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# ROTAVIRUSES: IMMUNOLOGICAL DETERMINANTS OF PROTECTION AGAINST INFECTION AND DISEASE

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## I. INTRODUCTION

Rotaviruses are one of the most important causes of infectious diarrhea and death in infants and young children throughout the world. Each year, in Asia, Africa, and Latin America, an estimated 3–5 billion cases of gastroenteritis account for 5–10 million deaths (Walsh and Warren, 1979). Since their initial identification as a human pathogen in 1973 (Bishop *et al.*, 1973), rotaviruses have been found to account for 10–20% of gastroenteritis-associated deaths (Snyder and Merson, 1982; Walsh and Warren, 1979). In the United States 70,000–120,000 infants are hospitalized (Ho *et al.*, 1988a; Matson and Estes, 1990) and as many as 200 infants die each year with rotavirus-induced disease (Ho *et al.*, 1988b). Virtually all children, in both developed and

developing countries, are infected with rotaviruses by 2–3 years of age (Urasawa *et al.*, 1984; Yolken *et al.*, 1978a).

The worldwide impact of rotavirus infections has excited interest in disease prevention by immunization. Unfortunately, for the past 20 years, research in the areas of rotavirus immunology and pathogenesis has not yielded a safe, effective, and inexpensive vaccine for universal use. Although development of a successful rotavirus vaccine will depend on addressing a number of issues, this chapter focuses on four important questions that remain unanswered: (1) What is the importance of virus serotype in formulating an optimal vaccine? (2) Which immunological effector arm most likely protects against rotavirus disease? (3) By what means is virus antigen best presented to the host to elicit a protective immune response? (4) What are the advantages and disadvantages of replicating agents (e.g., live, attenuated human or animal rotaviruses, rotavirus reassortants, or vectors expressing individual rotavirus proteins) as compared to nonreplicating agents (e.g., inactivated virus, purified virus proteins, or peptides) as candidate rotavirus vaccines?

## II. ROTAVIRUS STRUCTURE

Rotaviruses are a genus within the family Reoviridae. These viruses are nonenveloped and contain an outer capsid and core (reviewed in Estes and Cohen, 1989; Bellamy and Both, 1990). The viral genome consists of 11 separate segments of double-stranded RNA (dsRNA); translation is monocistronic. Two proteins (vp4 and vp7) comprise the rotavirus outer capsid. vp4 is an 88-kDa protein that is the virus hemagglutinin and probable cell attachment protein. vp4 projects as a spike from the virus surface and comprises approximately 2.5% of the viral mass. Cleavage of vp4 by trypsin into two proteins of  $M_r$  60,000 (vp5\*) and 28,000 (vp8\*) is associated with virus entry into cells. vp7 is a 37-kDa glycoprotein that comprises the smooth outer capsid shell and represents approximately 30% of the viral mass. Four proteins (vp1, vp2, vp3, and vp6) comprise the virus core, with vp6 accounting for 50% of the viral mass and 80% of the viral core. Six nonstructural proteins (NS53, NS34, NS35, NS28, NS26, and NS12) are also produced during rotavirus infection (Mattion *et al.*, 1991).

Similar to other members of the Reoviridae, rotaviruses contain a virus-associated RNA transcriptase that synthesizes RNA transcripts that act as mRNA and as templates for the formation of dsRNA. However, rotaviruses are unique among the Reoviridae in that maturation of rotavirus particles occurs in the rough endoplasmic reticulum

(RER). Immature, single-shelled rotavirus particles bud across the RER, transiently acquiring a membrane. This budding event is mediated by an interaction between vp7 and NS28, the latter acting as a membrane-bound receptor. Both vp7 and NS28 are directed to the ER via specific targeting sequences (reviewed in Both *et al.*, 1993b).

### III. IMMUNE RESPONSE IN EXPERIMENTAL ANIMALS

#### A. *Animal Models of Infection and Disease*

Rotaviruses infect the young of most species of domestic and laboratory animals (reviewed in Estes *et al.*, 1983). Studies of the histopathological changes induced after rotavirus infection of both large (i.e., calves, lambs, pigs, and dogs) and small (i.e., mice) animals inoculated with homologous host viruses have yielded remarkably similar findings (reviewed in Greenberg *et al.*, 1994). Rotaviruses infect mature villus epithelial cells located at the small intestinal mucosal surface. Infection of epithelial cells causes vacuolization and denudation with replacement of columnar by cuboidal cells. As a result, villi become stunted and shortened. Neither crypt epithelial cells of the small intestine nor mature villus epithelial cells of the large intestine appear to support rotavirus replication. Although rotaviruses may replicate in specialized membranous epithelial cells ("M" cells) that overlay Peyer's patches (Buller and Moxley, 1988), there is scant evidence for an inflammatory response in either Peyer's patches or the intestinal lamina propria during acute infection. The mechanism by which rotavirus induces diarrhea remains unclear (reviewed in Greenberg *et al.*, 1993).

The finding of infectious rotavirus particles only in small intestinal epithelial cells is consistent with the observation that histopathological changes are limited to the intestinal mucosal surface. Rotavirus antigen has been detected by immunofluorescence in Peyer's patches and mesenteric lymph nodes of mice (Dharakhul *et al.*, 1988) and infectious virus has been detected in the mesenteric lymph nodes of calves (Mebus, 1976) after homologous host infection. However, infectious virus has not been clearly detected in the blood or sites distant from the intestine after homologous host infection. Therefore, a successful rotavirus vaccine must induce an immune response that is active at the intestinal mucosal surface.

A number of animal models have been used to study the immune response to infection as well as the capacity of immunization to protect against disease. The ideal model to study the immunological determi-

nants of protection against rotavirus challenge does not exist. Large animals (i.e., calves, pigs, and lambs) infected with cell culture-adapted, homologous or heterologous host rotavirus strains (Bohl *et al.*, 1984; Gaul *et al.*, 1982; Snodgrass and Wells, 1976; Snodgrass *et al.*, 1980; Vonderfecht and Osburn, 1982; Woode *et al.*, 1983) were among the first groups of animals to be studied. The use of cell culture-adapted rotavirus strains allowed for reproducible quantitation of infectious virus and production of adequate quantities of purified virus for *in vitro* immunological assays. In addition, the extended window of disease in large animals was suitable for studies of active immunization. Unfortunately, the expense and relative inaccessibility of large animals limited the size of studies. The initial choice of large animal models for study was necessitated by problems associated with the use of small animals. Although suckling mice were known to develop rotavirus-induced gastroenteritis (Adams and Kraft, 1967; Banfield *et al.*, 1968), immunological studies in mice were limited by the fact that murine rotaviruses were not adapted to growth in cell culture. This problem was obviated when primate strains (e.g., SA11, RRV) well adapted to growth in cell culture were found to induce clinical symptoms, small intestinal histopathological changes, and a rotavirus-specific immune response similar to that found in large animals (Offit *et al.*, 1984). Because mice were inexpensive and easily maintained, large numbers of animals could be studied. In addition, the use of genetically defined strains of mice allowed for studies of cytotoxic T lymphocytes. Unfortunately, the narrow window of infection in mice (about 10 days) precluded an active immunization scheme. In addition, because induction of disease required large quantities of virus, and disease occurred in the absence of either viral amplification or multiple cycles of virus replication, the degree to which infection of mice with heterologous host (i.e., nonmurine) strains was predictive of events occurring after natural (or homologous host) infection was questioned. The adaptation of murine rotaviruses to growth in cell culture (Greenberg *et al.*, 1986) and the large window of infection (i.e., virus shedding) in adult mice now allow for studies of active immunization in mice (Ward *et al.*, 1990). Similarly, oral inoculation of rabbits with cell culture-adapted lapine strains results in virus shedding over a period of time long enough to include studies of active immunization (Connor *et al.*, 1988). These models allow for determination of immunological correlates of protection by active immunization, using large numbers of genetically defined animals. However, adult mice and rabbits are models for virus shedding but not disease. The degree to which the adult mouse and rabbit models will be predictive of immunological correlates of protection against disease in humans in part depends on

understanding the mechanisms by which virus shedding but not disease is induced in these animals. In retrospect, perhaps the best approach to study the immunological determinants of protection against rotavirus challenge would be to go back to the future. Although limited by expense, large animals (i.e., calves, pigs, and lambs) clearly allow for studies of protection against disease using an active immunization scheme with cell culture-adapted homologous host viruses.

