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11. IMMUNOMODULATION OF THE GASTROINTESTINAL MUCOSA

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IMMUNOMODULATION OF THE INTESTINAL MUCOSA: A CHALLENGE AND AN OPPORTUNITY

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SUMMARY

Immunomodulation of the intestinal mucosa offers a major challenge to medical science. With progressive realisation of this goal, mankind can at last gain control of diseases which have caused suffering and death throughout history. Diarrhoeal diseases, cancers, parasitic diseases, systemic diseases and diseases of other mucosal surfaces caused by overt and opportunistic pathogens, and autoimmune conditions may all be someday alleviated through effective manipulation of the immune system of the alimentary canal. This tract is naturally protected by non-specific and specific immune components. New approaches are being developed which enhance and direct these components through delivery of antigens and immunomodulators to the intestinal tract. Research to better elucidate the intestinal immune system and integrate new immunomodulation approaches offers an unprecedented opportunity to eliminate scourges that have persisted too long.

INTRODUCTION

The importance of gastrointestinal defences to human health

Diseases involving the gastrointestinal tract are a major cause of human suffering. Gastrointestinal cancers, non-specific inflammatory bowel disease, schistosomiasis and other conditions are serious medical problems. By far, however, the greatest cause of gastrointestinal diseases are the bacteria and viruses causing diarrhoeal illnesses (*Farthing* and *Keusch*, 1988). The mortality from diarrhoea-causing pathogens exceeds 4 million people, mostly children, annually. The morbidity and malnutrition associated with these diseases accounts for an even greater number of victims.

Many infectious agents not causing diarrhoea also exploit the gastrointestinal tract. In the last decade it has been learned that more than half of the world's population harbours *Helicobacter pylori*, making it perhaps the most common bacterial infection on earth (*Cover* and *Blaser*, 1995). This organism can cause gastritis, ulcers, and stomach cancer, which is currently the second leading cause of cancer deaths world-wide. Other infectious diseases affecting various body sites, from polio to typhoid fever, begin with colonisation of the intestine by a pathogen.

Primary pathogens are not the only threat to intestinal mucosal surfaces. The biggest problem on the rise is due to opportunistic infections by microorganisms ordinarily controlled by existing defence mechanisms. During situations in which those defences are diminished by injury or immunosuppression, opportunistic pathogens, which are normally transient colonisers, can cause

Table 1: Mucosal barriers

- Mucus coat	- Peristalsis	- Lymphoepithelium
- Microvillus membrane	- Gastric acidity	- Phagocytes
- Colonisation resistance	- Proteolysis	- Immunoglobulin

systemic septic infections. Septic shock is the leading cause of death in hospital intensive care units and has a prevalence that has more than doubled in a ten-year period (*Reitschel* and *Wagner*, 1996). Despite intensive efforts, mortality associated with septic shock remains at 40-60%. Diseases such as AIDS and the appearance of antibiotic-resistant bacterial strains are contributing significantly to the increase in cases of bacterial sepsis.

While the gastrointestinal tract can be a source of many human afflictions, it also offers the key to the alleviation of diseases of the organ itself, as well as a host of maladies affecting other parts of the body. Immunomodulation of mucosal surfaces is important because relatively few pathogens, such as yellow fever and malaria, enter the host by direct penetration. Instead, most pathogens are mucosal pathogens at some stage in their pathogenesis. Thus the fact that the immune system of the gastrointestinal tract is linked to other mucosal sites such as the respiratory and urogenital systems becomes very important. Further, immunogens acting primarily at mucosal surfaces can also enhance systemic immunity. In short, immunisation of the gastrointestinal tract can have far-reaching effects for human benefit. Modulation of the immune system of the gastrointestinal tract will have the most profound social, political and medical impact of the late 20th or early 21st centuries.

DEFENCES OF THE GASTROINTESTINAL TRACT

The human body has developed many defences against disease. This is particularly true for the gastrointestinal tract probably because this site is the most heavily colonised area of the body. Over 10^{14} bacteria colonise this surface, beginning with 200 species that are indigenous to the oral cavity (*Berg*, 1996). The number of organisms increases distally with 10^{8} per ml in the ileum and 10^{10}-10^{11} per gram in the colon. Opposing these indigenous populations are transient flora that displace normal flora and cause disease. Faced with these microbial challenges as well as a multitude of other antigens from food and the environment, it is not surprising that the gastrointestinal tract has evolved a variety of defensive mechanisms.

Non-immunologic defences

Non-immunologic defences in concert with the local mucosal immune system collectively comprise the mucosal barrier (*Walker*, 1985) (Table 1). The gastric acid barrier and peristalsis are major physical deterrents to microbial colonisation of the intestine. Proteolysis by pancreatic enzymes within the intestine limits penetration by bacterial toxins. The mucous coat and the microvillous membrane it covers also protect the host from pathogens. The mucus barrier, a 450 μm thick gel, is the major site for retaining microorganisms and regulating potential pathogens, and protects the epithelium from injury by microorganisms. Non-indigenous flora are controlled significantly by antimi-

crobial metabolites produced by normal enteric flora, especially anaerobic or colonisation-resisting flora. Host cells are also important as active agents of defence. For example, mucosal leukocytes phagocytise bacteria and other particles.

The importance of colonisation resistance by indigenous microorganisms as a defensive barrier in the intestine has been demonstrated by *van der Waaij* et al. (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 normal defences or defences impaired by injury or disease. In lethally irradiated rats, in which indigenous populations are altered, for example, 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). These studies showed that in sublethally irradiated animals overgrowth by opportunistic pathogens did not occur. Resistance to bacterial overgrowth was associated a population of indigenous flora, the segmented filamentous bacteria, which were maintained in sublethally irradiated animals in contrast to their permanent loss in lethally irradiated animals. These bacteria may actually contribute to colonisation in various ways. Recently, for example, segmented filamentous bacteria were reported to stimulate both local and systemic humoral immune systems of postweaning mice (*Lee* and *Cebra*, 1997).

Oral immunisation

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 microorganisms 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 (Figure 1). The follicles beneath the M cells contain lymphocytes and macrophages essential for processing antigens 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), whereas others enter the general circulation to act at other mucosal sites such as the respiratory or genitourinary tracts. Thus, immunisation via the oral route can protect other mucosal sites outside the gastrointestinal tract. Likewise, immunisation of other mucosal sites provides immunity to the gastrointestinal tract. For example, nasal immunisation against *H. pylori* can protect mice against gastric colonisation by *H. felis* (*Weltzin* et al., 1997).

The production of secretory immunoglobulin A at mucosal surfaces may be a key immune defence. This was demonstrated in experiments using monoclonal antibodies against either *Salmonella typhimurium* or *V. cholerae* (*Winner* et al., 1991; *Michetti* et al., 1992). Hybridomas secreting IgA against either organism were implanted subcutaneously into mice as 'backpacks'. Immunoglobulin in this manner protected the animals against an oral challenge with either the invasive or non-invasive pathogen. The protective activity against *S. typhimurium* was achieved at the mucosal surface because the IgA-secreting hybridoma did not protect against this pathogen when it was administered intraperitoneally (*Michetti* et al., 1992).

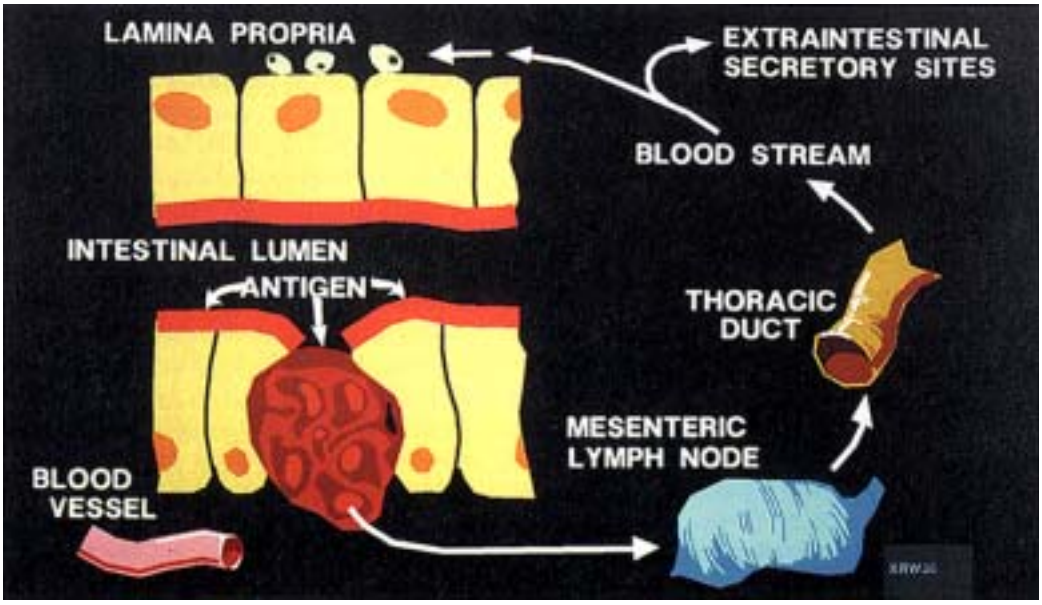

Figure 1: Stimulation and homing of IgA producing plasma cells

Mucus and antibodies could contribute to the rapid clearance of *Campylobacter* seen in immunised rabbits (*McSweegan* et al., 1987). When epithelial cells *in vitro* were overlayed with mucus from non-immune rabbits, *Campylobacter* penetration of the mucus 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 further reduced. 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 were not sufficient to retard bacterial interaction with epithelium. If the lavage fluid was mixed with mucus from non-immune animals, however, then penetration was significantly inhibited.

While antibodies can be important and in some cases pivotal for protection, cellular defences associated with Th1 responses can also be important. For instance, it has been shown that immunity of mice and people to *Shigella* infection correlates with an early and strong interferon gamma response (*van der Verg* et al., 1995; *Raquib* et al., 1995). However, the importance of Th1 and Th2 responses in mucosal defence requires further study.

APPROACHES TO ACHIEVING INTESTINAL IMMUNOMODULATION

Mucosal immunisation

Until recently, it has been problematic to successfully immunise mucosal surfaces. The last decade, however, has seen development of a variety of strategies for using mucosal vaccination to

achieve more effective immunisation (*Walker*, 1994).

Effective immunity usually results from gastrointestinal infections, so it is not surprising that most attempts towards intestinal immunomodulation have used living organisms. Two living but attenuated oral vaccines are now licensed in the U.S. for polio and typhoid fever. An attenuated vaccine for cholera, known as CVD103-HgR (not yet licensed in the U.S.) can be administered as a single dose with bicarbonate buffer. This vaccine was shown to be immunogenic in children, had a 62-87% efficacy in volunteer studies, and was associated with minimal reactogenicity (*Levine* et al., 1998; *Tacket*, 1992; *Sharyono* et al., 1992). Attenuated organisms are not only potential immunogens against the pathogen itself, but are also means to vaccinate against other pathogens by being genetically engineered to express protective antigens of other organisms. However, a problem with the use of attenuated organisms is that they are often not as safe as would be desired.

Subunit antigens are generally safer for use as mucosal immunogens than live microorganisms, but often have not evoked protective immune responses unless certain immunomodulating steps are taken. Subunit antigens may be damaged by the gastrointestinal environment. Some, however, such as urease or OspA (*Lee* et al., 1995; *Luke* et al., 1997) not only withstand degradation in the gastrointestinal tract, but have particulate qualities which make them reasonable mucosal immunogens.

The immunogenicity of subunit antigens is not solely associated with their resistance to degradation. If antigens are adhesive to epithelial surfaces, they make better vaccines (*de Aizpurua* et al., 1988). Proteins exhibiting adhesive properties, such as pili, the B subunit of the heat-labile enterotoxin of *Escherichia coli* (LT) and the HA surface antigen of inactivated influenza virus elicited serum antibody responses when given orally. Other protein antigens and polysaccharides lacking adhesive characteristics did not generate an immune response when given orally, but were as immunogenic as the adhesive antigens when administered intramuscularly. On the other hand, it was shown that orally administered horseradish peroxidase (HRP, a poor adhesin), coupled with the B subunit of cholera toxin (CTB, a strong adhesin), presented a much stronger IgA anti-HRP response in gut washes than HRP alone, or HRP and CTB mixed together (*McKenzie* and *Halsey*, 1984).

Another approach to making subunit antigens practical for oral immunisation is to enclose them in microparticle carrier systems. One such microparticle carrier system is the DL-lactide-coglycolide microsphere (*Morris*, *Steinhoff* and *Russell*, 1994). These particles are taken up in Peyer's patches where they degrade into lactic and glycolic acids. The rate of this process, and thus antigen release, is controlled through alteration of the ratio of the lactide and glycolide present in the polymer (*Miller*, *Bracy* and *Cutwright*, 1977). This approach has been used with many antigens (*Eldridge* et al., 1991). For example, *Eldridge* et al. (1989) showed that Staphylococcal enterotoxin B (SEB) given orally in microspheres to mice, elicited a strong plasma IgM and IgG antibody response whereas no effect was seen with soluble enterotoxoid. Anti-SEB IgA was found in lung, saliva and gut secretions only from the encapsulated group.

Inactivated microorganisms can also be used to immunise the intestinal mucosa. As vaccines, they can be relatively quickly developed compared to other vaccine approaches, are inexpensive to produce and possess multiple antigens which can be important for protection. They are generally safe for mucosal immunisation.

Whole cell vaccines may be effective

immunogens without adjuvants or special delivery systems. An adequate dose of antigen is important to successful immunisation. For instance, an effective whole cell vaccine against *V. cholerae* is administered as two doses, each having $2.5x10^{10}$ particles of different strains of the pathogen, given for a total of 10^{11} cells (*Holmgren* and *Svennerholm*, 1990).

The immunogenicity of inactivated microorganisms can be optimised by genetic engineering, which could be used to enhance or delete an antigen prior to inactivation of the microorganism. Another approach, one that is used at Antex Biologics, is to optimise immunogenicity through the use of NST (Nutriment Signal Transduction) technology, which involves growth of the organisms under conditions which maximise expression of key antigens. Tow vaccine preparations of inactivated whole cells using this technology are currently being evaluated in human clinical studies, *Campylobacter* and *Helicobacter*. Altered antigen expression of *Pasteurella haemolytica* A2 prior to inactivation has been reported by others (*Gilmour* et al., 1991). In this report, *Pasteurella haemolytica* A2 were manipulated to alter expression of iron-regulated proteins. A vaccine made from the outer membrane proteins of these organisms gave better protection against experimental pasteurellosis in lambs than did a preparation from cells that had not been manipulated to enhance expression of these proteins.

It is important to inactivate the cells in such a way that key antigens are preserved. It has been shown that non-viable preparations of a *Salmonella dublin* strain which codes for production of the binding subunit of the heat-labile enterotoxin of *Escherichia coli* (LT-B), when inactivated with heat, ultraviolet light, ethanol, or acetone can elicit serum and mucosal anti-LT-B antibody responses equivalent to those in animals immunised orally with the same number of viable organisms (*Cardenas, Dasgupta* and *Clements*, 1994).

Co-administration of mucosal adjuvants offer a means to alter the magnitude and, possibly, the quality of the immune response to non-living microbial antigen preparations such as inactivated whole cells and subunits. Much work in this area has focused on the heat-labile protein enterotoxins of *V. cholerae* (*Dertzbaugh*, 1990; *Elson*, 1984, 1989) and enterotoxigenic *E. coli* (*Clements*, 1988; *Lycke*, 1992; *Rollwagen*, 1993; *Walker*, 1993; *Majde*, 1994). Mucosal stimulation with these enterotoxins generates both systemic IgG and mucosal IgA responses to an unrelated antigen administered simultaneously (*Clements*, 1988; *Elson*, 1989). The LT has recently been mutated in the A subunit of the toxin in an effort to dissociate enterotoxicity from adjuvanticity (*Dickenson* and *Clements*, 1995; *Douce* et al., 1995; *Tommaso* et al., 1996).

Future adjuvant effects may also be obtained if the cytokines released in response to the enterotoxin adjuvants can themselves be delivered to the appropriate sites as components of vaccines. This has already been achieved where living microorganisms expressing cytokines and specific antigens have been used as mucosal vaccines (*Robinson* et al., 1997; *Ramsay* et al., 1994). Recently, recombinant murine IL-12 complexed to liposomes was given orally to mice along with tetanus toxoid (*Marinaro* et al., 1996, 1997) which resulted in shifts to IgG2a, IgG3 and low IgE antibodies concomitant with enhanced interferon gamma.

The importance of adjuvant administration may vary, depending on the pathogen involved. One pathogen for which an adjuvant is essential is *H. pylori*. Animals immunised with as little as 5 μg of recombinant urease were significantly protected against *H. felis* chal-

lenge when CT adjuvant was co-administered (*Lee* et al., 1995). Strong local and systemic immune responses were also obtained. However, when urease was administered without CT, no protection was seen, even at antigen doses up to 5 mg.

C. jejuni is a major enteric pathogen which infects the colon of man (*Walker* et al., 1986). It is believed that a whole cell vaccine is a logical approach to managing this disease (*Haberberger* and *Walker*, 1994). In preclinical studies the adjuvant, LT, induced intestinal IgA responses to an inactivated *Campyobacter* antigen similar to IgA responses obtained with life organisms (*Rollwagen* et al., 1993). In subsequent work it was established that co-administration of the adjuvant with the antigen was necessary to protect rabbits against challenge with the organism (*Pavlovskis* et al., 1991; *Walker*, *Rollins* and *Burr*, 1992).

Antex has prepared a vaccine consisting of inactivated whole cells of *C. jejuni* produced using Antex's proprietary NST technology. Based on promising preclinical results, these cells were tested in clinical trials, administered either alone or co-administered with an adjuvant. The data from two Phase I trials show that the vaccine is safe and immunogenic and that the adjuvant both improves and broadens the nature of the immune responses elicited by the vaccine. Volunteers responded to the vaccine in a dose-dependent manner. Blood samples from the volunteers were collected and analysed for both humoral and cellular responses. Data from IgA antibody secreting cell assays (indicative of humoral immunity) show that the vaccine produces a *Campylobacter*-specific response. Further, measurements of cytokine responses showed that the vaccine group had greatly increased interferon gamma levels as compared to placebo recipients. Interferon gamma production typifies a Th1-type T-cell response, which is predominantly indicative of active cellular immunity.

Non-specific immunomodulation

Although oral immunisation can be a powerful tool to control infections, non-specific immunomodulation also has potential merit. Non-specific immunomodulation of effector cells with microbial products activates a cascade of mediators which have profound effects on the immune and haematopoietic systems. Systemically administered immunomodulators such as trehalose dimycolate and glucan can non-specifically enhance resistance to a variety of infections, even in immunocompromised subjects (*Madonna* et al., 1989; *Patchen* et al., 1993). The effect of these immunomodulators is probably realised through the release of cytokines and other regulatory factors. Evidence is now accumulating that this approach can also be applied to mucosal surfaces. For example, modulation of mucosal resistance against *Campylobacter jejuni* can be achieved with orally administered cytokines (*Baqar*, *Pacheco* and *Rollwagen*, 1993). Mice given recombinant IL-5 and IL-6 before and shortly after infection with *C. jejuni* displayed up to a 3-log-unit reduction in the number of organisms shed in the faeces.

APPLICATIONS OF IMMUNOMODULATION OF THE INTESTINAL MUCOSA

Protection of mucosal sites

While gastrointestinal immunisation can be used to protect against primary enteric pathogens, immunomodulation at this site can also be important for less obvious applications. As already suggested, oral immunisation can protect distant mucosal sites. For example, oral

delivery of an inactivated influenza vaccine with LT elicited humoral and cell-mediated immune responses in BALB/c mice critical for protection against subsequent infection with influenza virus (*Katz* et al., 1997). Oral co-administration of LT with inactivated influenza vaccine increased serum IgG and mucosal IgA antiviral responses compared with oral influenza vaccine given alone.

Mucosal immunomodulation may not only prevent infections, but may be used to disrupt mucosal colonisation by a pathogen before it causes disease. An important finding with *H. pylori* vaccine preparations is that immunisation of mice colonised with *H. felis* results in reduction of the pathogen to undetectable levels (*Doidge* et al., 1994; Walker, R.I., unpublished data). The preclinical studies performed using the inactivated whole cell *H. pylori* vaccine developed at Antex Biologics showed that two or more doses of vaccine are effective in reducing the *H. felis* below detectable levels and that the amount of adjuvant affects the immunologic response to the treatment. Further, prevention or disruption of colonisation via mucosal vaccination is encouraging for management of other diseases. For example, pathogens associated with otitis media may asymptomatically colonise the nares. Oral (or nasal) immunisation against these pathogens could prevent or disturb the colonisation which ultimately leads to disease.

Induction of systemic immunity

Mucosal immunisation can enhance systemic immune responses, a fact that is not only important for vaccination against those pathogens which invade from mucosal surfaces, but also for those pathogens which enter the bloodstream as a result of a penetrating inoculation. A good example of the latter is a recent report by *Luke* et al. (1997), which showed that, due to its special properties, oral delivery of purified outer surface lipoprotein OspA protects mice from systemic infection with *Borrelia burgdorferi*, the aetiologic agent of Lyme disease. Protection against systemic infection has previously been achieved in mice and other animals when OspA was delivered orally as a recombinant protein in *E. coli*, bacille Calmette-Guerin or *Salmonella typhimurium* (*Fikrig* et al., 1991; *Dunne* et al., 1995; *Langermann* et al., 1994). In the present study, Luke and her colleagues administered OspA or another surface protein, OspD, orally without cell carrier or adjuvant to mice. In comparison to OspD, OspA is highly resistant to trypsin and forms regular complexes of 17-25 nm in size. These complexes could be more efficiently taken up by M cells in the gut epithelium, and thereby stimulate antibody production. As shown in Table 2, OspA, but not OspD, elicited a specific antibody response. Moreover, the orally delivered purified protein protected the mice against infection with *Borrelia*.

Control of infection by opportunistic pathogens

As stated earlier, opportunistic infections are a growing problem. Although many approaches have been tried, real success in protecting immunocompromised individuals against these pathogens is yet to be achieved. Since it is often possible to predict those individuals likely to succumb to such infections, it may become possible to immunise them by oral administration of killed organisms with a mucosal adjuvant. As healthy individuals have been primed by encountering opportunistic pathogens (i.e. *Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus* and others) in the environment, it is possible that they may very rapidly respond to intestinal immunisation with these organisms. Alternatively, non-specific immunomodulation, such as with cytokines or microbial products,

Table 2: Reciprocal ELISA titres and protection in mice immunised orally with rOspA or rOspD

	Immunogen	Serum ELISA titres		Culture positive
		IgG	IgA	
Exp. 1:	rOspD, 4 μg	<20	N.D.	3/3
	rOspA, 4 μg	640	N.D.	0/3
	rOspA, 2 μg	320	N.D.	1/2
Exp. 2:	rOspD, 4 μg	<20	<20	5/5
	rOspA, 4 μg	1470	320	0/5
	rOspA, 2 μg	485	80	0/5

may also be adapted to control opportunistic pathogens on mucosal surfaces.

Regulation of noninfectious disease processes

Some noninfectious diseases may also benefit from mucosal immunomodulation. Mucosally induced immunological tolerance is an attractive strategy for preventing or treating illnesses resulting from untoward inflammatory immune reactions against self- or non-self-antigens. A promising example of this was recently reported by *Bergerot* et al. (1997), who showed that oral administration of microgram amounts of antigen, coupled to cholera toxin B subunit (CT-B), can suppress systemic T cell reactivity. They applied this principle by feeding mice with small amounts (2-20 μg) of human insulin conjugated to CT-B. This procedure can effectively suppress beta cell destruction and clinical diabetes in adult non-obese mice. This protective effect could be transferred by T cells from CT-B-insulin treated animals and was associated with reduced lesions of insulinitis. This finding suggests that not only can infectious diseases be regulated by gastrointestinal immunomodulation, but many non-infectious diseases with an immunologic component may someday lend themselves to this treatment approach.

CONCLUSION

The human gastrointestinal tract is a remarkable organ which can hold the key to control many diseases threatening mankind. This organ has numerous non-specific and specific defence mechanisms which are only now being elucidated. Efforts to manipulate these mechanisms for human benefit are progressing, as suggested in this report. The challenge is great but the rewards should justify the extensive effort put forth.

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BACTERIAL REGULATION OF IMMUNITY

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INTRODUCTION

The gut-associated lymphoid tissue is confronted with a vast array of antigens, ranging from food antigens to pathogenic microorganisms. There is evidence that the gut immune system has the capacity to distinguish between potentially harmful antigens (microbial antigens) and harmless antigens (food antigens), the former inducing strong mucosal and systemic immunity, the latter inducing immunologic tolerance (oral tolerance). The difference in immunogenicity between bacterial and food antigens is probably related to how they are presented by antigen presenting cells. Thus, microbial antigens are probably mainly presented by macrophages capable of phagocytosing whole bacteria. Phagocytosis stimulates the macrophage to express surface-bound T cell costimulatory molecules and secrete cytokines capable of activating T cells. Soluble food antigens are chiefly presented by non-phagocytic dendritic cells and cannot stimulate the expression of costimulatory molecules or cytokines. Presentation of protein antigen by dendritic cells in the absence of such signals leads to T cell anergy (non-reactivity) or may stimulate T suppressor cells. Paradoxically, at the same time as bacteria enhance strong immunity to themselves, they seem to be able to down-regulate immune responses to other antigens, such as food proteins. Thus, animals lacking a normal intestinal microflora display enhanced immunity to fed proteins compared with conventional animals. This may be explained by the fact that interaction of macrophages with LPS or other bacterial antigens induces the release of a series of mediators which decrease antigen presentation by dendritic cells in the vicinity. Failure to develop oral tolerance to food antigens leads to allergies and hypersensitivity reactions, diseases which are constantly increasing in incidence in the Western world. The paper discusses the hypothesis that the increased incidence of allergies is related to a changed pattern of neonatal intestinal colonisation which has occurred during the last decades, for example a delayed colonisation with enterobacteria.

The gut-associated lymphoid system comprises the largest part of the immune system. This is not surprising, considering that most foreign substances reach us via the gut. Not only do we swallow food antigens and food-borne microbes, but also inhaled particles, which become trapped in the respiratory mucus and are transported to the pharynx (cilia beat upwards from the trachea and downwards from the nasal cavity). Furthermore, the gut-associated lymphoid system has intimate contact with the normal intestinal microflora which contains ten times more cells that there are eukaryotic cells in the body. The aim of the present paper is to discuss the regulation of the immune responses engendered against bacterial and food antigens and the impact of the normal microbial microflora on this regulation.

THE GUT IMMUNE SYSTEM

Bacterial and viral infections of the gastrointestinal tract generally induce strong immune responses. Intestinal immunity against gut microbial antigens is induced in the Peyer's patches, which are groups of lymphoid nodules in the gut wall of the small intestine. The epithelium overlying the Peyer's patches contains M cells, epithelial cells specialised in transporting antigen from the gut lumen to the underlying lymphoid cells (*Gebert* et al., 1996). In the Peyer's patches, bacteria are degraded and bacterial antigens are presented to B and T cells, which, provided they have specificity for the antigens in question, start to proliferate and mature. The activated cells leave the Peyer's patches via the lymphatics, circulate for a few days in the blood stream, and finally settle in the lamina propria of the gut and other mucosa, a process called "homing" (*Craig* and *Cebra*, 1971; *Guy-Grand* et al., 1978; *Parmely* and *Manning*, 1983).

Most of the homing B cells develop into plasma cells that produce dimeric IgA. The IgA dimers are held together by a protein named joining chain, which also permits the molecular complex to bind to secretory component, an integral membrane protein exposed on the basolateral side of epithelial cells of the intestine, as well as on ductal cells in salivary and respiratory glands, and lactating mammary glands (*Goldblum* et al., 1996). The complex between dimeric IgA and secretory component is transported through the epithelial cell to the lumenal side, where the secretory component is cleaved off from its transmembrane portion. In this way, secretory component remains attached to the IgA dimer, forming secretory IgA, which is highly resistant to low pH and proteolytic enzymes, and is therefore optimally designed to persist at mucosal surfaces (*Mostov* and *Blobel*, 1983; *Brandtzaeg*, 1985). Secretory IgA is the predominant antibody isotype produced in the intestine, but cells producing IgG and IgM antibodies are also found in the lamina propria of various mucosae (*Goldblum* et al., 1996) and are also part of the homing system (*Dahlgren* et al., 1990).

The T cells which are present in the gut lamina propria also originate in the Peyer's patches (Guy-Grand et al., 1978) and are mainly of the CD4 phenotype (*Selby* et al., 1981). They show signs of activation (*Schieferdecker* et al., 1992; *deMaria* et al., 1993) and spontaneously secrete cytokines (*Hauer* et al., 1997). Interspersed among the epithelial cells are the intraepithelial lymphocytes, which are mainly CD8-positive T cells with cytotoxic potential and unknown function (*Lundqvist* et al., 1996).

There are thousands of single lymph nodules in the colon, but their role in triggering gut immune responses has not been much studied. However, colonic lymphoid nodules seem to be able to function as induction sites for local immunity, since locally applied virus induces a secretory IgA response in the colon (*Ogra* and *Karzon*, 1969).

THE RESPONSE OF THE GUT IMMUNE SYSTEM TO MICROBIAL ANTIGENS

The presence of microbial pathogens in the gut does not only stimulate vigorous mucosal immune responses, but also strong systemic immunity, e.g. high titres of specific IgG and IgM antibodies in the blood (*Waldman* and *Ganguly*, 1974; *Bienenstock* and *Befus*, 1980).

A strong immune response is also seen when non-pathogenic gut bacteria colonise germ free animals (*Wold* et al., 1989; *Shroff* et al., 1995). In a conventional animal which already harbours a normal microflora, the capacity of a novel bacterial strain to induce immunity to itself depends on its ability to persist in high enough numbers for a period of time, i.e. to colonise. Dead microorganisms, or microorganisms transiently passing through the gastrointestinal tract without colonising it, may be taken up into the Peyer's patches and induce moderate immune responses (*Ogra* et al., 1968; *Goldblum* et al., 1975; *Ogra* et al., 1980; Wennerås et al., 1994), but the response will be much stronger with a colonising or invading strain (*Hohmann* et al., 1979). This is probably only a function of the amount of bacterial antigen reaching the immune system.

IMMUNE RESPONSE TO FOOD ANTIGENS

As opposed to microbial antigens, food proteins elicit a very weak and slow antibody response. The response is dominated by serum antibodies of the IgG4 and IgG2 subclasses (*Husby* et al., 1985a), which are poor in fixing complement and interacting with phagocytes. The normal immune response to food antigens is thus characterised by a low inflammatogenic potential. The weak responsiveness to food antigens is not due to their exclusion by the gut mucosal barrier, because an estimated 0.1% of ingested food proteins are taken up into the circulation in an intact, theoretically fully immunogenic form (*Kilshaw* and *Cant*, 1984; *Husby* et al., 1985b). In laboratory animals, the feeding of high doses of protein antigens does not result in a secretory IgA antibody response, but only in the formation of serum IgG antibodies (*Peri* et al., 1982; *Wold* et al., 1987).

EVIDENCE FOR A DIFFERENCE IN RESPONSIVENESS AGAINST FOOD AND BACTERIAL ANTIGENS

To be able to directly compare the responsiveness to food and bacterial antigens experimentally, we gave germfree rats a feed containing egg and milk whey powder (to which they had never been exposed before), and simultaneously colonised them with an E. coli strain (*Wold* et al., 1989). Antibodies of the IgG, IgA and IgM isotypes directed against E. coli LPS and type 1 fimbrial antigens rapidly occurred in secretions, concomitantly with IgM and IgG antibodies in serum. At a later stage, serum IgA antibodies occurred. In contrast, the food antigens induced only a very weak and slow IgG response in serum, and no IgA antibodies in secretions.

LPS and type 1 fimbriae are large polymers and could thus be expected to be more immunogenic than moderately sized monomeric food proteins such as ovalbumin and β-lactoglobulin. However, rat dams monocolonised with *E. coli* during pregnancy and lactation, displayed quite substantial titres of antibodies against the internal bacterial protein β-galactosidase in the milk, while antibody titres against food proteins were low or absent (*Wold* et al., 1989). Thus, a small protein within a bacterium induced better immunity than a protein of similar size consumed in large amounts via the food. This suggested that the "packaging" of an antigen was

crucial to its immunogenicity. To prove this, we transformed *E. coli* with an ovalbumin-encoding plasmid and colonised germ-free rats with this bacterium. Indeed, these rats made secretory IgA antibodies against ovalbumin, whereas rats fed ovalbumin did not (*Dahlgren* et al., 1991).

MECHANISM BEHIND STRONG IMMUNE RESPONSE TO BACTERIAL ANTIGENS

All protein antigens must be taken up, processed and presented on class II MHC molecules by antigen-presenting cells in order to stimulate T cells. Antigen-presenting cells derive from bone marrow precursors which differentiate to various types of macrophages (for example Kupffer cells in the liver, alveolar macrophages in the lungs, microglia in the brain), to dendritic cells in lymphoid tissue, or to Langerhans cells in the skin and buccal mucosa (*Szabolcs* et al., 1996). Whereas some antigen-presenting cells are good phagocytes (for example tissue macrophages), others are poor phagocytes, but excellent presenters of soluble antigens (dendritic cells).

Nevertheless, it is not enough for a T cell to recognise its specific antigen peptide on the proper MHC molecule, in order for it to be activated. The antigen presenting cell must also deliver additional, stimulating signals to the T cell, which are necessary for triggering naive T cells to produce IL-2 and IL-2 receptor, and hence to start proliferating. Certain cytokines produced by antigen presenting cells are stimulatory, e.g. IL-1 (*Rosenwasser* et al., 1979). In addition, antigen presentation becomes much more efficient if the T cell and antigen-presenting cell bind to each other via so called co-stimulatory molecules (*Geppert* et al., 1990). There are a number of co-stimulatory molecules on macrophages and dendritic cells, e.g. ICAM-1 which binds to LFA-1 on T cells, LFA-3 which binds T cell CD2 (*Geppert* et al., 1990), and CD80 (B7-1) and CD86 (B7-2) which bind to CD28 on T cells (*Hathcock* et al., 1994).

