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POTENTIAL BENEFIT FROM INACTIVATED WHOLE CELL VACCINES

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INTRODUCTION

The potential for the development of new and better vaccines against infection is greater today than at any time in the past two centuries since Jenner's initial vaccinations against smallpox. This opportunity has been fuelled by the great strides made in immunology and biotechnology. One potentially useful approach to achieve immune protection through immunisation at mucosal surfaces is the use of inactivated whole cell vaccines. As will be described below, new technologies are making this approach a very promising means to alleviate human suffering.

Infectious diseases continue to be the leading cause of morbidity and mortality world-wide. With the increasing occurrence of antibiotic resistance to many infectious agents, new vaccination strategies for mucosal immunisation may provide efficacious preventive and therapeutic treatments. Mucosal membranes are the most frequent portals of entry for pathogenic organisms. These facts have prompted studies aimed at developing vaccination protocols to increase protection of mucosal surfaces. Systemic immunisations are of limited effectiveness in the induction of mucosal protection (*Bakke* et al., 2001); however, ingestion or inhalation of antigens results in a generalised mucosal immune response. Mucosal immunity will not only protect the surfaces against colonisation, but also has the potential to reduce existing colonisation by pathogens inhabiting mucosal surfaces. Further, mucosal immunisation can also increase systemic immunity (*Bakke* et al., 2001; *Stiles* et al., 2001) which could make the mucosal route appropriate for needle-less delivery of many current and future vaccines.

Inactivated micro-organisms offer potential advantages as vaccines for mucosal immunisation. Physically, they are naturally occurring microparticles and possess multiple antigens that should enhance interactions between the antigens they carry and mucosal lymphoid tissues. Presentation of multiple antigens may be particularly important for pathogens for which protective antigens are not known, or are not readily available in purified or recombinant forms. Also, whole cell vaccines can often be developed relatively quickly and produced and administered economically. A killed whole cell cholera vaccine made in Vietnam, for example, consists of four cell types, 2.5×10^{10} cells per type, and costs just 10 cents per dose (*Trach* et al., 1997). Inactivated whole cell vaccines are generally safe for mucosal administration and, in clinically useful combinations, may be safer than similar combinations of attenuated micro-organisms.

Several considerations are paramount for successful development of inactivated whole cell vaccines. One, the bacteria grown in vitro must express important antigens associated with pro-

tection. Second, the antigens so expressed must be retained upon inactivation. Here, both the process of inactivation and the method of preservation are important. Other considerations include immunomodulary techniques and delivery formulations, which will facilitate intact delivery and maximise the appropriate host immune response to antigens presented to the mucosal surface. In short, the key to successful mucosal vaccination is to induce the appropriate mediated responses to provide strong protective immunity without associated illness.

MECHANISMS OF IMMUNITY ASSOCIATED WITH ORAL VACCINATION

A mucosal immune system exists whereby antigens encounter specialised epithelial cells and then the underlying lymphoid cell aggregates (*Craig* and *Cebra*, 1971). In the gastrointestinal tract, these lymphoid aggregates are the Peyer's patches. The predominant pathway of lymphocyte effector cells within the intestinal immune system is from the gut-associated lymphoid system to other mucosal sites (*Rudzik* et al., 1975). Other pathways do, however, exist (*McGhee* et al., 1992). Lymphoid tissue throughout the mucosal system exists either as well defined and clearly organised tissue such as the lingual, palatine and pharyngeal tonsils, and the Peyer's patches along the intestine, or it exists as diffuse collections of lymphocytes, plasma cells, and phagocytes such as in the lung and the lamina propria of the intestinal wall. In the intestine, antigen entering the Peyer's patches does so in pinocytotic vesicles across specialised epithelial cells (M cells). Within the follicle, antigen is processed by antigen presenting cells to stimulate antigen sensitive T and B lymphocytes. These subsequently drain into lymph, migrate via the mesenteric lymph nodes and the thoracic duct lymphatics, and pass from the blood into the mucosal lamina propria where they differentiate into mature effector T cells and predominantly IgA producing plasma cells (*Craig* and *Cebra*, 1971).