### B. B Cell Response

The intestine is a rich source of T and B lymphocytes. Intestinal lymphocytes are located either at the intestinal mucosal surface among villus epithelial cells (intraepithelial lymphocytes), among lymphatic capillaries of the villus below the basement membrane (lamina propria lymphocytes), within lymphatic nodules underlying specialized epithelial cells (M cells) at the base of the villus (Peyer's patch lymphocytes), or within lymph nodes that drain the small intestine (mesenteric lymph nodes). Approximately 50–60% of intraepithelial lymphocytes are T cells, most of which have surface markers consistent with the functions of cytotoxicity and suppression (CD8) (Cerf-Bensussan *et al.*, 1985; Petit *et al.*, 1985); less than 10% of intraepithelial lymphocytes are B cells. In contrast, in the lamina propria and Peyer's patch B cells outnumber T cells by factors of 2:1 and 3:1, respectively. The percentages of T lymphocytes in lamina propria and Peyer's patch bearing CD4 (helper/inducer) or CD8 on their surface are similar.

The humoral immune response, using animals infected with homologous host rotavirus strains, has been the primary focus of a number of studies. Studies of calves, rabbits, and pigs orally inoculated with cell culture-adapted strains (Conner *et al.*, 1991; Corthier and Vannier, 1983; Saif, 1987; Saif *et al.*, 1992; Vonderfecht and Osburn, 1982) and mice with murine strains poorly adapted to growth in cell culture (Sheridan *et al.*, 1983) have produced important information on the site of rotavirus-specific antibody-bearing cells (i.e., B cells and plasma cells) and the isotype and neutralization capacity of these antibodies. Within 10 days of infection, rotavirus-specific binding and neutralizing antibodies of all isotypes are detected both in the serum and in small intestinal fluids; in both sites the appearance of rotavirus-specific IgM precedes the appearance of virus-specific IgA and IgG. At the intestinal surface, rotavirus-specific secretory IgA (sIgA) is the predominant isotype. This finding is consistent with the observation that rotavirus-specific IgA-bearing cells in the lamina propria and mesenteric lymph node outnumber virus-specific IgG- or IgM-bearing

cells by a factor of at least 10:1 (Saif *et al.*, 1992; Vonderfecht and Osburn, 1982). Indeed, in the lamina propria of suckling mice orally inoculated with murine rotavirus, 50% of *all* IgA-bearing cells are rotavirus specific (Dharakul *et al.*, 1988).

There are also a number of studies examining the immune response to animals orally inoculated with heterologous host viruses (Merchant *et al.*, 1991; Offit and Clark, 1985a,b; Shaw *et al.*, 1993). In mice, for example, heterologous host rotaviruses are better adapted to growth in cell culture than murine strains. The availability of large quantities of purified virus and the capacity to readily evaluate the immune response *in vitro* with well-characterized strains and large numbers of genetically defined animals has afforded an opportunity to examine the humoral and cellular immune response in detail. However (as stated above), because the biology of heterologous host virus infection differs from that of homologous host virus infection, the results of these studies must be interpreted with caution. Induction of rotavirus gastroenteritis in mice with nonmurine rotaviruses requires an inoculum  $10^5$ - to  $10^6$ -fold greater than that required for cell culture-adapted murine rotaviruses (Greenberg *et al.*, 1986). In addition, virus amplification and extended cycles of replication are observed after homologous but not heterologous host infections. Despite these caveats, mice orally inoculated with heterologous host rotaviruses develop a vigorous immune response. Suckling mice orally inoculated with rhesus rotavirus strain RRV develop rotavirus-specific IgM, IgG, and IgA antibody-secreting cells in Peyer's patch and the small intestinal lamina propria in greater numbers than those detected in spleen and mesenteric lymph nodes (Merchant *et al.*, 1991). Virus-specific IgA-secreting cells are detected at far greater frequency in lamina propria and Peyer's patch than are virus-specific IgG- and IgM-secreting cells. Similar to findings with murine viruses in mice, virus-specific IgA accounted for an astounding 50% of all IgA-secreting cells in the lamina propria. Dominance of rotavirus-specific IgA-secreting cells was long-lasting; 1 year after inoculation 17% of all IgA-secreting cells were rotavirus specific (Shaw *et al.*, 1993). Similarly, adult mice orally inoculated with simian strain SA11 generate SA11-specific neutralizing antibodies of both IgA and IgG isotype in both serum and milk (Offit and Clark, 1985a).

Clearly oral inoculation of experimental animals with live homologous or heterologous strains of rotavirus induces antibody-secreting cells among intestinal lymphocytes as well as rotavirus-specific IgA and IgG at the intestinal mucosal surface and in breast milk. However, inoculation by the oral route and inoculation with live rotavirus may

not be required for induction of a vigorous immune response among intestinal lymphocytes or at the intestinal mucosal surface. Adult mice parenterally inoculated with simian strain SA11 develop high titers of virus-specific IgG in milk (Offit and Clark, 1985a). [Because lymphocytes sensitized in the gastrointestinal tract are selectively transported to the mammary gland, milk antibodies are a window to the antibody response occurring at the intestinal mucosal surface (Goldblum *et al.*, 1975).] In addition, parenteral inoculation of mice with noninfectious RRV induced circulating, RRV-specific neutralizing antibodies as well as virus-neutralizing antibodies (presumably IgG) in milk (Offit and Dudzik, 1989a). A direct proof that rotavirus-specific B cells are induced among intestinal lymphocytes (e.g., by B cell enzyme-linked immunospot assay) after parenteral inoculation awaits further experimentation. However, these results are consistent with those previously observed after inoculation of mice with cholera toxin (Fuhrman and Cebra, 1981). Mice parenterally inoculated with cholera toxin develop toxin-specific B cells in the lamina propria at frequencies similar to that observed after oral inoculation; parenteral inoculation induces primarily toxin-specific IgG and oral inoculation induces primarily toxin-specific IgA-bearing cells.

### C. T Cell Response

Mice orally inoculated with heterologous host strains of rotavirus develop a vigorous, virus-specific cytotoxic T lymphocyte (CTL) response. Unlike the immune response induced by most virus infections in experimental animals, rotaviruses induce a primary CTL response (i.e., lymphocytes taken directly from the host lyse virus-infected target cells). Primary, rotavirus-specific CTLs are detected at the intestinal mucosal surface (among intraepithelial lymphocytes), in Peyer's patch, lamina propria, mesenteric lymph nodes, and spleen acutely after oral or parenteral inoculation of mice with RRV (Offit and Dudzik, 1989b); this response is inducible, virus specific, and mediated by Thy-1<sup>+</sup>, major histocompatibility complex (MHC)-restricted cells. Similarly, rotavirus-specific memory CTLs (CTL precursors) appear among intestinal and nonintestinal lymphocyte populations after oral or parenteral inoculation (Offit *et al.*, 1991a). Although route of inoculation determines the frequencies of CTL precursors within a given site acutely after infection (frequencies of RRV-specific CTL precursors were 25- to 30-fold greater after oral than after subcutaneous inoculation), CTL precursors are distributed throughout the lymphoid system several weeks after inoculation (Offit *et al.*, 1991a). Therefore, analo-



gous to induction of virus-specific B cells, oral inoculation of rotavirus may not be necessary to induce virus-specific CTLs or CTL precursors among intestinal lymphocytes.

Activation and differentiation of CD3<sup>+</sup>CD8<sup>+</sup> virus-specific CTL precursors to CTLs is enhanced by exposure to interleukin 2 (IL-2) secreted primarily by CD4<sup>+</sup> cells (i.e., helper T cells). Similarly, activation and differentiation of B cells to antibody-secreting cells is enhanced by exposure to IL-4 or IL-5 or both. If T cell help for rotavirus-specific CTL precursors or B cells occurs locally, virus-specific helper T cell activity should be detected in lamina propria and Peyer's patch. However, this may not be the case. After oral inoculation of mice with live rotavirus strain RRV or parenteral inoculation with live or noninfectious RRV, virus-specific helper T cell activity was detected among CD4<sup>+</sup> cells by lymphoproliferation assay (LPA) in mesenteric lymph nodes and spleen, but not in lamina propria and Peyer's patch (Riesen and Offit, 1992). Therefore, T cell help for either CTL precursors or B cells may not occur locally but rather in central sites of antigen concentration and presentation (i.e., mesenteric lymph nodes and spleen). Because cytokine secretion may occur in the absence of lymphoproliferation (Evavold and Allen, 1991), these results must be interpreted with caution.

