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Flagella-Induced Immunity Against Experimental Cholera in Adult Rabbits

R. J. YANCEY,† D. L. WILLIS, AND L. J. BERRY*

Department of Microbiology, The University of Texas, Austin, Texas 78712

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The adult rabbit ligated ileal loop model was used to evaluate the prophylactic potential of a crude flagellar (CF) vaccine produced from the classical, Inaba strain CA401. A greater than 1,000-fold increase in the challenge inoculum was required to induce an intestinal fluid response in actively immunized adult rabbits equivalent to that produced in unimmunized animals. Similar protection was afforded against challenge with classical and El Tor biotypes of both Inaba and Ogawa serotypes. Highly virulent ^{35}S-labeled vibrios were inhibited in their ability to associate with the intestinal mucosa of CF-immunized rabbits. The protection conferred by CF immunization was found to be superior to that of a commercial bivalent vaccine and also to that of glutaraldehyde-treated cholera toxoid. The critical immunogenic component of CF appears to be a flagella-derived protein. The immunogenicity of CF was destroyed by heat treatment, and absorption of CF-immune serum with aflagellated mutant vibrios did not diminish its ability to confer a high level of passive protection. The intestinal protection of CF-immunized rabbits was completely reversed by the introduction of both goat anti-rabbit immunoglobulins A and G, but by neither alone.

Problems involved in the induction of protective immunity against cholera are mostly related to the noninvasive nature of the pathogen (17). Throughout the course of infection, *Vibrio cholerae* is confined to the intestinal lumen and mucosal surface, where it is partially isolated from the full array of host defense mechanisms that would normally be directed against an invasive organism. Vibrios produce a well-characterized enterotoxin, which binds to an epithelial ganglioside receptor (7, 20) and causes the fluid outpourings characteristic of cholera. It is reasonable to assume that a humoral response directed against either the bacteria or toxin confers protection only if specific antibody is present within the intestinal lumen or at the epithelial surface of the host's small intestine.

In cholera-endemic areas, a lower rate of reinfection and a decrease in incidence of disease has been correlated with increasing age (26). This indicates that a significant degree of specific immune protection is acquired through exposure to *V. cholerae* antigens. Further evidence from volunteer studies (5, 28) shows that convalescents from induced cholera were resistant to homologous challenge for up to 12 months. Nevertheless, conventional cholera vaccines consisting of parenterally administered killed cells have proven relatively ineffective in field trials (1, 8, 26, 27). There is a protection rate of about 40 to 60% during the first 2 months, but this falls to between 10 and 20% after 6 months. The level of protection afforded by killed-cell vaccines seems to correlate with a rise in the titer of serum vibriocidal antibody (25-27). The inadequacy of these vaccines is possibly related to their inability to elicit substantial coproantibody. Freter and Gangarosa (14, 15) have advocated the use of orally administered killed vibrios to elicit and maintain gut immunity. However, prolonged protection by this route has not been demonstrated, and the large quantities of antigen that must be ingested render the feasibility of this technique unlikely.

Antitoxic as well as antibacterial antibodies are produced during the immune response to cholera infection in humans (4, 6, 30). Although there is no direct evidence that antitoxin plays a role in recovery from the disease, the prophylactic potential of antitoxic immunity is obvious. Finkelstein and Hollingsworth (12, 13) induced antitoxic immunity in rats and rabbits with subcutaneous injections of a formalinized toxoid vaccine. Although somewhat variable, this immunity was protective against both homologous and heterologous challenge. Northrup and Chisari (29), in subsequent studies with monkeys, found that the formalinized toxoid vaccine reverted to toxin in vivo and caused a severe localized reaction to subcutaneous injection.

† Present address: Department of Microbiology, University of Texas Southwestern Medical School, Dallas, TX 75235.