Different co-stimulatory molecules preferentially stimulate selected subsets of T cells, and thus direct the immune response into various pathways (*Shahinian* et al., 1993). Similarly, the cytokine milieu which prevails when a naive T cell first encounters its antigen determines the future cytokine repertoire of that T cell (*Mosmann* and *Sad*, 1996). Since different antigen presenting cells have different cytokine and co-stimulatory molecule repertoires, they are likely to deviate the immune responses in different directions. For example, macrophages, but not dendritic cells produce the T cell stimulatory cytokine IL-1 (*Steinman*, 1991). Differences in antigen presenting cell types in various organs is probably the reason for the varied immune responses evoked by a single antigen when given via different routes. For example, intradermal injection of an antigen is the superior mode of delivery to evoke delayed type hypersensitivity.

Bacterial products stimulate macrophages to produce IL-1 (*Keller* et al., 1994; *Hauschildt* and *Kleine*, 1995), and to express several co-stimulatory molecules (*Hathcock* et al., 1994; *Keller* et al., 1995). In this way, their antigen presenting capacity of the macrophage is increased (Ding et al., 1993). This enables all antigens contained in or on a bacterium to be presented to T cells in a fashion which leads to maximal stimulation, and hence immunity. Thus, ovalbumin present within a bacterium will be regarded by the gut immune system as a

bacterial, and hence potentially dangerous, antigen. By this mechanism, the immune system functions more economically in that it wastes little resources on harmless substances, and the risk of undesirable inflammatory reactions also decreases.

ORAL TOLERANCE

Food proteins are not only poor immunogens, but often induce a state of specific unresponsiveness to themselves, termed oral tolerance (*Weiner* et al., 1994; *Telemo* et al., 1997). If an orally tolerised animal is parenterally immunised with the same antigen to which it is tolerant, it will not respond with the expected activation of T helper cells. Thus, local swelling will not be elicited after intradermal injection of the antigen (so called delayed type hypersensitivity) and circulating T cells will not proliferate *in vitro* after addition of the antigen. In some cases, antibodies are formed after parenteral immunisation, but in other cases the antibody response is tolerised as well.

T helper cells may be divided into two more or less well defined subsets, according to their function. Th1 cells are T helper cells which preferentially secrete the cytokines IL-2 and IFN-γ and promote delayed type hypersensitivity reactions (such as the tuberculin reaction). Th2 cells, on the other hand, preferentially secrete IL-4, IL-5 and IL-10 and help B cells mature to antibody-producing cells (*Mosmann* and *Sad*, 1996). Th2 cells secreting IL-4 and IL-5 have been implicated in the pathogenesis of allergy, since IL-4 stimulates IgE production and IL-5 promotes the maturation of eosinophils in the bone marrow (*Jirapongsananuruk* and *Leung*, 1997). Th1 cells are more easily made tolerant than Th2 cells (*Burstein* et al., 1992; *de Wit* et al., 1992; *Melamed* and *Friedman*, 1994). Therefore, delayed type hypersensitivity reactions are more easily tolerised than antibody production, but both may be subject to the induction of oral tolerance (*Garside* et al., 1995b; *Lundin* et al., 1996; *Sudo* et al., 1997).

The original observations of oral tolerance were made in guinea-pigs (*Wells*, 1911; *Chase*, 1946), rats and mice (*Thomas* and *Parrott*, 1974; *Hanson* et al., 1977; *Kagnoff*, 1978), but a few years ago, it was shown that human beings may also become tolerant to ingested antigens (*Husby* et al., 1994). In the human experiment, tolerance was only demonstrated among T cells (i.e. T cells from tolerant individuals did not proliferate upon the addition of antigen) but not among B cells (cells producing antibodies to the fed antigen could still be demonstrated in the circulation).

MECHANISMS FOR TOLERANCE

To uphold a state of tolerance to certain antigens is an equally important task of the immune system as to respond with immunity to other antigens. Without mechanisms of tolerisation, we would react vigorously to our own tissues, as well as to harmless antigens such as food proteins which would create inflammatory and hypersensitivity states. The following mechanisms for achieving tolerance to autoantigens have been described:

1) Clonal deletion. This is the "classical" means by which autoreactive T cell clones are eliminated in the thymus during development (*Ramsdell* and

Fowlkes, 1990). T cells which bind strongly to antigens exposed in the thymus undergo apoptosis. Hence, autoreactive T cells are eliminated from the repertoire of mature T cells.

2) Anergy. Although it was for long thought that all autoreactive T cell clones were eliminated in the thymus, it was later found that there are T helper cells capable of reacting with self antigens in the circulation. However, when they encounter their antigens, they become paralysed du to inability to produce IL-2 and IL-2-receptor and hence to proliferate (IL-2 is an autocrine growth factor for T cells).
3) Active suppression by suppressor cells. According to this mechanism of tolerance, a helper T cell encounters its antigen, but is prevented to proliferate and develop into an effector cell by a regulatory, or suppressor, T cell, which secretes inhibitory cytokines. Suppressor cells may be CD8-postive (*Khoury* et al., 1992; *Miller* et al., 1992) or CD4-positive (*Chen* et al., 1995b; *Garside* et al., 1995a, 1995b), and often produce TGF-β (*Taguchi* et al., 1994; *Sakaguchi* et al., 1995; *Taguchi* and *Takahashi*, 1996).

All the above mechanisms have been shown to mediate oral tolerance in different experimental systems: clonal deletion (*Chen* et al., 1995a), anergy (*Whitacre* et al., 1991; *Melamed* and *Friedman*, 1993, 1994), and active suppression (*Miller* and *Hanson*, 1979; *Miller* et al., 1992). It has been suggested that a high dose of ingested protein tends to favour anergy and a low dose active suppression (*Weiner* et al., 1994), but there is also data to indicate that in young rats, anergy prevails, whereas adults rely more on active suppression (*Lundin* et al., 1996).

Anergy can result if the antigen presenting cell does not provide the proper co-stimulatory signals to the T cell, for example stimulating cytokines and co-stimulatory molecules (*Schwartz*, 1990). Hence, it is easy to understand how food antigens which lack the capacity to stimulate antigen presenting cells will tend to induce anergy and thereby tolerance, whereas bacteria induce immunity. Probably, similar mechanisms exist for the induction of suppressor cells. Thus, presentation of antigen by antigen presenting cells from the lamina propria has been shown to preferably stimulate the generation of CD8-positive cells (which may constitute suppressor cells), while antigen presenting cells from other organs preferentially promote the generation of CD4- positive cells (*Williams* et al., 1992).

CAN BACTERIA REGULATE IMMUNITY TO OTHER THINGS THAN THEMSELVES?

An interesting question is whether bacteria might affect antigen presenting cells in such a profound way that immune reactivity to other antigens, e.g. food antigens or autoantigens could be altered. The answer is "Yes". The presence or absence of a bacterial normal flora affects the immune response to food antigens. Thus, it is difficult to achieve oral tolerance in germ-free animals lacking a normal intestinal microflora (*Moreau* and *Corthier*, 1988; *Sudo* et al., 1997). Further, the administration of LPS together with food antigens increases the tolerising effect of feeding (*Kim* and *Ohsawa*, 1995). Conversely, cholera toxin and *E. coli* heat labile toxin may break oral tolerance to food antigens (*Elson* and *Ealding*, 1984; *Gaborieau-Routhiau* and *Moreau*,

1996). Thus, it is clear that the presence of bacteria or their products not only promotes immunity to themselves, but may in addition strongly influence immune responses to other antigens occurring concomitantly in a human being or experimental animal.

How does this come about? The presentation of soluble protein antigens is probably chiefly a function of dendritic cells, which are antigen presenting cells with high density of class II MHC antigens on their surface, and with a great capacity to present protein antigens, but with no or very poor phagocytic ability (*Steinman*, 1991). Macrophages, on the contrary, are good presenters of bacteria (*Ziegler* et al., 1987), but poor presenters of soluble proteins antigens (*Crowley* et al., 1990). However, it is conceivable that dendritic cells are regulated by neighbouring macrophages. A number of macrophage-derived products have been shown to decrease the antigen presenting capacity of the dendritic cell, for example the cytokines TNF-α (*Holt* et al., 1993) and IL-10 (*Koch* et al., 1996), the prostaglandin PGE2 (*Chouaib* et al., 1985), and nitrous oxide (*Holt* et al., 1993). All these mediators are secreted by macrophages upon phagocytosis of bacteria or interaction with bacterial products. Holt and co-workers have shown that alveolar macrophages profoundly suppress the antigen presenting function of the dendritic cells of the lung (*Holt* et al., 1993). In fact, if macrophages are removed from the lung alveoli of rats, the rats display greatly enhanced immune responses to all inhaled antigens (*Holt* et al., 1993). Similarly, depletion of macrophages from a preparation of dendritic cells from gut lamina propria also enhances their antigen presenting ability (*Pavli* et al., 1990). Thus, if we were to lack macrophages (or if our macrophages were not activated by microbial products), it is likely that our immune system would overreact to many harmless environmental antigens, including food proteins and airborne allergens.

Intestinal colonisation of germ-free rats, or even feeding of large numbers of bacteria to conventional mice, induces a state of activation of the macrophages in the peritoneal cavity as evidenced by an up-regulation of lysosomal enzymes (*Morland* and *Midtvedt*, 1984; *Perdigón* et al., 1986) and altered cytokine production (*Nicaise* et al., 1995). It is, thus, clear that the normal flora has the capacity to regulate the immune reactivity of the whole organism, including the handling of dietary antigens in the small intestine, despite the fact that the normal flora itself chiefly inhabits the large intestine. It is likely that the constant uptake of bacterial products via the portal blood, and the more or less constant translocation of low numbers of bacteria does prime a global low grade inflammatory response, which affects e.g. antigen presentation.

We can guess that certain bacterial groups in the intestinal microflora have a greater potential to affect the immune system than others. Bacterial species which are able to translocate, i.e. pass viable over the epithelial barrier, e.g. E. coli and other enterobacteria, lactobacilli and staphylococci (*Berg*, 1983), will presumably have a greater chance to influence the cells of the gut immune system than most obligate anaerobes, which do not translocate. Certain non-translocating bacteria may affect the immune system in an indirect fashion, e.g. by triggering mediator release from epithelial cells or by stimulating cells of the enteric nervous system. For example, lactic acid bacteria, which change the physical milieu in the intestine by production of H_2O_2 and hydrogen ions may affect enterochromaffin cells which are constantly monitoring the intestinal physico-chemical milieu, and which in turn interact with the enteric nervous system. A number of neuropeptides are

likely to interfere with immune functions, e.g. substance P which generally enhances inflammatory reactions and VIP and somatostatin which inhibits them.

Clearly, different bacterial groups will have different impact on the immune system. Thus, Gram-positive and Gram-negative bacteria have different ability to affect various co-stimulatory molecules on macrophages, and to stimulate the secretion of different cytokines (*Keller* et al., 1994). We have recently observed that colonisation of germ-free rats with *Lactobacillus plantarum* and *E. coli* altered T cell populations of the intestinal lamina propria, as compared with *E. coli* colonisation alone, and that *Lactobacillus plantarum*-colonised rats displayed reduced T cell proliferative responses to Con-A (*V. Herías*: Personal communication).

IS THE RISE IN THE FREQUENCY OF ALLERGIES ASSOCIATED WITH ALTERATIONS OF THE NEONATAL INTESTINAL MICROFLORA?

The incidence of atopic allergy, i.e. IgE-mediated hypersensitivity, is steadily on the increase in Western European countries, and vastly supersedes the incidence in the former socialist countries and in the third world (*Strachan*, 1989; *Björkstén*, 1994; *von Mutius* et al., 1994). Simultaneously, the intestinal bacterial colonisation pattern of neonates has changed gradually over the last decades. For example, in a study of the intestinal microflora of Swedish newborn infants born during the early eighties, 25% of the infants did not acquire any enterobacterial species during their first week of life (*Adlerberth* et al., 1991). In earlier studies (*Gareau* et al., 1959; *Bettelheim* et al., 1974), and in studies performed in developing countries (*Mata* and *Urrutia*, 1971; *Rotimi* et al., 1985; *Adlerberth* et al., 1991), infants are as a rule always colonised by day 3 with E. coli or other Gram-negative enterobacteria. It is probably a combination of the strict hygiene applied during Western hospital deliveries, and a reduced spread of enterobacteria in the hospital which results in the low enterobacterial colonisation rate. Later on, the Western babies continue to lead an overly hygienic life, with a low exposure to bacteria via the food or the environment in general. This will certainly reduce the risk of neonatal infections, and thus infant mortality, but may also result in an "abnormally" stable microflora. Thus, Swedish infants often carry a single *E. coli* strain in their microflora for months and years (*Kühn* et al., 1986), while Pakistani infants are colonised with a multitude of different *E. coli* strains in a rapid succession during their first six months (*Adlerberth* et al., manuscript in preparation).

Instead of enterobacteria, whose spread is severely restricted in a very clean milieu, Western infants will be colonised by other bacteria, which are less affected by hygienic measures. *Staph. epidermidis* and *Staph. aureus* are part of the normal flora of the skin and are transferred to the infants from the caretakers. They are also present on the nipple, and breast-fed infants suckle staphylococci together with their milk. Therefore, staphylococci, which are traditionally thought of only as inhabitants of the skin and nasal cavity, are nowadays the dominant species in the intestinal microflora of quite a few newborn infants, for example in Sweden (*Bennet* et al., 1991) and in the USA (*El Mohandes* et al., 1993). Other bacteria whose presence in the normal flora has increased, are the clostridia (*Sepp* et al., 1997), which are spore-formers and

therefore survive the hygienic measures applied in a modern hospital.

The slow colonisation of the intestine of Swedish children with enterobacteria may drastically reduce the exposure of the developing immune system to LPS. When the Swedish infant is finally colonised with enterobacteria, the anaerobes may already have become established, in which case the enterobacteria cannot reach equally high numbers as they do in the absence of competition (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982). This, in turn, will hamper their ability to translocate across the intestinal mucosal barrier and influence the developing intestinal immune system (*Berg*, 1980; *Herías* et al., 1995). Furthermore, translocation occurs only during the establishment of a particular bacterial strain in the intestine. Once a specific IgA response to the bacterium has evolved, coating of the bacteria with secretory IgA will namely prevent further translocation (*Shroff* et al., 1995). The extended periods of carriage of individual E. coli strains among Swedish children will therefore contribute to the low exposure of their immune systems to LPS. As pointed out above, LPS favours the induction of oral tolerance to food antigens, and consequently, too little LPS may impede the ability of the infant's immune system to mature into being capable of distinguish between harmful and harmless antigens.

Equally possible is that the bacteria which have in part replaced E. coli and enterobacteria as dominant in the newborn infant's intestine may have untoward effects on the capacity to react with oral tolerance. One can speculate that Clostridium difficile, via its elaboration of toxins might be able to break oral tolerance, even if the toxin of this specific bacterium has not been tested in this respect. Many *Staph. aureus* strains elaborate toxins which function as so called superantigens, i.e. molecules which bind to and activate a large fraction of the T cells (*Herman* et al., 1991). All *Staph. aureus* strains also possess the immunoglobulin-binding molecule protein A, which may function as a B cell superantigen and activate a broad range of B cells (*Seppälä* et al., 1990; *Silverman*, 1992). It remains to be tested whether any of the above changes in the neonatal colonisation pattern is responsible for the continuous rise in allergies in the Western world.

As outlined above, the normal intestinal microflora is not an innocent bystander of the intestinal immune system, but an important modulator of intestinal immune functions. This opens the possibility that hypersensitivity and autoimmune phenomena may be related to disturbances in intestinal colonisation. Fortunately, it also opens the possibility of a remedy to these conditions - a controlled colonisation by probiotic bacteria.

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HOST INTERACTIONS WITH INTESTINAL *LISTERIA*: EXCEPTION OR THE RULE?

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SUMMARY

Listeria monocytogenes normally infects the host by translocating from the intestinal lumen. Experiments were carried out to determine if, when and where tumour necrosis factor (TNF) and gamma interferon (IFN-γ) function in antibacterial resistance during enteric listeriosis. Groups of normal mice and severe combined immunodeficient (SCID) mice were injected with monoclonal antibodies (MAb) specific for either cytokine and then inoculated intragastrically with *L. monocytogenes*. The course of infection was monitored by enumerating listeriae in gut associated lymphoid tissues (GALT), livers and spleens. By the third day of infection, bacterial numbers in infected tissues and organs were greatly exacerbated in normal mice and SCID mice treated with anti-TNF Mab, whereas bacterial numbers in the organs of mice treated with anti-IFN-γ Mab did not differ from those present in the respective organs of control mice. However, by the fifth day of infection, bacterial numbers in the organs of anti-IFN-γ Mab-treated normal mice and SCID mice were much greater than in the corresponding organs of control mice. Experiments using immune mice revealed that TNF and IFN-γ are involved in the expression of anti-*Listeria* memory immunity, however, it was also found that the anti-IFN-γ Mab was relatively ineffective in inhibiting the expression of anti-*Listeria* immunity whereas, a monospecific polyclonal anti-IFN-γ was quite effective. This paradox between the ability of the anti-IFN-γ Mab to inhibit IFN-γ-mediated effects in innate antibacterial resistance and its inability to inhibit IFN-γ-mediated effects in anti-*Listeria* memory immunity suggest that the IFN-γ molecule possesses functional domains which mediate distinct activities.

INTRODUCTION

Carter and Pollard, Berg and Savage, Adrian Lee, and many others have shown that lactobacilli and other members of the mouse autochthonous flora do not induce an immune reaction when they colonise the intestinal tract. A close relative of members of the genus *Lactobacillus* is *Listeria monocytogenes*, an enteric organism which induces a somewhat unusual immune response. Mouse listeriosis is a widely used model for the study of host resistance mechanisms that are expressed against intracellular bacteria. Mackaness reported that

host cells, not humoral factors, effected anti-*Listeria* immunity in mice (*Mackaness*, 1962). Mackaness also established the importance of both lymphocytes and macrophages in the expression of anti-*Listeria* immunity (*Mackaness*, 1964, 1969). Newly generated and sensitised immune lymphocytes conferred the specific nature of immunity to listeriae, however, resolution of infection required the activation of macrophages which exhibit a heightened state of non-specific bactericidal activity. Later, *Lane* and *Unanue* (1972) and *North* (1973) established that T lymphocytes mediated the expression of specifically acquired anti-*Listeria* immunity. Since this time, considerable information concerning the importance and interactions of various host effector cells and cytokines in non-specific antibacterial resistance (innate immunity) and in specifically acquired T cell-mediated antibacterial immunity has come about using mouse models of listeriosis.

The identification of cytokines involved in host antimicrobial resistance has come largely from the use of mouse models of infectious diseases in which the action of a cytokine is inhibited. Inhibition of a cytokine-mediated effect has generally been accomplished by either blocking the action of an endogenously produced cytokine with specific anti-cytokine antibodies or using cytokine gene knockout mice which lack the capacity to synthesise functional cytokines or cytokine membrane receptors. Indeed, both approaches have been used to demonstrate the importance of IFN-γ and TNF in host anti-*Listeria* resistance in mice. *Buchmeier* and *Schreiber* (1985) showed that listeriosis was exacerbated in mice treated with monoclonal anti-IFN-γ Mab. Work carried out in our laboratory (*Havell*, 1987, 1989) and by others (*Nakane* et al., 1988) showed that anti-TNF antibody treatment of mice converted a normally immunising infection initiated by the intravenous injection of a sublethal *L. monocytogenes* inoculum into a lethal infection. Likewise, anti-TNF antibody treatment exacerbated listeriosis in immunoincompetent nude (*nu/nu*) mice (*Havell*, 1989; *Hauser* et al., 1990). Gene knockout mice lacking functional membrane receptors for either IFN-γ or TNF were found to be considerably more susceptible to listeriosis than the wild type mice (*Huang* et al., 1993; *Rothe* et al., 1993; *Pfeffer* et al., 1993). Similar experimental approaches have shown both that TNF and IFN-γ are also important components of host resistance against a variety of other microorganisms and that these cytokines are important both in innate antimicrobial resistance and in the generation and/or expression of specifically acquired antimicrobial immunity (*Nakane* et al., 1989; *Kindler* et al., 1989; *Chen* et al., 1992, 1993; *McCafferty* et al., 1994; *Aguirre* et al., 1995).

Most studies investigating host cells and cytokines in anti-*Listeria* resistance have used mice infected by parenteral routes of inoculation, however, *L. monocytogenes* normally infects the host by translocating from the intestinal lumen and then spreading to internal organs. *MacDonald* and *Carter* (1980) reported that listeriae present in the lumen of the gastrointestinal tract of mice were capable of infecting Peyer's patches. Peyer's patch-associated listeriae were shown capable of entering mesenteric lymph nodes, from where these bacteria can spread to other internal organs, including the liver and spleen. Like *L. monocytognes*, *Salmonella typhimurium* present in the intestinal lumen was also shown to invade the Peyer's patches In view of the reports that IFN-γ is found in S. *typhimurium*-infected Peyer's patches (*George*, 1996) and that intracellular IFN-γ is detected in intraepithelial lymphocytes in mice following the intragastric *Listeria* inoculation, experiments were carried out to deter-

mine whether IFN-γ and TNF play roles in innate resistance and specifically acquired antibacterial resistance in the GALT and other infected organs following the translocation of listeriae from the intestinal lumen of mice.

MATERIALS AND METHODS

Mice

Male BALB/c mice 8 to 12 wk of age were purchased from either Charles River Laboratories (Wilmington, MA) (BALB/c Crl) or Taconic Farms (Germantown, NY) (BALB/c Tac). C.B-17 SCID mice were purchased from Jackson Labs (Bar Harbor, ME). BALB/c mice were maintained under pathogen-free husbandry conditions while immunoincompetent SCID mice were maintained in autoclaved microisolator cages provided with sterile food and water.

Listeria monocytogenes

Listeria monocytogenes (strain EGD, serotype 1/2a) was grown overnight at 37°C in Trypticase soy broth (BBL Microbiology Systems, Becton Dickinson, Cockeysville, MD). The culture broth was centrifuged at 800 x g/20 minutes and the pelleted bacteria were re-suspended in Dulbecco's phosphate-buffered saline (DPBS) pH 7.4. The stock culture having a titre of 6.6 x 10^9 CFU/ml was aliquoted in tubes and stored at -70°C. Immediately before use, stock preparations were quick-thawed and diluted in DPBS (pH 7.4). The intravenous LD_{50} for *L. monocytogenes* in BALB/c Crl mice was determined to be 4 x 10^3 CFU. The standard intagastric (i.g.) inoculum was 2 x 10^8 CFU in 0.2 ml of DPBS. Mice were gavaged intragastrically with an 18 gauge feeding needle (Popper, Long Island City, NY).

Enumeration of organ-associated bacteria

Organ homogenates of livers, spleens, mesenteric lymph nodes and Peyer's Patches were prepared by grinding organs suspended in iced sterile saline (0.85%) with a Teflon motorised pestle. Enumeration of bacterial CFU in the organ homogenates were determined by plating serial 10-fold dilutions of liver, spleen, or mesentery/mesenteric lymph nodes homogenates on trypticase soy agar (TSA, BBL Microbiology Systems, Becton Dickinson, Cockeysville, D). Bacterial CFU in homogenates of the Peyer's patches were plated on *Listeri*a-selective phenylethanol (PEA) agar consisting of 1.5% Noble agar, 1.5% trypticase peptone, 0.5% phytone peptone, 0.5% NaCl, 1.0% Glycine, 0.05% LiCl, and 0.25% Phenylethanol (*MacDonald* and *Carter*, 1980). *Listeria monocytogenes* colonies on PEA agar were identified by their characteristic light blue colour when illuminated with oblique light. Tests for esculin, catalase, and/or motility were performed to insure that questionable colonies on PEA agar were indeed *L. monocytogenes* colonies.

Anti-cytokine antibodies

The R4-6A2 hybridoma (ATCC, HB170) which secretes a rat anti-murine IFN-γ Mab (IgG1) and the XT3.11 hybridoma (DNAX Research Institute, Palo Alto, CA) which secretes rat anti-murine TNF-α MAb (IgG1) were grown as ascites in the peritoneal cavities of pristane-primed $CB6F_1$ mice, according to our published procedures (*Havell*, 1986a; *Aguirre* et al., 1995). The R4-6A2 anti-IFN-γ MAb and the XT3.11 anti-TNF Mab were purified from ascitic fluids according to previously published procedures (*Havell*,

1986a). A rabbit anti-IFN-γ polyclonal IgG antibody was generated by immunising a New Zealand White female rabbit with pure recombinant mouse IFN-γ having a specific activity of 10^7 antiviral units/ml (u/ml) which was the kind gift of Genentech, Inc. (South San Francisco, CA). The rabbit anti-IFN-γ IgG or rabbit control IgG was purified from serum according to published procedures (*Havell* et al., 1988). The various purified antibody preparations were assayed for endotoxin concentrations by means of a quantitative chromogenic *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD).

The quantitation of the anti-IFN-γ antibody neutralising activity was performed as previously reported (*Spitalny* and *Havell*, 1984). Briefly, serial two-fold dilutions of sample (50 μl) were incubated with an equal volume of IFN-γ (20 antiviral units/ml) in wells of a 96-well flat bottom plate for 1 hour at 37°C. At the end of this time, $2x10^4$ L929B mouse fibroblasts in 100 μl were added to each well, and the plates were incubated at 37°C. Eighteen hours later, 10^3 PFU of vesicular stomatitis virus (VSV) were added to each well. The plates were incubated for 48 hours, after which viral cytopathic effect was scored. The neutralising titre (neutralising units/ml [NU/ml]) of the anti-IFN-γ Mab is defined as the reciprocal of the highest dilution of the sample that, when mixed with an equal volume of IFN-γ (concentration, 20 antiviral units/ml), neutralises 50% of the antiviral activity, as judged by the development of VSV cytopathic effect in the L929B cell monolayer.

Quantitation of anti-TNF Mab neutralising activity was also performed as previously reported (*Havell* et al., 1988). Basically, the anti-TNF Mab assay procedure is the similar to that outlined above for anti-IFN-γ antibody assay procedure, except the titration of anti-TNF Mab activity measures the neutralisation of TNF cytotoxic activity on actinomycin D-treated L929B cell monolayers.

Anti-cytokine treatment of mice

Mice were injected intraperitoneally with a given antibody preparation 4 hours prior to the intragastric inoculation of bacteria. Mice were injected with 10^5 neutralising units of the R4-6A2 rat anti-IFN-γ Mab (sp act $1.8x10^5$ NU/mg) in PBS (pH 7.4). Mice injected with the XT3.11 rat anti-TNF Mab received $2x10^4$ NU (sp act 6 $x10^3$ NU/mg) in PBS (pH 7.4). The mice that were injected with the rabbit anti-IFN-γ IgG were given $2x10^4$ NU (sp act $2x10^3$ NU/mg) while the corresponding control mice were injected with an equivalent amount (mg) of control rabbit IgG.

At the time of sacrifice, antibody-treated mice were anaesthetised, bled by cardiac puncture, and the serum collected. The sera were assayed to determine the anti-cytokine antibody neutralising titres in order to insure that excess amounts of the anti-cytokine were present in the blood throughout the course of the experiments. In all cases, antibody titres exceeded 10^3 NU/ml of blood.

Statistical analysis

The experimental results were compared using Student's t-test, which requires that the populations be normally distributed and have equal variances. A significant difference between experimental groups was defined by a p value of <0.05. Experiments involving statistical comparisons were performed using 3 to 5 mice per group.

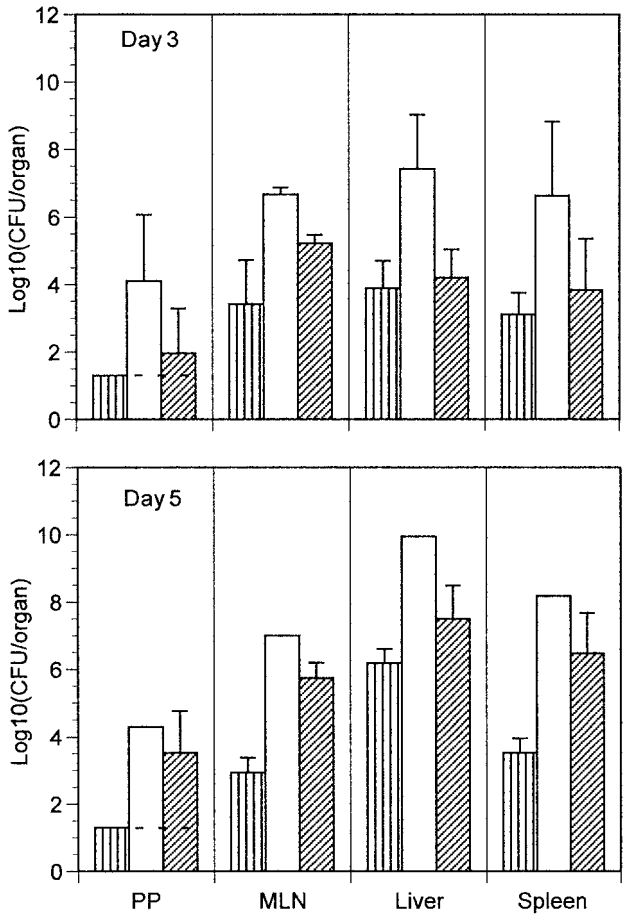

Figure 1: The effect of anti-TNF Mab or anti-IFN-γ Mab treatment on the course of enteric listeriosis. BALB/c mice were injected intraperitoneally with 10^5 NU of anti-IFN-γ Mab, $3x10^4$ NU of anti-TNF, or PBS and 4 hours later, all mice were inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. Organ *L. monocytogenes* CFU were determined on days 3 and 5 following the intragastic inoculation of bacteria. Data are presented as the means (bars) +/- standard deviations of organ CFU for an experimental group. Means lacking standard deviations indicate that either bacterial CFU were below detection limits in one or more organs from an experimental group, or insufficient numbers of mice survived treatment, as was the case on day 5 for the anti-TNF Mab-treated group (1 survivor). ▥: control; □: anti-TNF; ▨: anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

RESULTS

The importance of TNF and IFN-γ in resistance to an immunising *Listeria* enteric infection

The inhibition of cytokine-mediated effects *in vivo* by administering specific antibodies has proven an effective means for establishing the importance of a cytokine in host resistance to infectious agents (*Buchmeier* and *Schreiber*, 1985; *Havell*, 1987, 1989; *Chen* et al., 1993). Experiments were carried out using specific anti-TNF or anti-IFN-γ Mab treated mice to determine if TNF and IFN-γ are involved in resistance to enteric listeriosis. Groups of BALB/c mice were treated intraperitoneally with

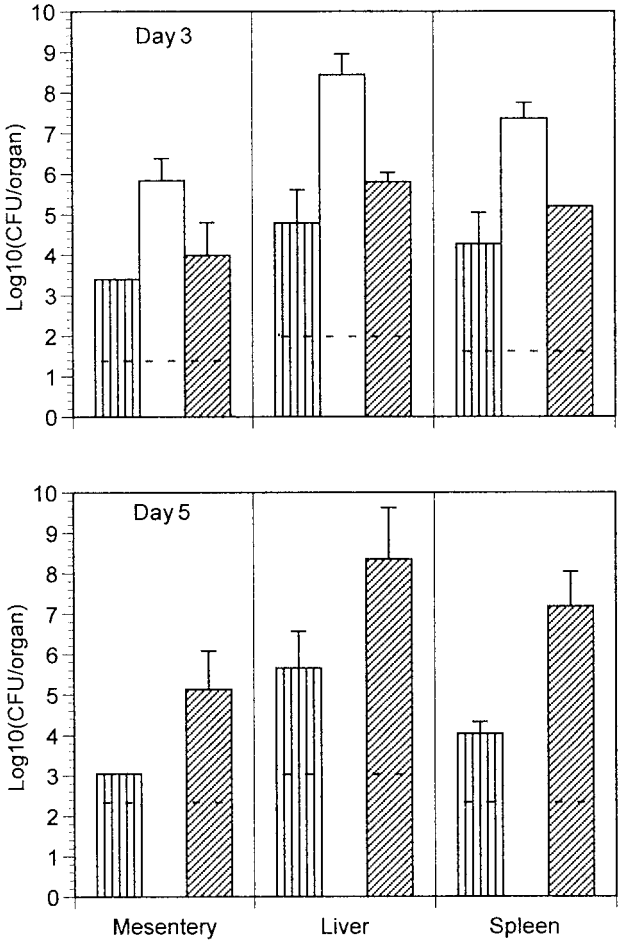

Figure 2: The effect of anti-TNF Mab or anti-IFN-γ Mab treatment on the course of enteric listeriosis in immunoincompetent SCID mice. C.B-17 SCID mice were injected intraperitoneally with 10^5 NU of anti-IFN-γ Mab, $3x10^4$ NU of anti-TNF, or PBS and 4 hr later, all mice were inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. Organ *L. monocytogenes* CFU were determined on days 3 and 5 following the intragastric inoculation of bacteria. All anti-TNF Mab-treated mice were dead by day 5 of infection. Data are presented as the mean (bars) +/- standard deviations of organ CFU. Means lacking standard deviations indicate that bacterial CFU were below detection limits in one or more organs from an experimental group of mice. ▥: control; □: anti-TNF; ▨: anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

anti-TNF Mab or anti-IFN-γ Mab and 4 hr later, inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. The course of enteric listeriosis was then monitored at progressive times by enumerating listerial CFU in the PP, MLN, livers and spleens of the treated mice and control mice. It was found that by the end of the first day of infection, no significant differences existed in numbers of listeriae present in the corresponding organs (MLN, livers, and spleens) of mice in the different experimental groups of mice (results not presented). Moreover, based on the limits of detection of the assay, listeriae were absent from the PP of control mice groups (results not presented). However, by day 3 of infection (Figure 1) the numbers of listeriae in the organs of

the anti-TNF Mab-treated mice were greatly exacerbated, whereas numbers of listeriae were elevated only in the MLN of the anti-IFN-γ-treated mice. Also, at this time of infection, anti-TNF Mab-treated mice were lethargic and hypothermic, and most died by day 5 of infection. Enumeration of listeriae in the organs of the one remaining anti-TNF Mab-treated mouse on day 5 of infection revealed overwhelming numbers of listeriae in the organs (Figure 1). On day 5 of infection, the infected organs of the anti-IFN-γ Mab-treated mice had greater numbers of listeriae than did the corresponding organs of the control mice. These results establish that TNF has an effect that is important in antilisterial resistance during the first 3 days of enteric listeriosis, whereas IFN-γ mediates an important effect in resistance following this time.

