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Evaluation of Formalin-Inactivated *Clostridium difficile* Vaccines Administered by Parenteral and Mucosal Routes of Immunization in Hamsters

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***Clostridium difficile* produces toxins that cause inflammation, necrosis, and fluid in the intestine and is the most important cause of nosocomial antibiotic-associated diarrhea and colitis. We evaluated *C. difficile* antigens as vaccines to protect against systemic and intestinal disease in a hamster model of clindamycin colitis. Formalin-inactivated culture filtrates from a highly toxigenic strain were administered by mucosal routes (intranasal, intragastric, and rectal) with cholera toxin as a mucosal adjuvant. A preparation of culture filtrate and killed whole cells was also tested rectally. The toxoid was also tested parenterally (subcutaneously and intraperitoneally) and by a combination of three intranasal immunizations followed by a combined intranasal-intraperitoneal boost. Serum antibodies against toxins A and B and whole-cell antigen were measured by enzyme-linked immunosorbent assay, neutralization of cytotoxic activity, and bacterial agglutination. The two rectal immunization regimens induced low antibody responses and protected only 20% of hamsters against death and 0% against diarrhea. The intragastric regimen induced high antibody responses but low protection, 40% against death and 0% against diarrhea. Hamsters immunized by the intranasal, intraperitoneal, and subcutaneous routes were 100% protected against death and partially protected (40, 40, and 20%, respectively) against diarrhea. Among the latter groups, intraperitoneally immunized animals had the highest serum anticytotoxic activity and the highest agglutinating antibody responses. Hamsters immunized intranasally and revaccinated intraperitoneally were 100% protected against both death and diarrhea. Protection against death and diarrhea correlated with antibody responses to all antigens tested. The results indicate that optimal protection against *C. difficile* disease can be achieved with combined parenteral and mucosal immunization.**

Clostridium difficile is recognized as the most important single identifiable cause of nosocomial antibiotic-associated diarrhea and colitis. It is responsible for 10 to 25% of all cases of antibiotic-associated diarrhea and for almost all cases of pseudomembranous colitis (4). In recent years, an increase in the incidence of *C. difficile* diarrhea has been observed (11, 39). Nosocomial outbreaks are frequent in hospitals and nursing homes, are difficult to control, and may occur even after a hospital ward has been closed and decontaminated (6, 31). In tertiary-care hospitals, up to 37% of patients become infected, and 7.8% become ill (39). Although most patients with *C. difficile* diarrhea respond to therapy, relapses may occur at a frequency of up to 55% (4).

C. difficile grows in the intestine of patients whose normal microflora has been disrupted by antimicrobial or antineoplastic drugs. An adhesin composed of surface proteins probably is involved in colonization (21). Once it successfully colonizes, the organism grows and produces two exotoxins. Toxin A induces inflammation, necrosis, and fluid accumulation in the gastrointestinal tract (29, 33, 44). Toxin A is chemotactic for human neutrophils and stimulates the release of inflammatory mediators from macrophages, neutrophils, and mast cells (14, 32, 38). Toxin B does not cause necrosis in the intestine but may induce hypersecretion in intestinal loops in rats (44) and also disrupts barrier function in human intestinal epithelial cell lines (17). Recently, toxin B was shown to cause necrosis in human colon tissue when biopsy specimens in Ussing chambers were exposed to the toxin (24a). Toxin B also induces the release of inflammatory mediators from human monocytes and increases cell-mediated cytotoxicity (14, 40). Diarrhea and colitis result from the action of *C. difficile* toxins and the stimulation of local inflammation.

The protective role of antibodies against *C. difficile* toxins is controversial. Hamsters experimentally immunized against both toxins were protected better against clindamycin-induced colitis than those immunized with only one toxin. Protection in hamsters immunized with toxin A has been reported to vary from 0 to 100%, and protection after immunization with toxin B varies from 0 to 30% (13, 24, 26). In intestinal loops of rabbits immunized parenterally with toxin A, mucosal damage and secretion caused by toxin A challenge were significantly reduced; however, less protection was observed when loops were challenged with both toxins A and B (23). These reports suggest that full protection may require immunization against both toxins.

In patients with *C. difficile* colitis, a significant increase of specific immunoglobulin G (IgG) and IgM antibodies against toxins A and B in convalescent-phase sera was reported, and this response correlated with reduced severity or fewer relapses (25, 45, 46). However, others have reported a lack of correlation between immunity and clinical response (25). Secretory IgA has been found in colonic aspirates of over 50% of an adult population, and these antibodies blocked the binding of toxin A to rabbit ileal brush border membranes (22). Administration of pooled intravenous gamma globulin to children with chronic relapsing *C. difficile* colitis produced clinical re-

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TABLE 1. Schemes of immunization of hamsters with formalin-inactivated *C. difficile* cultures

Antigen	Route of immunization (group)	Dose per immunization	Adjuvant and dose per immunization
Culture filtrate toxoid	Intranasal for first three immunizations, intraperitoneal and intranasal for fourth immunization (i.n.i.p.)	5 μg of toxin A, 5 μg of toxin B	Cholera toxin, 5 μg for i.n.; RIBI adjuvant, 0.3 ml for i.p.
Culture filtrate toxoid	Intranasal (i.n.)	5 μg of toxin A, 5 μg of toxin B	Cholera toxin, 5 μg
Culture filtrate toxoid	Intragastric (i.g.)	100 μg of toxin A, 100 μg of toxin B	Cholera toxin, 10 μg
Culture filtrate toxoid	Rectal (r.)	50 μg of toxin A, 50 μg of toxin B	Cholera toxin, 10 μg
Culture filtrate toxoid and formalin-killed *C. difficile* cells	Rectal (w.c.r.)	50 μg of toxin A, 50 μg of toxin B, 5×10^8 cells	Cholera toxin, 10 μg
Culture filtrate toxoid	Intraperitoneal (i.p.)	5 μg of toxin A, 5 μg of toxin B	RIBI adjuvant, 0.3 ml
Culture filtrate toxoid	Subcutaneous (s.c.)	5 μg of toxin A, 5 μg of toxin B	RIBI adjuvant, 0.3 ml
Phosphate buffer	Intranasal (c.i.n.)	15 μl	Cholera toxin, 5 μg
Phosphate buffer	Subcutaneous (c.s.c.)	0.3 ml	RIBI adjuvant, 0.3 ml

mission (25). Experimental passive immunization was also successful in preventing disease in hamsters and mice (7, 27).