Similar reactions were experienced by human volunteers inoculated with 25 μg of toxoid vaccine, which is less than the amount believed necessary for the induction of effective immunity (32). The probability of harmful side effects due to toxoid reversion is apparently reduced by oral immunization (16). However, antitoxic immunity by this route in mice required up to 100 times more antigen to attain a level of protection comparable to that induced parenterally. Rappaport et al. (31) produced a stable toxoid vaccine by treating purified toxin with glutaraldehyde. The product was reported to be somewhat less immunogenic than formalinized toxoid, but its antigenicity was enhanced by combining the toxoid with an alum adjuvant. However, a recent report from field trials in Bangladesh with the glutaraldehyde-treated toxoid vaccine indicates disappointing results (G. T. Curlin, J. Chakravorty, K. M. A. Aziz, R. J. Levine, and W. F. Verwey, *in* Proceedings of the 14th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Cholera Panel, in press). The risk of cholera infection in an endemic area was found to be more closely related to such factors as the source of domestic water for bathing and drinking than to the serological status of toxoid-immunized individuals. Experiments presented here compare the level of protective immunity afforded adult rabbits by the toxoid vaccine versus that conferred by a motility-related immunogen described below.

Previous studies from this laboratory have demonstrated the importance of motility as a virulence factor in cholera (10, 18, 35). Experiments with the infant mouse model (18) and in rabbit ligated ileal loops (35) strongly suggest that an intimate association of vibrios with the intestinal mucosa is necessary for efficient toxin delivery. Nonmotile but fully toxinogenic and prototrophic mutant derivatives of motile, highly virulent parental strains showed a greatly reduced ability to associate with the intestinal mucosa and to produce disease symptoms. Since motility (or some factor related to the expression of motility [23]) is essential for the induction of cholera, Eubanks et al. (11) prepared crude flagella (CF) from sheared vibrios and used this antigen to passively immunize infant mice. The level of passive protection with the CF vaccine was shown to be significantly higher than that obtained with the commonly employed commercial bivalent vaccine (CV).

The present investigations are a continuation of studies with the CF vaccine. The rabbit ligated ileal loop technique was used to confirm the high degree of protection against both homologous and heterologous challenge provided by active and passive immunization with the CF vaccine.

MATERIALS AND METHODS

Rabbits. Adult rabbits weighing 2 to 4 kg were obtained from local breeders.

Bacterial strains. The same highly virulent strains of *V. cholerae* described previously were used in these studies (18). They are: CA401 (classical, Inaba), CA411 (classical, Ogawa), 8233 (El Tor, Inaba), HK-1 (El Tor, Ogawa), and the hypertoxinogenic strain 569B (classical, Inaba). Cultures were maintained in the lyophilized state and restored as needed. Bacteria for ileal loop inoculation were grown at 37°C on brain heart infusion agar for 18 h. They were removed from the surface, washed once by centrifugation, and diluted in phosphate-buffered saline (pH 7.2) containing 0.1% gelatin (PBSG).

Vaccines. Commercial bivalent cholera vaccine (CV) was obtained from the Health Center, the University of Texas at Austin. Sclavo control no. VC 49/D (prepared by I. S. V. T. Sclavo, Siena, Italy) was administered in doses equivalent to about 1 mg (wet weight) of protein (two injections of 0.5 ml of vaccine) unless otherwise specified.

Glutaraldehyde-treated cholera toxoid (lot 20201, Wyeth Laboratories) was provided by Carl Miller, National Institute of Allergy and Infectious Diseases. Toxoid for subcutaneous injection was rehydrated to a concentration of 200 μg/ml with a protamine sulfate-aluminum chloride diluent containing 3.75 mg of aluminum chloride per ml, 0.5 mg of protamine sulfate per ml, and 0.05 mg of thimerosal per ml. This alum-precipitated toxoid solution was administered in 0.5-ml doses.

CF was prepared from the highly motile strain CA401 by a modification of the method of Eubanks et al. (11). Cells from an 8-h brain heart infusion agar slant were suspended in PBSG and inoculated into a 1-liter flask containing 200 ml of brain heart infusion broth. The broth culture was incubated at 37°C on a rotary shaker (150 rpm) for 14 to 18 h, and these cells (early stationary phase) were inoculated into a 20-liter carboy containing 10 liters of brain heart infusion broth. This large culture was incubated at 37°C with aeration for 4 h, after which the cells were harvested by centrifugation (6,000 × *g*) and resuspended in 180 ml of 1 mM potassium phosphate buffer (pH 7.2) containing 1 mM $CaCl_2$ (PC buffer). Flagella were sheared from the cells at low speed in a Waring blender for 20 s, and cells and large debris were removed by centrifuging twice at 16,000 × *g* for 30 min. Flagella and smaller debris were collected from the supernatant fluid by centrifuging at 85,000 × *g* for 1 h and resuspending the pellet overnight in fresh PC buffer. Differential centrifugations were repeated three times, and the final pellet was concentrated by resuspending in 5 ml of PC buffer. Final protein concentration of CF preparations was about 2 mg/ml. Flagella were stored at 4°C in PC buffer containing 0.5% Formalin (to kill residual bacteria) and were used within 2 weeks of preparation.