The importance of TNF and IFN-γ in innate immunity to enteric listeriosis

Results from the foregoing experiment established that an IFN-γ-mediated effect is expressed in *Listeria*-infected organs of mice after the time (day 3) when the host normally begins to generate a T cell-mediated anti-*Listeria* immune response that is capable of effecting the resolution of infection (39-41). In an attempt to dissociate IFN-γ- or TNF-mediated effects in innate antibacterial immunity from possible effects which could be important in the generation and/or expression of specifically acquired anti-*Listeria*-immunity, immunoincompetent SCID mice were used in an experiment to determine the importance of these cytokines in innate antibacterial immunity to enteric listeriosis. Groups of C.B-17 SCID mice were inoculated intraperitoneally with anti-TNF Mab or anti-IFN-γ Mab and 4 hr later, inoculated intragastrically with $2x10^8$ CFU of *L. monocytogenes*. Since SCID mice lack discernible PP and MLN, listeriae were enumerated in the mesentery, livers and spleens of groups of the Mab-treated SCID mice and control SCID mice on days 3 and 5 of listeriosis. It can be seen in Figure 2 that anti-TNF Mab treatment, but not anti-IFN-γ Mab treatment of SCID mice greatly enhanced numbers of listeriae in the mesentery, liver, and spleen on day 3 of infection. By day 5 of infection, all anti-TNF Mab-treated SCID mice had succumbed to overwhelming infection whereas, infected SCID mice treated with anti-IFN-γ Mab were only beginning to show signs of morbidity associated with overwhelming bacterial infection. Bacterial numbers present in the mesentery, liver and spleens of the anti-IFN-γ Mab-treated SCID mice and control SCID mice on day 5 of listeriosis are also presented in Figure 2, where it can be seen that the numbers of listeriae in the organs of the anti-IFN-γ-treated mice are much greater than in the respective organs of control mice. Thus, the collective results presented in Figure 1 and Figure 2 establish that the magnitude and temporal manifestation of TNF- and IFN-γ-mediated antibacterial effects are, respectively, similar in the organs of both immunocompetent mice and immunoincompetent mice during enteric listeriosis.

The importance of TNF and IFN-γ in the expression of anti-*Listeria* memory immunity in the intestine

The results of the preceding experiments do not allow conclusions to be made as to whether TNF and IFN-γ function in the expression of anti-*Listeria* immunity because of the similar results which were obtained with normal mice and SCID mice undergoing a primary *Listeria* enteric infection. To determine whether TNF or IFN-γ plays a role in anti-*Listeria* immunity in the intestine, *Listeria*-immune mice, immunised by an intragastric inoculation of

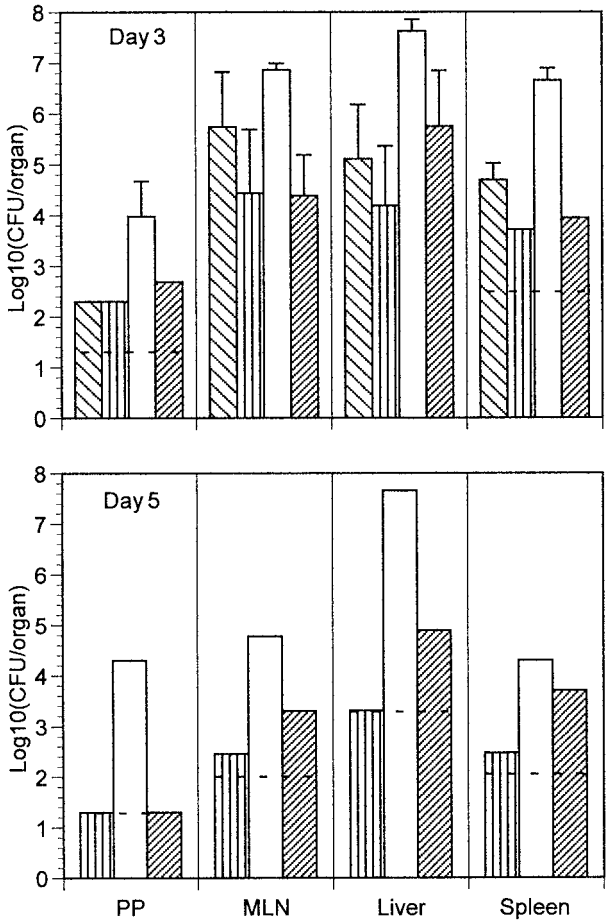

Figure 3: The effect of anti-TNF Mab or anti-IFN-γ Mab treatment on the expression of memory anti-*Listeria* immunity. BALB/c mice rendered *Listeria* immune by intragastric inoculation of $2x10^8$ *L. monocytogenes* 28 days earlier were injected intraperitoneally with 105 NU of anti-IFN-γ Mab, $3x10^4$ NU of anti-TNF, or PBS. Four hours later, all treated *Listeria* immune mice and a group of non immune mice were challenged with an intragastric inoculum of $6x10^9$ CFU of *L. monocytogenes*. On days 3 and 5 following rechallenge, organ CFU were enumerated. Data are presented as means (bars) and +/- standard deviation. Means lacking standard deviations indicate that bacterial CFU were below detection limits in one or more organs from an experimental group of mice, or insufficient numbers of mice survived treatment, as was the case on day 5 for the anti-TNF Mab-treated group (1 survivor). ▧: non-immune controls; ▥: immune controls; □: anti-TNF; ▨: anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

$2x10^8$ CFU of *L. monocytogenes* 28 days earlier, were injected intraperitoneally with either anti-TNF Mab or anti-IFN-γ Mab and, 4 hr later, challenged with an intragastric dose of $6x10^9$ CFU of *L. monocytogenes*. In Figure 3 are presented the CFU present in the PP, MLN, livers and spleens of the Mab-treated *Listeria*-immune mice on days 3 and 5 of a secondary challenge. It can be seen that on day 3 of infection listerial numbers were elevated in the organs of the anti-TNF Mab-treated immune hosts relative to the listerial numbers in the corresponding organs of immune control mice ($p<0.01$). At this time of infection, listerial numbers in the respective organs of non-

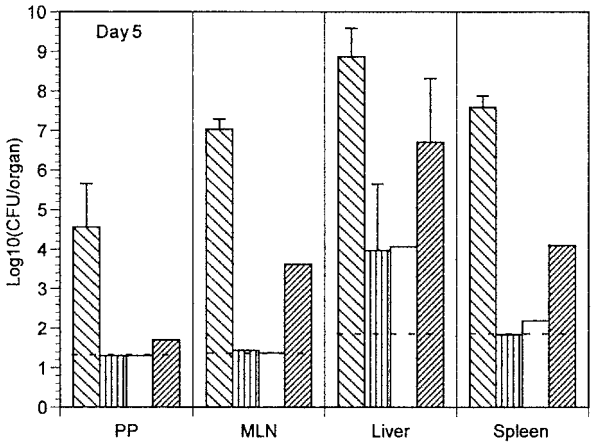

Figure 4: A comparison of the abilities of the R4-6A2 anti-IFN-γ Mab and a rabbit anti-IFN-γ IgG preparation to inhibit the expression of memory anti-*Listeria* immunity. BALB/c mice rendered *Listeria* immune by intragastric inoculation of $2x10^8$ *L. monocytogenes* 30 days earlier were injected intraperitoneally with 10^5 NU of anti-IFN-γ Mab, $2.5x10^4$ NU of a rabbit polyclonal anti-IFN-γ IgG, or control rabbit IgG. Four hours later, all treated *Listeria* immune mice and group of non immune mice were challenged with an intragastric inoculum of $7.2x10^8$ CFU of *L. monocytogenes*. Five days later, organ CFU (mean +/- standard deviation) were enumerated. Means lacking standard deviations indicate that bacterial CFU were below detection limits in one or more organs from an experimental group of mice. ▨: non-immune controls; ▥: immune controls; □: Mab-anti-TNF; ▨: Pab-anti-IFN. Dashed horizontal lines represent the detection limits of the assay.

immune control mice, *Listeria*-immune control mice, and the anti-IFN-γ Mab-treated and *Listeria*-immune mice were similar. However, by day 5 of infection listeriae were not detected in the organs of the control *Listeria*-immune mice whereas, substantial numbers of listeriae were present in the corresponding organs of the non-immune control mice which indicates that memory anti-*Listeria* immunity is not expressed until after the first 3 days of infection. All anti-TNF Mab-treated *Listeria*-immune mice were dead by day 5 of infection. On day 5, the bacterial numbers in the organs of the anti-IFN-γ Mab-treated and *Listeria*-immune mice were only marginally higher than those of the *Listeria*-immune control mice, but were not as high as the bacterial numbers in the organs of the non-immune control mice. Moreover, the anti-IFN-γ Mab treatment of immune mice did not completely prevent the expression of immunity, since these mice survived the secondary challenge. This was not due to a lack of serum anti-IFN-γ Mab neutralising activity during the course of the experiment, for it was found that substantial quantities of anti-IFN-γ Mab were present in the peripheral circulation of treated mice on day 5 of infection (results not presented).

Comparative analysis of the ability of different anti-IFN-γ antibody preparations to block the expression of anti-Listeria memory immunity

The failure of the anti-IFN-γ Mab treatment of *Listeria*-immune mice to completely subvert the expression of immunity (Figure 3) conflicts with previous reports showing that anti-IFN-γ Mab treatment of *Listeria*-immune mice converted what would be normally a sublethal infection initiated by an extravascular challenge into a lethal one

(*Tripp* et al., 1995). One possible explanation for the apparent discrepancy between the results reported in this publication and those reported elsewhere, is that anti-IFN-γ antibody preparations may have specificities for different molecular domains mediating distinct IFN-γ activities (*Schreiber* et al., 1985; *Caruso* et al., 1993). In view of such a possibility, a comparison was made as to abilities of the anti-IFN-γ Mab used in the preceding experiments and a monospecific rabbit anti-IFN-γ polyclonal antibody (Pab) to inhibit the expression of memory immunity in *Listeria*-immune mice. The results presented in Figure 4 show *Listeria* CFU in the PP, MLN, livers, and spleens of immune mice treated with anti-INF-γ Mab, rabbit anti-IFN-γ Pab, or control rabbit IgG on day 5 following the intragastric inoculation of listeriae. The *Listeria* CFU in the organs from the immune host treated with the anti-IFN-γ Mab did not differ from the immune control mice whereas, immunised mice treated with the anti-IFN-γ Pab preparation possessed greatly enhanced numbers of bacteria in the organs as compared to listerial numbers in the corresponding organs of either the immune control mice or anti-IFN-γ Mab-treated immune mice. This indicates that the anti-IFN-γ Pab is more effective than the anti- IFN-γ Mab in inhibiting the expression of anti-*Listeria* immunity in memory immune hosts.

DISCUSSION

Listeria monocytogenes is capable of infecting man and animals following its ingestion in great numbers (*Burn*, 1936; *Osebold* and *Inouye*, 1954). Immunocompromised individuals, women in the first trimester of pregnancy, and neonates are at the greatest risk for infection. Following ingestion, this facultative intracellular bacterium moves rapidly through the intestinal tract and normally does not become a permanent component of the microflora (*Zachar* and *Savage*, 1979). To establish enteric infections in mice, great numbers of listeriae have to be deposited intragastrically, even in mouse strains (e.g., BALB/c) which are highly susceptible to infections initiated by para-enteral routes of inoculation. Peyer's patches are known to be a translocation route of for listeriae present in the intestinal lumen (*MacDonald* and *Carter*, 1980). However, results presented in this paper show that SCID mice become infected following intragastric inoculation of listeriae (Figure 2). This suggests the possibility of an alternate route of translocation route for listeriae, since immunoincompetent SCID mice lack PP. With regards to such a possibility, *Racz* et al. (1972) reported the presence of listeriae in mucosal epithelial cells following the intragastric inoculation of bacteria into guinea pigs. Moreover, the results of unpublished experiments carried out in this laboratory have shown that listeriae are capable of entering, proliferating and destroying the mouse Mode K small intestinal epithelial cell line (*Vidal* et al., 1993). Collectively, these observations and the *in vivo* observations of *Racz* et al. (1972) suggest the possibility that invasion of intestinal epithelial cells may constitute the first step in a PP-independent translocation route. Listeriae present in epithelial cells are capable of multiplying, transiting through the cytoplasm by polymerising actin, and penetrating into neighbouring cells (*Havell*, 1986b; *Dabiri* et al., 1990). Ultimately, the parasitised host cells are destroyed and internalised listeriae are released. Such a sequence of events in enterocytes could result in lis-

teriae entering the intestinal lamina propria and spreading to draining lymph nodes, from where listeriae are free to access the peripheral circulation and spread to other tissues and organs.

The results of experiments presented in this paper clearly establish roles for TNF and IFN-γ in anti-*Listeria* resistance mechanisms that are brought into play during primary and secondary infections caused by listeriae translocating from the gut lumen. TNF and IFN-γ are detected in organs within hours of listerial implantation (*Ehlers* et al., 1992; *Poston* and *Kurlander*, 1992). Previously, we reported that TNF is important in anti-*Listeria* resistance during the first 3 days of infection following the intravenous inoculation of *L. monocytogenes* into normal mice, immunodeficient mice, and *Listeria*-immune mice (*Havell*, 1989). Similarly, the results presented in this paper also establish that TNF is important in anti-*Listeria* resistance mechanisms in these same hosts during the first 3 days of an infection caused by bacteria translocating from the intestine. *Buchmeier* and *Schreiber* (1985) established the importance of IFN-γ in resistance to listeriosis by showing that anti-IFN-γ Mab treatment of mice infected by intraperitoneal inoculation caused an exacerbation of infection and the death of the host. The results presented in this paper also show that anti-IFN-γ Mab treatment exacerbated listeriosis in normal mice and immunoincompetent SCID mice. Moreover, the results of these experiments establish not only the importance of IFN-γ in anti-*Listeria* resistance during an enteric infection in either immunocompetent or immunoincompetent hosts, but also reveal that the IFN-γ-mediated effect occurs in the infected organs following the time when the TNF-mediated effect is expressed. In addition, TNF- and IFN-γ-mediated effects in antibacterial resistance occurred, respectively, at corresponding times during both a primary infection in naive mice and a secondary infection in *Listeria*-immune mice.

Evidence presented in a earlier publication from this laboratory suggested the importance of TNF in focusing host cells having antibacterial function at infectious sites (*Havell*, 1989). A histological examination of infected livers of mice given anti-TNF IgG revealed great numbers of listeriae in hepatocytes and a paucity of both neutrophils and macrophages at infectious foci. Indeed, TNF has activities that would be important in directing the migration of neutrophils, monocytes and lymphocytes to sites of inflammation in infected organs. For example, TNF causes the upregulation of ICAM-1 on endothelial cells (*Gamble* et al., 1985) and such an effect would result in adherence of neutrophils and other leukocytes to the vascular endothelium of infected organs and the subsequent extravasation of these cells into infectious foci. The work of Conlan and North (*Conlan* et al., 1993; *Conlan* and *North*, 1991) showed that neutrophils play an important role in antilisterial resistance by destroying *Listeria*-infected hepatocytes. TNF is known to cause the activation and degranulation of neutrophils (*Ferrante* et al., 1993) and such an effect in the vicinity of *Listeria*-infected non-professional cells could ultimately lead to the destruction of the infected host cells. This effect could serve the host by terminating the intracellular infection in cells incapable of coping with intracellular bacteria, thus allowing host cells having antibacterial function access to the previously internalised bacteria. Monocytes newly recruited from the peripheral circulation begin to supplant neutrophils at infectious foci following the first day of listeriosis (*Mackaness*, 1962). TNF has been shown to be important in granuloma formation, granuloma maintenance, and in triggering antimicrobial actions of macrophages (*Kindler* et al., 1989;

Oswald et al., 1992). Indeed, these effects of TNF early in listeriosis would be important in the expression of antibacterial resistance mechanisms in infected organs and tissues in either immunocompetent or immunoincompetent hosts.

TNF and IFN-γ regulate the synthesis of one another. TNF induces IL-12 which elicits the secretion of IFN-γ from natural killer cells and T lymphocytes (*D'Andrea* et al., 1993). In turn, IFN-γ is capable of greatly augmenting the host's potential for TNF production (*Havell*, 1993) . Natural killer cells produce IFN-γ during the first 24 hr of listeriosis in mice (*Dunn* and *North*, 1991). However, based on the results of experiments presented in this paper using anti-IFN-γ Mab to neutralise IFN-γ in *Listeria*-infected immunocompetent or immunoincompetent hosts, the anti-*Listeria* effect mediated by this cytokine is not evident until after the third day on infection. This observation raises the question as to whether IFN-γ is involved in the implementation and/or expression of the anti-*Listeria* resistance mechanism(s). Since the IFN-γ mediated effect occurs when macrophages populate infectious foci, it seems reasonable to assume that these phagocytes are involved in the IFN-γ-mediated antilisterial effect. On the one hand, it is possible that IFN-γ functions in events that result in the recruitment of monocytes/macrophages at these cells at sites of inflammation. With regards to such a possibility, IFN-γ alone, or in combination with other cytokines, induces the expression of certain beta chemokines (C-C) which can function to focus monocytes at sites of infection (*Proost* et al., 1996; *Cassatella* et al., 1997). On the other hand, IFN-γ is believed to prime macrophages for enhanced listericidal activity (*Buchmeier* and *Schreiber*, 1985; *Denis* and *Gregg*, 1990). Indeed, such a function may result in maintaining the chronic infection that characterises listeriosis in immunoincompetent mice (*Emmerling* et al., 1975).

During a sublethal immunising infection in mice, *Listeria monocytogenes* is rapidly eradicated following the appearance of specifically sensitised T cells that are capable of adoptively transferring anti-*Listeria* immunity to naive mice. The numbers of these sensitised T cells increase and decrease in concordance with the host's capacity to produce IFN-γ during listeriosis (*Havell* et al., 1982). In addition to having effects in innate antibacterial resistance, IFN-γ has actions that could be important in the generation and expression of specifically acquired T cell-mediated antibacterial immunity. With regards to effects in the generation of T cell-mediated immunity, IFN-γ causes the upregulation of MHC class II antigen expression on antigen presenting cells (*Stein* et al., 1984; *Inaba* et al., 1986). This cytokine is also capable of regulating the induction of Th1 helper T cells which regulate T cell immunity (*Belosevic* et al., 1989; *Swain* et al., 1991). As to possible roles for IFN-γ in the expression of T cell-mediated immunity, this cytokine is capable of augmenting the activity of specifically sensitised $CD8^+$ cytolytic T cells either directly, by enhancing the activity of these cells (*Blanchard* et al., 1988) or indirectly, by increasing the expression of MHC class I expression on infected target cells (*Halloran* et al., 1992). However, it is also important to mention both that *Harty* and *Bevan* (1995) have reported that $CD8^+$ T cells capable of adoptively transferring anti-*Listeria* immunity are generated during *Listeria* infection in IFN-γ gene knockout mice, and *Harty* et al. (1992) also found that anti-*Listeria* $CD8^+$ T cells can protect the host in an IFN-γ-independent manner. These findings would seem to suggest that IFN-γ may not be important in the mediation of anti-*Listeria* resistance by $CD8^+$ T cells, however,

this does not exclude the possibility that IFN-γ may be important in the mediation of anti-*Listeria* resistance by other phenotypically distinct T cells which have also been reported to be protective against this intracellular pathogen (*Kaufmann* et al., 1987, 1988; *Rakhmilevich*, 1994). Following the generation of a primary anti-*Listeria* immune response the numbers of T cells capable of adoptively transferring immunity rapidly decline, however, a state of long lived-state of memory immunity ensues. The T cells that are responsible for immunological memory are both physiologically and phenotypically distinct from those that mediate resistance during a primary *Listeria* infection (*Orme*, 1989; *North* and *Deissler*, 1975).

In order to establish the importance of IFN-γ in anti-*Listeria* T cell-mediated memory immunity, *Listeria* immune mice were treated with an anti-IFN-γ Mab preparation and challenged with an intragastric dose of *L. monocytogenes*. It was found that treatment with an anti-IFN-γ Mab had little, or no effect on the expression of *Listeria* memory immunity (Figures 3,4). Both in view of this finding and the knowledge that the monoclonal anti-IFN-γ Mab exacerbated a primary *Listeria* infection in either immunocompetent mice (Figure 1) or immunoincompetent SCID mice (Figure 2), it seems reasonable to assume that IFN-γ does not function in the expression of anti-*Listeria* memory immunity. However, the finding that listeriosis was exacerbated during a secondary infection in immune mice treated with a monospecific anti-IFN-γ Pab establishes the importance of this cytokine in the expression of memory immunity. Of interest was the finding that while the anti-IFN-γ Pab treatment caused an increase in listerial CFU in all organs examined, however, the extent of the increase was not as great as the increase in CFU in the organs of anti-IFN-γ Mab-treated mice during a primary infection (Figure 1). These findings indicate that both IFN-γ-dependent and IFN-γ-independent resistance mechanisms serve to resolve the secondary infection. This conclusion is similar to that reached by *Samsom* et al. (1995) who concluded that IFN-γ played only a minor role in the expression of anti-*Listeria* immunity against a secondary infection initiated by intravenous inoculation of bacteria. This conclusion was based on results showing that in anti-IFN-γ Mab-treated memory immune mice the liver bacterial CFU were only ~1 $\log_{10}$ higher than in control memory immune mice.

The apparent contradiction between the capacities of the anti-IFN-γ Mab and anti-IFN-γ Pab to interfere with the expression anti-*Listeria* memory immunity may be explained by different specificities of the two antibody preparations for distinct molecular domains on the IFN-γ molecule. Indeed, anti-IFN-γ Mab preparations have been shown to differ in abilities to neutralise certain IFN-γ activities, which indicates the presence of different molecular domains involved in signal transduction, which could account for the multiple activities of IFN-γ (*Schreiber* et al., 1985; *Caruso* et al., 1993).

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INFLAMMATORY BOWEL DISEASE IN SEVERE COMBINED IMMUNE DEFICIENT (SCID) MICE: HISTO- AND IMMUNO-PATHOGENESIS

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SUMMARY

Over the recent years several murine models for the study of inflammatory bowel disease (IBD) have been developed. These models play an important role in the search for new insights in mucosal immunology and for the development of curative regimes in humans.

Transplantation of immunodeficient SCID mice with low numbers of $CD4^+$ T-cell from immunocompetent donors leads to the development of a chronic, lethal IBD. The histopathological changes of this disease resembles closely those of human IBD. The present review will focus on the histopathology of IBD in SCID recipients of $CD4^+$ T-cells, the accompanying changes in the recipients innate immune system and characterisation of the disease-inducing cell type in this murine model of IBD.

INTRODUCTION

Over the last decades the incidence and prevalence of the idiopathic human inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) have shown a inclining tendency (*Langholz* et al., 1991; *Munkholm* et al. 1992). This fact combined with the increased cancer risk of people suffering from IBD (*Gillen* et al., 1994; *Langholz* et al., 1992) make the inflammatory bowel diseases major hazards for the public heath in the industrialised world. Despite a massive research effort in the field of mucosal immunology during the later years, the aetiology of IBD remains obscure (*Podolsky*, 1991a,b). The currently accepted hypothesis is that IBD results from an uncontrolled or inadequately down regulated immune response in the gut towards a hitherto unknown pathogenic agent or parts hereof, probably derived from the gut flora (*Reinecker* et al., 1994; *MacDermott*, 1996). Also, genetic predisposition might play a role in the onset and development of the diseases (*Satsangi* et al., 1996; *Polito II* et al., 1996). A wider understanding of the immune modulatory and disease promoting mechanisms in the gut is obviously necessary for the development of therapeutically regimes in the treatment of IBD. Recently, a reductionistic approach to the investigation

of IBD has been made possible through the development of several animal disease models, for review see (*Elson* et al., 1995; *Bregenholt* et al., 1997a). These include mice with deletion in genes encoding certain cytokines (*Shull* et al., 1992; *Sadlack* et al., 1993; *Kühn* et al., 1993), T cell receptor chains (*Mombaerts* et al., 1993), signal transducing molecules (*Rudolph* et al., 1995), mice spontaneously developing IBD (*Sundberg* et al., 1994) and SCID mice reconstituted with T-cells from immunocompetent donors (*Powrie*, 1995; *Claesson* et al., 1996).

As the result of an autosomal recessive mutation in the gene encoding the recombinase necessary for B and T cell antigen receptor rearrangement (*Bosma* et al., 1983), SCID mice do not contain mature lymphocytes in their central and peripheral lymphoid organs, including mucosa and gut-associated lymphoid tissue (GALT).

Due to the lack of an active adoptive immune system, SCID mice are well suited as host for transplantation of various tissues, organs and cell types (*Bosma* and *Carroll*, 1991). We have used SCID mice to study the re-population of the central and peripheral lymphoid organs (*Reimann* et al., 1991) and the development of IBD following injection of T-cell subsets from allogeneic, semi-syngeneic or syngeneic immunocompetent donors. Also, this model of murine IBD has been used to characterise the disease-inducing cells phenotypically and functionally *ex vivo*. This review will describe the induction and development of IBD in SCID mice, and focus on the histo- and immunopathological features in the colon of diseased animals.

INDUCTION OF IBD IN SCID MICE

Initially, IBD in SCID mice was reported to be inducible by transfer of purified $CD45RB^{high}$ virgin type $CD4^+$ T-cells from congenic immunocompetent donor mice (*Powrie* et al., 1993; *Morrissey* et al., 1993), whereas transfer of both $CD45RB^{high}$ cells and $CD45RB^{low}$ cells would reconstitute the SCID mice but not lead to disease. Subsequently, we demonstrated that IBD can be induced in SCID mice by intraperitoneal injection of limited numbers ($<10^5$) of syngeneic highly purified $CD3^+$ $CD4^+$ spleen T-cells containing both the $CD45RB^{high}$ and $CD45RB^{low}$ subsets (*Claesson* et al., 1996). Thus, in both experimental models, non-fractionated spleen cells are unable to induce IBD (*Claesson* et al., 1996; *Powrie* et al., 1993; *Morrissey* et al., 1993). The tissue origin of the injected cells does not seem to be important as both $CD4^+$ T-cells isolated from thymus, spleen, lymph nodes and lamina propria also have disease-inducing potentials (*Reimann* et al., 1994). Non-traumatic transplantation of small pieces of gut-wall from syngeneic donors onto the back of SCID mice, likewise induces an IBD indistinguishable from the disease induced by purified $CD4^+$ T cells (*Rudolphi* et al., 1994). Furthermore, IBD can be induced in SCID mice by injection of purified $CD3^+$ $CD4^+$ T-cells expressing a transgenic T cell receptor (TCR) specific for the H-Y male epitope (*Reimann* et al., 1995), suggesting that the disease develops independently of specific antigen recognised by the disease inducing T-cells.

In agreement with this suggestion, attempts to clone the disease-inducing cell type by adoptive transfer of lamina propria T-cells from diseased mice into normal SCID hosts have proven unsuccessful, as IBD in secondary or tertiary

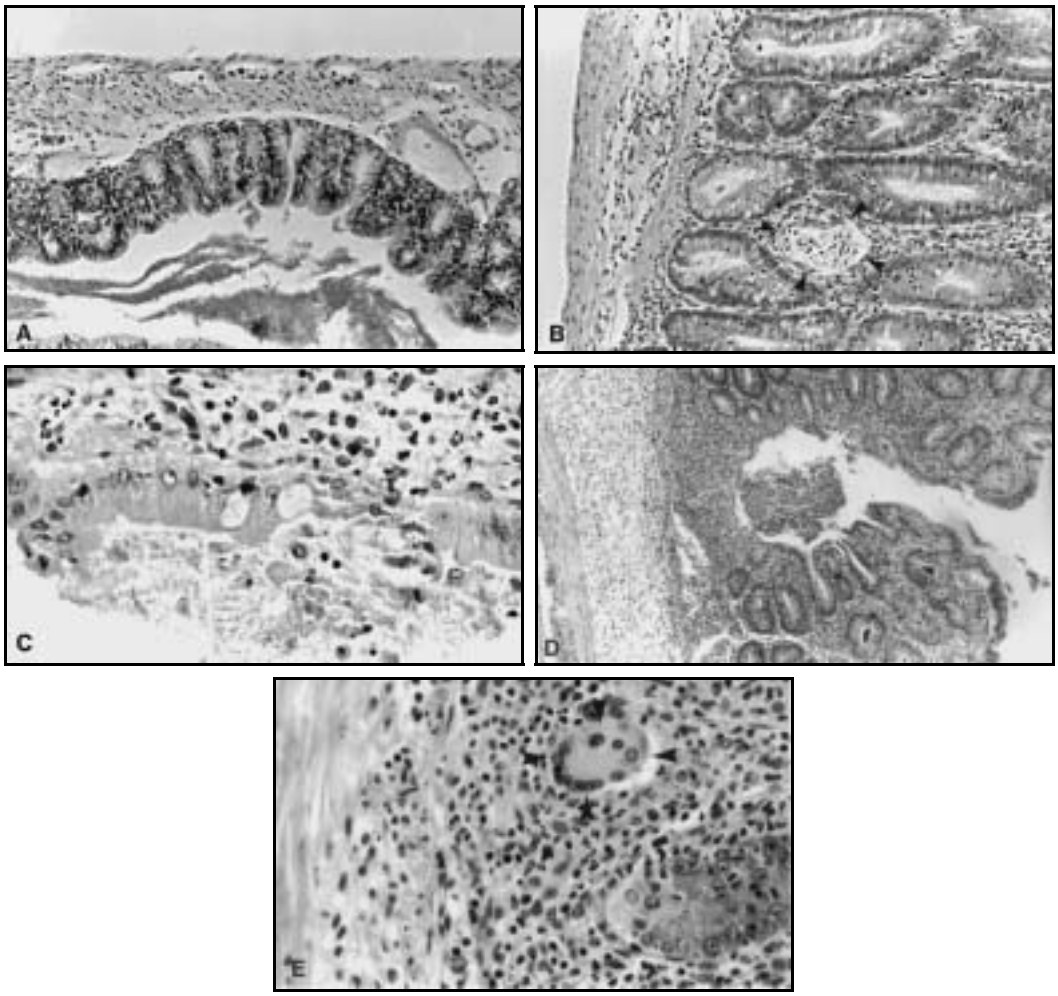

Figure 1: Histopathology of T-cell induced IBD. (A) Colon from a normal SCID mouse, magnification x100. (B) Mononuclear cell infiltration and epithelial proliferation in the colon of a SCID mouse with IBD. Arrowheads show crypt abscesses (x100). (C) Epithelial lesion in the colon of a SCID mouse with IBD, notice the many polymorphonuclears in the subepithelial lamina propria (x200). (D) Large ulcerative lesions in the colon of a SCID mouse with IBD (x50). (E) Giant cell formation (arrowheads) in the deep colonic lamina propria of a SCID mouse with IBD (x200).

transplanted SCID mice did not develop faster than in the primarily transplanted animals (*Reimann* et al., 1995).

However, the colonic microbial flora of the recipient plays a major role, because under specific pathogen free conditions, $CD4^+$ T-cell transplanted SCID mice fail to develop IBD (*Aranda* et al., 1997).

RECONSTITUTION OF LYMPHOID ORGANS

Upon transplantation, the $CD4^+$ T-cells selectively repopulate the spleen, mesenteric lymph node, peritoneal cavity, and the GALT of SCID recipients, whereas the thymus and regional peripheral lymph nodes are not repopulated (*Rudolphi* et al., 1991a,b; *Reimann* et al., 1991a, 1993a). A similar re-population pattern is seen when $CD4^+$ T-cell clones specific for the make H-Y antigen are transplanted into SCID mice (*Rudolphi* and *Reimann*, 1993).

Other organs such as such as liver, lungs, gonads, and adrenals do not get infiltrated or show signs of damage post $CD4^+$ T-cell transplantation (*Reimann* et al., 1991).

CLINICAL FEATURES OF IBD IN SCID MICE

Within three to six months following injection of purified $CD4^+$ T-cells SCID mice begin to show signs of a chronic inflammatory bowel disease, although some mice stay disease free for up to eight months. The mice exhibit a hunched back appearance, ruffled fur, and distended abdomen. The clinical disease is characterised by a prominent weight loss and softened stools. In the later stages of disease development further weight loss, diarrhoea, and in severe cases prominent rectal prolaps and bloody diarrhoea are seen. The disease is lethal within two months after onset.

HISTOPATHOLOGY OF IBD IN SCID MICE

On gross examination, the entire large intestine is enlarged and exhibits a whitely inflamed appearance. Occasionally, in severe cases the distal parts of the small intestine is macroscopically affected.

Histologically, a massive infiltration of the colonic lamina propria with mononuclear cells as well crypt elongation and crypt abscesses are observed (Figure 1B). The predominant mononuclear cell type in this infiltrate is donor derived $CD4^+$ T-cells, but increased numbers of host derived macrophages and dendritic cells are also present. At later stages in the disease development, all layers of the colon display pronounced hypertrophy and infiltration. Polymorphonuclears may dominate the lamina propria and the epithelia show spot wise signs of destruction (Figure 1C) (*Claesson* et al., 1996). The proliferative nature of the infiltrating cells is made evident by a large fraction of cells staining positive for proliferating cell nuclear antigen (PCNA) (*Claesson* et al., 1996). The massive proliferation of the colonic epithelia parallels a reduction in the number of goblet cells (see below). At the latest stages of the disease, and large transmural ulcerations are encountered (Figure 1D) accompanied by mononuclear and polymorphonuclear cell infiltration and Mac-3 positive giant cell formation (Figure 1E) (*Reimann* et al., 1995; *Rudolphi* et al., 1994; *Claesson* et al., 1996). Although lamina propria of the small intestine is densely infiltrated with donor derived $CD4^+$ T-cells, ulcerations are never found. In general, the disease is segmental, as the most severely affected animals display areas of the colon only being slightly affected by infiltration and mild hyperplasia (*Claesson* et al., 1996). The histopathological charac-

teristics described here are similar to those observed in SCID mice transplanted with CD45RB^{high} virgin T-cells (*Leach* et al., 1996). Based on the histopathology, this murine model of IBD resembles in some of the lesions human UC, in other lesions some features of CD such as crypt abscesses and transmural inflammation.

The reduction in numbers of goblet cells described above, is followed by a change in the nature of the secreted mucins. Thus, sulphomucins containing goblet cells dominate in the colon of non-transplanted SCID mice. This pattern changes towards expression of neutral mucins and subsequently to a decline in mucin containing goblet cells in severely diseased SCID mice (*Delbro*, personal communication). This observation is in agreement with a recent rapport showing, that patients suffering from UC express an altered pattern of mucin throughout the entire colon (*Smithson* et al., 1997). The cause of the change in the mucin pattern in the course of IBD in CD4$^+$ T-cell transplanted SCID mice is currently unknown, but it might reflect changes in microbial enzymatic activity such as increased sialyase activity of the luminal bacteria flora associated with disease development.

