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Passive Protective Effect of Chicken Egg Yolk Immunoglobulins against Experimental Enterotoxigenic *Escherichia coli* Infection in Neonatal Piglets

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Passive protection of neonatal piglets against fatal enteric colibacillosis was achieved with powder preparations of specific antibodies against K88, K99, and 987P fimbrial adhesins of enterotoxigenic *Escherichia coli*. The antibody powders were obtained by spray drying the water-soluble protein fraction of egg yolks from immunized hens after the lipid components were precipitated with an aqueous dispersion of acrylic resins (Eudragit L30D-55; Rohm pharma). The anti-K88, -K99, and -987P antibody preparations reacted specifically against the corresponding fimbrial antigens in an enzyme-linked immunosorbent assay. The orally administered antibodies protected in a dose-dependent fashion against infection with each of the three homologous strains of *E. coli* in passive immunization trials with a colostrum-deprived piglet model of enterotoxigenic *E. coli* diarrhea. Scanning electron microscopy revealed adherence of enterotoxigenic *E. coli* in intestinal epithelial surfaces of control piglets, whereas in treated piglets treated with high-titer antibodies, a resistance to bacterial adhesion was observed. An enzyme immunoassay with avidin-biotin complex demonstrated specific local antibody activity in target areas of the small intestines. In vitro, *E. coli* K88$^+$, K99$^+$, and 987P$^+$ strains adhered equally to porcine duodenal and ileal epithelial cells but failed to do so in the presence of homologous anti-fimbrial antibodies. Absorption of egg yolk antibodies with fimbrial immunosorbent removed the anti-fimbrial antibody fraction and reduced significantly the protective nature of the antibody preparation in a passive immunization experiment, suggesting that anti-fimbrial antibodies were the active components.

Diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC) is by far the most common enteric colibacillosis encountered in neonatal piglets (22). Previous investigations indicated that colonization of the small intestine of the piglet by ETEC adhering to the epithelium accounts for most gastrointestinal disorders (1, 4, 5, 26). The fimbrial K88, K99, and 987P antigens of porcine ETEC that are associated with intestinal colonization have been extensively investigated with respect to their genetic background, protein chemistry, and immunological properties (7, 16, 21). They have been widely employed with promising results as vaccine antigens in controlling porcine colibacillosis. In passive immunization experiments, antibodies raised against these fimbrial antigens have been administered orally to piglets and have offered potential therapeutic value in controlling the disease.

Oral administration of antibodies derived from serum and colostrum and even with monoclonal antibodies has been very successful; however, it is prohibitively expensive to obtain the large amounts of antibodies required (18). Of particular veterinary interest is the use of chicken egg yolk antibodies for the treatment of porcine colibacillosis. Vaccination of laying hens provides a cheaper and good alternative antibody source; the eggs are collected after a high level of antibodies is reached in the egg yolk. This principle is not new; some authors have used chicken egg yolk antibodies in the prevention or control of rotaviral infection in mice and cats (3, 11), and their promising results have led to the suggestion that egg preparations might serve as a source of antiviral antibodies for humans (28). There has been no report so far on the use of chicken egg yolk antibodies in the prevention and control of enteric colibacillosis in swine, although the use of egg yolk antibodies in the treatment and prevention of diseases in other animals and even humans has been described. To our knowledge, the present study describes the first clinical use of spray-dried chicken egg yolk antibodies against ETEC infection in piglets.

A number of methods for extraction of antibodies from egg yolk have been described (2, 6, 10, 14, 23, 27). Traditionally, organic solvents were used to extract antibodies from egg yolk, but because bioactive proteins are denatured with these methods and because of the health hazards of solvents like chloroform used during production, alternative methods have been sought. Polson et al. (23) and Jensenius et al. (14) successfully used polyethylene glycol and sodium dextran sulfate, respectively, as protein precipitants in the isolation of pure immunoglobulin fractions from egg yolks, but their methods, like others, were time-consuming and the agents used have not been approved as food additives. We tried using an aqueous dispersion of acrylic resins to isolate the water-soluble proteins from egg yolk and to extract antibodies; the advantages of this method include the absence of toxic effects and compatibility of the aqueous medium with spray-dry processing.

The objective of the present study was to evaluate a crude chicken egg yolk immunoglobulin fraction as a treatment agent for experimentally induced ETEC diarrhea in colostrum-deprived piglets reared in an isolated environment.

MATERIALS AND METHODS

Animals. A total of 76 colostrum-deprived, newborn Large White pigs were utilized in protection trials with antibody

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powder preparations and absorbed antibody solutions. Five-month-old White Leghorn chickens (strain Hyline W36) were utilized for immunization, and New Zealand White rabbits (3 kg) and Japanese Black cattle (4 years old) were used for antiserum production.

Bacteria and cultivation conditions. ETEC strains 19304 (O157:K88ac:NM, LT^+), 431 (O101:K30:K99:NM, ST^+), and 987 (O9:K103:987P:NM, ST^+) were obtained from Salsbury Laboratories Inc. (Charles City, Iowa). $K88^+$ and $K99^+$ ETEC were cultured in 20 liters of minca broth (9), and $987P^+$ ETEC were cultured in Trypticase soy broth of equal volume. After incubation for 18 h at 37°C with shaking (200 rpm), the cells were harvested by centrifugation at 12,000 × *g* for 20 min. The cells were used for preparative extraction of fimbriae. $K88^+$ and $987P^+$ ETEC were suspended in 200 ml of sterile phosphate-buffered saline (PBS; pH 7.2) supplemented with 0.01% Tween 80. The suspensions were then homogenized by using a Polytron homogenizer (Kinematica, Luzern, Switzerland) for 30 min on an ice bath to detach pilus fractions from the bacterial cells. $K99^+$ ETEC was suspended in PBS, and the pilus fraction was detached from the bacterial cells by heating in a water bath at 60°C for 30 min with stirring. Then each suspension of treated bacteria was centrifuged as described above, and the supernatant was filtered through a 0.45-μm-pore-size membrane filter to remove any remaining whole cells. The pilus concentration in the crude extract was determined by using an enzyme-linked immunosorbent assay (ELISA).

A fraction of the crude extract was saved for pilus purification. The K88 and K99 fimbriae were further purified by affinity column chromatography by the method of Kuzuya et al. (19), and 987P pili were purified by the method of Isaacson and Richter (13) with the following slight modification of the crystallization procedure. After the crystallizing buffer was added to solubilizing buffer containing pili, the mixture was stirred for 30 min at room temperature and then allowed to stand for 48 h at 4°C. The purity of each pilus preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% acrylamide gels (20) with prestained standards (Bio-Rad Laboratories, Richmond, Calif.) and examined by using a transmission electron microscope (H-300; Hitachi, Tokyo, Japan) and negative staining (19). For protein measurement, a Bio-Rad protein assay system was used with bovine plasma albumin as the reference protein. The purified pili were used for hyperimmunization of chickens, cattle, and rabbits and for the ELISA.

