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Analysis of the Roles of Antilipopolysaccharide and Anti-Cholera Toxin Immunoglobulin A (IgA) Antibodies in Protection against *Vibrio cholerae* and Cholera Toxin by Use of Monoclonal IgA Antibodies In Vivo

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Secretory immunoglobulin A (IgA) antibodies (sIgA) directed against cholera toxin (CT) and surface components of *Vibrio cholerae* are associated with protection against cholera, but the relative importance of specific sIgAs in protection is unknown. A monoclonal IgA directed against the *V. cholerae* lipopolysaccharide (LPS), secreted into the intestines of neonatal mice bearing hybridoma tumors, was previously shown to provide protection against a lethal oral dose of 10^7 *V. cholerae* cells. We show here that a single oral dose of 5 to 50 μg of the monoclonal anti-LPS IgA, given within 2 h before *V. cholerae* challenge, protected neonatal mice against challenge. In contrast, an oral dose of 80 μg of monoclonal IgA directed against CT B subunit (CTB) failed to protect against *V. cholerae* challenge. A total of 80 μg of monoclonal anti-CTB IgA given orally protected neonatal mice from a lethal (5-μg) oral dose of CT. Secretion of the same anti-CTB IgA antibodies into the intestines of mice bearing IgA hybridoma backpack tumors, however, failed to protect against lethal oral doses of either CT (5 μg) or *V. cholerae* (10^7 cells). Furthermore, monoclonal anti-CTB IgA, either delivered orally or secreted onto mucosal surfaces in mice bearing hybridoma tumors, did not significantly enhance protection over that provided by oral anti-LPS IgA alone. These results demonstrate that anti-LPS sIgA is much more effective than anti-CT IgA in prevention of *V. cholerae*-induced diarrheal disease.

Vibrio cholerae organisms are gram-negative, uniflagellate, motile bacteria which are spread via the fecal-oral route. After ingestion, *V. cholerae* enters the small intestine, adheres to mucosal surfaces, and forms colonies that secrete cholera toxin (CT), the agent responsible for the massive secretory diarrhea that is the hallmark of the disease (7, 14, 22). Colonization of the intestine by *V. cholerae* evokes a mucosal immune response in the host, including secretion of immunoglobulin A (IgA) antibodies (sIgA) that are thought to be involved in limiting the duration of the primary infection and in imparting resistance to subsequent oral challenge (10, 13, 16–20, 33). This response includes polyclonal sIgA antibodies directed against both CT and bacterial surface components including the outer membrane lipopolysaccharide (LPS) (10, 15, 19).

There is limited information about the relative efficacies of specific sIgA antibodies in protection (14). Systemic immunization of rabbits with both CT and LPS provided more effective protection against *V. cholerae* challenge than immunization with either antigen alone (34). Although the mechanism of this synergistic protection was not defined, it was suggested that anti-LPS and anti-CT antibodies together could enhance protection by interfering with two separate events in *V. cholerae* pathogenesis, colonization and CT action (34). The relative abilities of anti-LPS sIgA alone, anti-CT sIgA alone, or both to protect against *V. cholerae*, however, have not been systematically studied.

We previously demonstrated that the specificity of mucosal immune responses to enteric viruses and bacteria can be analyzed by mucosal immunization followed by generation of hybridomas from Peyer's patch cells and characterization of the monoclonal IgA antibodies produced in hybridoma cell culture (21, 38, 39). Monoclonal sIgA antibodies produced in this way were tested for their abilities to protect against oral challenge with enteric pathogens in syngeneic mice with a hybridoma backpack tumor method that results in secretion of monoclonal IgA antibodies into the intestinal lumen via transepithelial transport (21, 39). With this approach, we demonstrated that secretion of a monoclonal IgA directed against an Ogawa strain-specific carbohydrate epitope of the LPS of *V. cholerae* can protect suckling mice against oral challenge with a lethal dose of these organisms, presumably by preventing epithelial colonization (39). In addition, we recently produced and characterized three hybridoma cell lines that secrete monoclonal IgA antibodies directed against two distinct epitopes on the binding (B) subunit of CT (CTB) (1). These antibodies were shown to protect cultured monolayers of human intestinal enterocytes against CT binding and action in vitro, but their capacity for protection against live *V. cholerae* in vivo was not assessed.

In the present study, we have directly compared the abilities of anti-CTB and anti-LPS monoclonal IgA antibodies to protect mice against lethal oral doses of *V. cholerae* and CT. These experiments, in which the antibodies were

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either administered as a single oral dose or secreted continuously from hybridoma tumors, demonstrate that anti-LPS IgA is much more efficient than anti-CTB IgA in protection of suckling mice against *V. cholerae*.

MATERIALS AND METHODS

Hybridoma cells. The hybridoma cell lines producing monoclonal IgA antibodies used in this study were generated in this laboratory and have been described previously (1, 38, 39). All produce dimeric and polymeric IgA. Monoclonal IgA RB3, used as a control in some experiments, is directed against the sigma 3 protein of reovirus (38). Monoclonal IgA 2D6 is directed against an Ogawa-specific carbohydrate epitope of the *V. cholerae* LPS (39). Three monoclonal IgAs directed against CT recognize two distinct epitopes on the B subunit, neither of which corresponds to the GM_1 binding site (1). Hybridoma cell lines were maintained in Serum Free Protein Free Medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with penicillin and streptomycin (GIBCO, Grand Island, N.Y.).

Concentration and quantitation of IgA antibodies. Supernatants from hybridoma cultures were concentrated in a stirred ultrafiltration cell at 4°C (Amicon, Beverly, Mass.), resuspended in phosphate-buffered saline (PBS), and again concentrated. Concentrated IgA in PBS was sterilized by passage through a 0.22-μm-pore-size μSTAR filter (Costar, Cambridge, Mass.) and stored at 4°C until use.