The common mucosal immune system can be differentiated into two areas. Initial responses are induced at the site where the antigen is first encountered by the lymphoid cells (inductive site). The second and larger area of induced immunity occurs at distant mucosal sites where the lymphocyte effector cells migrate and thus activate IgA production and cell-mediated immunity for local immune protection. The distant mucosal site commonly has a predominance of T cells of the CD4+ helper type. It is enriched in B cells and plasma cells, usually of an IgA isotype, and is covered by epithelial cells producing the polymeric immunoglobulin receptor secretory component that transports polymeric IgA into external secretions (e.g., saliva, mucus) (*McGhee* et al., 1992). Exposure to most environmental antigens is usually via the respiratory or gastrointestinal tracts, which therefore act as the first lines of defence. Thus, the common mucosal immune system provides the network for the distribution of specific humoral and cellular immune responses from inductive sites, particularly in the gastrointestinal tract, to peripheral mucosal sites. The generation of protective mucosal immune responses by oral presentation of antigen is therefore a feasible vaccine approach.

At least two criteria determine the efficacy of antigens in the gastrointestinal tract for the induction of mucosal immune responses: firstly, the protection

of antigenic components from the gastric acid and digestive components and, secondly, access to the mucosal immune apparatus which preferentially interacts with particulate matter. The first of these criteria can now be achieved by use of protective enteric formulations or co-administration of buffer. The second requires consideration of particle size, shape, and sufficient antigen delivery and immunomodulation at the mucosal surface. Traditionally, live organisms were thought to give a more effective mucosal immune response compared with whole killed organisms. An appropriately formulated inactivated whole cell vaccine, however, which safely presents an array of protective antigens should be able to induce a mucosal immune response that is equivalent to that obtained with live vaccine delivery systems. Evidence, for this possibility is available for at least one organism (*Cardenas* et al., 1994). *Salmonella dublin* strains expressing the *Escherichia coli* heat-labile enterotoxin B subunit were inactivated with several different methods and used to orally immunise mice. Immunoglobulin G and IgA responses to B subunit on the inactivated cells were equivalent to the immune response following infection with live organisms expressing the same labile toxin B-subunit. More recently, orally administered formaldehyde-killed recombinant bacteria expressing a mimic of the shiga toxin receptor were found to protect mice from a normally fatal challenge with shiga-toxigenic *E. coli* (*Paton* et al., 2001). These data suggest that inactivated whole cells may be suitable as vectors to deliver foreign antigens that have been cloned into them.

The efficacy of a mucosal vaccine delivery system may be dependent on the type of immune response induced. It has been shown that oral immunisation with attenuated *Salmonella typhi* strains carrying foreign genes elicits a strong systemic cell-mediated immunity that is of a Th1-type, but not Th2-type (*Sztein* et al., 1994). Oral delivery of a *Salmonella* vector having a recombinant tetanus toxin C induces a dominant Th1-type response, whereas tetanus toxin C co-administered orally with cholera toxin (CT) induced a predominantly Th2-type response in CD4+ T cells (*Xuamamo* et al., 1994; *Jackson* et al., 1996). This suggests that the immune response to the recombinant antigens expressed in *Salmonella* strains may be directed by the *Salmonella* carrier. Interestingly, both vaccine delivery systems elicited high levels of mucosal secretory IgA. It has been suggested that following oral immunisation with recombinant *Salmonella*, antigen-specific Th1 cells producing IFN-gamma and IL-2, Th2-derived IL-10, and IL-6 from macrophages, all provide important signals for the development of mucosal IgA in the absence of a dominant Th2 response (*Vancott* et al., 1996).