"Deactivated" CF was prepared by heating for 15 min at 100°C in a water bath. This was sufficient to

denature the protein components without affecting the lipopolysaccharides.

Immunization. Rabbits were immunized subcutaneously in both flanks on day 0 and day 7. They were challenged via ligated ileal loops on days 14 and 18. Animals vaccinated with CF or toxoid received 100 μg of protein per injection. CV was administered in two 1-mg doses.

Preparation of ligated ileal loops. Ileal loops were prepared by a modification of the method of De and Chatterje (9). Rabbits were fasted for 48 h to allow elimination of fecal pellets. One-half milliliter of a vibrio suspension, appropriately diluted in PBSG, was injected into segments of the ligated ileum, each approximately 6 to 9 cm in length and each separated by an uninoculated segment of about 2 cm. Control loops received 0.5 ml of PBSG or 0.5 μg of cholera toxin in 0.5 ml of diluent. After 18 h (or at shorter intervals, if required), the rabbits were sacrificed and the ileum was excised. Loops were carefully measured, and the ratio of the volume of fluid accumulated (in milliliters) to the length of the segment (in centimeters) was calculated and expressed as the fluid accumulation (FA) ratio.

Adsorption of ^{35}S-labeled vibrios. Vibrios were labeled by the procedure of Baselski and Parker (2). ^{35}S-labeled cells were injected into the ligated loops of normal and CF-immune rabbits and assayed after 3 h of incubation, as described previously (35). The number of counts associated with tissue sections was compared with the number remaining unattached in the gut fluid. From 70 to 98% of the total injected counts were recoverable by this technique.

Absorption of anti-CF antiserum with aflagellated cells of *V. cholerae*. Complement-inactivated (56°C for 30 min) antisera from rabbits immunized with CF were pooled and sterilized by passing through a 0.45-μm filter. Preimmune sera collected from the same rabbits prior to immunization were used for control. One-half of each pool was absorbed with aflagellated cells by the procedure below, and the other half was reserved for comparison.

Approximately 10^{10} Formalin-killed cells of the aflagellated mutant strain CA401 M-6 were washed three times with sterile saline and suspended in 5 ml of immune serum. After incubation at 37°C for 30 min, the cells were pelleted by centrifugation. The serum was decanted and added to an equal volume of washed, Formalin-killed cells. Again after incubation at 37°C for 30 min, the cells were removed by centrifugation. The remaining cells were removed by passing the serum through a 0.45-μm filter.

Serum titers. Anti-O (somatic) and anti-H (flagellar) titers of serum from immunized rabbits were determined by the quantitative tube agglutination test. Serial dilutions of complement-inactivated serum were made in sterile saline (0.5 ml per tube). Cells of *V. cholerae* CA401 were grown at 37°C for 14 h on Trypticase soy agar slants. Vibrios to be used for anti-O determination were harvested in normal saline and killed by heating for 30 min in a boiling water bath. Cells for H-agglutination were killed by suspending in normal saline containing 1% Formalin for 30 to 60 min. The turbidity of these suspensions was adjusted to match a 100 Klett unit standard, and 0.5 ml of the appropriate antigen suspension was added to each tube of diluted antiserum. Preimmune serum at the lowest dilution was used as a control. The tubes were incubated for 2 h at 50°C, and H-agglutination was read using a concave mirror. O-agglutination was also read in the same manner after refrigeration at 4°C overnight. Titers were defined as the reciprocal of the highest dilution at which obvious (++) agglutination occurred.

Passive immunization with absorbed and unabsorbed serum. Unabsorbed serum and serum absorbed with aflagellated cells were diluted 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} in PBSG. Preimmune serum was diluted 10^{-1}. Diluted sera were mixed with virulent CA401 cells (2×10^8 colony-forming units [CFU] per ml) immediately before surgery and approximately 15 to 30 min before injection. Test loops were injected with 0.5 ml of this mixture, and control loops received PBSG or cells only. The degree of passive protection was determined by calculating FA ratios after 18 h.