MACROPHAGES AND ANTIGEN PRESENTATION IN THE GUT OF DISEASED MICE

In human IBD a massive macrophage infiltration of the lamina propria is observed (*Rugtveit* et al., 1994). The macrophages are thought to play a central role in initiating and maintaining the inflammatory process (*Mahida* and *Jewell*, 1990).

As mentioned above, one of the first histopathological changes observed in CD4$^+$ T-cell transplanted SCID mice, is a massive mononuclear cell infiltration of the lamina propria. Immunohistochemical characterisation of this infiltrate has shown that initially, Mac-1$^+$ polymorphonuclear cell precursors, dendritic cells and activated, MHC class-II expressing, Mac-2$^+$ macrophages are infiltrating the submucosa and the basal lamina propria. In more severely affected mice, infiltration of Mac-1$^+$ polymorphonuclears is scattered through all layers of the mucosa, and clusters of activated macrophages and dendritic cells are found in the lamina propria (*Bland* et al., 1997).

In the normal gut mucosa, MHC class-II antigen-presenting molecules are expressed mainly on lamina propria macrophages and dendritic cells. During the development of IBD in SCID mice, MHC class-II expression is induced on epithelial cells whereas the MHC class-II expression on lamina propria macrophages is down regulated (*Bland* et al., 1997). The shift in antigen presentation away from the lamina propria towards the epithelium, might suggest a shift in the antigen handling of the diseased gut. This could be a determining factor in the maintenance of the intestinal inflammation as it might allow a direct presentation of luminal antigens to lamina propria CD4$^+$ T-cells.

PHENOTYPE OF DISEASE INDUCING T CELLS

IBD in SCID mice can be induced only by purified CD4$^+$ T-cells. CD8$^+$ T-cells are neither able to reconstitute the lymphoid organs of the mice nor to in-

Table 1: Characteristic of IBD-inducing T-cells in SCID mice

Surface marker / function	Suggests
TCR$\alpha\beta$ (diverse vβ repertoire), $CD4^+$, $CD8^-$	Polyclonal T-helper cells
$CD25^+$, $CD44^+$, $CD69^+$	Activated cells
$CD3^+$, $CD45RB^{low}$, L-$selectin^{low}$	Memory cells
$\alpha 4\beta 7$-$integrin^+$	Mucosa seeking
$CD2^+$, $CD28^+$	Receptive to co-stimulation
$CD95^+$ (Fas)	AICD-sensitive
Fas-L^+	AICD inducing (potentially suicidal)
IFN-γ^+, TNF-α^+, IL-2^+, IL-10^-	Th1 cells

duce IBD in SCID mice (*Rudolphi* et al., 1991b).

The general characteristics of the IBD-inducing T-cells is shown in Table 1. The lamina propria infiltrating $CD4^+$ T-cells express L-PAM-1 and L-PAM-2 (*Reimann* and *Rudolphi*, 1995; *Rudolphi* et al., 1994), as a reflection of a mucosa seeking cell type. Obviously, this is an important feature of disease inducing T-cells, since blockade of mucosal homing can reduce intestinal inflammation in $CD45RB^{high}$ $CD4^+$ T-cell reconstituted SCID mice (*Picarella* et al., 1997). The T-cells also display high levels of CD3 and low levels of CD45RB and L-selectin, a phenotype typical for memory peripheral lymphocytes (*Reimann* and *Rudolphi*, 1995; *Reimann* et al., 1993; *Rudolphi* et al., 1994, 1996). Moreover, these cells are activated as they express interleukin (IL)-2-receptor -chain (CD25) and high levels of the activation markers CD44 and CD69 (*Rudolphi* et al., 1994). Also the cells express CD2 and CD28 which increase their responsiveness to co-stimulatory signals (*Rudolphi* et al., 1996; *Reimann* and *Rudolphi*, 1995), and CD95 and CD95-ligand making them prone to activation-induced cell death (AICD, see below) (*Bonhagen* et al., 1996).

FUNCTIONAL ANALYSIS OF DISEASE-INDUCING $CD4^+$ T CELLS

The mononuclear cells infiltrating the lamina propria of $CD4^+$ T-cell transplanted mice are highly proliferative, as shown by immunohistochemical staining for PCNA (*Claesson* et al., 1996). Likewise, a high proportion of purified lamina propria $CD4^+$ T-cells have a DNA content of >2n, indicative of mitotic activity (*Rudolphi* et al., 1996). Analysis of the T-cell receptor repertoire, shows that this in vivo proliferation leads to a polyclonal expansion of $CD4^+$ T-cells (*Rudolphi* et al., 1996), arguing against one or a few single pathogenic T-cell epitopes as the driving force in this murine model of IBD. This is in concordance with human IBD where the $CD4^+$ T-cell pool appears to be selectively but, polyclonally expanding (*Probert* et al., 1996; *Gulwani-Akolkar* et al., 1995, 1996).

When isolated, lamina propria infiltrating $CD4^+$ T-cells proliferate spontaneously *in vitro* (*Bonhagen* et al., 1996; *Rudolphi* et al., 1991). This proliferative response could be co-stimulated by exogenous IL-2 and IL-7 in combination (*Bonhagen* et al., 1996). The fact that

isolated lamina propria T-cells are responsive to IL-7 *in vitro* is interesting, since epithelial cell-derived IL-7 is though to be a central regulator of mucosal T-cells (*Watanabe* et al., 1996).

The surface molecule Fas (CD95) and its counter-receptor Fas-ligand are thought to be involved in the maintenance of the homeostasis in the immune system via the induction of AICD (*Lynch* et al., 1997). As mentioned above, our previous studies suggest that freshly isolated lamina propria infiltrating $CD4^+$ T-cells express both the Fas and Fas-ligand (FasL) molecules thus, making them sensitive to AICD. In fact, AICD can be provoked *in vitro* by ligation of the CD3 molecule on $CD4^+$ T-cells in the presence of exogenous IL-2 and Il-7, a phenomenon which might explain the large number of activated but apototic mononuclear cells seen in the lamina propria of diseased mice (*Bonhagen* et al., 1996). Since colonic epithelial cells express Fas (*Moller* et al., 1994), it could be speculated that infiltrating $FasL^+$ $CD4^+$ T-cells play a central role in the destruction of epithelial cells, by induction of Fas-mediated apoptosis, resulting in the epithelial lesions (see Figure 1C). In addition, cytotoxic reactivity of lamina propria $CD4^+$ T-cells have also been reported in IL-2 knock out mice suffering from IBD (*Simpson* et al., 1995).

$CD4^+$ T-cells can be divided into two functional subsets based on their cytokine production. The pro-inflammatory Th1 subset produces interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and interleukin (IL)-2, IL-12, and IL-17 whereas the anti-inflammatory subset produces IL-4, IL-5, IL-10, and IL-13 (*Mosmann* and *Sad*, 1996). Under normal circumstances this balance is strictly regulated, however, a distorted Th1/Th2 balance is observed in many human auto-inflammatory diseases (*Romagnani*, 1996; *De Carli* et al., 1994). In the normal colonic mucosa, Th2 $CD4^+$ T-cells are the dominating cell type (*Kiyono* and *McGhee*, 1994), facilitating a B-cell-mediated, rather than a T-cell-mediated immune response. Especially, IFN-γ is speculated to be a key mediator of mucosal inflammatory reactions (*Strober* et al., 1997).

By staining for intracellular cytokines in $CD4^+$ T-cells we have shown that the Th1/Th2 balance found in healthy control animals is distorted in transplanted SCID mice (*Bregenholt* and *Claesson*, 1998a,b). The levels of all the inflammatory cytokines tested (IFN-γ, TNF-α and IL-2) are increased in diseased mice. The fraction of $CD4^+$ T-cells producing IFN-γ is increased by a factor of five to six in moderately and severely diseased mice compared to healthy controls. The fractions of TNF-α and IL-2 producing $CD4^+$ T-cells are increased by a factor of two to three in moderately and severely affected mice. The production of IFN-γ and IL-2 in colonic tissue of transplanted SCID mice has also been demonstrated by PCR technique (*Rudolphi* et al., 1993). In addition to an increase in the fraction of Th1-type cells, a decrease in Th2 cell-derived cytokines is also observed in diseased mice. IL-10 producing $CD4^+$ T-cells are almost absent from moderately and severely diseased mice and the fraction of IL-4 producing $CD4^+$ T-cells is generally decreased in diseased mice as compared to healthy controls. Taken together, this means that the Th1/Th2 ratio in diseased mice is increased by up to 20 fold compared to healthy controls.

Skewing of the cytokine pattern towards a Th1-like phenotype is a common pattern of murine models of IBD (*Hörnquist* et al., 1997; *Simpson* et al., 1997; *Davidson* et al., 1996; *Mizoguchi* et al., 1996). In the human forms of IBD, CD is generally thought to be a Th1-like disease, whereas UC is a Th2-like disease (*Fuss* et al., 1996; *Breese* et al., 1993; *Niessner* and *Volk*, 1995). Thus, although the histopathology of the

SCID IBD model resembles human both UC and CD, the cytokine pattern strictly resembles that of human CD.

MUCOSAL PLASMA CELLS IN SCID MICE WITH IBD

Involvement of auto-antibodies in the immunopathogenesis of IBD has been suggested by the finding of antibodies reacting towards components of the colonic epithelium in human UC patients (*Halstensen* et al., 1990, 1993; *Biancone* et al., 1995).

Immunohistochemical staining has shown that the development of IBD is followed by infiltration of immunoglobulin containing cells in the colonic lamina propria which are totally absent in normal SCID mice (*Claesson* et al., 1996). We have attempted to correlated the local level of plasma cell infiltration with the local histopathology in individual segments of the colon: the levels of IgM, IgA, and the anti-inflammatory isotypes IgG1 and IgG2b are significantly increased in areas showing severe histopathology as compared to areas showing no, mild, or moderate histopathology (*Bregenholt* et al., 1997b, 1998). It is noteworthy that the number IgG1 and IgG2b containing cells are higher than the numbers found in normal syngeneic C.B.-17 mice (*Claesson* et al., 1996; *Bregenholt* et al., 1997b, 1998). The specificity of the produced antibodies is currently under investigation.

Induction of immunoglobulin leakiness in T-cell transplanted SCID mice and the subsequent occurrence of serum IgM has been observed previously (*Riggs* et al., 1991, 1992; *Rudolphi* et al., 1992). Transfer of $CD4^+$ T-cells from congenic dm2 mice (IgM-allotype) into SCID mice (IgM-allotype) have shown that the resulting plasma cells are of the host IgM-allotype (*Rudolphi* et al., 1992). Thus, the plasma cells in the mucosa of $CD4^+$ T-cell transplanted SCID mice, most probably originate from leakiness in the SCID-mutation and not from donor B-cells contaminating the inoculated $CD4^+$ T-cells.

Although a possible role for B-cells in other animal models of IBD has been severely questioned (*Ma* et al., 1995; *Davidson* et al., 1996), these results in combination with several other reports (*Hörnquist* et al., 1997; *Mizoguchi* et al., 1996a,b) suggest a role for B-cells in the immunopathology of IBD.

THERAPEUTIC CONSIDERATIONS

In this and several other animal models of IBD, $CD4^+$ T-cells are shown to be the disease-inducing cells type (*Powrie* et al., 1993; *Morrissey* et al., 1993; *Simpson* et al., 1995; *Davidson* et al., 1996; *Bregenholt* et al., 1997a). $CD4^+$ T-cells also play a key role in human IBD (*Probert* et al., 1996; *Gulwani-Akolkar* et al., 1995, 1996), making them or their products obvious targets for immune therapies.

Immune therapy with infusion of monoclonal antibodies against the CD4 molecule, have shown varying results (*Emmrich* et al., 1991; *Canva-Delcambre* et al., 1996; *Stronkhorst* et al., 1997) and might today be considered as a questionable approach (*Nielsen* et al., 1997). Instead, the neutralisation of inflammatory mediators and the restoration of the Th1/Th2 balance should be brought into focus. Neutralisation of pro-inflammatory mediators such as IL-1, IL-8 and TNF-α

have shown promising results in animal models of intestinal inflammation (*Powrie* et al., 1994; *Casini-Raggi* et al., 1995). In human IBD, the blockade of TNF-α have recently shown good clinical results (*van Dullemen* et al., 1995). Treatment of IBD by reestablishment of the Th1/Th2 balance has proven useful in a number of animal models. This has been accomplished either by neutralisation of the pro-inflammatory cytokine mediators or by reconstitution of the anti-inflammatory cytokine pool (*Ehrhardt* et al., 1997; *Halstensen* et al., 1990; *Neurath* et al., 1995; *Powrie* et al., 1994). This approach has not yet been tried in human IBD, but might prove useful especially in CD, which is thought to be a Th1-mediated disease (*Romagnani*, 1996). Until the potential disease-inducing pathogen, agent or antigen have been isolated and the involvement of genetic factors have been established, cytokine directed therapy might be one of the only alternative candidates to conventional drug therapy (*Murch* and *Walker-Smith*, 1994).

CONCLUDING REMARKS

The development of several new animal models has made a reductionistic approach towards studies of the disease-inducing and -maintaining mechanisms of IBD possible. This might be valuable in defining cell types, cytokines and pathogens involved in human CD and UC.

In the present murine model of IBD, activated, mucosa seeking, memory $CD4^+$ T-cells of the Th1 type have been defined as the disease-inducing cell type. In this model the development of therapeutic regimes to restore the immunological balance of the gut mucosa should be possible. Germ-free techniques combined with the adoptive transfer of $CD4^+$ T-cells might prove very useful in the characterisation of exogenous agents important for the development of IBD.

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MECHANISMS OF INTESTINAL IMMUNITY TO NEMATODE PARASITES AND THE CONSEQUENCES TO INVASION BY OPPORTUNISTIC BACTERIA

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SUMMARY

Gastrointestinal nematode parasites can evoke dramatic and stereotypical changes in the intestinal milieu of the infected mammalian host. These changes may be inconsequential or result in protective immunity, pathology or an alteration in the immune response to opportunistic organisms that inhabit the intestine. The immune response is driven by the induction of a pattern of cytokines derived from $CD4^+$ helper T cells categorised as Th2. These cells, along with other T cell subsets and cells of the innate immune system, are initially stimulated by worm infections to produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. These cytokines evoke a type 2 immune response that is defined by an increase in mucosal mast cells, eosinophilia, and reaginic antibodies. There are also structural and physiological changes in the intestine that include increases in the quantity and composition of mucus secretions, a net accumulation of fluids into the lumen, smooth muscle contractility and alterations in lumenal content transit time, as well as changes in epithelial cell function and proliferation. Certain combinations of these effectors can limit parasite development or cure the host of worm infestation. This response can be quite polarised because of the additional feature of counter-regulation of some type 2 cytokines on the type 1 response. Production of IL-4 can limit the expansion of $CD4^+$ T helper cells of the Th1 type, and IL-10 can down-regulate macrophage activities that are largely responsible for expression of an IFN-γ-induced type 1 response directed at intracellular microorganisms and for delayed-type hypersensitivity responses. This interplay of cytokines can work in both directions so that intracellular parasites, bacteria and viruses that elicit a strong Th1 response can down regulate a type 2 response primarily through the growth limiting activity of IFN-γ on Th2 cells. Infections that strongly shift an immune response in one direction or another can predictably result in restricted immune flexibility that can be exploited by opportunistic infections. The current report describes changes in intestinal immunity induced by gastrointestinal nematode parasites and a spe-

cific situation where natural infection of pigs with *Trichuris suis* enhances susceptibility of colonic epithelial cells and gut-associated lymphoid tissue (GALT) in the distal colon to invasion by *Campylobacter jejuni*.

STEREOTYPICAL RESPONSE PATTERN TO GASTROINTESTINAL NEMATODE INFECTION

Mammals respond to gastrointestinal (GI) nematodes with a classical immediate-type hypersensitivity response where IgE antibody, mucosal mast cells, and tissue and blood eosinophils are markedly elevated (*Urban* et al., 1989), and the quantity and composition of goblet cell mucins are altered (*Ishiwata* et al., 1998). A general requirement for $CD4^+$ T cells in resistance to GI nematodes has been observed in several rodent models of infection (*Finkelman* et al., 1997). Neutralisation of $CD4^+$ T cells can either block worm expulsion, enhance worm fecundity by reducing immunity and inflammation or generally convert host resistance to susceptibility. Nematodes induce the development of $CD4^+$ Th2 cells that synthesise a cytokine pattern that includes IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (*Mosmann* and *Coffman*, 1989). Cytokines are pluripotent and, in the context of a parasitic infection, drive a generalised type 2 response. Production of IL-4 increases synthesis of IgE (*Finkelman* et al., 1990) and serves as a co-factor with IL-3 and IL-9 for development of intestinal mucosal mast cells (*Madden* et al., 1991); nematode-induced eosinophilia is dependent on the expression of IL-5 (*Sher* et al., 1990). Infective larvae stimulate IL-5 and IL-9 mRNA synthesis in duodenal Peyer's patch within 3 to 6 hr after per os inoculation (*Svetic* et al., 1993), but type 2 cytokine gene expression is not completely $CD4^+$ T cell-dependent until 6 days after inoculation (*Svetic* et al., 1993). Blocking of CD8 T cell function is generally without consequence in rodent models of GI nematode infections (*Katona* et al., 1991).

IL-4/IL-13 PROMOTE PROTECTIVE IMMUNITY TO GASTROINTESTINAL NEMATODES

There are shared aspects of a protective response to GI nematodes. Neutralisation of IL-4 that completely inhibits IgE production in *Nippostrongylus brasiliensis*-infected mice does not affect worm expulsion. In addition, expulsion is normal in IL-4 deficient gene knock-out (KO) mice (*Lawrence* et al., 1996), and treatment with anti-IL-4 receptor (R) mAb delays but does not interfere with expulsion (*Urban* et al., 1998a). Although IL-4 is not required for resistance to *N. brasiliensis*, it conditionally evokes a protective response. Adult *N. brasiliensis* do not self- cure in immunocompromised SCID or anti-CD4-treated mice, but the infection is cured by treatment with a long-lived formulation of IL-4-anti-IL-4 complexes (IL-4C) (*Urban* et al., 1995) which lengthens the half-life of IL-4 in vivo from <30 minutes to >24 hr (*Finkelman* et al., 1993). Blocking the IL-4R with a specific mAb prevents IL-4C-induced cure. However, IL-4 does not directly affect worms in situ because injection of IL-4C into Stat6-KO or IL-4R -chain-KO

mice, in which IL-4-induced cell signalling is decreased or absent, respectively, (*Shimoda* et al., 1996; *Takeda* et al., 1996) does not expel adult worms (*Urban* et al., 1998a). The observations that: 1) an intact IL-4R -chain and a functional Stat6 molecule is needed to activate expulsion of *N. brasiliensis*; and 2) IL-4 is not required to induce worm expulsion in immunocompetent mice, suggests that another cytokine that binds to the IL-4R -chainand activates Stat6, most likely IL-13, can also induce worm expulsion. This is supported by the fact that anti-IL-4R mAb can effectively block expulsion of *N. brasiliensis* in IL-4 KO mice, but not in normal IL-4 intact mice, and a specific antagonist for IL-13 blocks expulsion in normal and IL-4 KO mice (*Urban* et al., 1998a). In addition, IL-13 KO mice fail to efficiently clear *N. brasiliensis* adults even though a strong Th2 cytokine response is evident (*McKenzie* et al., 1998). Thus, expulsion of *N. brasiliensis* requires signalling via IL-4R -chain and Stat6, and IL-13 may be more important than IL-4 as an inducer of Stat6 signaling that precedes expulsion of *N. brasiliensis*.

Changes in the in vivo cytokine environment of the intestine of *Trichuris muris*-infected mice can reverse the outcome of an infection (*Else* et al., 1994). Chronic *T. muris* infection in susceptible mice is characterised by a predominant Th1 cytokine response pattern, but neutralisation of IFN-γ increases Th2 cytokines and the expulsion of worms. Conversely, the Th2-dominant cytokine response pattern of mice resistant to *T. muris* is reversed by treatment with anti-IL-4R mAb and results in continued worm development and a chronic infection. In addition, injection of IL-4C cures susceptible mice, while treatment of resistant mice with IL-12, during the second week of infection, induces an IFN-γ-dependent increase in host susceptibility (*Bancroft* et. al., 1997). There is redundancy in the protective response to *T. muris* because both IL-4 KO and IL-13 KO mice are susceptible to infection. (*Bancroft* et al., 1998).

The injection of IL-4C also effectively cures a chronic primary infection of mice with *Heligomosomoides polygyrus*, a nematode related to *N. brasiliensis*. Infection of immune system- deficient SCID mice is cleared less efficiently by injection of IL-4C (*Urban* et al., 1996). Protective immunity to a challenge *H. polygyrus* infection in mice previously drug-cured of a chronic primary infection is completely blocked by anti-IL-4R mAb in a way similar to the conversion of *T. muris*-resistant mice to susceptibility. IL-4 KO mice are susceptible to a secondary challenge infection with *H. polygyrus*, but the role of IL-13 in protective immunity has not been examined (*Urban* and *Finkelman*, unpublished results). Recent studies have shown, however, that Stat6 KO mice are susceptible not only to infections with *N. brasiliensis*, but also to *H. polygyrus*, *T. muris*, and *Trichinella spiralis* (*Urban* and *Finkelman*, unpublished results). Thus, there appears to be a common IL-4/IL-13-dependent protective mechanism to GI nematodes that proceeds through IL-4R -chain signalling via Stat6.

IL-4-DEPENDENT MECHANISM OF RESISTANCE TO GASTROINTESTINAL NEMATODES

Injection of IL-4C into either immunocompetent or immunodeficient mice infected with *H. polygyrus* alters the feeding behaviour of worms *in situ*. Rhodamine dye injected intravenously into mice with a primary *H. polygyrus* infection is ingested by the worm and appears in the intestine of explanted

worms viewed microscopically (*Urban* et al., 1998b). However, a single intravenous injection of IL-4C into an infected mouse greatly reduces uptake of the dye by the worm. A similar response in detected in IL-4C-treated mice infected with *N. brasiliensis.* The inhibition of feeding is probably related to the observed drop in *H. polygyrus* egg production (EPG) after IL- 4C treatment, suggesting that elevations in IL-4 can affect subtle changes in the intestine that affect worm metabolism. The mechanism remains largely unknown, but IL-4 induces changes in murine intestinal physiology that are partially mediated by products of mucosal mast cells (MMC) (*Goldhill* et al., 1997). Increased small intestinal smooth muscle contractility is observed during a secondary *H. polygyrus* challenge infection (*Goldhill* et al., 1997), but not during a primary infection when immunity is less intense. Contractility is induced in normal mice by injection with IL-4C, and blocked in mice challenged with *H. polygyrus* after injection with anti-IL-4R mAb. IL-4C-induced changes in contractility are also blocked by an inhibitor of leukotriene D_4 (LTD_4), and are not observed in SCID mice, in 5- lipoxygenase(LO)-deficient KO-mice, or in mast cell deficient W/W^v mice (*Goldhill* et al., 1998). SCID mice have virtually no gastrointestinal MMC following infection with *H. polygyrus*, but develop MMC after prolonged treatment with IL-4C (*Urban* et al., 1995). The MMC are a source of LTD_4 which is absent in 5-LO-KO mice. In addition, secondary *H. polygyrus*-challenge infection disrupts normal intestinal peristaltic activity, so that lumenal contents are evenly rather than segmentally distributed in the gut (*Goldhill* et al., 1997). However, this phenomenon does not appear to be completely IL-4-dependent. Nevertheless, IL-4-dependent changes in smooth muscle contractility can create a localised spastic action that could dislodge adult worms from their niche.

There are distinct IL-4-dependent differences in small intestine epithelial cell function during a primary *H. polygyrus* infection compared to a secondary challenge infection (*Sullivan* et al., 1998). Physiological measurements of segments of small intestine mucosa derived from mice with a secondary *H. polygyrus* infection, but not a primary infection, have significant increases in basal short circuit current (SCC) and tissue resistance, which measure net ion flux and changes in tissue permeability, respectively. Secretagogues like prostaglandin E_2 (PGE_2) enhance SCC in mucosa derived from mice infected with a secondary but not primary *H. polygyrus* infection. Similar changes in the response to PGE_2 were observed in normal mice treated with IL-4C over a 6 day period. In addition, injection of anti-IL-4R mAb at the time of a secondary challenge infection blocked *H. polygyrus*-induced changes in SCC, resistance, and responses to PGE_2. Thus, IL-4 appears to mediate increases in ion flux, decreased tissue permeability, and responses to secretagogues during a secondary infection with *H. polygyrus*. These results were substantiated by the observation that IL-4-KO mice, which are relatively susceptible to a secondary infection with *H. polygyrus* (*Shea-Donohue*, *Urban* and *Finkelman*, unpublished results), have unaltered SCC, resistance, and responses to secretagogues when challenge-infected with *H. polygyrus* (*Sullivan* et al., 1998). In addition, primary and secondary infections of mice with *H. polygyrus* and a single injection of IL-4C into mice with a primary *H. polygyrus* infection decrease glucose absorption. Thus, there are significant physiological differences in the host small intestine responding to a primary and secondary in-

fection with *H. polygyrus* that are attributable to IL-4. The ability of IL-4 to augment both intestinal secretion and contractility suggests that IL-4 can alter the concentration of critical nutrients in the intestinal lumen upsetting the microenvironment and provide host effectors that interfere with the metabolism and/or attachment of the infective organism (*Finkelman* et al., 1997).

THE COUNTER-REGULATORY PROPERTIES OF TH1/TH2 CYTOKINES CAN MODULATE IMMUNITY DURING AN INFECTION

A polarised cytokine response would inherently down regulate the reciprocal Th1/Th2 cytokine pattern (*Pearce* et al., 1991; *Acton* et al., 1993; *Scott* and *Kaufman*, 1991). Therefore, the cytokine status of the host could influence the process of active immunisation or immunity to an infection. Induction of a strong Th1 response would likely interfere with resistance to GI nematodes that are sensitive to Th2-dependent protective mechanisms. This was demonstrated experimentally when mice treated with exogenous rIL-12 (*Finkelman* et al., 1994), rIFN-γ or rIFN-α (*Urban* et al., 1993) became susceptible to *N. brasiliensis*. The stereotypical type 2 cytokine gene expression pattern was completely reversed by treatment with IL-12 via an IFN-γ-dependent down-regulation. As a result, there is a general decrease in effectors related to type 2 cytokines and an inhibition of the protective response. Once IL-12 treatment is terminated during the course of the infection, however, expulsion of worms follows after a period of increasing type 2 cytokine gene expression. Endogenous activators of IFNs can also down-regulate type 2 responses in *N. brasiliensis*-infected mice and temporarily inhibit parasite expulsion. Injection of mice with killed-*Brucella abortus* cells (*Urban* et al., 1993) or inoculation with live *Eimeria ferrisi* oocysts, a natural protozoan parasite of mice that invades the intestinal mucosa, will temporarily block parasite expulsion (*Urban* et al., 1993, 1996). This effect is reversible by neutralisation of IFN with specific mAb.

Worm-induced polarisation towards type 2 responses and down-regulation of Th1 immunity may be significant to both human and animal populations that have a propensity to acquire chronic worm infections. A normal in vitro Th1-like response to tetanus toxoid (TT) of PBMCs from vaccinated humans is more Th2-like when the PBMCs come from individuals infected with *Schistosoma mansoni* (*Sabin* et al., 1996). The amount of TT specific IFN-γ produced by PBMC from *S. mansoni*-infected individuals decreases inversely with the intensity of the infection compared to the response of PBMC from uninfected, TT-vaccinated controls. These results suggest that the type 2 milieu created by a *S. mansoni* infection affects the direction of a response to a non-parasite related antigen. Therefore, the parasite status of an individual may be of considerable importance in determining the cytokine pattern one is likely to achieve when an individual is exposed to a subsequent infection or to toxic materials.

The skewing of a cytokine pattern in response to an infectious agent could also have severe consequences when complex disease interactions exist. *Trichuris suis* in pigs interact with resident bacterial flora in the colon to induce mucohaemorrhagic enteritis (*Mansfield* and *Urban*, 1996). When the infected pigs are maintained in standard confinement housing indoors, they have a

parasite-induced 10-100 fold increase in IL-10 gene expression in the MLN draining the site of infection, but no increase in IL-12 gene expression (*Mansfield* et al., 1998). This is similar to the *T. muris*- and *H. polygyrus*-induced increases in Th2 cytokine gene expression that follows inoculation of mice (*Svetic* et al., 1993; *Else* et al., 1994). It is also of interest that increases in IL-12 gene expression in the MLN of *T. suis*-infected pigs are only observed when secondary bacterial invasion in colonic epithelial cells and GALT are evident (*Mansfield* et al., 1998). Interleukin-12 is induced as a consequence of infection with intracellular parasites and by exposure to microbial-derived products (*Biron* and *Gazzinelli,* 1995). Stimulation of IFN-γ synthesis by IL-12 leads to activation of macrophage accessory cell function through elevated expression of class II MHC antigen and the production of microbicidal products such as H_2O_2, proteases, and nitric oxide. This process can also activate non-immune cells such as intestinal epithelial cells to express cell surface ß$_2$-integrins that allow neutrophil adhesion and class II MHC molecules to mediate interactions with $CD4^+$ T-cells (*Colgan* et al., 1994). In contrast, IL-10 down regulates the production of inflammatory cytokines by mononuclear phagocytes and neutrophils and reduces or prevents macrophage activation by IFN-γ (*Cassatella* et al., 1994).

SPECIFIC INTERACTIONS BETWEEN *T. SUIS* AND *C. JEJUNI*

Confinement reared pigs inoculated with *T. suis* eggs exhibit diarrhoea, and mucosal oedema, inflammatory cell infiltration, and bacterial accumulation at the site of worm attachment in the proximal colon. There is also localised thickening of the muscularis and mucosa at the site of worm attachment, destruction of the absorptive cells on the surface of the colon, crypt destruction with loss of goblet cells, and an increase in inflammatory cells in the lamina propria. Bacteria are detected by Warthin-Starry stain in close proximity to adult worms. Notable also is the appearance of severe bacterial lesions in the lymphoglandular complexes (LGC) in the distal colon far from the site of worm attachment. Bacterial isolates from the LGC include *C. jejuni, C. coli, C. lari,* as well as single isolates of *Escherichia coli, E. fergusonii, Enterobacter intermedium, E. cloacae, Pseudomonas fluorescens,* and *Lawsonia intracellularis.* The most prominent change in LGC from *T. suis*-infected pigs is an increase in size. The LGCs are enlarged partly due to an increase in cells in germinal centres of the LGC nodule which include lymphocytes, macrophages, and neutrophils surrounded by eosinophils. There is a follicle-associated membrane overlying the follicle that contains cells with M cell morphology. Some of the bacteria in the LGCs are within the entrapped mucosal crypts that appear as crypt abcessation with purulent debris. Bacteria are in the submucosa and muscularis below the follicle suggesting the LGC is a route of invasion and dissemination of pathogenic organisms. Bacteria with morphology consistent with *C. jejuni* are within M cells of follicle-associated epithelium. No pathogenic bacteria were isolated from the tissues of pigs that were not *T. suis*-infected, although many of these pigs had *C. jejuni* in the stool with no clinical signs of disease. The severity of *T. suis*-induced mucohaemorrhagic enteritis is inhibited by broad spectrum antibiotics or by anthelmintic clearance of the worm infec-

tion (*Mansfield* and *Urban*, unpublished results). These observations demonstrate that *T. suis* alters conditions proximal and distal to worm attachment in the colon that affect susceptibility to opportunistic bacteria.

Supporting this concept are recent observations made in gnotobiotic pigs inoculated with either *T. suis* or *C. jejuni* alone or in combination (*Mansfield* et al., manuscript in preparation). Pigs derived germ-free by caesarean section of sows were placed in germ-free incubators and treated as four groups:

1) untreated and uninfected,
2) inoculated with *T. suis* eggs at 250 eggs/kg body weight,
3) inoculated with 10^6 colony forming units of log phase *C. jejuni* (ATTC strain 33292 isolated from a human with enteritis), or
4) inoculated with both *T. suis* eggs and *C. jejuni*.

Pigs with combined infections had significantly more frequent and severe signs of diarrhoea. All pigs inoculated with *C. jejuni* had transient fever, depression and diarrhoea for 24 hr after inoculation, and shed *C. jejuni* in the stool. However, severe clinical signs and pathology were present only in the colon of pigs inoculated with both *T. suis* and *C. jejuni*. In addition, only pigs inoculated with both *T. suis* and *C. jejuni* had bacterial invasion in the colonic epithelium and the epithelial cells of the follicles associated with LGCs in the distal colon. Macrophages stained immunohistochemically in the LGC also stained for intracellular *C. jejuni* with a specific stain suggesting that the macrophages were invaded by *C. jejuni*.

POTENTIAL MECHANISMS OF *T. SUIS*-FACILITATED *C. JEJUNI* COLONISATION OF PIG INTESTINAL CELLS

Campylobacter jejuni are attracted to an infection site, penetrate the mucus layer, and associate with the base of the crypt or adhere to the mucosal surface to initiate colonisation and infection (*Wallis*, 1994). *Campylobacter spp*. are opportunistic pathogens that multiply in the gastrointestinal tract of their hosts when immune defences are compromised (*Bernard* et al., 1989, *Sorvillo* et al., 1991). Pathogenicity of the organism is dependent on virulence traits and host susceptibility is secondary to agents that disrupt the host immune response or change the microenvironment of the mucosal surface. Nematodes are clearly able to modulate the immune response and alter the microenvironment with consequences that could affect bacterial colonisation. Penetration of the intestinal epithelial barrier by *C. jejuni* is dependent on adhesins binding to host substrates. Flagella (*McSweegan* and *Walker*, 1986; *Szymanski* et al., 1995), as well as other bacterial structures that bear adhesins (*Ofek* and *Doyle*, 1994) may be involved in the adhesive process. Receptors for bacterial adhesins are found on animal cell membranes (*Ofek* and *Doyle*, 1994) as well as extracellular matrix molecules including fibronectin, laminin, and collagens (*Gravis* et al., 1997). Glycoconjugates associated with glycoproteins and/or glycolipids can serve as receptors for bacterial adhesins. Nematode secretions that are important to parasite feeding and invasion mechanisms could modify bacterial adhesin/receptor interactions. Both a metalloprotease and cysteine protease has been identified in adult *T. suis* (*Hill* et al., 1993; *Hill* and *Sakanari*, 1997), and the metalloprotease is implicated in tissue breakdown during worm invasion of the mucosa. A serine protease inhibitor from secretions of *T. suis* has been shown to inhibit trypsin, chymotrypsin and elastase that

could alter the extracellular matrix during worm development (*Rhoads* et al., personal communication). Secreted products from *T. suis* (*Abner* et al., 1998), and a related parasite *Trichinella spiralis* (*Butcher* et al., 1998), have been shown to affect epithelial cell proliferation, and possibly enhance endocytosis or membrane permeability. Peroxidation of epithelial cell membrane lipids has been associated with host protective mechanisms against parasites in the gut, while parasite-derived catalase, glutathione reductase, and superoxide dismutase salvage oxygen radicals produced at the site of infection. Adhesive substances have recently been identified from nematodes that could facilitate epithelial cell attachment and invasion (*Maruyama* and *Nawa*, 1997). The nematode adhesins are heavily glycosylated proteins that have an affinity for mast cell proteoglycans like heparin and sulphated mucins secreted by goblet cells (*Ishiwata* et al., 1998). Changes in the sugar side chains of mucins present during the expulsion of *N. brasiliensis* suggest both a qualitative as well as quantitative alteration in mucus production coincident with goblet cell hyperplasia (*Oinuma* et al., 1995). Parasite-induced changes in intestinal epithelial cell permeability and especially the accumulation of glucose and other nutrients in the lumen could affect the adhesin quality of commensal bacteria. The impact of parasite product-induced changes of the intestinal microenvironment on bacterial adhesins and host receptor sites could be an intriguing target of facilitated bacterial adherence and invasion, especially at the site of worm attachment in the intestine.