EXPERIENCE WITH KILLED WHOLE CELLS FOR ORAL IMMUNISATION

Vaccines for Enteric Pathogens

Campylobacter Vaccine Development

During the past decade *Campylobacter jejuni* has become the focus of growing attention due to its increasing frequency of isolation (*Nachamkin* et al., 1992; *Taylor* and *Blaser*, 1991; *Stern* and *Kazmi*, 1989). No vaccine is currently available to protect against this pathogen. American adult volunteers challenged with *C. jejuni* developed serum and intestinal antibodies and were

protected from subsequent illness, but not against infection, following challenge (*Black*, 1988). This observation is consistent with reports documenting prolonged excretion or symptoms, or both, in patients with IgA deficiency (*Johnson* et al., 1984; *Melamed* et al., 1983). An in vitro mucus assay was used to study the role of sIgA in preventing attachment of *C. jejuni* to INT 407 cells. Mucus from rabbits previously colonised with *C. jejuni* completely blocked adherence to the underlying cells (*McSweegan* et al., 1987). Anti-*Campylobacter* sIgA was readily detected in these mucus samples and was responsible for eliminating bacterial adherence. This was shown by loss of inhibition after mucus absorption with *Campylobacter* cells.

Since natural infection confers IgA-associated immunity against *Campylobacter*, both living and non-living vaccine approaches against *Campylobacter* have been considered as means to safely confer immunity. The use of inactivated *Campylobacter* whole cells (CWC) offers a practical approach to vaccination against the pathogen and, at present, this has been the most studied approach to development of a vaccine against this pathogen.

A formalin-inactivated whole cell vaccine for *C. jejuni* has been extensively studied in humans and animals. Mice have been orally immunised with a 3-dose primary series of particles of CWC (48-hour intervals) at doses of 10^5, 10^7, or 10^9 cells (*Baqar* et al., 1995). The vaccine was given to mice with or without the mucosal adjuvans consisting of the heat-labile enterotoxin of *Escherichia coli* (LT) (*Walker* and *Clements*, 1993). Baqar's studies showed that the *Campylobacter*- specific intestinal IgA response was dependent on the use of LT, whereas serum immunoglobulin responses were not. Upon subsequent oral challenge, colonisation resistance was found to be induced over a broad range of vaccine doses when LT was included. However, only the highest dose (10^9) of CWC alone gave comparable levels of protection against colonisation. Both the adjuvanted and unadjuvanted formulations of CWC given at the intermediate dose (10^7) provided equivalent protection against systemic spread of challenge organisms.

The CWC vaccine is currently being evaluated in a series of clinical trials by a team from Antex Biologics Inc and the Naval Medical Research Center. Studies completed so far indicate that the vaccine is safe and immunogenic (D. Tribble, personal communication). Future answers concerning the optimal dosing and the contribution of adjuvans to the vaccine are needed.

Shigella Vaccine Development

Shigellae are enteric pathogens that invade and spread between the epithelial cells of the colonic mucosa, thereby causing inflammation and ulceration, resulting in diarrhoea or dysentery. Vaccine development for this disease has focused on oral administration of invasive attenuated strains. For many of these vaccine candidates it has been difficult to balance protective efficacy with reactogenicity. To date, no licensed vaccine for *Shigella* spp. exists. Although not previously thought to be effective (*Hale*, 1995), it has recently been shown that heat-killed *S. flexneri* 2a could induce protective immunity in guinea pigs if co-administered with mutant labile toxin of enterotoxigenic *Escherichia coli* (*Hartman*, 1999).

Hartman's studies (*Hartman*, 1999) used a 2-dose (days 0 and 14) oral regimen containing 10^{10} killed bacteria per dose. No protection was seen with this regimen unless the adjuvans LT_{R192G} [an attenuated LT (*Dickenson* and *Clements*, 1995)] was included. It

remains to be seen whether an increased dosing regimen could overcome the need for the adjuvans. In support of this idea, Hartman also found that the 2-dose regimen (5 x 10^7 CFU) did produce protection without the adjuvans if given nasally. Further support for the concept of increased vaccine dosing comes from work with an attenuated oral vaccine for typhoid fever, Ty21a. Although not a killed cell preparation, the typhoid vaccine studies showed that a fourth dose was significantly more protective than a three dose regimen which was much better than a 2-dose regimen (*Levine* et al., 1989).

