Protection Against Colibacillosis in Neonatal Piglets by Immunization of Dams with Procholeragenoid

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Protection against colibacillosis in neonatal piglets was obtained by immunization of pregnant dams with procholeragenoid. Procholeragenoid is a stable high-molecular-weight aggregate of cholera toxin formed during the heating of cholera toxin. Procholeragenoid retained approximately 1% of the toxicity of native toxin as determined in the rabbit ileal loop and Y-1 adrenal cell assays and 5% of the activity in the rabbit skin assay. Immunization of pregnant dams with 50 µg of procholeragenoid 5 and 2 weeks before the expected delivery date elicited high titters of antitoxic immunoglobulin G and toxin-neutralizing antibody in both the colostrum and serum. In three independent field trials, immunization with procholeragenoid resulted in a substantial decrease in diarrhea (73% in controls versus 11% in immunized) and death (4.7% in controls versus 0.77% in immunized) in neonatal piglets. The protection rate in the immunized population was approximately 85% for both diarrhea and death. In the following gestation period, reinmunization of dams with a single dose of procholeragenoid (50 µg) 2 weeks before delivery elicited titters of antitoxic immunoglobulin G and toxin-neutralizing antibody comparable to those obtained during the primary immunization. The death rate in neonatal piglets (0.86%) was comparable to that seen after immunization during the first gestation period (0.77%). These results indicate that substantial protection of neonatal piglets against colibacillosis can be obtained by immunization of dams with procholeragenoid. Protection was found to be based solely on antitoxic immunity.

Enteric colibacillosis is a leading cause of death in neonatal piglets (26, 39). The ability of certain strains of *Escherichia coli* (termed enteropathogenic *E. coli*) to cause disease in piglets is dependent primarily upon two factors: the ability to colonize and multiply in the small intestine (38) and the production of enterotoxins (1, 18, 24, 38). Colonization is dependent upon the presence of pili which mediate attachment to the mucosal surface (16, 21, 23, 24, 33). Most enteropathogenic *E. coli* strains are hosts for plasmids which code for either a heat-labile enterotoxin (LT) or a heat-stable enterotoxin or both (17, 38). LT has been isolated and characterized (5, 9, 27). LT and cholera toxin (CT) have been shown to be structurally (5, 7), functionally (5, 11, 15), and immunologically (3, 4, 19) related. Furthermore, cross-neutralization and cross-protection between CT and LT have been demonstrated (5, 10, 19).

Efforts to protect piglets against colibacillosis by immunization of pregnant dams have been attempted with a variety of immunogens. The most promising results were achieved with vaccines directed against either colonization factors (30, 36, 29) or LT (8, 10).

In this study we have used procholeragenoid, a toxoid of CT, to immunize dams (12, 13). Procholeragenoid was selected in place of LT because CT can be obtained in higher yields (5, 27). In addition, procholeragenoid is far less toxic than CT or LT on a weight basis (12, 13) and has been shown to be a good immunogen (13, 14).

MATERIALS AND METHODS

Preparation of CT, procholeragenoid, and cholera- genoid. CT and choleragenoid were produced and purified as previously described (13, 28). CT was converted to procholeragenoid in the following manner: CT was suspended in either phosphate-buffered saline, pH 7.4 (PBS), or Tris-EDTA buffer, pH 7.5 (50 mM Tris, 1 mM EDTA, 0.2 M NaCl, 3 mM NaNO3) (12). CT was heated at 60°C for 30 min in PBS or for 20 min in Tris-EDTA buffer.

Procholeragenoid preparations were analyzed by chromatography on a Sepharose 4BCL column, 1 by 38 cm (Pharmacia Fine Chemicals, Uppsala, Sweden). A 3-mg sample was applied in 0.5 ml and eluted with Tris-EDTA buffer at a flow rate of 17 ml/h. Fractions (1.7 ml) were collected, and absorbance at 280 nm was measured. Based on the absorbance at 280 nm, both procholeragenoid preparations contained approxi-
imately 90% of the large-molecular-weight component (peaks I and II, Fig. 1) and 10% of the lower-molecular-weight component (peak III, Fig. 1).

