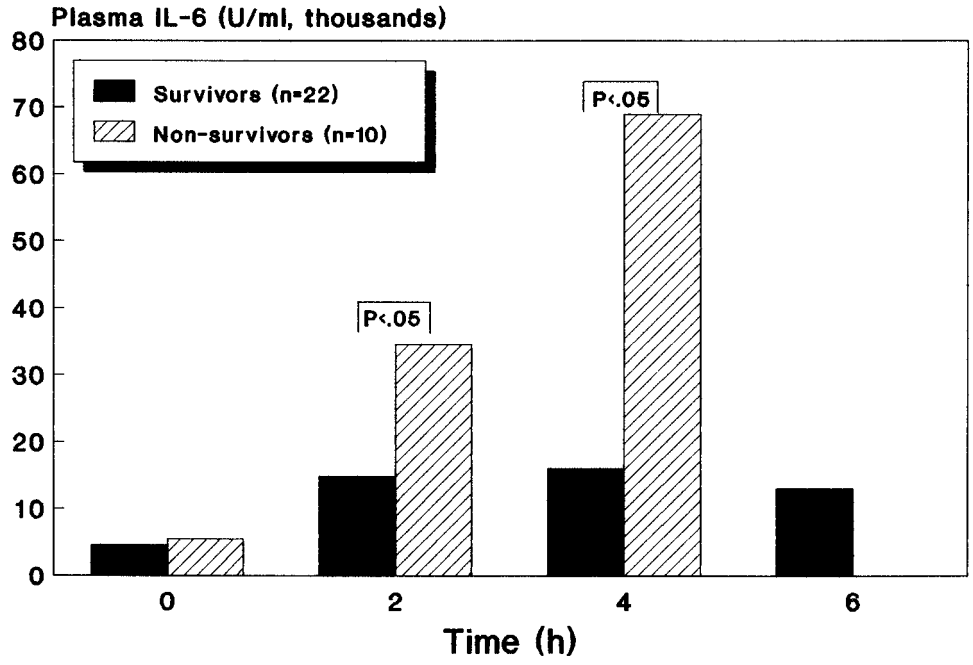

Figure 3: Plasma IL-6 levels in septic rats, surviving versus non-surviving animals.

Correlations between viable counts, plasma levels of free endotoxin and plasma IL-6 levels in septic rats

At the start of the treatment, we found a strong correlation between viable counts and the levels of free endotoxin ($r=0.82$, $p < 0.001$). There was also a correlation between the plasma levels of IL-6 and viable counts ($r=0.51$, $p=0.01$), and between levels of IL-6 and levels of free endotoxin ($r=0.53$, $p < 0.01$). During the 6 hours following the administration of saline in the control animals, we found a correlation between viable counts and the levels of free endotoxin ($r=0.88$, $p < 0.001$) and between levels of IL-6 and free endotoxin ($r=0.87$, $p < 0.001$). In the animals treated with taurolidine alone, similar correlations were found. In the animals treated with ceftazidime or imipenem only a weak correlation was found between IL-6 levels and the levels of free endotoxin, while in the rats treated with aztreonam such a relation was not found.

Mortality

Of the 28 animals tested, 10 died before the end of the study period (= 6 hours after the administration of the antibiotics or the saline). Three out of the five animals treated with saline died at 5 hours of treatment. Two of the five animals treated with aztreonam died at 4 and $5^1/_4$ hours of treatment, respectively. Treatment with taurolidine or the combination taurolidine with aztreonam resulted in death of 3 and 2 animals, respectively, at approximately 4 hours of treatment. In the animals treated with either ceftazidime or imipenem, no mortality occurred.

Morphological studies

In the animals treated with saline normal Gram-negative bacteria were

seen in the abdominal fluid, while after treatment with ceftazidime only cellular debris was seen. After treatment with imipenem we noticed cellular debris together with normal looking Gram-negative bacteria. Treatment with aztreonam resulted in the formation of long filaments of non-septating bacteria.

DISCUSSION

In the present study we demonstrate the release of free endotoxin during the treatment with both placebo or different antibiotics of rats with experimental *E. coli* septical peritonitis. In the control rats, the levels of free endotoxin remained more or less proportional to the level of blood viable counts as observed in previous studies (*Shenep* et al., 1985; *Dofferhoff* et al., 1991a). Median levels of free endotoxin increased concordantly after the administration of the different antibiotics in spite of decreasing levels of bacteraemia. Although in the imipenem treated animals levels of endotoxin tended to be highest, we found no significant differences in the extent of endotoxin release between the different antibiotic treated groups, the taurolidine group or the control group. However, we did find significant differences in the IL-6 production between the different treatment groups. All animals had elevated IL-6 levels at the start of the therapy, and median IL-6 levels correlated with the level of viable counts and the level of endotoxin. In the control group and in the rats treated with taurolidine alone, the level of IL-6 remained proportional to the level of viable counts and the level of endotoxin.

In the antibiotic treated groups there was a rise in IL-6 levels from the start of the treatment to two hours after the start of the antibiotic treatment. At this time, imipenem had caused the greatest rise in IL-6. From this point IL-6 levels started to decrease again in the imipenem and ceftazidime treated rats, while in the rats treated with aztreonam IL-6 levels further increased. The increase in IL-6 levels in the imipenem group between 4 and 6 hours after the start of the therapy is probably the result of an increase in viable counts. The difference in IL-6 production between aztreonam treated rats and the rats treated with either ceftazidime or imipenem is unexplained, but may be related to the formation of bacterial filaments in the aztreonam treated animals. The antibiotics used in this study cause different modes of bacterial cell damage by binding to and inhibition of the different penicillin-binding proteins (PBP's). The three penicillin-binding proteins of *E. coli* that are essential for antibacterial activity have been assigned PBP-1, PBP-2, and PBP-3. It has been shown that inhibition of PBP-1 is associated with rapid killing and lysis, whereas inhibition of PBP-2 produces spherical non-growing cells and that of PBP-3 produces long filaments (*Neu*, 1985; *Tuomanenet* al., 1986).

In previous studies, we have demonstrated that treatment of *in vitro* cultured *E. coli* with ceftazidime (high dose) or imipenem resulted in the formation of non-growing spherical cells and rapid bacterial cell lysis, which resulted in a rapid increase in the levels of free endotoxin, while treatment with aztreonam (or cefuroxime) resulted in the formation of long filaments, which resulted in a more gradual but much higher increase in the levels of free and total endotoxin. The observed effects of cefuroxime and aztreonam and low dose ceftazidime are consistent with their reported binding to PBP-3, while the effects of ceftazidime (high dose) and

imipenem are consistent with binding to PBP-1 and PBP-2, respectively (*Neu*, 1985; *Hanberger* et al., 1990). In the present study, the higher initial rise in IL-6 levels in the imipenem group may be the result of rapid cell lysis within the abdominal cavity, while the rise in IL-6 levels between 4 and 6 hours of therapy probably results from the increase in viable counts due to low antibiotic concentrations. The persistent rise in IL-6 levels in the aztreonam treated groups may be the result of the formation of long filaments of non-septating bacteria within the abdominal cavity. These long filaments may have caused the prolonged activation of the cytokine cascade. In a previous study (*Dofferhoff* et al., 1991b) we have demonstrated that treatment with antibiotics that cause the formation of long filaments, like aztreonam or cefuroxime can cause higher levels of TNF from *E. coli* stimulated human monocytes than treatment with drugs that cause rapid cell lysis or the formation of non-growing spherical cells like ceftazidime or imipenem.

We have also studied the effects of the endotoxin-binding agent taurolidine in the management of Gram-negative sepsis. *Thomas* and colleagues (1985) demonstrated a dose dependent inactivation of endotoxins by taurolidine. They showed that the endotoxin inactivation by taurolidine was irreversible and that there was no interaction of taurolidine with the chromogenic limulus lysate assay they used. We have also studied the effects of taurolidine on the production of TNF by *E. coli* human monocytes and demonstrated a decrease in TNF production by these monocytes as the result of a nearly complete neutralisation of the endotoxin released upon treatment with aztreonam and imipenem and that this neutralisation lasts for at least 24 hours. In the present study we found no influence of the administration of taurolidine on the release of endotoxin but did find a dramatic increase in IL-6 levels upon treatment with taurolidine, alone or in combination with aztreonam, which was associated with an increased mortality.

From the beginning of the antibiotic era it has been suggested that antibiotics may cause the massive liberation of endotoxins in the bloodstream as the result of bacterial lysis and this may actually aggravate the endotoxin shock (*Galpine*, 1949; *Spink* et al., 1948; *Hopkin*, 1977; 1978; 1985). So far, the endotoxin liberating effect of penicillin (*Andersen* and *Solberg*, 1984), moxalactam (*Shenep* et al., 1985), cefotaxime (*Tauber* et al., 1987), ceftriaxon (*Mustafa* et al., 1989), cefuroxime, ceftazidime, aztreonam and imipenem (*Dofferhoff* et al., 1991a; 1991b), the aminoglycosides kanamycin (*Johnston* and *Greisman*, 1984), tobramycin (*Dofferhoff* et al., 1991a; 1991b) and gentamicin (*Shenep* et al., 1985; *Rokke* et al., 1988), several quinolones (*Cohen* and *McConnell*, 1986; 1985; *McConnel* and *Cohen*, 1986) and chloramphenicol (*Shenep* et al., 1985; *Tauber* et al., 1987; *Dofferhoff* et al., 1991a) has been studied *in vitro*. There are, at present, little data on the effect of antibiotic treatment on the release of mediators like TNF and/or IL-6 *in vivo*. In rabbits with experimental *Haemophilus influenzae* type b meningitis, treatment with ceftriaxon resulted in increased concentrations of endotoxin as well as TNF in the cerebrospinal fluid, which was associated with an enhanced meningeal inflammatory response (*Mustafa* et al., 1989a). *Mustafa* and colleagues (1989b) also demonstrated that in children with Gram-negative meningitis treated with a combination of ampicillin intravenously and gentamicin intraventricular, cerebro-spinal fluid concentrations of endotoxin, interleukin-1β were significantly higher than

in children treated with ampicillin i.v. alone. In these children high levels of IL-1β were associated with poor outcome.

In conclusion, we have demonstrated antibiotic-induced release of endotoxin from *E. coli* by the different antibiotics *in vivo*. We also found an antibiotic type dependent release of IL-6 during the treatment of experimental Gram-negative septic peritonitis, in which high levels of IL-6 were associated with mortality. These data suggest that the effects of antibiotic treatment on the release of endotoxin from Gram-negative bacteria and the subsequent increase in the release of other mediators like IL-6 (and probably TNF and IL-1 as well) may be of clinical importance. In this study, the addition of taurolidine could not prevent the release of endotoxin and/or IL-6, but rather enhanced IL-6 production.

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NATURAL AND ARTIFICIAL LIPOPROTEINS MODULATE LIPOPOLYSACCHARIDE-INDUCED TUMOUR NECROSIS FACTOR PRODUCTION *IN VITRO* AND *IN VIVO*

JAN EVA DORAN, PETER LERCH, and ALPHONSE P. HUBSCH

Central Laboratory, Swiss Red Cross Blood Transfusion Service, Bern, Switzerland

SUMMARY

The role of serum and serum proteins in lipopolysaccharide (LPS) binding and detoxification has been studied extensively during the course of the last decade. It is now well established that lipoproteins are able to react with and modify LPS functional activity. We have prepared and tested a spectrum of natural as well as artificial lipoproteins for their ability to modulate LPS-induced tumour necrosis factor (TNF) production *in vitro*. Our experiments show that the natural lipoproteins had a pronounced influence on TNF production, decreasing it by as much as 33%. However, these effects were easily surpassed through the use of artificially constructed lipoproteins. Lipoproteins prepared by the admixture of apolipoprotein A-I (the major protein component of high density lipoprotein) and phosphatidylcholine (designated ApoLipo) were far more efficacious in reducing TNF production *in vitro* than were the natural lipoproteins (TNF reductions >85% were achieved). The supplemental addition of triglycerides or cholesterol to the artificial lipoprotein mixture further diminished TNF production in a dose dependent manner. ApoLipo's influence on TNF production *in vitro* did not require precomplexation with LPS, and was observable even after LPS stimulation had begun.

We have also tested ApoLipo (apoA-I: Lipid at a 1:200 molar ratio) in an *in vivo* rabbit endotoxin shock model. ApoLipo dose dependently reduced LPS-induced TNF production, and attenuated the metabolic and cellular changes associated with endotoxin shock.

INTRODUCTION

The experimental infusion of purified bacterial lipopolysaccharide (LPS) into animals in sufficient doses results in the development of pathophysiological changes, collectively known as endotoxin shock. Manifestations of endotoxin shock include fever, hypotension, alterations in cellular adhesiveness and activation leading to leukopenia and thrombocytopenia as well as metabolic disturbances (e.g., acidosis) and disseminated intravascular coagulation (DIC). If sufficient in magnitude and duration, these pathophysiological changes may lead to multiple organ failure and ultimately to death. In small doses, the injection of LPS stimulates immune and phagocytic system function, resulting in an increased resistance to subsequent challenge.

Although LPS can interact directly with host systems, it mediates most of

its toxic and also its beneficial effects by stimulating the release of mediators, principally tumour necrosis factor (TNF or cachectin) from host cells. The kinetics of LPS induced TNF in plasma are virtually identical between species: Plasma TNF levels peak at 90 - 120 minutes post infusion of LPS, returning to baseline by 4 to 6 h (*Mathison* et al., 1988; *Beutler* et al., 1985; *Hinshaw* et al., 1990; *Feuerstein* et al., 1990; *Michie* et al., 1988). The evidence that TNF is one of the major mediators in the pathogenesis of endotoxin shock comes from two lines of investigation. The first is that the infusion of recombinant human TNF into research animals produces many of the same sequelae as seen in endotoxin shock (*Tracey* et al., 1986; *Lehmann* et al., 1987; *Natanson* et al., 1979; *Remick* et al., 1987). Second, monoclonal antibodies against TNF are protective in model systems of endotoxin shock and gram negative bacterial sepsis (*Beutler* et al., 1985a; *Tracey* et al., 1987; *Mathison* et al., 1988; *Sanchez-Cantu*, 1989; *Hinshaw* et al., 1990; *Silva* et al., 1990). This is not to say that TNF is the only mediator in endotoxin shock, but it clearly plays a central role in the pathogenesis of endotoxin and septic shock.

Methods of reducing or neutralising LPS activity *in vivo* have long been sought. When LPS is exposed to plasma or serum, a number of its biological activities are lost or diminished (*Rall* et al., 1957; *Ulevitch* and *Johnston*, 1978; *Ulevitch* et al., 1979; *Freudenberg* et al., 1980). LPS has been shown to interact with a multitude of plasma proteins [reviewed in: *Doran*, 1991); however, it is only the interactions of LPS's with lipoproteins that will be discussed in this report. Their role in LPS binding and detoxification has been extensively studied in the last decade. *Ulevitch* et al. (1979, 1981), *Mathison* and *Ulevitch* (1979), *Freudenberg* et al. (1980), *Munford* et al. (1981) and their respective co-workers showed that LPS forms complexes predominantly with high density lipoprotein (HDL) in normal animals. Other lipoprotein fractions including low density lipoprotein (LDL), very low density lipoproteins (VLDL) and chylomicrons have all been examined for their interactions with LPS (*Ulevitch* et al, 1979; *van Lenten* et al., 1986; *Harris* et. al., 1990). The binding of LPS by HDL or other lipoproteins has been shown to modify the biological activity of LPS *in vitro* and *in vivo*. Lipoprotein binding decreases LPS induced Interleukin 1 (IL-1) (*Warren* et al., 1988; *Flegel* et al., 1989; *Cavaillon* et al., 1990), Interleukin 6 (IL-6), and TNF production by mononuclear cells (*Cavaillon*, 1990). LPS-lipoprotein complexes are also less reactive *in vivo*: pyrogenicity, leukopenia and thrombocytopenia induced by HDL-LPS complexes is reduced in comparison to LPS alone (*Ulevitch* and *Tobias*, 1988). Although a protective capacity of lipoproteins has been demonstrated *in vitro* and *in vivo*, benefits are usually only seen when the LPS has been precomplexed with lipoproteins *ex vivo* (*Harris* et al., 1990). The extent and significance of lipoprotein-mediated LPS detoxification in the natural setting *in vivo* is not known.

We have investigated the ability of an artificial lipoprotein designated ApoLipo to modulate cytokine production *in vitro*. We further investigated if the prophylactic infusion of ApoLipo prevents TNF production *in vivo* and the pathophysiological manifestations of endotoxin shock in a rabbit endotoxaemia model. One critical difference between these studies and those found in the literature is that the *in vitro* and *in vivo* studies were performed without prior complexation of our artificial lipoprotein with LPS.

MATERIALS AND METHODS

Lipoproteins and apoA-I

Chylomicrons and the lipoprotein fractions VLDL, LDL, and HDL were prepared from normal human plasma by ultracentrifugation (*Schumaker* and *Puppione*, 1986). Apolipoprotein A-I (apoA-I), the major protein component of HDL was purified from human HDL by delipidation and size exclusion chromatography or was purified from precipitates obtained by cold ethanol fractionation of human plasma (*Lerch* et al., 1989). ApoLipo was prepared by a method similar to that of *Chen* and *Albers* (1982). Briefly, apoA-I was mixed with phosphatidylcholine (PC) (Molar ratio of apoA-I to PC was 1:200) and sodium cholate, followed by extensive dialysis against buffer (1 mM $NaHCO_3$, 10% Saccharose). Additional artificial lipoproteins were prepared in which other components (triglycerides and/or cholesterol were added to the apoA-I:PC mixture prior to dialysis (see results section for ratios used). Total protein (*Markwell* et al., 1978), phospholipid (*Chen* et al., 1956), triglyceride (Merkotest Triglycerides #14354), and cholesterol content (Boehringer Mannheim Monotest Cholesterin # 290319) of each of the natural lipoprotein preparations were also measured. The apoA-I and ApoLipo preparations used in these studies were shown to be sterile and nonpyrogenic.

Tumour Necrosis Factor Production and Assay

The whole blood assay introduced by *Desch* and colleagues (1989) was adapted for use in these studies. Whole heparinised blood or ACD blood from single donors (200 μl) was incubated with LPS (25 μl of 100 μg LPS/ml) in the presence of various concentrations of apoA-I, ApoLipo or other synthetic lipoproteins, natural lipoprotein fractions, saline, or other controls (25 μl) at 37°C. After 6 h, 750 μl of pyrogen free saline was added to the reaction mixture; TNF containing supernatants were harvested by centrifugation and tested for immunoreactive TNF (Medigenix, Belgium).

Bioassayable TNF was measured in samples of rabbit serum by the L929 cytotoxicity assay (*Ruff* and *Gifford*,1981). Care was taken to avoid any microbial contamination of the serum samples. It was shown that the addition of ApoLipo to standard concentrations of recombinant human TNF does not influence the L929-cytotoxicity assay. Recombinant mouse TNF (Genzyme, Boston, MA, USA) was used as a standard.

LPS Preparations

A mixture of 13 smooth strain LPS (all Difco: including *E. coli*, *Salmonella*, *Serratia*, *Klebsiella*, and *Pseudomonas* variants) was used as the stimulant in all of the *in vitro* assays. *E. coli* O111:B5 (a generous gift of Prof. B. Urbaschek) was used throughout the *in vivo* rabbit studies.

Animals

In these preliminary studies, outbred rabbits of both sexes with an average weight of 3.2 ± 0.4 kg (mean ± SD) were used. These studies were approved by the Animal Protection Committee of the Canton of Bern, Bern, Switzerland. In brief, arterial and venous catheters were implanted in 4 groups of anaesthetised rabbits for measurements of physiologic parameters, blood sampling and infusions. All animals remained under anaesthesia during the entire course of the experiment. Animals received either a prophylactic infusion of ApoLipo [250 mg/kg (n=3) or 75 mg/kg (n=6)] or control protein

[Physiogel 250 mg/kg, n=4], followed by a 6 h continuous infusion of *E. coli* LPS (4.17 μg/kg/h). These groups are designated as ApoLipo250-LPS ApoLipo75-LPS, and Control-LPS respectively. The fourth group [ApoLipo75-Control (n=4)] represents the non-endotoxin treated control. Here ApoLipo was administered at a dose of 75 mg/kg, the endotoxin infusion was replaced with pyrogen free saline.

Blood samples were withdrawn at specified intervals for the measurement of bioreactive TNF, blood gas and haematological analyses (leukocyte and platelet count, haematocrit, and haemoglobin determinations), simultaneous with the recording of physiologic parameters (e.g., temperature, heart rate, blood pressure, respiratory parameters). Only the results from the TNF and blood gas analyses, and from the leukocyte and platelet determinations will be presented in this report.

Data Presentation

The *in vitro* data presented in Figures 1 - 6 are from single experiments but are representative of multiple experiments.

Spearman rank correlation analysis was also performed. The *in vivo* results presented are as mean ± standard error of the mean (SEM), unless otherwise specified. Where appropriate, comparison with baseline values were made by Student's t test for paired samples; comparisons between groups was made by Student's t test for unpaired samples. TNF levels were compared with the Mann-Whitney U test. In all instances, probabilities less than 0.05 are considered significant.