At present no clinical data are available regarding inactivated whole cell vaccines for *Shigella* spp. The fact that human challenge models for this pathogen do exist should facilitate eventual clinical studies.

Cholera Vaccine Development

Inactivated cholera vaccines now under development rely on oral delivery of the mucosal immunogen. The best studied of these vaccines is a preparation composed of a mixture of the non-toxic B sub-unit of cholera toxin and killed whole *V. cholerae* cells (WC-BS). There were no adverse events among volunteers in a clinical safety trial of the WC-BS cholera vaccine administered orally at 5 x 10^{10} bacterial cells per dose (*Svennerholm* et al., 1984). A random, double-blind placebo-controlled field trial, involving 63,000 individuals in rural Bangladesh, established the safety, immunogenicity and efficacy of the WC-BS vaccine. Two and three doses of the WC-BS vaccine conferred 85% protection against cholera for the first six months in all age groups tested, and 51% overall protection after three years (*Clemens* et al., 1990). No adverse events attributable to the vaccine were reported. More recently, a new formulation of the WC-BS cholera vaccine containing a recombinantly produced cholera toxin B sub-unit was also found to be non-reactogenic (*Sanchez* et al., 1993) and gave high levels of protective immunity (protective immunity = 86%) against symptomatic cholera in Peruvian military recruits (*Sanchez* at al., 1994). These vaccines provide proof of principal that orally administered mucosal vaccines can be a safe and effective means to protect against disease.

ETEC Vaccine Development

An approach similar to that used with the cholera vaccine has been used to develop a vaccine against enterotoxigenic *E. coli* (ETEC). The vaccine consists of a combination of 5 strains (2 x 10^{10} particles of each strain) that expresses the most important colonisation factor antigens (CFA) on their surface and also contain the main O antigens (*Holmgren* and *Svennerholm*, 1990). The bacteria are grown under conditions to maximise the expression of CFAs, then inactivated with a mild formalin treatment. The CFAs are resistant to degradation in gastric juice when presented in this whole cell complex, rather than as free macromolecules (*WHO*, 1990). The B sub-unit of cholera toxin, which cross-reacts with the heat labile toxin of ETEC, is included in the vaccine to provide antitoxin immunity. This vaccine, when given to Egyptian school children as two doses two weeks apart, is safe and immunogenic. The vaccine also induced a prompt mucosal immune response to multiple CFA antigens (*Savarino* et al., 1997). Further trials to demonstrate protection are needed.

Helicobacter pylori Vaccine Development

Helicobacter pylori infects nearly half of the world's population, resulting in chronic active gastritis which persists throughout life unless the organism is

eradicated (*Drumm*, 1993; *Fiocca* et al., 1987). Most infected individuals remain asymptomatic, but some develop peptic ulcer disease as well as run an increased risk of gastric cancer (*Graham* et al., 1992; *Rauws* and *Tytgut*, 1990; *Correa* et al., 1990; *Forman* et al., 1991). Treatment of *Helicobacter* infections is difficult and the use of vaccines for treatment and prevention of *H. pylori* infections is being actively explored.

Helicobacter seems to differ from other enteric pathogens in that colonisation persists in spite of a strong host immune response (*Crabtree*, 1993). Conflicting data are available regarding the importance of different components of the immune response in clearing this pathogen. One consistent observation, however, is that an immune response induced mucosally by *Helicobacter* antigens alone is not sufficient to affect colonisation. Protective responses are seen only if mucosal adjuvans such as cholera toxin (*Lee* and *Chen*, 1994) or LT are used with antigens from *Helicobacter*.

