INFECTION AND IMMUNITY, Oct. 1982, p. 74–79
0019-9567/82/100074-06$02.00/0

Vol. 38, No. 1

Synergistic Protective Effect of Antibodies Against *Escherichia coli* Enterotoxin and Colonization Factor Antigens

CHRISTINA M. ÅHRÉN AND ANN-MARI L. SVENNERHOLM*

Department of Medical Microbiology, University of Göteborg, S-413 46 Göteborg, Sweden

Received 12 March 1982/Accepted 18 June 1982

We studied the ability of antisera against different *Escherichia coli* surface antigens, both alone and in combination with anti-enterotoxin, to decrease fluid secretion induced by intestinal challenge with enterotoxigenic *E. coli* in rabbits. Antiserum against lipopolysaccharide protected significantly against O group homologous bacteria. Monospecific antisera against pilus-associated colonization factor antigens CFA/I and CFA/II were also effective, giving highly significant protection against enterotoxigenic *E. coli* strains bearing the corresponding colonization factor antigens. Protection was also observed with Fab fragments of the CFA/I antibodies. Addition of the anti-lipopolysaccharide serum to a protective antiserum against purified heat-labile enterotoxin resulted in an antisecretory effect which slightly exceeded the sum of the effects obtained with each preparation alone. The combination of antiserum against CFA/I or CFA/II with anti-enterotoxin gave protection that equaled the product of the effects obtained with each antiserum alone; i.e. the antisera cooperated synergistically.

In previous studies we have shown that *Vibrio cholerae* lipopolysaccharide (LPS) and toxin antigen cooperate synergistically in inducing immune protection against experimental cholera infection (20). The protection induced is probably due to action of the antibodies at different pathogenic levels (i.e., adhesion of the bacteria to intestines and binding of enterotoxin to specific membrane receptors) rather than to an adjuvant action of either of the antigen components (12, 20). Recently, we have also demonstrated synergistic cooperation between antibodies against whole bacteria and heat-labile enterotoxin (LT) for protection against challenge with enterotoxigenic *Escherichia coli* belonging to not only homologous but also heterologous O groups (18).

Colonization of small intestines seems to be necessary for enterotoxigenic *E. coli* to cause diarrhea (6). The ability to colonize is due to adhesive structures on the bacterial surface, and two such adhesins, colonization factor antigens (CFAs) CFA/I and CFA/II, have been identified on many enterotoxigenic *E. coli* strains isolated from humans (3, 5). Controversy exists as to the prevalence of these adhesins on different *E. coli* strains, but in certain areas they seem to be present on 40 to 80% of the enterotoxigenic *E. coli* strains isolated from patients with acute diarrhea (1, 6; Gothefors et al., manuscript in preparation). Whereas CFA/I has been recognized on *E. coli* strains belonging to different O groups (e.g., O15, O63, and O78) (10, 14), CFA/II is usually associated with O groups 6 and 8 (3). The two adhesins are immunogenic and do not cross-react in immunodiffusion analyses (3). Antibodies against CFA/I have been found to be effective in protecting infant rabbits against experimentally induced enterotoxigenic *E. coli* diarrhea by interfering with bacterial colonization (7). Recently, antibodies against CFA/II were also shown to inhibit fluid secretion in rabbit loops challenged with different CFA/II-bearing enterotoxigenic *E. coli* strains (19).

The aim of the present study was to evaluate the capacity of antibodies against bacterial structures, such as LPS and CFAs, to cooperate synergistically with anti-enterotoxin for protection against enterotoxigenic *E. coli*. This was studied in rabbits by determining the protective effect against intestinal challenge with LT-producing *E. coli* of antibodies against these surface antigens, both alone and in combination with anti-LT antibodies.

MATERIALS AND METHODS

Bacterial strains. The following enterotoxigenic *E. coli* strains were used: H10407 (O78:K80:H11; CFA/I); H10407P, a CFA/I-negative mutant of strain H10407; E1392-75 (O6:K15:H16; CFA/II); 408-3 (O78:K nontypable:H12; CFA/II); CFA/II-negative mutants of strains E1392-75 and 408-3 (19); and 286-C2 (O8:K nontypable:H nontypable). All strains except strain 286-C2 were suspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% (wt/vol) glycerol and stored in portions at −70°C. Before use the CFA-possessing strains were grown on CFA agar (5) and checked for

the presence of the CFA property by hemagglutination (HA) with different species of erythrocytes and agglutination with CFA/I- or CFA/II-specific antisera, as described below. *E. coli* 286-C2 was maintained on agar slants at room temperature until it was used.