#### *D. Serotype and Structural Specificities of B and T Cell Response*

Several studies have determined the rotavirus protein or proteins that evoke antibodies that neutralize virus infectivity *in vitro* (i.e., determine neutralization phenotype or serotype). Using reassortant rotaviruses made by *in vitro* coinfection between either two animal strains (Offit and Blavat, 1986; Offit *et al.*, 1986a) or between two human strains or an animal and a human strain (Hoshino *et al.*, 1985), both outer capsid proteins [vp4 (P type) and vp7 (G type)] were found to segregate independently with neutralization phenotype. Although the greatest quantity of rotavirus-specific antibodies is directed against inner capsid proteins (Shaw *et al.*, 1991), there is no evidence that these antibodies are important in neutralization of virus infectivity. Therefore, characterization of rotavirus serotypes is dependent on a definition of both surface proteins (in a manner analogous to the influenza virus hemagglutinin and neuraminidase). To underline further the importance of vp4 and vp7, inoculation of animals with vp4 or vp7 expressed in either a baculovirus, herpes simplex virus, or vaccinia virus vector has been found to elicit virus-specific neutralizing antibodies (Andrew *et al.*, 1990; Dormitzer *et al.*, 1992; Nishikawa *et al.*, 1989). Although studies of reassortant rotaviruses have contributed

to our understanding of B cell specificities, there are two reasons why data generated using this approach should be interpreted with caution. First, rotavirus serotypes were determined in these studies after oral or parenteral inoculation of experimental animals with large quantities of heterologous host strains (in some cases with oil-based adjuvants). Because infection in the wild does not occur either via the parenteral route, with adjuvant, or with large quantities of heterologous host viruses, these studies may not accurately predict the relative importance of vp4 and vp7 in inducing neutralizing antibodies after natural infection. Second, interactions between surface proteins of different strains used to generate reassortants may alter neutralization epitopes present on parental strains (Chen *et al.*, 1992).

The epitopes on rotavirus surface proteins vp4 and vp7 associated with virus neutralization have been identified by sequencing variant viruses selected with monoclonal antibodies (reviewed in Matsui *et al.*, 1989a). Both vp4 and vp7 contain regions that evoke serotype-specific or cross-reactive neutralizing antibodies. However, most monoclonal antibodies directed against vp7 and the vp8\* region of vp4 are serotype specific, whereas those directed against the vp5\* region of vp4 are cross-reactive (Kitaoka *et al.*, 1986; Mackow *et al.*, 1988; Taniguchi *et al.*, 1988; Matsui *et al.*, 1989b).

Rotavirus-specific CTLs are not serotype specific. Lymphocytes obtained from mice orally inoculated with different rotavirus strains lyse target cells infected with different rotavirus G (vp7) types (Offit and Dudzik, 1988). Although one would have predicted that cross-reactive, rotavirus-specific CTLs would be directed against rotavirus inner capsid proteins (which are antigenically conserved among different rotavirus serotypes), this is not the case. Using vaccinia virus recombinants expressing individual rotavirus proteins, cross-reactive, rotavirus-specific CTLs recognized vp7 better than vp4 or vp6 (Offit *et al.*, 1991b). Similarly, mice orally inoculated with vaccinia virus recombinants expressing vp7 develop rotavirus-specific, cross-reactive CTLs, whereas virus-specific CTLs are not induced after inoculation with recombinants expressing vp4 or vp6 (Offit *et al.*, 1994). Using peptides representing regions on bovine rotavirus vp7, an immunodominant peptide associated with cross-reactive, rotavirus-specific CTL activity was mapped to amino acids 30–41 (Franco *et al.*, 1992). All of these studies were performed in *H-2<sup>b</sup>* haplotype mice with a limited number of rotavirus proteins or peptides; the extent to which these findings are predictive of important CTL structural specificities of all rotavirus proteins or of animals or humans representing different genetic backgrounds remains to be determined.

*E. Protection against Disease by Passive Immunization*

The development of a successful rotavirus vaccine will depend on an understanding of the rotavirus structural and serotype specificities of the humoral and cellular immune response associated with protection against challenge. Many studies have used a passive immunization model of protection to answer these questions. Although passive models of immunization allow for studies of individual effector arms of the immune system, the degree to which passive immunization is predictive of events that occur after natural infection or active immunization is unclear.

Rotavirus-induced gastroenteritis is a common disease among the young of most species of domestic and feral animals (reviewed in Estes *et al.*, 1983). Therefore, in nature, despite the presence of rotavirus-specific neutralizing antibodies in colostrum and milk, breast-feeding does not appear to protect completely against rotavirus-induced disease. However, many studies in experimental animals found that immunization with live or inactivated rotaviruses or rotavirus-specific proteins can induce a level of rotavirus-specific neutralizing antibodies in colostrum and milk that passively protects against disease.

Colostrum and milk antibodies probably protect against relatively severe disease under natural conditions of infection. Rotavirus replication in mature villus epithelial cells of the small intestine induces virus-specific B cells among intestinal lymphocytes that travel to the mammary gland (Goldblum *et al.*, 1975). Therefore, colostrum and milk from animals exposed to wild-type viruses contain rotavirus-specific binding and neutralizing antibodies (Woode *et al.*, 1975; Saif *et al.*, 1983). Colostrum obtained from cows previously exposed to wild-type bovine rotavirus protected calves against rotavirus disease when fed in large quantities; smaller quantities of colostrum did not protect against severe disease (Snodgrass and Wells, 1976, 1978a,b; Saif *et al.*, 1983). Protection occurred in the absence of circulating, rotavirus-specific antibodies.

Rotavirus-specific antibodies induced in colostrum and milk after oral or parenteral inoculation of experimental animals clearly protect against disease induced by challenge with a homotypic strain. Cross-fostering studies in mice found that gastrointestinal but not circulating antibodies protected against disease induced by heterologous host rotavirus challenge (Offit and Clark, 1985a). However, presentation of virus antigen to the intestinal mucosal surface was not necessary to induce virus-specific B cells in the mammary gland. For example, parenteral or oral immunization of mice with heterologous host rotaviruses induced high titers of virus-specific neutralizing antibodies

in milk; oral immunization induced primarily rotavirus-specific sIgA and parenteral immunization induced primarily virus-specific IgG (Offit and Clark, 1985a). Although rotavirus-specific sIgA was approximately 10-fold more potent than IgG *in vivo*, both clearly protected against rotavirus-induced disease (Offit and Clark, 1985a). Similarly, parenteral inoculation of cows with inactivated bovine rotavirus, or of mice with "empty capsid" preparations of SA11 (i.e., containing inner and outer capsid rotavirus proteins but lacking viral genome), or of mice with vaccinia virus or adenovirus vectors expressing SA11 vp7 induced a level of virus-neutralizing antibodies in milk that protected against homotypic rotavirus challenge (Mebus *et al.*, 1973; Sheridan *et al.*, 1984; Snodgrass *et al.*, 1980; Andrew *et al.*, 1992; Both *et al.*, 1993a).

The capacity of rotavirus antibodies to passively protect against challenge by strains heterotypic to the immunizing strain is dependent on the route of inoculation used to generate virus-specific antibodies. Repeated oral inoculation of mice with human and nonmurine animal strains induced a level of homotypic neutralizing antibodies that was approximately 80-fold less than that induced after parenteral inoculation (Offit and Clark, 1985b). Similarly, the level of heterotypic neutralizing antibodies was greater after parenteral than oral inoculation. Protection by passively acquired rotavirus-specific antibodies in milk was induced against both homotypic and heterotypic virus challenge after parenteral immunization but only against homotypic challenge after oral "hyperimmunization." The capacity of parenteral immunization to induce protection against heterotypic virus challenge has been demonstrated by a number of investigators (Lecce *et al.*, 1991; Losonsky *et al.*, 1986a; Schaller *et al.*, 1992; Snodgrass *et al.*, 1977). In addition, parenteral immunization with various combinations of baculovirus-expressed whole vp4, vp6, and vp7, or vp4- or vp7-specific peptides linked to vp6, can induce antibodies that passively protect against homotypic or heterotypic rotavirus challenge (Ijaz *et al.*, 1991; Mackow *et al.*, 1990; Redmond *et al.*, 1993). Presumably, the capacity to induce antibodies directed against broadly cross-reactive epitopes on either vp4 or vp7 or both is dependent on the way in which antigen is initially processed by the immune system (i.e., peritoneal vs intestinal antigen-presenting cells) or the quantity of virus available to the immune system (e.g., absence of inactivation of rotavirus by gastric acid after parenteral as compared to oral inoculation).

