POTENTIAL EFFECTS OF IMMUNIZATION ON THE HOST DEFENSE SYSTEMS IN HUMAN MILK

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INTRODUCTION

Diarrheal disease mortality data from the Pan American Health Organization (1,2) indicate that in 1976 in North America, diarrhea accounted for 1.4% and 0.9% of infant deaths in children less than 1 year of age and children 1 to 4 years of age, respectively. In tropical areas of America, diarrhea accounted for 15 to 23% of all infant deaths and 15 to 26% of deaths in children 1 to 4 years of age (table 1). In many areas of the world diarrhea remains the leading cause of death in infants and young children (2).

The number of enteric bacteria, viruses and parasites that are recognized as causing acute diarrheal disease has increased greatly since the early 1970's. Not only have new enteropathogens been described, but new pathophysiologic mechanisms of disease production have been defined (3). Table 2 shows the causes of acute infectious diarrhea in the United States and in tropical developing areas of the

Table 1. Diarrheal disease mortality in children four years of age and less in the Americas

	Age (years)			
	less than 1		1-4	
Area	Rate	%	Rate	%
North America	19	1.4	0.6	0.9
Central America	1078	22.8	154	25.8
South America (temperate)	496	10.9	20	9.1
South America (tropical)	1066	20.3	151	21.5

Modified from references 1 and 2.

M. Hamosh et al. (eds.), *Human Lactation 2*

Table 2. Causes of Acute Infectios Diarrhea

	Approximate Percentage	
	North America	Tropical Developing Areas
Bacteria		
Enterotoxigenic *E. coli*	5	25
Campylobacter	5	5-15
Shigella	1-5	5-10
Salmonella	1-5	1-5
Others	1-3	1-5
Viruses		
Rotavirus	10-50	5-20
Norwalk	10-20	Unknown
Enteric adenovirus	5-10	Unknown
Parasites		
Giardia lamblia	3-10	5
Cryptosporidium	Unknown	Unknown
E. histolytica	< 1	2-5

From references 3-8.

world (4-8). These enteropathogens produce diarrhea by enterotoxins released in the intestinal lumen or by mucosal invasion with or without release of proteolytic enzymes, cytotoxin or other humoral factors. Identification and characterization of these processes are important for the biologic control of enteric infection, particularly with respect to vaccine development. A brief description of various enteropathogens and their mechanisms of disease production will follow.

MECHANISMS OF DISEASE PRODUCED BY ENTEROPATHOGENS

Escherichia coli. Many new developments have occurred which have permitted clarification of the role of *E. coli* as a cause of diarrhea. *E. coli* that produce diarrhea can be divided into several categories based upon their mechanism of disease production and clinical manifestations (table 3). *E. coli* that produce diarrhea have been classified as enteropathogenic *E. coli* (EPEC), cytotoxin producing *E. coli*, enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (8,9).

For many years all *E. coli* that were associated with diarrhea were referred to as EPEC. Outbreaks of diarrheal disease in infants were recognized as being associated with a small number of *E. coli* serogroups defined by the O somatic antigen. These serogroups included 026, 055, 086, 0111, 0114, 0119, 0125, 0126, 0127, 0128, and 0142. However, with

Table 3. Categories of Escherichia coli that produce diarrhea

Category	Mechanism of disease
enteropathogenic	? adherence ? cytotoxin production
enterohemorrhagic	cytotoxin production
enterotoxigenic	adherence enterotoxin production
enteroinvasive	? invasion ? cytotoxin production

the recognition of invasiveness and production of heat stable (ST) and heat labile (LT) enterotoxins as important virulence properties of pathogenic E. coli (10), the role of EPEC as a cause of diarrhea became less clear. These EPEC have been shown to be non-invasive and ST^-/LT^-. With the demonstration that serogroup defined ST^-/LT^- EPEC can cause diarrhea in volunteers (11), there has been a resurgence of interest in EPEC (12). In some parts of the world EPEC are a frequent cause of endemic diarrhea in infants and young children (13). In Canada and Japan, EPEC occur more commonly in infants with endemic diarrhea than in well infants (14,15). EPEC are considered to be pathogenic because of epidemiologic studies as well as production of disease in volunteers (11,16).