Introduction of anti-IgA and anti-IgG into the ligated loops of CF-immune rabbits. Goat anti-rabbit immunoglobulin G (IgG) was obtained from Miles Laboratories, Elkhart, Ind. Normal goat serum and goat anti-rabbit IgA were provided by William J. Mandy, Department of Microbiology, The University of Texas at Austin. The anti-IgA was absorbed twice with rabbit IgG affixed to Sepharose 4B, and the specific anti-immunoglobulin activities of both antisera were confirmed by Ouchterlony gel immunodiffusion against purified rabbit IgA and IgG.

Decimal dilutions (10^{-1} to 10^{-4}) of goat anti-rabbit IgA, goat anti-rabbit IgG, or both antisera were mixed with CA401 cells (2×10^8 CFU/ml) immediately before surgery. Ligated ileal loops of CF-immune rabbits were injected with 0.5 ml of this mixture. Control loops received normal goat serum plus cells or cells only. Loops were assayed after 18 h for FA.

Statistics. Statistical significance was determined by the Wilcoxon rank sum test (33).

RESULTS

Protection against homologous and heterologous challenge in CF-vaccinated rabbits. The importance of motility in experimental cholera has been demonstrated by Guentzel and Berry (18) and, more recently, by Yancey et al. (35). In both studies, a loss of motility resulted in a decreased capacity of the vibrios to associate with the ileal mucosa. Immunity directed primarily against the flagellum might, therefore, protect the host by preventing association. Eubanks et al. (11) tested this assumption by preparing a CF vaccine from sheared cells of *V. cholerae*. This vaccine was shown to confer excellent protection against cholera in passively immunized infant mice. The following experiments were designed to test active immunity to this vaccine in adult rabbits.

CF was prepared from the highly motile strain CA401 by a modification of the method of Eubanks et al. (11), as described above. Examina-

tion of CF preparations by transmission electron microscopy revealed a large number of sheathed flagella and some contamination with vesicular debris. Rabbits immunized with this material showed a high degree of protection against challenge with CA401, CA411, and 569B. The results are presented graphically in Fig. 1. Whereas maximal FA ratios were produced in normal animals after challenge with 10^6 CFU, FA was effectively eliminated under the same conditions in immune animals. FA ratios remained significantly depressed in immune animals challenged with 10^8 CFU. With this number of cells, FA ratios in loops injected with the heterologous strain CA411 and the highly toxinogenic Inaba strain 569B were approximately equal. Similar protection was afforded against challenge with the El Tor biotypes 8233 and HK-1 (data not shown). Immunized rabbits required a challenge increase of 100- to 1,000-fold to yield an FA ratio equivalent to that seen in control animals. The immunity was not antitoxic, since control loops receiving as little as 0.2 μg of cholera toxin were maximally positive. These results emphasize the ability of CF prepared from the classical Inaba strain CA401 to protect against challenge by classical and El Tor strains of both the Inaba and Ogawa serotypes.

Association of vibrios with the ileal mucosa of normal and immune rabbits. Guentzel et al. (19) used fluorescent antibodies to demonstrate the inability of motile, highly virulent vibrios to successfully associate with the ileal mucosa of infant mice passively immunized to CF. The results were similar to those obtained with nonmotile mutant strains of *V. cholerae* in normal infant mice (19) and, more recently, with nonmotile mutant strains in nonimmune adult rabbits (35). The results presented in Fig. 2 extend this comparison to the association of motile, virulent, ^{35}S-labeled bacteria with the mucosa of normal and CF-immune adult rabbits. About 50% of the total recoverable counts were associated with ileal sections in normal rabbits. This level of tissue association decreased to between 11 and 23% in immune animals. The difference is statistically significant with the El Tor strains (8233 and HK-1) as well as classical strains of both serotypes ($P < 0.01$).

Comparison of the efficacy of CF, CV, and cholera toxoid. The effectiveness of CF as an immunogen was compared to those of a commonly employed CV and cholera toxoid. Rabbits were immunized, as described above, with subcutaneous injections of CV diluted to a protein concentration 10 times greater than that of CF vaccines and equivalent to approximately one-thirtieth of the prescribed dose for an adult human being. CF and Wyeth toxoid were each administered in two doses totaling 200 μg of protein.