A formalin-inactivated whole cell preparation of *H. pylori* (HWC) has been tested as a vaccine candidate. A Phase I clinical trial with the HWC + LT_{R192G} vaccine was conducted to establish its safety and immunogenicity in humans (*Kotloff* et al., 2001). An initial dose response study was conducted among 23 volunteers to determine whether increasing inocula of HWC, co-administered with 25 µg of LT_{R192G}, were well tolerated, and to evaluate whether increasing HWC inocula enhanced the immune response. It was anticipated that the optimal dose would contain 2.5 x 10^{10} HWC + 25 µg LT_{R192G}. Groups of 3-10 *H. pylori* infected and *H. pylori* uninfected subjects were assigned in an unblinded fashion to receive three oral doses of vaccine, on days 0, 14, and 28, at an inoculum of either 2.5 x 10^{6}, 2.5 x 10^{8}, or 2.5 x 10^{10} HWC plus 25 µg LT_{R192G}. Safety was established at each dose level before a new group of volunteers received a higher inoculum of vaccine. For the purpose of characterising the dose-response, the eight *H. pylori* infected subjects who received 2.5 x 10^{10} HWC plus 25 µg LT_{R192G} as part of the Randomised Safety and Immunogenicity Study included in this trial were also included in this analysis.

A randomised study was conducted among *H. pylori*-infected subjects to investigate in a preliminary fashion the safety and immunogenicity of the oral HWC vaccine administered with and without the adjuvans. Twenty *H. pylori*-infected subjects were randomly assigned, in a double blind, placebo controlled fashion, to receive, on days 0, 14, and 28, either 2.5 x 10^{10} HWC plus placebo-adjuvans, placebo-vaccine plus 25 µg LT_{R192G}, placebo-vaccine plus placebo-adjuvans, or 2.5 x 10^{10} HWC plus 25 µg LT_{R192G}.

Of the 41 subjects who participated in the trial, six experienced diarrhoea (three who had baseline *H. pylori* infection); one subject received placebo-vaccine plus LT_{R192G} and the remaining five received 2.5 x 10^{10} HWC plus LT_{R192G}. Thus, diarrhoea was seen only among subjects who received LT_{R192G} (with or without vaccine), and only following the highest (2.5 x 10^{10}) HWC dose. Diarrhoea followed the first inoculation in all but one subject. The episodes lasted for 1-3 days, during which time these subjects passed a total of 3 to 17 loose stools.

Immunisation elicited rises in geometric mean serum and mucosal anti-HWC antibodies only among subjects who received the highest (2.5 x 10^{10} HWC) vaccine dose. Whereas post-vaccination increases in geometric mean peak serum IgA and IgG titres were marginal ($p=0.06$), and only seen among *H. pylori*-infected subjects, the

faecal and salivary IgA responses were statistically significant and occurred in both *H. pylori*-infected and uninfected volunteers. Anti-HWC ASC responses were meagre (none exceeded 10 cells per 10^6 PBMC), and so were not subjected to statistical analysis.

Immunisation with 2.5 x 10^{10} HWC plus LT_{R192G} resulted in significant ($p<0.05$) increases among *H. pylori*-uninfected volunteers in mean group IFN-gamma production to the *H. pylori* sonicate at 2 µg/ml; significant rises were observed in 7 of the 10 volunteers studied. No significant increases in mean interferon gamma production were observed when PBMC were incubated with either recombinant catalase or BSA. In contrast, significant increases in mean interferon gamma production to the *H. pylori* sonicate were not observed following immunisation of *H. pylori*-infected volunteers.

Vaccines for Non-Enteric Pathogens

Neisseria gonorrhoeae Vaccine Development

A systemic-oral vaccination approach has been used to vaccinate mice against *Neisseria gonorrhoeae* (*Arko* et al., 1997). In this approach, systemic administration of a gonococcal synthetic peptide was followed by oral administration of killed gonococcal cells made deficient in protein III, which interferes with protective immune responses. These studies showed that vaginal clearance of gonococci from mice was faster in the vaccinated group than in control animals.