Biological assays for CT activity. The biological activities of CT, procholeragenoid, and choleragenoid were measured in the rabbit ileal loop (25), Y-1 adrenal cell (37), and skin vascular permeability (6) assay systems.

Adsorption of procholeragenoid to aluminum hydroxide. Procholeragenoid (2 mg/ml in PBS) was diluted with an equal volume of 10% lactose. The solution was diluted either 1:20 (100 μg/2-ml dose) or 1:40 (50 μg/2-ml dose) in a 1% suspension of aluminum hydroxide containing 0.01% thimerosol. Greater than 99% of the procholeragenoid was found to be tightly bound to the aluminum hydroxide as quantitated by enzyme-linked immunosorbent assay (see below). The final solution was stored at 4°C, under which condition the preparation remained stable for up to 14 months.

Quantitation of antitoxic IgG in serum and colostrum. An enzyme-linked immunosorbent assay for detection of antitoxic immunoglobulin G (IgG) was developed by modification of the method described by Holmgren and Svennerholm (20). Polystyrene tubes (13 by 100 mm; Falcon Plastics, Oxnard, Calif.) were coated with CT by placing 1 ml of PBS containing 20 μg of CT into each tube. Tubes were incubated for 3 h at 37°C and then stored at 4°C. Protein A from Staphylococcus aureus (Pharmacia Fine Chemicals) was coupled to horseradish peroxidase, type VI (Sigma Chemical Co., St. Louis, Mo.), by the method of Nakane and Kawaoi (32). Horseradish peroxidase activity was determined by using H₂O₂ as substrate and 2,2'-azino-di(3-ethylbenzylthiozoline)sulfonic acid-6 (ABTS) (Boehringer Mannheim, Mannheim, West Germany) as a hydrogen donor. ABTS solution was prepared daily by dissolving 10 mg of ABTS in 50 ml of 0.1 M NaH₂PO₄-HCl buffer, pH 4.0. Before use, 0.125 ml of 10% H₂O₂ was added (substrate solution).

The enzyme-linked immunosorbent assay was performed as follows. CT-coated tubes were washed three times with 10 ml of PBS containing 0.02% Tween 20 (PBS-T). To each tube was added 1 ml of serially diluted serum or colostrum (dilutions in PBS-T). Tubes were incubated at 22°C for 6 h and washed three times with PBS-T. Horseradish peroxidase-protein A complex in 1 ml of PBS-T was added, and the tubes were incubated overnight at 4°C. Tubes were then washed three times with PBS-T, 1 ml of substrate solution was added, and tubes were incubated for 1 h at 22°C. The reaction was stopped by the addition of 0.1 ml of 0.04% NaNO₂. The samples were then measured at 415 nm. Titers were expressed in comparison with porcine reference antisera (PAS-1) (400 antitoxin units per ml). Titers for each serum were assigned the reciprocal of the serum dilution which gave 50% of maximal absorbance of PAS-1 in the assay.

Porcine reference antisera. Three swine were immunized intramuscularly with 250 μg of aluminum hydroxide-adsorbed procholeragenoid 5 and 2 weeks before collection of sera. Sera were pooled and small samples were lyophilized. The toxin-neutralizing titer of the pooled porcine antisera (PAS-1) was determined in the Y-1 adrenal cell assay by comparison with the

FIG. 1. Chromatography of procholeragenoid on Sepharose 4BCl. V₀ = 2 × 10⁷ daltons. Symbols: (●) procholeragenoid generated by heating choleragen for 30 min at 60°C in PBS, pH 7.4; (○) procholeragenoid generated by heating choleragen for 20 min at 60°C in Tris-EDTA buffer, pH 7.5. A₂₈₀. Absorbance at 280 nm.
TABLE 1. Biological properties of procholeragenoids and choleragenoid