Figure 3 shows that CV protection against challenge with CA401 was slight but not significant in loops injected with 10^5 or 10^6 CFU and nonexistent in loops injected with 10^4 or 10^8 CFU. As in previous experiments, CF afforded highly significant protection ($P < 0.005$) at all doses tested. Therefore, on a protein-weight basis, CF was more than 10 times as effective as CV. As with CF, toxoid immunogenicity appeared to be at least 10 times greater than that of CV on a protein-weight basis. However, toxoid

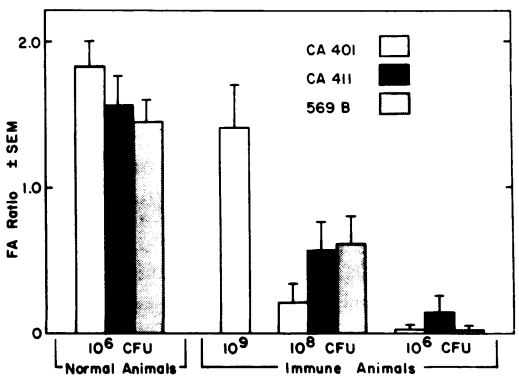

FIG. 1. *FA response in the ligated loops of normal rabbits and in rabbits immunized with CF from CA401, after challenge with different doses of homologous and heterologous strains. Each value represents the mean FA ratio of at least five rabbits. Small bars indicate the standard error of the mean for this response.*

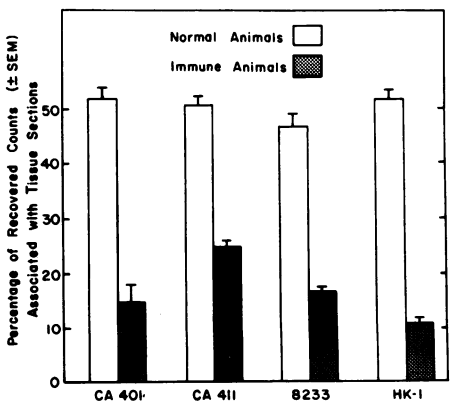

FIG. 2. *Association of ^{35}S-labeled vibrios with the ileal mucosa of normal and CF-immunized rabbits. This association was determined as described in the text. Each value represents the average counts adsorbed to at least five cross sections of the rabbit ileum.*

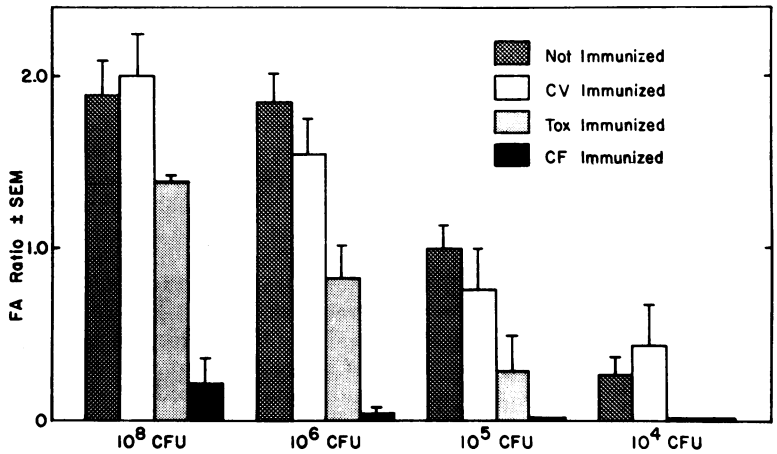

FIG. 3. *Comparison FA responses in the ligated ileal loops of normal and immune rabbits after challenge with various doses of strain CA401 (classical, Inaba). Animals were immunized as described in the test. CF and Wyeth toxoid (Tox) were each administered in two 100-μg doses. CV was injected in two 1-mg doses.*

immunization was significantly less effective than CF immunization, especially at the higher challenge doses. After challenge with 10^4 CFU of CA401, FA ratios were reduced to zero in rabbits immunized with either toxoid or CF.