Although the mechanism of production of gastroenteritis by EPEC is not known, both adherence and cytotoxin production have been suggested. The first involves factors that allow EPEC to adhere to enterocytes. Pathologically, these enterocyte adherent E. coli have been associated with morphologic changes on electron microscopy of small intestinal biopsies, including loss of microvilli with tightly adherent bacteria (18). The second potential mechanism that has been suggested is that EPEC produce a cytotoxin, variously referred to as Vero toxin (19) or Shiga-like cytotoxin (20), which damages cells by inhibition of peptide elongation during protein synthesis. Studies are being conducted to determine if either adherence or Shiga-like cytotoxin production is involved as the mechanism by which EPEC cause disease.

Both adherence and cytotoxin production appear to be important in E. coli diarrheal disease caused by serogroups not belonging to the classic EPEC. Non-EPEC enteroadherent E. coli, as defined by HEp-2 cell adherence, recently have been recognized as an important cause of travelers' diarrhea (21). In addition, high level Shiga-like cytotoxin production by E. coli is now recognized as the mechanism of a disease referred to as hemorrhagic colitis produced by E. coli 0157:H7 (22). Although high level toxin-producing organisms can cause disease, the threshold for the amount of toxin production needed to produce symptoms remains to be defined.

As many as 25% of episodes of diarrhea in children in Mexico are caused by enterotoxigenic E. coli (ETEC) (5). In addition ETEC are responsible for approximately 40% of diarrhea in adults who travel from industrialized countries to developing countries. In North America enterotoxigenic E. coli have occasionally been recognized as a cause of diarrhea in children (4,5). Most enterotoxigenic E. coli produce ST only or ST plus LT (23). The genetic material that codes for production of

enterotoxin may be located in plasmids (as is the case of E. coli) or in chromosomes (as in V. cholerae).

The initial step in production of disease by ETEC involves adherence of bacteria to intestinal mucosa, often mediated by fimbrial colonization factor antigens (CFA) (9). Although CFA-I and CFA-II are carried on the same plasmids that encode for ST and LT, many toxin producing strains do not possess these fimbrial antigens. LT works like cholera toxin through increased cyclic nucleotide (cAMP) levels in enterocytes. ST mediated fluid loss occurs through stimulation of guanylate cyclase with increased cGMP in enterocytes.

Invasive E. coli infrequently cause diarrheal disease, but when disease occurs, it clinically resembles shigellosis. Invasive E. coli have been shown to have a high molecular weight plasmid, similar to the high molecular weight plasmid that codes for invasiveness in Shigella species (25). Invasive E. coli are biochemically and serologically closely related to Shigella (49,50). These virulence factors can be transferred via plasmids between bacterial species (24). These similar properties may be useful in formulating vaccines that are effective against several organisms.

Shigella. There are four serogroups of shigella containing over 40 serotypes. S. sonnei is currently the most common cause of bacillary dysentery in the United States and Europe while S. flexneri serotypes account for a majority of the remaining cases. Shigellosis is currently believed to be a disease resulting from two major virulence properties, cytotoxin production and invasiveness. These factors determine clinical manifestations.

Salmonella. Currently, three species of Salmonella are differentiated on the basis of biochemical characteristics: S. cholerae-suis, S. typhi, and S. enteritidis. Nearly all of the 1700 serotypes of Salmonella are now grouped under S. enteritidis. Determining serotypes remains a useful epidemiologic tool for outbreak situations, although simple definition of one of the three species is still adequate for the vast majority of patients. The mechanism of Salmonella gastroenteritis is throught to involve penetration of organisms through the gut mucosa (26), a cholera-like enterotoxin (27) and a protein synthesis inhibiting cytotoxin. The enterotoxin appears to be more closely related to E. coli LT than to cholera toxin (28).

Campylobacter. Campylobacter jejuni and C. coli are enteric pathogens that are common causes of diarrhea world wide (29). A thermophilic strain of Campylobacter has been distinguished from C. jejuni by its nalidixic acid resistance and halotolerance. The species name C. lardis has been proposed because the great majority of isolates were from seagulls. It is a rare cause of diarrhea in humans. C. fetus produces fever and bacteremia in immunocompromised hosts. Clinically, C. jejuni frequently resembles bacillary dysentery; bloody diarrhea has been a common finding in this illness. This organism also may produce a heat labile enterotoxin (30). In many areas of the world Campylobacter is increasingly being recognized as the most commonly documented cause of bacterial diarrhea.