Invasion of LGCs by *C. jejuni* distal to worm attachment sites in the proximal colon is more easily explained by immune modulation of GALT in the intestinal mucosa. Increases in pig IL-10 gene expression resulting from *T. suis*-induced stimulation of mucosal immunity maybe indicative of a more typical Th2 pattern (*Svetic* et al., 1993) that includes IL-4-induced changes in intestinal physiology (*Goldhill* et al., 1997) and down regulation of IFN-γ-dependent responses to intracellular organisms. It is therefore of considerable interest that macrophages in the LGCs of gnotobiotic pigs inoculated with both *T. suis* and *C. jejuni* were invaded by bacteria (*Mansfield* et al., 1998).

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HELICOBACTER PYLORI AND LONG TERM SURVIVAL IN THE GASTRIC MUCOSA: IS IMMUNOMODULATION THE KEY?

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SUMMARY

The discovery of *Helicobacter pylori,* a bacterium that inhabits the mucus and epithelium of the stomach, revolutionised the discipline of gastroenterology and resulted in a paradigm shift in the management of gastroduodenal disease. *H. pylori* causes 95 % of duodenal ulcers, 60-70% of gastric ulcers, 60-70% of gastric adenocarcinomas and most if not all gastric B cell lymphomas. These diseases are relatively new human afflictions and it is suggested that in earlier times *H. pylori* actually evolved as a member of the normal microbial flora just as many other commensals evolved to inhabit the ecological niches provided by the mucus of the lower intestinal tract. Symptomatic disease is a consequence of recent environmental changes in the human host including possibly an increased acid output in the stomach. As with other normal flora, *H. pylori* inhabits its chosen niche, the gastric mucosa, for life and thus must have acquired sophisticated mechanisms to survive. These include an ability to withstand gastric acidity via the enzyme urease. The immune responses against the bacterium are evaded by a number of possible strategies including antigen mimicry via the manufacture of the blood group antigens Lewis x and y on the bacterial surface, antigen variation and a shedding of decoy bacterial antigens into the lamina propria. Immune responses are likely to be modulated in a number of ways including the production of a lipopolysaccharide of reduced biological activity. The Th lymphocyte profiles may also be modulated with infection directing the lymphocyte profile towards a predominantly inflammatory Th1 phenotype with the inflammation not only being unable to remove the mucosal coloniser but actually benefiting the bacterium. The move away from a Th2 response which would be more likely to result in removal of the *H. pylori* is a deliberate strategy. Understanding of these mechanisms of immunomodulation may have relevance beyond gastric disease. The mucus surfaces of most animals and probably some human populations are now known to be colonised with other *Helicobacter* species. The impact of these bacteria on lower bowel disease has yet to be determined.

INTRODUCTION

In Perth in 1982, an event occurred which was to revolutionise the field of gastroenterology. Plates were removed from an incubator after a prolonged in-

cubation over the Easter long weekend (*Marshall* et al., 1984). These plates had been inoculated with material from gastric biopsies in an attempt to grow bacteria which had been observed by an histopathologist, Robin Warren, who was convinced they were associated with gastritis, inflammation of the gastric mucosa (*Warren*, 1983). The culture had been coordinated by a young medical registrar, Barry Marshall, who had teamed up with Warren as part of a short research project. The first Perth culture attempts incubated for shorter periods had failed, yet now the plates revealed small round colonies and so the bacterium we now know as *Helicobacter pylori* was born (*Goodwin* et al., 1989). Marshall and Warren then tried to convince the world that this bacterium was not only a cause of the gastritis but was indeed associated with peptic ulcer disease. Given the current wisdom was that ulcers were caused by acid, the suggestion that a bacterium could somehow be involved in ulcer formation was greeted with sceptiscm and frank disbelief by the community of gastroenterologists. While accepting the association, it was considered the bacterium was no more than an opportunistic coloniser attracted by the damaged gastric mucosa. However, over the years the evidence has accumulated to support the role of *Helicobacter pylori* in gastroduodenal disease and this has resulted in a paradigm shift in the management of these diseases. Anti *Helicobacter* therapy is now a mandatory component in the treatment of peptic ulcer disease (*Yamada* et al., 1994).

H. PYLORI, A MAJOR GLOBAL PATHOGEN

Following ingestion of *Helicobacter pylori*, an acute infection most likely occurs in the gastric mucosa with infiltration of large numbers of polymorphonuclear leukocytes. In those where early infection has been monitored e.g. Marshall himself who courageously swallowed the organism in an attempt to fulfil Koch's postulates, Arthur Morris an intrepid New Zealander who did the same and a number of individuals accidentally infected at endoscopy, acute symptoms were observed such as nausea vomiting etc. (*Debongnie* and *Bouckaert*, 1993; *Marshall* et al., 1985; *Morris* et al., 1991) However in most infected persons, this early episode of symptoms is uninvestigated as the gastritis progresses to what was previously called type b gastritis. This is an asymptomatic inflammation of the gastric mucus associated with the infiltration of both polymorphs and mononuclear cells, an active/chronic gastritis (*Dixon* et al., 1996). Importantly for the thesis being generated in this article, most persons with *H. pylori* infection remain asymptomatic for life although if biopsied all would show gastritis. In one subset of infected individuals, the bacterium may move into the duodenal bulb where it infects small areas of gastric type metaplastic tissue, induces a duodenitis and causes duodenal ulcer as a result of excess acid coming in from the stomach. In others, the gastritis in the stomach proper induces damage such that the mucosa becomes susceptible to acid attack and a gastric ulcer results (*Graham*, 1996). The involvement of acid in peptic ulceration was proven when it was found that the ulcers completely healed if patients were given acid suppressive therapy such as the H_2 receptor antagonists or the proton pump inhibitors. However, after cessation of acid suppression the ulcers recurred in 80% of cases after one year. The major contribution of the underlying gastritis to ulceration was proven when it was

demonstrated that successful cure of *H. pylori* infection with antimicrobial drugs resulted in resolution of the inflammation and the ulcers did not come back, i.e. for the first time peptic ulcer disease could be cured (*Bell* et al., 1996). Thus the symptomatic *H. pylori*-associated diseases are essentially immunopathologies. While peptic ulcer disease results in significant morbidity and mortality, the major global impact of *H. pylori* infection comes from another consequence of long term inflammatory damage i.e. gastric malignancy. By a series of indirect and possibly direct effects on the gastric mucosa, the bacterium is responsible for at least 60-70% of gastric adenocarcinomas (*Goldstone* et al., 1996). This form of gastric cancer remains one of the world's major tumours, killing up to one million per year. *H. pylori* is also responsible for the majority of another gastric cancer, low and high grade B cell MALT lymphoma (*Wotherspoon*, 1996).

H. PYLORI AS PREHISTORIC NORMAL FLORA OF THE GASTRIC MUCOSA

Our interest in microbial ecology began decades ago when one of us (AL) became fascinated with the bacteria that inhabit the mucus of the intestinal tract (*Lee*, 1985). This mucus provides a niche for a highly adapted group of spiral shaped bacteria in conventional mice and a range of other animal species. The caecal and colonic crypts are packed with these bacteria. We started to study *H. pylori* soon after its discovery because we reasoned it might be closely related to these lower bowel bacteria, as the also spiral/helical shaped organism occupied a similar ecological niche in the human stomach. Interestingly, many of these lower bowel bacteria have subsequently been shown to be *Helicobacter* species (*Lee* et al., 1992). Early on we described *H. pylori* as "almost normal flora", commenting that as with the commensals of lower bowel mucus the bacterium had evolved to inhabit gastric mucus. However, in this case the *H. pylori* always caused inflammation which we felt might be beneficial to the organism (*Hazell* et al., 1986). While not doubting the role of *H. pylori* as a gastric pathogen, we have more recently commented that the bacterium did indeed originate as a harmless commensal and it is only recently that it has become a pathogen (*Lee*, 1997). The *H. pylori*-associated symptomatic diseases, peptic ulcer disease and gastric cancer are relatively recent with ulcers only becoming common in the last two centuries. We have hypothesised that prior to 1700, gastric cancer was rare because people rarely lived to the age when it commonly occurs. Ulcer disease did not happen because acid output was lower than it is today due to co-infection with parasites which reduce acid production, nutritional and other unknown factors. Thus we claim that historically *H. pylori* is normal flora of the human body, and that it is only due to changes in environmental factors that it became able to cause symptomatic disease. From the large proportion of *H. pylori* infected persons in the world who are asymptomatic, we know that active/chronic gastritis of itself is not a debilitating condition. Ulcer disease and cancer are almost accidental consequences of infection. The bacterium certainly did not evolve to cause ulcer or malignancy as these conditions provide no advantage to the organism. Significantly nearly all other animal species examined have their own highly adapted gastric *Helicobacter* species which appear to colonise for life without

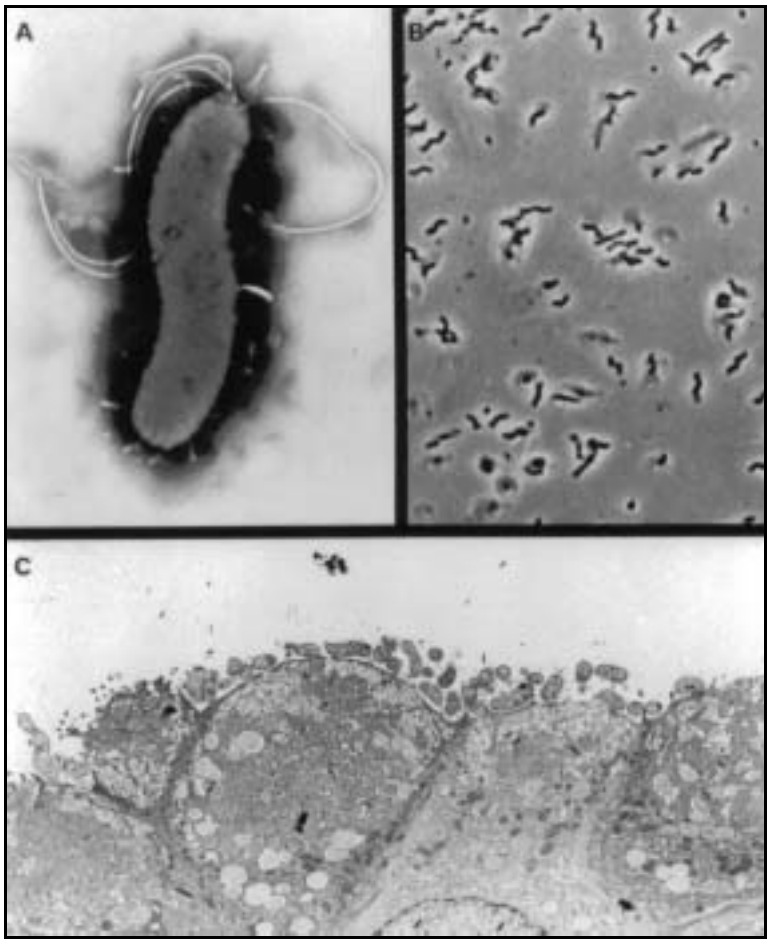

Figure 1:
A : Negative stain of *H. pylori* showing its spiral morphology and multiple polar flagella (x 18000).
B : Phase contrast view of a pure culture of *H. pylori* (x 1100).
C : Transmission electron micrograph showing H. pylori aligning along, and in some instances attaching directly to, the epithelial surface (x 3200).

significant consequence and which therefore fulfil the normal criteria for an autochthonous microflora (*Lee* and *O'Rourke*, 1993).

These concepts are particularly relevant to the topic of this symposium which aims to develop knowledge on the interactions between the immune mechanisms of the intestinal tract and the microorganisms of human and animal gut ecosystems. Understanding how this bacterium survives in the stomach despite a massive immune response mounted against it will provide information not only relevant to the colonising ability of other bowel flora but also might provide insights as to how other environmental factors might impact on the normal microbial flora of the lower bowel resulting in the appearance of symptomatic inflammatory conditions. Thus, the goal of this paper is to consider those factors that allow *H. pylori* to colonise the stomach whereas no other bacterium can. Possession of the enzyme urease is the characteristic

that allows it to survive gastric acidity via the breakdown of endogenous urea in the gastric mucosa thus providing ammonia to neutralise the local acid (*Lee* et al., 1996; *Meyer Rosberg* et al., 1996; *Mobley*, 1996).

Of more relevance here are the factors that permit long-term survival. *H. pylori* is acquired in early childhood and the infection remains for life (*Hazell* et al., 1994). This is despite an intense cellular response against the bacterium which manifests as the active/chronic gastritis which is also present lifelong and which sometimes increases in severity setting the scene for gastric cancer. A major humoral immune response also accompanies infection. Serology is an excellent predictor of active infection due to the high levels of IgG which are continually present (*Mendall*, 1997). The bacteria have been shown to be coated with IgA in the gastric mucosa (*Wyatt* and *Rathbone*, 1988). The working hypothesis of our group is that long term survival of *H. pylori* in the human gastric mucosa is due to immune evasion and immunomodulation. The aim of this chapter is to consider the strategies this highly evolved gastric bacterium has acquired over the millennia to evade and modulate the immune responses mounted against it.

STRATEGIES FOR IMMUNE EVASION

Antigen mimicry

The lipopolysaccharide (LPS) of the *H. pylori* cell wall has been shown to contain structures identical to host cell antigens and these will differ in different strains of the organism. Thus analysis of the chemical structure revealed that the LPS O antigen and core polysaccharide regions from three different *H. pylori* were all distinct (*Aspinall* et al., 1996). In each case, the O chain terminated in either Lewis y or Lewis x antigens. These antigens are structures commonly found on erythrocytes of persons of a certain blood group but are also expressed in the human gastric and other mucosae; it is possible that the expression of these antigens at the surface of the bacteria may act as a form of molecular mimicry - disguising the organism with the hosts own antigens to evade detection from the immune system. Two fucosyltransferases have been identified which may play a role in the molecular mimicry of Lewis antigen by *H. pylori* LPS (*Chan* et al., 1995). With possible relevance to the pathogenesis of *Helicobacter*-related diseases, some *H. pylori* infected patients have been found to produce autoantibodies directed against Le^x antigens (*Negrini* et al., 1991) and sera from *H. pylori* infected pigs and humans were shown to contain autoantibodies reactive against carbohydrate and peptide epitopes expressed on both the gastric pump and human intrinsic factor (*Appelmelk* et al., 1997). Perhaps the production of these autoantibodies is an inadvertent side effect of an attempt by the bacterium to avoid detection, but one which is costly in the long run for the host.

A study of 152 clinical strains of *H. pylori* from various geographical locations showed 12 different serotypes based on the expression of Lewis antigens; 85% of strains could be typed based on their expression of one or more of Le^x, Le^y or the related H1 antigen, with 77% of all strains expressing Lewis x (*Simoonssmit* et al., 1996). Interestingly, the majority of the Lewis non-typeable strains were of Chinese origin; given the high incidence of gastric cancer in this country, if fewer Chinese strains of *H. pylori* genuinely express Lewis antigens than those of Western countries, this does not support

the concept that molecular mimicry and/or production of autoantibodies targeted to Lewis antigens play a role in the progression from chronic *Helicobacter*-induced gastritis to more serious pathology. However, the non-typeability may have been due to loss of the O side chain as can occur following a number of *in vitro* passages (*Mills* et al., 1992).

Host binding: Another disguise?

Studies on the surface of *H. pylori* reveal that it is coated with a number of large protein molecules such as the enzymes urease and catalase (*Phadnis* et al., 1996). Both these proteins have been shown to be protective antigens in animal vaccine studies also confirming their surface location. It has been suggested that they originate from the lysis of other *H. pylori* cells. This would suggest that the bacterium has a particularly "sticky" surface for protein. In the protein rich milieu of gastric mucus it is not unlikely that host proteins could coat the organisms, once again hiding it from immune surveillance.

Protein shedding: Evasion by decoy antigens

The extreme lability of *H. pylori* which results in the release of proteins such as urease could also benefit the bacterium in a different way. In an important paper, Mai demonstrated urease antigen deep in the lamina propria of gastric biopsies from *H. pylori*-infected patients, far removed from the bacteria themselves (*Mai* et al., 1992). Given that the urease molecule has been shown to be chemotactic, it is possible that this and other antigens could act as a decoy stimulating the influx of phagocytic cells to a site removed from the actual site of infection. This is consistent with the observations that the zone of peak inflammation is often far removed from the bacteria which tend to be located in the outer mucus or attached to the outer surfaces of the gastric pits (*Fiocca* et al., 1994).

The *H. pylori* genome: Evidence for antigenic variation

A major leap forward in the study of *H. pylori* has recently occurred with the release of the complete genome sequence (*Tomb* et al., 1997). It is still much too early for all the implications of this genome to be determined but several observations have already been made. Firstly, as might be expected, there are many similarities between *H. pylori* and other Gram-negative bacteria such as *Escherichia coli*. However, there are also some significant differences which probably reflect functional modification. Most relevant to the current theme was the detection of a range of nucleotide sequences, which based on comparison with other well defined systems, indicates that *H. pylori* is equipped with a sophisticated machinery for extensive antigenic variation.

Alterations in antigenic epitopes is an important mechanism by which pathogens evade the immune system. A classical example of this occurs in the influenza virus. Two major antigens of this virus are the envelope expressed proteins neuraminidase (NA) and haemagglutinin (HA) which occur in several subtypes. It is mainly against these virulence factors that the immune response is mounted and infection with one strain of influenza confers protection against other strains possessing the same subtype of antigens. However periodically every 10-20 years, HA and less frequently NA undergo antigenic shift often leading to a new pandemic as all previous vaccines and acquired immunity become ineffective. Bacteria showing antigenic variation include *Neisseria meningitides, Neisseria gonorrhoeae* and *Haemophilus influenzae* (*Deitsch* et al., 1997).

The mechanisms of adaptive antigenic variation suggested by the *H. py-*

lori genome are listed below:

i) Slipped strand mispairing:
The genome sequence of *Helicobacter* has revealed a number of repeated sequences, some of which reside within open reading frames. This suggests that slipped strand mispairing may occur which could lead to the generation of phenotypic variation in molecules which have critical interactions with the host including surface structures such as pilins, lipoproteins or enzymes responsible for the production of LPS.

ii) Phase variation:
Synthesis of LPS involves glycosyltransferases which are enzymes involved in the transfer of saccharide moieties. Analysis of the genome has indicated that several of these may be subject to phase variation (*Tomb* et al., 1997). In phase variation, the bacteria possesses two or more highly homologous but not identical genes at different sites which encode antigenically distinct products; only one of these is expressed at any one time. An example of this occurs with the flagellin of *Salmonella* spp. where a gene encoding one flagellin protein is associated with a repressor for a second flagellin gene located at a separate site (*Simon* et al., 1980). When the first gene is transcribed, the second gene is repressed. During phase variation, a point mutation occurs in the promotor region of the first gene which is therefore not transcribed; this removes the repression on the second gene which is thus expressed.

iii) Another property possessed by *H. pylori* which can lead to antigenic variation is that of recombination events which result in mosaic organisation of a single loci. This means that different strains of *H. pylori* possess one type of allele which may influence the encoded proteins biological activity, e.g. the vacA protein (*Atherton* et al., 1995).

All of the above mechanisms have the potential to cause modifications which can induce antigenic variation to assist the *Helicobacter* in immune evasion. A more detailed discussion of the above mechanisms in other bacteria may be found in an excellent review published recently (*Deitsch* et al., 1997).

IMMUNOMODULATION

Down regulation of cellular activity

i) Lymphocyte stimulation by Helicobacter antigens

Helicobacter antigens have mitogenic capability; *in vitro* culture of isolated lymphocytes with antigens from *H. pylori* stimulate cells to undergo increased proliferation and produce elevated levels of cytokine. Several studies have reported that the response of human PBMC (*Karttunen* et al., 1990; *Karttunen*, 1991) and gastric T lymphocytes (*Fan* et al., 1994), from *H. pylori*-infected persons were stimulated significantly less *in vitro* by fixed *H. pylori* or bacterial sonicate respectively, than cells isolated from non-infected persons. In these cases, the responses to other non-*Helicobacter* mitogens such as Concanavalin A or pokeweed mitogen were unaffected. There was also a reported increase in the numbers of CD8+ T-cells in infected persons and T-lymphocytes expressing the CD8 molecule can possess suppressor activity. The induction of oral tolerance (a systemic non-responsiveness to an orally delivered antigen) is believed to be mediated by CD8+ cells by a mechanism involving the production of the cytokine, transforming growth factor β (*Miller* et al., 1992).

Thus it was suggested that the reduced stimulation following oral infection with *H. pylori* may have been caused by the production of antigen-specific suppressor cells.

Another group found that *in vitro* proliferation to both *Helicobacter* antigen and non-related mitogen was inhibited by infection with *H. pylori* which they associated with an activity of the cytoplasmic fraction (CF) of the bacteria on both monocytes and isolated T-cells (*Knipp* et al., 1994). During mitogenic activation of purified T-cells, expression of the receptor for the cytokine Interleukin 2 (IL-2) was down-regulated on the cell surface if stimulation occurred in the presence of a CF of *H. pylori*. These authors related these observations to a bacterial protein with an apparent molecular weight of 100±10 kDa which inhibited cell-proliferation (*Knipp* et al., 1996).

These studies propose that *Helicobacter* infection in some way downregulates lymphocyte proliferative responses in an antigen-specific manner. However, the majority of these studies looked at cells from the periphery and the few investigations of cells isolated from the actual site of infection presented unconvincing data. Thus it is still unclear as to the effect of *Helicobacter* infection on the immune response at the mucosal level, but the heavy inflammatory response that occurs is perhaps the best indicator that immune suppression is not actually occurring. Perhaps alternative explanations are that during infection there is a change in the frequency of antigen-specific cells in the peripheral blood as they are localised at sites of infection or there is a qualitative change in the recirculating lymphocytes which alters their responsiveness to mitogens; terminal effector cells for example would be expected to respond to stimulation in a different manner than a naive cell. This may not explain the observed reduction in response to other mitogens which has been reported by some workers.

ii) The LPS of H. pylori shares properties with the lower bowel normal microflora

The surface expression of LPS is an important factor in the pathogenesis of Gram-negative bacteria. LPS is the signal which the human immune system has evolved to recognise as the first sign of bacterial invasion and its presence stimulates the non specific and specific defences such as the inflammatory response. However with well adapted pathogens which cannot be quickly removed from the host, responses to excess LPS can actually contribute to the disease process. Thus endotoxin causes abortion of animals with brucellosis, the characteristic fever of typhoid, and the syndrome of septic shock which occurs during systemic infection with many Gram-negative bacteria. Binding of LPS to the host surface molecule CD14, a receptor found on the surface of monocytes and polymorphonuclear granulocytes leads to cell activation. Macrophages are stimulated to secrete lysosomal enzymes and proinflammatory cytokines such as IL-1 and Tumour Necrosis Factor alpha (TNF-α). Neutrophils undergo lysosomal degranulation with release of kallikrein (which causes formation of mediators of acute inflammation such as bradykinin), proteases which act on complement to produce anaphylatoxins, and cationic proteins which stimulate release of histamine from mast cells. These all contribute to the generation of an inflammatory response by Gram-negative bacteria.

In the case of *Helicobacter* infection of the stomach, evidence suggests that it may be of advantage to the bacteria to tone down the stimulatory activity of its endogenous LPS. Infection of various strains of mice with *H. felis*, showed that certain strains of mice give an in-

tense, inflammatory response with atrophy (*Sakagami* et al., 1996). Mice, such as SJL, which suffer severe atrophic gastritis actually have a reduction in their bacterial infection; this is probably due to atrophy causing a decrease in acid secretion with resulting changes in the microenvironment, creating a habitat less favourable for Helicobacter colonisation. Thus an overzealous inflammation can be detrimental to bacterial survival. We have recently reported that whereas the C3H/He mouse responds to *H. felis* infection with a strong atrophic gastritis, infection of its derivative C3H/HeJ strain, which has a well characterised mutation in the LPS-response gene, produced no atrophy and little or no inflammation (*Sakagami* et al., 1997). This suggests, therefore, that the *Helicobacter*-associated gastritis is LPS-driven, although this is partly challenged by the evidence of *Mai* et al., (1991) who found that soluble *H. pylori* surface proteins with no detectable LPS could activate human monocytes *in vitro*.

If severe gastritis leads to a reduction in colonisation and LPS is a major factor leading to this gastritis, then it would be advantageous to the bacteria to reduce the immunomodulating activity of its endotoxin and this indeed does appear to be the case. Several studies have shown that LPS from *H. pylori* has much lower biological activity than that of other Gram-negative bacteria such as *E. coli* (*Birkholz* et al., 1993).

These observations may be explained by numerous biochemical studies which have revealed some unusual structural properties of *H. pylori*-LPS compared with endotoxin from other bacteria. *Helicobacter pylori* was originally called *Campylobacter pylori* but was eventually excluded from the *Campylobacter* genus; a major reason for this was the unusual fatty acid profile of *H. pylori* and *H. mustelae* (*Goodwin* et al., 1989). Interestingly, *C. jejuni* - a common cause of human gastroenteritis - also has LPS with low biological activity (*Moran*, 1995). The biological activity of LPS is related to its lipid A component which consists of chains of fatty acids; changes in these fatty acids would therefore be expected to alter the activity of the LPS and this has been proposed for the reduced activity of LPS from *Rhodopseudomonas sphaeroides* (*Henricson* et al., 1992); interestingly, *Geis* et al. (1990) have also reported unusual fatty acid substitutions in *H. pylori* - LPS. Relevant to the concept of *H. pylori* as almost normal flora is the observation that the low biological activity of LPS is also a feature of the LPS of *Bacteroides* species, members of the normal flora of the human lower bowel (*Lee* and *Moran*, 1994).

Another mechanism by which *H. pylori*-LPS may be modified to reduce its proinflammatory activity involves CD14. The attachment of LPS to the CD14 receptor is facilitated by a host serum protein termed Lipopolysaccharide Binding Protein (LBP) which increases in concentration during an infection. It has been shown that activation of CD14-transfected cell lines by *H. pylori* -LPS requires the presence of LBP but again had lower activity than that of LPS from *E. coli*, being unable to stimulate IL-8 secretion by an epithelial cell line (*Kirkland* et al., 1997). Kinetic studies have shown that the transfer of *H. pylori*-LPS to CD14 by LBP is greatly reduced compared with other bacterial LPS and this is likely due to poor binding of *H. pylori*-LPS to LBP (*Cunningham* et al., 1996). Additionally, expression of CD14 by monocytes was downregulated following incubation with a cytoplasmic fraction of *H. pylori* (*Knipp* et al., 1994). Both the percentage of CD14 positive cells as well as the density of CD14 expression on those cells were downregulated. This effect was not mediated by *H. pylori*-LPS. This is very similar to

what has been reported for *Mycobacterium avium-M. intracellulare* complex induced inhibition of T-cell proliferation to mitogen and antigen, which also demonstrated a reduction in CD14 expression with no effect on other important immunological surface molecules such as MHC-class II (*Tsuyuguchi* et al., 1990).

CD14 is a key molecule involved in the recognition of bacteria by the innate immune system and it is tempting to speculate that it has evolved in the host particularly to detect LPS-expressing Gram-negative bacteria. If so, then modification of the LPS by bacteria which survive chronically in the host could be an evolutionary step to reset the balance in the constant battle between host and pathogen.

In conflict with this oft quoted and appealing hypothesis of the low biological and proinflammatory activity of the *H. pylori* LPS is the fact that in reality infection is nearly always associated with a marked inflammatory response that is the active/chronic gastritis.

iii) Other LPS related effects

As mentioned above, *H. pylori* may manufacture a molecule which mimicks the Lewis x antigen and the fact that in the human host this molecule appears to play some regulatory role in immune and inflammatory responses raises some fascinating paradoxes. During inflammation, IL-1, TNF and/or LPS induces an increase in the surface expression of a range of adhesion molecules on local endothelial cells, including E selectin (*Bevilacqua*, 1993). E-selectin is a membrane glycoprotein mainly expressed by endothelial cells which interacts with structures containing sialyl Lewis x and its expression following cytokine activation has been linked to increased adhesion of blood neutrophils, monocytes, some memory T-cells and possibly eosinophils and basophils (*Bevilacqua*, 1993). It is normally only transiently expressed but this expression can be increased by IFN-γ, a ubiquitous cytokine at sites of *Helicobacter* infection. Similarly, L-selectin is constitutively expressed on most circulating lymphocytes, neutrophils and monocytes, is involved in cell adhesion and also appears to interact with sialyl Lewis x.

Sialyl Lewis x is obviously structurally different from Lewis x; however, a pentasaccharide containing Lewis x can block the interaction of a platelet form of selectin (P-selectin) with its sialyl Lewis x expressing ligand. In addition, it has been suggested that the immunosuppressive property of an endometrial protein, glycodelin, may be related to its expression of a Lewis x analogue. It was proposed that the glycan moiety may block B-cell activation via the CD22 receptor (*Dell* et al., 1995)

Finally, binding of the T-cell receptor CD2 (another structure involved in cell adhesion) to its ligand on monocytes and neutrophils is inhibited by monoclonal antibodies specific for Lewis x, plus the same antibody has been shown to inhibit the killing of target cells by NK cells (*Warren* et al., 1996) This means that in addition to a role in controlling the trafficking of leukocytes, Lewis x associated structures may also be important in the effector functions of immune cells.

At a recent meeting in Lisbon, the complexity of the relevance of Lewis antigens in *Helicobacter* associated diseases was made even more apparent with a report that infected subjects who lacked anti- Le^x antibodies actually had a higher incidence of atrophic gastritis than those with antibodies (*Kuipers* et al., 1997). However, it is not clear whether the observed difference was due to the absence of anti-Lewis x antibodies in the infected host or bacterial strain variation; perhaps strains of Helicobacter which do not express Lewis x produce greater atrophy. All

this evidence implies a significant role for different forms of the Lewis x antigen in many different aspects of the host's inflammatory and immune response. Thus, when this molecule is expressed at the surface of a pathogen, as is the case with *H. pylori*, it raises the possibility of a mechanism of immunomodulation.

Complement and *H. pylori* associated inflammatory effects

Helicobacter localising in the pit openings of the gastric mucosa have been found to be coated with activated complement, whereas those located within the foveolae were generally not (*Berstad* et al., 1997). This suggests the bacteria can evade the activity of complement by its localisation. This could be related to the exposure of the bacteria in their various niches to mucosally secreted antibodies as has been shown by *Wyatt* et al. (1986), although *in vitro* studies have shown that *H. pylori* can also activate complement in the absence of antibodies (*Bernatowska* et al., 1989). Urease is an important molecule released by the bacteria during infection and can be found in the gastric mucosa. Recently, evidence has been presented which suggests that urease from *H. pylori* inhibits the alternate pathway of complement activation *in vitro* (*Rokita* et al., 1997), which may imply an immunomodulatory role.

Immunomodulation via manipulation of Th phenotype towards Th1

The host's acquired immune response has many varied components which can give a variety of responses, mainly controlled and directed by Helper T-lymphocytes. The production of T-cell clones revealed that these cells can be broadly divided into two subsets based on the cytokines that they produce (*Mosman* and *Coffman*, 1989). The Th1 cells produce Interferon gamma (IFN-γ) and IL-12, amongst others, which drives the immune response towards a cell mediated profile. This would be the response mounted against an invading microorganism that lives in an intracellular location. In contrast, Th2 cells produce IL-4 and IL-10 leading to a more humoral response with production of an antibody based reaction. The Th2 response, for example is responsible for the production of IgA-secreting plasma cells, the main effectors of mucosal immunity which localise in the lamina propria and secrete antibodies into the lumen of mucosal surfaces such as the intestine. This is the mechanism of defence against bacterial pathogens which invade mucosal surfaces and would be most likely to confer protection against an organism living in the stomach in mucus and on the epithelial surface i.e. a location inaccessible to cellular immunity.

The type of Th response generated *in vivo* in response to an infection can be vital to the outcome of the struggle between host and parasite and this is well illustrated in the classic oft quoted example of *Leishmania* infection in inbred strains of mice. C57BL/6 mice infected with *L. major* mount a Th1 type cell-mediated response which is effective against the intracellular parasite; the mice survive and have long lasting immunity. In stark contrast, challenge of the *Leishmania*-sensitive BALB/c mice induces a Th2-type response and these mice succumb to the infection and die (*Heinzel* et al., 1989; *Hill* et al., 1989).

It is therefore of great interest that infection with *H. pylori* actually produces an ineffective Th1 response (*Bamford* et al., 1997). *Kartunnen* et al. (1995) found that there is a predominance of IFN-γ secreting cells in lymphocytes isolated from the gastric mucosa of *Helicobacter*-infected persons and elevated levels of messenger for the Th1-promoting cytokine IL-12 (*Karttunen* et al., 1997) when compared to non-in-

fected controls. Recently presented data also showed that both IgG subtype responses and T-cell clones produced by antigenic restimulation of cells isolated from infected persons indicated a Th1-type profile (*Bamford* et al., 1997; *Bamford*, 1997). We would submit that this predominant Th1 phenotype is not just coincidence, but a deliberate action of the bacterium to circumvent the effective branch of the host's immune response.