Development of Vaccines for Respiratory Pathogens

Oral administration of formalin-killed whole cell *Pseudomonas,* non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* has been shown to protect rats against acute respiratory infection (*Wallace* et al., 1989; *Yoshimura* et al., 1991; *Cripps* et al., 1994). Similarly, intestinal Peyer's patch immunisation with formalin-killed whole cell *Branhamella (Moraxella) catarrhalis* provides protection against acute respiratory infection in mice when intestinal immunisation is accompanied by an immunisation boost delivered to the lungs (Alan Cripps, personal communication). In human studies, adult patients were protected against acute bronchitis after receiving a series of immunisations with 10^{11} killed non-typeable *Haemophilus influenzae* in enteric-coated tablets (*Lehman* et al., 1991). In an earlier study (*Clancy* et al., 1985) in which patients were given this vaccine, a ten-fold reduction in the incidence of infection was noted. There was no clear correlation, however, between clinical protection in these studies and either carriage of *H. influenzae,* or the level of antibacterial antibody in saliva. Subsequent studies in a mouse model of acute respiratory infection (*Wallace* et al., 1991; 1995) have supported the hypothesis that the protective mechanisms induced in the lung include a combination of opsonic antibody and T-cell upregulation of polymorphonuclear cell recruitment and activation.

Oral immunisation of guinea pigs with formalin-killed *Streptococcus pneumoniae* in enteric capsules, following systemic sensitisation with the bacterial preparation, enhanced IgA mediated mucosal immunity (*Yoshimura* et al., 1991). This immunity was associated with reduction of histological changes in the middle ear mucosa compared to control animals. More recently intranasal immunisation with killed unencapsulated whole cells with cholera toxin as an adjuvans was used in rats to prevent colonisation and invasive disease by capsulated pneumococci (*Malley* et al., 2001). In this way, multitypic protection against unrelated pneumo-

coccal serotypes could be obtained associated with the production of systemic antibodies.

The possibility of acts of bioterrorism has led to intense interest in a vaccine against *Bacillus anthracis.* The non-capsulated attenuated Sterne strain of *B. anthracis* has been used to construct genetically detoxified derivatives, thus making a safer non-toxic vaccine candidate (*Mock* et al., 2001). A cellular vaccine may be useful because the cell-free protective antigen (PA) vaccine is less effective in animal models than live strains. This suggests that other components and/or various immune response mechanisms are required for optimal protection. Addition of formaldehyde-inactivated spores of the non toxic Sterne strain protected 100% of mice and guinea pigs against challenge with virulent *B. anthracis* strains under conditions in which PA alone is ineffective.

FUTURE CONSIDERATIONS

Human and animal data indicate that the inactivated whole cell vaccines are safe when administered orally. This characteristic may be made even more important if whole cell vaccines of various pathogens are eventually formulated in a multi-pathogen vaccine or administered simultaneously. The work with the CWC vaccine could lead to a vaccine against major enteric pathogens. Previous experience with cholera and ETEC suggest that this type of vaccine should be effective.

The CWC vaccine could be administered as part of an enteric diseases vaccine. The ETEC component of a combined enteric vaccine should consist of strains to include the major adhesin antigens. Colonisation factor antigen I (CFA/I), CFA/II and CFA/IV are individually expressed by a majority of ETEC isolates from most geographic regions (*Holmgren* and *Svennerholm*, 1990). CFA/I is antigenically homogeneous, whereas CFA/II is composed of coli surface antigen (CS) 3 alone or in combination with CS1 or CS2, and CFA/IV is composed of CS6 alone or in combination with CS4 or CS5. The most advanced ETEC vaccine is a group of inactivated whole cells representing the major CFAs (*Holmgren* and *Svennerholm*, 1990; *Savarino* et al., 1999). It is possible that other organisms, such as *Shigella,* can serve as a platform for passenger antigens, which could include the colonisation factor antigens of ETEC. For example, *Noriega* et al. (1996) used an attenuated *S. flexneri* 2a as a live vector for ETEC antigens CFA/I and CS3. This preparation induced immune responses to the ETEC passenger antigens in mice and guinea pigs. Whether this could also be accomplished with a non-living whole cell preparation of this organism remains to be seen. The *Shigella* component of an enteric vaccine would not only contain *S. flexneri* 2a, but also *S. flexneri* 3a, and *S. flexneri* 6 which would cover most clinically significant strains of this Shigella species (*Noriega* et al., 1999), *S. sonnei,* and *S. dysenteriae*.