The protein and peptide specificities of rotavirus-specific antibodies that passively protect against challenge parallels the specificities of *in vitro* neutralizing antibodies. Animals orally inoculated with reassortant rotaviruses containing either vp4 or vp7 from different parents

induced antibodies that passively protected mice against disease induced by either parental serotype (Offit *et al.*, 1986a). Similarly, monoclonal antibodies directed against regions on vp4 or vp7 that were serotype specific or cross-reactive passively protected animals against challenge with one or several rotavirus serotypes, respectively (Offit *et al.*, 1986b; Matsui *et al.*, 1989b). The parallel between *in vivo* and *in vitro* observations with monoclonal antibodies is due to the manner in which studies were performed. Large quantities of rotavirus-specific antibodies derived from ascitic fluids were administered either 30 min before or up to 2 hr after rotavirus challenge. The question of which epitope or epitopes on vp4 or vp7 induce virus-specific memory B cells likely to afford protection against challenge remains unanswered by studies using a passive immunization scheme.

Rotavirus-specific CTLs also passively protect animals against disease. Adoptive transfer of splenic lymphocytes from adult mice orally inoculated with homologous or heterologous host rotavirus strains protected suckling mice against murine rotavirus challenge (Offit and Dudzik, 1990). Protection was MHC restricted, abrogated after elimination of Thy-1- or CD8-bearing cells, and occurred in the absence of circulating, rotavirus-specific antibodies. Similarly, CD8-bearing splenic or small intestinal intraepithelial lymphocytes from adult mice inoculated parenterally with murine rotavirus ablate virus shedding in SCID (severe combined immunodeficiency) mice chronically infected with murine rotavirus; ablation of virus shedding was MHC restricted, broadly cross-reactive among different rotavirus serotypes, and occurred in the absence of neutralizing antibodies in the serum (Dharakul *et al.*, 1990, 1991). In addition, CD8-bearing lymphocytes obtained after inoculation of mice with baculovirus recombinants expressing vp1, vp4, vp6, or vp7 but not vp2, NS53, NS35, or NS28 abrogated murine rotavirus shedding in SCID mice. Rotavirus-specific CTLs induced after oral inoculation with heterologous host strains were directed primarily against vp7 (Offit *et al.*, 1991b; Franco *et al.*, 1992). Last, *in vivo* depletion of CD8-bearing but not CD4-bearing cells increased rotavirus excretion in calves (Bridger *et al.*, 1992). There remains much to be learned about the memory, trafficking, function, structural specificities, and distribution of rotavirus-specific CTLs after natural infection or immunization.

#### *F. Protection against Disease by Active Immunization*

Passive transfer of either rotavirus-specific antibodies or CTLs can protect animals against rotavirus disease. A passive immunization model allows for the evaluation of the protective capacity of individual

effector arms of the immune response. However, the host does not acquire high-titered monoclonal or polyclonal rotavirus-specific neutralizing antibodies or adoptively transferred virus-specific CTLs at or before natural challenge with wild-type virus. Therefore, analyses of the immunological determinants of protection against challenge are best performed in an active immunization scheme. Unfortunately, studies of active immunization have not provided a clear picture of which immunological effector arms are protective against challenge. For example, studies in both calves and mice found that protection against disease induced by homologous host virus after active immunization occurred in the absence of challenge virus-specific neutralizing antibodies in either serum, feces, or intestinal washes (Bridger and Oldham, 1987; Woode *et al.*, 1987; McNeal *et al.*, 1992; Ward *et al.*, 1992a). Fecal antibodies may not accurately predict antibody responses in the small intestine. Alternatively, virus-specific CTLs (which were not measured in these studies) may play an important role in protection after active immunization. In either case, the absence of a reliable and easily obtained immunological marker for protection against disease is a recurrent theme in immunological studies of children and experimental animals and has clearly hampered efforts to develop a successful rotavirus vaccine.

Three important observations made in studies of passive immunization were confirmed using an active immunization scheme. First, protection against homotypic challenge can be induced after oral immunization. Mice, pigs, calves, and rabbits were protected against disease induced by homologous host viruses after oral inoculation with strains homotypic but not heterotypic to the challenge virus (Woode *et al.*, 1978, 1983, 1987, 1989; Gaul *et al.*, 1982; Bohl *et al.*, 1984; Hoshino *et al.*, 1988; Hambraeus *et al.*, 1989; Ward *et al.*, 1990, 1992a; Conner *et al.*, 1991). However, ablation of virus shedding after heterotypic challenge by active oral immunization has also been demonstrated (Burns *et al.*, 1992). Second, neither inoculation with live rotavirus nor presentation of antigen to the intestinal mucosal surface is necessary for induction of protection against rotavirus disease. Intraperitoneal inoculation of mice with purified, inactivated murine rotavirus without adjuvant ablated rotavirus shedding after murine rotavirus challenge (McNeal *et al.*, 1992). In addition, parenteral inoculation of virus-like particles containing baculovirus-expressed vp2, vp4, vp6, and vp7 ablated rotavirus shedding after heterotypic challenge (Crawford *et al.*, 1992). Third, protection against heterotypic challenge is probably best induced after parenteral inoculation (Wyatt *et al.*, 1979, 1983; Zissis *et al.*, 1983; McNeal *et al.*, 1992; Ward *et al.*, 1992a).

The structural specificities of the humoral immune response associ-

ated with protection against challenge after active immunization have been defined (Hoshino *et al.*, 1988). Similar to studies *in vitro* and those using a passive immunization scheme with polyclonal or monoclonal antibody preparations, both vp4 and vp7 independently evoke antibodies that are associated with protection against challenge. Oral inoculation of piglets with reassortant rotaviruses containing genes that encoded vp4 or vp7 from two serotypically distinct (and not mutually cross-protective) porcine rotaviruses protected against challenge with either parent.

#### IV. IMMUNE RESPONSE IN INFANTS AND YOUNG CHILDREN

##### *A. Infection and Disease in Humans*

The clinical presentation of children admitted to the hospital with rotavirus-induced disease is not clearly distinct from other infectious causes of gastroenteritis. Rotavirus disease affects children primarily between 6 and 18 months of age (Kapikian *et al.*, 1976) and in temperate climates occurs almost exclusively during the winter months (LeBaron *et al.*, 1990). The disease is characterized by the sudden onset of watery diarrhea, fever, and vomiting. The presence of vomiting and moderate to severe dehydration is significantly more common in patients excreting rotavirus than in those who are not (Rodriguez *et al.*, 1977); this fact accounts in large part for the disproportionate number of hospitalizations for rotavirus-induced gastroenteritis as compared to gastroenteritis caused by other agents.

The epidemiology and clinical presentation of rotavirus-induced disease provide a number of clues to host factors associated with amelioration of acute infection and protection against reinfection. First, rotavirus replication probably occurs solely in small intestinal villus epithelial cells (Bishop *et al.*, 1973; Davidson *et al.*, 1975). Although there are a number of case reports claiming an association of rotavirus infections with diseases distant from the intestinal tract (Salmi *et al.*, 1978; Wong *et al.*, 1984; Rotbart *et al.*, 1983, 1988; Hattori *et al.*, 1992; Grunow *et al.*, 1985; Santosham *et al.*, 1983; Matsuno *et al.*, 1983; Yolken and Murphy, 1982; Whorwell *et al.*, 1977), there remains no clear evidence that rotavirus replication is supported by cells other than those that line the small intestine or that viremia is an important component in the pathogenesis of rotavirus-induced disease. Therefore, protection against reinfection is dependent on induction of an immune response active at the intestinal mucosal surface. Second,

children with immunodeficiency syndromes develop prolonged shedding of rotavirus after acute infection (Saulsbury *et al.*, 1980; Wood *et al.*, 1988). Therefore, amelioration of acute infection is at least in part mediated by immunological factors.