Immunization with fimbrial vaccine. Each dose of crude fimbrial vaccine, containing 0.5 mg of pilus antigen in emulsion oil mixed with 5% Arlacel 80 (Maine Biological Laboratories), was injected intramuscularly in the breast muscle of a chicken. Six weeks after the initial injection, the animals were boosted in the same manner, and the eggs were harvested 2 weeks later.

Purified pilus antigen (1 mg/ml) in complete Freund adjuvant was injected into each chicken as described above on day 0; two consecutive booster immunizations were given on days 14 and 28 with same dose of antigen in incomplete Freund adjuvant. The anti-pilus agglutinating titers and ELISA optical density (OD) values of several egg yolk samples were monitored regularly for 10 weeks after the first immunization.

Serological methods. Six rabbits and six cattle were used to produce specific anti-fimbrial antisera by injection of 1 mg of purified fimbriae per ml in complete Freund adjuvant into each animal. The antigens were administered by multiple intradermal injections into the backs of animals. The responses to this immunization were monitored regularly over a period of 5 weeks by determining anti-K88, -K99, and -987P agglutination titers in microdilution plates.

ELISAs. (i) Fimbrial concentration. Microdilution plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, Va.) were coated with 100 μl of a 7-μg/ml solution of the first antibody (bovine anti-pilus immunoglobulin G [IgG]) in 0.05 M carbonate buffer (pH 9.6) per well at 4°C for 18 h. The plates were emptied and blocked with 150 μl of PBS containing 3% bovine serum albumin (PBS-BSA) per well at 37°C for 1 h and then washed with 0.02% Tween 20–saline (T-S) three times. Then 100 μl of twofold serial dilutions of purified K88, K99, or 987P pili in 0.05% Tween 20–PBS (T-PBS) per well, at initial protein values of 0.4, 1.0, and 4.0 μg/ml, respectively, were applied to appropriate wells as reference antigens. Dilutions of pilus extract specimens (same volume per well) were then added at 37°C for 1 h, and the plates were washed as described above. A second antibody (100 μl of rabbit anti-pilus IgG per well) diluted 1:2,000 in 0.05% T-PBS was applied at 37°C for 30 min. The amount of pilus antigens binding with antibodies was measured colorimetrically by incubating samples at 25°C for 30 min with 100 μl of goat anti-rabbit IgG conjugated with horseradish peroxidase (Cappel, Organon Teknika Co., Pa.) diluted 1:16,000 in 0.05% T-PBS and (after five further washes with T-S) with o-phenylenediamine dihydrochloride substrate solution per well. The reaction was stopped with 3 N H_2SO_4, and the ELISA OD at 492 nm was determined in a microdilution plate reader (MR650; Dynatech).

(ii) Titer determination. Microdilution plates were coated overnight at 4°C with purified pilus antigen solution (at final concentrations of 5 μg/ml for K88 and K99 and 25 μg/ml for 987P). The plates were blocked with PBS-BSA as described above, and subsequent washings were done in the same manner. Sample antibody powders were reconstituted in PBS (1:10 dilution) to make working antibody solutions; 1:1,000 dilutions of antibody solutions were added, and the plates were incubated at 37°C for 1 h. Rabbit anti-chicken IgG conjugated with horseradish peroxidase (Cappel) diluted 1:8,000 in 0.05% T-PBS was applied and incubated at 25°C for 30 min; then the *o*-phenylenediamine dihydrochloride substrate was added. The color reaction was inhibited by the addition of 3 N H_2SO_4, and the ELISA OD values were obtained as described above. Titers were determined by plotting the OD values against a standard curve formed by correlated ELISA OD values (1:1,000 dilutions) and agglutination titers of several chloroform-extracted egg yolk antibodies (6) obtained from chickens hyperimmunized with purified fimbrial antigens (Fig. 1).

(iii) Chicken IgG. The ELISA was performed as described above, except the plates were coated with goat anti-chicken IgG (Cappel) at a final concentration of 5 μg/ml. Chromatographically purified chicken IgG (Cappel) was used as the reference antibody. A 1:1,000 dilution of peroxidase-conjugated rabbit anti-chicken IgG (Cappel) was utilized to measure colorimetrically the anti-chicken IgG antibody activity with the *o*-phenylenediamine dihydrochloride substrate.

Separation of antibodies from chicken egg yolk. The yolk was carefully separated from the egg white and the yolk membrane and mixed with 4 volumes of distilled water. An aqueous dispersion of 30% Eudragit L30D-55 (Rohm pharma, Darmstadt, Germany) was added to the diluted egg yolk to make a 5% (vol/vol) mixture. After centrifugation at 12,000 × *g* for 20 min, the water-soluble fraction was removed and passed through a 0.45-μm-pore-size membrane

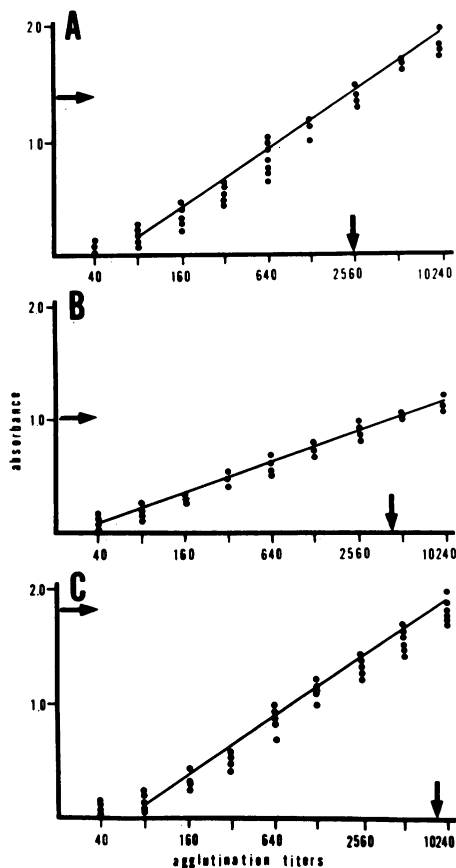

FIG. 1. Standard curve from ELISA OD on agglutination titers of egg yolk anti-purified pilus antibodies. The titers of the antibody solutions used corresponded to the following ODs: A, 1.44 for K88 (titer, 2,500); B, 1.03 for K99 (titer, 4,100); C, 1.842 for 987P (titer, 8,200).

filter to remove solid lipid materials and bacteria. The remaining lipid content of the fraction was determined by extracting with chloroform-methanol (3:1), evaporating to dryness, and weighing the dried lipid residues. For protein measurement, the Bio-Rad protein assay was used with bovine gamma globulin as the reference protein. In detecting egg yolk IgG, an indirect ELISA was performed with chromatographically purified chicken IgG as the reference protein. Egg yolk IgG was isolated and purified as described by Polson et al. (23) (Table 1).