The experimental and human data available on immunity and protection in *C. difficile* disease suggest that it might be possible to effectively prevent disease by active immunization. Since toxins mediate the disease, the stimulation of antitoxin immunity is a logical goal in selection of a vaccine candidate. Therefore, we evaluated a formalin-inactivated *C. difficile* toxoid in the hamster model. The possibility that antibacterial immunity might interfere with colonization and *C. difficile*-induced disease was investigated by using formalinized whole cells as an immunogen. Recently, significant advances have been made in the study of mucosal immunity and in the use of adjuvants and alternative routes of immunization to achieve a protective local immune response (41). We tested different mucosal and parenteral routes of immunization and determined the ability of vaccine to protect against both lethality and intestinal disease. Correlations between serum antibody levels against *C. difficile* antigens and protection were studied.

MATERIALS AND METHODS

***C. difficile* toxoid vaccine.** *C. difficile* culture filtrate was prepared and inactivated as described previously (26). Briefly, *C. difficile* VPI 10463 was grown for 3 days in dialysis flasks, centrifuged, and filter sterilized. One milliliter of formaldehyde was added to 100 ml of the culture filtrate, and the mixture was incubated at 37°C for 1 h. The culture filtrate had a concentration of approximately 50 μg of toxin A per ml as determined by enzyme-linked immunosorbent assay (ELISA) (30) and a cytotoxic titer of 10^6 for toxin B by cell culture cytotoxicity (12). The toxoid was washed with 3 volumes of phosphate-buffered saline (PBS; pH 7.4) by ultrafiltration through a 30-kDa membrane in a 500-ml cell concentrator (Amicon, Beverly, Mass.). Toxoid was concentrated 10 times (10× toxoid) filter sterilized, and stored at 4°C until used. On the basis of the size of the toxins (308 kDa for toxin A and 269 kDa for toxin B), we assumed no significant loss of toxin protein during concentration and estimated a concentration of 500 μg of each inactivated toxin per ml in the 10× toxoid solution.

Whole-cell vaccine. *C. difficile* VPI 10463 was grown in proteose peptone-yeast extract (PPY) medium (19) at 37°C under an anaerobic atmosphere, for 36 h, to minimize spore formation. Cultures were centrifuged, and cells were washed three times with PBS. After the last wash, pellets were resuspended in PBS containing 1% (vol/vol) formaldehyde and incubated overnight at 4°C for 24 h. Excess formaldehyde was removed by three washes with PBS. The formalinized *C. difficile* cell suspension was stored at 4°C. Inoculation of the equivalent of 10^9 *C. difficile* CFU (a cell suspension with an optical density [OD] of 1.0 at 550 nm) into PPY medium yielded no growth after 36 h at 37°C in an anaerobic atmosphere.

Animals. Female Syrian hamsters (*Mesocricetus auratus*; Charles River, Kingston, N.Y.) 6 to 8 weeks old at the time of the immunization, were used in all experiments. Animals were caged in groups of five during the immunization period and then caged individually during *C. difficile* challenge.

Immunization regimens. Seven different regimens of immunization were tested (Table 1). For intranasal (i.n.) immunization, 5 μg of each toxoid (inactivated toxin A and toxin B) in 10 μl of the 10× toxoid was mixed with 5 μl containing 5 μg of cholera toxin (Calbiochem, La Jolla, Calif.). The 15-μl antigen-adjuvant dose was delivered into the external nares with a micropipettor, half of the dose in each nostril. For intragastric (i.g.) immunization, 100 μg of each toxoid was mixed with 10 μg of cholera toxin, adjusted to 1 ml with PBS, and administered by gavage. For intraperitoneal (i.p.) and subcutaneous (s.c.) immunizations, 5 μg of each toxoid was mixed with 0.3 ml of RIBI adjuvant (RIBI ImmunoChem, Hamilton, Mont.). For rectal (r.) immunization, 50 μg of each toxoid in 100 μl of toxoid was mixed with 1 μl containing 10 μg of cholera toxin. For r. administration of whole cells (w.c.r.), 5×10^8 cells were mixed with 50 μg of each toxoid in 100 μl of toxoid plus 1 μl containing 10 μg of cholera toxin. For both the r. and w.c.r. groups, the sample was applied with a disposable feeding needle (20 by 1 in. [ca. 50 by 3 cm]) inserted 3 cm into the rectum. The i.n., i.g., i.p., and s.c. immunizations were performed in animals lightly anesthetized with isofluorane. The r. and w.c.r. immunizations were done in pentobarbital-anesthetized animals. A control intranasal (c.i.n.) group received 5 μg of cholera toxin i.n. A control subcutaneous (c.s.c.) group received 0.3 ml of RIBI adjuvant s.c. Groups of five animals were used for all immunization regimens. All groups received a total of four doses of the vaccine (or adjuvant control) on days 0, 7, 14, and 28.

To evaluate the immune response, samples (200 to 400 μl) of blood were obtained on days 0, 2, 4, 7, and 36 from the retroorbital sinus of hamsters under isofluorane anesthesia. Blood was left to clot overnight at 4°C, and the serum was obtained by centrifugation. Only serum antibodies were evaluated; secretory IgA was not measured because of the lack of a suitable anti-hamster IgA reagent. After *C. difficile* challenge, a sample of feces was obtained every other day from surviving animals and mixed with 2 volumes of PPY medium for evaluation of the degree of colonization and presence of toxins (see below).

***C. difficile* challenge.** All hamsters were challenged on day 38 (10 days after the fourth immunization) with 0.5 mg of clindamycin orogastrically, followed 3 h later by an orogastric inoculation of 10^5 CFU of viable *C. difficile* VPI 10463 washed with PPY medium to eliminate free toxins. After challenge, hamsters were observed daily for diarrhea and illness. The severity of diarrhea was scored as follows: 0, no diarrhea; 1+, loose feces but no wet tail; 2+, perianal and tail region wet; 3+, tail, paws, and lower abdomen wet (animals with this appearance were usually hunched and inactive).

Evaluation of tissue damage. Severely ill hamsters were euthanized. Samples of cecum from euthanized hamsters and from survivors from every immunization regimen taken 8 days after clindamycin challenge were fixed in 10% neutral buffered formalin. Formalin-fixed tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. Histologic grading criteria were as follows: 0, minimal infiltration of lymphocytes, plasma cells, and eosinophils; 1+, mild infiltration of lymphocytes, plasma cells, neutrophils, and eosinophils plus mild congestion of the mucosa with or without hyperplasia of gut-associated lymphoid tissue; 2+, moderate infiltrations of mixed inflammatory cells, moderate congestion and edema of the lamina propria, with or without goblet cell hyperplasia, individual surface cell necrosis or vacuolization, and crypt dilatation; 3+, severe inflammation, congestion, edema, and hemorrhage in the mucosa, surface cell necrosis, or degeneration with erosions or ulcers.