### *B. Protection against Disease by Breast-Feeding and Passive Immunization*

Virtually all women, independent of socioeconomic background, have rotavirus-specific binding antibodies (primarily sIgA) and rotavirus-specific neutralizing antibodies in colostrum and milk (Yolken *et al.*, 1978b; Cukor *et al.*, 1979; Otnaess and Orstavik, 1980; Bell *et al.*, 1988; Ringenbergs *et al.*, 1988). Levels of virus-specific binding and neutralizing antibodies appear to decline in milk during the first 6 months of life (Yolken *et al.*, 1978b; Ringenbergs *et al.*, 1988). The presence of virus-specific neutralizing antibodies may in part be responsible for the decreased incidence of symptomatic rotavirus infection in early infancy (Perez-Schael *et al.*, 1984; Chrystie *et al.*, 1978).

Breast-feeding appears to protect against relatively severe rotavirus disease in infants (Mata *et al.*, 1983; Duffy *et al.*, 1986a,b; Zheng *et al.*, 1992). Higher levels of rotavirus-specific neutralizing antibodies in colostrum and milk are detected in mothers of uninfected as compared to infected neonates (Zheng *et al.*, 1992). However, unlike enteric infections such as cholera (Glass *et al.*, 1983) and shigella (Stoll *et al.*, 1982) (in which the relationship between breast-feeding and protection against disease is clear-cut), a number of studies failed to demonstrate an association between breast-feeding and protection against rotavirus disease (Cushing and Anderson, 1982; Totterdell *et al.*, 1982; Weinberg *et al.*, 1984; Glass *et al.*, 1986; Blake *et al.*, 1993). Similar to passive protection studies performed in animals (Offit and Clark, 1985b), the capacity of colostrum or milk to protect against disease is probably dependent on the titer of serotype-specific neutralizing antibodies. Levels of neutralizing antibodies in milk that are protective against challenge may only occasionally be reached after natural infection. Therefore, studies including low numbers of infants may give conflicting results. In addition, part of the variance among these studies may be attributed to differences in (1) definition of protection against challenge (i.e., protection against infection vs protection against relatively severe disease), (2) sensitivity of assays used to correlate rotavirus-specific antibodies with protection (i.e., binding vs neutralizing antibodies), (3) nutritional status of the study population, and (4) study design. To date, studies of protection by breast-feeding



have not included an analysis of the relationship between serotype-specific neutralizing antibodies in milk and characterization of the P (vp4) and G (vp7) types of the infecting strains.

Protection against rotavirus infection and relatively severe disease can be afforded by oral administration of either serum immunoglobulins or bovine milk containing high titers of rotavirus-specific neutralizing antibodies (Barnes *et al.*, 1982; Davidson *et al.*, 1989). However, amelioration of acute disease in infants (as distinct from prophylactic protection against disease) is not afforded by passive administration of bovine milk containing high titers of rotavirus-neutralizing antibodies (Hilpert *et al.*, 1987). It is unclear whether passive protection is afforded by transplacental transfer of maternal, rotavirus-specific IgG (Totterdell *et al.*, 1980; Jayashree *et al.*, 1988; Bernstein *et al.*, 1990b). These findings are almost identical to those observed in mice; oral inoculation of mice with monoclonal antibody preparations containing high levels of neutralizing activity protected against disease when administered within 2 hr of infection, but did not ameliorate acute disease (Offit *et al.*, 1986b). In both mice and humans, rotavirus replication in the small intestine is limited to several days, and administration of antibodies at the time of clinical disease is probably too late to alter the clinical course. However, in children with immunodeficiency disorders (in whom rotavirus replication may occur over many weeks or months), rotavirus-specific immunoglobulin preparations administered orally may ablate shedding and ameliorate disease (Guarino *et al.*, 1991).

### *C. Response to Natural Infection*

The humoral immune response of infants and young children following rotavirus infection is similar to that observed in animals (Davidson *et al.*, 1983; Riepenhoff-Talty *et al.*, 1981; Grimwood *et al.*, 1988; Aiyar *et al.*, 1990). Within the first week of illness rotavirus-specific IgM is detected in the duodenal fluid and serum. Both 1 and 4 months after infection, rotavirus-specific IgG and sIgA are detected in the duodenal fluid and rotavirus-specific IgG and monomeric IgA are detected in the serum. Levels of salivary and fecal IgA are predictive of those obtained in duodenal fluid. One year after infection, rotavirus-specific IgG but not IgA is detected in the serum, and neither IgG nor IgA is detected at the mucosal surface. Because of its persistence in serum after natural infection, circulating rotavirus-specific IgG provides an excellent marker for previous exposure to rotavirus in older infants and children. In addition, fecal or duodenal IgA provides an excellent marker for recent infection (either primary infection or reinfection) because of

the relatively rapid disappearance of this isotype from the intestinal mucosal surface (Coulson *et al.*, 1990). Similar to infants and older children, neonates (i.e., infants less than 1 month of age) develop rotavirus-specific IgM and IgA in the circulation after either symptomatic or asymptomatic infection (Bishop *et al.*, 1990; Losonsky and Reymann, 1990).

Rotavirus-specific helper T cells are detected in the circulation within several weeks of primary, symptomatic infection (Offit *et al.*, 1993). This finding is consistent with the observation that lymphocytes originating in the murine small intestine migrate to the circulation after entrance through the thoracic duct (Guy-Grand *et al.*, 1978) and, therefore, similar to rotavirus-specific sIgA, rotavirus-specific T cells probably provide a window to intestinal T cell responses. There are no studies evaluating the relative contributions of T helper type 1 ( $T_h$ -1) or  $T_h$ -2 T cell or CTL responses in children after natural infection.

Although understanding may be critical to development of a successful vaccine, the P (vp4)- and G (vp7)-type specificities of the humoral immune response after natural infection remain unclear. Sera from animals parenterally inoculated with human rotaviruses have been used to define at least nine different human rotavirus G types (G types 1–4, 6, 8, 9, 10, and 12). G types 1–4 are the most important G types isolated in epidemiological studies in both developed and developing countries; G type 1 is the most common (Flores *et al.*, 1988; Matson *et al.*, 1990b; Padilla-Noriega *et al.*, 1990; Bishop *et al.*, 1991; Bingnan *et al.*, 1991; Ahmed *et al.*, 1991; Noel *et al.*, 1991; Woods *et al.*, 1992). Hybridization analysis has been used to distinguish at least five human rotavirus P types (Gentsch *et al.*, 1992; Gorziglia *et al.*, 1990; Estes and Cohen, 1989). The P- and G-type specificities of the humoral immune response after natural infection have been difficult to determine for a number of reasons. First, different human rotavirus P genotypes have only recently been identified. Second, rotavirus strains associated with infection are rarely characterized by both P and G type. Third, there may be important antigenic differences between P and G types of the infecting strains as compared to reference strains used in *in vitro* assays of neutralizing activity. Fourth, the specificities of the immune response may differ after primary versus subsequent infection. The presence of passively transferred maternal rotavirus-specific IgG has made it difficult to determine whether an infant has been previously exposed to rotavirus. Fifth, use of reassortant viruses to determine P- and G-type specificities may be undermined by differences in vp4 or vp7 phenotype determined by the parent (or background) strain (Chen *et al.*, 1992). Finally, use of epitope-blocking analysis to determine serotype specificities is hampered by the use of

monoclonal antibodies representing some but probably not all neutralization regions on vp4 or vp7. Therefore, epitope blocking assays may not accurately predict neutralization assays (Matson *et al.*, 1992). Despite these problems, a preponderance of evidence supports the hypothesis that vp4 may be more important than vp7 in evoking virus-specific neutralizing antibodies during natural infection (Clark *et al.*, 1985; Puerto *et al.*, 1987; Ward *et al.*, 1988; Gerna *et al.*, 1990; Brussow *et al.*, 1990; Offit *et al.*, 1993; Ward *et al.*, 1993). Studies of infants and young children found that neutralizing antibodies generated after natural infection were not specific for G type (Clark *et al.*, 1985; Puerto *et al.*, 1987; Gerna *et al.*, 1990) and were directed against vp4 (Brussow *et al.*, 1990; Offit *et al.*, 1993; Ward *et al.*, 1993). In addition, adults orally inoculated with a human rotavirus strain develop higher levels of neutralizing antibodies directed against vp4 than vp7 (Ward *et al.*, 1988). The capacity of the humoral immune response to distinguish different human rotavirus P types after natural infection has not been determined.