RESULTS

In Vitro Studies

Preliminary studies were performed which showed that our adaptation of the whole human blood assay for TNF was both LPS dose and time dependent (*Doran* et al., 1991). In the absence of exogenously added LPS, immunoreactive TNF levels were routinely less than 50 pg/ml. Following LPS stimulation, TNF production was shown to be time dependent, demonstrating an almost linear increase over the 6 h test period (*Doran* et al., 1991). TNF levels at the end of 6 h rose to levels between 2.5 and 12.5 ng/ml depending on individual donor, and LPS concentration used for stimulation (*Doran* et al. 1991).

Table I provides the total protein, triglyceride, phospholipid, and cholesterol contents of plasma, dialysed

Table 1: Protein, triglyceride, phospholipid and cholesterol content of plasma, dialyzed plasma, lipoprotein free plasma (LFP), HDL, LDL, VLDL, and chylomicrons

	Protein g/l	Triglyceride mM	Phospholipid mM	Cholesterol mM
Plasma	78.5	1.4	6.2	5.4
Dialysed Plasma	69.3	1.0	4.7	4.1
LFP	124.5	0.0	0.8	0.2
HDL	12.1	1.3	8.0	9.4
LDL	5.3	1.6	8.2	24.9
VLDL	1.0	4.8	2.1	4.8
Chylomicrons	70.0	1.1	2.5	4.1

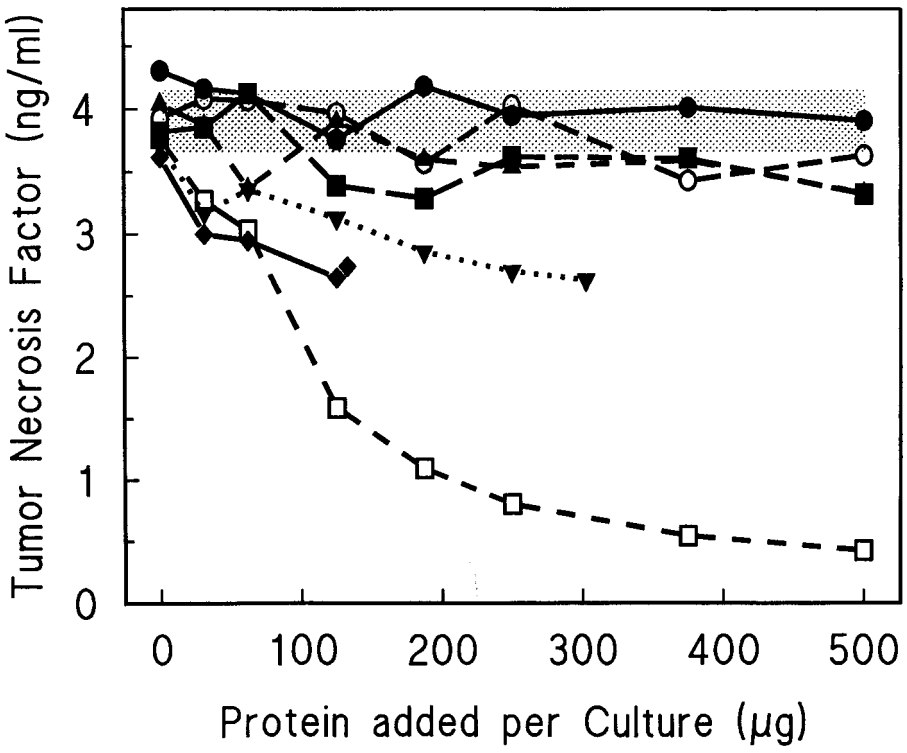

Figure 1: Inhibition of TNF production by natural and artificial lipoproteins. Whole blood cultures were incubated concomitantly with LPS and plasma (J), dialysed plasma (B) lipoprotein free plasma (LPF plasma; E), natural lipoproteins [HDL (P) , LDL (F)], chylomicrons (H), or ApoLipo [apoA-I:PC molar ratio 1:200; (G)] for 6 h at 37°C. The supernatants were tested for TNF activity. The hatched bar represents the mean ± SD of TNF produced by whole blood cultures in the presence of saline and LPS (n=8).

plasma, lipoprotein free plasma, and purified HDL, LDL and VLDL and chylomicron fractions. These preparations as well as our artificial lipoprotein ApoLipo (apoA-I:PC at a 1:200 molar ratio) were tested for their ability to modulate LPS induced TNF production. All preparations were added to the culture concomitantly with LPS and incubated for 6 h at 37°C. In Figure 1, TNF measured in the culture supernatants is plotted versus the total protein added per culture for each of the preparations tested. As seen in this figure, plasma, dialysed plasma, chylomicrons and lipoprotein free plasma had virtually no inhibitory effect on LPS stimulated TNF production, whereas LDL, HDL, and ApoLipo each demonstrated inhibitory effects. Because of the low protein content of the VLDL, it could only be tested at one protein concentration, and was not included in Figure 1. TNF production in the presence of LPS and 24 µg VLDL protein was 3 ng/ml. Clearly, phospholipid rich (e.g., ApoLipo), cholesterol rich (LDL, HDL), or triglyceride rich preparations (VLDL) all can decrease LPS-induced TNF production. Using data from all of these preparations, correlation analysis showed a significant negative association ($r = -0.899$, $p < 0.001$, $n = 32$) between TNF levels and lipid content.

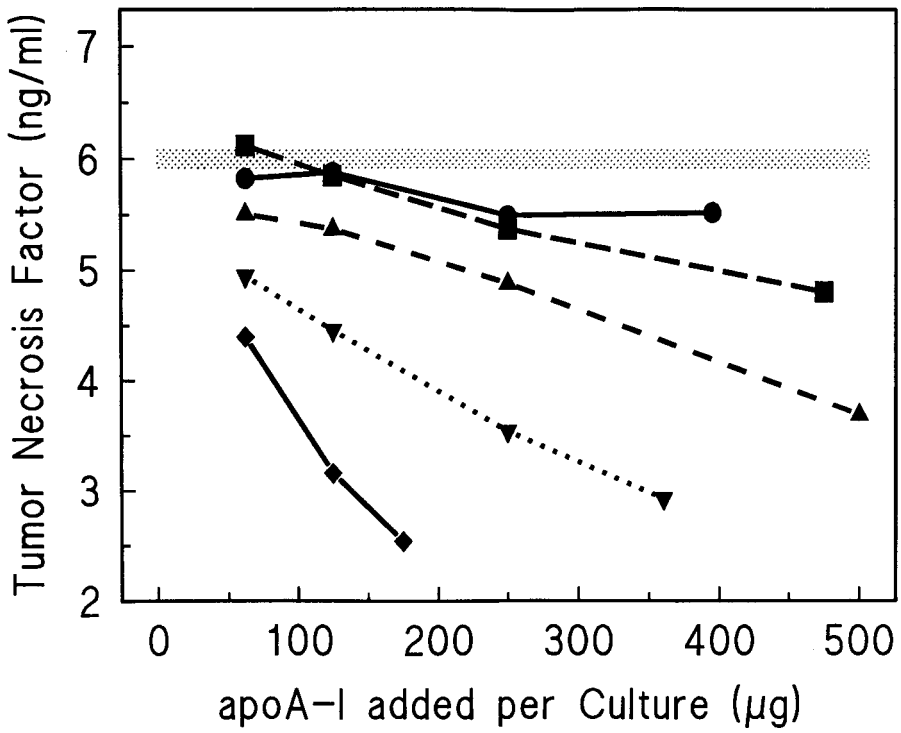

Figure 2: Influence of apoA-I:lipid ratio on TNF production. Artificial lipoproteins were prepared at apoA-I to phosphatidyl choline ratios of 1:20 (J), 1:50 (B), 1:100 (H), 1:200 (P) and 1:250 (F). Each of these preparations were then tested in the whole blood assay at different protein concentrations in the presence of LPS. TNF was measured in the supernatants collected at 6 h. The hatched band represents mean ± SD TNF production (n=3) in the presence of saline and LPS.

When the data from the ApoLipo preparation were removed from the analysis, negative correlations between TNF and cholesterol and triglyceride content of the preparations could also be discerned ($r = -0.54$, $p < 0.01$, and $r = -0.71$, $p < 0.001$ respectively). Clearly, triglycerides and cholesterol are not required for TNF inhibition, as ApoLipo is inhibitory in their absence, however they may still contribute to the inhibitory activity of the natural lipoproteins.

In order to more clearly show the importance and influence of lipids, cholesterol and triglycerides in this model system, artificial lipoprotein combinations with various compositions were prepared. The influence of lipid on TNF production and release is shown in Figure 2. A spectrum of apoA-I:PC ratios between 1:20 and 1:250 were tested for TNF inhibiting activity in the whole blood assay. These results clearly demonstrate a greater TNF inhibiting effect in preparations having more lipid. Supernatant TNF is further diminished by the addition of either triglycerides or cholesterol to the apoA-I: PC mixtures (shown in Figures 3 and 4 respectively). Analogous to the situation found with natural lipoproteins, the more triglyceride (in this case trimyristin) that is present (Figure 3), or the more cholesterol that is present (Figure 4), the greater the TNF inhibition. The influence of triglycerides is not triglyceride specific, as similar dose response curves were obtained when

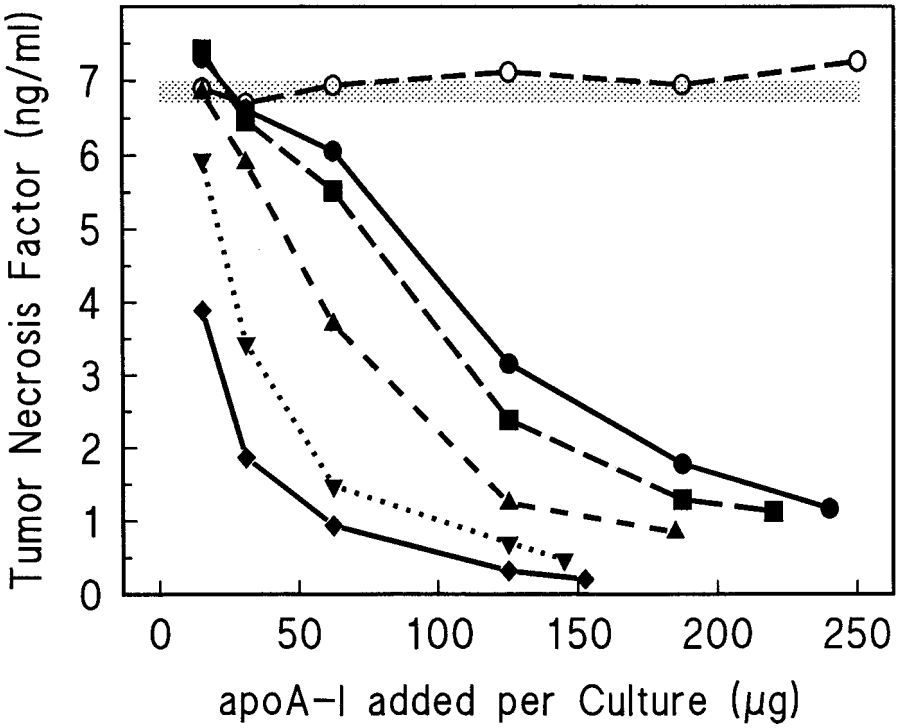

Figure 3: Effect of apoA-I:PC:triglyceride preparations on TNF production. Artificial lipoproteins at apoA-I : PC : trimyristin ratios of 1:100:0 (J), 1:100:10 (B), 1:100:20 (H), 1:100:50 (P) and 1:100:100 (F) were compared with apoA-I alone (1:0:0) (E) for TNF production inhibition.

triolein was used. It should be noted that none of these preparations required precomplexation with LPS to show TNF modulating effects.

The importance of lipid in these interactions must also be carefully considered. The evidence thus far presented points to a non-specific lipid-LPS interaction as the cause of the TNF inhibition. However, tests performed with apoA-I-PC mixtures prepared in the absence of cholate (Figure 5) or by mechanical means (Microfluidiser; data not shown) show that these preparations have markedly reduced TNF inhibiting activity. It is apparent from these studies that the configuration of the components involved may be critical for their biological activity.

We also wished to determine the temporal requirements for ApoLipo to mediate a beneficial effect. Using the whole blood assay system, TNF production inhibition was still evident when ApoLipo was given up to 1 h after LPS exposure (Figure 6). The inhibition is ApoLipo dose dependent (maximal inhibition shown with 500 μg added per culture). ApoLipo is able to diminish cellular TNF production/release even after stimulation has occurred. Data from additional studies suggest that ApoLipo may also have a direct effect on cells. Cells pulsed with ApoLipo and then washed, are refractory to LPS stimulation as assessed by TNF production (data not shown). This effect is clearly not attributable to early LPS tolerance.

In Vivo Studies

The ApoLipo preparation was also

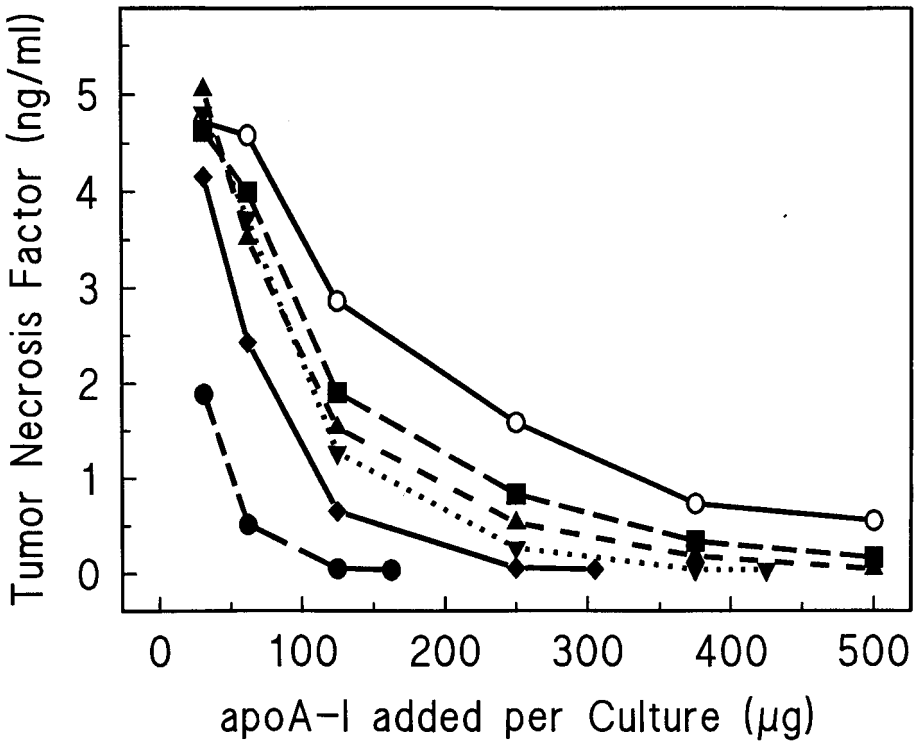

Figure 4: Effect of apoA-I:PC:cholesterol preparations on TNF production. Artificial lipoproteins at apoA-I:PC:Cholesterol ratios of 1:200:0 (E), 1:200:2 (B), 1:200:5 (H), 1:200:10 (P), 1:200:15 (F), and 1:200:20 (J) were tested for their ability to reduce TNF production by whole blood cultures in the presence of LPS.

used prophylactically in an *in vivo* rabbit endotoxaemia model system (described in detail by *Hubsch* et al., 1991). Control-LPS rabbits exhibit many but not all of the manifestations of endotoxin shock within the 6 h LPS infusion period. The Control-LPS group developed a marked leukopenia within 15 minutes of the beginning of LPS infusion (76% baseline). After 1 h of LPS infusion, leukocyte count continued to decrease (43% baseline), reaching its nadir (24% baseline) after 4 h of LPS infusion. Plasma TNF levels rose significantly above baseline levels after 1 h of LPS infusion, reaching their maximum (29.4 ng/ml) at 2 h. Metabolic acidosis as assessed by arterial base excess was clear-cut and statistically significant at 2 h (-5.3 ± 0.8), worsening as LPS continued to be infused. Thrombocytopenia began to appear at 30 minutes of LPS infusion (92% baseline), and progressed steadily during the period of LPS infusion.

Doses of ApoLipo approximating that used in the *in vitro* studies (75 mg/kg body weight) or above (250 mg/kg) significantly attenuated TNF production *in vivo*, and many of the manifestations of shock. At 2 h, TNF levels in LPS treated controls were 29.4 ± 6.5 ng/ml (n=4), as compared to 1.2 ± 0.2 ng/ml (n=6) in ApoLipo75-LPS treated animals and 0.7 ± 0.3 in ApoLipo250-LPS treated animals (n=3). Metabolic acidosis was also significantly attenuated by ApoLipo. At

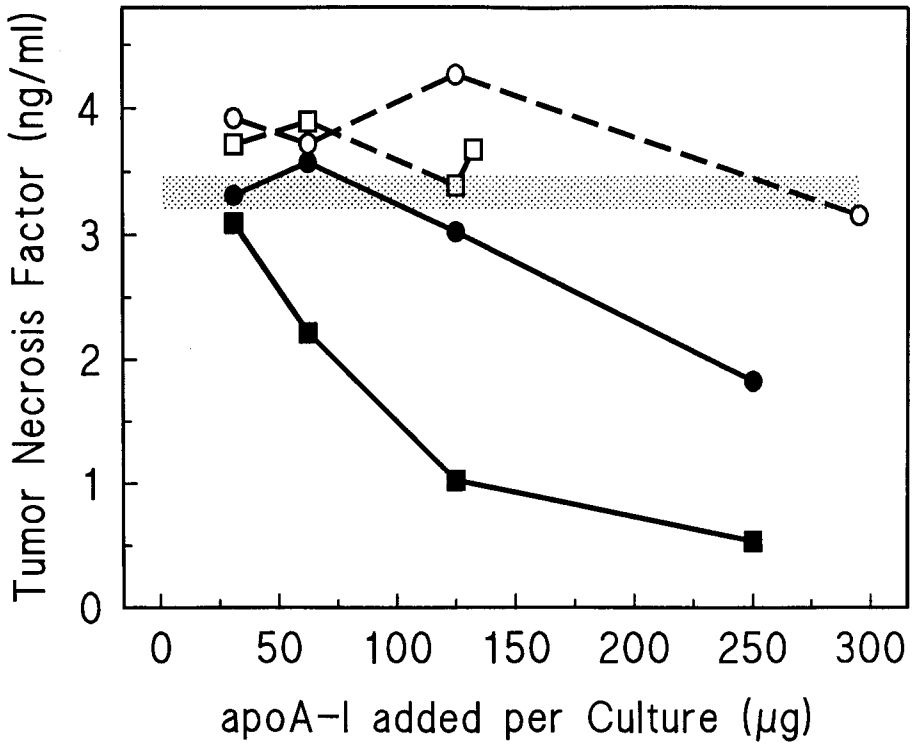

Figure 5: Importance of lipoprotein configuration for activity. ApoA-I:PC mixtures with ratios of 1:100 (squares) and 1:200 (circles) were prepared in the presence (solid symbols, solid line) and absence (open symbols, dotted line) of cholate. These preparations were incubated concomitantly with LPS in the whole blood assay. Supernatants were collected at 6 h and TNF determined. The hatched bar represents the mean ± SD (n=3) of TNF produced in the presence of saline and LPS.

5 h, the base excess levels reached their nadir (-9.0 ± 2.1) in LPS treated controls, whereas nadir levels were -4.1 ± 0.6 and -2.8 ± 0.2 in ApoLipo75-LPS and ApoLipo250-LPS treated animals respectively (also at 5 h). These base excesses correspond to arterial blood pH values of 7.22 ± 0.05 for LPS control animals and 7.33 ± 0.01 and 7.28 ± 0.01 for ApoLipo75 and ApoLipo250 groups respectively. The complete base excess and TNF data for the Control-LPS, ApoLipo75-LPS and ApoLipo250-LPS groups are shown in Figure 7. TNF values in the ApoLipo75-Control and ApoLipo250-LPS groups never statistically exceeded their background controls. Base excess for the ApoLipo75-Control group remained above -2.2 throughout the experimental course. The ApoLipo250-LPS group maintained their metabolic acidosis between that of the ApoLipo75-Control group and that of ApoLipo75-LPS. The cellular aberrations associated with endotoxin shock were also significantly attenuated (platelet decrease; Figure 8) or delayed (leukocyte drop; Figure 9) by ApoLipo infusion. It is only with the high doses of ApoLipo that these cellular changes associated with endotoxin shock are clearly attenuated.