Evaluation of the infection. Feces obtained after clindamycin challenge were studied for the presence of *C. difficile*. Tenfold dilutions in PPY medium were inoculated onto selective media containing cycloserine (125 μg/ml) and cefoxitin (8 μg/ml), and colonies were counted after 48 h of anaerobic incubation. The presence of toxin A in feces was determined with a toxin A kit (TechLab, Blacksburg, Va.) as described by the manufacturer. Fifteen minutes after addition of substrate, the OD was read at 450 nm and the concentration of toxin was estimated from a standard curve of toxin A prepared in each plate. The estimations were done by use of Softmax software (Molecular Devices, Sunnyvale, Calif.). For quantitation of toxin B, fecal suspensions were centrifuged and filter sterilized, and 10-fold dilutions of the samples were tested for cytopathic effect on IMR-90 fibroblast cell cultures, as described below.

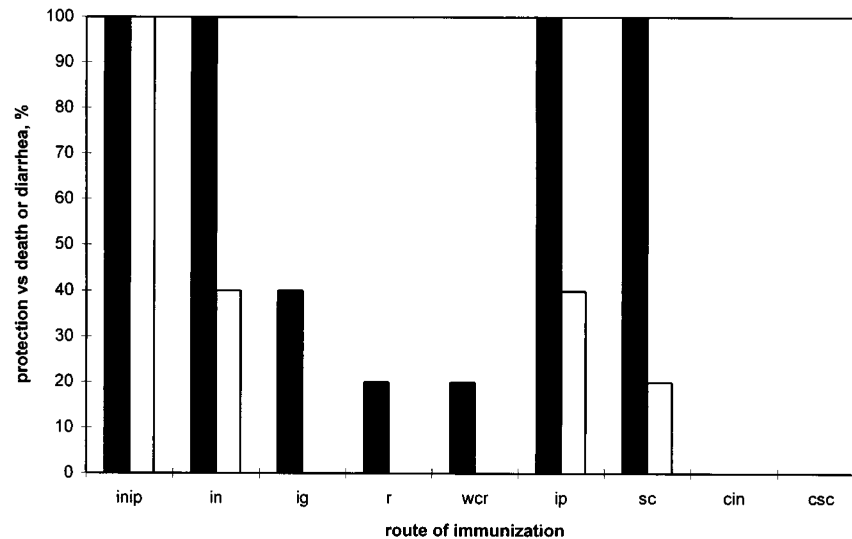

FIG. 1. Protection against *C. difficile* disease (death [■] or diarrhea [□]) in vaccinated hamsters. Hamsters were immunized with *C. difficile* antigens by the routes indicated, and the outcomes for systemic and intestinal disease after clindamycin challenge are shown. See Table 1 for description of routes of immunization.

ELISA for antibodies to toxins A and B. Microtiter plates (Corning, New York, N.Y.) were coated with 100 ng of purified toxin A or toxin B in carbonate-bicarbonate buffer (pH 9.3) per well and incubated overnight at 4°C. The plates were washed and blocked with 2.5% nonfat dry milk in PBS solution (pH 7.4). Serum samples were added at twofold dilutions from 1:500 to 1:64,000, and the plates were incubated for 1 h at 37°C. Anti-hamster IgG (1:1,000; Southern Biotechnology, Birmingham, Ala.) conjugated with alkaline phosphatase was added and the plates were incubated for 1 h at 37°C and washed prior to the addition of *p*-nitrophenyl phosphate substrate. A positive control was included in each plate; wells were coated with toxin A or toxin B in twofold dilutions from 100 to 0.8 ng/ml and reacted with specific goat antitoxin (TechLab), followed by anti-goat IgG alkaline phosphatase conjugate. Negative controls were wells coated with purified toxin and reacted with anti-hamster IgG alkaline phosphatase conjugate. The OD was read at 405 nm, and the titer was defined as the reciprocal of the highest dilution of sample giving an OD of ≥0.3.

ELISA for antibodies to whole-cell antigens. Plates were coated with 100 μl of a formalin-killed *C. difficile* suspension adjusted to an OD of 0.2 at 550 nm and incubated overnight in an orbital shaker at 150 rpm. Cells were then fixed to the plates by incubation at 70°C for 2 h. After washing, serum samples were added at twofold dilutions from 1:100 to 1:12,800, and the plates were incubated for 1 h at 37°C. Anti-hamster IgG and substrate were added as described above. A positive control was included in each plate by use of mouse *C. difficile* whole-cell antiserum at 1:500 to 1:64,000 (antiserum produced at OraVax against VPI 10463). Wells coated with whole cells were reacted directly with the anti-hamster IgG-alkaline phosphatase conjugate as negative controls.

Inhibition of cytotoxicity. IMR-90 fibroblast cells were grown to confluence in 96-well plates in Dulbecco modified Eagle medium (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum. The minimal dose of toxin A or toxin B needed to cause 100% rounding of the cells was defined as 1 cytotoxic unit (CTU_{100}). For toxins A and B, 6.3 and 125 pg/ml, respectively, were defined as 1 CTU_{100}. Twofold dilutions of the hamster serum samples, from 1:100 to 12,800, were mixed with 4 CTU_{100} of either toxin and incubated for 1 h at 37°C, and the mixture was added to the cells. Goat anti-toxin A or B served as a positive control. Cells were observed after 24 h, and the proportion of round cells was determined. The titer of the samples was defined as the reciprocal of the highest dilution of sera inhibiting >50% cell rounding.

Agglutination. Twenty-five-microliter samples of hamster serum were diluted from 1:25 to 1:3,200. The dilutions were prepared in 96 U-bottom microplates (Falcon, Oxnard, Calif.). The formalin-killed *C. difficile* suspension was adjusted to an OD of 1.0 at 550 nm, and 25 μl of the suspension was added to serum dilutions. Mouse anti-*C. difficile* whole-cell antiserum served as a positive control, and PBS served as a negative control. Plates were incubated overnight at 4°C, and the agglutination was scored. Endpoint titers were defined as the reciprocal of the highest dilution of serum causing agglutination.

