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ENHANCEMENT OF GUT WALL DEFENCES AGAINST ENTERIC PATHOGENS

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SUMMARY

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

INTRODUCTION

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

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

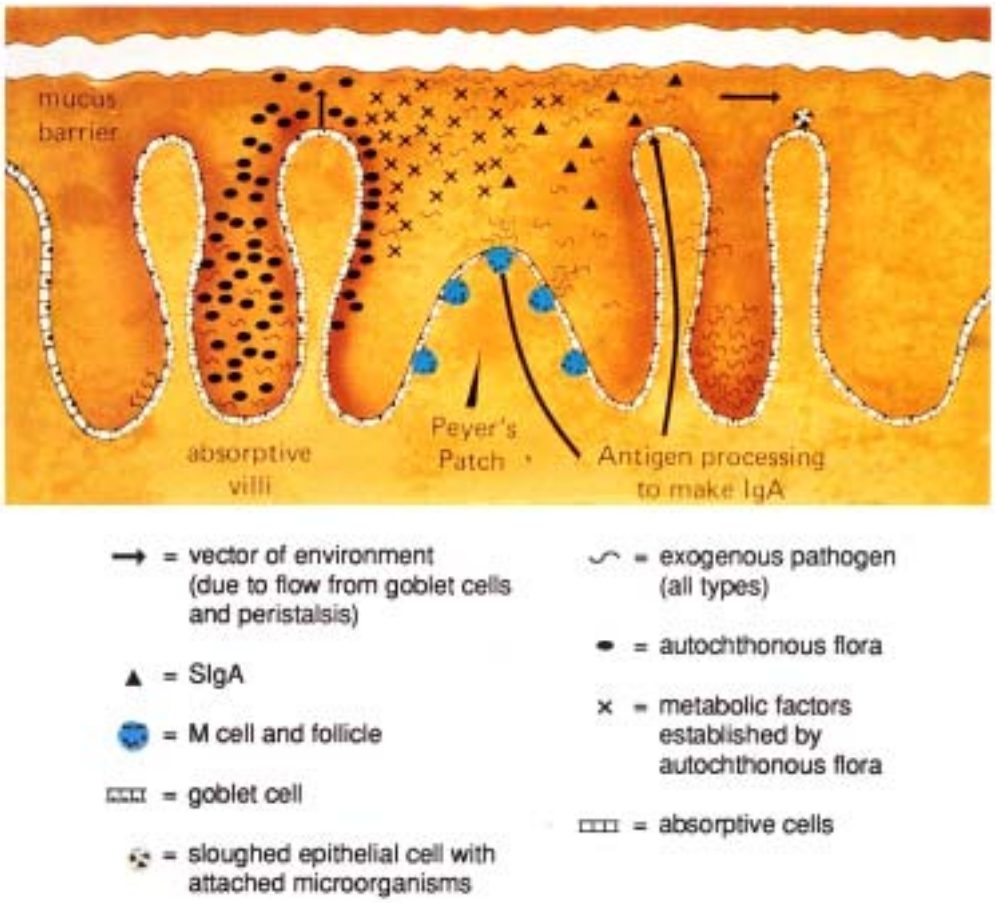

Figure 1: Diagram illustrating defence mechanisms whereby the intestine keeps potentially pathogenic micro-organisms from colonising sites where they may initiate disease. Mechanisms represented include mucus-antibody blockage of epithelial colonisation, colonisation resistance, peristalsis and cell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

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

NORMAL GUT WALL FUNCTION IN ANTIMICROBIAL DEFENCES

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

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

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

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

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

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

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

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

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

PROSPECTS FOR ENHANCEMENT OF GUT WALL DEFENCES WITH MUCOSAL VACCINES

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

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

Importance of humoral immunity

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

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

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

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

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

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

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

Considerations for a killed *Campylobacter* vaccine

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

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

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

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

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

Since there are antigenic similarities between cholera enterotoxin and the

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

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

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

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

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

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

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

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

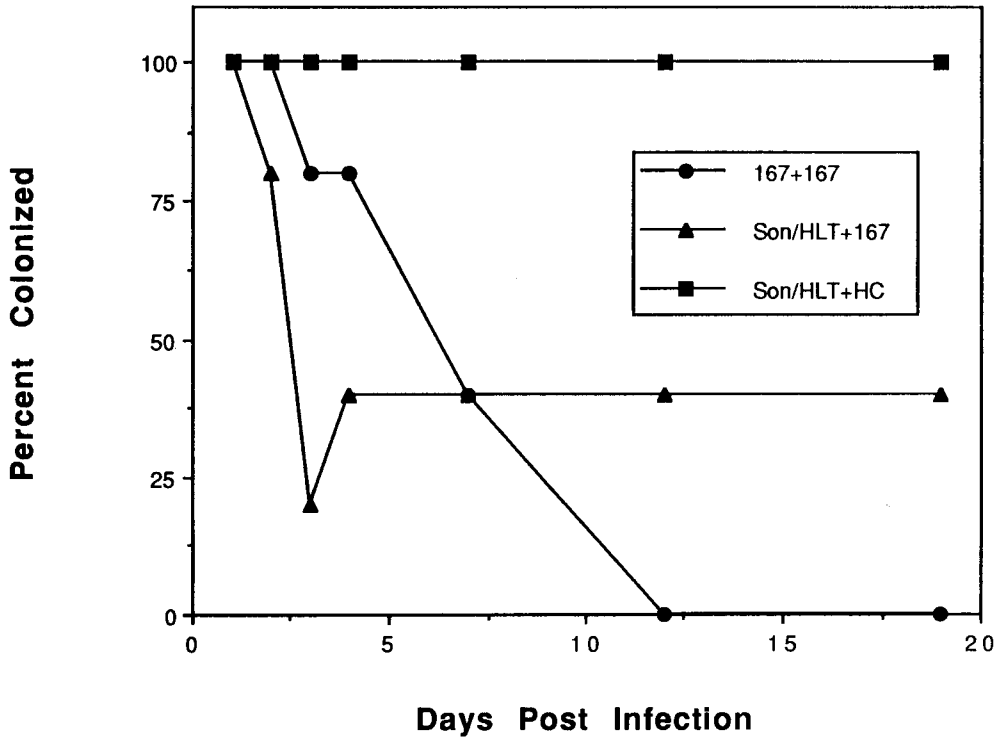

Figure 2: Rate of clearance of live homologous (strain 167) and heterologous (strain HC) serogroups of *Campylobacter* from intestines of mice immunised with live bacteria or killed 167 strain bacteria. The killed bacteria were administered with oral adjuvant (HLT). Information to the left of the plus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

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

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

HLT-immunised animals reacted to a lesser extent.

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

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

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

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

FUTURE PROSPECTS FOR ORAL MUCOSAL VACCINES

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

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

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

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

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

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

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

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