Total IgA in concentrated hybridoma supernatants or biological samples was quantitated by a sandwich enzyme-linked immunosorbent assay (ELISA) with a purified monoclonal IgA (MOPC-315) from myeloma cells as a standard (Sigma). Rabbit polyclonal antibodies directed against mouse IgA (Zymed, South San Francisco, Calif.) were applied as a coating to 96-well plates in carbonate-bicarbonate buffer, pH 9.6. Nonspecific protein binding sites were blocked by incubation with 5% nonfat dry milk in PBS. After extensive rinsing with PBS, IgA standard concentrations and unknowns were allowed to bind at 4°C overnight. Bound IgA was detected with rabbit anti-mouse IgA coupled with horseradish peroxidase (Zymed) and developed with *O*-phenylene diamine as substrate.

Specific anti-CT IgA antibody was quantified by ELISA, with, as standard, a preparation of anti-CTB IgA from hybridoma supernants whose concentration had been determined as described above. Ninety-six-well plates were coated by incubation with 1 μg of CT per ml (100 μl per well) in carbonate-bicarbonate buffer, pH 9.6, and nonspecific protein binding sites were blocked by incubation with a solution of 5% nonfat dry milk in PBS. After being washed in PBS containing 0.2% Tween (PBS-Tween), hybridoma supernatants or intestinal washes were incubated in these plates for either 2 h at room temperature or overnight at 4°C. Bound immunoglobulins were detected with rabbit anti-mouse IgA as described above. ELISA reaction products were read on an ASA 400 ELISA reader (SLT Instruments, Salzburg, Austria). Standard curves were generated (Cricket Graph; Cricket Software, Malvern, Pa.), and IgA concentrations were calculated.

Oral challenge of neonatal mice. BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were bred in the Children's Hospital animal facility. Pups in litters born on the same day were distributed equally among dams. Suckling mice aged 5 days and weighing 4.0 to 4.5 g were used in oral challenge experiments with CT or *V. cholerae*. *V. cholerae* organisms (Ogawa 395) were grown overnight in L broth, pH 6.5, at 30°C, conditions that favor the expression of pili (36). For challenge experiments, the bacteria were washed twice in PBS to remove secreted toxin and diluted to a concentration of 10^8 organisms per ml. A peroral dose of 10^7 *V. cholerae* cells (100 times the 50% lethal dose [LD_{50}] for mice of this age [33]) in 0.1 ml of PBS was delivered into the stomach with a blunt-tip feeding needle. Mice were maintained in a 30°C incubator for 24 h or until death, and all surviving mice were scored as well or ill at 24 h. Mice were considered ill if they met all of the following criteria: gray-blue skin coloring, markedly reduced skin turgor, and poor response to stimuli.

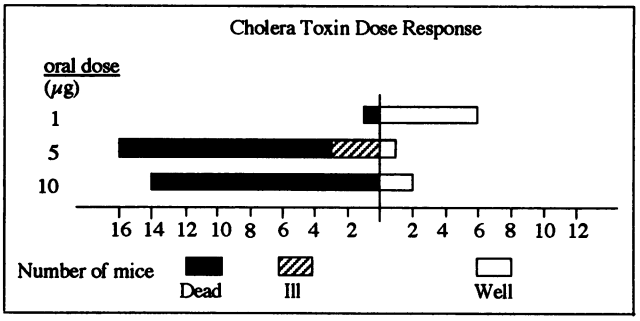

FIG. 1. Determination of a lethal oral dose of CT in 5-day-old suckling mice. Mice were given 1, 5, or 10 μg of CT by gastric tube, maintained in an incubator for 18 h, and scored. A dose of 5 μg of CT resulted in illness or death in over 90% of the mice.

For challenge with CT, suckling mice were given 0.1 ml of PBS containing 1, 5, or 10 μg of CT (Calbiochem, La Jolla, Calif.) by peroral gastric intubation. Neonates were maintained in a 30°C incubator for 18 h or until death and scored as described above. In a pilot experiment, 5 μg of CT caused illness or death in over 90% of the neonates tested (Fig. 1); therefore, this dose was used for all subsequent challenges. In experiments testing the protective effects of perorally administered monoclonal IgA antibodies, the mice received 0.1 ml of PBS containing known amounts of IgA via feeding tube and were placed in a 30°C incubator until challenge.

Production of IgA hybridoma tumors. To produce hybridoma tumors in suckling mice, cells were injected on the day of birth. Newborn pups were injected subcutaneously on the upper back with 10^6 hybridoma cells in 0.1 ml of PBS and were returned to their mothers. At 5 days of age, the mice bearing subcutaneous hybridoma tumors, like control mice, ranged in weight from 4.0 to 4.5 g and showed no ill effects from the tumor cells. They were then challenged with CT or *V. cholerae* as described above. To verify secretion of monoclonal IgA into the intestines of 5-day-old tumor-bearing mice, the small intestine from a test 4-g mouse was excised, and the contents were flushed with 150 μl of PBS into an Eppendorf tube. Solid matter was pelleted by centrifugation, and the supernatant was tested without further dilution in the CT ELISA described above. Optical density readings of anti-CT IgA in the gut wash supernatant were over 0.4 (controls, 0.01 or less); this was in the same range as that in a 1:50 dilution of serum from tumor-bearing mice.