Similar results were obtained when CV-immunized rabbits were challenged with CA411. Fig. 4, which summarizes the fluid response to two challenge doses (10^6 and 10^8 CFU) of the classical Ogawa strain, shows that FA ratios in CV-immunized rabbits were at least as great as in control rabbits. Heterologous protection in CF-immunized rabbits is evidenced by significantly lower FA ratios at both challenge doses.

Mean FA ratios for two challenge doses were slightly lower for CA411 than CA401 (Fig. 3) in toxoid-immunized rabbits. However, maximal FA ratios in control animals were also lower for the Inaba strain, so actual protection was about the same. The FA ratio in toxoid-immunized rabbits challenged with 10^6 CFU was approximately equal to the FA ratio in CF-immunized rabbits challenged with 10^8 CFU. This indicates that, on a dose basis, CF protection by this immunization schedule was about 100 times more effective than toxoid immunity.

Passive protection of ileal loops in normal rabbits with anti-CF antiserum. Normal rabbits were passively immunized with various dilutions of complement-inactivated anti-CF antiserum introduced into ligated ileal loops along with 10^8 virulent CA401 cells. The upper portion of Fig. 5 demonstrates passive protection at serum dilutions of 10^3 or less, as evidenced by significantly reduced ($P < 0.03$) FA ratios. This protection coincided roughly with the anti-flagellar (anti-H) titer of the serum. The anti-H

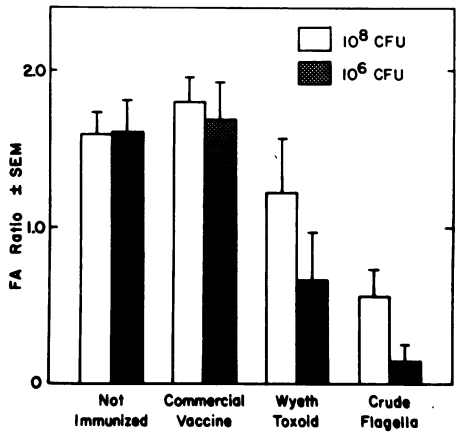

FIG. 4. *Comparison of FA ratios in normal and CF-immunized rabbits challenged with two doses (10^8 and 10^6 CFU) of the classical Ogawa strain CA411. Animals were immunized as described in the text.*

titer of the serum employed was 1,280, whereas the anti-O titer was 256. At a dilution of 10^{-1}, the immune serum completely eliminated fluid accumulation in the loops. Preimmune serum from the same rabbits afforded no protection against the 10^8 CFU challenge, as indicated by maximal FA ratios.

Evidence that passive protection might be due to anti-flagellar antibody was obtained by absorbing the serum twice with an aflagellated derivative of CA401, as described in Materials and Methods. The data presented in the lower part of Fig. 5 show that no appreciable loss in passive immunity resulted. The anti-O titer of the serum was reduced below a detectable level by absorption, while the ability to reduce fluid

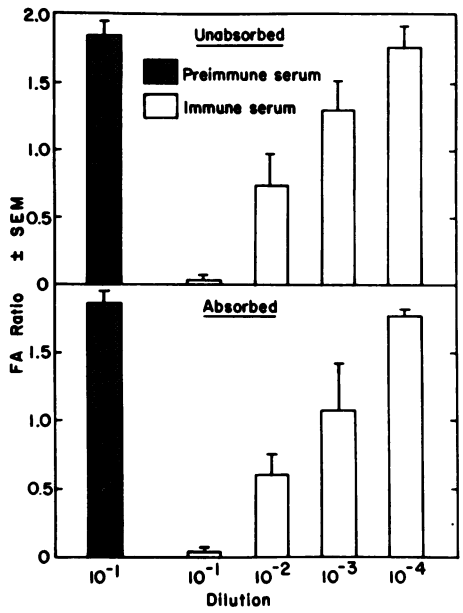

FIG. 5. *FA in ligated ileal loops of normal rabbits passively immunized with anti-CF antiserum. Decimal dilutions of serum were mixed with virulent CA401 cells and injected into the loops at an inoculum of 10^8 CFU per loop. The lower graph shows the results obtained using serum that had been absorbed with the aflagellated mutant CA401 M-6 to remove anti-O activity.*