This hypothesis is supported by the observation that effective protection against Helicobacter infection is indeed possible in mice, following immunisation with various bacterial products plus an adjuvant such as cholera toxin or heat labile toxin from *E. coli* (*Czinn* et al., 1993; *Ferrero* et al., 1995; *Lee* et al., 1995; *Michetti* et al., 1994; *Nedrud* et al., 1997; *Radcliff* et al., 1996). If immunisation can work then evidently the host's immune system has the capability to eject the pathogen but is incapable of doing so without some outside help.

The mechanisms of how immunisation actually achieves protection is still unknown, but the current favoured hypothesis is that oral exposure of the antigen in the presence of adjuvant causes a switch towards a Th2-type response (*Ernst* et al., 1996). Several studies have supported this, such as the adoptive transfer of *Helicobacter*-specific cloned Th1 cells into infected mice which led to an exacerbation of the gastric inflammation, whereas transfer of Th2 clones actually produced a reduction in bacterial colonisation (*Mohammadi* et al., 1996). However, it is becoming clear that the situation is much more complex than a simple switch to a Th2-type response. From current understanding of mucosal immunity, the most likely effector mechanism for clearance of a *Helicobacter* infection would involve an IL-4-dependent (Th2) antibody responses mediated via the secretion of IgA into the lumen of the stomach. Studies using oral pretreatment of mice with monoclonal IgA (*Czinn*, et al., 1993) and infection of knock-out mice lacking IL-4 (*Radcliff*, et al., 1996) certainly seem to support this. But this is where things get more complicated. Although the IL-4 deficient mice had reduced protection following immunisation, there was still a certain degree of protection even in the apparent absence of Th2 driven immunity. In addition, knock-out mice incapable of producing IgA (the main effector of Th2/mucosal immunity) were as well protected as wild-type mice following immunisation, indicating that protective immunity is possible in the absence of IgA (*Nedrud*, et al., 1996). Recent data showing that infection of mice lacking receptors for IFN-γ are not protected following immunisation have suggested an important role for Th1 cytokines, thus it may be that a mixed Th2/Th1 is necessary for effective protection (*Radcliff* et al., 1997).

If an immune response can be mounted which clears infection then why is over half the population of the world chronically infected with *H. pylori* ? To many, this clearly suggests that the bacteria manipulates the Th profile to its own advantage. This may not only be of benefit with regard to protection from immunity, but it has been shown that the Th1 cytokine IFN-γ can increase the membrane permeability of intestinal epithelial cells (*Madar* and *Stafford*, 1989) and this may be of benefit to the bacteria by allowing the release of nutrients into the stomach lumen. We have previously proposed that as inflammation is likely to benefit the organism, the move to an inflammatory Th1 response would thus be a benefit (*Hazell* et al., 1986).

Other infectious organisms have been shown to actually modify the hosts immune response to its own advantage. An example of this is the obligate intracellular protozoan *Toxoplasma gondii*. During the acute phase of infection with

this parasite there is a downregulation of the proliferative response of PBMC to both parasite antigen and non-specific mitogenic activation (*Chan* et al., 1986).*T. gondii* preferentially infests macrophages and it has been shown that during acute infection in mice, the parasite induces the macrophage to secrete IL-10 and nitric oxide which inhibits the cell mediated immunity which is so effective against intracellular pathogens (*Khan* et al., 1995). Eventually, over a number of weeks the host immune system overcomes this immunomodulation and clears peripheral tachyzoites - the multiplying, invading form of the pathogen. However, this window allows the parasite to multiply in the host and when effective immunity kicks in, it converts to the bradyzoite, or cyst form and hides in immunoprivileged sites such as the brain.

A similar story occurs with the causative agent of Chagas disease, *Trypanosoma cruzi*. During acute infection there is suppressed immunity in both humans and in animal models with reduced proliferative responses to antigen and mitogen plus diminished numbers of T-cells in the spleen. In human PBMCs, this has been related to a parasite-induced suppression of expression of the receptor for the crucial cytokine IL-2 on T-cells (*Beltz* et al., 1988). Without this receptor, T-cell activation is severely impaired.

Possibly the most intriguing precedent for manipulation of an immune response by a pathogen is shown in animal studies of schistosomiasis as yet again it features the Lewis x antigen, a molecule that has figured prominantly in the discussion above. A Lewis x trisaccharide has been found expressed on a surface antigen from the eggs of the parasitic worm, *Schistosoma mansoni* (*Velupillai* and *Harn*, 1994). This Lewis x antigen stimulated a B-cell enriched population of spleen cells to secrete IL-10 (a Th2 cytokine) and prostaglandin E2 which both downregulate Th1 cell mediated immunity. This is exactly the opposite to what would be advantageous to *Helicobacter* and our hypothesis, yet it demonstrates the potential immunomodulating capability of Lewis molecules. Could it be that in humans the Lewis x does the opposite to what it does in the *Shistosoma*-infected mice?

CONCLUSION

Diseases caused by *H. pylori* remain one of the world's major killers. Thus, in a recent article on the causes of death of any type in the world, gastric cancer was ranked 14th for the year 1990 (*Murray* and *Lopez*, 1997). Moreover, due to the age profile of the globe, it was predicted that this *H. pylori* linked malignancy would move up the ladder of death to 8th in the year 2002. Better understanding of the mechanisms of survival of this pathogen in the gastric mucosa as discussed above should point the way to novel approaches to both prevention and cure. Understanding the interaction of this organisms in its mucus niche with the host's immune system may also provide pointers to other diseases lower down in the bowel. Unlike in the stomach where there is only one bacterial inhabitant, in the lower bowel a consequence of high species diversity is that the complex microflora play a protective role against invading bacterial pathogens. In animals, the intestinal mucus is inhabited by a myriad of spiral bacteria that are closely related to *H. pylori*. Could it be that these bacteria use similar evasive and immunomodulatory mechanisms? Could these mechanisms be protective of the intestinal mucosa? This may not

appear relevant to human disease as we have no lower bowel spirals, but remember the world is losing *H. pylori* from its gastric mucosae. In the developed world, children no longer become infected with *H. pylori* and it is likely that even if there were no intervention strategy the bacterium would be lost from whole populations in 100 years. Could not the same have happened with the lower bowel spirals? There is anecdotal evidence in some underdeveloped countries such as India there still may be many spiral bacteria on the lower bowel surface. (*Mathan* and *Mathan*, 1985) What would be the consequence of the removal of these potentially immunmodulatory commensals from the surfaces of the lower bowel? Could this be relevant to the observation that inflammatory bowel disease (IBD) is a new disease of the developed world? Recent animal experimentation has showed that other *Helicobacter* species can, as pure cultures, induce IBD (*Ward* et al., 1996). We need to learn much more about the interaction of the host with the mucus associated microbiota of our mucosal surface.

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DIETARY COMPONENTS: EFFECTS ON MUCOSAL AND SYSTEMIC IMMUNITY

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SUMMARY

It is a major function of the immune system to control and down-regulate reactivity to food components. An important mechanism is to develop specific immunological tolerance to foods. This seems to mainly occur through mucosal membranes and via several mechanisms like suppressor cells and anergy in and elimination of specific lymphocyte clones.

Deficiencies of nutrients is a large global problem. Undernutrition is usually not a direct cause of death in spite of its many untoward effects on mucosal and systemic immunity, but is a contributing factor adding to the risk of dying from infections. Undernutrition and frequent infections are most often consequences of the pathology of poverty. Prevention of infections and food supplementation are both required and can save the lives of millions of children every year, which as a paradox contributes to decreased population growth. This is due to the fact that a lower infant mortality is usually followed by a decreased birth rate.

INTRODUCTION

Central tolerance in the thymus limits the number of auto-aggressive T cell clones. Mucosal tolerance develops to diminish or avoid unnecessary inflammatogenic immune reactivity to antigenic material in our foods and our normal microbial flora on mucosal membranes, especially in the gut. What determines whether immunological tolerance or immune reactivity is to occur against e.g. foods at the mucosal level is not well understood.

FOOD ANTIGENS AND THE MUCOSAL AND SYSTEMIC IMMUNE SYSTEM

Most new-borns who are fed formula or meet food antigens from the mother's diet in her milk will not react clinically to these food antigens. Still it has been shown that there is an antibody response to e.g. cow's milk proteins during the first year of life which slowly decreases (*Lippard* et al., 1936). This response often includes IgE antibodies as well (*Hattevig* et al., 1984). Normally, however, this response does not cause any untoward effects and van-

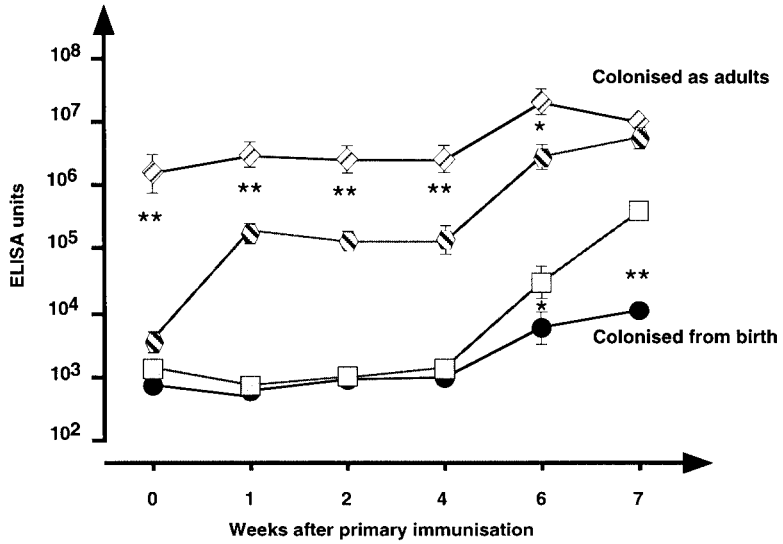

Figure 1: Serum IgG anti-LPS antibodies in young and adult colonised animals. The rats were subcutaneously immunised with a mixture of killed *E. coli* 06:K13 and Freund's complete adjuvant in the hind leg six weeks (week 0 in the figure) after the colonisation, and booster immunised 6 after the primary immunisation. The p-values (Mann-Whitney U test) are obtained by comparison with the age matched non-colonised rats, * = $p<0.05$, **= $p<0.01$.

ishes. A further increase, or remaining levels of IgE antibodies are mainly found in atopic children, many of whom later develop allergic reactions. This would suggest that mucosal tolerance normally develops early in life.

Two studies suggest that the secretory IgA antibodies to cow's milk proteins in the mothers' milk can affect the breastfed infants reactivity: the higher the levels of breastmilk antibodies to cow's milk the smaller the risk to develop cow's milk allergy (*Machtinger* and *Moss*, 1986; *Casimir* et al., 1989). It is not clear whether breastfeeding can enhance development of tolerance.

Cow's milk allergy is common in early childhood, but some 80-90% spontaneously loose this hyperreactivity by the age of 2-3 years, which could be interpreted as development of immunological tolerance. In the so called "allergy march" allergic children start with food allergy and a few years later they become allergic to inhalant allergens developing hayfever and asthma. Does this indicate that tolerance develops more efficiently in the gut than via the mucosa of the respiratory tract?

In fact we do not know very well how oral tolerance develops. It seems that the intestinal flora may be important, since it is more difficult to induce tolerance in germfree than conventional animals (*Wannemuehler* et al., 1982). There are also observations suggesting that whereas LPS given parenterally breaks tolerance, it enhances development of tolerance if given in the gut (*Khoury* et al., 1990; *Gaboriau-Routhiau* and *Moreau*, 1996).

Our recent studies show that animals colonised early in life with a transgenic *E. coli* producing ovalbumin (OvA) develop tolerance both to a T cell independent antigen, LPS, and T cell dependent antigens, type 1 pili and OvA. This was seen by decreased levels of specific antibodies to LPS, OvA and type 1 pili and a lower delayed type hypersensitiv-

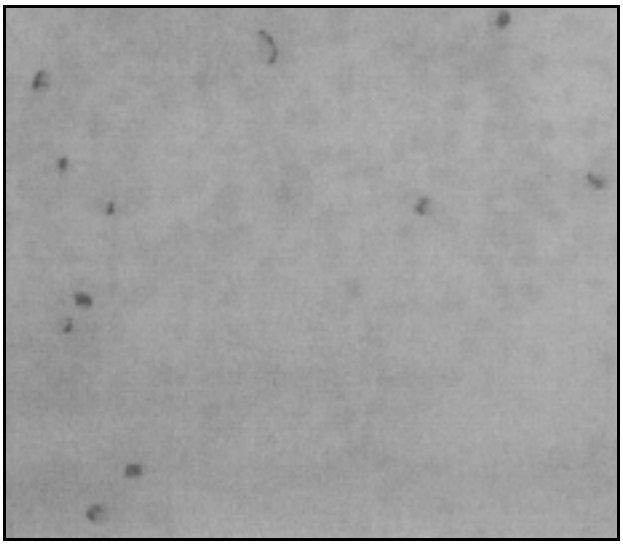

Figure 2: TGF-β positive cells (stained dark) in the T-cell area of the draining lymph node in a young colonised rat. The rats were subcutaneously immunised with a mixture of killed *E. coli* 06:K13 and Freund's complete adjuvant in the hind leg six weeks after the colonisation, and booster immunised 6 weeks later. No TGF-β positive cells were found in non-colonised rats (not shown).

ity reaction (OvA) compared to a non colonised control group (Figure 1). The reverse was seen in adult colonised animals. Further, TGF-β positive cells were found in the draining lymph nodes in the young colonised and tolerised animals after immunisation with dead bacteria (Figure 2). This indicates that the tolerance against bacterial components is fully or partially mediated by active suppression (*Karlsson* et al., 1997a).

Our information about how immune responses or mucosal tolerance develops in the intestinal mucosa is also limited. It has been demonstrated that dendritic cells from the intestinal mucosa can present antigens to naive T cells (*Liu* and *MacPherson*, 1993; *Liu* and *MacPherson*, 1995). The epithelial cells have been proposed to be antigen presenting, but this is still an open question. Our work with rat epithelial cells shows that MHC class II molecules are not found on their surface, but in vacuoles in the cytoplasm. The lamina propria contains a network of dendritic cells which may well be the central antigen presenting cells (APCs), possibly after having met the MHC class II from the epithelial cells in complex with peptides from e.g. food proteins taken up by the epithelial cells. The dendritic cells could present antigen to the local memory $CD4^+$ T cells located centrally in the villi, and/or migrate to the mesenteric lymph nodes and there appear as APCs for presentation to naive T cells. As these APCs normally lack the ”danger” signals the activated T cells turn into regulatory T_H3 cells producing TGF-β upon restimulation.

As mentioned above it might be that the MHC class II molecule-containing vacuoles in the gut epithelium are of importance for the development of oral tolerance. Rats start to express MHC class II vacuoles in the gut epithelium at four weeks of age. This coincides in time with the possibility to induce oral tolerance with active supression against fed soluble protein antigens indicating a re-

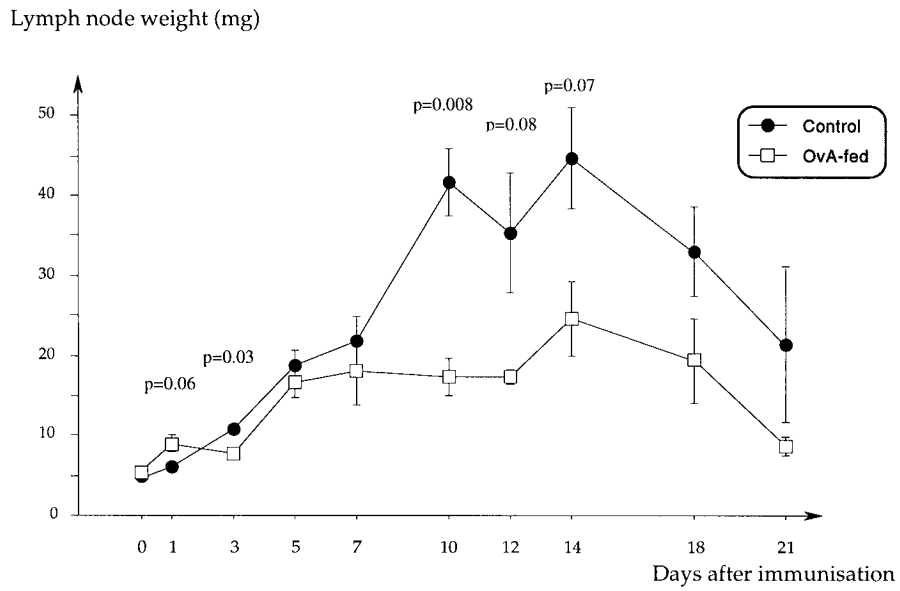

Figure 3: Rats made tolerant to OvA, and control rats, were immunised s.c. in the hind leg with a mixture of OvA and HSA in Freund's complete adjuvant. At different intervals after challenge, the draining lymph nodes were removed, and weighed. Three animals from each group was sacrificed at every time-point. The results are shown as mean ± SEM; three experiments with similar results have been performed.

quirement for MHC class II expression by enterocytes for the induction of oral tolerance (*Miller* et al., 1994; *Karlsson* et al., 1995). A fully developed intestinal proteolytic system also seems to be required to generate the tolerogenic peptides from the fed proteins (*Whitacre* et al., 1991; *Hanson* et al., 1993a; *Hanson* et al., 1993b). It is interesting to note as well that both bacterial colonisation of germfree rats and treatment with LPS dramatically increase the expression of MHC class II molecules particularly in the epithelium, but also in the lamina propria dendritic cell compartment.

It is likely, but not definitely demonstrated, that the intraepithelial lymphocytes have a regulatory role for immune reactivity in the intestinal mucosa, possibly related also to the development of tolerance.

SENSITISATION OR TOLERANCE TO OVALBUMIN (OvA) IN THE GUT OF RATS

It is quite easy and efficient to induce tolerance in rats by feeding them OvA. After immunisation and colonisation with *E. coli* genetically manipulated to produce OvA the gut mucosa of the tolerised animals contain few mast cells coated with IgE, few eosinophils and the goblet cells are not activated and emptied. Centrally in the villi these tolerant animals have a $CD4^+$ T cell population carrying CD25, the IL-2R α-chain (*Dahlman-Höglund* et al., 1996), which might be responsible for the down regulation of the immune response to OvA. Such cells were not found in the non tolerant animals.

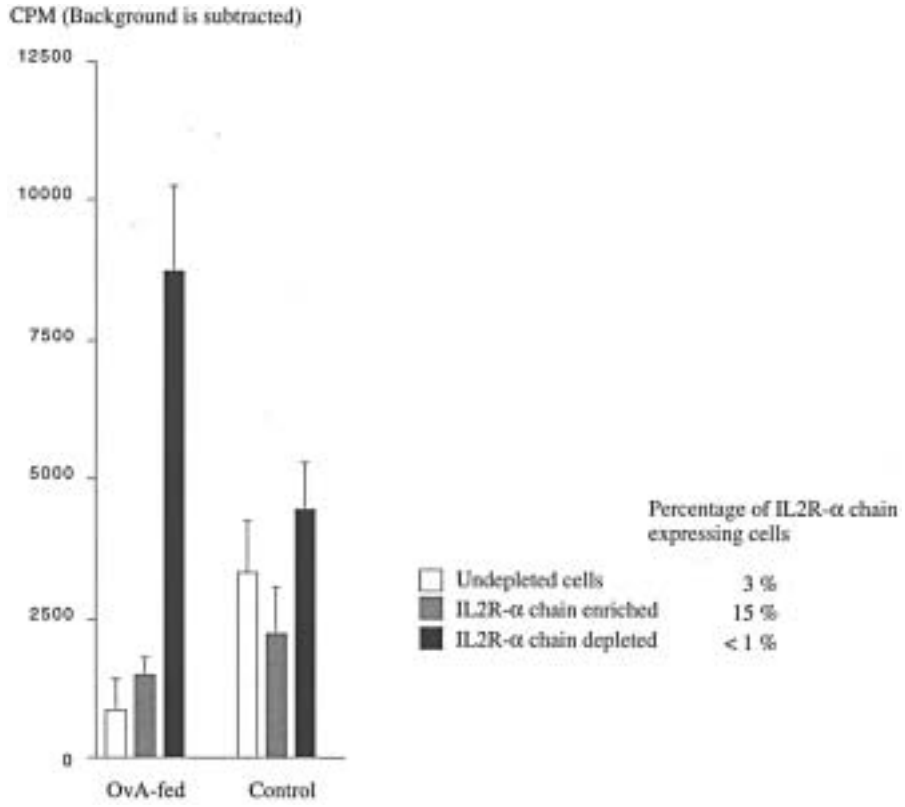

Figure 4: Proloferation of MLN-cells after OvA stimulation. 10 weeks old rats were given ovalbumin (OvA)-containing pellets for two weeks; control rats were given ordinary pellets throughout the experiment. Two weeks after the removal of the OvA-pellets all rats were immunised perorally with 20 μg Cholera toxin, 20 mg OvA and 20 mg Human serum albumin in 2 ml PBS. The rats were subsequently immunised another two times in the same way, with a 5-day interval (in total 3 times in 15 days). One day after the last immunisation, the rats were sacrificed, and the mesenteric lymph nodes were taken out. The lymph node cells from each group of rats (three rats per group) were pooled, and either enriched for or depleted of CD25-expressing (IL2-Roc chain-expressing) cells, using superparamagnetic beads (MACS). The cell populations were stimulated with OvA *in vitro* for 4 days, and the proliferation was measured.

$CD25^+$ cells isolated with magnetic beads from draining lymph nodes in the tolerised animals are shown to suppress T cell reactivity to OvA *in vitro*, as well as B cell responsiveness measured as IgE and IgG antibody production to OvA upon transfer (*Lundin* et al., 1997a) (Figure 3). That these cells are generally present was evident from the fact that after immunisation in the food pad with OvA together with an unrelated antigen in Freunds complete adjuvant the OvA tolerant animals showed no swelling of the draining popliteal lymph nodes despite the use of such a very strong adjuvant (*Lundin* et al., 1997b) (Figure 4). Furthermore, TGF-β production was more frequently detected in the lymph nodes from the tolerised than from the sensitised animals (*Karlsson* et al., 1995; *Lundin* et al., 1997b).

Another striking finding was that in tolerised animals the suppression of immune reactivity would include also a

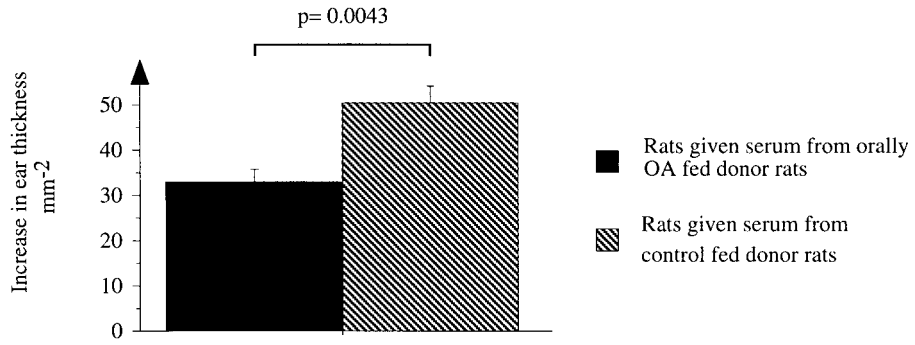

Figure 5: DTH reaction against human serum albumin (HSA) measured as increase in ear thickness 24 hours after challenge with 50 μg HSA in 20 μl PBS. All rats were subcutaneously immunised with a mixture of OvA and HSA in Freund's complete adjuvant in the hind leg two weeks prior to the DTH challenge. The p-value (Mann-Whitney U test) are obtained by comparison with rats receiving control serum.

second unrelated antigen given simultaneously, so called bystander tolerance (*Dahlman-Höglund* et al., 1995; *Lundin* et al., 1996). This is presumably due to the non-specific suppression mediated by e.g. TGF-β from the $CD4^+$ suppressor cells.

Oral tolerance may be due to suppressor cells, like those described above, as well as anergy in or deletion of antigen specific cell clones. The latter two mechanisms would obviously not result in bystander tolerance. There were also indications that the oral tolerance induced in young and older individuals may differ in that active suppression and bystander tolerance dominates in adult animals, but anergy and/or deletion in young animals. This may be due to the different levels of MHC class II expression in the gut epithelium observed in young and adult animals as discussed above (*Lundin* et al., 1996).

We have also observed that tolerance to a soluble protein antigen can be transferred with a serum factor from antigen fed donors. This factor is also capable of inducing an actively suppressed immune response in the recipients as shown by bystander tolerance to an unrelated antigen, human serum albumin (*Karlsson* et al., 1997b), (Figure 5). The nature of this serum factor has not been fully described. We are currently investigating the possibility that it consists of preformed MHC class II-peptide complexes possibly emanating from the gut epithelium. Interestingly SCID mice that lack MHC class II in the epithelium are unable to produce the tolerogenic serum factor (*Furrie* et al., 1994).

THE CONSEQUENCE OF DEFICIENCY OF VARIOUS DIETARY COMPONENTS ON THE MUCOSAL AND SYSTEMIC IMMUNITY

Above has been described the consequences of the normal exposure of the immune system to dietary components.

Below will follow a description of some of the consequences for the immune system of deficiencies in various food components, like proteins and micronutrients. The clinical consequences of such undernutrition will be debated since they are not always so clear-cut,

although many forms of undernutrition may lead to various abnormalities of the immune system.

Mostly undernutrition, especially in field studies, is poorly defined. Undernutrition is often labelled as protein-calory undernutrition, PEM, but simultaneous deficiencies of various important micronutrients like vitamin A, zinc and iron are usually not determined. This makes it difficult to evaluate many earlier studies of undernutrition and the effect on the immune system.

EFFECTS OF UNDERNUTRITION ON THE IMMUNE SYSTEM

PEM has been reported to cause "nutritional thymectomy", as well as diminished spleen and lymph nodes. In cases of kwashiorkor (mainly protein deficiency) and marasmus (mainly energy deficiency) there is less lymphoid tissues in the tonsils, appendix and the Peyer's patches. In severe PEM $CD4^+$ T cells seem to be more reduced than $CD8^+$ cells. In experimental PEM in rodents there is impairment of mesenteric lymph node cells (*Chandra*, 1992; *Gupta*, 1993; *Keusch*, 1993; *Hanson* et al., 1998).

Proliferative responses of lymphocytes to mitogens are reduced, as are delayed type hypersensitivity reactions (DTH). B cells are mostly normal. However, in children before the age of 7 months there is panhypoimmunoglobulinaemia, but later polyclonal hyperimmunoglobulinaemia.

Undernutrition of lactating mothers decreased the S-IgA levels in milk, although within the normal range, whereas avidity of certain specific S-IgA antibodies seemed to be impaired (*Herías* et al., 1993). Severe malnutrition results in decreased secretory component (SC) and S-IgA; also S-IgA responses to measles and poliovirus vaccines are decreased (*Chandra*, 1975; *Watson* et al., 1985).

Complement levels are reduced in PEM and bacterial killing by phagocytes may be impaired. So is production of cytokines like IL-1, IL-2 and IFN-γ, as well as the responsiveness of T cells to cytokines (*Chandra*, 1992).

UNDERNUTRITION AND FREQUENT INFECTIONS

The many effects of undernutrition on the mucosal and systemic immune system should be expected to result in impaired host defence and decreased capacity to inflammatory reactivity. However, that is not so clear from clinical studies. A major reason for this is that undernutrition and frequent infections are both part of the pathology of poverty.

Early work assumed that the frequent infections were the consequences of the undernutrition and the impairment of host defence. Therefore large programmes with food supplementation were initiated in poor populations. They had very limited effects because the frequent infections continued and they are a major cause of undernutrition. This is due to the reduced appetite during infections, increased losses of nutrients due to vomiting and diarrhoea, impaired digestion and uptake of nutrients, mucosal changes including inflammation caused by released cytokines, NO and activated inflammatory cells on the vast mucosal surfaces of the respiratory tract and gut consuming much energy (*Mata*, 1978, 1992).

Mata, who did much of the classical

work in this field, showed that repeated infections were the main cause of kwashiorkor and marasmus. He also estimated that as much as 21% of yearly calories and 24% of total yearly protein was not consumed by children in Guatemala because of frequent diarrhoea (*Mata*, 1992). The effects of the repeatedly disturbed intestinal flora by all these infections are not quite clear.

On this basis it was understood that undernutrition must be fought as part of the pathology of poverty: prevention and treatment of infections, food supplementation, and education, especially health education, are all essential parts of preventive programmes.

Later it has been demonstrated that although undernutrition per se does not kill, it can add to the problem of repeated infections. Thus underweight children (<70% weight for age) had a higher risk of diarrhoea, especially of chronic diarrhoea (*Bhandari* et al., 1989). Skin test anergy (DTH) related to a higher attack rate and longer duration of diarrhoea (*Koster* et al., 1987). Although in other studies the relation between undernutrition and diarrhoea was modest (*Baqui* et al., 1993), it must be realised that so many confounding factors are at play in the situation of the children studied that it becomes very difficult to define causative relationships. Still *Pelletier* (1994) claims a definite relation between undernutrition and child mortality. Although infections are the major cause of death, undernutrition is a contributing factor. These two risk factors potentiate each other, according to Pelletier 8-10 times more than previous more conservative measures.

It must be stressed again that the undernutrition in field studies of children mostly are not well defined. *Good* and *Lorenz* (1992) could not reproduce the severe immunodeficiency caused by PEM in experimental animals. Only if there was also a zinc deficiency then cell-mediated immunity was impaired. They showed that mice on chronic calorie restriction – undernutrition without malnutrition – had a prolonged life, less cancer and autoimmune diseases (*Good* et al., 1991).

MICRONUTRIENT DEFICIENCIES AND THE IMMUNE SYSTEM

Vitamin A deficiency

While about 250.000 children turn blind from xerophtalmia caused by severe vitamin A deficiency every year, some 125 million children have subclinical vitamin A deficiency. Vitamin A deficiency has numerous effects on the host defence mechanisms and it is clear that it should make a major difference if lack of this micronutrient is part of the deficiency pattern in cases of undernutrition.

Vitamin A deficiency and its effect on host defence

Vitamin A deficiency and its effect on host defence has been mainly studied in experimental animals (*Hanson* et al., 1998). The deficiency impairs serum IgM, IgG and IgE antibody responses to most antigens, but for T cell independent antigens of type 1 (*Pasatiempo* et al., 1990; *Wiedermann* et al., 1993b). The S-IgA response in the bile of rats is reduced by 90% and is linked to fewer antibody producing cells in the mesenteric lymph nodes (*Wiedermann* et al., 1993a). SC is not clearly decreased.

The vitamin A deficient rats are underweight by some 25% due to loss of appetite. However, pair-fed animals repleted with vitamin A have normal antibody responses, which is also seen in deficient rats supplemented with retinoic acid (*Wiedermann* et al., 1993a). This suggests that the immune abnormality is

really related to the vitamin A deficiency, not to the general undernutrition - PEM.

B lymphocytes from vitamin A deficient rats have recently been demonstrated to increase proliferation of activated T cells (*Bjersing* et al., 1997). T cells from vitamin A deficient rats seem to be activated as T_H1 cells, producing increased amounts of IFN-γ and IL-12, but less IL-5 and IL-6 than control animals (*Carman* and *Hayes*, 1991; *Wiedermann* et al., 1993b, 1996b). As a result B lymphocytes get less support presumably contributing to the reduced antibody production.

Delayed type hypersensitivity can be impaired, or increased under different circumstances (*Smith* and *Hayes*, 1987; *Wiedermann* et al., 1993b, 1996a). Children with vitamin A deficiency show reversible disturbances of their T cell populations with reduced $CD4^+$ and $CD45Ro^+$ T cells (*Semba* et al., 1993).

Vitamin A deficiency has effects on numerous other mechanisms which are important for host defence. NK cells are fewer and with lower cytotoxic capacity (*Zhao* et al., 1994). However, their ability to produce IFN-γ seems undisturbed. Uptake of bacteria, as well as killing by macrophages and neutrophils is impaired (*Ongsakul* et al., 1985; *Wiedermann* et al., 1995, 1996b).

Since the differentiation of mucosal epithelium, including goblet cells, are dependent on vitamin A it is obvious that the deficiency impairs the epithelial as well as the mucus barrier (*Rojanapo* et al., 1980; *De Luca* et al., 1994).

Colonising the intestine of vitamin A deficient rats with an *E. coli* of low virulence, these bacteria appeared in large numbers all through the intestinal tract (*Wiedermann* et al., 1995). These rats showed increased levels of serum antibodies to the *E. coli* 0 antigen of the isotypes IgM, IgG and IgE. But the number of IgA secreting B cells in the lamina propria were lower than in vitamin A replete rats. There was also an increased translocation of the *E. coli* to mesenteric lymph nodes, kidneys and joints, inducing arthritis.

Adherence of bacteria to intestinal and respiratory epithelium is increased in vitamin A deficient animals (*Gabriel* et al., 1990; *Schoeb* et al., 1993). This might enhance translocation. Gnotobiotic rats with vitamin A deficiency showed increased translocation to mesenteric lymph nodes after colonisation with *E. coli* strains compared to controls. The expression of P fimbriae did not contribute to the increased translocation. The translocation also occurred in spite of the fact that the vitamin A deficient rats had higher levels of serum IgG and IgM antibodies to the bacteria than the vitamin A replete controls. It might be that these antibodies are not protective, but rather inflammatogenic.

A model of arthritis caused by *Staphylococcus aureus* applied in vitamin A deficient rats showed a worse course of the arthritis (*Wiedermann* et al., 1996b). The T cell response was increased in these animals, while B cell reactivity was unchanged. Phagocytic uptake and killing by phagocytes was decreased as was complement lysis activity.

Measles as well as several other infections are well known to reduce vitamin A levels. Vitamin A supplementation should always be given to children with measles, whether in developed or developing countries.

Rotavirus infections in mice with vitamin A deficiency destroyed the villus tips and exposed the lamina propria to the small intestinal content (*Ahmed* et al., 1990). DTH of these animals was reduced and so was the antibody response to the rotavirus.

The consequences of subclinical vitamin A deficiency in man

The consequences of subclinical vitamin A deficiency in man has been

much debated. A meta-analysis of several vitamin A supplementation studies in poor communities comes to the conclusion that mortality is significantly reduced (*Fawzi* et al., 1993). Several studies find reductions of some 30 per cent (*Anonymous*, 1993; *Fawzi* et al., 1994, 1995).