Electron microscopy. *V. cholerae* (Ogawa 395) organisms in growth phase were pelleted, resuspended in PBS, and injected into ligated loops of ilea of anesthetized adult BALB/c mice as previously described (39). After 4 h, the loop was removed, rinsed, and fixed by infusion with a solution consisting of 2% formaldehyde–2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4. After fixation

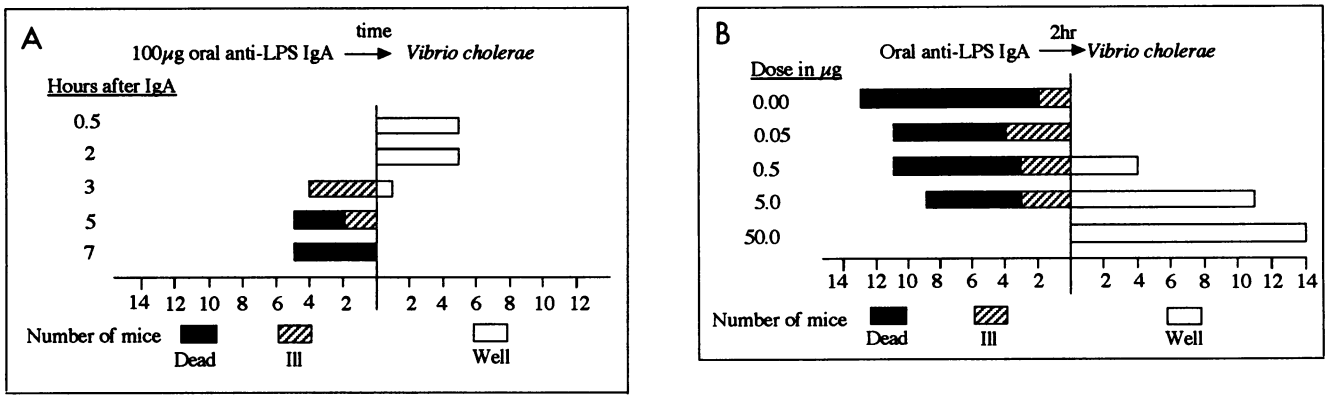

FIG. 2. Protection against *V. cholerae* challenge provided by perorally delivered monoclonal anti-LPS IgA: time and dose dependency. (A) Neonatal mice were given 100 μg of monoclonal anti-LPS IgA perorally and challenged at various subsequent times with 10^7 virulent *V. cholerae* (Ogawa 395). A single dose of IgA protected mice for 2 h. At later times, protection was lost. (B) Neonatal mice were given peroral doses of monoclonal anti-LPS IgA ranging from 0.05 to 50 μg and were challenged 2 h later with 10^7 *V. cholerae* cells. A total of 50 μg consistently protected the mice, while 0.5 to 5 μg provided partial protection.

for 4 h at 23°C, blocks of mucosal tissue were further fixed with 1% osmium tetroxide and 0.5% uranyl acetate and processed for electron microscopy by methods standard in this laboratory (26). Ultrathin sections were examined and photographed with a JEOL 100CX electron microscope.

RESULTS

Anti-LPS monoclonal IgA delivered orally protects suckling mice against *V. cholerae*. We previously showed with 5-day-old (4.0- to 4.5-g) BALB/c mice that an oral challenge of 100 LD_{50} (10^7 organisms) resulted in severe illness or death within 36 h whereas mice bearing hybridoma backpack tumors secreting monoclonal anti-LPS IgA were protected (39). To test the ability of the same anti-LPS monoclonal IgA delivered orally to protect neonatal mice against *V. cholerae*, five groups of suckling mice were given a single 100-μg oral dose of anti-LPS IgA and challenged 30 min or 2, 3, 5, or 7 h later with 10^7 *V. cholerae* cells. Health status was scored 24 h after the challenge. Mice that received anti-LPS IgA within 2 h prior to challenge were consistently protected, and partial protection was observed at 3 h (Fig. 2A).

In some experiments, an intragastric dose of PBS containing a small amount of blue food coloring was given and the mice were sacrificed 1 to 5 h later. Examination of the gastrointestinal tract revealed that most of the dye-containing fluid was cleared from the stomach by 1 h, and dye was still present in the upper small intestine at 2 h. At 3 h and later, however, the small intestine was progressively cleared. This indicates that the loss of protection against *V. cholerae* observed after 2 h was likely to be due to clearance of monoclonal IgA by peristalsis.

To determine the minimal dose of monoclonal anti-LPS IgA necessary for protection, four groups consisting of 11 to 20 mice each were given 0.05, 0.5, 5.0, or 50 μg of anti-LPS IgA intragastrically 2 h before *V. cholerae* challenge. A control group of 13 mice was given PBS with no antibody. In these experiments, 5 μg of anti-LPS IgA was found to protect 11 of 20 mice (55%), while 50 μg of antibody protected all of the mice from disease and death (Fig. 2B).

Anti-CTB monoclonal IgA delivered orally provides partial protection against CT. The three monoclonal anti-CT IgA antibodies used for these studies (anti-CTB IgA-1, IgA-2, and IgA-3) recognize the CTB, are produced in dimeric and polymeric form, and are able to block CT binding and action on polarized T84 human enterocyte monolayers in a dose-dependent manner (1). All three IgAs recognized a preparation of CTB derived from organisms of the Inaba serotype, and IgA-2 and -3 also recognized CTB derived from a strain of the Ogawa serotype (1). In in vitro studies, 20 μg of monoclonal anti-CTB IgA completely inhibited binding of 1 μg of CT to T84 intestinal epithelial cells, a molar ratio of four to five dimeric IgA antibodies per CT molecule (1). To determine whether the monoclonal anti-CTB IgA antibodies can passively protect neonatal mice against oral CT challenge, five groups of 10 or 11 mice were given oral doses of 80 μg of anti-CTB IgA (IgA-1, -2, or -3), anti-LPS monoclonal IgA, or no antibody and were challenged 1 h later with 5 μg of CT. Health status was scored after 18 h. In this experiment, the oral dose of CT resulted in diarrheal disease or death in 90% of control mice receiving no antibody and in 73% of mice receiving anti-LPS IgA. In contrast, only 20 to 34% of the mice that received 80 μg of any one of the three anti-CTB monoclonal IgA antibodies were sick or dead at 18 h (Fig. 3A).

Anti-CTB IgA delivered orally does not protect against *V. cholerae*. To test the ability of orally administered anti-CTB monoclonal IgA to protect against *V. cholerae* challenge, 50 or 100 μg of anti-CTB IgA-3 (or 50 μg of anti-LPS IgA as a control) was delivered intragastrically and followed 1 h later by 10^7 *V. cholerae* cells. The mice were scored 24 h later. As in previous experiments, 50 μg of anti-LPS IgA was sufficient to protect all of the mice from disease following *V. cholerae* challenge. In contrast, anti-CTB IgA, even at levels double that of the anti-LPS IgA, had almost no protective effect (Fig. 3B).