Antigens. Highly purified *E. coli* LT was prepared from strain 286-C2. The bacteria were grown in Casamino Acids yeast extract medium (8) in a fermentor at 37°C for 9 h, and the bacterial sediment obtained after centrifugation at 9,000 × *g* for 30 min was subjected to ultrasonication at 22 kHz for 1 min. The supernatant obtained after centrifugation (23,500 × *g*, 30 min) of the sonicated material was used for the purification of LT, as described by Clements and Finkelstein (2), except that 0.5 M galactose instead of 0.2 M galactose was used to elute the toxin from the agarose column. The purity of the material was ascertained by immunodiffusion. Moreover, immunization of experimental animals with the LT preparation did not result in significant titer increases to homologous (O8) LPS. Highly purified cholera toxin was purchased from Schwarz/Mann, Orangeburg, N.Y. Purified LPS was prepared from *E. coli* strain H10407 (O78) by the hot phenol-water extraction procedure, followed by repeated ultracentrifugation (13). The preparation contained 0.9% protein as measured by a protein assay (Bio-Rad Laboratories, Munich, Germany) but no detectable levels of *E. coli* LT and CFA/I as demonstrated by comparative immunodiffusion analyses, using monospecific antisera against *E. coli* LT and CFA/I, respectively. Purified CFA/I was prepared from a flagellum-deficient mutant of strain H10407 (kindly provided by Dr. D. G. Evans), as described previously (4).

Antisera. Immune sera were produced in New Zealand white rabbits that weighed about 3 kg at the onset of immunization. The injections were given at 2- to 3-week intervals, and 1 to 2 weeks after the immunizations were completed, the animals were bled by heart puncture. The sera obtained were stored at −30°C until they were used.

Anti-LT serum was prepared in rabbits given two subcutaneous injections of 30 μg of highly purified *E. coli* LT in Freund complete adjuvant. Antiserum against LPS was obtained from animals given three subcutaneous immunizations of 1.5 mg of O78 LPS; the first two injections were given in Freund complete adjuvant. Antisera against CFA/I were prepared by immunizing rabbits three to five times subcutaneously with 1×10^9 to 5×10^9 washed cells of strain H10407 and then extensively absorbing the sera collected with live and boiled strain H10407P cells, as described previously (7). Antisera against CFA/II were prepared similarly from bleedings of rabbits given three to five subcutaneous injections of 1×10^9 to 5×10^9 washed strain 408-3 and 1392 cells and then absorbing the sera with the CFA/II-negative mutants of strains 408-3 and 1392-75, respectively (3). Absorption was continued until more than 99.9% of the homologous anti-LPS antibodies of the serum were eliminated, as determined by the enzyme-linked immunosorbent assay (ELISA). Most of the remaining anti-enterotoxin antibody activity was then removed by repeatedly passing the sera through immunosorbent columns with cholera toxin covalently coupled to Sepharose (21).

Fab fragments. Fab fragments of antibodies against CFA/I were prepared essentially as described by Porter (15). A serum with a very high anti-CFA/I titer (anti-CFA/I:2) (see Table 1) was precipitated with ammonium sulfate added to 40% saturation, and a solution containing 5 mg of immunoglobulin per ml was treated with papain (final concentration, 50 μg/ml) at 37°C for 16 h.

ELISA. Antibodies against LPS and cholera toxin were titrated by using a microplate ELISA, as previously described (11). For assaying antibodies against LT a micro-modification of the GM1 ELISA method (22) was used. Antibodies to CFA/I were determined by an ELISA method, using plates coated at 37°C for 18 h with 5 μg of purified CFA/I per ml.

Agglutination tests. Agglutination of bacteria by CFA/I- and CFA/II-specific antisera was performed on glass slides. HA tests of human group A and bovine erythrocytes for identification of CFA/I- and CFA/II-bearing strains, respectively, were performed on slides, using 3% erythrocyte suspensions containing 1% D-mannose, (3, 5). The HA inhibitory effect of antibody was studied by mixing bacteria with antibody for 1 min before the erythrocytes were added (5, 19).