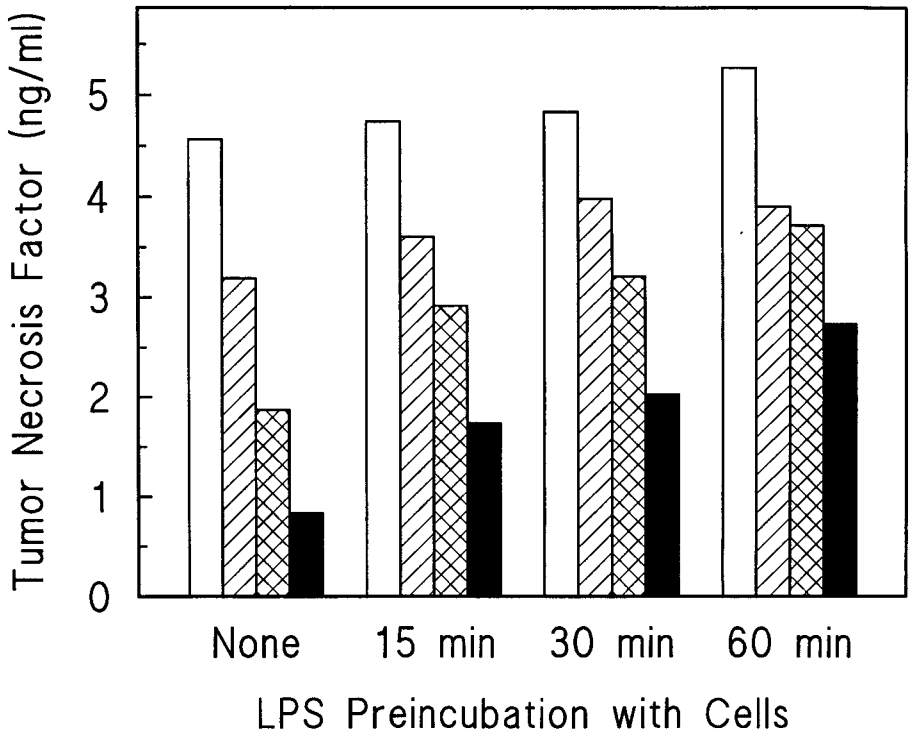

Figure 6: Influence of ApoLipo on TNF production after LPS Stimulation. Whole blood cultures were preincubated with LPS for 0, 15, 30, or 60 minutes, before ApoLipo or saline was added to the cultures. The effects of saline (open) and ApoLipo at three different protein concentrations (125, 250, and 500 μg; hatched, crosshatched and solid, respectively) on TNF production were assessed.

DISCUSSION

It is well established that the binding of LPS by HDL results in altered/diminished LPS functional activity. However, LPS's functional activities are not always reduced to the same extent. *Ulevitch* and *Tobias* (1988) reported that Re595 LPS complexation with HDL reduces LPS induced complement activation and leukopenia 1000 fold; pyrogenicity and thrombocytopenia were reduced to a lesser extent (100 and 50 fold respectively). In their hands, the development of shock and DIC in rabbits was diminished, but mitogenicity of murine B cells was virtually unaffected by LPS's complexing with HDL. It is quite likely that lipoprotein binding to LPS effects the systemic manifestations of LPS through the regulation or modulation of cytokine production. Lipoprotein binding to LPS reduces LPS induced IL-1 (*Warren* et al., 1988; *Flegel* et al., 1989; *Cavaillon* et al., 1990), IL-6, and TNF production by mononuclear cells (*Cavaillon* et al., 1990; Figure 1).

Other lipoprotein fractions have also been examined for LPS binding and modulating activities, including LDL, VLDL and chylomicrons (*Ulevitch* et al., 1979; *van Lenten*, 1986; *Harris* et al., 1990). In contrast to the earlier reports that LPS bound only to HDL, *van Lenten* et al. (1986) reported that all

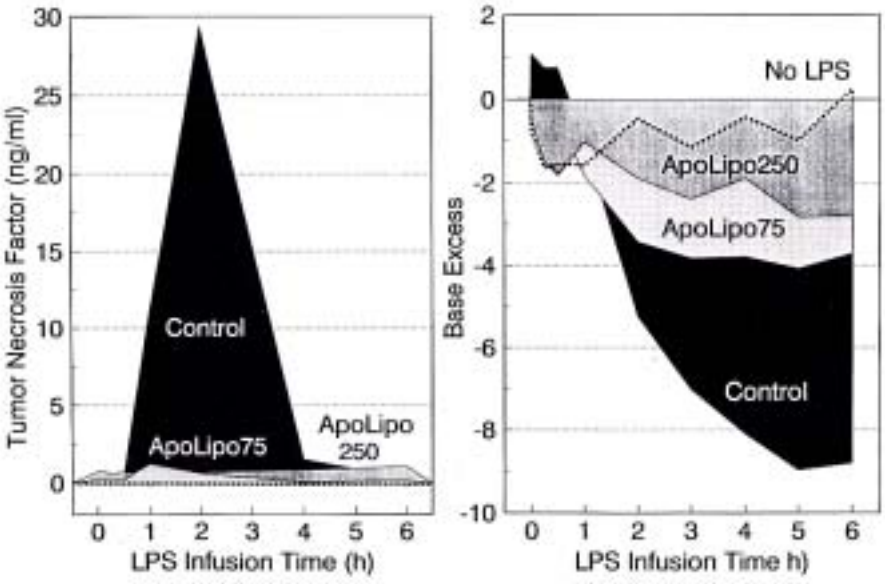

Figure 7: Influence of ApoLipo on rabbit TNF production *in vivo* and metabolic acidosis (Base Excess) in a rabbit endotoxin shock model. Bioreactive TNF and base excess are displayed for the 4 rabbit groups. The control-LPS group is shown in black, ApoLipo75-LPS as dotted, and ApoLipo250-LPS group as grey. The ApoLipo75-Contol is shown as a dotted line. At 1 and 2 h of LPS infusion, TNF in the control-LPS treated group was significantly increased over the ApoLipo treated groups ($p<0.05$). Statistically significant ($p<0.05$) differences in Base Excess between control-LPS and one or both ApoLipo-LPS treated groups were demonstrated at 4, 5, and 6 h.

lipoprotein classes were able to bind LPS; binding was in direct proportion to their cholesterol concentration. Binding was also shown to lead to endotoxin detoxification. Using an *ex vivo* incubation system, *Harris* et al. (1990) demonstrated that cholesterol rich LDL and HDL, triglyceride rich VLDL and chylomicrons, and a commercial lipid emulsion (Soyacal) were all able to protect mice against endotoxin induced lethality, albeit at vastly different concentrations. Interestingly, protection required small amounts of lipoprotein free plasma, and was dependent on the incubation time and lipoprotein lipid concentration. These data would support our observations on the importance of lipid for ApoLipo functionality (Figure 2). Although *Harris* and colleagues (1990) demonstrated a protective capacity of all tested lipoprotein fractions, the systems they used required a 6 h pre-incubation of LPS with lipoproteins *ex vivo* for efficacy to be demonstrated. Judging by the requirement for precomplexation in these systems, one might hypothesise that the natural lipoprotein system may only be able to detoxify small amounts of LPS (e.g. that naturally crossing the intestine). The significance and extent of lipoprotein mediated LPS detoxification in bacteraemia and sepsis remains unknown.

In previous studies (*Doran* et al., 1991), we have demonstrated that ApoA-I, the major protein component of HDL does not appear, by itself, to neutralise or substantially modulate endotoxin activity. However, in association with lipids (as ApoLipo), it clearly

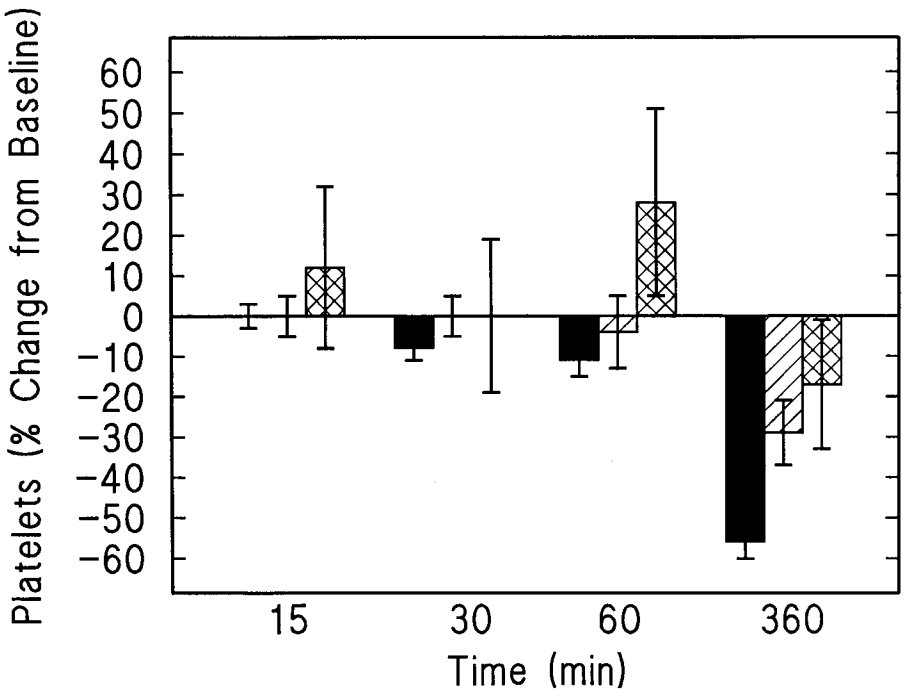

Figure 8: Change in platelet count with LPS administration. The percent change of platelet count from baseline for each of the LPS treated groups are shown. Control-LPS, ApoLipo75-LPS, and ApoLipo250-LPS are shown as solid, hatched and crosshatched bars respectively. The platelet decrease in the Control-LPS group is significantly different ($p<0.05$) from that of the ApoLipo75-LPS and ApoLipo250-LPS groups only at 4h (data not shown) and at 6 h (last set of bars) .

diminishes TNF production by monocytic cells in response to LPS exposure. The *in vitro* studies presented in this report have focused on TNF, but other cytokines (IL-1, IL-6) are similarly diminished (data not shown). In contradistinction to what is reported in the literature for the natural lipoproteins, precomplexation with LPS is not required for ApoLipo to modulate LPS's biolological activity. The influence of lipids, triglycerides, and cholesterol are clearly shown in Figures 2-4. Caution, however, must be exercised in the interpretation of these results. High concentrations of artificial lipoproteins at Apo:PC ratios above 1:200 caused demonstrable erythrocyte lysis. Lysis was increased by the addition of triglycerides or cholesterol, and was demonstrable at lower lipoprotein concentrations as the cholesterol or triglyceride concentrations increased. The role which cholate plays in the formation of efficacious preparations still needs to be elucidated. Clearly, particle size and/or protein-lipid conformation is important for TNF modulating activity to be present. Experiments are in progress to better delineate the particle size and composition necessary for maximal protective effects.

What is perhaps most important, is that the effects mediated by ApoLipo *in vitro* were also realised *in vivo*. TNF production *in vivo*, as well as some of the pathophysiological changes associated with endotoxin infusion were attenuated in our model system by the prophylactic administration of ApoLipo

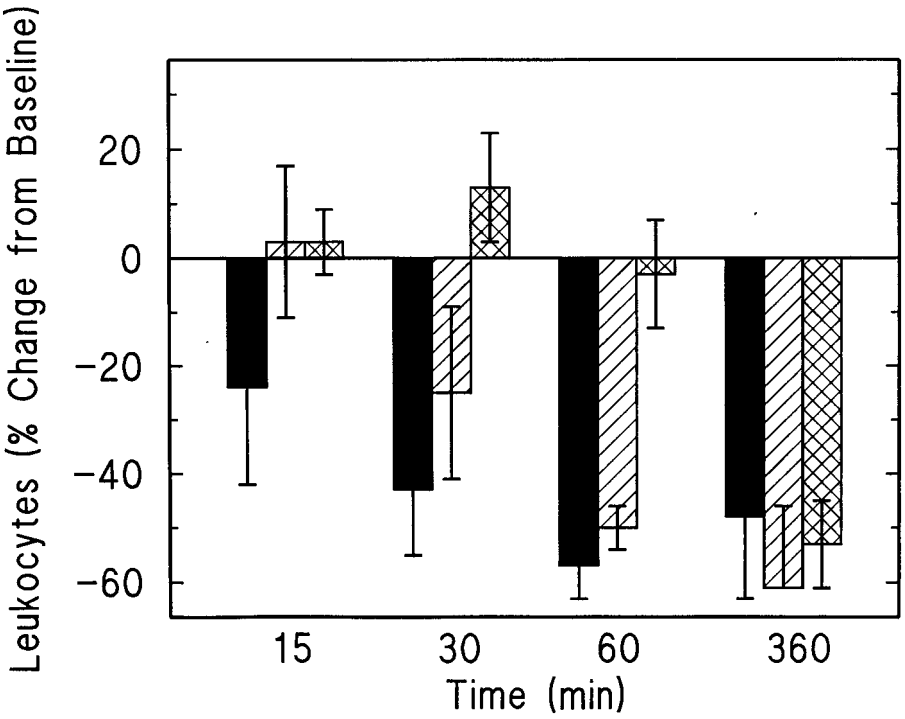

Figure 9: Change in leukocyte count with LPS administration. The percent change of leukocyte count from baseline for each of the LPS treated groups are shown. Control-LPS, ApoLipo75-LPS, and ApoLipo250-LPS are shown as solid, hatched and crosshatched bars respectively. The leukocyte drop of the Control-LPS and ApoLipo75-LPS groups are significantly different from that of the ApoLipo250-LPS group at 60 min ($p<0.05$).

(*Hubsch* et al., 1991; Figures 7-9). In our rabbit model, LPS induces high levels of TNF 2 h after the start of its infusion (Figure 7), a severe metabolic acidosis and significant decreases in leukocyte and platelet counts (Figures 7-9). Except for the leukopenia, all of these derangements were significantly attenuated by the prophylactic infusion of ApoLipo. The TNF levels observed in this study are consistent with the findings by *Mathison* et al. (1988), who reported peak TNF levels of 20 to 30 ng/ml, from 45 to 100 min after a bolus injection of 10 μg of LPS in rabbits (comparable to our Control-LPS group). In this same model system, the injection of plasma precomplexed LPS induced only a minimal (< 2.5 ng/ml) TNF response, comparable to our ApoLipo-LPS groups. One can argue that the TNF produced acts in concert with other biological mediators to alter metabolic status and cellular adhesiveness. Thus, in our system, ApoLipo most likely modulates metabolic status (acidosis) as a consequence of its action on TNF production. The fact that other sequelae are not affected to the same extent by ApoLipo (e.g., leukopenia), suggest that these sequelae are due to the direct effects of LPS as opposed to a dependence on TNF. Although high doses of ApoLipo were able to reduce and delay leukopenia, it should be noted that the leukocyte drop occurred very early in the course of LPS infusion, at a time when TNF was not measurable in

the circulation. The leukocytes may be reacting with LPS directly via their LPS receptors (*Wright* et al., 1989).

The mechanisms by which ApoLipo mediates its effects *in vivo* require further clarification. At present, we speculate that ApoLipo may function through two vastly different mechanisms of action: the formation of ApoLipo-LPS complexes and a down regulation of macrophage and perhaps other cells' reactivity. Our cell pulse experiments and the lack of requirement for precomplexation with LPS supports the second hypothesis. In each case, ApoLipo treatment is consistent with decreased TNF production. It is still too early to speculate on the clinical utility of ApoLipo. A greater understanding of the mechanisms of protection are clearly needed and experiments to this end are in progress.

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INTESTINAL IMMUNE REGULATION EXEMPLIFIED BY COELIAC DISEASE

HELGE SCOTT, TROND S. HALSTENSEN, and PER BRANDTZAEG

Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, University of Oslo, The National Hospital, Oslo, Norway

SUMMARY

Polymeric IgA is produced by 80-90% of the plasma cells in normal intestinal mucosa. It is transported externally by the epithelial secretory component and acts in a first line of mucosal defence by blocking antigen penetration. IgG, IgE and T cell mediated responses against soluble non-replicating luminal antigens are normally downregulated, perhaps by mechanisms involving interactions between the gut epithelium, T cells and macrophages leading to anergy or suppression collectively termed "oral tolerance". It has been claimed that IgA-producing cells escape suppression due to a specific effect of contrasupressor cells but the mechanisms remain unclear. Abrogation of oral tolerance may lead to increased activation of mucosal T lymphocytes with elevated lymphokine production. An altered lymphokine profile may induce aberrant epithelial HLA class II expression, increased epithelial permeability, crypt hyperplasia and disproportionate local overproduction of IgG. Expansion of a particular TCRγ/δ^+ intraepithelial T cell subset may also be involved in break of oral tolerance. Immunecomplex-mediated subepithelial complement activation together with local production of tumour necrosis factor and interferon-γ may cause epithelial damage and increased epithelial desquamation. Coeliac disease appears to be an example of break in oral tolerance to gluten peptides where the immunogenetics is at least partially defined.

INTRODUCTION

The human gastrointestinal tract is exposed to an enormous amount of antigens from food and micro-organisms. Despite this persistent bombardment of the mucosal membrane, adverse immunological responses to dietary antigens are relatively uncommon while effective immunity to infectious agents is usually elicited. The homeostatic mechanisms that explain a state of intestinal hyporesponsiveness to soluble non-replicating agents is known as "oral tolerance" and involve both humoral and cell-mediated immunity.

THE NORMAL STATE

Humoral immunity

There is a striking preponderance (80-90%) of IgA-producing blasts and plasma cells in the intestinal mucosa, and it has been estimated that at least 80% of all Ig-producing cells of the body are located in the gut (*Brandtzaeg* et al., 1989). The IgA cells produce

mainly dimers and larger polymers of IgA containing a disulphide-linked polypeptide called the J ("joining") chain (*Brandtzaeg*, 1985; *Mestecky* and *McGhee*, 1987). Polymeric IgA is transported externally along with J chain containing pentameric IgM by an epithelial glycoprotein called secretory component (SC) or poly-Ig receptor (*Brandtzaeg*, 1985). The secretory immunoglobulins (SIgA and SIgM) act in a first line of mucosal defence (*Brandtzaeg* et al., 1989). IgA is probably crucial for immunologic homeostasis also within the lamina propria because it lacks potent effector functions such as classical complement activation and hence may block triggering of non-specific biological amplification mechanisms (*Brandtzaeg* et al., 1987). At the same time antigen binding to SIgA blocks antigen penetration and thereby dampens potentially phlogistic IgG and IgE reactions and T cell mediated hypersensitivity against harmless luminal antigens (*Brandtzaeg* et al., 1987).

Cellular immunity

The normal intestinal mucosa contains numerous intraepithelial lymphocytes (IEL). Human IEL are mainly T lymphocytes ($CD3^+$) and 80-90% express CD8 whereas the lamina propria T cells are mainly $CD4^+$ (*Brandtzaeg* et al., 1989). IEL express the marker HML-1 that originally was thought to be unique for mucosal lymphocytes (*Cerf-Bensussan* et al., 1987) but later shown to be an activation antigen (*Schieferdecker* et al., 1990). However, IEL are negative for markers of recent activation such as MHC class II and CD25 (p55 IL-2 receptor) (*Selby* et al., 1983; *Trejdosiewicz* et al., 1987). Human IEL show no spontaneous cytotoxicity (*Cerf-Bensussan* et al., 1985) and they are negative for the H366 antigen which is a putative marker of MHC restricted cytotoxic $CD8^+$ T cells (*Trejdosiewicz* et al., 1987). These results and data from *in vitro* studies suggest that human IEL are mainly suppresser cells although this concept remains quite controversial as discussed later. In the normal state, intestinal IEL are found mainly along the basement membrane, apparently crossing it in either direction (*Marsh*, 1975b). Thus, after immune stimulation they may return to the lamina propria where their immunoregulatory function(s) perhaps are exerted.

Much interest is focused on intraepithelial T cells that express the γ/δ T cell receptor (TCR) (discussed in more detail later). In humans, 0-39% (median 2%) of the intraepithelial T cells express this alternative antigen receptor while few $TCR\gamma/\delta^+$ cells are seen in the lamina propria (*Halstensen* et al., 1989). About 75% of the intraepithelial $TCR\gamma/\delta^+$ lymphocytes are $CD8^-$ (*Halstensen* et al., 1989) and a remarkably large fraction (median 67%) express the Vδ1/Jδ1 encoded epitope revealed by monoclonal antibody δTCS1 (*Spencer* et al., 1989; *Halstensen* et al., 1989).

It has been claimed that the intestinal IgA production escapes suppression because of antisuppressor or contrasuppressor cells that preferentially support IgA responses (*Suzuki* et al., 1988; *Ernst* et al., 1988; *Green* et al., 1988), but the mechanisms remains unclear.

Migration of mucosal lymphocytes

Regulation and effector mechanisms of mucosal immunity depend on the migration of specifically primed T and B lymphocytes from organised gut associated lymphoid tissue (GALT) through lymph and peripheral blood primarily to the intestinal lamina propria. Extravasation of lymphoid cells appears to be mediated by receptors for endothelial

determinants ("vascular addressins") which to some extent are tissue specific (*Jalkanen* et al., 1986, 1988; *Streeter* et al., 1988). Additional adhesion molecules and other local factors are also involved (*Duijvestijn* and *Hamann*, 1989; *Bienenstock* et al., 1983). While B cells localise in the lamina propria and proliferate and differentiate there, many T cells find their way into the intestinal epithelium. The latter phenomenon is partially antigen-independent because intraepithelial lymphocytes are observed even before birth (*Spencer* et al., 1986), but luminal antigens clearly determine the magnitude of the intraepithelial migration (*Ferguson* and *Parrott*, 1972). The follicle associated epithelium covering the human PP contains particularly many T cells, especially near the antigen-transporting M ("membrane") cells (*Bjerke* et al., 1988).