Neonatal mice bearing anti-CTB IgA hybridoma backpack tumors are not protected against live *V. cholerae* or CT. A single oral dose of monoclonal antibody is cleared from the intestine by peristalsis and does not provide a widespread, uniform distribution on mucosal surfaces analogous to that of sIgA. We therefore tested the ability of anti-CTB IgA, delivered into the intestine from subcutaneous IgA-producing hybridoma tumors, to protect against CT and *V. cholerae*. On the day of birth, three groups of mice were injected subcutaneously with hybridoma cells secreting anti-LPS, anti-CTB, or antireovirus (control) IgA. Secretion of monoclonal anti-CT IgA into the intestine of a test mouse was

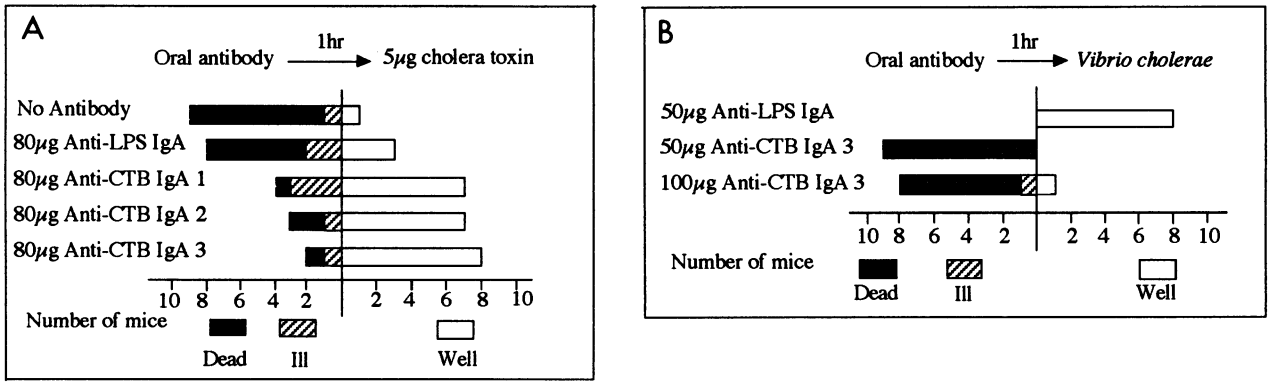

FIG. 3. Perorally delivered monoclonal anti-CTB IgA: protection against CT but not against *V. cholerae*. (A) A single dose of 80 μg of anti-CTB monoclonal IgA, given perorally, partially protected neonatal mice from a lethal dose of 5 μg of CT given 1 h later. Neonates receiving the control monoclonal anti-LPS IgA or no antibody were not protected. (B) A single oral dose of 50 μg of monoclonal anti-LPS IgA consistently protected neonates against a lethal oral dose (10^7 cells) of live *V. cholerae*, but monoclonal anti-CTB IgA at doses of 50 or 100 μg failed to provide protection.

verified on day 5 by ELISA. On day 5, all mice were challenged perorally with 10^7 *V. cholerae* cells. Neonates bearing anti-LPS IgA hybridoma tumors were fully protected against *V. cholerae*, as observed in previous studies (39). Those bearing anti-CTB or antireovirus IgA hybridoma tumors, however, were not protected (Fig. 4A).

To test the ability of anti-CTB sIgA from hybridoma tumors to protect against CT, two groups of neonatal mice bearing tumors producing either anti-CTB IgA or anti-LPS IgA were challenged orally with 5 μg of CT. Mice bearing anti-LPS IgA hybridoma tumors were not protected against toxin challenge. Unexpectedly, those bearing anti-CTB IgA hybridoma tumors were also unprotected (Fig. 4B).

Anti-CTB IgA does not significantly enhance the protection provided by anti-LPS IgA. Previous oral immunization studies had concluded that sIgA directed against CT and LPS can provide synergistic protection against oral challenge with *V. cholerae* (29, 33). The availability of monoclonal anti-CTB and anti-LPS IgA antibodies allowed us to directly test their protective capacities alone and together in the absence of confounding factors introduced by immunization such as the adjuvant effects of CT and LPS. These experiments could not be done in mice bearing both anti-LPS and anti-CTB IgA hybridoma tumors, because anti-LPS IgA tumors alone provided complete protection.

Thus, to test whether anti-CT IgA can enhance the protective capacity of anti-LPS IgA, a marginally protective (5-μg) oral dose of anti-LPS IgA, alone or mixed with 100 μg of anti-CTB IgA, was given perorally to two groups of 5-day-old suckling mice 1 h before oral challenge with 10^7 *V. cholerae* cells. The combined IgAs provided no increase in protection against *V. cholerae* over anti-LPS IgA alone (Fig. 5A). To test whether anti-CTB IgA secreted into the intestine via transepithelial transport might add to the protective efficacy of an orally delivered dose of anti-LPS IgA, three groups of suckling mice bearing anti-CTB IgA hybridoma tumors, antireovirus IgA hybridoma tumors, or no tumors were given a 5-μg oral dose of anti-LPS IgA and challenged 1 h later with *V. cholerae*. The results of this experiment showed no dramatic increase in protection in the group bearing anti-CTB IgA hybridoma tumors (Fig. 5B).