Protection tests. The capacities of the various immune sera to protect against experimental enterotoxigenic *E. coli* infection were evaluated in ligated small bowel loops in rabbits, essentially as described previously (17, 18). The bacteria used for challenge were initially grown on CFA agar at 37°C for 18 h and then in a Casamino Acids yeast extract medium at 37°C overnight. After the suspension was washed in phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH 7.2), it was adjusted to the desired bacterial concentration according to optical density. Serial 5- or 10-fold dilutions of the bacterial cultures in 1-ml volumes were incubated for 15 min at room temperature and for 30 min at 4°C with 0.1 ml of a dilution of immune serum. The anti-LT serum was used at a final concentration of 1/100, which corresponded to an antibody concentration which was 1,000 times the GM1 ELISA titer against LT (see Table 1). The anti-LPS serum was used at a final dilution of 1/40, which corresponded to 5,000 times the ELISA titer against homologous LPS, and the anti-CFA sera were used at final concentrations of 1/10, unless otherwise stated. The antibody-bacterium mixtures were then injected into four to eight 5-cm small bowel loops randomly positioned in at least four different animals. After 18 to 20 h the animals were sacrificed and the ratio of volume of fluid accumulation to length was determined. For each animal the bacterial dose that gave half-maximum fluid accumulation (50% effective dose [ED_{50}]) in the presence of antiserum or PBS was determined. The protection factor (PF) was then calculated as the ratio between the geometric means of individual ED_{50} values in the presence of antiserum and in PBS, as follows: PF = (mean ED_{50} in antiserum)/(mean ED_{50} in PBS).

To evaluate the bacterial growth in the loops after challenge, intact loops were homogenized in an Omnimixer homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.) at 8,000 rpm for 1 min, and the viable counts were determined on serially diluted homogenates.

RESULTS

Specificity of antibody preparations. As shown by ELISA titrations against different *E. coli* antigens, both the anti-LT and anti-LPS sera at

TABLE 1. Antibody titers to different *E. coli* antigens in the dilutions of the sera used in the protection tests

Antiserum	ELISA titer against:		
	LT	O78 LPS	CFA/I
Anti-LT	1,000	<5	<5
Anti-O78 LPS	<5	5,000	<5
Anti-CFA/I:1	50	30	500
Anti-CFA/I:2	<5	5	800
Anti-CFA/IIA	50	35	30
Anti-CFA/IIB	50	50	20

the dilutions used contained high titers against the homologous antigens but no significant titers against any of the other antigens assayed (Table 1). The diluted anti-CFA sera contained low levels of antibodies to *E. coli* LT and O78 LPS, but, as determined in preliminary experiments, these titers were too low to be protective. The specificities of the anti-CFA/I sera were also ascertained by their capacity to agglutinate strain H10407 and other CFA/I-bearing strains but not the CFA/I-deficient mutant H10407P or the two CFA/II-positive strains used. Furthermore, the sera did not contain significant levels of type 1 pilus antibodies, as demonstrated by their inability to agglutinate CFA-negative, heavily piliated type 1 pilus strains (16). Similarly, both antisera against CFA/II (from strains E1392-75 and 408-3) were specific for CFA/II. Thus, these sera both agglutinated strains 408-3 and E1392-75 but not the CFA/II-negative mutants of these strains or any of several enterotoxigenic *E. coli* strains with defined CFA/I or the type 1 pilus property.

Protection by anti-enterotoxin. The protective effect against *E. coli* strains H10407 and 408-3 of anti-LT antibodies was tested in ligated small bowel loops in rabbits. We found that antiserum against purified LT was highly effective against both of these strains. Thus, the ED_{50} values of the challenge bacteria were significantly ($P < 0.01$; Student's t test) higher if the bacteria were pretreated with a dilution of anti-LT serum than if they were mixed with PBS (Fig. 1 through 3). The protection seemed to be due to specific antibodies because neither normal rabbit serum nor sera from bleedings taken before immunization had any antisecretory effect when they were tested in concentrations 10-fold higher than the concentration of the immune serum.