Western blots (immunoblots). *C. difficile* VPI 10463 and the strains isolated from hamsters after clindamycin challenge were grown in 5 ml of PPY medium at 37°C under an anaerobic atmosphere for 36 h. Cultures were centrifuged, and the pellet was washed three times with PBS. Pellets were resuspended in 250 μl of 3% sodium dodecyl sulfate (SDS) in PBS. The lysate was electrophoresed in a preparative SDS–12% polyacrylamide gel (Bio-Rad, Hercules, Calif.) at 200 V for 1 h. Proteins were then transferred to nitrocellulose at 150 V for 1.2 h in a Bethesda Research Laboratories Mini-V 8-10 chamber (Life Technologies, Grand Island, N.Y.). Membranes were blocked with 5% nonfat dry milk in PBS for 1 h, washed, and mounted in a multiscreen apparatus (Bio-Rad). A 1:200 dilution of each hamster serum sample was added, and the membranes were incubated for 1 h. After washing, membranes were incubated with anti-hamster IgG-alkaline phosphatase conjugate for 1 h, and the reaction was developed with NBT-BCIP (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium; Gibco, Gaithersburg, Md.). Mouse anti-*C. difficile* VPI 10463 whole-cell serum served as a positive control. To type *C. difficile* strains isolated from feces, SDS lysates from isolates were electrophoresed, transferred, and reacted with whole-cell mouse antiserum as before.

Statistical analysis. The immune response to the different *C. difficile* antigens was studied for possible significant correlation to the outcome of the hamsters after clindamycin challenge by the Kruskal-Wallis test (Quick-STATISTICA software; StatSoft, Tulsa, Okla.).

RESULTS

Outcome after clindamycin challenge. Hamsters were challenged with clindamycin and *C. difficile* 10 days after the last immunization. The outcome of the clindamycin challenge in all vaccine groups is presented in Fig. 1. Mucosally immunized animals that received toxoid vaccine by the i.g. or r. route or whole-cell toxoid vaccine by the r. route (w.c.r.) were minimally protected against death, and all had diarrhea. The r. and w.c.r. immunization regimens protected only 20% of hamsters, and the i.g. regimen protected 40% of hamsters against death. Parenterally immunized (i.p. and s.c.) animals were fully protected against death; 40% of the i.p. immunized and 20% of the s.c.-immunized animals were protected against diarrhea. A similar outcome was observed in animals immunized i.n. When i.n. immunized animals received a booster dose of toxoid i.p. (group i.n.i.p.), 100% protection was achieved against both death and diarrhea.

All sham-immunized animals, both c.i.n. and c.s.c. groups, died within 48 h of challenge, most with severe (grade 3+) diarrhea. Acute, diffuse, necrohemorrhagic typhlitis, grade 3+, was found on pathologic examination. Crypt epithelium was hyperplastic, and dilated crypts were filled with neutrophils. Lymphocytes, plasma cells, and neutrophils infiltrated the lamina propria. These changes are similar to those previously reported for hamsters challenged with clindamycin and *C. difficile* (26). Vaccinated hamsters that succumbed to challenge also died within the first 48 h, and most had grade 3+ diarrhea

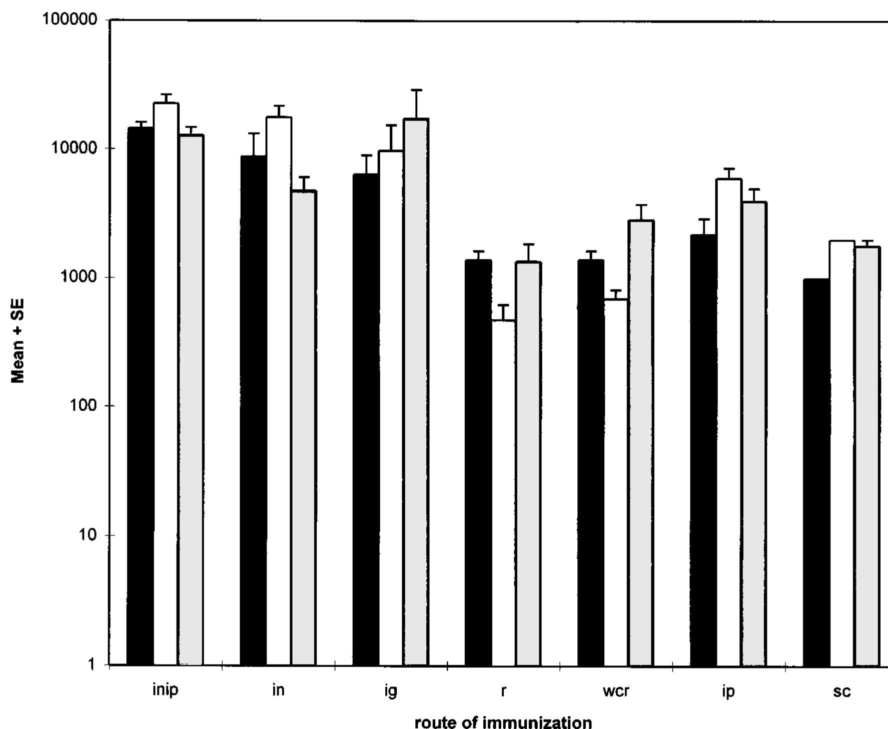

FIG. 2. Mean (+standard error [SE]) antibody titers to *C. difficile* toxin A (■), toxin B (□), and whole-cell antigen (□) in sera from hamsters after three doses of vaccine administered by different routes (see Table 1 for description of routes of immunization), as determined by ELISA. Serum from hamsters after three doses of vaccine was assayed for specific IgG; titer was defined as the maximum dilution with an absorbance of >0.3. Each bar represents the mean + standard error of five animals.

and grade 3+ typhlitis on histopathologic examination. Animals that survived challenge either had no diarrhea or had diarrhea ranging in severity from 1+ to 3+. The severity of diarrhea correlated with the severity of typhlitis on pathologic examination. Animals with 3+ diarrhea had subacute, diffuse, mucopurulent typhlitis of grades 2 to 2.5+. Neutrophils, lymphocytes, and plasma cells infiltrated the lamina propria, and multifocal crypt abscesses also were noted. Those with 2+ diarrhea had subacute to chronic moderate typhlitis of grade 1.5 to 2+. Animals with mild diarrhea (grade 1+) had a mild lymphocytic typhlitis of grade 1.0 to 1.5+. Mild lymphocytic typhlitis, grade 1+, was also evident in hamsters without diarrhea.