***V. cholerae* adheres to the glycocalyx of intestinal enterocytes in BALB/c mice.** To visualize the relationship of adherent *V. cholerae* to intestinal epithelial cells in BALB/c mice, we examined apical surfaces of epithelial cells on villi and follicle-associated epithelium in the ilea of adult mice after a 4-h exposure to *V. cholerae*. Although adult mice are resistant to *V. cholerae*-induced disease, organisms adhered to the surfaces of small intestinal villi as described previously for other species (24). At sites of adherence, the bacterial

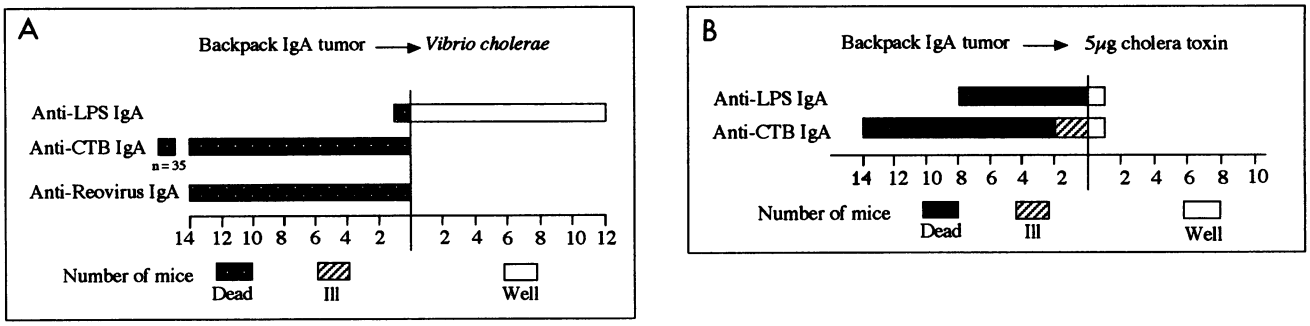

FIG. 4. IgA hybridoma tumors secreting anti-CTB monoclonal IgA failed to protect neonatal mice against *V. cholerae* challenge. (A) Anti-CTB IgA secreted into the intestines of mice bearing hybridoma tumors failed to protect neonatal mice against oral challenge with 10^7 live *V. cholerae* cells. In contrast, mice bearing anti-LPS IgA hybridoma tumors were fully protected as previously reported (39). (B) Both monoclonal anti-CTB and anti-LPS IgA antibodies delivered perorally before CT challenge failed to protect neonatal mice from illness and death.

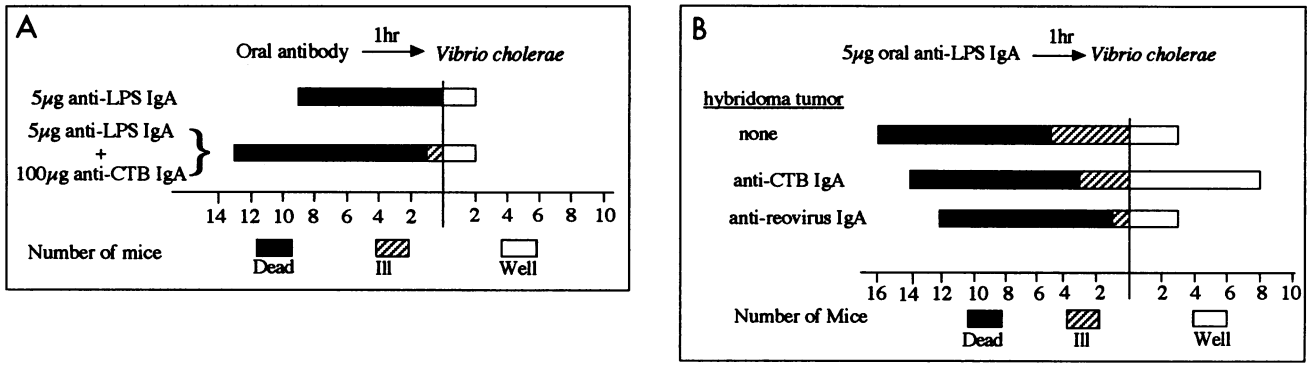

FIG. 5. Effect of anti-CTB and anti-LPS monoclonal IgA antibodies administered together. (A) Addition of 100 μg of anti-CTB monoclonal IgA to a 5-μg oral dose of anti-LPS monoclonal IgA did not provide protection for neonatal mice against an oral challenge of 10^7 live *V. cholerae* cells. The majority of this group of mice, unlike those shown in Fig. 2B, were not protected by 5 μg of anti-LPS IgA alone. (B) Anti-CTB IgA secreted into the intestines of neonatal mice bearing hybridoma tumors did not clearly enhance the ability of 5 μg of anti-LPS IgA delivered perorally to protect against *V. cholerae* challenge.

outer membrane was separated from the tips of enterocyte microvilli by a gap measuring 50 to 100 nm (Fig. 6). This apparent space is known to contain the enzyme-rich glycocalyx associated with enterocyte apical membranes (9). The space between adherent *V. cholerae* and microvilli in infected rabbits was shown to be occupied by a dense meshwork composed of cell surface components of both enterocyte and bacterium (24). *V. cholerae* adhered to M cells in large numbers as previously noted for BALB/c mice (39) and for rabbits (27). On M cells, the *V. cholerae* outer membrane came within 15 to 20 nm of the M-cell plasma membrane and formed broad areas of close interaction (Fig. 6B). In mice, as in rabbits (25), organized actin filaments were associated with the M-cell plasma membrane at these sites, reflecting the initiation of phagocytic uptake and transcytosis.