Anti-LPS antibodies. Similarly, the protective effect of antibodies to purified O78 LPS against O group homologous strain H10407 bacteria was studied. Significantly ($P < 0.05$) more bacteria were needed to induce half-maximum fluid accumulation in the loops in the presence than in the absence (PBS controls) of anti-LPS serum (Fig. 1). Extensive absorption of the serum with O78 LPS completely eliminated its protective effect. Addition of anti-LT serum to the anti-LPS preparation resulted in increased protection against

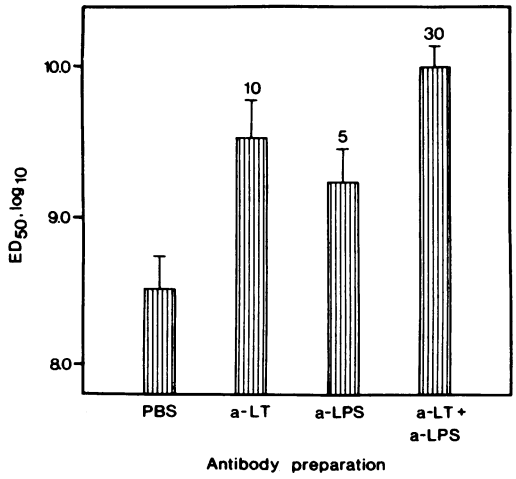

FIG. 1. Protective effects of antisera against purified *E. coli* LT (a-LT) and *E. coli* O78 LPS (a-LPS), both alone and in combination, against intestinal challenge with enterotoxigenic *E. coli* strain H10407. The bars indicate the geometric mean ± standard error of the mean of the ED_{50} values obtained for each animal. Protection factors (i.e., the ratio between the ED_{50} values of the bacteria in the presence of antisera and in PBS) are indicated on top of the bars.

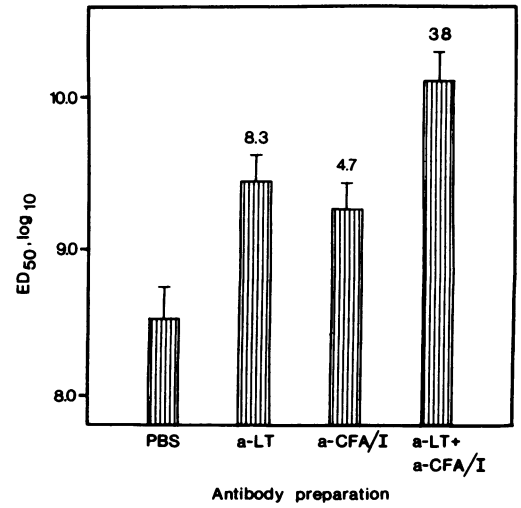

FIG. 2. Protective effects of anti-LT serum (a-LT) and monospecific anti-CFA/I:1 serum (a-CFA/I), both alone and in combination, against challenge with CFA/I-bearing strain H10407. The bars indicate the geometric mean ± standard error of the mean of the ED_{50} values obtained for each rabbit; protection factors are indicated on top of the bars.