Presence of *C. difficile* and toxins in feces. Fecal samples after clindamycin challenge were studied for the presence of *C. difficile* and toxins A and B. Similar patterns were observed in all surviving animals. Two days after challenge, *C. difficile* reached approximately 10^9 CFU/ml of feces; thereafter, colonization decreased slightly and remained at about 10^8 CFU/ml for at least 9 days, regardless of the presence of diarrhea. In contrast, levels of toxins A and B steadily decreased, and by day 9, almost no toxins were found in feces in spite of continuous colonization. No clear correlation was found between the levels of toxins in feces and disease. All isolated *C. difficile* strains were typed by Western blotting with whole-cell antiserum against the VPI 10463 strain. Strains isolated from different animals and from different immunization groups were tested, and all were found to be similar to each other, suggesting that only one strain was colonizing all hamsters. However, contrary to what we expected, this strain was different from VPI 10463, the strain used for challenge (data not shown).

Immune response. Serum antibodies against *C. difficile* antigens were measured in hamsters from all experimental groups. Immune responses to toxin A after the priming immunization were studied by an ELISA in some of the groups. No specific IgG was detected in animals vaccinated by the i.n.i.p., r., and w.c.r. routes 2, 4, and 7 days after the initial vaccine dose. In the parenterally immunized animals (i.p. or s.c.), no response was evident after 2 and 4 days, but at day 7, a slight rise in antibody titer was observed. In contrast, the antibody responses measured after the last vaccine dose (day 36) demonstrated seroconversion in all groups. The absence of an early (anamnestic) antibody response to the first vaccine dose suggested that the animals were immunologically naive and had not been primed previously with toxin A.

Three approaches were used to study the systemic antibody responses to *C. difficile* antigens: (i) recognition of immobilized antigens by ELISA; (ii) inhibition of cytotoxicity in cell cultures; and (iii) agglutination of bacteria. Toxin A, toxin B, and whole cells were used as antigens. Antibody responses determined by ELISA to toxin A, toxin B, and whole-cell antigen were present in all groups (Fig. 2). Hamsters immunized by the i.n.i.p., i.n., i.g., and i.p. regimens had higher responses against toxin A and toxin B than those immunized by the rectal (r. and w.c.r.) and s.c. routes. Antibody levels against whole-cell antigens showed a pattern similar to that observed with toxins. Antibodies to whole-cell antigens were further characterized by Western blotting with whole-cell lysates from the *C. difficile* strain isolated from the hamsters after clindamycin challenge (Fig. 3). Animals in all immunization regimens developed antibodies to a 70-kDa protein and to proteins with sizes of >200 kDa (likely to be toxins); animals immunized by the i.n.i.p. route had the strongest responses. A variety of other proteins were recognized by animals immunized by the i.n.i.p., i.p., and s.c. routes, but these proteins were not apparent or were less prominent in animals immunized mucosally (r. and w.c.r.

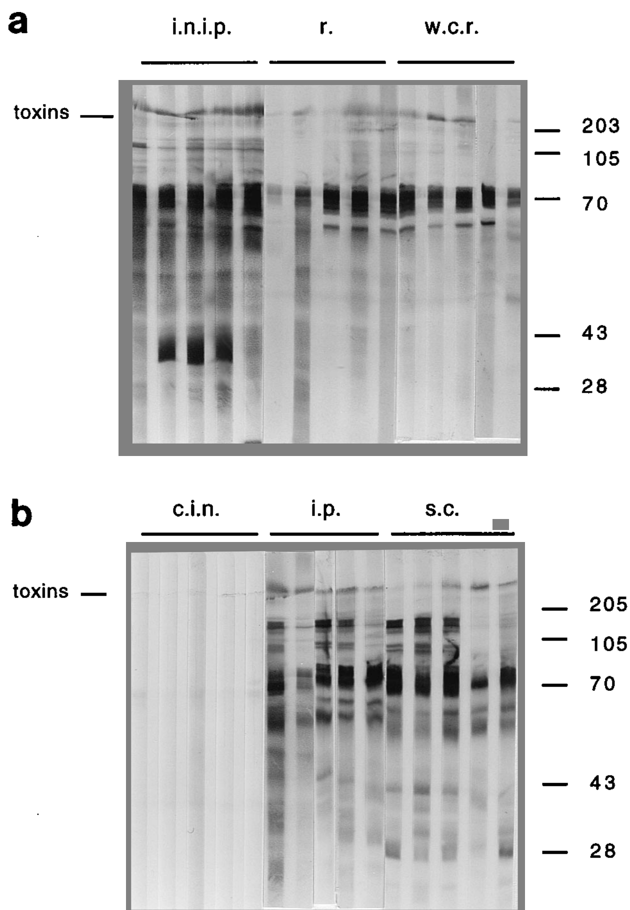

FIG. 3. Recognition of whole-cell lysate antigens by serum from hamsters after three doses of vaccine administered by different routes (see Table 1 for description of routes of immunization). The *C. difficile* strain isolated from hamsters was lysed with SDS, electrophoresed in a preparative 12% polyacrylamide gel, and transferred to nitrocellulose membranes. Antigens were reacted with antiserum from each immunized hamster. Six groups are presented, i.n.i.p., r., w.c.r., c.i.n., i.p., and s.c.; the five animals per group are presented from left to right for each group. The band corresponding to toxins is indicated at the left of the membranes. Molecular mass markers (in kilodaltons) are shown on the right.

groups). In other tests, hamster sera were immunoblotted in parallel against purified toxins and whole-cell lysates, demonstrating that proteins with molecular masses <70 kDa reacting with hamster sera were not toxin fragments and that the bands of >200 kDa corresponded to the toxins, as suggested above (data not shown).

Serum antibodies with biologic function showed a different pattern from that obtained by ELISA (Fig. 4). Antibodies inhibiting cytotoxicity by toxin A were elicited in all animals. Hamsters immunized by the i.n.i.p. and i.p. routes developed the highest anti-toxin A activity (titers of 22,000 ± 4,900 and 18,000 ± 2,000, respectively [mean ± standard error]), whereas mucosally immunized animals (i.n., i.g., r., and w.c.r.) had lower activities (titers of 580 ± 280, 280 ± 146, 1,720 ± 560, and 2,760 ± 290, respectively). High anti-toxin B responses were obtained in all groups except in r. immunized animals. Agglutinating antibodies were elicited only in animals that received toxoid vaccine parenterally (i.p. or s.c.) or by a combined mucosal-parenteral route (i.n.i.p.).