DISCUSSION

sIgA antibodies directed against CT and surface components of *V. cholerae*, including LPS, are thought to be important in prevention of diarrheal disease due to *V. cholerae* infection (8, 10, 20, 33). Our goal has been to directly test the roles that specific sIgA antibodies may play in this protection. We have previously shown that subcutaneous hybridoma tumors producing monoclonal IgA directed against *V. cholerae* LPS, presumably secreted into the intestines of suckling mice, protected the mice against a lethal oral dose of *V. cholerae* (39). In the present study, we demonstrate that an oral dose of the same IgA, if given within 2 h before *V. cholerae* challenge, is also protective. This anti-LPS antibody is known to bind to the bacterial

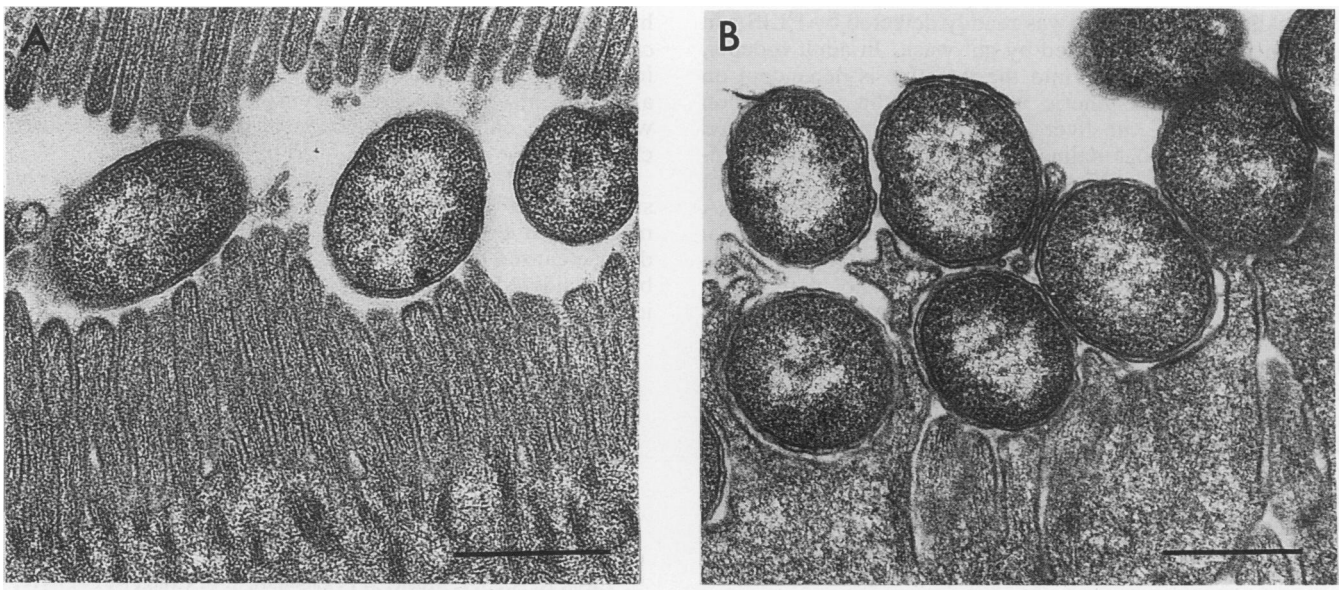

FIG. 6. Interaction of *V. cholerae* with apical membranes of mouse intestinal epithelial cells. (A) Vibrios adhere to absorptive cell surfaces by attaching to components of the membrane-associated glycocalyx (not stained in this preparation). Secreted toxin would readily diffuse across the gap between bacterial and host cell membranes. Bar = 0.5 μm. (B) Vibrios that adhere to M-cell surfaces interact closely with the apical membrane and induce rearrangement of submembrane actin filaments. Bar = 0.5 μm.

surface and to aggregate live *V. cholerae* in vitro (39), and dye-clearance experiments indicated that the 2-h window of protection provided by orally delivered IgA corresponded to the time period during which the antibody was present in the proximal small intestine. Since *V. cholerae* must colonize the proximal small intestine in order to produce sufficient toxin to cause illness, our results suggest that aggregation of the bacteria in the lumen by the anti-LPS IgA and clearance of aggregates by peristalsis prevented disease.

IgA antibodies directed against CT have also been implicated in protection against *V. cholerae*-induced disease (7, 10, 16, 19), but the relative importance of anti-CT IgA in protection has not been well defined (13, 14). In several experimental systems, the presence of anti-CT IgA antibodies in secretions was associated with protection of the intestine against orally administered CT (16–18, 28–30, 37). Although the monoclonal anti-CTB IgA antibodies used in this study are not specifically directed against the GM_1 binding site, they completely prevented CT binding and the CT-induced Cl^- secretory response of T84 human enterocyte monolayers in vitro (1). In the present study, we have confirmed the protective role of IgA against CT in vivo by showing that an 80-μg oral dose of monoclonal anti-CTB IgA can partially protect suckling mice against a lethal (5-μg) peroral dose of CT. Both in vitro and in vivo, however, high doses of IgA (corresponding to about four to five dimeric IgA antibodies per molecule of CT) were required for CT neutralization. Binding of dimeric or polymeric anti-CT IgA antibodies to toxin B subunits may have protected by causing aggregation and clearance of toxin from the intestinal lumen, by inhibiting toxin binding to apical cell membranes by steric hindrance, or by both.

In contrast, suckling mice secreting the same monoclonal anti-CTB IgA from hybridoma tumors were not protected against oral challenge with a lethal dose of CT. Levels of circulating monoclonal IgA in these mice were presumably high, as previously documented for adult mice with backpack tumors (21, 39). Although we were not able to determine exact IgA concentrations in the small intestinal lumens of sucklings, specific IgA was readily detected by ELISA in intestinal contents collected by gut wash. In adult rodents, selective sIgA secretion into the intestine is dependent on the presence of polymeric immunoglobulin receptors on epithelial cells of the liver and intestine (6, 12, 23), and polymeric immunoglobulin receptors are especially abundant on hepatocytes (35). In adult mice, bile is an important source of intestinal IgA (31), and in mice bearing IgA hybridoma backpack tumors, about 80% of the monoclonal IgA recovered by intestinal wash is from bile (6a). Although polymeric immunoglobulin receptors were not detected in intestinal epithelium of suckling rats by immunoassay (2), their presence in suckling mouse intestine or liver has not been examined. Circulating monoclonal IgA could also enter the bile by fluid-phase vesicular transport across hepatocytes (32), and transport can be enhanced by binding of IgA to asialoglycoprotein receptors (11). The relative importance of the various mechanisms or mechanism by which circulating dimeric or polymeric IgA may enter the intestines of suckling mice is not known. Nevertheless, we previously demonstrated that, in suckling mice bearing anti-LPS IgA hybridoma tumors, sufficient monoclonal IgA from the circulation entered the intestines to protect against lumenal colonization by *V. cholerae* (39), and it seems safe to assume that comparable amounts of monoclonal anti-CT IgA also entered the intestines of the mice in this study. The amount transported, however, was not sufficient for protection against a lethal dose of CT.