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Activity as % CTa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit ileal loop (U/mg of protein)</td>
</tr>
<tr>
<td>CT</td>
<td>100 (6,900)</td>
</tr>
<tr>
<td>Procholeragenoid (Tris-EDTA; peak I)</td>
<td>1</td>
</tr>
<tr>
<td>Procholeragenoid (PBS; peak II)</td>
<td>1</td>
</tr>
<tr>
<td>Peak IIIc</td>
<td>NDd</td>
</tr>
<tr>
<td>Choleragenoid</td>
<td>ND</td>
</tr>
</tbody>
</table>

a CT = 100%. LBs, Amount of toxin giving a 4-mm-diameter increase in the permeability of rabbit skin in the presence of 1 U of antitoxin.
b MRD, Minimal rounding dose (smallest quantity of toxin necessary to cause rounding in approximately 50% of cells).
c Peak III from either PBS or Tris-EDTA material gave comparable results.
d ND, Not determined.

international standard antiserum EC-3 (Swiss Serum and Vaccine Institute, Berne, Switzerland) (13). EC-3 has been shown to contain 4,470 antitoxin units per ml based on the neutralization of CT in the skin vascular permeability assay (13). By comparison, PAS-1 possessed approximately 10-fold less CT-neutralizing capacity in the Y-1 adrenal cell assay than EC-3 and was therefore assigned a titer of 400 antitoxin units per ml.

CT or LT neutralization assay. The activity of serum and colostrum to neutralize CT or LT was determined in the Y-1 adrenal cell assay. Purified LT was kindly provided by B. Withold, University of Groningen, Groningen, The Netherlands. Serum or colostrum was diluted 1:50 in PBS, and 0.5 ml was placed in a 13- by 100-mm tube. To a constant amount of diluted antiserum various amounts of CT or LT (4 ng to 4 µg, in twofold serial dilutions) in 0.5 ml were added. The mixtures were incubated for 45 min at 37°C and diluted 1:20 with Hams F-10 medium, and 0.2 ml was added to each well of a 96-well microtiter plate containing a confluent monolayer of Y-1 adrenal cells. Each plate contained controls of CT and medium alone. Cultures were incubated for 6 h at 37°C and scored microscopically. Titer were assigned based on comparison to PAS-1 antiserum (400 antitoxin units per ml).

Vibriocidal antibody determination. Vibriocidal titers were determined by a slight modification of previously published methods (13, 14). Colostrum was filter sterilized and heated at 56°C for 30 min. The test strain used was NIH 35 (Inaba). Vibriocidal titers are expressed as the reciprocal of the highest colostrum dilution which inhibited bacterial growth by 50%.

RESULTS

Physical and biological properties of procholeragenoid. Prior studies have demonstrated that, in the presence of Tris-EDTA, CT was converted to procholeragenoid in an almost quantitative manner (12, 13). However, upon analysis by chromatography on Sepharose 4BCI, the product was found to be heterogeneous, consisting of multiple-sized aggregates (peak I, Fig. 1). Efforts were then directed towards selecting conditions under which a more homogeneous procholeragenoid could be produced. The heating of CT in PBS (pH 7.4) for 30 min at 60°C resulted in a far more homogeneous material (peak II, Fig. 1). Present in both preparations of procholeragenoid was a peak of lower molecular weight (peak III, Fig. 1).

The biological activities of peaks I, II, and III from the two procholeragenoid preparations were determined in several assay systems (Table 1). Procholeragenoid, produced in either PBS or Tris-EDTA, gave similar results in all three assays. Both preparations possessed approximately 1% of the activity of CT in the rabbit ileal loop and Y-1 adrenal cell assays and 5% in the skin permeability assay. The activity of peak III was comparable to that of the procholeragenoid preparations. Choleragenoid showed less than 0.1% of the activity of CT in all assays (Table 1). Analysis of peak III by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed only a single band which comigrated with choleragenoid (data not shown). These results indicate that peak III is composed primarily of choleragenoid but probably contains trace quantities of CT To account for residual toxicity. Removal of peak III from procholeragenoid before use was not deemed necessary given the comparable toxicities displayed by peak III and procholeragenoid. For all of the following experiments procholeragenoid produced in PBS was used.