Studies of the humoral and cellular immune response in infants and young children after natural infection provide three important pieces of information. First, circulating rotavirus-specific IgA and virus-specific helper T cells probably provide a window to immune responses occurring among intestinal lymphocyte populations. Detection of rotavirus-specific sIgA in duodenal fluid and feces simultaneous to detection of rotavirus-specific monomeric IgA in the serum is compatible with the observation that IgA-bearing, antigen-specific B cells and plasma cells are detected concomitantly in the lamina propria and circulation acutely after oral inoculation of adult volunteers with cholera or *Streptococcus mutans* (Quiding *et al.*, 1991; Czerkinsky *et al.*, 1987). Second, the rotavirus-specific sIgA response induced at the intestinal mucosal surface after natural infection may be short lived (Davidson *et al.*, 1983; Coulson *et al.*, 1990). This finding has enormous implications on the timing of administration of rotavirus vaccine candidates. Third, vp4 is perhaps the most important protein in evoking neutralizing antibodies after natural infection. It is, therefore, of great importance to determine whether different human rotavirus P types distinguished by hybridization analysis are also distinguished by neutralizing antibodies generated after primary infection.

#### D. Protection against Disease by Natural Infection

Natural infection with rotaviruses protects against relatively severe disease caused by reinfection. Neonates infected within the first 2

weeks of life are protected against relatively severe disease but not reinfection (Bishop *et al.*, 1983). Similarly, infants and young children are protected against symptomatic disease after primary infection, independent of whether the primary infection was symptomatic or asymptomatic (Bernstein *et al.*, 1990a; Bishop *et al.*, 1990); protection lasted for at least 2 years. On the other hand, symptomatic reinfection 1 year following primary infection (even with the same serotype) is well described (Yolken *et al.*, 1978a; Black *et al.*, 1982; Bishop *et al.*, 1983; Mata *et al.*, 1983; Chiba *et al.*, 1986; Ward *et al.*, 1986, 1989; Linhares *et al.*, 1988; Georges-Courbot *et al.*, 1988; Friedman *et al.*, 1988; Grinstein *et al.*, 1989; Reves *et al.*, 1989; O'Ryan *et al.*, 1990; De Champs *et al.*, 1991). Similarly, 10–20% of adults in contact with rotavirus-infected infants and young children develop rotavirus-induced gastroenteritis (Kim *et al.*, 1977; Haug *et al.*, 1978; Rodriguez *et al.*, 1979, 1987). The attack rate in adults is similar to that observed in infants and young children. Therefore, although the data are somewhat contradictory, protection against rotavirus disease induced by natural infection may in many cases be short lived. It remains unclear whether serotype is important in protection against reinfection (Chiba *et al.*, 1986; Ward *et al.*, 1992b).

There remains no definitive immunological correlate of protection against rotavirus disease. However, protection is probably best predicted by the immunological response occurring at the intestinal mucosal surface. High levels of fecal, rotavirus-specific IgA correlate with protection against disease (Coulson *et al.*, 1992; Matson *et al.*, 1993). Of interest, high levels of rotavirus-specific IgG in serum also correlate with protection against relatively severe disease (Black *et al.*, 1982; Ryder *et al.*, 1985; Clemens *et al.*, 1992). However, although the quantity of serotype-specific neutralizing antibodies in serum directed against the challenge virus has been found to correlate directly with protection against disease (Chiba *et al.*, 1986) this has not been a consistent finding (Ward *et al.*, 1992b). Possibly, high levels of rotavirus-specific IgG in serum are predictive of persistence of rotavirus-specific sIgA at the intestinal mucosal surface. The short-lived sIgA response (in contrast to the relative persistence of circulating, rotavirus-specific IgG) is consistent with the often short-lived nature of protection against disease induced by natural infection, and further supports the correlation between rotavirus-specific sIgA at the intestinal surface and protection against disease. It would be of interest to determine the correlation between the serotype specificity of intestinal, rotavirus-specific IgA and protection against challenge of known serotype.

*E. Response to and Protection against Disease  
by Active Immunization*

Over the past 10 years thousands of infants and young children have been orally inoculated with candidate rotavirus vaccines in trials of protective efficacy (see Table I). Similar to the use of cowpox to prevent smallpox infection and *Mycobacterium bovis* (i.e., bacillus Calmette-Guérin) to prevent *Mycobacterium tuberculosis* infection, heterologous host rotaviruses have been used to prevent rotavirus infections. Non-human strains have included a primate strain [rhesus rotavirus strain MU18006 (RRV)] isolated from a rhesus monkey, and bovine strains that have been passaged many [Nebraska calf diarrhea virus (NCDV), strain RIT 4237] or few [Wistar calf 3 (WC3)] times in cell culture. In addition, reassortant rotaviruses containing 1 gene encoding outer capsid protein vp7 from human rotaviruses and 10 genes from either the bovine or simian rotavirus parent have been studied.

Similar to natural infection, oral inoculation of infants and young children with NCDV (strain RIT 4237) induces rotavirus-specific IgG and IgM and virus-specific neutralizing antibodies in serum (Vesikari *et al.*, 1985a, 1986; Maldonado *et al.*, 1986). In infants not previously exposed to rotavirus, immunization induces antibodies that neutralize NCDV but not human serotypes G1 or G2 (Vesikari *et al.*, 1983). In Finland, 40–50% of infants were protected against rotavirus-induced disease and 80–90% were protected against relatively severe disease within 1 year after oral inoculation with one or two doses of NCDV (Vesikari *et al.*, 1984, 1985b, 1991a). Similarly, three doses of NCDV in Peru induced protection against severe disease (Lanata *et al.*, 1989). On the other hand, studies in Rwanda (DeMol *et al.*, 1986) and Gambia (Hanlon *et al.*, 1987) failed to show protection against disease.

WC3, like NCDV, is not well adapted to growth in the human intestinal tract. Only about 20% of infants orally inoculated with  $10^7$  plaque-forming units (PFU) of WC3 shed virus in the feces and none develop signs or symptoms of gastrointestinal infection. Similar to NCDV, WC3 induces WC3-specific but not human serotype G1-, G2-, G3-, or G4-specific neutralizing antibodies in the serum (Clark *et al.*, 1986; Bernstein *et al.*, 1990b). In addition, WC3 inoculation induces a rotavirus-specific IgA response in serum and feces (Bernstein *et al.*, 1990b). In Philadelphia, 75% of infants orally inoculated with one dose of WC3 were protected against rotavirus disease induced by serotype G1. One hundred percent of these infants were protected against severe disease; protection occurred in the absence of G1-specific neutralizing antibodies in serum (Clark *et al.*, 1988). Statistically significant protection against disease was not observed in trials performed in

**TABLE I**  
**PROTECTION AGAINST ROTAVIRUS DISEASE INDUCED BY ACTIVE IMMUNIZATION WITH EITHER ANIMAL OR HUMAN ROTAVIRUSES**  
**OR ANIMAL × HUMAN REASSORTANT ROTAVIRUSES**

Candidate vaccine	Site	Number of subjects	Number of doses	Protection against severe disease (%)	Protection against all disease (%)	Ref.
<b>Bovine</b>						
NCDV	Finland	178	1	88	50	Vesikari <i>et al.</i> (1984)
NCDV	Finland	328	2	82	58	Vesikari <i>et al.</i> (1985b)
NCDV	Rwanda	245	1	0	0	DeMol <i>et al.</i> (1986)
NCDV	Gambia	185	3	31	0	Hanlon <i>et al.</i> (1987)
NCDV	Peru	391	3	75	40	Lanata <i>et al.</i> (1989)
NCDV	Finland	252	2	89	43	Vesikari <i>et al.</i> (1991a)
WC3	Philadelphia, PA	104	1	100	76	Clark <i>et al.</i> (1988)
WC3	Cincinnati, OH	206	1	41	17	Bernstein <i>et al.</i> (1990b)
WC3	Central African Republic	472	2	36	0	Georges-Courbot <i>et al.</i> (1991)
<b>Bovine × human reassortants</b>						
WC3-G1	Philadelphia	77	2	100	—	Clark <i>et al.</i> (1990)
<b>Human</b>						
M37	Finland	282	1	—	0	Vesikari <i>et al.</i> (1991b)