For production of standard egg yolk antibody solutions, egg yolk antibodies were separated from chickens hyperimmunized with purified fimbrial antigens by chloroform extraction (6); the anti-K88, -K99, and -987P agglutination titers were determined in microdilution plates. Dilutions (1:1,000) of chloroform-extracted egg yolk antibodies were assayed by ELISA, and the OD values were plotted against their corresponding agglutination titers.

TABLE 1. Purification of IgG from egg yolk

Prepn	Total amt (mg) of:			
	Protein	Lipid	IgG	Purity of IgG (%)
Egg yolk[a]	1,240 (100)[b]	3,470 (100)	65 (100)	5.2
Supernatant	323 (26.0)	9 (0.3)	40 (61.5)	12.4
Polson method[c]	16 (1.3)	0 (0.0)	15 (23.1)	93.8

[a] A 10-g egg yolk was used.
[b] Numbers within parentheses indicate the mean yield count as a percentage of that of the original volume.
[c] Precipitation by polyethylene glycol (22).

TABLE 2. K88 antibody products with the spray dry method at different temperatures

Prepn and air inlet temp (°C)	Air outlet temp (°C)	Wt of powder[a] (g)	Moisture content (%)	Antibody titer[b] by ELISA
Supernatant			99.1	250
Spray dry				
140	72	170	4.0	2,500
150	76	170	3.7	2,580
155	79	165	3.2	2,510
160	82	165	3.3	2,540
165	85	155	2.9	2,360
170	88	150	2.4	1,980
Freeze-dry		19	0.7	2,590

[a] The amounts of supernatant used were 20 kg for the spray dry method and 2 kg for the freeze-dry method.
[b] Antibody titer of antibody solution (1:10 dilution of antibody powder).

Production of antibody powders by spray drying. The water-soluble protein fraction of egg yolk was used in a protection experiment after it was assayed for immunoglobulin content and converted to powder by using a spray dryer (model L-12; Ohkawara kakohki, Kanagawa, Japan) at an air inlet temperature of 140°C and an air outlet temperature of 72°C. The material was introduced into a feeder at the rate of 5 liters per h with a pump and then sprayed in rotary fashion at a high speed (22,000 rpm) in the application zone, where it was mixed with temperature-controlled air. At the bottom of the dryer, the dried material was transported by a flow of lower-temperature air to the collection vat. Several temperature settings were tried to evaluate effects on antibody potency, moisture content, and consistency of the antibody products; the results were compared with those with an antibody powder obtained by freeze-drying (Labconco LL-12, Labconco Corp.) (Table 2). The antibody activities against the three fimbrial antigens of *E. coli* were analyzed by using the ELISA, and the moisture contents of the powders were determined by the Karl Fischer volumetric titration method with moisture analyzer MKS-3p (Kyoto Electronics, Tokyo, Japan).

Absorption of anti-fimbrial antibodies. A 500-mg sample of purified pili was linked to 100 g of Formyl-Cellulofine (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) in 0.7% sodium cyanoborohydride as recommended by Seikagaku kogyo Co. The pilus-linked gels were packed into a column (2.5 by 50 cm) and washed with 0.2 M PBS (pH 7.2). Then 150 ml of a 1:100 dilution of the antibody powder was applied to the column. The column was washed with 10 column volumes of starting buffer to remove unbound substances before elution with 0.2 M glycine–HCl buffer (pH 2.5). Unbound substances were pooled and concentrated 10-fold and are referred to as the absorbed yolk antibodies (Table 3). Their anti-O agglutinating titers were determined as described by Gross and Rowe (8), with a slight modification. The agglutinating titers in microdilution plates were obtained by using O-antigen suspensions prepared from vaccine strains by growing overnight broth cultures at 37°C and then heating them for 1 h at 100°C.

TABLE 3. Absorbed antibody preparation

Antibody	Pilus titer by ELISA[b]	O-antigen titer by agglutination
K88 solution[a]	2,500	8
After absorption[c]	30	8
K99 solution	4,100	16
After absorption	50	16
987P solution	8,200	8
After absorption	100	8

[a] A 1:10 dilution of antibody powder was used.
[b] A 1:1,000 dilution of antibody solution was used for the ELISA.
[c] Absorption of anti-fimbrial antibodies by immunosorbent.

Infection procedure and antibody treatment. Large White piglets were collected at birth, deprived of colostrum, and infected 4 h after birth. Trial 1 consisted of 32 piglets challenged with $K88^+$ ETEC at dose of 10^{12} CFU of viable organisms per piglet, trial 2 consisted of 20 piglets infected with $K99^+$ ETEC at the same challenge dose, and trial 3 consisted of 24 piglets receiving 10^{10} CFU of viable $987P^+$ ETEC each. The bacterial inoculum was delivered orally to each piglet with a 5-ml syringe attached to a length of silicone tube that was held in place in the piglet's oral cavity. Before infection with ETEC was started, the piglets were randomly distributed into three antibody powder treatment groups and one control group. At the onset of diarrhea, piglets in treatment groups 1, 2, and 3 were treated with antibody solutions specific against the challenge bacteria at titers of 156, 625, and 2,500, respectively, whereas control groups received placebo treatment. Antibody solutions were prepared from K88-, K99-, and 987P-specific antibody powders of known antibody activity. Another group of piglets designated as treatment group 4 were treated with absorbed egg yolk antibody preparations with highly reduced anti-fimbrial immunoglobulin. Treated piglets each received 4 ml of an antibody solution of a given titer and with specificity against the infecting strain of ETEC three times a day for 3 consecutive days after the occurrence of diarrhea. The clinical response of each piglet was noted throughout the experiment in terms of fecal consistency score, weight loss, enumeration of infecting strains from rectal swabs, and mortality rate. Fecal scoring was based on the following index used by Sherman et al. (25): 0, normal (i.e., feces firm and well formed); 1, soft consistency (i.e., feces soft and formed); 2, mild diarrhea (i.e., fluid feces, usually yellowish); 3, severe diarrhea (i.e., feces watery and projectile). The degree of colonization of the small intestine (duodenum, jejunum, and ileum) with ETEC strains in piglets that died with the diarrheal disease and in surviving piglets that were sacrificed on day 6 of the experiment period was evaluated by culturing intestinal swabs taken at the time of necropsy. Sections of small intestines were also taken at necropsy from a representative number of surviving piglets in the different antibody treatment groups and from control piglets for histopathological studies. Experimental animals subsisted on Borden SPF-LAC formula milk given three times daily throughout the trial.