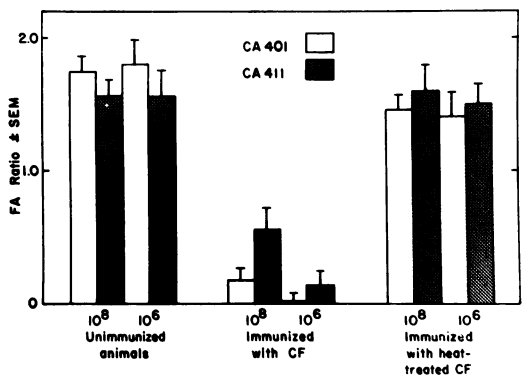

FIG. 6. *The FA responses of rabbits immunized with heat-treated CF. Heat treatment, as described in the text, destroyed proteins but not lipopolysaccharides.*

accumulation was not affected.

Immunogenicity of the heat-treated CF vaccine. Evidence that the immunogenic component of the CF preparation was not contaminating lipopolysaccharide is indicated by the results presented in Fig. 6. In this experiment, the CF vaccine was heated at 100°C for 15 min (a temperature capable of denaturing protein but not lipopolysaccharide), and the rabbits were immunized with this material as before. The FA response to challenge with homologous (CA401) and heterologous (CA411) strains in rabbits immunized with heat-treated CF was not significantly different from that of unimmunized controls. Data from rabbits immunized with unheated CF are included for comparison.

Role of IgA and IgG in intestinal anti-CF immunity. Goat anti-rabbit IgA and goat anti-rabbit IgG were introduced into the ligated ileal loops of CF-immune rabbits along with virulent CA401 cells as described above. Initial results indicated optimum reversal of immune protection with goat anti-rabbit serum at a dilution of about 10^{-2}. There may have been a slight prozone effect at the lower (10^{-1}) dilution, since reversal of protection was not as evident at this dilution of anti-immunoglobulin serum. Data obtained at the optimal dilution are presented graphically in Fig. 7. The high level of protective immunity was indicated by the almost negligible fluid response in loops challenged with 10^8 virulent vibrios. The addition of normal goat serum had no observable effect on this response. Protection appeared to be partially reversed by the addition of either anti-IgA (alpha) or anti-IgG (gamma) to the loops, as evidenced by slightly increased FA ratios. Immune protection was completely reversed by the simultaneous injection of both anti-IgA and anti-IgG into the loops along with the challenge inoculum. This is dem-

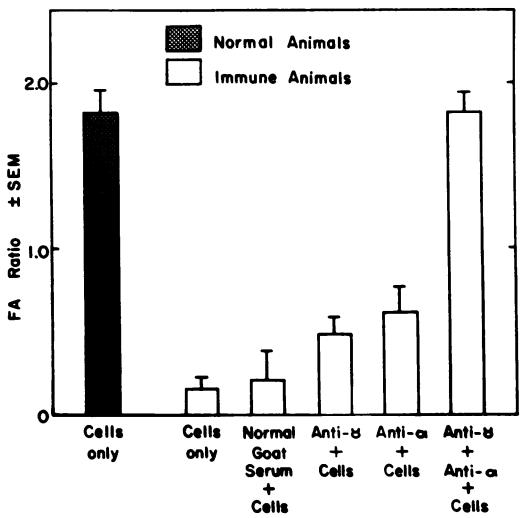

FIG. 7. *Reversal of the protection provided by CF immunization with goat anti-rabbit IgA (anti-α) and goat anti-rabbit IgG (anti-γ). The goat antisera were injected with the challenge organism (10^8 CFU of strain CA401). The bars represent the standard error of the mean for three rabbits.*

onstrated by an average fluid accumulation response as high as that in nonimmune animals receiving the same challenge dose. Apparently, the intestinal protection afforded by anti-CF immunity requires the combined participation of both immunoglobulin types.

DISCUSSION

Antibacterial immunity and antitoxic immunity have proven marginally effective as prophylactic measures against cholera in field trials. The continued quest for effective prophylaxis should be based, therefore, on different routes of immunization and/or a more effective immunogen. This report extends the use of a CF vaccine originally introduced by Eubanks et al. (11). Subcutaneous injection of a relatively small amount (200 μg) of the antigen produced highly significant active protection against homologous and heterologous challenge in rabbit ligated ileal loops (Fig. 1) and excellent passive protection (Fig. 5). CV given by the same route but with 10 times as much protein (and even more lipopolysaccharide) did not induce significant protection (Fig. 3 and 4). The glutaraldehyde-treated Wyeth toxoid gave some protection at a dose of 200 μg but less protection than that provided by the same amount of CF vaccine.