Oral immunisation with inactivated whole cells shows promise as a vaccination strategy. Some pathogens, however, have mechanisms, which challenge the success of immunisation. Evidence presented above suggests that *H. pylori* is one of these pathogens. While reduction in colonisation is fairly easily achieved, culture data in animals suggests complete eradication is rarely achieved (*Lee* et al., 1999; *Sutton* et al., 2000). This finding illustrates the need to develop better ways to present anti-

gens and modulate the immune system. Indeed, improved clearance of *Helicobacter* from rodent models has been described in recent papers using a variety of immunisation strategies (*Sutton* et al., 2000; *Guy* et al., 1999; *Eaton* and *Mefford*, 2001). Even reduction of colonisation may be sufficient to relieve symptomatic aspects of infection. *H. pylori* colonisation may protect against diarrhoeagenic gastrointestinal infections (*Rothenbacher*, et al., 2000), indicating that in some circumstances, infection may not be detrimental to the host.

Several associated technologies may be useful with whole cell vaccines. The role of the adjuvans LT_{R192G} in orally administered vaccines remains to be determined. It can certainly serve as an antigen to protect against certain enteric toxins. The adjuvans seems to also enhance the local IgA response in mice (*Rollwagen* et al., 1997; *Baqar* et al., 1995), but this is yet to be shown in humans. Protection in mice could be obtained without adjuvans if the dose of CWC was sufficient (*Baqar* et al., 1995). In contrast, no protection was seen in *Helicobacter* - challenged mice unless the adjuvans was present. Future human trials with inactivated whole cell vaccines should consider the immune responses to a multiple dose, 10^{10} cells per dose, regimen which would include groups with and without a mucosal adjuvans. In this way the IFN-gamma and faecal IgA responses in the presence of adjuvans could be monitored and assessed.

The observation that inclusion of LT-based adjuvans induce a Th1 response (*Bowman* and *Clements*, 2001) may not only be important for protection against the specific pathogen. By inducing immune responses closer to those seen with live organisms, the adjuvans could affect the Th1-Th2 balance that develops after birth. A current view is that Th2-mediated immunity exists in the foetus, but gradually changes to a Th1-mediated immunity (*Rook* and *Stanford*, 1998). This shift is thought to be due to childhood infections, which, through this induction process, could provide significant protection from atopy. It may be prudent to use appropriate adjuvans to ensure that vaccines do not merely protect from infections, but actually replace them as immunologic stimuli (*Walker* and *Zuany-Amorim*, 2001).

In addition to mucosal adjuvans, the method for inactivation could be important in optimising responses to whole cell vaccines. Antibody responses obtained with *Salmonella dublin* varied markedly depending on whether the cells were inactivated with heat, formalin, acetone or ethanol (*Cardenas* et al., 1994). Another approach to inactivating cells is to use expression of cloned PhiX174 gene E in Gram-negative bacteria to cause lysis of the bacteria by formation of an E-specific transmembrane tunnel structure through the cell envelope complex (*Eko*, et al., 1994). Bacterial ghosts prepared with this approach do not suffer from possible denaturation of relevant immunologic determinants obtained with other inactivation procedures.

Another associated technology for use of whole cell vaccines involves their delivery to the mucosal immune system. Frequently buffers have been used to protect antigen from gastric acidity (*Sack* et al., 1997; *Clemens* et al., 1986). These have been particularly important for protein antigens. A less-studied approach is the use of enteric-coated microparticles to induce antibodies against orally administered antigens. Heat-killed *E. coli* were encapsulated in microcapsules coated with a pH-sensitive enteric coating (*Flanagan* et al., 1996). The whole killed bacteria encapsulated by an enteric coating and administered orally to mice effectively

induced an antibody response to its LPS.

Although encouraging data are accumulating, the foregoing illustrates that there is much work to be done to fully realise the potential benefits of orally administered inactivated whole cell vaccines. The safety factor, multi-antigen local and systemic immunity obtained and relative ease of vaccine production and delivery associated with oral immunisation with inactivated whole cell vaccines certainly makes it worthwhile to meet these challenges.

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