Anti-CTB IgA also failed to protect suckling mice against a lethal oral dose of *V. cholerae*, whether the antibodies were delivered perorally or secreted intraintestinally in mice bearing backpack tumors. Whereas a single oral dose of 50 μg of anti-LPS IgA consistently protected the mice, doses of anti-CT IgA as high as 100 μg did not. Our results corroborate previous human studies in which volunteers were orally immunized with multiple large doses (up to 4 mg) of purified CT (15). Although they responded with high titers of circulating antitoxin antibodies and presumably secreted anti-CT sIgA onto mucosal surfaces, they were not protected against an oral dose of 10^5 *V. cholerae* cells, a smaller dose than that used here for 4-g mice. In contrast, human volunteers immunized orally with live *V. cholerae* organisms were completely protected from rechallenge with 10^6 organisms (15). Since CT is not a component of the bacterial surface, anti-CT IgA alone is not likely to prevent *V. cholerae* colonization. Secretion of toxin from adherent organisms and colonies on the mucosal surface could deliver high levels of CT directly into the enterocyte glycocalyx and onto apical membranes. Our results suggest that anti-CT IgA, either delivered orally or transported (primarily by crypt epithelial cells) into the intestinal lumen, may not effectively intercept toxin in this microenvironment.

Animal immunization studies have indicated that antibacterial and anti-CT immunity may cooperate in protection by interfering with both colonization and toxin binding (7, 8, 29, 34). In vaccination studies, however, the effects of anti-CT sIgA cannot be distinguished from the potential adjuvant effects of the toxin. The availability of specific monoclonal IgA antibodies against LPS and CT allowed us to test for possible synergy in the absence of other immune and nonimmune responses to *V. cholerae* or CT. In our mouse model, addition of anti-CTB IgA either as an oral dose or secreted in mice bearing hybridoma tumors failed to enhance the protection provided by marginally effective oral doses of anti-LPS IgA. Our results thus do not demonstrate cooperation of anti-LPS and anti-CT IgAs, but are consistent with human vaccine trials in which oral immunization with a *V. cholerae* whole cell plus CTB vaccine provided only a 15% increase in protection over that of the whole-cell vaccine alone (4, 5). The possibility remains that anti-CTB IgA would have enhanced protection in our mice if a smaller challenge dose of *V. cholerae* had been used.

In summary, the use of monoclonal IgA antibodies in suckling mice has demonstrated that anti-LPS IgA is much more effective than anti-CT IgA in prevention of diarrheal disease caused by *V. cholerae*. Thus, anti-LPS sIgA should be considered an important component of the mucosal immune response evoked by oral cholera vaccines.

REFERENCES

1. **Apter, F. M., W. I. Lencer, R. A. Finkelstein, J. J. Mekalanos, and M. R. Neutra.** 1993. Monoclonal immunoglobulin A antibodies directed against cholera toxin prevent the toxin-induced chloride secretory response and block toxin binding to intestinal epithelial cells in vitro. Infect. Immun. **61**:5271–5278.
2. **Buts, J.-P., and D. L. Delacroix.** 1985. Ontogenetic changes in secretory component expression by villous and crypt cells of rat small intestine. Immunology **54**:181–187.
3. **Cash, R. A., S. I. Music, J. P. Libonati, J. P. Craig, N. F. Pierce, and R. B. Hornick.** 1974. Response of man to infection with *Vibrio cholerae*. II. Protection from illness afforded by previous disease and vaccine. J. Infect. Dis. **130**:325–333.
4. **Clemens, J. D., J. R. Harris, D. A. Sack, J. Chakraborty, F.**

Ahmed, B. F. Stanton, M. U. Khan, B. A. Kay, N. Huda, M. R. Khan, M. Yunus, M. R. Rao, A.-M. Svennerholm, and J. Holmgren. 1988. Field trial of oral cholera vaccines in Bangladesh: results of one year of follow up. J. Infect. Dis. **158:**60–69.

5. **Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Stanton, B. A. Kay, M. U. Khan, M. Yunis, W. Atkinson, A.-M. Svennerholm, and J. Holmgren.** 1986. Field trial of oral cholera vaccines in Bangladesh. Lancet **ii:**124–127.
6. **Delacroix, D., G. N. Malburney, and J. P. Vaerman.** 1985. Hepatobiliary transport of plasma IgA in the mouse: contribution to clearance of intravascular IgA. Eur. J. Immunol. **15:**893–899.

6a. **Haneberg, B., D. Kendall, and M. R. Neutra.** Unpublished data.

7. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292:**413–417.
8. **Holmgren, J., and A. M. Svennerholm.** 1977. Mechanisms of disease and immunity in cholera: a review. J. Infect. Dis. **136:**S105–S112.
9. **Ito, S.** 1974. Form and function of the glycocalyx on free cell surfaces. Philos. Trans. R. Soc. Lond. B Biol. Sci. **268:**55–66.
10. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** 1986. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination. J. Clin. Microbiol. **24:**203–209.
11. **Jones, A. L., and S. J. Burwen.** 1985. Hepatic receptors and their ligands: problems of intracellular sorting and vectorial movement. Semin. Liver Dis. **5:**136–146.
12. **Kraehenbuhl, J.-P., and M. R. Neutra.** 1992. Molecular and cellular basis of immune protection of mucosal surfaces. Physiol. Rev. **72:**853–879.
13. **Levine, M. M., R. E. Black, M. L. Clements, L. Cisneros, D. R. Nalin, and C. R. Young.** 1981. Duration of infection derived immunity to cholera. J. Infect. Dis. **143:**818–820.
14. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47:**510–550.
15. **Levine, M. M., D. R. Nalin, J. P. Craig, D. Hoover, E. J. Bergquist, D. Waterman, H. Preston Holley, R. B. Hornick, N. P. Pierce, and J. P. Libonati.** 1979. Immunity to cholera in man: relative role of antibacterial versus antitoxic immunity. Trans. R. Soc. Trop. Med. Hyg. **73:**3–9.
16. **Lycke, N., A. K. Bromander, and J. Holmgren.** 1989. Role of local IgA antitoxin-producing cells in intestinal protection against cholera toxin challenge. Int. Arch. Allergy Appl. Immunol. **88:**273–279.
17. **Lycke, N., L. Eriksen, and J. Holmgren.** 1987. Protection against cholera toxin after oral immunization is thymus-dependent and associated with intestinal production of neutralizing IgA antitoxin. Scand. J. Immunol. **25:**413–419.
18. **Lycke, N., and J. Holmgren.** 1987. Long-term cholera antitoxin memory in the gut can be triggered to antibody formation associated with protection within hours of an oral challenge immunization. Scand. J. Immunol. **25:**407–412.
19. **Majumdar, A. S., P. Dutta, D. Dutta, and A. C. Ghose.** 1981. Antibacterial and antitoxin responses in the serum and milk of cholera patients. Infect. Immun. **32:**1–8.
20. **Majumdar, A. S., and A. C. Ghose.** 1981. Evaluation of the biological properties of different classes of human antibodies in relation to cholera. Infect. Immun. **32:**9–14.
21. **Michetti, P., M. J. Mahan, J. M. Slauch, J. J. Mekalanos, and M. R. Neutra.** 1992. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen *Salmonella typhimurium*. Infect. Immun. **60:**1786–1792.
22. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243:**916–921.
23. **Mostov, K. E., J. Kraehenbuhl, and G. Blobel.** 1980. Receptor mediated transcellular transport of immunoglobulin: synthesis of secretory component as multiple and larger transmembrane forms. Proc. Natl. Acad. Sci. USA **77:**7257–7261.
24. **Nelson, E. T., J. D. Clements, and R. A. Finkelstein.** 1976. *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. Infect. Immun. **14:**527–547.
25. **Neutra, M. R., P. Michetti, and J.-P. Kraehenbuhl.** Secretory immunoglobulin A: induction, biogenesis and function. *In* L. R. Johnson (ed.), Physiology of the gastrointestinal tract, 3rd ed., in press. Raven Press, New York.
26. **Neutra, M. R., T. L. Phillips, E. L. Mayer, and D. J. Fishkind.** 1987. Transport of membrane bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patch. Cell Tissue Res. **247:**537–546.
27. **Owen, R. L., N. F. Pierce, R. T. Apple, and W. C. J. Cray.** 1986. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanisms for antigen sampling and for microbial transepithelial migration. J. Infect. Dis. **153:**1108–1118.
28. **Pierce, N. F., W. C. Cray, and P. R. Engel.** 1980. Antitoxic immunity to cholera in dogs immunized orally with cholera toxin. Infect. Immun. **27:**632–637.
29. **Pierce, N. F., W. C. Cray, Jr., and J. H. Saci, Jr.** 1982. Oral immunization of dogs with purified cholera toxin, crude cholera toxin, or B subunit: evidence for synergistic protection by antitoxic and antibacterial mechanisms. Infect. Immun. **37:**687–694.
30. **Pierre, P. G., A. Langendries, and J. P. Vaerman.** 1989. Cholera toxin-induced fluid secretion in rat gut ligated loops: influence of bile from normal or cholera toxin-immunized rats. Immunology **68:**319–324.
31. **Russell, M. W., T. A. Brown, and J. Mestecky.** 1982. Preferential transport of IgA and IgA-immune complexes to bile compared with other external secretions. Mol. Immunol. **19:**677–682.
32. **Scharschmidt, B. F., J. R. Lake, E. L. Renner, V. Licko, and R. W. Van Dyke.** 1986. Fluid phase endocytosis by cultured rat hepatocytes and perfused rat liver. Implications for plasma membrane turnover and the vesicular trafficking of fluid phase markers. Proc. Natl. Acad. Sci. USA **83:**9488–9492.
33. **Svennerholm, A., M. Jertborn, L. Gothefors, A. M. M. M. Karim, D. A. Sack, and J. Holmgren.** 1984. Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combined B subunit-whole cell vaccine. J. Infect. Dis. **149:**884–893.
34. **Svennerholm, A.-M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
35. **Sztul, E. S., K. E. Howell, and G. E. Palade.** 1985. Biogenesis of the polymeric immunoglobulin receptor in rat hepatocytes. I. Kinetic studies of its intracellular forms. J. Cell Biol. **100:**1248–1254.
36. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987.Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
37. **Vaerman, J. P., A. De Rijck-Langendries, M. Rits, and D. L. Delacroix.** 1985. Neutralization of cholera toxin by rat biliary secretory IgA antibodies. Immunology **54:**601–603.
38. **Weltzin, R., P. Lucia-Jandris, P. Michetti, B. N. Fields, J. P. Kraehenbuhl, and M. R. Neutra.** 1989. Binding and transepithelial transport of immunoglobulins by intestinal M cells: demonstration using monoclonal IgA antibodies against enteric viral proteins. J. Cell Biol. **108:**1673–1685.
39. **Winner, L. S., III, J. Mack, R. Weltzin, J. J. Mekalanos, J.-P. Kraehenbuhl, and M. R. Neutra.** 1991. New model for analysis of mucosal immunity: intestinal secretion of specific monoclonal immunoglobulin A from hybridoma tumors protects against *Vibrio cholerae* infection. Infect. Immun. **59:**977–982.