Vibrio cholerae. Cholera remains a public health problem primarily in Asia and Africa, although a focus of cholera is present in the Gulf Coast of the United States (31). Epidemic cholera is caused by O-1 V. cholerae. However, non O1 or non agglutinating V. cholerae also can cause disease. V. cholerae O1 clinical isolates from the United States have been hemolytic, biotype El Tor, serotype Inaba and have had the

same unique phage type and toxin gene sequence. The major mechanism by which this organism causes fluid loss is the production of a heat labile enterotoxin which has the ability to bind the terminal galactose of GM 1 receptors on intestinal epithelial cells with subsequent internalization of an active subunit capable of hydrolysing NAD to ADP ribosylate, a GTP binding regulatory protein of adenylate cyclase. This results in an increase in intracellular cAMP and fluid loss into the gut.

Clostridium difficile. Antimicrobial associated diarrhea (AAC) or pseudomembranous colitis represent the clinical spectrum of illness associated with C. difficile and its toxins (32). Many patients have diarrhea that is mild and self limited if antibiotics are discontinued. In others the illness is characterized by fever, bloody diarrhea, and pseudomembranes on protoscopy. Healthy infants can harbor this organism and its toxins in their stools and yet demostrate no illness (33). Healthy children also can be demonstrated to have serum antibodies to one or both toxins. The frequency of colonization with this organism (34) is higher in formula fed infants when compared to breast fed infants. There is evidence for both antibody and non antibody factors in milk that may be important in protecting infants from this organism (35,36). Two toxins, designated A and B, have been described. It is unclear which is more important in producing disease. Cytotoxin is usually demonstrated in tissue culture systems in which the neutralization of toxin by antiserum raised to Clostridium sordellii toxin (a toxin closely related to that made by C. difficile) is taken as demonstration of presence of this cytotoxin.

Viruses. Rotavirus, Norwalk-like virus and enteric adenovirus are recognized causes of acute infectious gastroenteritis (37). Other viruses which may cause infectious gastroenteritis include enteroviruses, astroviruses, calicivirus, coronavirus, and minirotavirus. These viruses exhibit different epidemiologic and clinical features, although most episodes are self limited and characterized by various combinations of diarrhea, nausea, vomiting, abdominal cramps, headache, myalgia and low grade fever. Rotavirus is the only one of these agents for which a vaccine is being developed.

Rotavirus is one of the most important enteric pathogens throughout the world, particularly in infants. The agent is a double stranded RNA virus. The precise significance of asymptomatic shedding of rotavirus is unclear at present, although it is a common event (38). Illness is particularly prevalent in temperate climates in winter months and may account for as much as 50-60% of diarrhea in infants and young children (37). In those symptomatic with rotavirus infection, nearly 100% have both diarrhea and vomiting. Because of increased insensible losses with fever, intestinal fluid losses, and emesis interferring with rehydration, it is common for rotavirus to be associated with significant dehydration. Because there are multiple serotypes, reinfection can occur (39). Circumstantial evidence suggests that human milk is useful in preventing rotavirus illness due to the presence of specific IgA. The data for prevention of rotavirus infection in animals by breast milk feeding is more convincing than that currently available from human studies. Rotavirus is the only enteropathogen against which vaccines are being developed.

Norwalk-like virus agents remain inaccessible to comprehensive study because of the difficulty in their diagnosis. They cannot be cultivated and therefore development of a vaccine is not yet possible. This same problem has skewed the epidemiologic data toward finding this agent only in outbreaks. Norwalk infection has been shown to be common in all age groups and has a worldwide distribution.

A group of fastidious adenoviruses with a distinct set of antigenic determinants and specific tissue culture growth characteristics has been linked with acute gastroenteritis. Illness is characterized by low grade fever, vomiting and watery stools in infants. These organisms fail to grow on conventional tissue culture cell lines, although they will grow on 293 cells, an adenovirus type 5 transformed line. These agents appear to be endemic in certain areas and infants may be especially susceptible. More information is needed on the epidemiology, extent of clinical involvement and immunology before vaccines can be considered.

Parasitic enteropathogens. Three parasites are the most frequent causes of parasitic diarrhea in persons in the United States. New information has become available about the pathogenic mechanisms and immunology of Giardia lamblia and Entamoeba histolytica. Another agent, Cryptosporidium, has been described as a cause of gastroenteritis in both immunocompromised and immunocompetent hosts. Immunization agents against these parasitic enteropathogens are not available.

G. lamblia is a flagellated protozoan that exists in trophozoite and cyst forms. Disease due to this organism is common in certain high risk populations and in persons who travel to hyperendemic areas. Children appear to be more susceptible to infection than adults. Infection with Giardia may range from asymptomatic excretion to acute explosive, watery, foul smelling diarrhea with abdominal distention, flatulence, nausea and anorexia. Chronic diarrhea with malabsorption and significant weight loss over many months also may occur. Asymptomatic colonization in young children is common.