COELIAC DISEASE

Coeliac disease is a small intestinal disorder characterised clinically by malabsorption, histologically by villous atrophy and crypt hyperplasia (*McNeish* et al., 1979), and genetically by a strong association with certain HLA class II allotypes (*Sollid* et al., 1989). It is precipitated by ingestion of gluten in wheat and some other grains and may be regarded a model for a break of oral tolerance.

Immunopathology of coeliac disease

T lymphocytes and macrophages. After gluten challenge in treated coeliac patients, there is a rapid and dose dependent migration of lymphocytes into the jejunal epithelium (*Rosenkrans* et al., 1981; *Leigh* et al., 1985). The proportion of $CD3^+CD45R0^+$ IEL is significantly raised and the density of such putative memory T cells is strikingly increased both in the surface and crypt epithelium of untreated patients compared with normal controls (*Scott* et al., 1987). There is also an elevated mitotic activity and blastoid transformation of many IEL (*Marsh*, 1975; 1988). However, the CD4 to CD8 ratio is unchanged compared with normal (*Selby* et al., 1983; *Malizia* et al., 1985). The proportion and absolute numbers of IEL $TCR\gamma/\delta^+$ cells are significantly increased both in untreated and partially treated coeliac patients (*Halstensen* et al., 1989; *Savilathi* et al., 1990). In untreated patients an increased fraction of the lamina propria $CD4^+TCR\alpha/\beta^+$ cells express IL-2 receptors (*Halstensen* et al., 1991) and there are raised circulating levels of soluble IL-2 receptor (*Crabtree* et al., 1989). An increase of IL-2R positive T cells is also detected after gluten stimulation *in vitro* for 24 h of small intestinal biopsy specimens from coeliac patients (*Scott* et al., 1991). Moreover, in such specimens cultivation with gluten induces IL-2R expression by macrophages (Mø) in significantly higher numbers than in controls (*Scott* et al., 1991). This strongly suggests that mucosal Mø play an important role in the pathogenesis of coeliac disease, perhaps by being a key link between gluten and genetic (HLA class II determined) disease susceptibility.

Epithelial SC and HLA class II expression. Increased epithelial expression of both SC and HLA class II determinants is seen in the active coeliac lesion (*Scott* et al., 1981). Bright immunohistochemical staining for SC extends from the crypts to the surface epithelium, whereas DR positivity extends from the surface deep into the hyperplastic crypts (*Scott* et al., 1981). In

addition, there is an increased expression of HLA-DP by the surface epithelium (*Scott* et al., 1987; *Marley* et al., 1987). HLA-DQ is only slightly or not at all expressed by epithelial cells in coeliac disease but strongly by Mø situated just beneath the surface epithelium (*Scott* et al., 1987).

The increased epithelial HLA class II expression in coeliac patients takes place quite rapidly after gluten challenge (*Ciclitira* et al., 1986), appears to be related to the number of intraepithelial $CD45R0^+$ T cells (*Scott* et al., 1987), and is most likely caused by cytokines released from primed T cells and perhaps activated Mø (*Cerf-Bensussan* et al., 1984). This possibility is supported by studies of the intestinal HT29 epithelial cell line which shows differential HLA class II expression (DR>DP>DQ) in response to interferon-γ (*Sollid* et al., 1987) with an additive stimulatory effect of tumour necrosis factor-α (*Kvale* et al., 1988). Local stimulation of T cells may therefore be an early event in the coeliac lesion. A consequence of this is probably also cytokine-induced crypt hyperplasia (*MacDonald* and *Spencer*, 1988) and increased epithelial permeability (*Madara* et al., 1988) with ensuing overstimulation of the B cell system.

Immunoglobulin production. In untreated coeliac disease the average numbers of jejunal IgA, IgM and IgG immunocytes per mucosal tissue unit are raised 3, 3.3 and 4.7 times, respectively (Figure 1). These results are supported by tissue culture studies (*Wood* et al., 1987). The local immune response is thus dominated by a typical SIgA and SIgM response with preserved J chain expression by mucosal plasma cells (about 90% positive) and a relatively high proportion of the IgA2 subclass (*Kett* et al., 1990). SC expression is also enhanced, and immunohistochemical staining indicates increased epithelial IgA and IgM transport (*Brandtzaeg* and *Baklien*, 1977).

Only trace amounts of IgG antibodies to gluten and other food antigens are detected in intestinal juice in contrast to IgA and IgM antibodies (*Labrooy* et al., 1986). However, untreated coeliac patients have substantial amounts of IgG antibodies to gluten and other food antigens in peripheral blood (*Scott* et al., 1984). An imbalanced humoral immune response to gluten is likewise suggested by the finding that a much larger percentage of jejunal IgG than IgA cells produce antibodies to gluten (*Brandtzaeg* and *Baklien*, 1976), and there is a significant inverse correlation between time to clinical relapse and the number of IgG producing cells in jejunal mucosa of coeliac children after gluten challenge (*Scott* et al., 1980). The gluten antibodies disappear more slowly from gut fluid than from serum during gluten restriction and persist for prolonged periods within jejunal IgM in treated adults (*O'Mahoney* et al., 1991).

Complement activation. Activated complement has recently been detected beneath the surface epithelium in untreated coeliac lesions (*Halstensen* et al., 1991a), being well correlated with the numbers of mucosal IgG cells and the serum level of gluten specific IgG and IgM. Furthermore, following gluten challenge, degranulation of mast cells is observed (*Horvath* et al., 1990) and also release of histamine and increased mucosal permeability (*Lavo* et al., 1989; 1990), phenomenona that may be initiated by split products of the complement cascade (especially C3a and C5a). These findings support the putative immunopathological role of IgG and suggest that gluten antigens are part of the complement activating immune complexes. Such complexes containing dimeric IgA may contribute

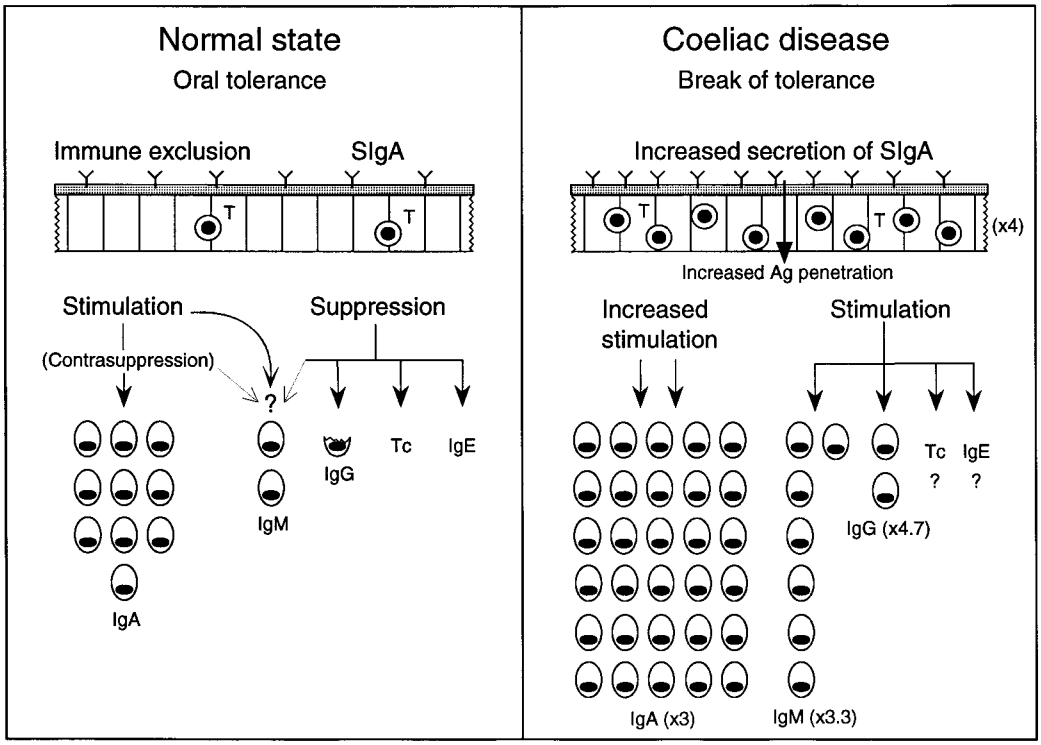

Figure 1: Schematical depiction of the intestinal mucosal immune system in coeliac disease compared with normal. Left: In the normal state a first line of defence is mediated by secretory antibodies, mainly SIgA. Oral tolerance is induced by suppression of systemic (phlogistic) types of immunity - that is, IgG and IgE antibody responses and T cell mediated delayed type hypersensitivity against non-adherent soluble luminal antigens. Contrasuppressor cells may release IgA-promoting helper cells from suppression so that a prominent mucosal IgA response can develop. Right: Coeliac disease represents a break in oral tolerance. Both secretory antibodies, IgG production and mucosal T cells are stimulated, the median number of IgA, IgM and IgG cells per mucosal tissue unit being raised 3, 3.3 and 4.7 times, respectively. The median number of T cells per mm of surface epithelium is raised 4 times and there is increased antigen penetration.

to degranulation of eosinophils since SIgA has been shown to exert this effect by itself *in vitro* (*Abu-Gazaleh* et al., 1989).

PUTATIVE MECHANISMS INVOLVED IN INTESTINAL IMMUNE REGULATION

In addition to immune exclusion performed by SIgA antibodies, animal experiments suggest that suppressive mechanisms protect the intestinal mucosa against potentially harmful systemic types of immune responses elicited by IgG, IgE or T-cell-mediated delayed type hypersensitivity. Such hyporesponsiveness in the gut may involve clonal anergy, suppressor T lymphocytes, cytotoxic T lymphocytes or suppressor Mø, but may also to some

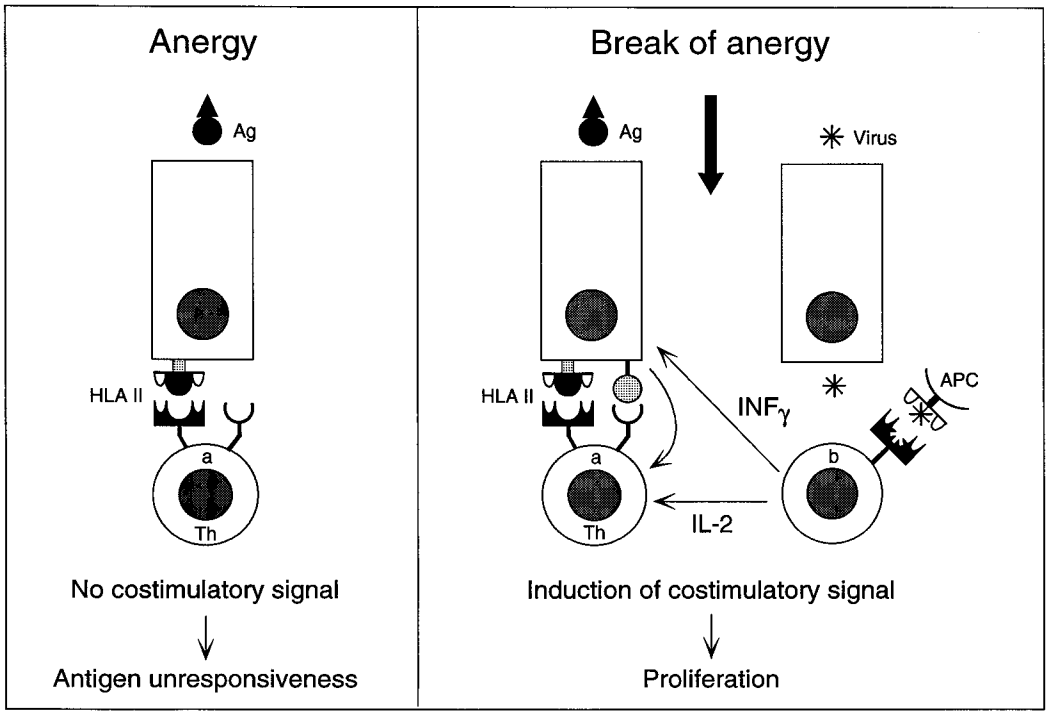

Figure 2: Hypothetical scheme for induction and abrogation of locally induced tolerance. Left: Antigen taken up by the intestinal epithelial cell is degraded and presented in the context of HLA class II to antigen specific $CD4^+$ Th cell in lamina propria. Lack of costimulation (IL-1 and/or adhesion molecules) results in antigen unresponsiveness (anergy). Right: Virusinfection induces activation of T cell (b) that secretes IL-2 and INF-γ. The latter cytokine induces increased expression of HLA determinants and adhesion molecules on epithelial cells, enabeling them together with IL-2 to provide sufficient costimulatory signals for induction of antigen-specific response of T cell (a).

extent depend on the liver (*Brandtzaeg* et al., 1989). Antigen handling by an intact gut epithelium seems to be critical (*Nicklin* and *Miller*, 1983) and direct injections of antigen into Peyer's patches is incompatible with the induction of oral tolerance (*Dunkley* and *Husband*, 1987).

Epithelial presentation of luminal antigens

Small intestinal epithelial cells express HLA class II determinants apically and basolaterally (*Scott* et al., 1980; *Selby* et al., 1983; *Scott* et al., 1987); they may therefore in theory be able to present immunogenic peptides to T lymphocytes which respond to stimuli both by producing effector molecules (lymphokines) and by proliferation (*Schwartz*, 1990). However, complete T cell activation requires two signalling events, one through the antigen specific receptor (TCR) and one through the receptor for a costimulatory molecule (*Schwartz*, 1990). In the absence of the latter signal (Figure 2), the T cells mount only a partial response and, more importantly, are subjected to clonal anergy in which state they do not produce their own growth factor interleukin 2 (IL2) on restimulation (*Schwartz*,

1990). In some cases costimulation can be provided by soluble mediators such as IL-1 (*Durum* et al., 1985). In other cases, cellular interactions are required (*Jenkins* et al., 1988). The two possibilities are difficult to distinguish because a close proximity is sometimes required to deliver high concentrations of labile mediators and such soluble ligands can act indirectly to increase the activity of the interacting cells (*Koide* et al., 1987). Activated keratinocytes induce anergy in T cell clones *in vitro*, presumably because they lack the ability to deliver a costimulatory signal (*Gaspari* et al., 1988). Murine intestinal epithelial cells expressing a relatively low level of MHC class II molecules, were unable to present bovine serum albumin (BSA) to antigen-specific T helper (Th) cells (*Zhang* and *Michael*, 1990). However, INF-γ treatment of the animals strongly enhanced epithelial MHC expression thus enabling antigen presentation to Th cells (*Zhang* and *Michael*, 1990).

Relatively low expression of MHC class II on intestinal epithelial cells may thus contribute to immunosuppression/anergy induced by oral administration of a protein antigen. Down-regulation of TCR and CD8 molecules on the antigen-specific T cells have also been suggested as a mechanism by which peripheral tolerance may occur (*Schonrich* et al., 1991; *Rocha* and *von Boehmer*, 1991). Nevertheless, anergic cells expressing IL-2R may be triggered *in vivo* by IL-2 from adjacent T cells responding to other antigens (*Schwartz*, 1990).

Virus infection may cause T cell activation with increased lymphokine production and increased epithelial MHC class II expression (Figure 2). Under these circumstances epithelial cells may be able to activate T cells because sufficient costimulatory signals are available (Figure 2). A virus infection may therefore be important in the initiation of coeliac disease by enabling epithelial cells in the presence of costimulatory signals to activate antigen specific T cells with gluten peptides.

Suppressor lymphocytes

The numerous intraepithelial $CD8^+$ T cells may be involved in oral tolerance. Bland and *Warren* (1986a) found that MHC class II positive columnar cells from rat villous epithelium presented ovalbumin *in vitro* to primed lymph node T cells, which thereby were induced to proliferate. This apparently class II restricted immune response led to antigen specific suppression and involved a $CD8^+$ subset (*Bland* and *Warren*, 1986b). The presence of T suppressor inducer (TSI) cells in the test system was not excluded. It has recently been shown that one chain of TCR of a particular $CD4^+$ clone can be secreted and contribute to induction of $CD8^+$ suppressor cells (*Zheng* et al., 1988). This finding suggests that $CD4^+$ TSI cells may be derived from $CD4^+$ Th cells and thus have identical specificity. *Mayer* and *Shlien* (1987) applied human colon epithelial cells in autologous or allogeneic mixed lymphocyte responses and included also macrophages pulsed with tetanus toxoid. They observed preferential stimulation of $CD8^+$ T lymphocytes. This might be due to crosslinking of CD8 molecules by CD1d (*Blumberg* et al., 1991a; 1991b) or another unique MHC class I-like epithelial molecule (*L. Mayer*, personal communication). The responding cells did not express a putative cytotoxicity marker (9.3) and showed no cytotoxic effect but caused nonspecific suppression (*Mayer* and *Shlien*, 1987).

It remains unclear how $CD8^+$ cells mediate suppression. T suppressor cells that recognise B or T cell idiotypes have been described (*Nisonoff* et al., 1977; *Lynch* et al., 1979; *Mohagheghpour* et

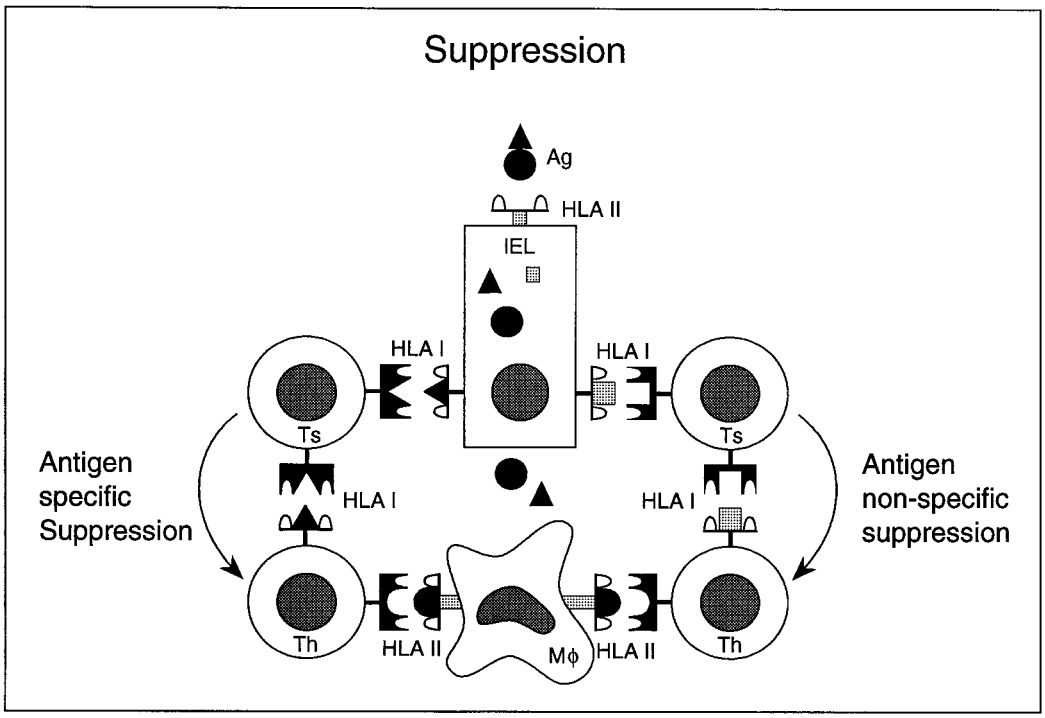

Figure 3: Putative mechanisms for T cell mediated suppression in small intestinal mucosa. Antigen is taken up by epithelial cell bound to apical HLA class II molecule. Antigen is further degraded to peptides with helper determinant (J) and suppressor determinant (H). The latter is presented to antigen specific $CD8^+$ T suppressor cell in the antigen binding grove of HLA class I molecule. The stimulated T suppressor cell returns to lamina propria where it interacts with $CD4^+$ Th cell expressing the suppressor determinant in the context of HLA class I (left). Alternatively, T suppressor cell is activated by fragment of HLA class II molecule presented in the context of HLA class I. T suppressor cell then returns to lamina propria and downregulates $CD4^+$ Th cell expressing the same HLA class II peptide in the antigen binding grove of a similar HLA class I molecule (right side of figure).

al., 1986; *Ben-Nun* et al., 1981; *Batchelor* et al., 1989; *Janeway*, 1989). It has also been found that antigens contain helper determinants and suppressor determinants which generally do not overlap (*Sercartz* and *Krzych*, 1991). Perhaps peptide binding to TCR is followed by endocytosis of the complex, processing and then loading of MHC class I molecules, which appear in the T cell endosomes (*Tse* and *Pernis*, 1984). T suppressor cells may also respond to MHC-class II peptides (*Sercartz* and *Krzych*, 1991) in the antigen binding grove of MHC class I molecules on Th cells (Figure 3). Moreover, soluble CD8 is released in response to lymphocyte activation (*Tomkinson* et al., 1989) and may function as an inhibitory ligand, thereby mediating the immunoregulatory activity of $CD8^+$ cells (*Hambor* et al., 1990). However, only cells undergoing primary but not secondary antigen stimulation through their TCR are susceptible to CD8 dependent inhibition (*Hambor* et al., 1990).