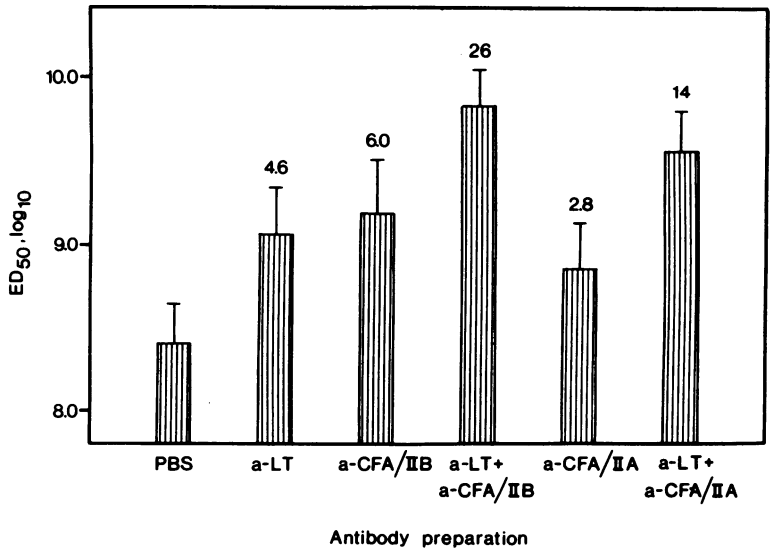

FIG. 3. Protective effects of antisera against CFA/II from strain 408-3 (a-CFA/IIA) and strain E1392-75 (a-CFA/IIB), both alone and in combination with anti-LT serum (a-LT), against challenge with CFA/II-bearing strain 408-3. The bars indicate the geometric mean ± standard error of the mean of the ED_{50} values obtained for individual rabbits; protection factors are indicated on top of the bars.

strain H10407, which slightly exceeded the sum of the effects induced by each antiserum alone.

Anti-CFA/I antibodies. The capacity of antibodies against CFA/I, both alone and in combination with anti-enterotoxin, to decrease the fluid accumulation induced by CFA/I-bearing *E. coli* strains was also tested. As shown in Fig. 2, the anti-CFA/I serum was effective in preventing fluid secretion induced by strain H10407 in intestinal loops ($P < 0.02$). High-titer immune serum against CFA/II had no effect on this challenge strain.

To evaluate whether the inhibitory effect of anti-CFA/I was due to interference with binding of the bacteria to mucosal receptor structures or to agglutination of the bacteria in the intestinal lumen, we analyzed the protective effect of Fab fragments of CFA/I antibodies. Such fragments did not agglutinate any of a number of CFA/I-bearing strains, including strain H10407, but did inhibit HA of strain H10407. In relation to the HA-inhibiting effect, the Fab fragments were almost as effective as the noncleaved immunoglobulin fraction of the serum in inhibiting fluid secretion by strain H10407 in small bowel loops (Table 2).

The addition of anti-LT serum to the anti-CFA/I preparation resulted in markedly increased protection against strain H10407. This protection corresponded to the product rather than the sum of the effects obtained with each antiserum alone (Fig. 2).

Anti-CFA/II antibodies. Similarly, antisera against two serological subtypes of CFA/II (19) were produced and analyzed for protection and for synergistic cooperation with anti-enterotoxin against CFA/II-bearing *E. coli* strains. The monospecific antiserum against strain 408-3 CFA/II (anti-CFA/IIA) gave significant protection ($P < 0.01$) against challenge with homologous bacteria (Fig. 3).

Antiserum against strain E1392-75 CFA/II (anti-CFA/IIB), which contained higher levels of specific antibodies (as shown by HA inhibition), was even more effective ($P < 0.01$) in decreasing the fluid accumulation induced by strain 408-3 (Fig. 3).

The addition of anti-enterotoxin to either of the two anti-CFA/II sera resulted in a protective effect against the challenge strain, which in both instances corresponded to the product of the effects obtained with the anti-enterotoxin and the anti-adhesin alone (Fig. 3).

TABLE 2. HA inhibition and protection by anti-CFA/I and Fab fragments of anti-CFA/I

Antiserum	HA inhibition titer	Protection	HA inhibition/protection
Anti-CFA/I:2	600[a]	530[b]	1.13
Papain-cleaved anti-CFA/I:2	12	14	0.86

[a] Reciprocal of the dilution that completely inhibited HA of human A erythrocytes by strain H10407 (10^8 cells per ml).

[b] Protection factor against challenge with strain H10407 multiplied with reciprocal of dilution used (150 for anti-CFA/I and 5 for Fab fragments).

Effect of antisera on bacterial growth. To determine whether the protective effect of the antibodies was due to inhibition of bacterial growth, the viable counts of bacteria in antiserum-containing loops were compared with the number of bacteria in PBS control loops. At the time of sacrifice, there was no significant reduction in the number of bacteria after treatment with either anti-LT and anti-LPS sera or anti-CFA sera.