However, from clinical studies it is not clear how this comes about. They do not clearly show that supplementation saves numerous children from dying in pneumonia or diarrhoea as expected to explain the 30 per cent. However, it was found that there is a reverse relationship between vitamin A deficiency measured as blood retinol and diarrhoea (*Tafesse* et al., 1996). Supplementation reduced the incidence and the severity of diarrhoea as well as mortality (*Fawzi* et al., 1995; *Ross* et al., 1995). However, other studies found no effect, or even an increase in the incidence of diarrhoea of young children during the two first weeks after supplementation (*Bloem* et al., 1990; *Stansfield* et al., 1993; *Dibley* et al., 1996).

The reason for this complex picture is not clear, but may relate to the numerous and various microbes which can cause diarrhoea, the many other factors like the mode of feeding of the child such as partial or exclusive breast feeding, the extent of microbial exposure, the likely complexity of the food deficiencies and the multiple damages on host defence caused by vitamin A deficiency.

This reasoning may hold also for the morbidity in respiratory tract infections which shows a relation to vitamin A deficiency (*Sommer* et al., 1987). However, a relation to mortality has only been suggested (*Fawzi* et al., 1994). Supplementation with vitamin A has shown a reduction in incidence in some studies, but not in others (*Bloem* et al., 1990; *Ramakrishnan* et al., 1995).

Iron deficiency

Iron deficiency, as well as overload, have been shown to cause impaired immune functions (*Bryan* and *Stone*, 1993). Iron deficiency, which is the most common micronutrient deficiency in the world, impairs macrophage function, lymphocyte blastogenesis, NK cell activity and reduces numbers of circulating T cells and thymus size (*Srikantia* et al., 1976; *Rothenbacher* and *Sherman*, 1980; *Kuvibidila* and *Wade*, 1987; *Hallquist* and *Sherman*, 1989). Iron supplementation has been claimed to decrease respiratory and gastrointestinal infections by 50 per cent in anaemic children compared to non supplemented controls (*Mackay*, 1928). Such an effect is supported by later studies (*Latham* et al., 1990; *Sherman*, 1992).

Zinc deficiency

Zinc deficiency was mentioned above as a likely important component in undernutrition, to which it may add further to by decreasing appetite (*Krebs* et al., 1984). Zinc deficiency causes splenic atrophy and decreases T cell responsiveness to antigens and mitogens (*Chandra*, 1985; *Sherman*, 1992). The deficiency also causes reduced IL-4, IFN-γ, peripheral eosinophil levels and serum IgE, as well as IgG1 (*Shi* et al., 1994).

Supplementation with zinc reduces incidence of diarrhoea in children and enhances recovery from persistent diarrhoea (*Tomkins* et al., 1993). There are other studies to support these observations (*Rosado* et al., 1997; *Ruel* et al., 1997).

Deficiencies of other micronutrients like copper, selenium, vitamin E, D, K, B1, B6 and B12 also have consequences for the immune system and it is important to study them further to define when supplementation can be helpful (*Hanson* et al., 1998).

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MODULATION OF IMMUNE RESPONSES TO BACTERIAL VACCINE ANTIGENS IN MICE: USE OF CYTOKINES AS ORAL MUCOSAL ADJUVANTS

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SUMMARY

Oral administration of exogenous cytokines may modulate immune responses, and hence may act as an adjuvant to enhance the efficacy of a co-delivered vaccine. The adjuvant capacity of IL-2 and IFN-γ for inactivated Campylobacter or cholera whole vaccines were investigated in mice. Cytokines delivered via oral route was not toxic, and was able to augment vaccine induced humoral, as well as cellular immune responses. This augmentation varied depending upon the organ system (local vs. systemic), and the type of immune response (cellular vs. humoral) induced, and was a function of the cytokine delivered. This observation suggests that, to some extent, these cytokines induce and regulate immune responses by distinct yet interdependent pathways.

INTRODUCTION

Appropriately prepared and orally delivered, vaccines consisting of dead bacterial cells are safe, but generally lack sufficient immunogenicity to stimulate long-lasting protection against disease. Some examples of these vaccine candidates currently being developed include, *Vibrio cholera* (*Sanchez* et al., 1994), enterotoxigenic *Escherichia coli* (*Wenneras* et al., 1994), and *Campylobacter jejuni* (*Baqar* et al., 1995). Recent attempts to produce improved mucosal vaccines against these bacterial diarrhoeal diseases has focused on the use of inactivated whole bacterial cells co-administered orally with potential mucosal adjuvants.

An effective vaccine against an infection must be capable of inducing the appropriate protective immune responses. It is now known that cytokines have an important role in inducing, regulating and augmenting protective immune responses following infection or vaccination. Therefore, the outcome of a subsequent infectious challenge after vaccination or prior infection with the same agent may be the result of limiting or preferentially producing one or more of these immunological mediators. This suggests that the use of exogenous recombinant cytokines as vaccine adjuvants may provide a mechanism(s) whereby the magnitude and the characteristics of vaccine-specific immune responses could be favourably modulated. Various cytokines administered parenterally or orally, have been shown to be effective immunological adjuvants in animal or human models augmenting protection induced by viral (*Cummins* and *Rosenquist*, 1980; *Schijns* et al., 1994), bacterial (*Miller* et al., 1996) or parasitic (reviewed in *Correlissen* and

Schetters, 1996) vaccines, as well as enhancing anti-tumour immunisation in clinical trials (*Kirchner* et al., 1995). The systemic use of cytokines as therapeutics or vaccine adjuvant has been limited primarily due to its associated toxicity at effective doses; however, this draw back may potentially be overcome by administering these molecules orally. Cytokines delivered orally have also been shown to alter the kinetics and the immune response to bacterial/viral infections in animals (*Chong* 1987; *Baqar* et al., 1993; *Cummins* and *Rosenquist*, 1980;) as well as acted as therapeutics in humans (*Koch* and *Obe*, 1990; *Jordan*, 1994). We have previously reported that selected cytokines administered orally have no apparent side effects, retain their biological activity (*Rollwagen* et al., 1997), and can alter the course of infection, as well as augment immunity to *C. jejuni* infection in mice (*Baqar* et al., 1993). Here we report the results of studies evaluating IL-2 and IFN-γ, both Th1 type cytokines (*Finkelman* et al., 1988; *Fiorentino* et al., 1989), as mucosal adjuvants for co-administered inactivated bacterial whole cell vaccines.

MATERIALS AND METHODS

Mice

BALB/c mice (females, 10-week old) were purchased from Jackson Laboratories Bar Harbor, ME, and housed in laminar flow cages for 10-12 days before being used in these experiments.

Vaccines and adjuvants

The formalin inactivated *C. jejuni* whole cell (CWC) vaccine, prepared as previously described (*Baqar* et al., 1995), was used at 10^8 vaccine particles per dose. The *V. cholerae* whole cells (VWC) vaccine (National Bacteriological Laboratory, Stockholm, Sweden, obtained from the U.S. Army under IND#3842) was delivered at 10^9 vaccine particles/dose. Murine recombinant Interleukin-2 (IL-2) and Interferon gamma (IFN-γ) were purchased from Genzyme Diagnostics, Cambridge, MA.

Enterotoxicity assays

All cytokines or CT was delivered at the indicated concentration using 0.5 ml PBS pH 7.4 supplemented with 0.1% bovine serum albumin (PBS-BSA). After neutralising stomach pH with 5% sodium bicarbonate buffer, 0.5 ml of PBS-BSA alone or containing 50 U to 500 U of IL-2, 40 ng to 160 ng of IFN-γ or 5 mg of *V. cholera* toxin (CT, Swiss Serum and Vaccine Institute, Berne, Switzerland) was delivered orally to 5-7 mice. The details of the oral feeding procedure are reported previously (*Baqar* et al., 1993). Six to 8 h after feeding, animals were euthanised and weighed. The weights of the GI tracts, and remaining carcasses from individual animals were then also determined. Enterotoxicity was directly related to the amount of fluid accumulated in the intestine of mice following oral dosing, and the amount of fluid was calculated as the ratio of the GI tract weight to the remaining carcass weight as:

$$\frac{\text{GI tract weight}}{\text{Body weight - GI tract weight}}$$

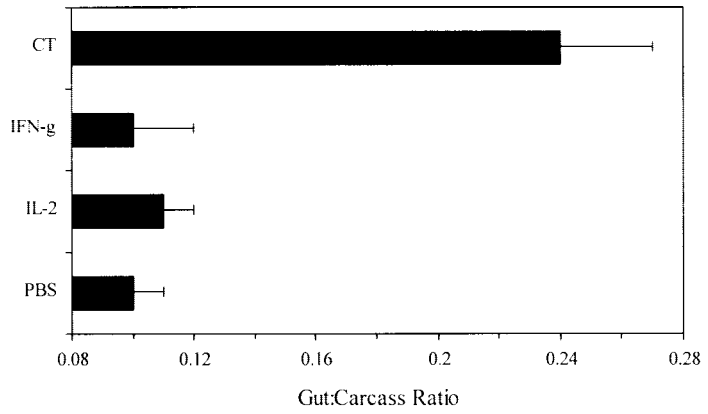

Figure 1: Comparative enterotoxicity of orally delivered cytokines and cholera toxin. Five mice per group were fed 0.5 ml of PBS-BSA alone or containing 500 units of IL-2, or 160 ng of IFN-γ, or 5 mg of cholera toxin. At 6-8 hrs after feeding, gut:carcass ratios for individual mice were determined. The figure presents mean and standard deviation values for these ratios derived from the indicated groups.

Vaccination

Mice (10-15 per group) received two oral doses at 8 day intervals, of 0.5 ml PBS-BSA only, or PBS-BSA containing 500 U of IL-2, or 10^8 CWC vaccine particles, or the same amount of vaccine delivered in combination with 500 U of IL-2. The VWC was tested using a similar vaccination regimen, except that 10^9 vaccine particles were used. The VWC vaccine was also delivered with 160 ng of IFN-γ.

Humoral immune responses

Campylobacter specific sIgA (intestinal lavage collected 7 days after vaccination; *Baqar* et al. 1993) were measured using an isotype-specific enzyme-linked immunosorbent assay (ELISA). Mononuclear cell suspensions prepared from Peyer's patches, lamina propria (*Hornqvist*, 1991), peripheral blood, spleen and mesenteric lymph nodes (*Baqar* et al., 1996), were used to enumerate vaccine antigen specific IgA and IgG antibody secreting cells (ASC). Details of the ELISA procedure (*Baqar* et al., 1993) and the methods employed to determine ASC in tissues (*Baqar* et al., 1996) are published.

Cell mediated immune responses

Mononuclear cells isolated from mesenteric lymph nodes and spleens were used to determine vaccine antigen specific *in vitro* lymphocyte proliferation 28 days after vaccination. Cells (10^5) were stimulated in triplicate in the presence of 10^5 inactivated bacterial cells for 7 days, at which time radioactive thymidine was added. After an additional 16 hrs incubation, tritium containing DNA was determined using standard liquid scintillation procedures. The procedures used for lymphocyte replication assays and data analysis are published elsewhere (*Murphy* et al., 1987, 1989).

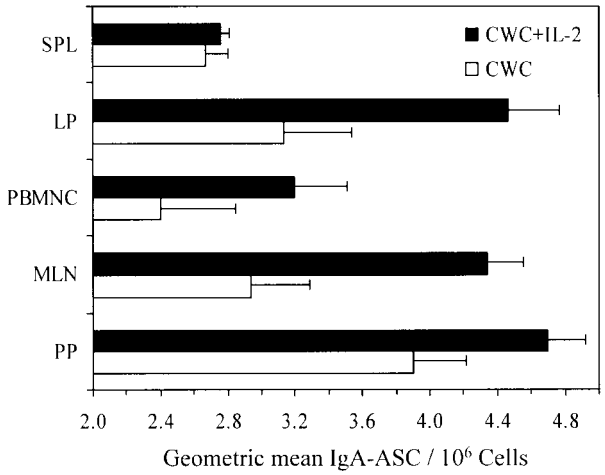

Figure 2: *Campylobacter* specific IgA-ASC responses after immunisation with inactivated CWC vaccine. Seven days after immunisation cells from 5-6 mice were separated individually from the indicated tissues and organs. The number of glycine extract antigen specific ASC/10^6 mononuclear cells was determined for each mouse. The figure presents geometric mean (loge) and standard deviations for the indicated groups. Abbreviations used; PP Peyer's patches; MLN mesenteric lymph nodes; PBMNC peripheral blood mononuclear cells; LP lamina propria; SPL spleen.

RESULTS

Enterotoxicity of orally delivered cytokines

The enterotoxicity of orally delivered cytokines were evaluated at a range of doses that were potentially thought or previously reported (*Baqar* et al., 1993) to have adjuvant action in mice. IFN-γ was tested across a 40 to 160 ng/mouse dosing range, and IL-2 at 50-500 units/mouse. In addition, a group of mice received 5 mg of CT as a positive control for enterotoxicity. The data presented in Figure 1 demonstrate that mice receiving only PBS-BSA exhibited no significant fluid accumulation as evidenced by a mean gut:carcass ratio of 0.10±0.01 (mean±standard deviation). CT stimulated a substantial amount of fluid secretion (ratio of 0.24±0.03), while the doses of IL-2 and IFN-γ examined failed to show any enterotoxicity giving gut:carcass ratios (IL-2: 0.11±0.01; IFN-γ: 0.10±0.02, data shown are for the highest dose only) which were essentially identical to those seen in control (PBS) mice.

C. jejuni vaccine

Inactivated CWC vaccine given alone or in combination with IL-2 was well tolerated with no apparent vaccine associated side effects being observed in mice.

Immune response to *Campylobacter* antigen

Separate groups of mice were immunised with PBS, IL-2, CWC or CWC+ IL2 and *Campylobacter* specific IgA-ASC were determined in tissues and peripheral blood samples collected 7-10 days after immunisation (Figure 2). No antigen specific ASC were detected in animals receiving PBS or IL-2. *Campylobacter* whole cell vaccine alone induced only a marginal ASC response, whereas, the response was augmented when the vaccine was delivered

Table 1: Murine IL-2 as an oral adjuvant for inactivated *Campylobacter* whole cell vaccine

Campylobacter specific[a] sIgA			*Campylobacter* colonisation[b]		
Immunisation	Titre	% Responder	log10/mg	Excretion (%)	Efficacy (%)
PBS	2.86±0.73	0	3.11±0.4	100	0
IL-2	3.06±0.88	0	2.40±0.8	100	0
CWC	3.44±0.89	14	2.04±0.5	57	43
CWC+IL2	4.75±0.48	71	1.47±0.5	28	72

[a]: *C. jejuni* 81-176 glycine extracted proteins (*Logan* and *Trust*, 1982) were used as antigens. Data are presented as geometric mean ($\log_e$) and standard deviation (stds). Responders are animals whose end-point titres were >4.32 (geometric mean + 2 stds of PBS mice = 4.32).
[b]: Four weeks after vaccination mice were orally challenged with $8x10^9$ CFU of *C. jejuni* 81-176, 6 days following challenge, faecal samples from individual mice were collected and CFU of *C. jejuni* / mg of faeces were determined. Vaccine efficacy was calculated as:

$$\frac{\text{\% control colonised - \% vaccinee colonised}}{\text{\% control colonised}} \times 100$$

with IL-2. The adjuvant effect of IL-2 was differentially expressed within the various tissues examined. The most pronounced immune enhancing effect of IL-2 was seen in mesenteric lymph nodes (2.9±0.35 vs. 4.3.4±0.21) and the lamina propria of vaccinated mice (compare the CWC vs. CWC+IL2 ASC responses in Figure 2). The detection of similar ASC response levels in the spleens of animals immunised with CWC or CWC+IL2 may be, in part, due to the less than optimal time (7-10 days) that these cells were collected to enumerate ASC.

Intestinal lavage fluid collected from PBS or IL-2 immunised mice had no detectable levels of *Campylobacter* specific sIgA (Table 1). When CWC was delivered alone, only 14% of vaccinated mice mounted a significant antigen specific sIgA response. In contrast, administering the same dose of CWC vaccine with IL-2 resulted in a significant enhancement in the number of sIgA responders (71%) to this vaccine.

Acquired resistance to *Campylobacter* infection

Vaccine efficacy was determined by orally challenging mice with *C. jejuni* 28 days after primary immunisation. Six days following challenge, 100% of PBS or IL-2 immunised animals were shedding the challenge organisms in their faeces, whereas, only 57% of CWC immunised and 28% of CWC+IL2 immunised animals remained colonised at this time. Mice which were positive for colonisation were excreting bacteria at much lower level (3.11±0.4 vs. 1.47±0.5 $\log_{10}$CFU/mg of faeces). Vaccine efficacy based on intestinal colonisation was calculated to be 43% and 72% for CW or CWC+IL2 immunised groups, respectively (Table 1).

V. cholerae vaccine

Inactivated VWC vaccine alone or in combination with IL-2 or IFN-γ was well tolerated with no apparent vaccination associated side effect being seen in mice.

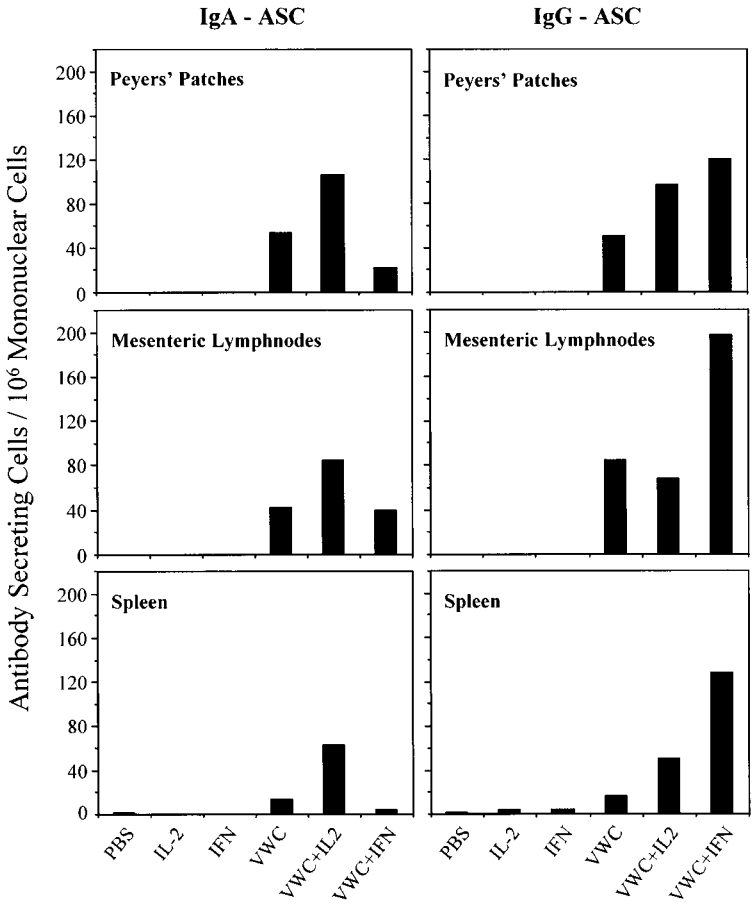

Figure 3: *V. cholerae* 01-LPS specific IgA- and IgG-ASC responses after immunisation with inactivated VWC vaccine. Seven days after immunisation cells from 5-6 mice were separated individually from the indicated tissues and organs. The number of 01-LPS specific ASC/10^6 mononuclear cells was determined for each mouse. The geometric mean (loge) and standard deviations for these values within the various group were determined. The figure presents these mean values after back-transformation to linear form. The standard deviations remained between 2-14%.

Humoral immune response to *Vibrio* antigen

Experiments were done to learn if humoral immune responses to cholera vaccine associated antigens could be enhanced by co-administering the VWC vaccine with IL-2 or IFN-γ, as oral mucosal adjuvants. Mononuclear cells isolated from Peyer's patches, mesenteric lymph nodes and spleens were used to detect cholera 01-LPS specific IgA- and IgG-ASC 7-10 days after vaccination (Figure 3). Oral administration of cholera vaccine alone resulted in a significant IgA-ASC response in Peyer's patches and mesenteric lymph nodes. These responses were further enhanced when the vaccine was delivered with IL-2, but not with IFN-γ. Compared to other tissues, the IgA-ASC response pattern in the spleen was different; VWC alone was a poor immunogen, but inclusion of IL-2 at the time of vaccination enhanced the response in this organ. In contrast to IgA responses, IL-2 had only a minimal adjuvant effect on

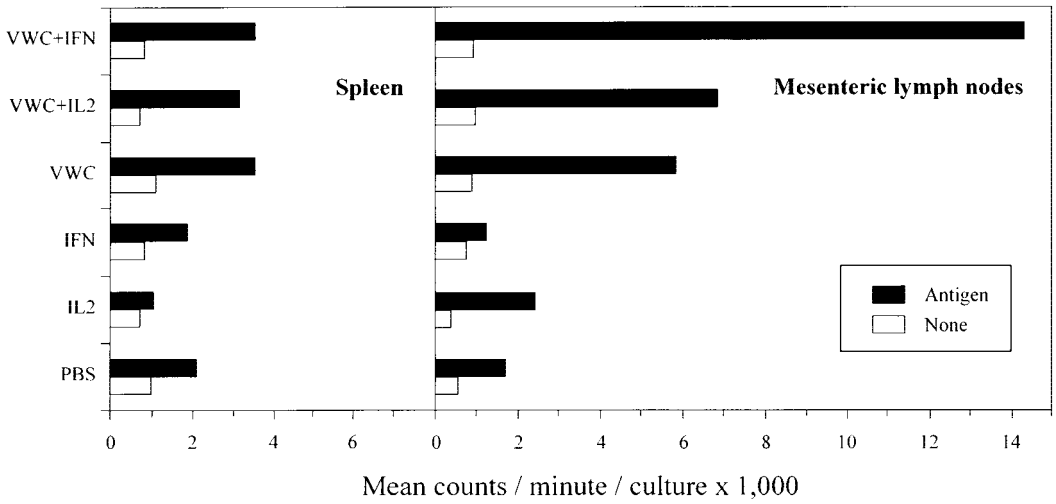

Figure 4: *Vibrio cholerae* whole cell antigen specific lymphocyte replicative responses after immunisation with inactivated cholera whole cell vaccine. The counts per minute for each culture well were loge transformed and geometric means were calculated. Data are presented as back transformed means, standard deviations were <13%.

vaccine specific IgG-ASC. However, IFN-γ substantially enhanced 01-LPS specific IgG-ASC in mesenteric lymph nodes and spleens.

Cellular immune response to *Vibrio* antigen

To determine the extent to which cell-mediated immune responses may develop following immunisation with the VWC vaccine alone or the IL-2 or IFN-γ supplemented formulations, spleen and mesenteric lymph nodes from individual (5 mice per group) animals were isolated and cultured in medium alone or medium containing VWC vaccine. Spleen and mesenteric lymph node cells from the various vaccination groups exhibited similar rates of basal lymphocyte replication (Figure 4: open bars). Cells from all the groups responded similarly to stimulation with Concanavalin A, thus vaccination did not cause non-specific stimulation or suppression of lymphocyte replication (data not shown). The proliferative response patterns differed substantially when lymphocytes from immunised and control (PBS, IL-2 or IFN-γ immunisation) mice were cultured *in vitro* in the presence of cholera whole cell antigens. Spleen and mesenteric lymph node lymphocytes from control mice showed low (mean CPM <3,000) and similar replication responses to vaccine antigens. Inclusion of either cytokine with VWC vaccine did not enhance the cholera antigen specific replicative responses of the splenocytes (mean CPM of 3,570; 3,170; 3,566 for VWC, VWC+IL2, VWC+IFN-γ respectively). In contrast, compared to spleen cells, mesenteric lymph node cells from VWC+IFN-γ vaccinated animals showed substantially enhanced proliferative responses to *Vibrio* antigens (mean CPM 14,3331 vs. 5,830 for VWC).

DISCUSSION

Recombinant IL-2 and IFN-γ retained their biological activity when orally delivered to mice. The doses used were sufficient to initiate a physiological/immunological cascade of action(s) to modulate protective immune responses without causing any detectable enterotoxicity. Some of these cytokines are known to be acid-stable and it has been suggested that recombinantly produced proteins that are glycosylated may resist proteolysis in the intestine (*Rollwagen* and *Baqar*, 1996). It has also been shown, that it takes at least 20 minutes in the presence of high concentration of trypsin or chymotrypsin to inactivate IL-6 *in vitro*. *In vivo* studies also showed that a longer incubation was necessary to inactivate IL-6 than the "normal" transit time through the gut. In this study where nonoglycosylated cytokines were used, administering these molecules with a carrier protein (BSA) after neutralising stomach acidity may have protected them from complete or partial digestion.

Previous work in our laboratory has shown the induction of various cytokines in response to *C. jejuni* infection in mice (*Baqar* et al., 1991). We have shown that exogenous orally delivered cytokines (IL-2, IL-5, IL-6) could modulate the course of *C. jejuni* infection and also enhanced protective immune responses in mice (*Baqar* et al., 1993). In the present study, we found that immune responses to cholera vaccine and protective immunity against *Campylobacter* colonisation in mice were induced only when inactivated bacterial whole cell vaccines were given with IL-2 or IFN-γ at the time of vaccination.

An adjuvant when delivered with an antigen, is capable of selectively enhancing specific immune responses *in vivo*. In mice immunised with vaccine alone (no cytokine added), immune responses and protection against infection were minimal, whereas, when the same vaccine was given with IL-2 or IFN-γ a distinct enhancement of vaccine-specific immune responses were observed. This augmentation varied depending upon the organ system (local vs. systemic), and the type of immune response (cellular vs. humoral) induced, and was a function of the cytokine delivered. This observation suggests that, to some extent, these cytokines induce and modulate immune responses by distinct yet interdependent pathways. We have previously (*Rollwagen* et al., 1997) shown, that ^{125}I-labelled IL-6 or IL-2 fed to mice have different distribution patterns *in vivo*; IL-6 remained in the gut for up to 6 hrs post administration whereas, IL-2 counts were more uniformly distributed among local and systemic compartments.

The mechanism(s) underlying the adjuvant effect of IL-2 or- IFN-γ remains to be determined. These T cell subset-specific cytokines are known to regulate and expand their own subset, although at times the activation of Th1 vs. Th2 subsets could be mutually exclusive (*Fiorentino* et al., 1989). Thus, administration of particular cytokines during immunisation might be expected to influence the physiological cytokine balance; and may serve to inhibit or stimulate antigen-specific immune reactions. The fact that these orally administered molecules can modulate humoral, as well as cellular immune responses both at the local site and systemically, suggests that they can also exert their adjuvant effect beyond the intestinal mucosa, the primary site of delivery. Although some biological activity is probably destroyed when cytokines are given by this route, a sufficient amount apparently remains to effect immunomodulatory functions which leads to enhanced protective immune responses to killed bacterial vaccines.

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SELECTIVE MODULATION OF MUCOSAL IMMUNE RESPONSES BY CYTOKINES ENCODED IN VACCINE VECTORS

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SUMMARY

We have developed vector systems encoding vaccine antigens and cytokines and have used these constructs to selectively modulate mucosal antibody and cell-mediated immune (CMI) responses. Information gained from studies in cytokine-deficient mice pointed to the importance of Th2-type factors, particularly IL-4 and IL-6, for the development of mucosal antibody responses, whilst type 1 factors, such as IL-12, would be expected to promote CMI. In this article, we describe the use of three distinct vectors, each constructed to encode cytokine genes along with the gene encoding a model vaccine antigen, the haemagglutinin (HA) of influenza virus, for the delivery of these proteins *in vivo* for mucosal immunomodulation. The vectors included DNA vaccines and poxviral vectors, including vaccinia virus (VV) and fowlpoxvirus (FPV). Each of these systems elicited measurable mucosal antibody responses when delivered locally and these were, in all cases, significantly enhanced by the co-expression of IL-6. In contrast, the mucosal delivery of IL-12 by DNA vaccine had little influence on mucosal antibody responses but stimulated good CMI responses. Indeed, mucosal CMI was not detected unless IL-12 was co-expressed with the vaccine antigen. The combined use of DNA and FPV vectors encoding the same vaccine antigen in a consecutive immunisation strategy gave rise to mucosal antibody responses of greatly enhanced magnitude and duration, even when the DNA priming dose was given by a systemic route. In addition, mucosal delivery of DNA vaccine primed not only for strong local responses but also for specific antibody production at distant mucosal tissues. Finally, the co-expression of IL-6 significantly further enhanced responses stimulated by consecutive immunisation. Thus, vector-driven cytokine delivery represents a powerful approach for the selective modulation of the mucosal immune system.

INTRODUCTION

Cytokines are hormone-like molecules produced by immune cells and play a critical role in intercellular communication, being important determinants of both the type and magnitude of immune responses. T cells which bear the CD4 marker from mice undergoing vigorous immune responses commonly display

one of two major patterns of cytokine synthesis (*Mosmann* and *Coffman*, 1989), and a similar pattern of T cell diversity may exist in humans. The so-called Th1-type cells, whose development is driven by interleukin-12 (IL-12) produced largely by macrophages, secrete interleukin-2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α). Functions mediated by these cells are largely concerned with cytotoxic activity, thus a Th1-based immune response would be most appropriate in defence against viruses and other intracellular pathogens. In contrast, though not exclusively, help for antibody production by B cells is readily provided by another major subset, the Th2-type cells. Cytokines produced by these cells include IL-4, IL-5, IL-6 and IL-10, all of which stimulate B cell growth and differentiation *in vitro*, preferentially inducing antibody responses of the IgG1, IgE and IgA isotypes. In this article, we describe the use of molecular approaches to study the role of cytokines, particularly the Th2-type factors, in the development of immune responses *in vivo*. We also describe our experiments showing that they may act as strong, natural B cell adjuvants capable of modulating antigen-specific antibody responses, particularly at mucosae. Similarly, IL-12 and Th1-type factors are potential adjuvants for cell-mediated immunity. Our approach has been to present these factors as components of delivery vectors, including vaccinia virus, fowlpox virus and DNA vaccines in order to selectively modulate mucosal immunity. We also describe the use of DNA and fowlpox virus vectors in consecutive immunisation strategies which elicit enhanced immune responses, both locally and at distant mucosal sites.

CYTOKINE REGULATION OF MUCOSAL IMMUNE RESPONSES

There is now a plethora of data from *in vitro* and *in vivo* studies supporting a major role for the Th2-type cytokines, IL-4, IL-5 and IL-6, in the development of mucosal IgA reactivity. Th2-type cells occur at high frequency in mucosal tissues (*Taguchi* et al., 1990; *Xu-Amano* et al., 1992), while cells expressing mRNA for Th2 cytokines predominate in the murine small bowel (*Bao* et al., 1993). Message for IL-4 and IL-5 is detected in Peyer's patches and all three factors are abundantly expressed in the lamina propria at sites of IgA production (*Bao* et al., 1993). IL-4 has been shown to promote the production of antibodies of the IgA isotype in murine B cell lines (*Lin* et al., 1991) and has been considered an essential factor for the switching of surface (s)IgM$^+$ mucosal B cells to sIgA expression in both mice (*Ehrhardt* et al., 1992) and humans (*Islam* et al., 1991). Murine IL-5 appears to have no activity on mucosal B cells which do not express sIgA, but promotes the development of activated mucosal sIgA$^+$ B cells *in vitro*. In this respect, IL-5 may act alone (*Beagley* et al., 1988) or in synergy with IL-4 (*Murray* et al., 1987), IL-6 (*Kunimoto* et al., 1989) or transforming growth factor (TGF)-β, (*Coffman* et al., 1989). Thus, IL-5 has been regarded as a terminal differentiation factor rather than a switch factor for sIgA$^+$ B cells, although there is no direct evidence that it normally plays such a role *in vivo*. Interleukin-6 also selectively and potently enhances IgA production *in vitro* by isotype-committed B cells but not sIgA$^-$ B cells (*Beagley* et al., 1989). The presence of T cells, macrophages and other cells known to produce IL-6 (*Fujihashi* et al., 1991; *Mega* et al., 1992). and the high incidence and widespread distribution of

cells containing IL-6 mRNA in intestinal mucosa (*Bao* et al., 1993), are also suggestive of an important role for this factor in regulating IgA responses. On the basis of these and many other published studies, it is thought likely that Th2-type cytokines, particularly IL-5 and IL-6, direct the development of IgA B cells arriving in the submucosa following their exposure to antigen in the organised mucosal lymphoid tissues.

The recent development of strains of mice with targeted disruption of genes encoding IL-4 (*Kopf* et al., 1993), IL-5 (*Kopf* et al., 1996) or IL-6 (*Kopf* et al., 1994) has provided a novel approach to *in vivo* studies of cytokine regulation of mucosal immunity, allowing analysis of the development of immune responses in the absence of these factors. These mice show varying degrees of immune dysfunction, although all appeared to develop normally. The IL-4-deficient mice (IL-4$^{-/-}$) had normal numbers of B cells and T cells and unaltered distribution of typical surface markers, however their ability to produce Th2-type factors with or without antigenic stimulation was severely impaired (*Kopf* et al., 1993). Serum antibody levels of the IgG1 isotype were markedly diminished and IgE antibody was not detected, presumably as a direct result of the IL-4-deficiency. It was concluded that immune responses dependent on Th2-derived cytokines were disrupted in these animals. Interleukin-6$^{-/-}$ mice produced normal levels of cytokines other than IL-6 and had unaltered numbers of B cells but a significant reduction in T cell numbers (*Kopf* et al., 1994). They were unable to control infection with intracellular parasites such as vaccinia virus and *Listeria monocytogenes*. While baseline serum immunoglobulin levels appeared normal in IL-6$^{-/-}$ mice, their ability to mount specific serum IgG antibody responses following virus infection was markedly impaired. Their acute phase responses after tissue damage or infection were also compromised. These mice provided an ideal opportunity to study the relevance of Th2-type factors for mucosal IgA development.