In mixed lymphocyte cultures with

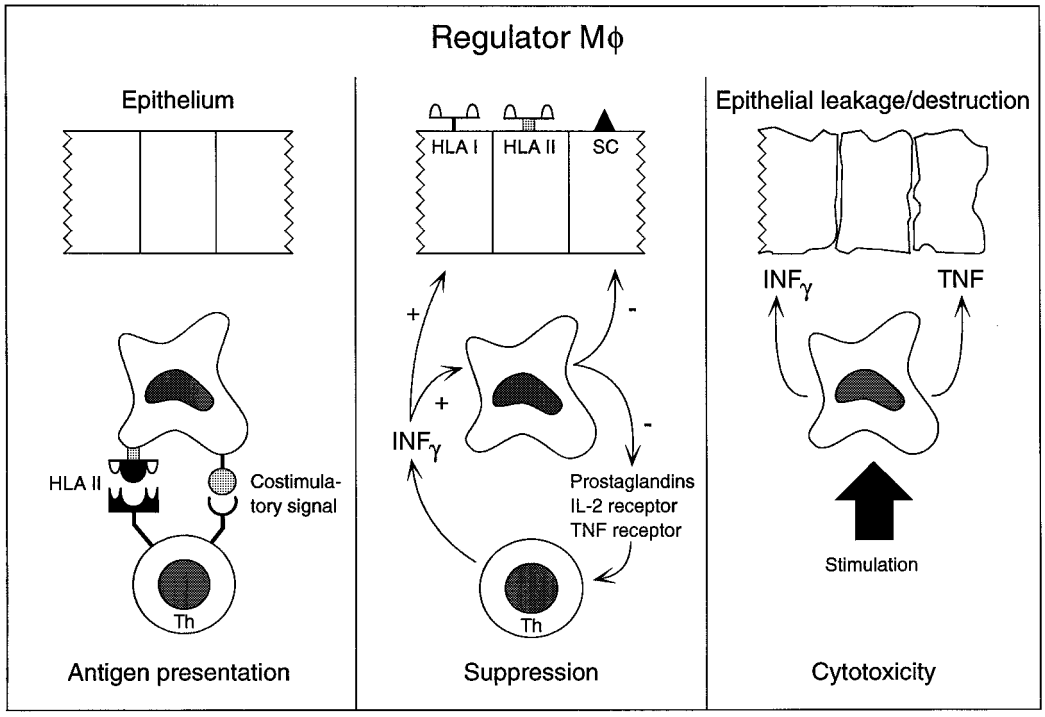

Figure 4: The subepithelial macrophage may have an important role in intestinal immune regulation: it may act as a professional antigen presenting cell (right); it may function as a suppressor cell by production of prostaglandins and soluble receptors for IL-2 and TNF (center); and it may also be a cytotoxic cell by producing TNF and INF-γ that synergistically cause epithelial damage (left).

peripheral blood mononuclear cells and intestinal epithelial cells from patients with inflammatory bowel disease, preferential stimulation of $CD4^+$ T cells is observed (*Mayer* and *Eisenhardt*, 1990). This difference from normal appears independent of the density of epithelial MHC class II molecules as shown by the fact that intestinal epithelial cells from other inflammatory disorders lead preferentially to stimulation of $CD8^+$ cells. There may thus be an intrinsic defect of certain surface molecules on epithelial cells from patients with inflammatory bowel disease, perhaps rendering crosslinking of CD8 molecules impossible. Similar experiments have not been performed in coeliac disease, but it would be very interesting if a preferential activation of $CD4^+$ cells takes place also in this disorder.

Cytotoxic "suppressor" cells

A "suppressor" cell may in theory downregulate an immune response by killing antigen presenting cells, B cells or Th cells. MHC class II restricted $CD8^+$ T suppressor cells efficiently inhibit specific antibody responses by lysing B cells with low levels of internalised and processed antigen (*Shinohara* et al., 1988). In addition, human T cells can process and present antigenic peptides in association with endogenous HLA class II molecules (*Lazavecchia* et al., 1988). Antigen specific Th cell may hence become the

target for cytotoxic T cells of the same specificity. However, studies of antigen specific unresponsiveness in humans with lepromatous lepra as model, suggest that specific $CD8^+$ T suppressor clones do not function by killing of antigen reactive Th clones (*Salgame* et al., 1989) and such cytotoxic cells may not operate *in vivo* (*Lanzavecchia*, 1989).

Regulatory macrophages

In the normal small intestine a population of large pleomorphic macrophages (Mø), positive for the myelomonocytic lysosomal antigen identified by Mab KPI but negative for the 36 KDa myelomonocytic L1 cytosol antigen or "calprotectin" (*Bjerke* et al., 1991) can be seen just below the surface epithelium. This subset is strongly HLA class II positive and also expressed constitutively HLA-DQ in contrast to the surface epithelial cells (*Scott* et al., 1987). Coeliac disease is notably associated with a particular HLA-DQ α/β heterodimer (*Sollid* et al., 1989). Because of the strategic location of these Mø, they may have an important role in intestinal immune regulation (Figure 4): A) They may act as "professional" antigen presenting cells, having the ability both to process antigens and give the necessary costimulatory signals for T lymphocytes. B) They may function as suppressor cells by production of prostaglandins (*Bray* et al., 1978; *Demenkoff* et al., 1980), and by release of blocking factors such as IL-2 receptors (*Loughnan* and *Nossal*, 1990) and tumour necrosis factor (TNF) receptors. In coeliac disease a large number of Mø express IL-2 receptors and this is seen to an even greater extent in small intestinal biopsy specimens from coeliac patients exposed for gluten *in vitro* (*Scott* et al., 1991). C) Activated Mø are effective killer cells; they may eliminate parasites and tumour cells by producing TNF and other toxic cytokines but may also contribute to the damage and increased turnover of surface epithelial cells seen in coeliac disease.

The function of the subepithelial Mø probably depends on the local cytokine profile. Increased antigen load may cause stimulation of T lymphocytes with enhanced interferon-γ (INF-γ) production. INF-γ may activate Mø to produce suppressive factors in a negative feed back system. However, on excessive stimulation of the intestinal immune system, mucosal Mø may change to killer cells with a predominant production of TNF (Figure 4).

T lymphocytes expressing TCRγ/δ^+ receptor

$TCR\gamma/\delta^+$ lymphocytes comprise a separate sublineage of T cells with mostly unknown specificity and restriction elements (*Bluestone* and *Matis*, 1989). Intestinal $TCR\gamma/\delta^+$ cells show a remarkable tropism for the epithelium and employ the variable $\delta 1$ gene ($V\delta 1/J\delta 1$) much more frequently than their counterparts in peripheral blood (*Spencer* et al., 1989; *Halstensen* et al., 1989; *Trejdosiewiz* et al., 1989). Intestinal $TCR\gamma/\delta^+$ IEL may primarily be directed against intestinal microorganisms or are perhaps specialised for elimination of transformed, infected or otherwise stressed autologous cells (*Janeway*, 1988; *Janeway* et al., 1988; *Lefrancois* and *Goodman*, 1989). The $TCR\gamma/\delta^+$ subset is also claimed to mediate contrasuppression in mice (*Fujihashi* et al., 1990).

The lymphokine profile of stimulated $TCR\gamma/\delta^+$ cells is at yet unknown. Both $TcR\alpha/\beta^+$ and $TCR\gamma/\delta^+$ cells may produce haemopoietic growth factors and granulocyte macrophage colony stimulating factor after stimulation, but IL-2 and IL-4 seem to be primarily produced by the $TcR\alpha/\beta^+$ cells (*Warren* et al., 1989). Stimulated $TCR\gamma/\delta^+$ IEL may

produce a different selection of lymphokines, perhaps depending on their actual CD45 isoform (*Halstensen* et al., 1990). Whether human TCRγ/δ IEL in coeliac disease are cytotoxic remains to be shown.

PUTATIVE IMMUNOPATHOLOGICAL MECHANISMS IN COELIAC DISEASE

Coeliac disease may be caused by a break in oral tolerance. A genetic predisposition may involve both HLA and the T cell receptor. Adenovirus 12 has been proposed as an additional environmental factor because of its cross-reactivity with A-gliadin (*Kagnoff* et al., 1987) but other studies have not supported this view (*Howdle* et al., 1989; *Carter* et al., 1989). However, a virus infection, perhaps combined with a high gluten intake, may supply the intestinal mucosa with sufficient costimulatory signals to change the local homeostasis from anergy/suppression to immunostimulation. HLA-DQ positive subepithelial Mø may present gluten peptides to gluten reactive T cells. Activated T cells may produce lymphokines that directly or indirectly increase the proliferation of epithelial crypt cells, increase intestinal permeability, activate B cells, transform B cells to plasma cells, and induce cytokine production in macrophages. Cytokines such as TNF, in addition to local complement activation, may exert a direct toxic effect on epithelial cells leading to increased desquamation and secondary crypt hyperplasia.

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ENHANCEMENT OF GUT WALL DEFENCES AGAINST ENTERIC PATHOGENS

RICHARD I. WALKER, FLORENCE M. ROLLWAGEN, and OLGERTS R. PAVLOVSKIS

Enteric Diseases Program, Naval Medical Research Institute, Bethesda, MD, USA

SUMMARY

Numerous micro-organisms can, under the right conditions, exploit the mucosal surface of the intestine. A healthy ecological balance at the gut wall is maintained by a combination of antimicrobial defences. These include the mucus coat which has both physical and physiological effects on gut micro-organisms, colonisation resistance due to the indigenous intestinal flora, and the induction of specific mucosal antibody responses. Advances in immuno- and biotechnology now provide opportunities to construct orally administered vaccines to safely enhance mucosal immunity. Studies towards development of a killed cell vaccine against *Campylobacter*, a major enteric pathogen world-wide, illustrate the problems and progress in constructing such vaccines. Recent work has used the heat-labile toxin of enterotoxigenic *Escherichia coli* to increase humoral immune responses to killed *Campylobacter* cells. The antibodies induced by mixing this adjuvant with the non-living *Campylobacter* antigen could be associated with protection of experimental animals from subsequent colonisation by living bacteria of the same serogroup. The approaches being used to construct a vaccine against *Campylobacter* may have application against a wide variety of mucosal pathogens.

INTRODUCTION

The intestine is a versatile organ. Not only does it perform a variety of physiologic functions responsible for digestion, absorption, and regulation of waste and electrolyte balance, but it also serves as the major microbial ecosystem on the human body. The number of micro-organisms in the intestine increases distally from 10^4/ml or less in the stomach and upper small bowel to about 10^8/ml in the lower ileum. In the colon there are up to 10^{11} organisms/gm of faeces. These micro-organisms exploit every type of relationship with their host to include commensalism, mutualism, neutralism, and parasitism (*Rusch*, 1989). The control of these relationships depends upon the maintenance of a balance between host and microbial factors. This balance can have nutritional and immunological benefits for the host, but its loss can have debilitating and deadly effects.

When normal gastrointestinal defence mechanisms are rendered ineffective by injury or disease, opportunistic micro-organisms can multiply on the gut wall and become serious problems (*Porvaznik* et al., 1979; *Walker* and *Porvaznik*, 1983). By far the largest

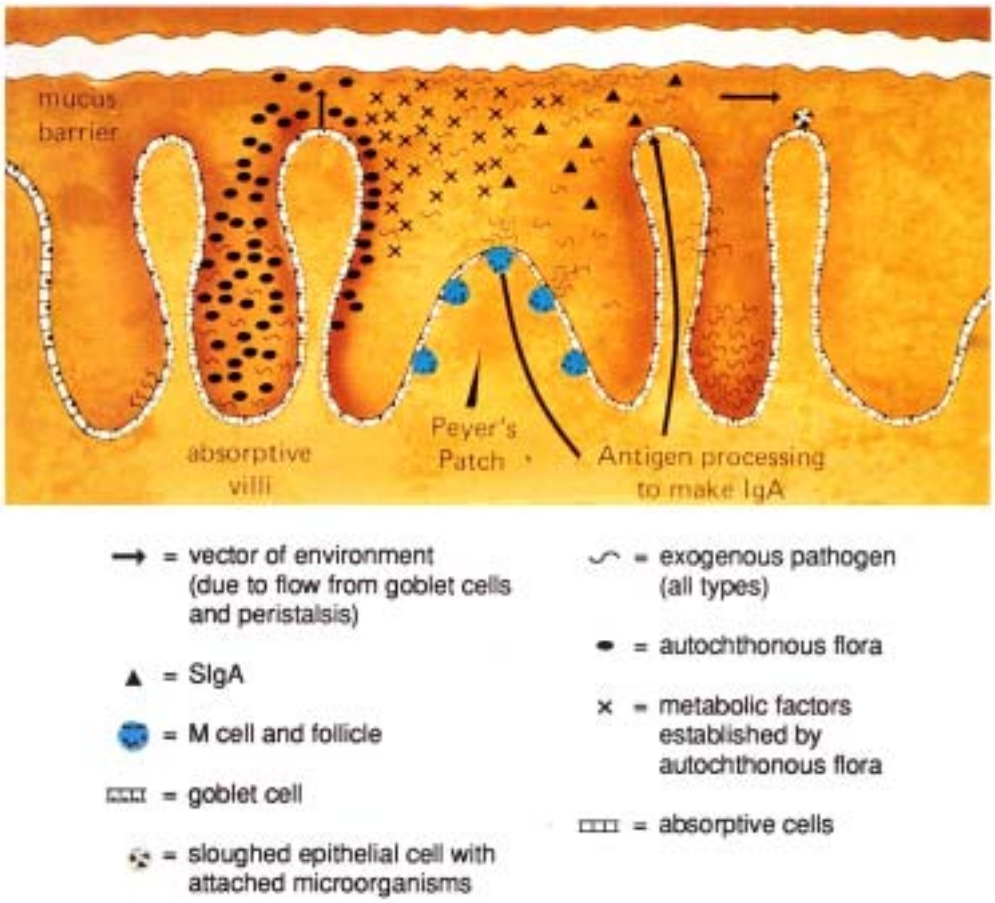

Figure 1: Diagram illustrating defence mechanisms whereby the intestine keeps potentially pathogenic micro-organisms from colonising sites where they may initiate disease. Mechanisms represented include mucus-antibody blockage of epithelial colonisation, colonisation resistance, peristalsis and cell sloughing. See text for details.

cause for concern about intestinal pathogens comes from those enteric organisms which can circumvent normal defences and cause diarrhoeal or dysenteric disease. These agents are second only to cardiovascular diseases as major killers world-wide, being responsible for an estimated 5,000,000 deaths annually with morbidity extending over a billion (*Sack* et al., 1991). Reduction of this problem will have social and political consequences reaching far beyond the obvious medical benefits. For this reason, attainment and distribution of vaccines against enteric and other mucosal pathogens is one of the greatest challenges facing medical science today. The following report will focus first on an overview of intestinal defence mechanisms and then, using *Campylobacter* as a prototype enteric pathogen, consider means by which the antimicrobial function of the gut wall can be made more effective through immunisation.

NORMAL GUT WALL FUNCTION IN ANTIMICROBIAL DEFENCES

A healthy microecology in the gut is maintained by a combination of host and microbial factors (Figure 1). The mucus barrier, a gel 450 μm thick, is the major site for microbial colonisation within the intestine (*Rozee* et al., 1982). By retaining and regulating potential pathogens at this site, the mucus can protect the epithelium from injury by micro-organisms (Table 1).

The mucus barrier can affect colonisation of mucosal surfaces by covering glycolipid and glycoprotein receptors on the surface of epithelial cells to which micro-organisms can attach (*Gibbons*, 1982). Alternatively mucus components can mimic receptors on the cell surface to competitively exclude pathogens. For example, *Escherichia coli* bound well to ileal epithelial cells after passing through newborn piglet ileal mucus, but it did not bind to these cells after passing through 35-day-old piglet ileal mucus (*Conway*, *Welin* and *Cohen*, 1990). Protection of epithelial cells by mucus may account for the relative resistance of guinea pigs to *Shigella* infections compared to monkeys. Guinea pig mucus inhibits the invasion of epithelial cells in vitro, but monkey mucus does not (*Dinari* et al., 1986). Since the mucus blanket is continuously secreted and removed through peristalsis, many bacteria contained in it rather than attached to the epithelial surface are flushed out of the intestine.

In addition to its role in blocking and/or flushing mechanisms, mucus can contribute to defence of the gastrointestinal tract by regulating physiologic activities of micro-organisms. *McCormack* and colleagues (1988, 1990) found that *Salmonella typhimurium* and *Escherichia coli*, which are motile in ileal mucus, become non-motile when grown in mouse coecal mucus. This change apparently occurs without the loss of flagella. Enhancement or suppression of bacterial growth and expression of some cell components are also affected by differential utilisation of mucus nutrients by micro-organisms (*Franklin* et al., 1990). Growth in mouse coecal mucus, for example, is accompanied by the appearance of several major outer membrane and periplasmic proteins with the concomitant loss of other proteins as opposed to organisms grown in broth (*Paul Cohen*, personal communication).

The importance of colonisation resistance by indigenous micro-organisms as a defensive barrier in the intestine has been demonstrated by *van der Waaij* and colleagues (1971, 1972). Metabolic factors or conditions generated by indigenous flora in the intestine are essential for helping to maintain the balance between the host and those organisms which can overwhelm either nor-

Table 1: The Effects of Mucus on Bacteria Entering the Intestine

Physical Effects:
· Blocks microbial interaction with receptors on epithelial cells
· With peristalsis, contributes to flushing micro-organisms from the intestine
Physiological Effects:
· Mucus from some parts of the intestine may render bacteria immobile
· May enhance or suppress microbial growth
· Can modify expression of surface and periplasmic proteins of bacteria

mal defences or those impaired by injury or disease. In irradiated rats in which indigenous populations are altered, overgrowth of the intestine by facultative bacteria is observed prior to their appearance in other organs and death of the host (*Porvaznik* et al., 1979; *Walker* and *Porvaznik*, 1983). The effectiveness of colonisation resistance in controlling other micro-organisms was shown by *Wells* and colleagues (1987) who found that, when anaerobic flora were eliminated in mice by metronidazole treatment, the animals experienced significant rates of dissemination of facultative intestinal bacteria into the mesenteric lymph nodes. *Brook* et al. (1988) accelerated the progress of opportunistic infections in irradiated mice by treating them with metronidazole.

One of the major components of gut defence against pathogens is specific immunity. Exposure of the intestine to microbial antigens begins after birth, so that resistance to many organisms found in the environment is eventually acquired. A major site for immunologic processing of micro-organisms is the Peyer's patch (*Walker* and *Owen*, 1990). The epithelium covering the Peyer's patch contains M cells which actively take up and transport antigens. The follicles beneath the M cells contain lymphocytes and macrophages essential for processing antigen for an immune response (*Keren*, 1989; *Azim*, 1991).

Lymphocytes travel from Peyer's patches to the mesenteric lymph nodes and spleen where further activation occurs. Some of these cells return to the intestinal wall to facilitate local defence through production of immunoglobulin A (IgA) at the gut wall, whereas others enter the general circulation to act at other mucosal sites.

PROSPECTS FOR ENHANCEMENT OF GUT WALL DEFENCES WITH MUCOSAL VACCINES

Development of vaccines against the many pathogens which exploit mucosal surfaces offers the best hope for defence against infection. Compared to parenteral vaccinations useful against systemic pathogens, relatively little progress toward successful mucosal vaccines has been made until recently. Now new advances in biotechnology and immunotechnology provide opportunities to safely enhance normal defence mechanisms of the gastrointestinal tract.

Campylobacter jejuni has recently been recognised as an important human enteric pathogen (*Cornick* and *Gorbach*, 1988) and can serve as a prototypic organism for exploration of the problems of developing mucosal vaccines. *C. jejuni* is a common cause of diarrhoea in both the developed and developing world as well as among travellers. With at least 2.4 million cases annually, this organism is more frequently isolated in the United States than either *Salmonella* or *Shigella* (*Taylor* and *Blaser*, 1991).

Importance of humoral immunity

Available data suggest that successful vaccination for *Campylobacter* enteritis is possible. Studies of the immune response of a cohort of 111 newborn infants with *Campylobacter* infections showed that nearly all of the children were naturally immunised by the age of two years (*Martin* et al., 1989). American adult volunteers challenged with *Campylobacter jejuni* developed serum antibodies and were protected from subsequent illness, but not against infection (*Black* et al., 1988). Immunisation of pathogen free *Macaca nemest*

Table 2: Comparison of the Effect of Matching Lior Serogroups on Cross-Protection Against Colonisation of Rabbits by *Campylobacter*

Immunisation[1]		Challenge[2]		Colonisation Time
Strain	Lior Group	Strain	Lior Group	(Days ± S.D.)
None	---	VC159	8	7.2 ± 2.6
VC159	8	VC159	8	1.0 ± 1.2
VC159	8	VC95	7	10.6 ± 3.9
None	---	VC95	7	11.5 ± 3.0
None	---	VC74	11	7.2 ± 0.8
VC74	11	VC74	11	1.2 ± 1.2
VC159	8	VC74	11	6.0 ± 1.9
None	---	VC91	11	6.2 ± 1.8
VC74	11	VC91	11	0.8 ± 0.8

[1]Rabbits given 10 ml of 10^9 live *Campylobacter* by naso-gastric tube
[2]Rabbits challenged via the RITARD procedure (see text) four weeks after naso-gastric immunisation. Challenge dose was 10 ml of 10^9 live cells.

rina monkeys with *C. jejuni* protects against rechallenge (*Russell* et al., 1989).