Correlation of the immune response and protection. The i.n.i.p. immunized animals were fully protected against death and diarrhea and had the highest serum immune responses when both ELISA and biologic activity were considered (Fig. 2 and 4). Complete protection against death was provided by all immunization schemes that included parenteral injection of vaccine or by i.n. immunization alone. In contrast, r. immunized animals had the lowest protection ratios and serum antibody responses, particularly, those of neutralizing antibody against toxin B (Fig. 4). Immunological correlates were not consistent, however, as illustrated by the similar antibody responses in the i.n. and i.g. groups (Fig. 2 and 4), despite the greater protection afforded by i.n. vaccine (Fig. 1). To better define immunologic correlates, animals from all groups were analyzed together, comparing the outcome of the challenge with the immune response (Table 2). Mean antibody levels in all tests except the whole-cell ELISA were significantly higher in survivor animals than in animals with a lethal outcome. Hamsters with severe diarrhea (grade 3+) had significantly lower serum immune responses by all assays compared with those without diarrhea (Table 3). Antibody responses in hamsters with mild to moderate diarrhea (grades 1+ and 2+) did not differ significantly from those without diarrhea, except for agglutinating antibody responses in those with 1+ diarrhea ($P < 0.05$).

Long-term protection. Four surviving animals from the i.n.i.p. group and four from the s.c. group were held for a period of 140 days after clindamycin challenge. On day 140, samples of blood and feces were taken, and the animals were rechallenged with clindamycin. Three of four animals (75%) in each group survived rechallenge. Two of four animals (50%) in the i.n.i.p. group and none of four (0%) in the s.c. group were protected against diarrhea. The immune responses before rechallenge were compared with the response obtained before the first challenge. In the ELISA, toxin A and toxin B antibodies were not reduced, although levels against whole-cell antigen were markedly decreased (Fig. 5). When the biologic activities were compared, marked decreases in anticytotoxin activity and in *C. difficile* agglutination were observed prior to rechallenge (Fig. 6).

DISCUSSION

Intestinal disease caused by *C. difficile* is due to the action of its toxins directly on the epithelium, and this damage is further enhanced by mediators induced by the proinflammatory activities of the toxins (18, 33). Immunological protection against diarrhea should thus require both local (secretory IgA) responses that would abrogate the effects of toxins on the epithelia and a systemic response that would inhibit the action of absorbed toxins on inflammatory cells. Since *C. difficile* toxins disrupt epithelial tight junctions, permitting leakage of serum proteins from the circulation into the intestinal lumen, serum (IgG) antibodies might also neutralize toxins at the mucosal level. The present study provides evidence that vaccination with *C. difficile* antigens may protect against both the intestinal disease and the systemic action of the toxins, when the appropriate immunization regimen is used. Protection against intestinal disease was determined by the absence of diarrhea and intestinal pathology, and protection against systemic action of toxins was measured by survival. It should be emphasized that the hamster is highly susceptible to *C. difficile* intoxication, which results in an acute lethal outcome (29), whereas in humans, acute death is rarely observed. The hamster model thus provides a severe test of vaccine-induced immunity.

The route of antigen administration is an important determinant of mucosal immune effector functions (16). In the case of *C. difficile*, the i.g. and r. routes of immunization were inef-

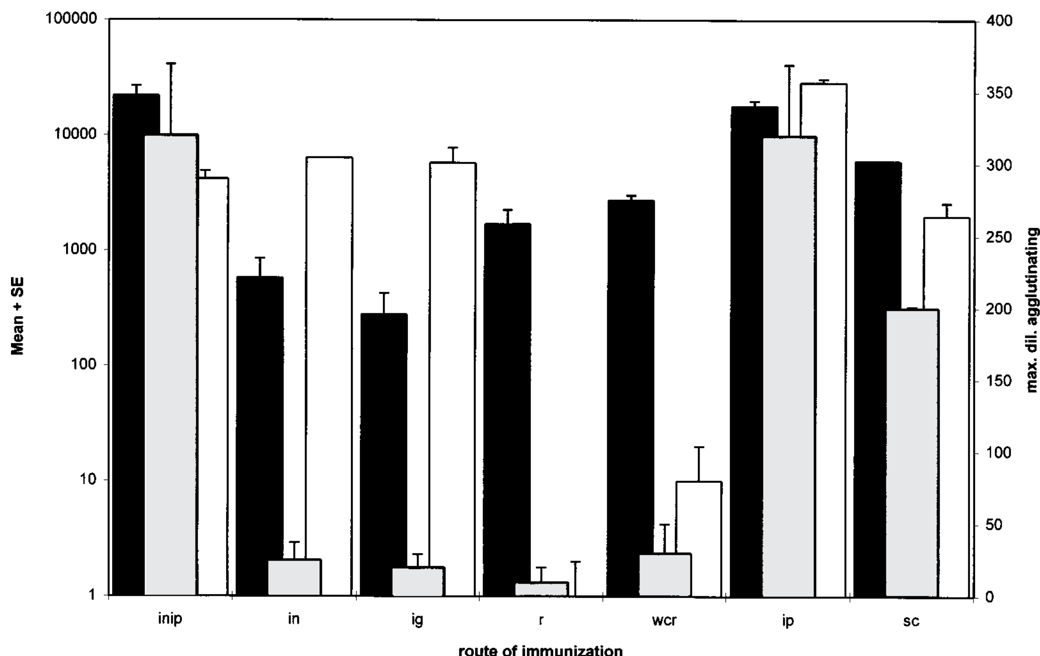

FIG. 4. Biologic activity of sera from hamsters after three doses of vaccine administered by different routes. Sera were tested for inhibition of cytotoxin A or B in IMR-90 cells (■ and □, respectively) and for agglutination of *C. difficile* cells (□); titers were defined as the maximal dilution with biologic activity. Each bar represents the mean + standard error (SE) of five animals. See Table 1 for description of routes of immunization.

fective, and minimal protection against death or diarrhea was observed. We initially reasoned that r. vaccination would result in protection against intestinal disease because this route induces a local and protective immune response against certain other enteric pathogens (15, 16, 34). Animals immunized by the r. route received a dose 10 times higher and those immunized i.g. received a dose 100 times higher than hamsters immunized by parenteral routes, to counterbalance expected antigen degradation by intestinal or gastric proteases, respectively. Cholera toxin was used as a mucosal adjuvant because it is known to potentiate secretory IgA responses (9). However, since we were not able to evaluate the mucosal (secretory IgA) immune response in hamsters, we were unable to determine whether failure to protect after r. or i.g. immunization was due to a poor local immune response. It should be noted that i.g. vaccine was given in the absence of $NaHCO_3$, and antigenicity may have been markedly compromised by acid peptic digestion of the vaccine. However, we have recently demonstrated in mice that immunization by the i.n. but not the i.g. route with $NaHCO_3$ elicits strong intestinal secretory IgA responses against toxins A and B (48). Thus, it is likely that measurement of secretory IgA responses in the hamster model would provide better correlations between immunity and protection.