*(continued)*

TABLE I (Continued)

Candidate vaccine	Site	Number of subjects	Number of doses	Protection against severe disease (%)	Protection against all disease (%)	Ref.
Simian						
RRV	Maryland	27	1	—	100	Rennels <i>et al.</i> (1986)
RRV	Nashville, TN	50	1	—	50	Wright <i>et al.</i> (1987)
RRV	Venezuela	247	1	100	68	Flores <i>et al.</i> (1987)
RRV	Rochester, NY	176	1	0	0	Christy <i>et al.</i> (1988)
RRV	Maryland	114	1	—	29	Rennels <i>et al.</i> (1990)
RRV	Finland	200	1	67	38	Vesikari <i>et al.</i> (1990)
RRV	Sweden	106	1	80	48	Gothefors <i>et al.</i> (1990)
RRV	Venezuela	320	1	90	64	Perez-Schael <i>et al.</i> (1990a)
RRV	Rochester, NY	223	1	—	66	Madore <i>et al.</i> (1992)
Simian × human reassortants						
RRV-G1	Finland	359	1	—	67	Vesikari <i>et al.</i> (1992)
RRV-G2			1	—	65	Vesikari <i>et al.</i> (1992)
RRV-G1	Rochester, NY	223	1	—	77	Madore <i>et al.</i> (1992)
RRV-G1	United States	903	1	—	65	Sack <i>et al.</i> (1992)
RRV-G1			1	—	63	Sack <i>et al.</i> (1992)
RRV-G2						
RRV (G3)						
RRV-G4						

Cincinnati (Bernstein *et al.*, 1990b) and Bangui (Georges-Corbot *et al.*, 1991). However, in both Cincinnati and Bangui protection against G1 and G3 rotavirus challenge was associated with high levels of WC-3-specific neutralizing antibodies in serum. Explanations for the differences in results between trials in Philadelphia and Cincinnati remain unclear. The poor results obtained in the trial of WC3 in Central Africa are similar to those for NCDV in Rwanda and Gambia. Difficulties in induction of virus-specific immunity and protection against disease in developing countries has also been observed with oral poliovirus vaccine and may relate to either interfering rotavirus-specific antibodies in breast milk, concomitant and competitive infections of the gastrointestinal tract, relative malnutrition of the host leading to a poorer virus-specific immune response, or other unknown causes.

In an attempt to enhance protection against disease by WC3, a gene encoding human rotavirus outer capsid protein vp7 (strain WI79, G1) was used to replace the gene encoding WC3 vp7 (G6) in a reassortant strain (WC3-G1). In a small trial performed in Philadelphia, 100% of infants were protected against rotavirus disease induced by G1 or G3 strains (Clark *et al.*, 1990). However, although 97% of vaccinees developed WC3-virus-specific neutralizing antibodies in serum, only 22% developed neutralizing antibodies to G1 rotavirus (strain WI79). The immunological basis of the heterotypic protection observed in this trial remains unclear.

Simian strain RRV is better adapted to growth in the human intestinal tract than either bovine strain NCDV or WC3 (Vesikari *et al.*, 1986). Strain RRV is consistently shed in the feces of orally immunized infants and young children. In addition, RRV is G type 3. Similar to the bovine strains, oral inoculation of infants and young children with RRV induced rotavirus-specific IgG, IgA and IgM in serum, rotavirus-specific IgA in feces, and RRV- but not G1-specific neutralizing antibodies in serum (Losonsky *et al.*, 1986b, 1988; Anderson *et al.*, 1986; Perez-Schael *et al.*, 1987; Rennels *et al.*, 1987; Christy *et al.*, 1988). Similar to WC3, oral inoculation of RRV in infants and young children inconsistently protected against rotavirus disease in developed countries (Rennels *et al.*, 1986, 1990; Wright *et al.*, 1987; Christy *et al.*, 1988; Vesikari *et al.*, 1990; Gothefors *et al.*, 1990). However, unlike WC3, RRV has been shown to protect against severe disease in developing countries (Flores *et al.*, 1987; Perez-Schael *et al.*, 1990a). Strain RRV induced protection against either the G1 or G3 strain (Vesikari *et al.*, 1990; Gothefors *et al.*, 1990). Similar to the bovine strains, the immunological basis of protection by RRV against heterotypic challenge is not associated with the presence of heterotypic neutralizing antibodies in serum. However, consistent with heterotypic protection,



blocking antibodies directed against a broadly cross-reactive epitope (on vp4) were found in infants orally inoculated with RRV (Shaw *et al.*, 1987); antibodies directed against heterotypic epitopes on either vp4 or vp7 were not found in other studies (Green *et al.*, 1990; Padilla-Noriega *et al.*, 1992).

Similar to the bovine strains, protection against challenge after immunization with RRV was induced against human serotypes (e.g., G1) distinct from RRV. To further enhance the response against human G types, single gene reassortant viruses were constructed that expressed vp7 from human G type 1, 2, or 4 (Midthun *et al.*, 1985, 1986). Oral inoculation of infants with RRV × human reassortant strains induced rotavirus-specific IgA and RRV-specific neutralizing antibodies in serum (Flores *et al.*, 1989; Midthun *et al.*, 1989; Perez-Schael *et al.*, 1990b; Wright *et al.*, 1991; Dagan *et al.*, 1992). However, neutralizing antibody responses directed against G type 1, 2, or 4 after immunization with reassortant viruses containing these G types were not dramatically greater than heterotypic neutralizing antibody responses obtained after immunization with RRV alone (Flores *et al.*, 1989; Perez-Schael *et al.*, 1990b; Dagan *et al.*, 1992). These data are consistent with previously discussed observations that vp4 may be more immunogenic than vp7 after either natural infection or immunization (see Section IV,C). Immunization with RRV reassortant rotaviruses expressing human G type 1, 2, or 4 induced protection against rotavirus disease (caused predominantly by G1 rotavirus strains) in 63–77% of vaccinees within the first year (Vesikari *et al.*, 1992; Madore *et al.*, 1992; Sack, 1992). Therefore, protection against disease induced by reassortant viruses was not significantly greater than that found after immunization with RRV (see Table I). Protection against disease 1–2 years after immunization was less than that observed during the first year (Vesikari *et al.*, 1992; Madore *et al.*, 1992; Sack, 1992).

The only human rotavirus studied as a possible vaccine candidate was isolated from an asymptomatic Venezuelan newborn (strain M37). Immunization of infants with M37 induced M37-specific neutralizing antibodies in serum (Midthun *et al.*, 1991; Vesikari *et al.*, 1991b). However, oral inoculation of infants with M37 did not induce protection against rotavirus disease (Vesikari *et al.*, 1991b).

Studies of adults orally inoculated and challenged with rotaviruses have provided important information on the immunological correlates of protection against challenge. Unlike studies of young infants, studies in adults inoculated with rotaviruses represent responses to reinfection and not primary infection. Similar to observations in infants and young children, reinfection induces neutralizing antibodies that broadly cross-react with rotavirus serotypes distinct from the immu-

nizing serotype (Kapikian *et al.*, 1983; Ward *et al.*, 1986); cross-reactive neutralizing antibodies generated in serum block a heterotypic epitope located on vp4 (Green and Kapikian, 1992). Protection against disease correlated with challenge virus-specific neutralizing antibodies in jejunal fluid (Ward *et al.*, 1989) and epitope-blocking antibodies in serum directed against either heterotypic epitopes (on vp4) or homotypic epitopes (on vp7) (Green and Kapikian, 1992).

Comparison of results of rotavirus vaccine trials (see Table I) is difficult for a number of reasons. Differences among studies include dose of virus administered, dosing schedules, age of child at time of inoculation, feeding regimens of mothers (breast-fed vs non-breast-fed), and biology of bovine, simian, and human rotaviruses. Despite these differences, a remarkably similar pattern of results allows one to draw the following conclusions. First, protection against disease caused by strains heterotypic to the immunizing virus is induced after immunization with either bovine or simian strains. These findings were not clearly predicted by studies in animal models. Second, protection against rotavirus-induced disease often occurs in the absence of virus-specific neutralizing antibodies in serum directed against the challenge virus. Third, infants previously exposed to rotaviruses are more likely to develop antibodies that neutralize rotavirus strains serotypically distinct from the immunizing strain than unexposed infants. Finally, animal  $\times$  human reassortant viruses (expressing human G types) do not clearly induce a level of protection against disease greater than that observed after immunization with the parent bovine or simian strains.