There is no definitive explanation for the high degree of active protection afforded by CF immunity. Bacterial motility is probably inhibited, though this would be difficult to demonstrate in vivo. The in vitro inhibition of motility by specific antiserum was used by Benenson et al. (3) to identify vibrios in stool samples of patients with acute diarrhea. Williams and his co-workers (34) used motility inhibition as a method for determining titers of immune serum raised against whole cells or purified somatic antigen. Motility inhibition titers of anti-CF antiserum (1:1,100) were not significantly higher than those reported for antibacterial antisera (34).

The association of motile, virulent vibrios with the ileal mucosa was significantly reduced in rabbits immunized with CF (Fig. 2). These results are similar to those reported by Guentzel et al. (19) in passively immunized mice and in nonimmune animals challenged with nonmotile vibrios (19, 35). Thus, the reduced association of vibrios with the ileal mocosa of immune animals might result from the inhibition of bacterial motility, but other data from normal and immune animals challenged with motile and nonmotile vibrios suggest that anti-CF immunity might involve something other than, or in addition to, motility inhibition. Nonmotile vibrios produce maximal FA ratios in normal rabbits at a challenge inoculum of 10^8 CFU of toxinogenic cells. This challenge dose with nonmotile vibrios resulted in maximal fluid accumulation, whereas the data in Fig. 1 and 3 show that a significant reduction in fluid accumulation occurred after challenge with 10^8 CFU of motile strain CA401 in CF-immune animals.

The decrease in attachment of radiolabeled vibrios to the mucosa of rabbits vaccinated with CF is about 60% (Fig. 2), whereas the increase in challenge dose required for maximum FA in vaccinated animals was 100- to 1,000-fold (Fig. 1). The two changes should not necessarily be expected to correlate, since the timing was different (3 h for attachment and 18 h for fluid accumulation) and, more importantly, attachment probably depends on physical phenomena while fluid loss depends on the catalytic activity of toxin.

The basis for CF immunity cannot be attributed to contaminating enterotoxin in the preparations, since the immunized rabbits produced maximal FA in loops challenged with as little as 0.2 μg of cholera toxin. Moreover, assays for toxin by the morphological change induced in Chinese hamster ovary cells indicated the presence of less than 1 ng/mg of protein in the CF preparations or less than 200 pg per immunizing dose (unpublished data of D. S. Schneider and R. J. Yancey). It is possible, however, that the CF vaccine contains vibrio surface components similar to adhesive K88 antigen of *Escherichia coli* (24). Cholera vibrios may possess a similar antigen (22, 23), which contaminates the CF vaccine and can then induce a response that simultaneously neutralizes the adhesive factor and impairs bacterial motility. This assumption cannot be confirmed, however, until the specific antigenic components of CF are determined.

High vibriocidal titers might account for the observed level of protection beyond that expected by motility inhibition alone. Vibriocidal titers of serum from CF-immune rabbits were found to be very high, but the data (not shown) varied greatly depending upon the assay. It would be difficult to correlate these titers with vibriocidal activity within the small intestine. The nature of the coproantibody response to CF immunization, as revealed by the use of goat anti-rabbit IgA and goat anti-rabbit IgG, indicated that both classes of immunoglobulins are required for effective protection at the site of infection (Fig. 7). It was not determined whether the IgG was serum derived or a normal component of intestinal immunity.

Holmgren et al. (21) recently reviewed the pathogenic mechanisms involved in cholera and the vaccines currently available. They recommended the use of an improved vaccine com-

prised of cholera toxin and lipopolysaccharide antigens. With this combined vaccine, they were able to demonstrate a 100-fold increase in immune protection of adult rabbits. The results presented here demonstrate that a similar level of protection in adult rabbits is attained by immunization with the subcellular CF vaccine alone. A combined CF-toxoid vaccine might prove to be optimal for human prophylaxis against cholera.

ACKNOWLEDGMENT

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