DISCUSSION

The ability to colonize the small intestine seems to be important for the virulence of enterotoxigenic *E. coli*, in addition to the production of LT or heat-stable enterotoxin. Since the two adhesins CFA/I and CFA/II are immunogenic (3, 7) and seem to be present on many of the diarrheogenic enterotoxigenic *E. coli* strains isolated from humans (1, 6), they are of potential interest for immunization against enterotoxigenic *E. coli*. Our results support previous findings that antibodies against these adhesins may decrease the fluid secretion induced in experimental animals by challenge with CFA-bearing enterotoxigenic *E. coli* strains (7, 19). Moreover, we found that antisera against CFA/I or CFA/II cooperate synergistically with anti-LT serum in protecting against CFA-piliated, LT-producing *E. coli* strains. Neither normal rabbit serum nor bleedings taken before immunization were protective, indicating that specific antibodies were responsible for the antisecretory effect of the immune sera. This was further supported by the observation that a high-titer serum against CFA/II had no effect on the fluid secretion induced by bacteria possessing CFA/I.

Recently, we demonstrated the existence of two serological subtypes of CFA/II (19). In that study we found that antibodies against the shared pilus structures and also (although to a lesser extent) antibodies to a subtype-specific structure(s) protected against bacteria possessing CFA/II of the homologous subtype. Our finding in the present study that anti-CFA/II cooperated synergistically with anti-enterotoxin for protection against bacteria possessing CFA/II of the heterologous subtype suggests that antibodies against the shared CFA/II structures are capable of such cooperation. Whether antibodies against the type-specific CFA/II structure(s) enhance the effect of anti-enterotoxin remains to be determined.

In the cholera system the synergistic cooperation between antibacterial and antitoxin antibodies seems to be due to action of the antibodies at different pathogenic levels (i.e., the mucosal adhesion of bacteria and binding of enterotoxin) (20). Against this background it is not surprising that antibodies against CFA/I and CFA/II, which may interfere with the colonization of CFA-piliated *E. coli*, cooperated with toxin-neutralizing antibodies for protection against enterotoxigenic *E. coli*.

The protective capacities of the different antibody preparations were evaluated in ligated loops in rabbit intestines. In such loops, the mechanical defense against bacterial colonization usually obtained by peristalsis is not operating. Therefore, adhesion of bacteria to the intestinal mucosa might not be necessary in order to deliver sufficient quantities of toxin to the mucosal cells. Alternatively, production of high levels of intraluminal toxin may have resulted in fluid secretion. Against this background we cannot exclude the possibility that the anti-CFA antibodies could have exerted their effect by mechanisms other than by interference with mucosal adherence. Since the sera used were complement inactivated and the levels of complement in intestinal secretions are very low (9), there is little evidence of a bactericidal effect of the antibodies. This is supported by the finding that the anti-CFA sera had no effect on bacterial growth in the loops, as shown by a comparison of the viable counts after incubation of bacteria with antibodies and with PBS.

The observation that in relation to their HA-inhibiting ability, Fab fragments of anti-CFA/I reduced the fluid secretion almost as effectively as the noncleaved immunoglobulin fraction of the serum argues against the possibility that all of the protective effect of the anti-CFA/I serum was due to agglutination of the bacteria in the intestine; rather, this finding supports the notion that anti-CFA antibodies could mediate protection in intestinal loops by direct blocking of bacterial adherence to the intestinal mucosa. However, agglutination of bacteria may be of some importance for protection in small bowel loops, and it is likely that this mechanism may be effective also in nonligated intestines by facilitating the expulsion of bacteria through peristalsis.

The mechanism of action of the anti-LPS antibodies may also be to prevent adhesion to the mucosa by steric hindrance or agglutination of the bacteria in the lumen or both, since these antibodies did not affect bacterial growth. Previous studies have suggested that antitoxin antibodies primarily act by preventing the binding of toxin to cell surface receptors, but they may also be effective by blocking the toxic sites on the enterotoxic molecule (12).

For protection against cholera, antibodies against the LPS component are capable of synergistic cooperation with anti-enterotoxin (20). In the enterotoxigenic *E. coli* system anti-LPS antibodies are also capable of enhancing the

protection by anti-enterotoxin. However, this enhancement is somewhat less pronounced than that obtained with anti-CFA preparations, whose protective capacities are similar to that of anti-LPS serum when it is tested alone. The reason for this stronger enhancement displayed by the anti-CFAs is not clear, but it may be related to the possibility that these antibodies can prevent close contact of the bacteria and thus of the enterotoxin with the intestinal cells.