Immunogenicity of procholeragenoid in dams. To determine the minimum quantity of procholeragenoid necessary to give an optimal antitoxic IgG and toxin-neutralizing response in serum and colostrum, dams were immunized with either 50 or 100 µg of procholeragenoid 5 and 2 weeks before expected delivery, and the antibody response was determined (Tables 2 and 3). Both doses of procholeragenoid were capable of eliciting high titers of antitoxic IgG and toxin-neutralizing antibody. Although in most cases the antibody titer was slightly higher at the higher immunizing dose (100 µg), these differences were not found to be statistically significant (P > 0.5). In all of the following experiments 50 µg of procholeragenoid was used as the immunizing dose.
PERSISTENCE OF ANTITOXIN IN THE COLOSTRUM OF IMMUNIZED DAMS. Most deaths in piglets due to colibacillosis usually occur within 72 h after birth. Since there is little transplacental transfer of antibody in swine (22), protective antibody during this critical period would be expected to be acquired via the colostrum. Antitoxic IgG and toxin-neutralizing antibody in the colostrum of immunized dams were followed during the first week postpartum (Fig. 2). Antitoxic IgG titers rapidly dropped during the first 3 days postpartum, reaching levels of <5% of those at day 0 (Fig. 2a, c, and e). Toxin-neutralizing antibody also decreased during this period, but remained at about 20% of the titers found on day 0 (Fig. 2b, d, and f). In contrast to antitoxic IgG levels, which returned to base-line levels by day 6 (Fig. 2a), toxin-neutralizing titers remained elevated for up to 7 days postpartum (Fig. 2b). These results indicate that the majority of toxin-neutralizing antibody present in the colostrum after 3 days postpartum is not IgG but is probably of the IgA class. Base-line antitoxin titers in unimmunized dams were found to be <1% of the titers noted at day 0 (data not shown).

Prior studies (13) have shown that procholeragenoid preparations contain trace amounts of vibrio somatic antigen(s) which elicits a serum vibriocidal antibody response upon parenteral immunization. Vibriocidal antibody titers were determined for six colostrum samples obtained at day 0 (those in Fig. 2a and c). Four of the six colostrum samples had undetectable levels of vibriocidal antibody, whereas two had titers of 4 and 8, demonstrating that parenteral injection of procholeragenoid results in little, if any, colostral vibriocidal antibody.

Dams were also boosted at 3 days postpartum with 50 μg of procholeragenoid to determine whether a rapid anamnestic response could be elicited. Two routes of booster immunization were tried, intramuscularly and via the mammary gland. No significant rise in antitoxic IgG was observed with either immunization route (Fig. 2a, c, and e). Similarly, there was no significant rise in toxin-neutralizing titers, although dams immunized via the mammary gland tended to have higher toxin-neutralizing titers than those not boosted or boosted intramuscularly (Fig. 2b, d, and f). These results suggest that a further immunization on or about the day of birth would probably not result in enhanced protection.

PROTECTION OF PIGLETS AGAINST COLIBACILLOSIS BY IMMUNIZATION OF DAMS. The incidence of diarrhea and death in piglets born to immunized and nonimmunized dams is shown in Table 4. The results are the total from three independent field trials, each conducted at a different breeding facility. Greater than 70% of the control group had episodes of diarrhea. Furthermore, approximately 5% died due to severe E. coli-induced diarrhea. In the immunized population only 11% had diarrhea, and the death rate was reduced to <1% (85% protection rate; Table 4). Bacteriologic examination of the stools from piglets of both groups having diarrhea demonstrated large numbers of E. coli serotype O149, which were found to give a positive Y-1 assay result, indicating LT production. It should be noted that this serotype was the only enterotoxigenic E. coli isolated in all three field trials.