The Removable Intestinal Tie Adult Rabbit Diarrhoea (RITARD) model has served as a good experimental system in which to study mucosal immunity to *Campylobacter*. In this model the cecum is ligated and the bacteria are injected into the ileum anterior to a slip knot placed temporarily on the ileum to block peristalsis (*Caldwell* et al., 1983). If rabbits are fed live organisms via a gastric intubation, they spontaneously clear the organism in 1-2 weeks (Table 2). If challenged with the RITARD procedure at 30 days post feeding, the immunised rabbits rapidly clear the homologous but not heterologous organism, generally in less than two days (*Burr* et al., 1988) Strong protection as manifested by rapid clearance can occur among *Campylobacter* strains only of matched Lior (heat labile cell surface components) serotypes (*Pavlovskis* et al., 1991). At present it appears that the Lior serotype predicts an associated flagellar structure which may contribute to cross strain protection.

McSweegan and colleagues (1987) demonstrated that mucus and antibodies may be responsible for the rapid clearance of *Campylobacter* from intestines of immunised rabbits. When epithelial cells in vitro were overlaid with mucus from nonimmune rabbits, penetration of the mucus by *Campylobacter* and subsequent attachment to epithelial cells were reduced, compared to preparations in which the mucus was replaced with bovine serum albumin. If the mucus came from immunised animals, the penetration was much further reduced compared to nonimmune mucus. This effect could be negated by absorption of the immune mucus with the homologous *Campylobacter* strain, but not with *E. coli*. Interestingly, antibodies collected by intestinal lavage (*Burr* et al., 1987) were not sufficient to retard bacterial interaction with epithelium. If the lavage fluid was mixed with mucus from nonimmune animals, however, then penetration was significantly inhibited (*McSweegan* et al., 1987).

Considerations for a killed *Campylobacter* vaccine

Immunisation with a safe vaccine is complicated by the fact that at present little is known about the genetics and virulence factors of *Campylobacter* and their regulation. For this reason, attenuated mutants or antigen carrier organisms are not available for use as vaccines, making *Campylobacter* a prime candidate for a killed whole cell vaccine.

Killed organism vaccines for mucosal immunisation have generally not been as effective as living vaccines. Two major problem areas may account for this fact. The first is antigen preparation. A living vaccine can adapt to the mucosal environment and, as the studies with growth in mucus containing media indicate (*Paul Cohen*, personal communication), modify its antigenic profile to one that will be more effective in colonising the host. This profile may induce protective antibodies, whereas antigenicity of bacteria grown in culture media not containing essential factors from the intestine may not be appropriate for optimal protection. Producing organisms for killed-cell vaccines that are antigenically similar to those growing *in vivo* will probably be best obtained by growing the organisms in media which closely mimic essential features of the intestinal environment.

Even if organisms are grown optimally, the way in which they are killed for vaccine preparation may influence the specificity of the antibodies they induce. Some treatments can alter or destroy critical antigens. Recently, heat shock proteins have been identified which significantly alter the antigenic structure of bacteria (*Kaufmann*, 1990). Thus the temperature at which an organism is grown and the heating regimen used to kill the organism may significantly alter the antigenicity of a pathogen.

Antigen presentation is another major problem area that should be considered when immunising mucosal surfaces with non-living materials. Means must be available to deliver critical antigens in their optimal state to the gut associated lymphoid tissue (GALT). There are several possibilities to accomplish effective antigen delivery which include encapsulation of material in liposomes (*Jackson* et al., 1990) or lactide-glycolide microspheres (*Eldridge* et al., 1991), conjugation of proteins (*Klipstein* and *Engert*, 1983; *Klipstein* et al., 1983; *McKenzie* and *Halsey*, 1984), or administration of whole killed bacteria as antigen bearing vehicles. For example, a whole cell killed cholera vaccine to which the B subunit of the cholera toxin has been added as an antigen for generation of anti-toxin activity has been tested with success in Bangladesh (*Svennerholm* et al., 1984; *Clemens* et al., 1988). Regardless of the delivery system, most non-living vaccines must be administered in several doses to achieve maximal effects.

Adjuvants have been used to greatly magnify systemic immune responses obtained with antigen alone. For this reason considerable interest has been directed toward finding adjuvants active at the mucosal surface. Among the substances that have been reported to have some adjuvant effects at the mucosal surface are avridine (*Anderson* et al., 1985; *Pierce* et al., 1985) and muramyl dipeptide (*Butler* et al., 1983; *Taubman* et al., 1983). Cholera toxin has also been found to have adjuvant properties (*Elson* and *Ealding*, 1984). Mice given minute amounts of cholera enterotoxin significantly below the levels causing fluid accumulation in the intestine increased the immune response to a protein antigen by 50 fold (*Lycke* and *Holmgren*, 1986).

Since there are antigenic similarities between cholera enterotoxin and the

Table 3: Characteristics of the Recombinant Heat-Labile Enterotoxin from enterotoxigenic *E. coli* (ETEC)

- · Has A and B subunits
  - A subunit stimulates adenylate cyclase activity
  - B subunit binds toxin to cell surface receptor
- · Immunologically and structurally related to cholera toxin
- · Enhances serum IgA and/or mucosal IgA against antigen with which it is delivered
- · Holotoxin probably required for best activity
- · Does not need to be bound to antigen, but must be given at the same time

heat labile toxin (HLT) of enterotoxigenic *E. coli* (*Clemens* et al., 1988), HLT has also been tested for adjuvant activity. The characteristics of recombinant HLT from enterotoxigenic *E. coli* (ETEC) are summarised in Table 3. Recombinant HLT was shown to influence induction and maintenance of tolerance in mice primed with orally administered antigen (*Clements* et al., 1988). During these studies it was noted that the mucosal IgA antibody, as well as serum IgG and IgA, response to ovalbumin was greatly enhanced with the simultaneous administration of HLT. The adjuvant does not need to be bound to the antigen, but must be given at the same time.

HLT, like cholera toxin, has A and B subunits (*Elson*, 1989; *Dertzbaugh* and *Elson*, 1991; *Sixma*, et al., 1991). The A subunit catalyses ADP-ribosylation of the stimulatory GTP-binding protein (Gs) in the adenylate cyclase complex resulting in increased intracellular levels of cyclic AMP. The B subunit binds the toxin to its cell surface receptor the Gm_1 ganglioside. The B subunit is the non-toxic portion of the adjuvant, but evidence suggests that the holotoxin is probably required for the best immunologic response. Nonetheless, as *Holmgren*'s data (*Lycke* and *Holmgren*, 1986) suggest, the margin of safety between a toxic dose and an effective adjuvant dose of the holotoxin could be large enough to make use of enterotoxic adjuvants practical.

Use of HLT adjuvant with killed cells to protect against infection with Campylobacter

The findings that certain bacterial enterotoxins can act as adjuvants raised the possibility that they can be used in vaccines against the many pathogens which attack these surfaces. For this reason we conducted oral vaccination studies in mice and rabbits by using sonicated whole cell *Campylobacter* preparations as antigen and HLT as an adjuvant (data to be reported elsewhere).

Bacteria (0.5ml containing 10^9 organisms) fed to mice colonise the intestine until cleared 4 weeks later with norfloxacin (200 mg/kg). Intestinal lavage fluid was taken from these animals 6-8 weeks after their initial feeding with bacteria as well as from animals given 300 μg of sonicated *Campylobacter* cells administered three times at weekly intervals. When the two groups were compared a potent antigen specific IgA immune response was seen only in those animals given live cells. When 25 μg of HLT was administered with each dose of antigen, then the non-living material induced an intestinal immune response comparable to that obtained with live cells.

Although *Clements* (1988) had found 300 μg of antigen optimal, his work used pure protein while the soni-

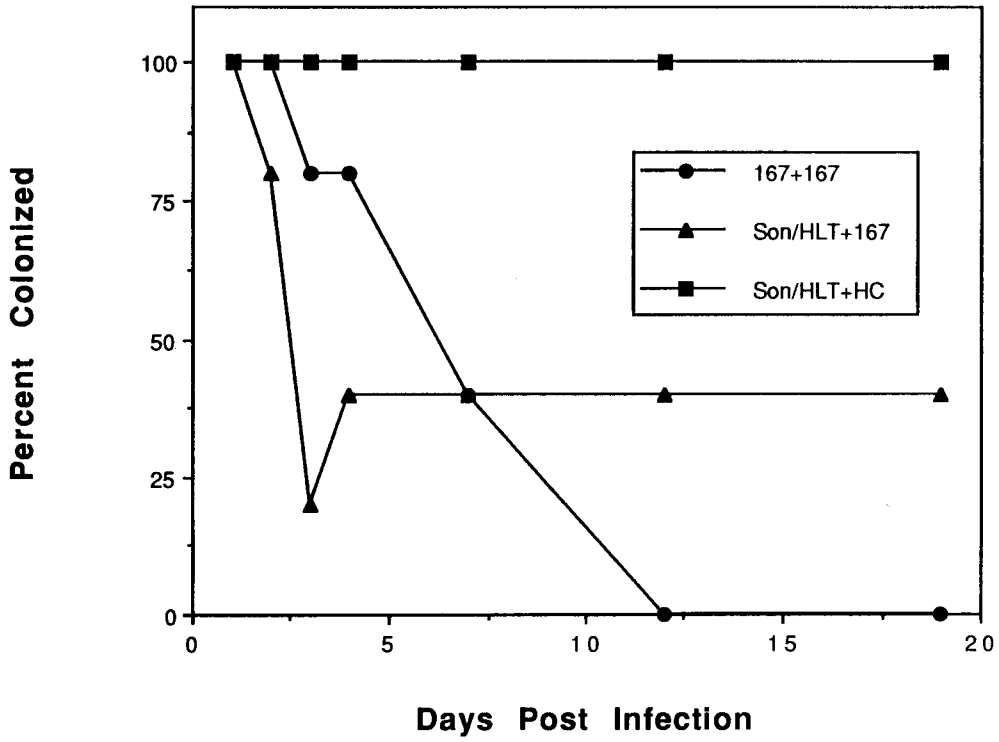

Figure 2: Rate of clearance of live homologous (strain 167) and heterologous (strain HC) serogroups of *Campylobacter* from intestines of mice immunised with live bacteria or killed 167 strain bacteria. The killed bacteria were administered with oral adjuvant (HLT). Information to the left of the plus sign is the immunising preparation and the material indicated on the right hand side of the plus sign is the challenge organism.

cated bacteria were made up of a complex mixture of antigens. For this reason the consequences of using smaller doses of the antigen were determined. The experiment was conducted so that intestinal lavages were collected from mice one week after each immunisation dose (live organisms were also administered three times even though the mice remained colonised in between doses). The most rapid response occurred in mice fed with live bacteria. Immune responses to the non-living material began to be seen after 2 weeks, with 300 μg and 100 μg dose groups responding faster than a 50 μg antigen group. By three weeks after the first inoculation, the major immune response was seen in animals given 300 μg of bacterial sonicate plus HLT.

Enterotoxin adjuvants are also antigens (*Elson*, 1989; *Dertzbaugh* and *Elson*, 1991). Thus when an ELISA plate was coated with HLT instead of sonicated bacteria, a strong IgA response to the toxin was detected in lavage fluid from animals immunised with HLT-sonicate combinations. No cross reactivity was seen with lavage fluid from animals given live bacteria. In contrast, lavage fluids from mice given cholera toxin-sonicate combinations showed moderate cross reactivity on the HLT-coated plates. This antigenic relatedness was also apparent when cholera toxin was used to coat the ELISA plates. Lavage from the cholera toxin immunised mice reacted strongest, while lavage from

HLT-immunised animals reacted to a lesser extent.

The biological activity of the two enterotoxin adjuvants was also similar. When cholera toxin was substituted for HLT in vaccine made of sonicated *Campylobacter*, it produced an immune response comparable in magnitude to that seen with live cells alone or killed cells given with HLT.

The enhanced immune response obtained when HLT was used with sonicated *Campylobacter* cells was found to be associated with increased resistance to challenges with live organisms. When mice fed live organisms were cleared with antibiotics and rechallenged with the homologous strain of *Campylobacter*, 100% of the animals cleared the bacteria in less than two weeks. Sixty percent of those given sonicate and HLT were also able to clear the infection. On the other hand when immunised mice were challenged with a *Campylobacter* strain of a different Lior serotype no protection was seen and all mice remained colonised through the three week period of the experiment (Figure 2). These data establish that the increase in clearance rate is due to specific rather than non specific effects of the immunisation procedure.

Challenge studies using HLT and sonicated antigen were also conducted in rabbits. Three weekly feedings with either the HLT or sonicates alone gave no protection. Rabbits still required about seven days to clear the organism. When the HLT and sonicate were administered together over the two week immunisation period, the colonisation time when the animals were challenged dropped to less than three days.

It was necessary to administer the HLT with each dose of sonicate. No protection was seen if the HLT was given with the first antigen dose, but not the subsequent two. Three doses of Lior serogroup 8 strain sonicate plus HLT provided no protection against challenge with a Lior serogroup 6 strain of *Campylobacter*. This suggests once again that a specific antibody response is induced by the HLT-sonicate immunisation which accounts for the rapid clearance of the pathogen from the intestine.

FUTURE PROSPECTS FOR ORAL MUCOSAL VACCINES

The studies reported above showed for the first time that HLT administered with sonicated *Campylobacter* cell fragments can be used to induce protective immunity. No protection was seen when antigen was administered without the adjuvant, but significant protection associated with increased humoral responses was seen when the adjuvant was added to the vaccine.

There are several possible approaches to enhancing the effectiveness of gut defence mechanisms through immunisation. Our findings with HLT and sonicated micro-organisms may provide one approach for development of oral vaccines against a variety of mucosal pathogens. Since most mucosal vaccines may benefit from inclusion of an adjuvant, the search for other oral adjuvants with at least the efficacy of the enterotoxins, but perhaps with greater safety, should continue. The possibility that other adjuvants may work by different mechanisms than the enterotoxin adjuvants and thereby be used synergistally should also be explored.

Oral adjuvants should also be useful with subunit vaccines whose immunogenicity may also be facilitated by delivery to the GALT by microspheres or li-

posomes. The killed cholera whole cell-B subunit vaccine (*Svennerholm* et al., 1984; *Clemens* et al. 1988) may also have greater effectiveness if used with adjuvants. This vaccine administered with HLT could have effectiveness against *Campylobacter* infections due to cross reactivity observed among the toxins of *Campylobacter*, entertoxigenic *E. coli* and cholera (*Gossens* et al., 1985; *Klipstein* and *Engert*, 1985; *Hariharan* and *Panigrahi*, 1990). The utility of adjuvants with attenuated mutants and carrier vaccine strains of micro-organisms expressing important antigens needs to tested.

In addition to using immunisation of the gut mucosa to control enteric pathogens, immunisation against certain facultative flora (i.e. *Pseudomonas aeruginosa*) by this route should also help control opportunistic pathogens. Numbers of these pathogens increase in the gut of immunocompromised individuals and, as shown by the work with maintenance of colonisation resistant flora this can lead to systemic infections (*van der Waaij* et al., 1971, 1972).

The common mucosal immune system should make it possible to vaccinate orally and achieve immunity against opportunistic organisms and other pathogens colonising distant mucosal sites. For example, *Jacqueline Katz* (personal communication) has used HLT with an inactivated influenza virus administered intragastrically to induce a vigorous IgA response in the lung.

Much work remains to done. The simplicity of oral administration, the relative safety of using complex (i.e. whole cell) vaccine preparations by the oral route, and the large number of pathogens which may be controlled as successful means for mucosal immunisation are perfected provide an impetus for development. It should be possible in the foreseeable future to begin to regulate gut wall defences with mucosal vaccines for the benefit of mankind.

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THE USE OF FLOW CYTOMETRY IN THE ANALYSIS OF LIPOPOLYSACCHARIDE EPITOPE EXPRESSION IN *ESCHERICHIA COLI* O26:B6 VARIANTS

MARTIN E. EVANS

Division of Infectious Diseases, Department of Medicine, Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, Bethesda, Maryland, USA

SUMMARY

Gram-negative bacteraemia and septic shock due to bacterial lipopolysaccharide (LPS) are important causes of morbidity and mortality in spite of the use of potent bactericidal antibiotics. Adjunctive therapy with antibodies directed against LPS may be of value. In the evaluation of binding of LPS core- and lipid A-specific monoclonal antibodies (MAbs) to smooth bacteria using an indirect immunofluorescence technique and epifluorescence microscopy, a small proportion of *E. coli* O26:B6 were observed to bind the core-specific MAb J8-4C10. To examine *E. coli* O26:B6 further, the cells were simultaneously stained with FITC-labelled O-antigen specific MAb E5-3A5, and biotinylated MAb J8-4C10 followed by a streptavidin R-phyco-erythrin conjugate and analysed by dual parameter flow cytometry. Sixty-four percent of bacteria bound O-antigen specific MAb, 21% bound core-specific MAb, and 13 % bound both MAbs consistent smooth, rough, and semi-rough LPS phenotypes respectively. Bacteria from the stock culture were stained with MAb J8-4C10 and smooth and rough/semi-rough bacteria isolated using a fluorescence-activated cell sorter. When the sorted cells were re-analysed, 96% of the smooth bacteria stained with MAb E5-3A5, and 93% of the rough/semi-rough bacteria stained with MAb J8-4C10 confirming that the bacteria had been sorted with high efficiency. When purified LPS from the isolates was compared by SDS-PAGE and immunoblot, few high molecular weight O-antigen bearing bands were seen in LPS from bacteria that had stained with MAb J8-4C10 confirming the rough/semi-rough phenotype of these bacteria. Cultures of smooth bacteria may contain subpopulations with rough or semi-rough LPS phenotype. Flow cytometry technology facilitates identification and isolation of these subpopulations for further analysis.

INTRODUCTION

Lipopolysaccharide (LPS), or endotoxin, is a major antigenic structure on the surface of Gram-negative bacteria, and a principal mediator of septic shock. This tripartite macromolecule consists of lipid A, which is inserted into the phospholipid bilayer of the bacterial outer membrane, the covalently attached core oligosaccharide, and, in the case of smooth LPS, the distally placed O-polysaccharide.

Antibodies to LPS may be useful

adjuvants in the therapy of septic shock if they neutralise endotoxin, or opsonise bacteria and facilitate the clearance of pathogens by phagocytes. In the evaluation of the binding of LPS core- and lipid A-specific monoclonal antibodies (MAbs) to bacteria using an indirect immunofluorescence antibody staining technique and epifluorescence microscopy, a subpopulation of purportedly smooth *E. coli* O26:B6 was observed that stained with the core-specific MAb J8-4C10. This suggested that a small proportion of cells lacked masking O-side chains and were rough or semi-rough.

In this study, cells in a stock culture of *E. coli* O26:B6 were simultaneously stained with MAbs directed against the LPS O-antigen and core oligosaccharide and analysed by dual parameter flow cytometry. Subpopulations of bacteria that bound O-antigen specific MAb (smooth), core-specific MAb (rough), or both MAbs (semi-rough) were observed. Smooth and rough/semi-rough bacteria were isolated by cell sorting, and their purified LPS compared by SDS-PAGE and immunoblot. Few O-antigen bearing, high molecular weight LPS moieties were seen in the LPS of bacteria that had bound MAb J8-4C10 consistent with the rough/ semi-rough LPS phenotype predicted by flow cytometry.

MATERIALS AND METHODS

Bacteria

E. coli O26:B6 was obtained from List Biological Laboratories (Campbell, CA) and maintained as a stock culture at -70°C. Smooth *E. coli* O26S and rough/semi-rough *E. coli* O26SR were isolated from this culture using a fluorescence activated cell sorter (see below).

Reagents and media

Paraformaldehyde, bovine serum albumin, and biotin N-hydroxysuccinimide ester (NHS) were purchased from Sigma Chemical Co. (St. Louis, MO). An affinity purified, FITC-labelled, goat anti-mouse Ig was purchased from Organon Teknika (West Chester, PA). Tryptic soy agar and broth were purchased from Difco (Detroit, MI). Fluorescein isothiocyanate (FITC) on celite 10% was purchased from Calbiochem (La Jolla, CA), and a streptavidin R-phyco-erythrin conjugate was obtained from Molecular Probes, Inc. (Eugene, OR).

LPS-specific monoclonal antibodies (Table 1)

Murine MAbs reactive with different structural elements of *E. coli* LPS were prepared employing previously described immunisation, fusion, screening, and cloning procedures (*Pollack* et al., 1989). MAbs E5-3A5 (IgG2a) and E5-3G12 (IgG2a) were specific for the O-antigen of *E. coli* O26:B6 based on reactivity with purified homologous LPS, staining of high molecular weight LPS moieties on immunoblot, and lack of reactivity with heterologous rough or smooth LPS or whole bacteria (unpublished data). MAb J8-4C10 (IgG2a) was specific for 3-deoxy-D-manno-octulosonic acid (KDO) based on reactivity with the incomplete core oligosaccharide of purified *E. coli* D31m4 (Re) LPS, loss of this reactivity when KDO was cleaved from lipid A by mild acid hydrolysis, and inhibition of reactivity in ELISA by purified KDO. MAb 13-17 (IgG2a), specific for an unrelated protein on the surface of *Para-*

Table 1: Lipopolysaccharide-specific monoclonal antibodies employed in flow cytometric analyses of *E. coli* O26:B6 variants.