Isolation and identification of ETEC. The procedure of Rutter and Anderson (24), with slight modifications, was followed. Rectal swabs were taken twice a day for 6 days after infection. Enumeration of the ETEC strain in swab was done by culturing on desoxycholate-hydrogen sulfide-lactose agar (DHL agar; Eiken Chemical Co., Ltd., Tokyo, Japan) plates and Trypticase soy agar with 5% defibrinated sheep blood and counting the number of colonies produced by enterotoxigenic strain as detected by a slide anti-pilus agglutination test in a total of 100 colonies. A piglet was considered to be excreting ETEC if the organism predominated in the culture.

Isolation of piglet small intestine epithelial cells. Isolated small intestine epithelial cells were prepared from newborn, colostrum-deprived piglets by a modification of the method of Knutton et al. (17). Piglets were sacrificed, and the entire small intestines were immediately exposed and removed. Segments (25 cm) of the duodenum and ileum were excised from the small intestines and processed individually. The intestinal contents were washed away from the intestine by repeated flushing with PBS. The distal end of the intestine was tied with a suture, and the intestine was filled with EDTA buffer solution (8 mM KH_2PO_4, 6.5 mM KCl, 10 mM EDTA [pH 6.8]). The proximal end of the intestine was secured with a knot, and the fully distended intestine was immersed in PBS and incubated at 37°C for 5 min with shaking. After the incubation, the intestine was gently massaged by pressing between the fingers to facilitate the exfoliation of epithelial cells, which were then collected by pouring the intestinal contents into a centrifuge tube. The intestinal segment was longitudinally split open, and the mucosal surface was gently scraped with a microscope slide to remove the remaining epithelial cells; the cell mass was added to the previous collection. Clumps of cells were disrupted and dispersed into single-cell suspensions by repeated passage through a pipette. Epithelial cells were then collected by centrifugation at 100 × *g* for 10 min at 4°C, washed once with Hanks balanced salt solution (HBSS), and centrifuged again. The concentration of the epithelial cells was determined by evaluation with a microscope, and the cells were immediately used in the binding assay.

In vitro adhesion assay. Piglet small intestine epithelial cells (1 ml; 10^6 cells per ml) were added to 1 ml of *E. coli* (10^8 CFU/ml) and incubated at 37°C for 30 min. Nonadherent bacteria were removed by centrifugation at 100 × *g* for 10 min for about four wash repetitions. The final cell pellet was suspended in 4 ml of HBSS, 1-ml aliquots of epithelial and bacterial cells were laid on a Lab-Tek chamber slide (Nunc, Inc.), and the cells were centrifuged as described above. The cells were fixed with ice-cold ethanol for 15 min and then stained with Giemsa for 30 min and examined under a light microscope (magnification, ×1,000). The number of bacteria attached to 25 epithelial cells was determined, and the average number of bacterial cells attached to a single epithelial cell was calculated. Only well-defined cells with visible brush borders were counted. This assay was repeated three times with each of the three piliated *E. coli* strains and with epithelial cells from either the duodenum or the ileum. Bacterial attachment to epithelial cells was inhibited with anti-fimbrial antibody preparations by preincubating the mixture of 1 ml of bacteria and 1 ml of antibody solution (titer, 2,500) for 15 min, adding 1 ml of epithelial cells, and then incubating at 37°C for 30 min.

Immunocytochemical ABC test. The small intestine of a piglet that survived infection with ETEC was examined. The tissues were fixed in 10% buffered Formalin and embedded in paraffin. Five-micrometer serial sections were cut, deparaffinized, and processed in alcohol as in routine processing. Sections were stained by the avidin-biotin complex (ABC) method (12) with rabbit anti-pilus (K88, K99, or 987P) IgG as the first antibody and horseradish peroxidase biotinylated goat anti-rabbit IgG as the second antibody (Vectastain ABC

TABLE 4. Clinical response of newborn piglets after challenge with ETEC K88$^+$, K99$^+$, and 987P$^+$ strains and treatment with antibody powder at various titers

Trial and strain	Antibody treatment (titer)	No. of piglets with diarrhea/total (FC score)[a] on day:			No. dead/ total
		1	3	5	
1. K88	0	7/7 (3.0)	4/4 (2.8)	1/1 (2.0)	6/7 (86)
	156	6/7 (2.6)	3/5 (1.6)	0/5 (0.4)	2/7 (29)
	625	5/7 (2.1)	0/7 (0.0)[b]	0/7 (0.0)[b]	0/7[b] (0)
	2,500	3/7 (1.3)[b]	0/7 (0.1)[b]	0/7 (0.0)[b]	0/7[b] (0)
	Absorbed	4/4 (3.0)	2/2 (3.0)	1/1 (2.0)	3/4 (75)
2. K99	0	4/4 (3.0)	0/0	0/0	4/4 (100)
	156	4/4 (3.0)	2/2 (3.0)	0/2 (0.0)	2/4 (50)
	625	4/4 (3.0)	4/4 (2.8)	1/4 (0.5)	0/4[c] (0)
	2,500	3/4 (1.5)	0/4 (0.5)	0/4 (0.0)	0/4[c] (0)
	Absorbed	4/4 (3.0)	1/1 (3.0)	0/0	4/4 (100)
3. 987P	0	5/5 (3.0)	1/1 (3.0)	1/1 (3.0)	4/5 (80)
	156	5/5 (3.0)	2/3 (2.0)	2/3 (1.3)	2/5 (40)
	625	5/5 (3.0)	0/5 (0.4)[c]	0/5 (0.0)[b]	0/5[c] (0)
	2,500	4/5 (2.0)[c]	0/5 (0.0)[b]	0/5 (0.0)[b]	0/5[c] (0)
	Absorbed	4/4 (3.0)	1/1 (3.0)	1/1 (3.0)	3/4 (75)

[a] The FC score is the mean fecal consistency score: 0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea.
[b] $P < 0.01$.
[c] $P < 0.05$.

kit; Vector Laboratories, Inc., Burlingame, Calif.) and poststained with hematoxylin stain.

Scanning electron microscopy. Tissues were fixed in Karnovsky fixative (15), which is composed of 1.6% paraformaldehyde and 1.7% glutaraldehyde in 0.08 M cacodylate buffer (pH 7.4), for 2 h at 4°C and then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 1 h. The tissue strips were dehydrated through a graded series of acetone, treated with isoamyl acetate, dried in a critical-point drying apparatus (JCPD-5; JEOL, Tokyo, Japan), coated with a gold layer 20 nm thick in an ion coater (IB-3; Eikoh Engineering, Tokyo, Japan), and examined with a scanning electron microscope (JSM-T300; JEOL).