EFFECTS OF REIMMUNIZATION OF DAMS DURING THE FOLLOWING GESTATION PERIOD. The above results demonstrated that immunization of dams with procholeragenoid 5 and 2 weeks before delivery conferred a substantial degree of protection against colibacillosis in piglets which correlated with antitoxic production. It was of interest to determine what levels of antibody could be obtained in the following gestation period. To investigate this aspect, dams immunized during the previous gestation period were given a single dose of procholeragenoid intramuscularly approximately 2 weeks before expected second delivery. Base-line antitoxin titers in unimmunized controls were <2. With a single immunization during the second gestation period, antitoxic IgG and toxin-neutralizing titers (380 and 600, respectively [range, 160 to 880 and 200 to 1,000, respectively]) were comparable to those obtained during the first gestation period with two doses of procholeragenoid (250 and 725, respectively). The death rate in piglets due to colibacillosis was also comparable, 0.77% after the first delivery and 0.86% (one death out of 116 piglets) after the second delivery.

TABLE 2. Antitoxic IgG titers in immunized dams at day of parturition

<table>
<thead>
<tr>
<th>No. of dams</th>
<th>Dosage (μg)</th>
<th>Geometric mean antitoxic IgG titer (range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colostrum</td>
</tr>
<tr>
<td>13</td>
<td>100</td>
<td>440 (150–880)</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>250 (50–640)</td>
</tr>
</tbody>
</table>

* Determined by enzyme-linked immunosorbent assay.

TABLE 3. Toxin-neutralizing titers in immunized dams at day of parturition

<table>
<thead>
<tr>
<th>No. of dams</th>
<th>Dosage (μg)</th>
<th>Geometric mean toxin-neutralizing titer (range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colostrum</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>1,250 (800–1,600)</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>725 (200–1,600)</td>
</tr>
</tbody>
</table>

* Determined in Y-1 adrenal cell assay. Colostrum and serum were titrated against LT.
FIG. 2. Duration of antitoxic IgG and toxin-neutralizing antibody in the colostrum of dams after delivery. Groups of three dams were immunized intramuscularly with 50 μg of procholeragenoid 5 and 2 weeks before the expected date of delivery. Dams were later immunized either intramuscularly (I.M.) or via the mammary gland (M.G.) with 50 μg of procholeragenoid 3 days postpartum (arrows). Antibody titers (geometric mean with ranges) are expressed as percentage of titer present at day 0 (delivery date). Colostrum was titrated against CT to obtain neutralizing titers.

DISCUSSION

Previous studies on the pathogenesis of E. coli infections in neonatal piglets have established that the majority of disease-causing strains possess two virulence factors: pili antigens, which allow for colonization of the small intestine, and the ability to produce enterotoxins (24, 26, 38). Although the routine use of antibiotics, either prophylactically or in treatment of colibacillosis, has lowered the overall mortality rate, the occurrence of antibiotic-resistant strains and a still unacceptably high death rate have made this approach unattractive.
TABLE 4. Incidence of death and diarrhea in piglets due to colibacillosis in immunized and nonimmunized dams

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of dams</th>
<th>No. of piglets</th>
<th>No. of piglets with diarrhea (%)</th>
<th>No. of deaths (%)</th>
<th>Protection rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmunized</td>
<td>167</td>
<td>1,678</td>
<td>1,225 (73)</td>
<td>79 (4.7)</td>
<td>85</td>
</tr>
<tr>
<td>Immunized</td>
<td>62</td>
<td>653</td>
<td>74 (11)</td>
<td>5 (0.77)</td>
<td></td>
</tr>
</tbody>
</table>

* Dams were immunized intramuscularly with 50 μg of procholeragenoid at approximately 5 weeks and again at 2 weeks before delivery.