MAb	Isotype	Specificity*
E5-3A5	IgG2a	O26:B6 O-Antigen
E5-3G12	IgG2a	O26:B6 O-Antigen
J8-4C10	IgG2a	KDO-disaccharide
13-17	IgG2a	Negative control

*Determined in ELISA and immunoblot

mecium multimicronucleatum, was employed as a negative control (*Pollack* et al., 1989).

MAbs were purified with a membrane affinity separation system (Nygene Corp., Yonkers, NY), and conjugated with FITC by a modification of a previously described procedure (*Rinderknecht*, 1962). Briefly, 10 mg MAb in bicarbonate buffer (pH 9.5) was mixed with 2 mg FITC on celite and incubated at room temperature for 4 hours. After removal of the celite by centrifugation, the mixture was passed over a Sephadex G-25 column to separate labelled MAb from unconjugated FITC. The protein content and amount of fluorescein bound to each antibody molecule in the final preparation were measured spectrophotometrically (*Goding*, 1983).

Purified MAbs were biotinylated by mixing 1 mg MAb in bicarbonate buffer (pH 8.3) with biotin NHS and incubating for two hours at 25°C (*Goding*, 1983). The mixture was then dialysed to remove excess unconjugated biotin.

Flow cytometric assays of MAb binding to viable bacteria

Bacteria were grown, harvested and then stained using a direct, or indirect immunofluorescence technique (*Evans* et al., 1990). In the former, the cells were mixed with FITC-conjugated MAb diluted in normal saline containing 1% (wt/vol) bovine serum albumin, and incubated at 4°C for 30 minutes before being washed and resuspended in 1% paraformaldehyde. In the indirect immunofluorescence assay, bacteria were stained with either unlabelled MAb followed by a FITC-labelled goat anti-mouse Ig antibody or biotinylated MAb followed by a streptavidin R-phycoerythrin conjugate. The bacteria were inspected under an epifluorescence microscope prior to flow cytometric analysis to determine adequacy of staining and the degree of cell aggregation. Aggregates were disrupted by passing the bacteria repeatedly through a 28-gauge hypodermic needle.

The fluorescence emissions of 5,000 stained bacteria were quantified in each sample using a FACScan® flow cytometer (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA). Live gates and forward scatter threshold were optimised to exclude large bacterial aggregates and cell debris from the analyses. Fluorescence data were collected, displayed, and analysed in log format.

Cell Sorting

The *E. coli* O26:B6 LPS phenotypic variants were sorted, as previously described (*Evans* et al., 1990), with a FACS II® cell sorter (Becton-Dickinson) on the basis of differential staining with the core-specific MAb, J8-4C10. Bacteria were sorted into cold tryptic soy broth and then subcultured on agar.

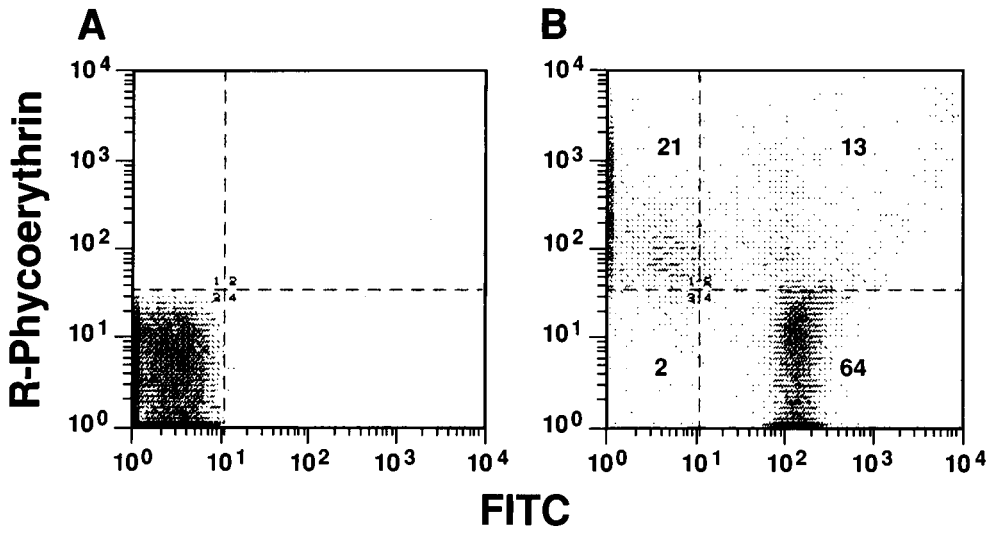

Figure 1: Dot plots of dual parameter flow cytometric analyses of cells from a stock culture of *E. coli* O26:B6 stained with A) FITC-labelled negative control MAb and streptavidin R-phyco-erythrin conjugate, or B) FITC labelled LPS O-antigen-specific MAb and biotinylated core-specific MAb followed by a streptavidin R-phyco-erythrin conjugate. Numbers refer to the percent of cells in each quadrant.

SDS-PAGE and immunoblot

LPS was prepared from proteinase K digests of whole cell lysates, and resolved by SDS-PAGE using a 4% stacking gel and a 14% separating gel according to the method of *Tsai* and *Frash* (1982). For immunoblots, LPS was transferred onto nitro-cellulose and exposed to MAbs as described previously (*Evans* et al., 1990).

RESULTS

Identification of smooth, rough and semi-rough sub-populations within a stock culture of *E. coli* O26:B6: A stock culture of *E. coli* O26:B6 was stained with the FITC-labelled, O-antigen-specific MAb, E5-3A5, and the biotinylated, core specific MAb, J8-4C10, followed by a streptavidin R-phyco-erythrin conjugate. As negative control, cells were stained with the FITC-labelled MAb 13-17, and the streptavidin R-phyco-erythrin conjugate. The bacteria were then analysed by dual parameter flow cytometry. Fully 99.8% of cells exposed to the negative control reagents fell into quadrant 3 (Figure 1A). Only 2% of the bacteria staining with MAb E5-3A5 and MAb J8-4C10 fell into this quadrant (Figure 1B). The majority of cells (64%) stained exclusively with MAb E5-3A5 suggesting that these bacteria maintained the smooth *E. coli* O26:B6 phenotype. Conversely, 21% of the cells stained only with the core-specific MAb J8-4C10 indicating that these cells had lost O-antigen and were rough. A third subpopulation (13%) stained with both

Table 2: Comparison of LPS phenotype of *E. coli* O26:B6 and sorted variants *E. coli* O26S and *E. coli* O26SR using dual parameter flow cytometric analyses.

Bacterium	% Population Binding MAb			
	E5-3A5*	E5-3A5 + J8-4C10	J8-4C10	Unstained
O26:B6	68	22	9	1
O26S	96	2	2	0
O26SR	0	93	0	7

*E5-3A5 specific for LPS O-antigen, J8-4C10 specific for core epitope

MAbs suggesting that these cells produced some O-antigen, but not enough to mask underlying core epitopes and were semi-rough.

Isolation of *E. coli* O26:B6 subpopulations binding O-antigen- or core-specific MAbs

To further examine the apparently smooth and rough/semi-rough subpopulations identified in dual parameter flow cytometric assays bacteria were stained with core-specific MAb J8-4C10, analysed, and sorted (Figure 2). Approximately 15% of the unsorted cells from the stock culture stained with the MAb (Figure 2A). Re-analysis of the sorted bacteria showed that the unstained (designated *E. coli* O26S) and stained (designated *E. coli* O26SR) cells were 97% and 92% "pure" respectively (Figure 2B and 2C). These bacteria were subcultured on agar and single colonies picked and regrown.

Sorted and unsorted bacteria were then stained with both FITC-labelled MAb E5-3A5 and R-phyco-erythrin conjugated MAb J8-4C10 and examined by dual parameter flow cytometry. The unsorted cells were comprised of four subpopulations as observed before (Table 2). Fully 96% of *E. coli* O26S stained with the O-antigen-specific MAb, and 93% of *E. coli* O26SR stained with the core-specific MAb. Bacteria staining with both MAbs were not seen.

SDS-PAGE and immunoblot analyses of *E. coli* O26S and O26SR LPS

Data from flow cytometric analyses suggested that the stock culture of *E. coli* O26:B6 consisted of smooth, rough, and semi-rough bacteria. Additional MAb binding studies of isolated bacteria suggested that *E. coli* O26S was smooth and *E. coli* O26SR was rough. Purified LPS from these bacteria was examined by SDS-PAGE and immunoblot to confirm the LPS phenotypes of these bacteria predicted by flow cytometry analyses. The LPS from both *E. coli* O26S and *E. coli* O26SR stained equally well with the core-specific MAb J8-4C10 on immunoblot (data not shown). Few high molecular weight bands staining with the O-antigen specific MAb E5-3G12 were apparent in the LPS of *E. coli* O26SR compared to that of *E. coli* O26S (Figure 3).

DISCUSSION

Flow cytometry has been an indispensable tool for the examination of immune mechanisms and pathophysiological events among eukaryotic cells (*Loken* et

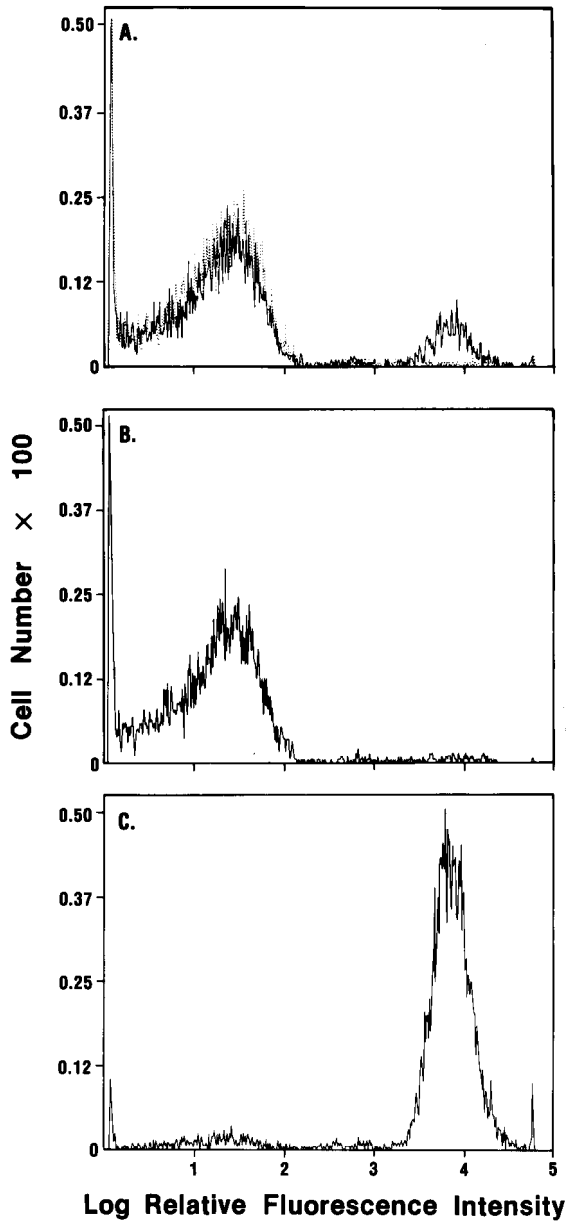

Figure 2: Subpopulations of *E. coli* O26:B6 identified and sorted with a FACS II® on the basis of differential staining by an LPS core-reactive MAb. Presort histograms (A) of nonstaining and staining cells. The fluorescence signals of cells stained with negative control MAb are shown as a dotted line overlying the larger histogram. Postsort histograms of nonstaining bacteria (B) with presumably smooth LPS, and staining cells (C) with presumably rough/semirough LPS.

al., 1982; *Herzenberg* et al., 1976; *Kruth*, 1982). With this technology, characteristics of individual cells can be measured at a rate of approximately 3,000 cells per second. Flow cytometry has rarely been applied to the study of bacteria and their interactions with host cells in infectious diseases (*Steen*,

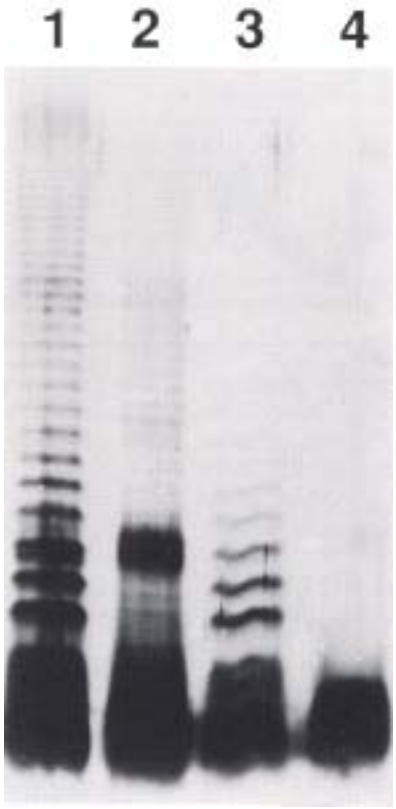

Figure 3: Immunoblots of bacteria sorted from a stock culture of *E. coli* O26:B6. LPS from proteinase K digests of whole cell lysates were separated on SDS-PAGE (*E. coli* O26S lanes 1, 3 and *E. coli* O26SR lanes 2, 4), transferred to nitro-cellulose, and stained with LPS O-antigen specific MAb. Lanes 1, 2 contained 10 μg LPS and lanes 3, 4 1 μg LPS.

1990; *Boye* et al., 1990). Instead, analyses in microbiology often rely upon measurements of mean values of large numbers of cells, as in antibiotic susceptibility testing, or the examination of a few select cells, as in studies employing electron microscopy. These analyses assume that variation among cells will centre around a single mean, and that subpopulations with markedly different phenotype do not exist.

In ELISA, we had previously documented the perplexing cross-reactivity of MAb J8-4C10 with rough, Re chemotype, LPS from *E. coli* D31m4, and presumably smooth LPS from *E. coli* O26:B6 (*Pollack* et al., 1989). We speculated that this cross reactivity was due either to shared epitopes in the LPS core and O-antigen of the respective LPS, or to the presence of both smooth and rough LPS in material purified from *E. coli* O26:B6. SDS-PAGE and immunoblot showed that MAb J8-4C10 reacted only with fast-migrating core structures and not O-antigen determinants of *E. coli* O26:B6 LPS, indicating that the MAb did not recognise an epitope in the O-antigen. This was consistent with data from other assays which suggested that MAb J8-4C10 recognised determinants in the KDO-disaccharide. The presence of cells in an *E. coli* O26:B6 stock culture with variable staining in immunofluorescence microscopy assays suggested that the LPS

purified from these cells and used in ELISA may have been a mixture of smooth and rough LPS. By staining the bacteria simultaneously with core-specific- and O-side chain-specific MAb and analysing the cells with dual parameter flow cytometry, we were able to document the presence of subpopulations of smooth, rough, and semi-rough bacteria within the stock culture. Smooth (*E. coli* O26S) and rough/semi-rough (*E. coli* O26SR) bacteria were differentially stained with MAb J8-4C10 and isolated by cell sorting. Dual parameter flow cytometric analyses of the sorted variants confirmed that the isolates were smooth and rough. The LPS of the isolated bacteria was then further characterised by SDS-PAGE and immunoblot. Although both LPS's appeared to contain the ladder-like pattern produced by LPS with progressively fewer O-side chain units, this pattern was less intense in LPS from *E. coli* O26SR compared to *E. coli* O26S. The difference between the two LPS's was even more marked in lanes which contained less LPS. These data confirmed that bacteria with variant LPS phenotypes were present in the original *E. coli* O26:B6 culture.

Since many of the pathophysiological events in infectious disease are due to host cell interaction with surface structures on bacteria, analysis of these structures on single cells and elucidation of variation within bacterial populations may be of value. Flow cytometry has proven useful for the detection of bacterial surface antigens, the identification of phenotypic variants, and the isolation of these cells for further analysis.

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OLD HERBORN UNIVERSITY SEMINAR ON EFFECTIVE AND INEFFECTIVE DEFENCE MECHANISMS OF THE GASTROINTESTINAL TRACT:

REVIEW OF THE INTERNAL DISCUSSION

HERMA Z. APPERLOO-RENKEMA, and DICK VEENENDAAL

Laboratory for Medical Microbiology, Groningen, The Netherlands

PARTICIPANTS (in alphabetical order)

G.L. Asherson, H. Cottier, S.J.H. van Deventer, A.S.M. Dofferhoff, J.E. Doran, P. Erb, M.E. Evans, J.-O. Gebbers, P.J. Heidt, B.D. Jankovic, R. Kraft, V.C. Rusch, H. Scott, S. Szabo, D. van der Waaij, T. Wadström, R.I. Walker, G.D. Wetzel.

INTRODUCTION

There is a growing interest in the notable interaction between the gastrointestinal immune system tract and the intestinal microflora. Studies dealing with this subject are rare, however, probably due to the complexity of the Gut Associated Lymphoid Tissue (GALT) and the diversity and high numbers of bacterial species colonising the gut. During the internal discussion among the speakers at the fifth Old Herborn University seminar some elements of the gut defence mechanisms were focused. In this overview several aspects have been grouped.

1. The first chapter deals with the immunological defence mechanisms of the gut mucosa, and the interaction between lymphocytes and the gut epithelium. Special attention has been given to the inflammatory bowel diseases ulcerative colitis (UC) and Crohn's disease (CD) and to coeliac disease.
2. In the second and the third chapter attention has been given to the functional role of cytokines e.g. TNF and interleukines, and endotoxin in health and disease.
3. Special notice has been given to monoclonal antibodies, to Lipopolysaccharide (LPS) and their potential use in the treatment of Gram-negative sepsis.
4. the fourth chapter deals with the defence mechanism of the stomach; the protective role of basic fibroblastic growth factor and in *Helicobacter pylori* the pathogenesis of gastric ulcer.
5. Finally some aspects among the neuro-immunological function of TNF and enkephalins are presented in the fifth chapter.

Histo-immunological areas and lymphocytes in the gut

The lymphoid system of the intestinal tract consists of solitary lymphoid follicles (LF), aggregated LF e.g. Peyer's patches (PP), and mesenteric lymph nodes (MLN) (the afferent limb); and isolated T and B lymphocytes and plasma cells in the lamina propria (LP) (the efferent limb).

LF are found along the mucosal lining of the colon up to a number of $2x10^4$. Since it is almost impossible to separate LF cells (afferent limb) from lamina propria cells (efferent limb), information on the cells of the LF cannot be obtained by Fluorescent Activated Cell Sorting (FACS) of single cell suspensions from colon, but can only be drawn from immunohistochemical investigations of cells in tissue sections from colonic mucosa. LF contain mainly B cells (61%). Other cells present in the LF are dentritic reticulum cells, which function as antigen presenting cells, and T cells (30% $CD4^+$, 8% $CD8^+$). The amount of $CD4^+$ and $CD8^+$ T cells in the LP is 56% and 30% respectively. The main proliferative activity is found in the centre of these follicles. The B cells of the LF mainly contain cytoplasmic IgG (78%) and IgM (14%), whereas in the surrounding LP the B cells mainly contain cytoplasmic IgA (92%). No secretory IgA is found in the follicle associated epithelium. This epithelium lacks secretory component (SC) and appears to resemble M cells, which are normally found at the site of PP.

Normally, M cells are responsible for the active uptake of (intestinal) antigens and the primary stimulation of Ig-producing cells takes place in the PP. Studies in chicken showed that the destruction of M cells in the bursa caused severe agammaglobulinaemia.

PP are only found in the small intestine and contain mainly thymus derived T cells. Intestinal antigens are handled primarily in the PP, whereas the secondary immune response is believed to occur in the regional lymph nodes. IgA plasma cells in the LP originate from the PP.

Intra epithelial lymphocytes (IEL) consist of several types of cells which are almost all $CD8^+$ (90%). Some carry the α/β TCR others the γ/δ TCR. The α/β cells are thymic dependent, are derived from the PP and require antigen for stimulation. On the other hand γ/δ cells are derived from the bone marrow and are thymic independent in the mouse; their origin is unknown in man. In man γ/δ T cells are mainly found in the colon, γ/δ IEL are considered to be end-stage cells. To our best knowledge there are no studies on the kinetics of the γ/δ T cells. The repertoire of γ/δ T cells is limited.

Production of IgA

Secretory IgA is the main defence mechanism in the digestive tract. Why IgA is produced in large amounts whereas other intestinal immune mechanisms are suppressed has not been fully elucidated. It might be due to the action of ill-defined contrasuppressor cells. The existence of these cells is still a subject of discussion. In mice intraepithelial γ/δ T cells may have a contrasuppressive function. The isotype switch from IgM to IgA is regulated by a so-called switch cell, originally thought to be a T cell but which in fact may be a macrophage. Switching from IgG directly to IgA is not reported, nor a switch from IgA to IgG. At present there is a lack of information about the actual regulation of the IgA immune response. However, transfer experiments with Ly1-B cells derived from geneti-

cally different donors showed that 50% of the IgA producing plasma cells in the lamina propria originated from Ly1-B cells. At what location in the body the isotype switch occurs is not known. It is unclear whether the IgA^+ B cells in the LP come from PP. Whereas the redistribution process itself is found to be antigen driven, the homing of B cells to lamina propria is regulated by vascular adhesion molecules so-called addressines.