Amebiasis is a major health problem in Asia, Latin America and Africa. Disease may remain localized to the intestine or may extend to other organs, most commonly the liver. E. histolytica adhere to the intestine by microfilament invasion and produce microabscesses, probably by production of enzymes and cytotoxin. The understanding of Entamoeba histolytica has improved with development of culture techniques for isolation and utilization of electrophoretic analysis of isoenzyme patterns. Multiple strains (zymodemes) have been identified on the basis of these characteristics. Identical zymodemes cause illness in different parts of the world and only pathogenic zymodemes elicit an immune reponse in infected patients. The vast majority of E. histolytica are nonpathogenic, although normal, healthy individuals can be asymptomatically infected with pathogenic zymodemes.

In 1976 the first human infections due to cryptosporidium were reported. With the onset of the AIDS epidemic, many patients with chronic malabsorption syndrome and severe protracted, watery diarrhea were recognized as having cryptosporidiosis; immunocompetent patients can also be infected. This pathogen probably accounts for less than 5% of diarrheal illness in most populations.

ENTERIC VACCINES

The prevention of infectious diarrhea by immunization is an attractive proposition, but is complicated by the number and diversity of enteropathogens that cause diarrhea. With regard to protection of infants from gastroenteritis by ingestion of milk containing specific antibodies, two methods of approach are available. One possible method is by oral administration of bovine milk containing immunoglobulins with specific antibody activity against infectious agents. Bovine milk immunoglobulin concentrations have been used in human infants against E. coli (40) and rotavirus (37). For bovine milk preparations to be

effective, the antibody activity must be preserved during processing and during gastrointestinal passage, and it must be specific and show protective activity against the targeted enteropathgen. Current technology will provide the basis for development of effective, safe enteric vaccines. Table 4 shows potential vaccines against enteric disease producing organisms including cholera, E. coli, S. typhi, shigella and rotavirus. Oral rather than parenteral immunization is more likely to stimulate the protective intestinal immune response of secretory IgA antibodies.

Cholera. Natural infection with cholera is followed by solid, long-lasting immunity (41), stimulating the search for a vaccine. The search for a safe and protective vaccine against cholera began in 1885. Parenteral, killed whole-cell vaccines have been the most intensively evaluated in the past, but they have been found to give variable protection of only 3 to 6 months (42). In addition, the antigen reaches the intestinal lymphoid tissue inefficiently and stimulates suppressive mechanisms that interfere with subsequent attempts to prime or boost the immune response orally (43). Vaccination of lactating women in Pakistan with killed whole cell vaccine given parenterally, produced a serum response as well as an increase in milk secretory IgA antibodies. These women had been naturally exposed previously, priming the gut mucosa. Swedish women without previous natural exposure did not respond with milk secretory IgA antibodies (44,45). Oral rather than parenteral immunization is the route most likely to stimulate protective intestinal immune response of the secretory IgA type (46). The oral vaccines currently under development are inactivated V. cholerae strains combined with altered, nonreactogenic toxin or purified toxin subunits, and attenuated V. cholerae strains (47).

E. coli. Approaches to vaccine development for enterotoxigenic E. coli infections will involve vaccines that stimulate antitoxic or antiadhesion immunity. ETEC pathogens include strains with many cell

Table 4. Promising vaccines against enteric disease

Organisms	Candidates
Vibrio cholerae	Inactivated V. cholerae strains combined with altered, nonreactogenic toxin or purified toxin subunits Attenutated V. cholerae strains
Escherichia coli	Stimulate antitoxic or antiadhesion immunity
Salmonella typhi	Live oral strain
Shigella	Recombinant vaccine
Rotavirus	Live virus (attenuated human strains, wild type animal strains, animal-human reassortment strains and vaccine-rotavirus hybrid) Inactive or subunit (killed whole virus, subunit neutralization proteins)

wall lipopolysaccharides, capsular and flagellar serotypes, and several enterotoxin phenotypes. ST is the toxin most commonly produced by virulent strains, but ST is not antigenic. Moreover, although LT is antigenic, volunteer studies suggest that antitoxin immunity is not the principal protective mechanism in humans (48). Killed vaccines composed of purified ETEC pili have not provided satisfactory protection in volunteers (9). Future ETEC vaccine strains will replicate in the intestine and contain antigens intended to stimulate both antibacterial and antitoxic immunity (9).