Parenteral routes of immunization efficiently protected against lethality. Partial protection against diarrhea was also observed, supporting the hypothesis that systemic immunity would decrease intestinal damage by inhibiting the proinflammatory action of the toxins or by transudation and intraluminal neutralization of toxins. Protection against diarrhea was higher in animals immunized by the i.p. than the s.c. route, perhaps because i.p. immunization more efficiently induces a mucosal immune response (37).

Others have shown that i.n. immunization with viral and bacterial antigens together with an appropriate adjuvant (e.g., cholera toxin) induces both systemic and mucosal immunity (35, 47). We found that i.n. vaccination with *C. difficile* antigens protects against intestinal disease and death. Animals primed by the i.n. route and boosted by the i.n. and i.p. routes demonstrated the highest protection of all the routes tested, possibly because this regimen elicited both mucosal and systemic immunity.

Vaccination did not prevent bacterial colonization, and an-

TABLE 2. Correlation between immune response against *C. difficile* antigens and protection against death

Immunoassay	Antibody titer (mean ± SE) of total studied (n = 34)	Antibody titer (mean ± SE) according to:		P value[a]
		Death (n = 11)	Survival (n = 23)	
Toxin A ELISA	5,210 ± 960	2,750 ± 1,360	6,390 ± 1,180	0.0290
Toxin B ELISA	8,610 ± 1,760	3,800 ± 2,840	10,910 ± 2,050	0.0063
Whole-cell ELISA	6,560 ± 1,890	8,363 ± 5,610	5,704 ± 980	0.1382
Anticytotoxin A	7,370 ± 1,620	1,470 ± 390	10,195 ± 2,170	0.0001
Anticytotoxin B	8,690 ± 1,900	1,790 ± 1,240	9,330 ± 2,610	0.0197
C. difficile agglutination	130 ± 25	25 ± 10	180 ± 31	0.0032

[a] By the Kruskal-Wallis test.

TABLE 3. Correlation between immune response against *C. difficile* antigens and the severity of diarrhea

Immunoassay	Antibody titer (mean ± SE) in hamsters with: No diarrhea (n = 10)	Antibody titer (mean ± SE) in hamsters with: Diarrhea of grade 3+ (n = 9)	*P* value[a]
Toxin A ELISA	9,400 ± 1,970	1,333 ± 160	0.0003
Toxin B ELISA	17,800 ± 3,430	870 ± 230	0.0001
Whole-cell ELISA	9,000 ± 1,660	1,820 ± 310	0.0001
Anticytotoxin A	15,840 ± 3,690	3,220 ± 610	0.0016
Anticytotoxin B	11,540 ± 4,770	670 ± 440	0.005
C. difficile agglutination	265 ± 49	55 ± 28	0.0098

[a] *P* values when compared with the no-diarrhea group by the Kruskal-Wallis test.

imals were infected regardless of the regimen of immunization; however, levels of toxins decreased with time. Similar results have been reported in hamsters parenterally immunized (24, 26), although no kinetic data have been reported previously. Levels of toxins might decrease as a result of local neutralization by antibodies; alternatively, antibodies might influence toxin production in vivo. No clear correlation was found between fecal toxin levels and diarrhea, probably because toxin measurements do not accurately reflect toxin concentrations at the level of the epithelium where mucosal damage is induced. Apparently, *C. difficile* strains colonizing hamsters were not affected by host immunity, since strains isolated on day 9 after challenge had the same immunoblot type as those isolated on day 2 and they produced similar amounts of toxins in vitro (results not shown).

We determined whether animals had previous experience with *C. difficile* by examining the response to toxin A after priming. No anamnestic antibody response was detected in the first 7 days after immunization, regardless of the regimen used, indicating that the animals had not been sensitized previously to toxin A. This is an important observation, since hamsters are susceptible to lethal clindamycin challenge without intentional inoculation of *C. difficile*, possibly due to intestinal carriage of these microorganisms. This is exemplified by the observation that after clindamycin challenge, hamsters in our study were colonized by a strain different from the challenge strain, suggesting that they were already carriers of another strain. Since the immune responses to the vaccines used in our study had characteristics of primary responses, it is likely that colonization and endogenous toxin production were at levels too low to provide immunological priming.

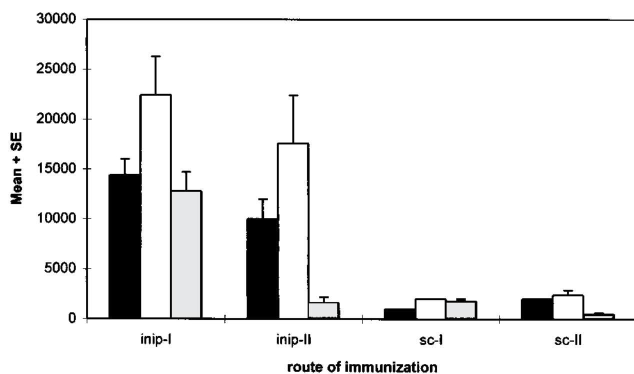

FIG. 5. Long-term antibody response in i.n.i.p. and s.c. immunized hamsters. A comparison of the responses before clindamycin challenge (i.n.i.p.-I and s.c.-I) with those 140 days after clindamycin challenge (i.n.i.p.-II and s.c.-II) is shown. Sera were tested in an ELISA against toxin A (■), toxin B (□), and whole-cell antigen (▫), and titers are expressed as the maximal dilution with an absorbance of >0.3; each bar represents the mean + standard error (SE) of five animals.

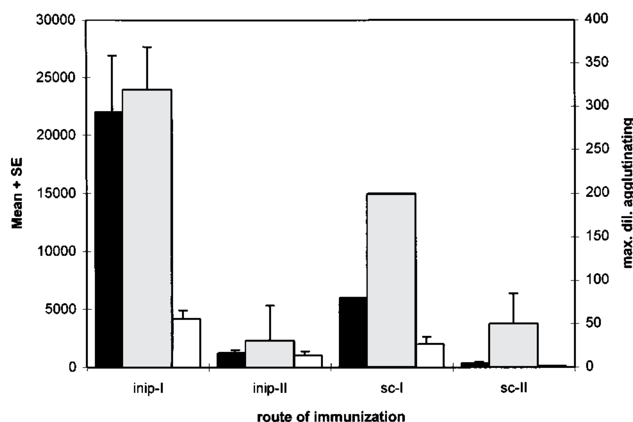

FIG. 6. Long-term antibody response in i.n.i.p. and s.c. immunized hamsters. A comparison of the responses before clindamycin challenge (i.n.i.p.-I and s.c.-I) with those 140 days after clindamycin challenge (i.n.i.p.-II and s.c.-II) is shown. Sera were tested for inhibition of cytotoxin A or B in IMR-90 cells (■ and □, respectively) and for agglutination of *C. difficile* cells (▫); the titer was the maximal dilution of serum with activity. Each bar represents the mean ± standard error (SE) of five animals.