Suppression

Possibly the most important immune mechanism in the digestive tract is suppression which prevents inflammatory reactions to food antigens and indigenous bacteria. Such intestinal hyporesponsiveness may involve clonal anergy, suppressor T lymphocytes, cytotoxic T lymphocytes and/or suppressor macrophages. Antigen handling by an intact gut epithelium seems to be critical. The numerous intraepithelial $CD8^+$ T cells may be involved in induction of hyporesponsiveness (oral tolerance). This type of immune response has been reported to be MHC class II restricted despite the well established MHC class I restriction of $CD8^+$ T cells; the presence of suppressor inducer T cells in the test system, however, was not excluded. MHC class II antigens are constitutively expressed on surface epithelial cells in the human small intestine, but occurs in colon epithelium only in inflammatory conditions.

One recent hypothesis is that down-regulation of immune responses in the gut mainly is caused by an interaction between gut epithelial cells expressing CD1 and T cells expressing CD8. Crosslinking of CD1 and CD8 is probably needed for suppression. Destruction of the gut epithelium, e.g. in inflammatory bowel disease, results in a lack of expression of CD1. This causes abrogation of oral tolerance and preferential stimulation of $CD4^+$ T cells. It has also been shown that $CD8^+$ γ/δ T cells may have helper function in the absence of CD1. The break in oral tolerance will lead to a systemic type of immune response.

There may be three approaches for study of the suppressor function in the gut.

1. One may focus on negative control systems. A lot of different phenomena are gathered under the umbrella of the suppressor cell. It is important to notice that there is not just one type of suppressor cell. Moreover, the function of 'suppressor cells' is very diverse and may depend on the stage at which they are activated during the immune response.
2. Functional studies can be carried out by eliminating parts of the immune system by thymectomy or administration of cytotoxic drugs e.g. cyclophosphamide. It is important to note that in case of athymic animals pre-T cells still occur. Cyclophosphamide has serious side effects including cytotoxicity, bone marrow suppression or possible induction of cytokines.
3. Another approach may be the elimination of single or multiple classes of cells of the immune system e.g. by using monoclonal antibodies directed against T afferent or T efferent cells.

Immunological aspects of Ulcerative Colitis, Crohn's Disease and Coeliac Disease

Examples of diseases in which the oral tolerance is abrogated are ulcerative colitis (UC), Crohn's disease (CD), and coeliac disease. As already mentioned the CD1 molecule is normally present on mucosal epithelial cells, whereas IEL carry the CD8 molecule. Crosslinking of both molecules, CD1 and CD8, is needed for suppression.

In UC there is a lack of epithelial expression of CD1. In mixed lymphocyte cultures with peripheral blood mononuclear cells and intestinal epithelial cells from patients with inflammatory bowel disease, preferential stimulation of $CD4^+$ T cells with helper function was observed. This may explain why polyclonal B cell activation occurs in UC, causing an increase of IgG. The IgG response, which is dominated by the IgG1 class, is strongly complement activating. Formation of IgG1 immune complexes with complement activation is probably the main effector mechanism in UC. High levels of antibodies to aerobic pathogenic bacteria such *E. coli*, *Pseudomonas*, *Yersinia*, *Str. bovis* and anaerobes like *Peptostreptococci* and *Bacteroides* spp. have been found in UC. These antibacterial antibodies are most likely not responsible for the primary lesions in UC, but secondary to the epithelial lesions. There is evidence that the lesions in UC are caused by an autoimmune reaction against a 40 kDa protein (Das-antigen) normally found in the mucus layer in the distal colon. Histological examination of the submucosal area underneath the destroyed epithelium in UC has shown that predominantly granulocytes, IgG plasma cells and macrophages are present but no T cells. In UC the whole colonic mucosa may be affected, whereas CD is characterised by skip lesions and primary lesions at the lymphoid follicle site. The pathogenesis of CD is different from that of UC in several aspects. First, CD appears to be T cell mediated. There is an increase in the total numbers of both $CD4^+$ and $CD8^+$ T cells, however, the CD4/CD8 ratio is unchanged. With respect to the function of T cells, it may be worthwhile to notify that the importance of phenotype assessment can tell us little of the actual functional capacity of these cells.

The aetiology of CD is unknown, however, the recorded immunological changes can be seen as a direct consequence of the destruction of the epithelium. In contrast to UC, this break in tolerance causes a disproportionate increase of IgG2 antibody levels. The target antigens responsible for the increased IgG2 response in CD are still unknown.

It has been shown that T cells and macrophages play an important immunoregulatory role in CD. It is not known whether the normal Ig class switch from IgM to IgA is redirected into a switch from IgM to IgG.

A high antibody response has been found in CD against *Mycobacterium paratuberculosis*. However, PCR studies failed to confirm a correlation between the presence of *M. paratuberculosis* and CD. It is therefore doubtful that *M. paratuberculosis* plays a direct role in the pathogenesis of CD. CD may occur due to an altered self caused by an infectious agent effecting the gut epithelium. Consequently, this may cause an alteration in the epithelial T cell interaction. The latter may functionally change from tolerance induction into activation of cytotoxic or helper-function.

The change in the epithelium has be speculated to be caused by a virus infection e.g. Herpes Simplex Virus (HSV) since lesions found in CD resemble apht-like lesions in the oral cavity, caused by HSV. Moreover such lesions have occasionally been reported in the stomach, in which case HSV was isolated. Until now, HSV has not been isolated from the gut epithelium in CD patients. It might be of interest, however, to search for HSV in the mesenteric lymph nodes in these patients. This may explain why thrombus formation in the small vessels in the submucosa occur. This process is possibly basic to the epithelial lesions in CD. It may be worthwhile to investigate the prevalence of HSV in CD by using PCR tech-

niques. On the other hand genetic predisposition may cause an altered immunoregulatory function of the epithelium.

For future studies on the comparison of UC with CD, it is important to note that one needs to define the degree of inflammation. Influx of granulocytes in inflammatory bowel disease lesions has been found to be regulated by adhesion molecules and upregulated by IFN-γ. Information on the time course and sequential induction of these responses remain subject for future study.

Coeliac disease is a small intestinal disorder characterised by villous atrophy and crypt hyperplasia. Unlike CD and UC, the immune dysregulation in coeliac disease is characterised by a slight break in oral tolerance. This break is reversible probably due to an intact IgA production; the number of IgA-producing cells in the small intestine is strongly increased in coeliac disease.

T independent antigens and superantigens

The categorisation of T independent (Ti) antigens is questionable. There are two classes of Ti antigens, Ti1 and Ti2. In fact the term Ti antigen is simply historic, since most antigens do require some help. This help may come from mast cells secreting IL-4 and IL-5, from stromal cells, or macrophages.

LPS is known to be the strongest stimulator of B cells. Proof is given by studies in germfree mice fed an antigen free diet. These mice only had a small B cell repertoire. In order to get a stimulation by T independent antigens, it is necessary to have crosslinking at the receptor site. Ti1 and Ti2 are both able to induce a primary response in B cells. Only Ti1 antigens also induce a specific secondary response in B cells.

Superantigens are bacterial products which display a special kind of increased immune stimulation compared to (normal) antigens. Examples of superantigens are *S. aureus* toxin and streptococcal M protein. Superantigens are able to bind specifically to the beta chain of the TCR and thereby induce a second signal on T cells. The simultaneous presence of MHC class II antigens increases the stimulatory function of superantigens. In this case superantigens are not presented on the MHC class II molecule after intracellular processing.

Antigen clearance by Kupffer cells

The liver plays an important role in Leishmaniasis. Liver macrophages known as Kupffer cells are not only the host cells which become infected but also play a role as effector cells. IFN-γ activated macrophages have been found to kill intracellular infectious agents up to 90-100% *in vitro*. The defence against *Leishmania* may be augmented *in vivo*, by administration of IFN-γ to the macrophages. Systemic IFN-γ therapy, however, is plagued by a great number of serious side effects, such as fever and increase of MHC class II expression which may result in autoimmune disease. To overcome these side effects, IFN-γ should be delivered directly to the macrophage. Packaging of IFN-γ in liposomes, however, does not serve this purpose properly, because liposomes are directly phagocytised by macrophages. After destruction of the liposomes by cytoplasmic liposomal enzymes, their contents are liberated into the cytoplasm of the macrophage. As a consequence, IFN-γ can not bind to the extracellular receptor on the macrophage and cannot perform its function. It is unknown whether macrophages express

a second messenger upon IFN-γ stimulation. In therapy, focusing on stimulating killing activity of macrophages, more experiments will be needed regarding the receptor signal transducing mechanisms in macrophages.

Kupffer cells are both stimulated by antigen coming from the gut portal vein liver, as well as from the route gut lymphatic vessels thoracic duct arteria subclavia lung blood liver. Uptake of endotoxin takes place via both routes. Because the flow in the thoracic duct is much larger compared to the portal vein, detection of endotoxin in the former correlates well with systemic endotoxaemia. During systemic endotoxaemia in humans, the liver and spleen are the most important sources of TNF. Downregulation of the macrophages in the liver leads to systemic disorders.

Finally, somatostatin is reported to stimulate macrophages in the liver. This may be a tool for future study on the role of macrophages in the immunological defence mechanism.

Mucosal vaccines

In order to get a good immune response by oral vaccines, presentation of the antigen in microspheres may be appropriate. Microspheres are taken up by M cells. Subsequent distribution depends on the size. Microspheres smaller than 3 microns pass the PP and enter the circulation. If their size is between 4 to 7 microns, they are phagocytised by macrophages. Microspheres of 7 to 10 micron stay in the PP and release their contents locally. Repeated doses are required in order to get an immune response. It is unknown whether there is a dose effect. This immune response, however, has only a short type of memory, if any. In order to get a long lasting immunity; i.e. memory, a persistent presence of the antigen is required.

An alternative for the vaccination with microspheres may be the administration of antigen in transgenic bacteria. However, these bacteria may only be present in the gut in limited numbers due to colonisation resistance.

CYTOKINES

Regulation

Cytokines are secreted upon stimulation of promoter genes. These genes all show a great similarity in their DNA sequence. This may be seen as the explanation why many cytokines are secreted by the macrophages, endothelial cells, and smooth muscle cells upon a single stimulus. Stimuli for the release of cytokines are not only endotoxins but also seen as an indirect effect of tissue damage. The type and kinetics of cytokine production and release vary according to stimuli. Upon pancreatectomy for example, IL-6 is produced by smooth muscle cells and endothelial cells. Upon LPS stimulation, first TNF and thereafter IL-1 and IL-6 are secreted by macrophages. This underlines that not all cytokines are released at the same time. In mice it has been found that TNF and IL-6 may act synergistically to modulate physiologic function.

TNF in granulomas

TNF plays an important role in necrotising as well as non-necrotising granulomas. Blocking of TNF by anti-TNF antibodies will inhibit granuloma formation e.g. in CD and in tuberculosis. In tuberculosis, this may result in the unlimited proliferation of micro-organisms. In a similar way cutaneous Leishmaniasis in mice can not be cleared if TNF production or release is blocked. In the lepromatous form of lepra remar-

kably less TNF is produced compared to the tuberculoid form. In addition to its role in granuloma formation, TNF also appears to be a potential stimulator of oxidative killing.

Cytokines in B cell differentiation

Cytokines also have an important function in B cell differentiation and proliferation. B cell maturation factors can be divided into secretion factors i.e. IL-2, IL-5, and IL-6 and switch factors i.e. IL-4, IFN-γ, TGF-β, IL-5, and IL-6. B cell growth factors can be divided into competence factors i.e. IL-1, IL-4, IL-5 and progression factors i.e. IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and C3a.

ENDOTOXIN

Intraluminal endotoxin in the gut

The number of LPS molecules per *E. coli* bacterium is about 10^6. This amount differs per strain and depends primarily on the growth phase of the organism. The concentration of endotoxin in the gut appears to show great variability between individuals. Whether LPS is needed for induction of oral tolerance remains unclear. Another physiological effect of endotoxin is its modulating effect on the gut motility.

Under pathological conditions like irradiation and trauma, intraluminal endotoxin may be responsible for the disruption of the tight junctions of the epithelial and goblet cells. Such alterations result in increased endotoxin in the liver and increase of intra-intestinal bacterial translocation.

Binding and clearance of endotoxin

LPS normally circulates in an aggregated form in blood. De-aggregation is necessary in order to get a response to LPS. Endotoxin interacts with and binds to a number of plasma proteins including LPS binding proteins (LBP) Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL). Upon stimulation with LPS, hepatocytes release LBP. Alpha 2 macroglobulin is thought to promote the clearance of endotoxin. Binding of LPS by LBP occurs at the Lipid A fragment of the LPS molecule. However, binding does not result in direct clearance of endotoxin. The LBP-LPS complex binds to the CD14 receptor on macrophages. Signal transduction may subsequently take place if crosslinking occurs with an ill-defined CD18 molecule. Possibly, CD18 is a binding protein. It is unknown whether CD18 plays a role in signal transduction in the macrophage.

LBP is an important factor in macrophage stimulation by LPS. In case of depletion of LBP by anti-LBP antibody, more LPS is needed in order to stimulate macrophages to TNF release. Binding of LPS by immunoglobulins and complement has been found *in vitro*. It is unknown whether this binding also takes place *in vivo*. HDL can in part compete with LBP for LPS-binding. When HDL is present, higher threshold of LPS is required for macrophage stimulation.

Clearance of endotoxin is orchestrated by antibodies which bind to the Fc-receptor or via complement binding the C3 receptor on macrophages, erythrocytes and neutrophil granulocytes. Granules of neutrophils contain enzymes that cleave off the fatty acids. This enzymatic cleavage does not result in clearance per se but rather in neutralisation. The clearance of the Lipid A part of the LPS molecule may be regarded as

most important since Lipid A displays high toxicity. LPS clearance occurs predominantly in the liver Kupffer cells. In the Kupffer cell, LPS is first degraded into free Lipid A and its polysaccharide chain. Degradation of Lipid A is a slow process. Biologically active Lipid A may persist in Kupffer cells for periods up to one week or more.

Future experiments should focus on signal transduction mechanisms after LPS stimulation, the intracellular clearance of endotoxin, the distribution of endotoxin clearance mechanisms in the body and the effect of immunoglobulins complexed with LPS.

Toxicity

Long lasting infection with endotoxaemia may result in the accumulation of Lipid A in Kupffer cells. This may act as an endogenous time bomb. This endogenous time bomb is best illustrated by experiments in rabbits which need decreasing doses of endotoxin in order to cause mortality when exposed to slow intoxication with endotoxin.

Anti-LPS antibodies

Most bacteraemias are caused by smooth bacteria instead of rough bacteria. Possibly, rough bacteria are easily lysed upon complement fixation whereas smooth bacteria cannot be lysed because the membrane attack complex cannot reach the membrane due to steric hindrance by the presence polysaccharide chains. Functional phenotype switching may be an important adaptation mechanism to foil the defence mechanism of the host. Phenotype switching of *E. coli* may occur at different levels. During rapid growth, smooth bacteria may switch to the rough phenotype. Bacteria may also switch off plasmid encoded cellular proteins e.g. pili after colonising the mucosa. Cell wall changes may also occur due to antibiotics. Beta lactam antibiotics have been found to increase cell wall permeability upon which fragmentation of bacteria occurs. Aminoglycosides on the other hand do not affect the cell wall permeability. Genetic control of *in vivo* variations of bacteria may be a field of future study.

Rough forms of bacteria e.g. *E. coli* are more susceptible to antibodies. Monoclonal anti-J5 antibodies are known to bind at the core region (Lipid A) of LPS *in vitro*. It is known that anti-J5 sera (HA1A) are protective in patients having endotoxaemia. Therefore, not bacteraemia but endotoxaemia is treated by HA1A. This may implicate that anti-J5 does not work by opsonisation of bacteria but by binding of endotoxin.

The endotoxin concentration in the blood does not always correlate with the number of viable bacteria found in the blood. This would suggest that LPS release either occurs from local sites, is readily bound and/or cleared or originates from dead bacteria. To estimate the severity of septicaemia in patients, TNF levels in serum are more indicative than endotoxin levels. Whether anti-J5 antibodies should be of IgM or IgG isotype remains a matter of controversy.

Use of anti-LPS antibodies for analysis of bacteria by flow cytometry; FACS

FACS not only enables the study of eukaryotic cells, but also of bacteria. Analysis of bacteria by flow cytometry may occur through the use of conjugated probes directed to the bacterial genome and conjugated monoclonal antibodies directed against cell wall determinants. Labelling at the genome level requires cell wall permeability without lysis. Detection of surface components of pure cultures by monoclonal antibodies reveals that within the bacterial population in pure culture not

all antibody binding sites may equally be present. Antigenic variation occurs in time, growth phase, and growth medium components. The antigenic expression should not be regarded as permanent mutations, as it may be due to varying expression of cell wall components. This is contradictory to the adagium that bacteria originating from one single parent cell are strictly homogeneous. This rapid reversible change of bacterial cell wall *in vitro* could perhaps be regarded as a virulence factor to escape the immune system *in vivo*.

Endotoxin in septic shock

It remains questionable whether the organ and physiologic dysfunction seen in septic patients is caused by release of endotoxin and cytokines. *In vitro* macrophages are shown to release a plethora of different cytokines upon endotoxin stimulation. These cytokines were also found in septic patients and showed a good correlation with the APACHE II score; i.e. severity of disease and clinical outcome. Animal model systems have also shown correlations between cytokine levels and physiologic function.

STOMACH

Protective mechanisms

Mucosal damage in the stomach may occur through ischaemia or chemical agents. Direct cellular protection may be provided by intracellular glutathion, intracellular pH, Ca^{2+}, ATP and the plasma membrane. Indirect protection is provided by accelerated gastric emptying, luminal and tissue dilution of damaging agents, and maintenance of blood flow and epithelial restitution.

Basic Fibroblast Growth Factor (b-FGF)

Failure of the protection may cause ulceration. B-FGF is a highly protective agent in experimentally cystamine-induced duodenal ulcer. B-FGF is administered orally and has been found to reduce the size of the ulcer lesions. Unlike cimetidine, b-FGF is not effective by reducing the acid or pepsin output. Instead, its beneficial effect is ascribed to the stimulation of angiogenesis in the normally hypovascular ulcer bed. Some glucocorticosteroids are also reported to have a b-FGF like effect.

Helicobacter pylori

Helicobacter pylori causes gastric ulcers and can be isolated in all patients with acute gastric ulcer. The habitat of *H. pylori* is the antrum. It is often difficult to isolate *H. pylori* from patients with chronic ulcers.

It is important to know that there are different strains of *H. pylori* having different degrees of virulence. For study of the pathogenesis of *H. pylori* induced ulcers an appropriate animal model is required. *H. pylori* does not grow in rodents. In very young pigs, *H. pylori* causes hardly any inflammation. Therefore, primates seem to be the experimental animals of choice. Urea in the gastric mucosa of the host is demolished into highly toxic ammonia by urease released by *H. pylori*. Strains which have lost their urease gene are not pathogenic. Once infected by *H. pylori*, these organisms may persist underneath the gastric epithelium for years. It is not yet clear so far what role is played in this respect by the coccoid dormant forms of *H. pylori*.

The route of transmission of *H. pylori* is unknown. Families being infected with one strain suggest an oral to oral route. Interestingly, there is also a higher incidence of *H. pylori* among gastroenterologists.

The treatment of choice in *H. pylori* gastric ulcer is amoxycillin or tetracycline, metronidazole, or bismuth. Bismuth penetrates and disrupts the *H. pylori* cell wall. Bismuth should not be combined with anti-acids because of disintegration when given simultaneously. Upon successful treatment, serum IgA antibodies against *H. pylori* decrease. The urease breath test also drops after successful treatment. The use of serodiagnosis in *H. pylori* infection is questionable. Commercial tests are not useful in this respect, because of the prevalence of too many serotypes and crossreactions.

It is important to have good parameters available for objective classification of *H. pylori* induced disease. Only histological inflammation markers are found to correlate with *H. pylori* infection.

NEURO-IMMUNOMODULATION

Enkephalins have an immunomodulating activity. There are two kinds of enkephalins (enk), namely Leu-enk and Met-enk; only Met-enk appears to be important. Met-enk injected systemically in high dose, may give suppression of antibody response and also has an anti-inflammatory activity. At low dose Met-enk, however, appears to have a potentiating effect on the immune response, e.g. an increase of $CD4^+$ cells has been described. Met-enk injected intracerebrally or intrathecally show similar effects as when they are injected intravenously. Enkephalins were studied instead of prolactin (pituitary hormones) because enkephalins also appear to be present in the gut. Lymphocytes are supposed to have receptors for enkephalins. Thus in all respects, enkephalins appear to have actions similar to cytokines.

Systemic cytokines e.g. IL1a in the adrenal medulla in rats may have a different effect as compared to cerebral produced cytokines. Similarly, TNF results in cachexia when produced and disseminated systemically, whereas intrathecally TNF only leads to anorexia without protein catabolism. Due to its size, TNF does not cross the blood-brain barrier. A local production of cytokines by neural and glial cells is just another example of the neuro-immune relationship.