The absence of IL-4 and the resultant down regulation of the Th2 phenotype in IL-4$^{-/-}$ mice suggested that these mutants may be impaired in their ability to produce mucosal IgA antibodies. However, we found that neither IgA antibody levels in lung lavage fluid nor numbers of IgA-secreting cells in the lungs differed in IL-4$^{-/-}$ mice and wild-type mice that had not been deliberately immunised (*Ramsay*, unpublished data). These results demonstrated that the ability of mucosal B cells to undergo isotype switching to IgA production was not dependent on the presence of IL-4. Nevertheless, we also found that IL-4$^{-/-}$ mice had smaller and fewer small intestinal Peyer's patches than wild-type mice and poor germinal centre development, a finding also reported by others (*Vajdy* et al., 1995). IL-4-deficiency also resulted in a marked inability to mount intestinal IgA responses following oral immunisation with soluble proteins in the face of a strong response to cholera toxin given as a component of the inoculum (*Vajdy* et al., 1995). This deficiency may have been due to a failure of IL-4$^{-/-}$ mice to develop the antigen-specific Th2 cells and B cells required to induce germinal centre activity in organised mucosal lymphoid tissues of the gut. We have found that antiviral IgA responses in the lung are also diminished but not ablated in IL-4$^{-/-}$ mice (*Ramsay*, unpublished data). Thus, the importance of IL-4 for the optimal development of Th2-driven immune responses is here illustrated in the context of mucosal immunity. Clearly however, isotype switching and mucosal IgA production is able to occur in the absence of this factor.

In contrast, IL-5$^{-/-}$ mice displayed little defect in systemic antibody or mucosal IgA responses despite the above

Table 1: Interleukin-6 regulates mucosal antibody responses *in vivo*

		Anti-HA antibody-secreting cells per 10^6 cells			
		IgA		IgG	
Mouse strain	Virus	day 8	day 15	day 8	day 15
IL-$6^{-/-}$	VV-HA	5 ± 1	2 ± 1	3 ± 1	6 ± 1
	VV-HA-IL-6	144 ± 15	96 ± 7	159 ± 10	98 ± 3
wild-type	VV-HA	32 ± 6	36 ± 6	15 ± 1	48 ± 3
	VV-HA-IL-6	76 ± 13	29 ± 7	40 ± 2	47 ± 2

Groups of five mice were given 10^7 plaque-forming units (PFU) rVV intranasally and lungs were removed on the days indicated for determination of numbers of HA-specific antibody-secreting cells by ELISPOT assay (*Ramsay* and *Kohonen-Corish*, 1993).

mentioned *in vitro* evidence to the contrary (*Kopf* et al., 1996). IgA B cell numbers in the intestinal lamina propria were similar in unimmunised IL-$5^{-/-}$ and wild-type mice and no significant differences were found in lung and small intestinal IgA responses in IL-$5^{-/-}$ and wild-type mice following local immunisation with recombinant vaccinia virus (rVV) constructs. In addition, specific lung antibody responses following sublethal intranasal infection with influenza virus were not affected by IL-5-deficiency (*Kopf* et al., 1996). Thus, whilst IL-5 is apparently abundantly expressed in mucosal tissues and may be used to stimulate mucosal IgA responses, it does not appear to play a crucial role in IgA production *in vivo*.

Interleukin-6, however appears to play a major role in the optimal development of IgA responses (*Ramsay* et al., 1994). In the absence of deliberate immunisation, IL-$6^{-/-}$ mice had fewer IgA plasma cells in mucosal tissues compared to wild type mice and these cells stained less intensely in the mutants. It is thought that over 40% of murine intestinal IgA cells are not conventional B2 cells, but B1 cells, many of which bear the CD5 marker and populate the gut from the peritoneal cavity rather than mucosal lymphoid tissues (*Pecquet* et al., 1992). Such cells do not require IL-6 for IgA secretion and may, therefore, account for some or all of the residual numbers of $sIgA^+$ cells in IL-$6^{-/-}$ mice (*Beagley* et al., 1995).

IL-6 deficiency also had major effects on the development of mucosal antibody responses to conventional B cell antigens (*Ramsay* et al., 1994). Mutants immunised locally with soluble protein mounted poor intestinal IgA responses. In addition, their production of anti-haemagglutinin (HA) IgA and IgG antibodies and numbers of HA-specific IgA and IgG ASC in lungs following intranasal immunisation with VV encoding HA (VV-HA) were dramatically lower than in wild-type mice. The ability of IL-$6^{-/-}$ mice to mount sustained mucosal antibody responses was dramatically restored when VV-HA-IL-6 was used to reconstitute the expression of IL-6 in the lungs (Table 1). These findings provide compelling evidence that IL-6 plays a major role in the development of mucosal antibody responses to virus infection and, perhaps, conventional B cell antigens in general. Indeed, deficient mucosal IgA responses and, in some cases IgG responses, were found in IL-$6^{-/-}$ mice challenged mucosally with *Candida albicans* (*Romani* et al., 1996), although not when given *Helicobacter*

felis or soluble protein together with the mucosal adjuvant cholera toxin (*Bromander* et al., 1996). IL-6 may be less important for the development of B1 cells, which supply half of the IgA-producing cells in the gut and other tissues and which apparently respond to a different set of antigens than conventional B cells (*Beagley* et al., 1995).

In summary, these studies have confirmed that Th2 factors are important for some but not all of the activities attributed to them in earlier published work. It should be remembered, however, that mutant mice develop in the absence of the factor encoded by the disrupted gene and also that alternative mechanisms may have compensated where no observable effect of a particular cytokine deficiency was observed. It appears that IL-4 is important for the optimal development of at least some mucosal IgA responses and of functional mucosal lymphoid tissues, but is not crucial for IgA isotype switching. IL-6 also seems to play an important role at mucosae, probably in the terminal differentiation of antibody-secreting plasma cells. Conventional mucosal IgA and IgG B cell responses, but not B1 cell development, are impaired in IL-$6^{-/-}$ mice.

Thus, both *in vitro* and *in vivo* studies in several species indicate that cytokines secreted by Th2-type cells play important roles in mucosal B cell development. Such findings suggest that these factors, particularly IL-4 and IL-6, are worthwhile candidates for testing as adjuvants for antibody responses at mucosae. The corollary of this assumption is that type 1 cytokines, including IL-12, may be useful adjuvants for cell-mediated immune responses. Thus, vector-directed expression of different cytokines may represent an effective approach to the selective immunomodulation of the mucosal immune response.

VECTOR DELIVERY OF CYTOKINES FOR MUCOSAL IMMUNOMODULATION

The above mentioned findings that type 2 cytokines are important for the development of B cell responses led us to examine their potential for selective manipulation of vector-driven mucosal immunity. Enhancement of the magnitude and longevity of specific IgG and mucosal IgA responses would have major implications for successful immunoprophylaxis, and to this end, we have established a murine model to test the ability of co-expressed cytokines to modulate immune responses to vaccine antigen. This has involved the construction and testing of a range of replicating and non-replicating vectors which co-express different cytokine genes along with the HA gene of influenza virus A/PR/8/34 as a model vaccine antigen (*Ramshaw* et al., 1992, 1997).

Attempts have been made to modify immune responses by the administration of recombinant cytokines, however this work has been hampered by difficulties in targeting these factors to sites of immune reactivity and by their short half-life *in vivo*. However, virus constructs produce the encoded factor and secrete it from infected cells such that the pattern of virus replication determines the level and sites of production of the cytokine. Using this system, we have studied the immunoregulatory and antiviral properties of a number of factors. Vaccinia and other poxvirus vectors now being developed have the capacity to carry enough heterologous DNA to encode multiple genes, to allow faithful tran-

Table 2: Sustained mucosal antibody responses elicited against vaccine antigen encoded in fowlpoxvirus vectors are enhanced by co-expression of IL-6

		Anti-HA antibody-producing cells / 10^6 lung lymphoid cells	
	Virus	IgG	IgA
Day 14	FPV-HA	5.5	3.0
	FPV-HA-IL-2	5.0	3.2
	FPV-HA-IL-6	38.9	30.6
Day 28	FPV-HA	11.4	9.5
	FPV-HA-IL-2	15.9	10.2
	FPV-HA-IL-6	93.3	15.6

Groups of five mice were given 10^7 PFU rFPV intranasally and lungs were removed on the days indicated for determination of HA-specific ASC numbers by ELISPOT.

scription and translation from these inserted genes and appropriate post-translational processing and transport (*Moss* and *Flexner*, 1987) and, therefore, to facilitate prolonged and enhanced production of desired antigens in host cells. We have expressed HA along with genes encoding cytokines in recombinant vaccinia virus (rVV), fowlpoxvirus (FPV) and DNA vaccine vectors in attempts to enhance mucosal and systemic antibody responses.

Initially we found that type 2 cytokines encoded in rVV vectors may increase mucosal IgA and IgG responses against HA five to ten-fold. Lung immunocytes secreting antibodies specific for the co-expressed HA glycoprotein were significantly enhanced in mice given VV-HA-IL-5 than in those given the control virus (*Ramsay* and *Kohonen-Corish*, 1993). The elevated response peaked on day 14 after infection at 4-fold greater than control levels but had declined by day 28, much later than in controls. Interleukin-5 given in this manner did not appear to affect local IgG responses or systemic reactivity. We also found four-fold increases in secretory IgA ELISA titres in lung fluids of mice given rVV expressing IL-5 by 28 days post-infection, and in those given VV-HA-IL-6 at both 21 and 28 days (*Ramsay*, 1995). Thus, both IL-5 (notwithstanding our findings in IL-5-deficient mice) and IL-6, when expressed in replicating rVV, were effective stimulators of antigen-specific mucosal IgA responses (Table 1).

Fowlpoxvirus is another poxvirus currently being tested as a vaccine vector. This agent has a highly restricted host range, conferring the potential advantage that its replication is blocked in mammalian cells, although heterologous genes under the control of early promoters are expressed, resulting in presentation of the encoded vaccine antigen to the immune system (*Somogyi* et al., 1993). This makes the virus extremely safe but nonetheless highly immunogenic. We have studied the capacity of rFPV to deliver IL-6 as an adjuvant for systemic IgG and mucosal IgA responses and found marked increases in these responses (*Leong* et al., 1994). Strong specific IgA and IgG responses were found in the lungs of mice given intranasal inocula of FPV-HA-IL-6 by 2 weeks after immunisation which were up to ten-fold higher than those detected in mice given control virus or FPV expressing IL-2 (Table 2). Responses at 4 weeks were still elevated in mice given

Table 3: Selective modulation of mucosal antibody and CTL responses by cytokines encoded in DNA vaccines

DNA vaccine	Antibody response in lungs (anti-HA ASC /10^6 cells)		Anti-HA CTL in lungs
	IgA	IgG	(% specific lysis)
pHA	94 ± 15	152 ± 35	<5
pHA + pIL-6	320 ± 47	510 ± 52	<5
pHA + pIL-12	72 ± 19	318 ± 30	32

Mice were immunised twice (day 0, day 21) intra-tracheally with DNA in lipofectin and sacrificed on day 35 following exsanguination of lungs. Lung cell isolates were assayed for specific antibody production (ELISPOT) and CTL (^{51}Cr release assay).

FPV-HA-IL-6, particularly numbers of specific IgG-secreting cells. Reactivity was further elevated when mice were boosted with FPV-HA-IL-6 or challenged with a sublethal dose of homologous wild-type influenza virus (*Leong* et al., 1994), suggesting that vector-driven IL-6 may both prime for enhanced responses as well as stimulating the development of mature IgA- and IgG-producing cells.

DNA plasmids (naked DNA or nucleic acid vaccines) represent novel vectors offering great promise as vaccines. The features of these constructs most relevant for improved vaccination are now well known (*Fynan* et al., 1993; *Pardoll* and *Beckerleg*, 1995; *Ramsay* et al., 1997). Briefly, DNA plasmids are non-replicating, non-infectious and non-integrating and are stable and easier to prepare at lower cost than other vectors or protein immunogens. Multiple genes may be expressed in these constructs and several different routes of inoculation have been shown to be effective (including direct mucosal administration). At least systemically, long-lived immune responses have been generated in many species following DNA immunisation, with the early development of high affinity antibodies. The major characteristic of DNA plasmids of relevance for vaccination and immunomodulation is an ability to persist in the host with sustained presentation of the encoded gene and the resultant potential for sustained immune responses. DNA vaccines have most often been administered via intramuscular injection or particle-mediated ballistic transfer into the dermal layer of the skin (the "gene gun" approach), but have also induced protection in mice when given intranasally prior to an otherwise lethal challenge with influenza virus (*Fynan* et al., 1993). We have confirmed that HA encoded in DNA plasmids elicits sustained serum antibody responses over several months in mice when given via the gene gun or intramuscularly (*Ramsay* et al., 1997). We have also encoded type 2 cytokines in DNA plasmids and observed a clear enhancement of these responses when given in a cocktail along with the HA-encoding DNA vaccine (unpublished data). More recently, we have administered DNA vaccines encoding either IL-6 or IL-12 and have administered these intra-tracheally in combination with DNA encoding HA in attempts to boost mucosal antibody and cell-mediated immune responses, respectively. In order to "target" the DNA constructs for uptake by mucosal cells, they were delivered in a lipid solution, as our previous studies had shown little evidence

Table 4: Mucosal anti-HA antibody responses after consecutive immunisation with DNA and FPV

Immunisation (i.m.)	Boosting (i.n.)	Anti-HA ASC per 10^6 cells (mean ± SD)	
		IgG2a	IgA
Week 1 post-boost			
pCMV/HA	FPV-HA	382 ± 46	195 ± 54
pCMV/HA	FPV-HA-IL6	1,324 ± 93	769 ± 48
pCMV/control	FPV-HA	145 ± 10	60 ± 21
pCMV/control	FPV-HA-IL6	359 ± 111	89 ± 40
Week 3 post-boost			
pCMV/HA	FPV-HA	135 ± 15	282 ± 23
pCMV/HA	FPV-HA-IL6	540 ± 63	793 ± 63
pCMV/control	FPV-HA	<2	<2
pCMV/control	FPV-HA-IL6	63 ± 20	87 ± 18

Mice were given FPV 28 days after priming with DNA and lungs were taken at 1 or 3 weeks thereafter for assessment of anti-HA antibody responses by ELISPOT.

for responsiveness following delivery in saline. Marked increases in mucosal anti-HA IgA and IgG antibody responses were observed in mice given DNA-IL-6 in lipid solution along with DNA-HA, whilst significant levels of specific cytotoxic T cell activity were found in the lungs of those given DNA-IL-12 together with DNA-HA (Table 3).

ENHANCEMENT AND MODULATION OF MUCOSAL IMMUNITY BY CONSECUTIVE VACCINATION WITH DNA AND FPV VECTORS

We have also devised a consecutive immunisation strategy involving intramuscular priming by DNA vaccination and boosting with poxvirus vectors encoding the same vaccine antigens in attempts to generate improved specific immune responses. The viruses used in these studies were rVV and FPV, which we have previously developed as vectors for the induction of long-lasting immune responses to heterologous vaccine antigens (*Ramshaw* et al., 1992; *Leong* et al., 1994). These responses have been enhanced, as described above, by the co-expression of genes encoding cytokines (*Ramshaw* et al., 1992; *Leong* et al., 1994; *Ramsay* et al., 1994). The rationale behind this consecutive vaccination strategy was that DNA immunisation, which elicits low-level but persistent immunity, may prime for greatly enhanced responsiveness following boosting with another persistent vector such as FPV, which expresses somewhat greater levels of vaccine antigen. In addition, immune responsiveness is likely to be directed almost entirely against the encoded vaccine antigens as the vectors themselves, which do not replicate, elicit poor responses.

Initially, mice given an intravenous booster inoculum of rFPV encoding the HA gene of influenza virus (FPV-HA)

Table 5: Priming for both local (lung) and distal (intestinal) mucosal antibody responses following consecutive immunisation with pCMV/HA and FPV-HA

DNA vaccine	Boosting	ASC per 10^6 cells in lung	Ab in faecal pellet (ELISA OD units)
pCMV/HA i.t.	nil	106 ± 16	<0.1
pCMV/HA i.n.	nil	NT	<0.1
pCMV/HA i.t.	FPV-HA i.n.	512 ± 48	0.94 ± 0.25
pCMV/HA i.n.	FPV-HA i.n.	NT	1.22 ± 0.36

Mice were given FPV 21 d after priming with DNA in lipofectin and lungs and faecal pellets were taken for assay 10 d later. ASC were <140 and OD readings were <0.15 units in mice given FPV-HA only. NT = not tested.

four weeks after intramuscular immunisation with DNA vaccine (pCMV/HA) exhibited high levels of anti-HA antibody within one week of boosting. Antibody titres peaked at extremely high levels (over 1 mg/ml, resembling those found in convalescent sera) by 3 weeks post-boosting and were maintained at significant titres for at least 15 weeks. We considered that this approach might offer prospects for improved mucosal vaccination, given that effective mucosal immunity has been notoriously difficult to achieve, particularly via systemic immunisation. Previous reports, however, have indicated that systemic DNA vaccination may, in fact, prime for immune responses at mucosae (*Fynan* et al., 1993; *Ulmer* et al., 1993). We have primed mice with pCMV/HA via the intramuscular route 4 weeks prior to intranasal boosting with FPV-HA in attempts to elicit strong, sustained mucosal responses. Although specific antibody-secreting cells (ASC) were not detected in the lungs of mice given pCMV/HA only, both mucosal anti-HA IgG (particularly IgG2a) and IgA antibody responses were markedly enhanced in DNA-primed animals given FPV-HA and were sustained for at least 3 weeks (Table 4). These augmented responses were further elevated in mice boosted with FPV-HA which co-expressed IL-6 (Table 4). Priming with pCMV/HA via gene-gun immunisation prior to intranasal delivery of FPV-HA was similarly effective for the development of strong mucosal immune responses, with IL-6 co-expression also significantly enhancing the levels of antibody which were secreted (data not shown).

Finally, we have studied the capacity of mucosal priming and boosting in our consecutive immunisation strategy to elicit enhanced mucosal responses both locally and at distant sites, particularly in the light of recent evidence that the intranasal route may more effectively prime for both systemic and distal mucosal antibody and cell-mediated immunity (*Porgador* et al., 1997; *Staats* et al., 1997). Whilst immunisation with DNA vaccine via the intra-tracheal route was superior to intranasal delivery for induction of mucosal antibody responses in the lung (data not shown), neither route elicited significant levels of specific intestinal antibodies (Table 5). However, both routes of immunisation primed for enhanced responses both in the lungs and intestines following intranasal boosting with FPV encoding the same vaccine antigen (Table 5). We are currently investigating the characteristics of this prime-boost strategy which underlie its capacity to elicit immune re-

sponses at distant mucosal sites and the ability of co-expressed cytokines to further enhance local and distal antibody and, perhaps cell-mediated immunity.

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OLD HERBORN UNIVERSITY SEMINAR ON IMMUNOMODULATION OF THE GASTROINTESTINAL MUCOSA: MINUTES AND REVIEW OF THE DISCUSSION

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DISCUSSION PARTICIPANTS (in alphabetical order):

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MINUTES AND REVIEW OF THE DISCUSSION

The seminar was opened by Richard Walker. He reviewed known mechanisms of gut/microbial interactions that could contribute to colonisation, "colonisation resistance" *vis a vis* other organisms, translocation, and stimulation of a specific or non-specific *local* inflammatory and/or immune response by the host. He also introduced various strategies for deliberately inducing specific gut mucosal immune responses and use of microbial products as adjuvants. The first speakers: Wold, Carter, and Claesson, addressed the modes by which gut food antigens and microbial flora may interact with the host to initiate:

1) specific or aspecific gut inflammatory/immune responses;
2) oral tolerance; and
3) gut inflammatory responses.

Subsequent discussion considered the enigma of why ingestion of soluble antigens - such as ovalbumin (OVA) - led to oral tolerance while expression of OVA by a recombinant, colonising bacteria led to an immune response (*Wold* et al., 1989; *Dahlgren* et al., 1991). Perhaps uptake at different sites by M-cells over Peyer's patches, enterocytes, dendritic cells in the intra-epithelial leukocyte (IEL) compartment could lead to different modes of antigen dissemination and presentation. Ordinarily, the weanling host does make a humoral mucosal immune response to gut organisms. This response appears to be "self-limiting" and excludes gut microbial antigens from further translocation and immune stimulation (*Shroff* et al., 1995). However, if the neonate is exposed early in life to a colonising microbe for which the nursing dam has no antibodies - such as 06:K13 *E. coli* expressing the OVA gene - it appears that oral tolerance results to both OVA and protein, carbohydrate, and LPS antigens of the bacterium. Probably, the various outcomes of gut microbial/host interactions depend partly on how commensal or pathogenic bacteria interact with the host's gut:

1) do they remain free in the lumen;
2) attach to the brush border of enterocytes or to mucus via "holdfast" organelles or pilli;
3) "swim" in the mucus of crypts, like spirilla (*Helicobacter muridarum*); or
4) produce special "invasins" or "internalins" that facilitate translocation via ligands of host cells?

A particularly detailed analysis of the latter has been made for *E. coli* (EPEC

strains) by *Donnenberg* et al. (1997). Certainly, artificial flushing of mucus, such as with $MgSO_4$, can upset the balance of gut micro-organisms and a particular microbe, *Bacteriodes* sp., can alter the expression of terminal sugars on glycoconjugates made by enterocytes (*Bry* et al., 1996) This may in turn influence the colonisation by other microbes.

Perhaps the importance of that part of the microflora which colonises the gut in the mucus layer and in the crypts has been overlooked. The goblet cells producing the mucus are T-cell dependent in their activity. In SCID mice with $CD4^+$ T cell induced IBD, there is an attenuation of sulphomucin secreting goblet cells in the colonic mucosa in the diseased animals (*Reimann* et al., 1995; *Bregenholt* et al., 1997)

The apparently paradoxical findings of Carter, that *Listeria monocytogenes* is a particularly effective gut mucosal pathogen, poor at colonising but efficient at causing a systemic disease after translocation - while failing to stimulate an appreciable mucosal immune response, was discussed at some length. Although transit of *Listeria* via M-cells has been detected, SCID mice - which lack demonstrable M-cells - are even more vulnerable than immunocompetent mice. Also, invasive *Listeria* express "internalin", which specifically interacts with a ligand, E-cadherin, on the basolateral surface of enterocytes to initiate uptake by enterocytes. The discussions turned to provocative findings by the group of Pringault (*Kerneis* et al., 1997) that the functional properties of cultured, transformed enterocytes could be modified to resemble some of those displayed by M-cells *in situ*. Nevertheless, it remains possible that the transient translocation of *Listeria*, mainly via enterocytes, allows it to "slip past" the gut mucosal follicles to disseminate and cause systemic diseases (CNS-listeriosis) without being detected by the mucosal immune system. Of some interest is that IFN-γ and TNF-α, probably made by cells in the IEL compartment, are elevated during infection via the gut route and appear to play an immunologically non-specific role in attenuating listeriosis. Finally, an avirulent, virulence-factor "knockout" mutant of *Listeria* (actA-negative) can chronically infect only the gut epithelium of both germ-free (GF) immunocompetent and GF SCID mice. The former mice do display an humoral mucosal immune response (*Manowar* and *Cebra*, unpublished observations). Perhaps either or both humoral and cellular mucosal immunity could, in principle, provide specific protection vs. gut mucosal infection and subsequent systemic disease.

Like *Listeria*, the vast majority of accidentally or deliberately introduced commensal or opportunistically pathogenic bacteria cannot readily colonise and establish themselves in the gut of an immunocompetent host already carrying a fully-established, rather stable gut microflora. However, certain members of the gut microflora such as segmented filamentous bacteria (*Snel* et al., 1998) seem particularly effective at stimulating the development of various elements of the gut mucosal immune system and maintaining their "activated" state. It also seems likely that the ready development of oral tolerance requires the presence of the gut microflora (*Moreau* and *Corthier*, 1988). Thus, the question arises as to whether it would be possible to deliberately administer particular benign bacteria that also were sufficiently competitive to establish themselves in the microenvironment of the gut and be effective there at chronically activating the "normal" or "natural" mucosal immune system? Two promising candidates, *E. coli* 083 (*Lodinova-Zadniková* et al.,1991*)* and *Lactobacillus plantarum* (*Johansson* et al., 1993; *Adlerberth* et al., 1996) were discussed

by Tlaskalová (Institute of Microbiology, Praque) and Wold respectively.

Other micro-organisms in the gastrointestinal tract have immunomodulating effects on the host depending on the nature of the pathology associated with the interaction. For example, *V. cholerae* can attach to epithelial cells and release a toxin which initiates the physiological effects associated with the disease, but also mediates an adjuvant effect which enhances the immune response to antigens being processed in the Peyer's patch. For this reason, cholera toxin and a related toxin of enterotoxigenic *E. coli* have been used as adjuvants for mucosal vaccines. Enteric pathogens eliciting a more damaging or invasive interaction with the epithelium can elicit different mediated responses which may also contribute to an improved immune response. Bacterial invasion of intestinal epithelial cells initiates a pro-inflammatory response by these cells that activates the underlying natural immune system (*Eckmann* et al., 1995). IL-8, for example is secreted by colonic epithelial cells in response to invasive bacteria, but not to non-invasive bacteria. In fact, IL-8 secretion normally induced by *S. dublin* is blocked in the presence of cytochalasin D, a drug that prevents bacterial invasion, but not attachment. This suggests that bacterial entry is required to stimulate IL-8 synthesis, and that neither bacterial attachment nor the presence of bacterial LPS causes IL-8 secretion by the epithelial cells (*Eckmann* et al., 1993).

The discussion moved to consideration of whether gut microbes (i.e., opportunistic pathogens, commensals) that lacked expression of specific molecules facilitating uptake either translocated randomly at a low frequency proportional to their density in the lumen, or translocated in a "gated" fashion that could be regulated by other external stimuli. Influences on the quality of the mucus stratum and the integrity of the epithelial cell layer - perhaps affected by enteric viruses, could indirectly alter frequency of bacterial translocation. Presently, we also do not know whether the rate of pinocytosis by M-cells can be externally regulated.

Claesson's presentation focused discussion on whether particular gut microorganisms or food antigens could play a role in the provocation or exacerbation of bowel lesions, especially when the host has a clearly dysregulated immune system. Certain subsets of $CD4^+$ T cells, when transferred to conventionally reared SCID mice, result in a version of IBD. The process is accompanied by a striking polyclonal expansion, cell turnover and accumulation of T cells in the GALT compartment of the recipient. At this stage the cells exhibit a typical mucosa-seeking, activated, memory/effector CD45RBlow $CD4^+$ phenotype. They induce severe inflammatory lesions of the colonic mucosa, leading to death of the animals. Apparently, under GF conditions, little expansion of T cells and no development of IBD results (*Kushnir* and *Cebra*, unpublished observations). In this model, the development of histopathology depends on a particular subset of T cells, influenced by the presence of gut microbes. Helena Tlaskalová and her co-workers presented another model of IBD, especially of celiac disease, in GF neonatal rats or conventionally-reared athymic mice (*nu/nu*) fed gliaden. A correlate of their development of bowel lesions is the appearance of anti-gliadin antibodies, cross-reactive with cytoplasmic elements in enterocytes. Apparently, some sort of molecular mimicry results in an autoimmune phenomenon (*Tuckova* et al., 1995).

Presentations by Urban and Lee focused on pathogen/host interactions, particularly those which indicated some direction - or "misdirection" - of the balance between the host's Th1 vs. Th2 responses and their characteristic cy-

tokines. The outcome of nematode infections caused by *H. polygirus* and *N. braziliensis* appears to be dependent on IL-4 production and can be manipulated via genetic "knock-out" or use of MAbs against IL-4 or IL-4R. There is some indication that IL-4R/IL-13 interactions, even in SCID mice can benefit the host and lead to control of worm burden. Vaccines based on recombinant DNA technology and combining expression of both particular cytokines and protective antigens may permit external distortion of the host's response to favour the most protective/therapeutic modes against a particular pathogen (see *Ramsay*, below). In contrast to infection by these worms, *Helicobacter* appears to distort a host's response in favour of Th1 predominance. Combination antibiotic therapy can be therapeutic, but in some animal models, a vaccine combining *Helicobacter* antigens and cholera toxin can also be therapeutic against gastritis and severe stomach ulcers. Generally, it has been difficult to totally eradicate *H. pylori* from the human host. Discussion initiated by Paul Simon (Neose Technologies, Inc.) concerned the relative roles of attached vs. free *Helicobacter* in the gastric mucosa. Use of polymeric sialoglycoconjugates, administered orally, to interfere with *Helicobacter* attachment, was suggested to complement antibiotic and vaccine therapy.

The presentations by Baqar and Ramsay continued the theme of combining microbial products and cytokines with protective antigens to enhance protective immune responses, particularly those benefiting from a mucosal component. Scarcely 20 years ago, *Campylobacter jejuni* was first recognised as an enteric pathogen of humans, yet today it is known as one of the major causes of diarrhoeal disease and is a frequent cause of Guillain-Barré syndrome. Present promising treatments are suggested by findings that IL-2, IL-4, IL-5 and IL-6 given orally, enhance clearance of *C. jejuni* from infected mice. Perhaps of more practical value were findings that IL-2 given orally with an inactivated whole cell *Campylobacter* vaccine, and CT or LT (*E. coli* heat labile exotoxin) given with the vaccine markedly enhance the gut humoral response and protected mice against oral challenge. Presently, a trypsin-resistant "variant" of LT, with decreased toxicity, seems to be the best adjuvant candidate. Ramsay discussed strategies for enhancing the mucosal immune responses in respiratory tissue and at other mucosal sites including the gut. Recombinant vaccinia virus (rVV), recombinant fowl pox virus (rFPV) and plasmids, each prepared to encode protective antigens and a CK IL-4, IL-5, or IL-6, seemed effective at enhancing the antibody response. Incorporation of the coding sequences for IL-12 showed promise in depressing asthmatic responses in the lungs. He also showed that a consecutive immunisation strategy involving intranasal priming by DNA vaccination and boosting with poxvirus vectors encoding the same vaccine antigens elicited enhanced mucosal responses both locally and at distant sites, particularly in the gut, whilst neither vector alone gave significant levels of specific intestinal antibodies.

The last two presentations, by Hanson and Björksten, concerned various aspects of food allergies, the modes by which we react to ingested antigens, the role of nutrition in influencing our full immune potential and our general well being and possible mechanisms by which previously acquired, suckled maternal antibodies may influence immune responsiveness of neonates.

Discussion centred around the enigmas raised by Björkstén:

1) that food allergies have rapidly increased in incidence in modern times;
2) that tobacco smoking can markedly increase respiratory allergies; and

3) that little correlation can be found between incidence of atopy and general air pollution in middle and eastern Europe.

Again, Th1 vs. Th2 balances and imbalances were suggested as determining the likelihood of food allergies: a predominant Th1 pattern of responsiveness appears to favour suppression of gut atopy. It was suggested that correlates of propensity towards food allergies should be sought in distribution of gut commensal bacterial species during neonatal life. One of the obvious deficiencies in our ability to better understand the mechanisms of development of food allergies is the lack of meaningful laboratory animal models for this set of maladies. Hanson offered the first new animal model for many years. If adult rats are colonised with *E. coli* 06:K13, carrying a plasmid that expresses the gene for OVA, the hosts develop an apparent mucosal and systemic immune response against OVA. Subsequent feeding of these sensitised rats with OVA leads to diarrhoea and to the appearance of gut mast cells coated with IgE.

Hanson next stressed the effects of under-nutrition on attenuating normal development and depressing responsiveness and normal functioning of many elements of the immune system. He emphasised in discussions that:

1) comprehensive treatment of children in impoverished regions required not only food-supplementation, but also countering and resolving the many infections to which undernourished children are susceptible; and
2) sub-clinical diet deficiencies, such as for vitamin A or essential fatty acids, could have severe consequences for susceptibility to infections if left undetected during early development.

Finally, the importance of breast feeding in providing passive immunisation and antibody specificities appropriate for the various opportunistic and frank pathogens in the neonate's environment was stressed. Hanson supported a more indirect benefit, namely that maternal antibodies against protective antigens may provide "idiotypes" to prime the neonate and facilitate active immunisation in advance of encounters with the relevant pathogens. Van der Waaij discussed this concept at some length: It is generally accepted that the first steps in the ontogeny of the immune system involve learning of self-nonself discrimination. This may be controlled from the "inside" through the *idiotype network* which produces so-called "natural" antibodies. These "natural" antibodies have been characterised from hybridomas derived from unstimulated lymphocytes of new-born mice. It turned out that many of these antibodies were directed against self-antigens and microbial determinants and were, in a large number of cases, multispecific. In addition, these antibodies appear highly connected through idiotype-anti-idiotype (Id-anti-Id) interactions. This type of recognition might very well explain how the Id-network might be broken in ontogeny:

1) Providing initial recognition signals to the T-cell compartment. This may begin to occur in ontogeny, leading later to the control of the newly emerging B-cell repertoire. Later on, whenever new non-connective monospecific B-cells emerge, all cells expressing one or the other of the original V_H and V_L genes would receive an amplification signal from the complementary Th cells. This would favour the amplification of quite a broad B-cell population. These "secondary type immature B-cells" would not be directly connected to the polyspecific Id-network through classical Id-anti-Id interactions, but indirectly connected to it through "idio-educated" T-cells; and
2) Maternal IgG (for example, directed

against a pathogen) which passes the placenta in a concentration sufficient to destroy the B-cells expressing the corresponding Id's (internal image and its antibodies). Absence of the primary B-cell clone would enable the foetus/new-born to respond after birth to the pathogen directly with Th induced IgG antibody response.

A brief review of the ontogeny of the "primary immune system" followed in a general discussion. Interactions of (high affinity) IgG antibodies produced in the dam to pathogens with homologous idiotypes on IgM of the primary network may help to understand the findings. In the foetus, maternal IgG may have destroyed the corresponding Id and anti-Id in primary B cell clones. This may have caused a T-cell dependent B-cell response to the antigen(s) involved in the foetus/new-born. After birth and gut colonisation (predominantly with maternal bacteria) and translocation, production of IgG to these translocating bacteria may have led to inflammatory responses in the submucosa (instead of a silent non-inflammatory clearance). An IgG response with complement activation/inflammation at the site, could also explain the histological findings. A comparable sequence of events, although less dramatic, may possibly occur in IBD patients. These patients may lack the capacity to respond to a number of "normal intestinal flora components" with clearance upon translocating of these bacteria in a non-inflammatory way. This could come into expression in cases where the innate immune system fails (for example due to antigen overloading of the capacity).

It was clear in this symposium, that the potential for achieving human benefit from learning to better immunomodulate the gastrointestinal tract is great. The task to understand and apply the immunomodulating process presents a challenge. The gastrointestinal tract, it must be remembered, must function in concert with other organs of the body. Neuro-endocrine factors play a role in intestinal immune function and it must be supposed that the liver is also an important part to this integrated system. While this seminar addressed a variety of factors germane to gastrointestinal immunomodulation, it is apparent that much further work and many more seminars will be required to more fully probe this fascinating and critically important subject.

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