Statistical analysis. The statistical significance of differences in mortality rates between the treated and control groups was assessed by using the Fischer exact test, and those of the fecal consistency score, bacterial count, and weight gain were assessed by using the variance *t* test.

RESULTS

Extraction of antibodies from egg yolk. Isolation of egg yolk immunoglobulin IgG by using a 30% aqueous dispersion of Eudragit was effective for the precipitation of yolk lipoprotein (Table 1). The lipid content in the supernatant after removal of precipitate was reduced to 0.3% of that of original egg yolk suspension. The IgG remaining in the supernatant (yield, 61.5%) was further purified by the method of Polson et al. (23). The purity of the IgG obtained was 93.8%. Because of lowered antibody yields after purification due to loss of antibodies during the process, the supernatants obtained after removal of lipoprotein with Eudragit were spray dried and used in the protection trials. The antibody titers of specific K88, K99, and 987P antibody solutions were 2,500, 4,100, and 8,200, respectively, and about 580 mg of powder was produced from one egg. No significant alterations in antibody potency and quality of powders were observed when several spray-drying temperatures were tried (Table 2).

Passive immunization of piglets with antibody powder. The clinical responses of piglets after challenge and treatment with antibodies at various titers are shown in Table 4. All piglets in the groups with antibody titers of 625 and 2,500 survived the infection, giving a 100% recovery rate from diarrhea caused by individual ETEC strains ($P < 0.05$). In each of the groups with the antibody titer of 156, two piglets died with the infection within the 3-day treatment period. Six of seven control piglets died with K88$^+$ ETEC infection, giving 86% mortality, and control piglets challenged with K99$^+$ and 987P$^+$ ETEC had mortality rates of 100 and 80%, respectively, at the end of the experiment.

All piglets had mild to severe diarrhea within 12 h after infection with ETEC strains. Treatment with antibodies with titers of 156 and 625 did not affect the incidence of diarrhea on day 1 after challenge with ETEC strains. There was no difference in the proportion of treated and control piglets that became diarrheic in any of the three trials, as judged from the mean fecal consistency scores of 2 or 3 (Table 4). However, there was significant difference in fecal consistency score between the control piglets and piglets treated with antibodies with a titer of 2,500 ($P < 0.01$). At the end of treatment period (day 3), there were significant differences in the incidence of diarrhea and fecal consistency scores between the treated and control piglets ($P < 0.01$). Our observation showed that piglets that received higher-titered antibodies had their fecal consistency returned to normal faster than did piglets that received lower-titered antibodies. In all piglets in the K88 and 987P antibody treatment groups treated with antibodies at titers of 625 and 2,500, the diarrhea ceased within 2 or 3 days after treatment. However, diarrhea persisted in one piglet in the K99 antibody group despite treatment with antibody with a titer of 625.

Excretion of ETEC strains in feces. Within 24 h of oral administration of bacterial suspension, ETEC was isolated in cultures from rectal swabs of all piglets (Table 5). Although there was no difference in the proportion of treated and control piglets excreting the ETEC strains on day 1 after challenge, the mean viable counts of organisms in cultures from rectal swabs of piglets treated with antibodies at a titer

TABLE 5. Rates of isolation of ETEC K88$^+$, K99$^+$, and 987P$^+$ strains from newborn piglets after challenge and treatment with antibody powder at various titers

Trial and strain	Antibody treatment (titer)	No. of rectal swabs positive/total (%) on day:		
		1	3	5
1. K88	0	7/7 (96)	4/4 (50)	1/1 (80)
	156	7/7 (79)	5/5 (43)	4/5 (34)
	625	7/7 (58)[a]	4/7 (14)	4/7 (14)
	2,500	4/7 (23)[a]	2/7 (1)[b]	0/7 (0)[a]
	Absorbed	4/4 (95)	2/2 (60)	1/1 (60)
2. K99	0	4/4 (80)	0/0	0/0
	156	4/4 (96)	2/2 (43)	0/2 (0)
	625	4/4 (81)	4/4 (25)	1/4 (1)
	2,500	3/4 (25)[a]	1/4 (3)	0/4 (0)
	Absorbed	4/4 (90)	1/1 (80)	0/0
3. 987P	0	5/5 (92)	1/1 (40)	1/1 (40)
	156	5/5 (95)	3/3 (47)	3/3 (25)
	625	5/5 (94)	3/5 (18)	0/5 (0)[a]
	2,500	5/5 (21)[a]	0/5 (0)[a]	0/5 (0)[a]
	Absorbed	4/4 (95)	1/1 (50)	1/1 (50)

[a] $P < 0.01$.
[b] $P < 0.05$.

of 2,500 were significantly lower ($P < 0.01$). As calculated from K88 and 987P antibody trials only, fewer piglets in the group receiving the antibody at a titer of 2,500, as compared with the control and other antibody titer groups, were excreting the ETEC strains at the end of treatment period (day 3). ETEC was not recovered from rectal swabs of piglets treated with high-titer (titer, 2,500) antibodies on day 5 postchallenge in all three antibody treatment groups. ETEC was also not isolated from rectal swabs of piglets challenged with 987P and treated with antibodies at a titer of 625. However, ETEC was persistently recovered from rectal swabs of piglets challenged with K88$^+$ and K99$^+$ ETEC and treated with antibodies at a titer of 625.

Enumeration of ETEC strains from small intestines. The ETEC K88$^+$, K99$^+$, and 987P$^+$ strains were recovered from all parts of the small intestine of dead piglets in the control group and in the groups treated with antibodies at a titer of 156 (Table 6); the exception was one dead control piglet in the 987P treatment group in which bacteria were not isolated from the duodenum. Only K88$^+$ and 987P$^+$ ETEC strains were recovered from the small intestines of surviving piglets in both groups. The K99$^+$ ETEC strain was not isolated from the small intestines of piglets that survived in the group treated with antibodies at a titer of 156. The counts of the 987P$^+$ ETEC strain recovered from the jejunums and ileums of surviving piglets in the control group and the group treated with antibodies at a titer of 156 were lower than those from piglets that died in these groups. Piglets treated with antibodies at titers of 625 and 2,500 in the three antibody trials successfully cleared the pathogenic bacteria from the alimentary tract. In light of these observations, the mortalities obtained from the protection experiment were generally encountered in piglets whose alimentary tract, including the anterior, middle, and posterior small intestine, had been extensively populated with the enteropathogenic bacteria.

