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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).

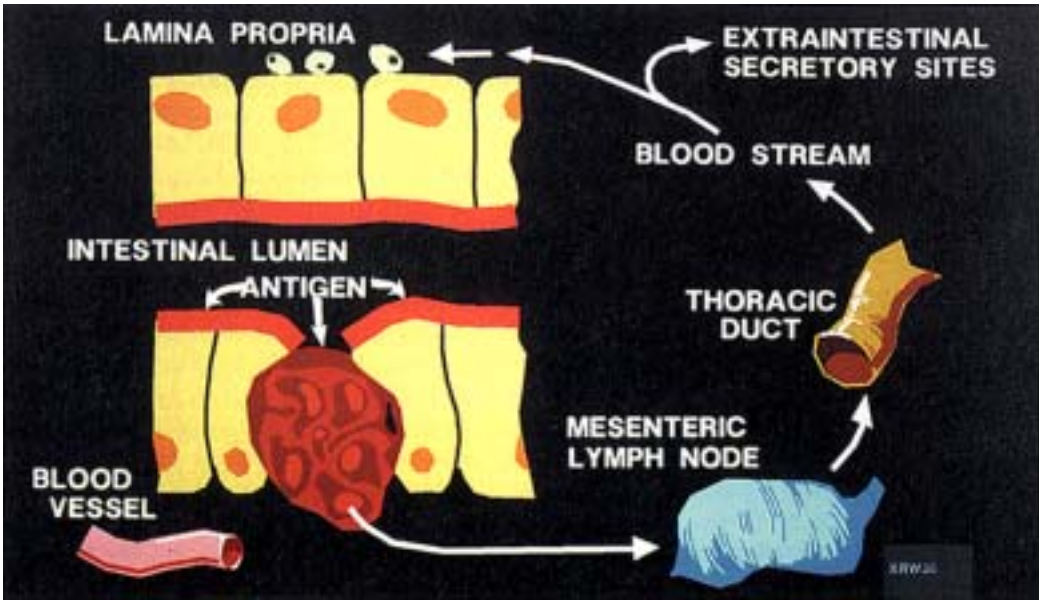

Figure 1: Stimulation and homing of IgA producing plasma cells

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 IgG	Serum ELISA titres IgA	Culture positive
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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