By ELISA, the highest serum immune responses against toxin A, toxin B, and whole-cell antigens were observed in the mucosally immunized groups (i.n., i.g., and i.n.i.p.). Animals immunized by the r. route had lower antibody responses than the i.n. and i.g. groups, possibly as a result of degradation of antigens by intestinal proteases. Alternatively, cholera toxin may have a low adjuvanticity for systemic responses when applied in the colon of hamsters. It should be noted that for r. injections, hamsters had to be anesthetized for 30 to 40 min to avoid rapid expulsion of the vaccine. Pentobarbital and other anesthetics stabilize membranes and might have had deleterious effects on the uptake of antigens, including cholera toxin.

A different pattern was observed when the biologic activity of antibodies was evaluated. Agglutinating antibody titers in mucosally immunized animals were about 1,000 times lower than the antibody titers obtained by ELISA. When the i.p. route (i.p. and i.n.i.p.) or s.c. route is considered, agglutination and whole-cell ELISA results are in closer agreement than the results for mucosally immunized animals. Thus, the antibacterial activity attained with this vaccine strongly depends on the route used for presentation of the antigen. This is relevant because of the possible protective role of the agglutinating activity against intestinal disease (see below).

Toxoid A elicited neutralizing antibodies when administered by any of the routes tested, with the highest mean titers observed after parenteral or a combination of mucosal (i.n.) and parenteral vaccination. Toxoid B is a poor immunogen when delivered by the r. route, possibly because of the instability of toxoid B in the colon rather than to destruction of immunogenicity by formaldehyde, since significant anti-toxin B values resulted after vaccination by the other mucosal routes (i.n. and i.g.). Agglutinating antibodies were elicited principally in response to parenteral vaccination. Overall, the i.n.i.p. and i.p. regimens were the most efficient for induction of antibodies, with both high anticytotoxic and agglutination activities.

Animals protected against death had significantly higher serum antibody levels against toxins and a significantly higher

agglutinating activity than the animals who were not protected. Toxoid A has been shown previously to induce a partial protective immune response against lethal enterocolitis (24, 26). Even a recombinant peptide comprising the C-terminal repeats of toxin A was able to partially protect against lethality (28). Toxoid B also induced partial protection against death (13). None of these studies was done with pure toxins, and other nontoxin antigens present in the preparations may have induced protective antibodies. Our results suggest that antibodies against both toxins are associated with protection against systemic disease and that antibacterial agglutinating antibodies may also play a role. Compared with animals without diarrhea, hamsters presenting with severe enterocolitis (grade 3+ diarrhea and typhlitis of grades $\geq$2) had a significantly lower antibody response to all *C. difficile* antigens. In animals with severe colitis, it is not possible to assign a specific role to antitoxins or antibacterial antibodies, but the poor immune response in this group indicates that levels of circulating anti-*C. difficile* antibodies may be used as indicators of an inadequate protective immune response.

We did not measure neutralization of enterotoxicity induced by toxin A nor did we determine the levels of antibodies with antitoxic activity in secretions. It is likely that these approaches would provide additional insights into the mechanism of protection afforded by vaccination. Studies are currently under way to elucidate these responses.

The role of the antibacterial antibodies in protection against *C. difficile* disease remains unclear. It should be emphasized that after clindamycin challenge, hamsters became colonized with a *C. difficile* strain different from the VPI 10463 strain used to prepare the vaccine or to challenge the animals. Even so, the antibodies elicited by vaccine recognized many proteins in SDS lysates of the strain infecting the hamsters. Major surface proteins from *C. difficile* have been shown to be highly immunogenic, and some of them, with sizes of about 35, 45, and 70 kDa, are cross-reactive and considered to be conserved *C. difficile* cell-surface proteins (42, 43). These proteins have been shown to be immunogenic in patients with *C. difficile*-associated disease (36). In our study, proteins in the range of 35, 45, and >100 kDa were recognized by sera from hamsters that were protected after vaccination, suggesting a protective role. A 70-kDa protein was recognized by all animals, including those immunized by the r. route and not protected by vaccination; the 70-kDa protein is immunodominant and may not be important for protection. To further define the role each specific antigen has in protection, immunization with purified antigens should be done.

Since phagocytosis of *C. difficile* by human polymorphonuclear leukocytes has been shown to be dependent on opsonization (8), antibacterial antibodies should help to clear infection. Additionally, the ability of polymorphonuclear leukocytes from elderly subjects, a population at high risk of developing antibiotic-associated disease, to kill *C. difficile* was significantly reduced when compared with that of young subjects (5). The defect was serum associated and might have been due to reduced opsonins; this would be explained in part by the observation that antibodies to *C. difficile* decrease with age (2). Although most bacterial enteric diseases are toxin mediated, prevention of colonization by antibacterial antibodies is protective and sometimes even more effective than that by antitoxin antibodies (1). In our work, hamsters were colonized regardless of the immune response and regardless of the disease outcome after clindamycin challenge. It is possible that in those animals protected against enterocolitis, antibacterial antibodies prevented colonization in the proximity of the epithelial layer, and thus, *C. difficile* growth and toxin production would be limited to the lumen and would be cleared in feces.

The i.n.i.p. and s.c. regimens were able to partially protect against lethality from a second clindamycin challenge 140 days after the first challenge, and 50% of the animals that received the i.n.i.p. immunization were also protected against diarrhea. Thus, the i.n.i.p. regimen confers relatively long-term protection against both lethality and enterocolitis.

In summary, we found that a combination of mucosal and parenteral immunization with *C. difficile* toxoid was the most effective regimen of immunization for induction of protective immunity against *C. difficile* disease in the hamster model and that i.n. but not i.g. or r. immunization fully protected against death and partially against diarrhea. The level of antibodies against toxins (determined by ELISA or neutralization of cytotoxin) and antibodies agglutinating *C. difficile* correlated with protection against both systemic and intestinal disease. To develop a vaccine more suitable for use in humans, studies are in progress to improve the quality of toxoid and the dosage; we are also exploring the use of alternative routes of immunization and adjuvants appropriate for use in humans.

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