In vitro adhesion to piglet small intestine epithelial cells. K88, K99, and 987P ETEC strains adhered to isolated porcine small intestinal epithelial cells from either the duodenum or the ileum. The average numbers of adherent bacterial cells per epithelial cell were 18.5 for strain K88, 13.5 for strain K99, and 11.7 for strain 987P. The piliated *E. coli* strains demonstrated the same degree of attachment to either duodenal or ileal epithelial cells, suggesting that no difference in epithelial cell receptor activities exists between the two. However, the attachment of bacterial cells to both duodenal and ileal epithelial cells was strongly inhibited when homologous anti-fimbrial antibody solutions were used in the in vitro adhesion assay (Table 7).

Figure 2 shows the in vitro adhesion of the 987P$^+$ ETEC strain to ileum epithelial cells from a piglet. The control epithelial cells (without pretreatment with antibody) and epithelial cells preincubated with K88 and K99 antibodies showed adherent bacteria on their surfaces. Epithelial cells preincubated with 987P antibody and challenged with 987P *E. coli* showed minimal bacterial adhesion on their surfaces.

Detection by the ABC method of bacterial adherence in small intestines of survived piglets. In K88 and 987P trials, piglets that were treated with lower-titer antibodies had

TABLE 6. Rates of isolation of K88$^+$, K99$^+$, and 987P$^+$ strains of ETEC from the small intestines of piglets

Trial and strain	Antibody treatment (titer)	No. of positive samples/total (%) from piglets that:					
		Died			Survived		
		Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
1. K88	0	6/6 (92)	6/6 (91)	6/6 (85)	1/1 (95)	1/1 (95)	1/1 (90)
	156	2/2 (100)	2/2 (98)	2/2 (98)	5/5 (81)	5/5 (73)	5/5 (70)
	625	0	0	0	1/7 (14)	1/7 (14)	3/7 (16)
	2,500	0	0	0	0/7 (0)	0/7 (0)	0/7 (0)
	Absorbed	3/3 (100)	3/3 (90)	3/3 (90)	1/1 (95)	1/1 (90)	1/1 (90)
2. K99	0	4/4 (54)	4/4 (81)	4/4 (83)	0	0	0
	156	2/2 (88)	2/2 (98)	2/2 (100)	0/2 (0)	0/2 (0)	0/2 (0)
	625	0	0	0	0/4 (0)	0/4 (0)	0/4 (0)
	2,500	0	0	0	0/4 (0)	0/4 (0)	0/4 (0)
	Absorbed	4/4 (60)	4/4 (100)	4/4 (100)	0	0	0
3. 987P	0	3/4 (30)	4/4 (100)	4/4 (100)	1/1 (20)	1/1 (80)	1/1 (95)
	156	2/2 (15)	2/2 (70)	2/2 (100)	0/3 (0)	2/3 (42)	3/3 (75)
	625	0	0	0	0/5 (0)	0/5 (0)	2/5 (16)
	2,500	0	0	0	0/5 (0)	0/5 (0)	0/5 (0)
	Absorbed	3/3 (40)	3/3 (100)	3/3 (100)	1/1 (10)	1/1 (90)	1/1 (95)

TABLE 7. In vitro adhesion of ETEC to piglet small intestinal epithelial cells

Challenge strain (pilus type)	Preincubation (antibody type)	No. of bacterial cells attached per epithelial cell[b]
19304 (K88)	Control[a]	18.5 ± 11.5
	K88	2.3 ± 1.3
	K99	17.1 ± 10.1
	987P	18.2 ± 12.2
431 (K99)	Control	13.5 ± 12.5
	K88	11.2 ± 10.2
	K99	2.4 ± 1.4
	987P	15.2 ± 10.2
987 (987P)	Control	11.7 ± 10.3
	K88	9.4 ± 7.4
	K99	12.8 ± 6.8
	987P	2.3 ± 1.3

[a] Control without preincubation with antibody.
[b] Average bacterial count from 25 well-defined epithelial cells.

TABLE 8. Detection with the ABC method of adhered $K88^+$, $K99^+$, and $987P^+$ bacteria in small intestines of surviving piglets

Strain	Antibody treatment (titer)	No. of positive samples/total		
		Duodenum	Jejunum	Ileum
K88	0	1/1	1/1	1/1
	156	5/5	5/5	5/5
	625	1/7	1/7	3/7
	2,500	0/7	0/7	0/7
	Absorbed	1/1	1/1	1/1
K99	0	0	0	0
	156	0/2	0/2	0/2
	625	0/4	0/4	0/4
	2,500	0/4	0/4	0/4
	Absorbed	0	0	0
987P	0	1/1	1/1	1/1
	156	0/3	2/3	3/3
	625	0/5	0/5	2/5
	2,500	0/5	0/5	0/5
	Absorbed	1/1	1/1	1/1

some intestinal colonization of ETEC bacteria in the three parts of small intestine examined. However, in the K99 trial, none of the antibody treatment levels yielded positive results by ABC method, suggesting that the infecting strain had been effectively passed out of the intestinal tract. All surviving control piglets and those treated with absorbed antibody preparation showed strong bacterial adhesion with this method (Table 8).

Figure 3 shows the ileum of a piglet infected with the $987P^+$ ETEC strain as examined by the ABC method. Ileal surfaces from control piglets, piglets treated with antibodies at a titer of 156, and piglets treated with absorbed antibody preparation were characterized by a dark surface coating upon staining by the ABC method, indicating the presence of adherent ETEC organisms. Intestinal sections from piglets treated with antibodies at titers of 625 and 2,500 had clear epithelial surfaces.

Figure 4 shows a scanning electron micrograph of ileal surfaces of antibody-treated and control piglets infected with the $987P^+$ ETEC strain. Intestinal sections from piglets treated with antibodies at titers of 625 and 2,500 revealed no adherent ETEC organisms along the intestinal epithelial surface, whereas adhering organisms were present along the entire length of villi in the small intestines of piglets in the control group, the group treated with antibody at a titer of 156, and the group treated with absorbed antibody.

Passive immunization of piglets with absorbed antibody. The results described above indicate that the antibody powder was effective. Because this preparation contained both anti-fimbrial antibodies and small amounts of contaminating antibodies with different specificities, e.g., anti-lipopolysaccharide antibodies (Table 3), it was not clear which was the protective component. This was studied later by testing the antibody preparation after absorption of the anti-fimbrial antibodies. The absorbed antibody preparation without the anti-fimbrial antibodies was inoculated into piglets in a passive immunization experiment (Tables 4, 5, 6, and 8). The protective capacity was poor, and mortality rates were from 75 to 100% at the end of the experiment, although the amounts of anti-O antibodies in the absorbed preparations were the same as that of the unabsorbed antibody powder.