## V. SUMMARY

Although studies of rotavirus immunity in experimental animals and humans have often yielded conflicting data, a preponderance of evidence supports the following answers to the questions initially posed.

1. What is the importance of virus serotype in formulating an optimal vaccine? Both vp4 and vp7 induce virus-neutralizing antibodies after either natural infection or immunization; the capacity of vp4 to induce rotavirus-specific neutralizing antibodies is probably greater than that of vp7. However, protection against disease after immunization of infants and young children is induced by strains heterotypic to the challenge virus (e.g., immunization with WC3 induces protection against disease induced by serotypically distinct human G1 strains). In

addition, oral inoculation of infants with primate or bovine reassortant rotaviruses containing genes that encode human vp7 has not consistently induced a higher level of protection against challenge than that induced by parent animal rotaviruses (see Table I). Therefore, although vp4 or vp7 or both are probably important in inducing protection against challenge, it has not been clearly demonstrated that inclusion of the epidemiologically important human (as distinct from animal) P or G type is important in protection against human disease.

2. Which immunological effector arm most likely protects against rotavirus disease? No immunological effector arm clearly explains protection against heterotypic challenge. Protection against disease is not predicted by rotavirus-specific neutralizing antibodies in serum. Rotavirus-specific, binding sIgA in feces [detected by enzyme-linked immunosorbent assay (ELISA)] induced after natural infection does correlate with protection against disease induced by subsequent infection. However, protection after immunization with WC3 may occur in the absence of a detectable fecal sIgA response. The relationship between rotavirus-binding sIgA and sIgA-mediated neutralizing activity directed against the challenge virus remains to be determined. Binding rotavirus-specific sIgA in feces detected by ELISA may only be a correlate of other events occurring at the intestinal mucosal surface. The presence of broadly cross-reactive, rotavirus-specific CTLs at the intestinal mucosal surface of mice acutely after infection is intriguing. It would be of interest to determine the degree to which the presence of cross-reactive, rotavirus-specific CTLs in the circulation is predictive of the presence of virus-specific CTLs among intestinal lymphocytes and protection against challenge. Unfortunately, studies of virus-specific CTLs are difficult to perform in children.

3. By what means is virus antigen best presented to the host to elicit a protective immune response? Oral inoculation may not be necessary to induce a protective, virus-specific immune response at the intestinal mucosal surface. Parenteral inoculation of experimental animals with rotavirus induces an immune response at the intestinal mucosal surface (probably virus-specific IgG) that is protective against challenge.

4. What are the advantages and disadvantages of replicating agents (i.e., live, attenuated human or animal rotaviruses, rotavirus reassortants, or vectors expressing individual rotavirus proteins) as compared to nonreplicating agents (e.g., inactivated virus, purified virus proteins, or peptides) as candidate rotavirus vaccines? Induction of a rotavirus-specific protective immune response is probably associated with the presence of virus-specific T and B cells in intestinal lymphoid tissues and virus-specific sIgA or IgG at the intestinal mucosal surface. There is some experimental evidence to support the hypothesis that

neither rotavirus replication nor presentation of rotavirus antigen to the intestinal mucosal surface is necessary to achieve this aim. Similarly, parenteral inoculation of children with inactivated poliovirus reduces virus shedding induced by subsequent oral inoculation with live, attenuated poliovirus (Faden *et al.*, 1990; Onorato *et al.*, 1991). Therefore, parenteral immunization of either replicating or non-replicating agents may prove to be an acceptable vaccine strategy.

## VI. FUTURE DIRECTIONS

Ten years of studying the immune response of infants and young children to natural infection and immunization with rotaviruses has yielded some good news and some bad news. The good news is that protection induced by natural infection is in many cases complete and long-lasting (i.e., for at least 2 years) (Bishop *et al.*, 1983, 1990; Bernstein *et al.*, 1990a). The bad news is that protection against disease by natural infection may also be short lived and incomplete (Yolken *et al.*, 1978a; Black *et al.*, 1982; Bishop *et al.*, 1983; Mata *et al.*, 1983; Chiba *et al.*, 1986; Ward *et al.*, 1986, 1989; Linhares *et al.*, 1988; Georges-Courbot *et al.*, 1988; Friedman *et al.*, 1988; Grinstein *et al.*, 1989; Reves *et al.*, 1989; O'Ryan *et al.*, 1990; De Champs *et al.*, 1991). Consistent with incomplete and short-lived protection after natural infection is the disappointing finding that oral inoculation with RRV (which occasionally induces mild disease and like natural infection clearly replicates in the intestine) does not consistently induce protection against challenge. The daunting task for rotavirus researchers may be to develop a vaccine that is in many cases better at inducing protection against disease than natural infection. There are currently about 20 vaccines used for active immunization in humans—only one (*Haemophilus influenzae* type B) induces protection against disease better than natural infection.

Why does infection with some viruses (e.g., measles, mumps, rubella, poliovirus) induce protection that is life-long and complete, whereas infection with others [e.g., rotavirus, influenza virus, respiratory syncytial virus (RSV)] induces protection that may be short lived and incomplete? The answer may be in part that rotavirus, influenza virus, and RSV are all superficial mucosal infections with short incubation periods (i.e., 2–5 days); virus is rarely detected in the blood, and replication at sites distant to the mucosal surface is not an important part of viral pathogenesis. In contrast, viremia (and replication distant from the site of primary replication) occurs in measles, mumps, rubella, and poliovirus infections; consequently, incubation periods are

long (i.e., 8–14 days). Because of these differences in incubation periods, activation and differentiation of virus-specific memory T and B cells are probably more important in protection against “systematic” infections than against “superficial” mucosal infections. Protection against superficial mucosal infections may simply be mediated by the level of virus-specific immunity (e.g., sIgA or IgG, or virus-specific CTLs) present at the mucosal surface at the time of infection.

The best rotavirus vaccine will probably be one that induces a protective level of virus-specific immunity at the intestinal mucosal surface from 6 months to 3 years of age. (Dehydration secondary to rotavirus-induced gastroenteritis is most commonly observed between 6 and 24 months of age and less commonly beyond 3 years of age.) There are several approaches that are currently being explored. (1) Oral inoculation of infants (beginning at 2–4 months of age) with either attenuated human, animal, or animal × human reassortant rotaviruses by schedules that would include booster immunizations immediately prior to the time at which children are likely to be exposed: If protection against disease induced by immunization or natural infection is short lived, immunization schedules similar to the oral poliovirus vaccine (i.e., 2, 4, and 6 months of age) may result in intervals between immunization and natural infection of up to 10 months. (2) Inclusion of genes that encode human vp4 (P type) in reassortant viruses: vp4 may more likely to induce rotavirus-neutralizing antibodies than vp7. Whether inclusion of vp4 in reassortant viruses induces protection better than that achieved with reassortants containing human vp7 remains to be determined. It should be noted that immunization of infants with bovine reassortant viruses containing both human outer capsid proteins vp4 and vp7 is probably less immunogenic than administration of bovine reassortants containing either human vp4 or vp7 (Clark *et al.*, 1992). (3) Immunization with attenuated human rotaviruses: Perhaps replication at the mucosal surface induced by a human virus will induce protection against disease better than that induced by a primate rotavirus (RRV) also adapted to growth in the human intestine. However, trials of protective efficacy with human strain M37 were disappointing (Vesikari *et al.*, 1991b). (4) Parenteral inoculation of inactivated rotaviruses or individual rotavirus proteins: Parenteral inoculation obviates concerns of inactivation of orally administered live virus by passively transferred maternal rotavirus-specific antibodies in milk (especially in developing countries) and by gastric acid, and may induce an immune response at the intestinal mucosal surface (e.g., virus-specific IgG) that is protective against challenge. (5) Oral or parenteral inoculation of rotavirus in a vehicle that allows for persistence of antigen (e.g., liposomes or micro-

capsules): This approach may prolong the normally short-lived sIgA response at the intestinal surface found after either natural infection or immunization.

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