* Protection rate was determined by dividing the percentage of piglets in the immunized group succumbing to colibacillosis by the percentage of deaths in the unimmunized group. Piglets were observed for 21 days postpartum before compilation of data.

* All piglets in the nonimmunized group which presented with diarrhea were immediately treated with antibiotics. No piglets in the immunized group received antibiotics.

Most fatalities due to colibacillosis occur within the first week postpartum (10, 39). This factor has made vaccination of pregnant dams, with the accompanying transfer of passive immunity via the colostrum, an appropriate approach (2, 8, 10, 35). Many antigens have been used to immunize dams. The most promising results have come from studies in which pil antigens or enterotoxin preparations have been used as immunogens (8, 10, 22, 29, 30, 31, 36).

Rutter et al. (36) were able to evoke a high degree of protection against colibacillosis in piglets which were suckled by dams immunized with a partially purified K88 preparation. The authors concluded that the protection afforded was predominantly attributable to anti-K88 antibody. Morgan et al. (30) demonstrated that protection followed by vaccination of dams with purified pil antigens was afforded only if the challenge strain was homologous in pil type. These findings were extended by Isaacson et al. (22), who found that immunization with K99 or 987P pil antigens was not effective when the immunology was not related to the pil type. A given pil type may also possess several serological variants (16, 34). It has been postulated that such variances are the response of K88-bearing strains to mass vaccination with K88 antigen (16). The above results suggest that vaccination with pil antigen may not be an ideal approach for the prevention of colibacillosis due to the numerous immunologically distinct pil types and evidence of antigenic variation in response to vaccination.

Alternatively, vaccination of dams with LT was shown to provide substantial protection in neonatal piglets when challenged with either LT or a virulent strain of E. coli (8, 10). Protection against both types of challenge was attributed to the passive transfer of colostral antitoxin to piglets. CT has also been shown to be an effective immunogen, indicating that a solely antitoxin-based immunity is protective against colibacillosis (10).

The present study, based on the above findings (8, 10), used procholeragenoid as an immunogen due to its reduced toxicity compared with LT or CT (13, 27) and its previously established immunogenicity in animals and humans (13, 14). The immunogenicity of procholeragenoid was confirmed in swine, with 2 × 50-μg doses giving rise to high titers of antitoxic IgG and toxin-neutralizing antibody in the serum and colostrum. It was somewhat surprising to find that there was a rapid fall in the colostral titers of both antibody types after birth. However, although antitoxic IgG titers returned to base-line levels within 4 days postpartum, toxin-neutralizing titers remained elevated for up to 6 days after delivery. These results indicate that the majority of colostral toxin-neutralizing antibody after 2 days postpartum is not IgG, but in all likelihood is of the IgA class, as has been found when LT was used as an immunogen (8).

Piglets, suckled by dams vaccinated with procholeragenoid, were protected to an equivalent degree against both diarrhea and death (protection rate, 85%). Reimmunization of dams with procholeragenoid during the following gestation period elicited colostral antitoxin titers comparable to those observed during the primary immunization. The incidence of fatal E. coli-induced diarrhea was also comparable (0.77% deaths in the immunized population after the primary immunization versus 0.86% after immunization in the second gestation period). Although an appropriate control group was not available to document the absolute protection rate after reimmunization, that antitoxin titers comparable to those seen during the initial immunization were achieved and that the death rate was similar suggest that a good level of protection was afforded.

In the present study, protection against fatal E. coli-induced diarrhea in piglets can be attributed solely to the passive transfer of colostral antitoxin to neonatal piglets. These findings are in agreement with previous studies in which
antitoxic antibody (either anti-LT or anti-CT) was also found to confer a substantial degree of protection (8, 10).

The use of an enterotoxin-based vaccine in place of pili antigen(s) may circumvent the problem of multiple antigen types noted with pili. In cross-neutralization studies (8), LT prepared from different strains of E. coli were found to be immunologically related.

ACKNOWLEDGMENTS

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LITERATURE CITED