DISCUSSION

The data presented herein show that antibodies prepared from the yolks of eggs from hens immunized with fimbrial antigens of ETEC are protective in piglets against challenge

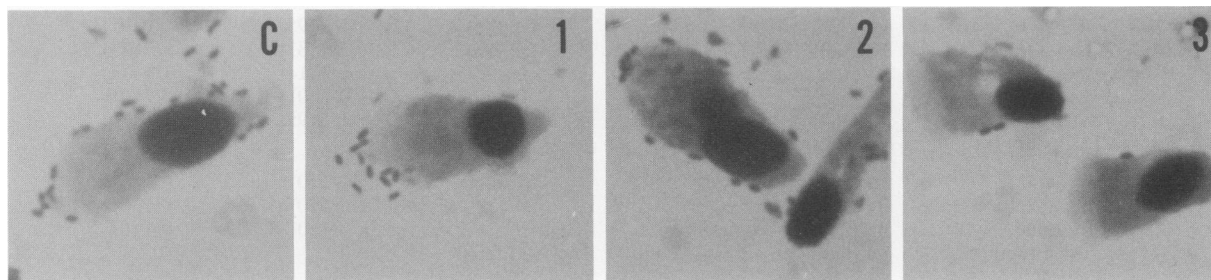

FIG. 2. In vitro adhesion of the $987P^+$ GL-148 strain to epithelial cells preincubated with antibodies and control ileal epithelial cells of piglet small intestines stained with Giemsa. Panels: C, control epithelial cells without antibody pretreatment showing rod-shaped adherent bacteria on the cell surface; 1 and 2, epithelial cells preincubated with K88 and K99 antibodies against $987P^+$ *E. coli* challenge showing similar adherent bacteria on the cell surface; 3, epithelial cells preincubated with homologous antibody against 987P challenge showing fewer adherent bacteria on the surface. Magnification, ×1,600.

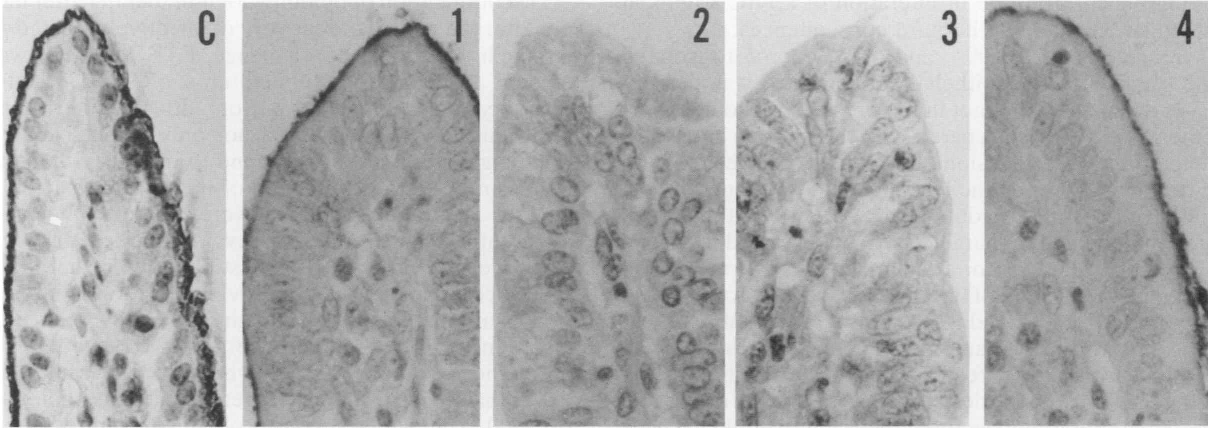

FIG. 3. Localization of $987P^+$ GL-148 strain in the small intestines of antibody-treated and control piglets by ABC method. Panels: C, villus from the ileum of a control piglet, indicating adhering bacteria on epithelial surface as a dark surface coating; 1, villus from the ileum of a piglet treated with antibody at a titer of 156, showing a moderate dark coating on the surface; 2, clear villus surface of a piglet treated with antibody at a titer of 625; 3, villus from the ileum of a piglet treated with antibody at a titer of 2,500, with no bacteria on the epithelial surface; 4, villus from the ileum of a piglet treated with the absorbed antibody preparation, indicating adherent bacteria on the surface. Magnification, ×480.

with homologous ETEC strains. The different therapeutic regimens with various titers of anti-K88, -K99, and -987P antibodies reduced considerably the severity of diarrhea in piglets receiving high-titer antibodies. Piglets treated with antibodies at titers of 625 or 2,500 had 100% survival after challenge with ETEC strains, whereas piglets in the groups treated with antibodies at a titer of 156 had mortality rates of 29 to 50%. When these differences in acquired resistance are taken into account, the optimum protection from mortality of piglets was achieved with oral administration of higher-titer antibodies (titers of 625 and 2,500) against all three challenge strains of *E. coli*. The difference in protection between the antibody-treated and control piglets was statistically significant ($P < 0.01$). Although treatments with homologous antibodies against all three challenge strains of *E. coli* at titers of 625 and 2,500 conferred protection from death, the efficacy achieved with antibodies at a titer of 2,500 proved superior in reducing the morbidity and severity of the disease. Piglets that received antibodies at a titer of 2,500 did not manifest severe watery diarrhea on day 1 after challenge, as did piglets that received other treatments. Moreover, the diarrhea was of shorter duration, and piglets in this group had recovered better body weight gain at the end of experiment (data not shown). The enterotoxigenic strains enumerated in cultures from rectal swabs of piglets treated with antibodies at a titer of 2,500 represented not more than 25% (mean bacterial count) of the aerobic bacterial flora, which is much lower than the percentages observed with piglets that received antibodies at titers of 625 and 156. Also, the degree of intestinal colonization was significantly reduced in piglets treated with this antibody level; ETEC strains were not detected in anterior duodenum, jejunum, and ileum samples taken at necropsy. On the basis of these findings, we consider the antibody titer of 2,500 as the most efficacious in