ACKNOWLEDGMENTS

The skillful technical assistance of B.-M. Lavstrand, G. Wiklund, and E. Olofsson is gratefully acknowledged.

Financial support was obtained from the National Defence Research Institute in Sweden (grant 506B601), from the Swedish Medical Research Council (grant 16X-3382), and from the Faculty of Medicine, University of Göteborg.

LITERATURE CITED

1. **Bäck, E., R. Möllby, B. Kaijser, G. Stintzing, T. Wadström, and D. Habte.** 1980. Enterotoxinogenic *Escherichia coli* and other gram-negative bacteria of infantile diarrhea: surface antigens, hemagglutinins, colonization factor antigen and loss of enterotoxigenicity. J. Infect. Dis. **142**:318–327.
2. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect. Immun. **24**:760–769.
3. **Evans, D. G., and D. J. Evans, Jr.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638–647.
4. **Evans, D. G., D. J. Evans, Jr., S. Glegg, and J. A. Pauley.** 1979. Purification and characterization of the CFA/I antigen of enterotoxigenic *Escherichia coli*. Infect. Immun. **25**:738–748.
5. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
6. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**:727–736.
7. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
8. **Evans, D. J., Jr., D. G. Evans, S. H. Richardson, and S. L. Gorbach.** 1976. Purification of polymycin-released heat-labile enterotoxin of *Escherichia coli*. J. Infect. Dis. **133**:S97–S102.
9. **Fubara, E. S., and R. Freter.** 1972. Source and protective function of coproantibodies in intestinal disease. Am. J. Clin. Nutr. **25**:1357–1363.
10. **Gross, R. J., A. Cravioto, S. M. Scotland, T. Cheasty, and B. Rowe.** 1978. The occurrence of colonization factor (CF) on enterotoxinogenic *Escherichia coli*. FEMS Microbiol. Lett. **3**:231–233.
11. **Holmgren, J., and A.-M. Svennerholm.** 1973. Enzyme-linked immunosorbent assays for cholera serology. Infect. Immun. **7**:759–763.
12. **Holmgren, J., A.-M. Svennerholm, I. Lönnroth, M. Fall-Persson, B. Markman, and H. Lundbäck.** 1977. Development of improved cholera vaccine based on subunit toxoid. Nature (London) **269**:602–604.
13. **Ørskov, F., I. Ørskov, K. Jann, E. Müller-Seitz, and O. Westphal.** 1967. Immunochemistry of *Escherichia coli* O antigens. Acta Pathol. Microbiol. Scand. **71**:339–358.
14. **Ørskov, I., and F. Ørskov.** 1977. Special O:K:H serotypes among enterotoxigenic *E. coli* strains from diarrhea in adults and children. Occurrence of the CF (colonization factor) antigen and of hemagglutinating activities. Med. Microbiol. Immunol. **163**:99–140.
15. **Porter, R. R.** 1959. The hydrolysis of rabbit immunoglobulin and antibodies with crystalline papain. Biochem. J. **73**:119–126.
16. **Salit, I. E., and E. C. Gotchlich.** 1977. Hemagglutination by purified type 1 *Escherichia coli* pili. J. Exp. Med. **146**:1169–1180.
17. **Svennerholm, A.-M.** 1975. Experimental studies on cholera immunization. IV. The antibody response to formalinized *V. cholerae* and purified endotoxin with special reference to protective capacity. Int. Arch. Allergy Appl. Immunol. **49**:434–452.
18. **Svennerholm, A.-M., and C. Åhrén.** 1982. Immune protection against enterotoxinogenic *E. coli*: search for synergy between antibodies to enterotoxin and somatic antigens. Acta Pathol. Microbiol. Immunol. Scand. Sect. C **90**:1–6.
19. **Svennerholm, A.-M., and C. Åhrén.** 1982. Serological subtypes of an Escherichia coli colonization factor antigen. Eur. J. Clin. Microbiol. **1**:107–111.
20. **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.
21. **Svennerholm, A.-M., and J. Holmgren.** 1977. Immunoglobulin and specific antibody synthesis in vitro by enteral and nonenteral lymphoid tissues after subcutaneous cholera immunization. Infect. Immun. **15**:360–369.
22. **Svennerholm, A.-M., and J. Holmgren.** 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. Curr. Microbiol. **1**:19–23.