Although antibodies to different O serogroups have been demonstrated in human milk, an understanding of the pathogenesis of EPEC in infants and children is necessary before a vaccine can be developed (22). It is known that unique entero-adhesive properties of some EPEC strains are associated with a plasmid (49), and it may be possible to identify the phenotypic gene products likely to be pili or outer membrane proteins and to prepare vaccines consisting of the purified gene products. The specific role that cytotoxin plays in the pathogenesis of EPEC diarrhea must be elucidated to determine if this aspect should be considered in vaccine strategy.

Typhoid. The available parenteral typhoid vaccines are neither entirely safe or fully protective (50). The development and successful trial of a live, oral S. typhi vaccine represents a major advance (51,52). In this vaccine a mutagenic strain of S. typhi (Ty21a) is incapable of utilizing galactose after galactose enters the bacterium. The strain proliferates in sufficient numbers to immunize the intestine before the galactose accumulates in the cell and kills it.

Shigella. Until recently oral, killed and attenuated vaccine candidates have lacked safety, stability or efficacy (53). Using genetic engineering plasmid genes coding for the protective antigen of S. sonnei have been inserted into the genome of the Ty21a vaccine strain of S. typhi. The resultant transconjugant strain manifested both S. sonnei and S. typhi antigens and was well tolerated and safe when fed to volunteers (54).

Rotavirus. Several types of rotavirus vaccine are being developed (47) (table 4). One type is an attenuated, otherwise virulent strain of human rotavirus. Attenuation was achieved by passing it repeatedly in gnotobiotic piglets and cell culture (55). This attenuated strain, referred to as WA, was fed to volunteers, but when questions of its safety arose, further trials were suspended. Cold-adapted human rotavirus also may provide future attenuated vaccine candidates. Strains obtained from newborn nurseries in which rotaviruses cause predominantly avirulent infection also may be potential vaccine strains since they may have become attenuated naturally (56).

A second strategy involves the use of rotavirus strains from animals. They need to infect humans and evoke cross-protective immunity without inducing illness. RIT 4237 is an attenuated animal strain that was derived from the Nebraska calf diarrhea virus strain of bovine rotavirus. It has been shown to be safe, immunogenic (57), and efficacious (58) in protecting infants against rotavirus diarrhea, but additional tests are necessary to substantiate effectiveness. Another oral candidate vaccine strain derived from animals has been isolated from a rhesus monkey with diarrhea. This rhesus strain of rotavirus has been safe and immunogenic in adult volunteers; tests in children and infants are underway. A third approach features fastidious human rotaviruses cultivated with less fastidious animal rotavirus strains in tissue culture to produce reassortment rotaviruses. The resulting virus

progeny grow efficiently in cell culture, and have the neutralizing specificity of the human rotavirus parent. Gene reassortment techniques may produce useful rotavirus vaccine candidates.

Availability of cloned rotavirus genes and the protein sequences of important rotavirus antigens should permit additional approaches to vaccine development, such as incorporation into a expression vector (45). If rotavirus antigens can be synthesized in such systems, a large amount of rotavirus antigen could be produced for subunit vaccines and for structure analysis necessary for construction of a synthetic vaccine. In another novel scheme, rotavirus genes are inserted into vaccine virus to create an infectious hybrid virus vaccine. Recent reports of new, serologically distinct human rotavirus strains (59,60) demand that field trials be carried out over many years and in different locales because of the possible variation in rotaviruses and their periodic occurrence.

HUMAN MILD PROTECTIVE FACTORS

Diarrheal disease occurs less frequently in infants who are breast fed compared to those who are bottle fed (61,62). Protective factors in human milk that modify or prevent disease include cells, nonspecific factors including all nonantibody factors and antibodies (63,64). Breast milk protective factors that are protective against one enteropathogen or its toxin may not afford protection against others, further complicating breast milk immunity. The major immunoglobulin in human milk is secretory IgA. This immunoglobulin is not absorbed in significant quantity from the human intestine and therefore acts at the mucosal surface of the intestine. Total IgA as well as specific IgA to many respiratory and gastrointestinal pathogens have been reported in human milk (2); however, demonstration of protective affects of these antibodies is often lacking. Addition information about specific protection afforded by antibodies in human milk is needed.

SUMMARY

Control and prevention of acute diarrhea involves improvement in environmental conditions with regard to hygiene, continued use of oral rehydration solutions when diarrhea occurs, breast feeding, and development of vaccines against various enteropathogens. Recent knowledge about causes of diarrhea and methods of vaccine development has rapidly advanced. Use of vaccines to confer immunity against diarrheal disease via breast milk is a future consideration.

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