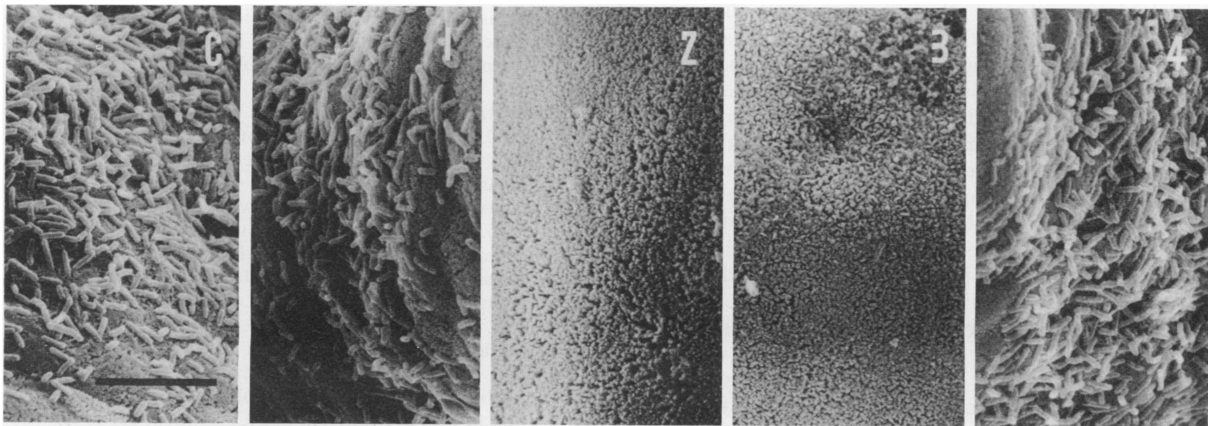

FIG. 4. Demonstration of bacterial adhesion by scanning electron microscopy of the $987P^+$ GL-148 strain in small intestines of antibody-treated and control piglets. Panels: C, villus from the ileum of a control piglet showing the rod-shaped morphology of adherent bacteria on the microvillous surface; 1, villus from the ileum of a piglet with antibody at a titer of 156, showing similar adherent bacteria on the microvillous surface; 2, villus from the ileum of a piglet treated with antibody at a titer of 625; 3, microvillous surface of a villus from the ileum of a piglet treated with antibody at a titer of 2,500, showing the absence of adherent rod shaped bacteria; 4, ETEC-laden villus surface of an ileum from a piglet treated with absorbed antibody preparation. Scanning electron microscopy was used. Bar, 10 μm.

protecting piglets from the proliferation of ETEC strains in the intestinal tract and its harmful consequences.

Adherence of K88$^+$, K99$^+$, and 987P$^+$ *E. coli* to isolated porcine small intestine epithelial cells in vitro was demonstrated in the absence but not in the presence of homologous anti-fimbrial antibodies prepared with the fimbrial antigen of each bacterium. The adhesion of *E. coli* was associated with specific attachment to epithelial cell surface receptors by the fimbrial structures. Both duodenal and ileal epithelial cell surfaces shared similar characteristics with regard to *E. coli* pilus receptors, allowing for attachment of the three piliated *E. coli* strains. Inhibition of bacterial attachment to epithelial cells by neutralizing the adhesive property of fimbriae with homologous anti-fimbrial antibodies demonstrates the specificity of the antibodies directed against ETEC pili in this in vitro system. It also provides evidence in support of the role of fimbriae in promoting bacterial adhesion to epithelial cells and the direct action of the antibodies against these adhesive structures. If the antibodies had no specific effect, no inhibition of bacterial attachment would occur. More convincing evidence in support of the specificity of these antibodies comes from inhibition studies with heterologous antibodies. There was no cross-inhibition observed among the three types of antibody used. Neither the K88 antibody nor the K99 antibody acted as an inhibitory agent against 987P adherence to epithelial cells or vice versa.

The antibody preparation contained small amounts of antibodies directed at other cellular components of *E. coli*. Agglutinating anti-O activity was detected to a slight degree. To test whether anti-O antibodies were important for the protection in our experimental system, we compared the protective capacity of the antibody preparation before and after absorption, which reduced the anti-fimbrial titer to 1/100 of its preabsorption value and retained the anti-O antibody activity. Since the protective capacity was reduced significantly, we consider it unlikely that the anti-O antibodies in our antibody preparation were responsible for the protection in our study.

Comparison of the isolation rates of pathogenic *E. coli* from the small intestines of piglets that died with clinical signs of diarrhea and surviving piglets revealed a correlation between the degree of intestinal colonization and mortality. Swabs taken from the small intestines of piglets treated with antibodies at a titer of 2,500 at a time when shedding of bacteria had ceased and clinical signs had disappeared always yielded negative isolation results, whereas intestinal swabs taken from dead piglets almost always yielded high concentrations of the challenge strains of *E. coli*. Surviving piglets treated with antibodies at lower levels also had fewer ETEC isolated from the small intestine or none at all. Very occasionally, however, large numbers of challenge organisms were isolated from surviving piglets, or pure colonies were isolated. It seems clear from our observations that, given the colonization and establishment of pathogenic *E. coli*, the final outcome of infection is primarily determined by the susceptibility of individual piglets to virulence mechanisms elicited by the thriving bacterial population. In the same manner, the survival of a piglet is primarily dependent upon its ability to prevent the colonization and subsequent proliferation of pathogenic bacteria in the intestinal tract. Therefore, the therapeutic value of orally administered antibodies against experimental ETEC infection in neonatal piglets lies in their ability to effect blocking of colonization of enteropathogenic strains. To achieve this antibody effect, apparently, a high antibody activity in the target site after oral administration of antibodies is needed.

Discontinuation of immunoglobulin therapy after day 3 did not alter the course of recovery of effectively treated piglets, although excretion of challenge ETEC strains was still evident from rectal swab cultures of some piglets. The recovery of infecting strains from either rectal or intestinal swab cultures was observed not only in persistently diarrheic piglets that did not respond to the 3-day immunoglobulin therapy but also in some of the piglets that responded well to the therapy. There were instances when piglets apparently recovered from severe diarrhea and regained body weight and appetite but still excreted the bacteria in their feces; some of these surviving piglets still harbored the organisms in their small intestines. However, the rates of isolation of ETEC from piglets that received lower-titer antibodies and that persistently harbored these bacteria were lower than those from piglets treated with antibodies at titers of 2,500. The bacterial counts obtained from rectal cultures of surviving piglets after day 3 did not show an increasing trend, but we are still studying whether these bacteria could recolonize the intestinal tract and cause a recurrence of the disease and whether continued immunoglobulin therapy is necessary.

Since piglets, particularly those deprived of maternal colostrum, appear to be most susceptible to ETEC infection during the first day of life, the challenge and therapeutic regimens were initiated within 4 h after birth to evaluate the efficacy of oral application of antibodies in countering the enteropathogenicity of *E. coli*. We have demonstrated that neonatal piglets can be protected from infection with enteropathogenic *E. coli* by repeated oral administration of soluble yolk antibody powder. This result raises the possibility of wide application of chicken egg yolk antibodies in the treatment of enteric diseases caused by other pathogens in animals and humans; the antibody powder can be added to feed or formula or applied as a separate therapeutic agent.

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