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Immunization of Suckling Pigs Against Enteric Enterotoxigenic *Escherichia coli* Infection by Vaccinating Dams with Purified Pili

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Pregnant swine (gilts) were vaccinated parenterally with a suspension of purified pili from the porcine enterotoxigenic *Escherichia coli* strain 987 (O9:K103:NM). Gilts injected with placebo served as controls. Suckling pigs born to gilts in both groups were challenged intragastrically with virulent strain 987. The percentage of deaths, incidence and duration of diarrhea, numbers of *E. coli* in the ilea, and *E. coli* attachment to the villous epithelia were significantly less in suckling pigs of vaccinated gilts than in those of controls. These results are consistent with the hypothesis that pili of some enterotoxigenic *E. coli* facilitate adhesion to intestinal epithelia. Vaccination of dams with pili appears to be a means of immunizing against diarrheal disease caused by enterotoxigenic *E. coli* in suckling neonates. This work confirms the role of somatic pili as colonization and virulence factors and provides another example of safe and effective purified pilus vaccines.

The two classes of bacterial pili are both important in infectious diseases. Sex pili (2, 3, 4, 7) are organelles of drug-resistant plasmid transfer, and somatic pili (1-3, 5, 6, 8, 20, 21) are colonization and virulence factors. The piliated phases of somatically piliated bacteria have a group of properties, the piliated phase syndrome, which appears to adapt them for growth on vertebrate epithelial surfaces (C. C. Brinton, *Proceedings of the 13th Conference on Cholera*, in press; C. C. Brinton, J. Bryan, J. Dillon, N. Guerina, L. Jacobson, A. Labik, J. McMichael, S. Polen, K. Rogers, S. Lee, C. Tempalski, and S. To, *Proceedings of the Symposium on Immunobiology of Neisseria gonorrhoeae*, in press). Among the properties in the syndrome are phase variation, adhesion to vertebrate cells, surface translocation by twitching motility, enhanced growth in limiting oxygen, altered colonial growth, and reduced flagellar swarming rate. Brinton has shown that pilus functions are neutralizable in vitro by specific antibodies (1, 3). Under the assumption that the virulence determining properties of somatic pili can be neutralized by pilus immunization, the laboratory at the University of Pittsburgh is conducting a broad program to develop purified pilus vaccines for bacterial diseases. Three somatic pilus vaccines have been found to be safe and effective in experimentally infected hosts: (i) a *Neisseria gonorrhoeae* pilus vaccine for gonorrhea evaluated in human volunteers (C. C. Brinton, J. Bryan, J. Dillon, P. Fusco, N. Guerina, A. Labik, S. Lee, A. Levine, J. McMichael, S. Polen, C. Tempalski, S. To, and C. To, *Proceedings of the Symposium on Immunology of Neisseria gonorrhoeae*, in press); (ii) a *Pseudomonas aeruginosa* pilus vaccine for burn infections evaluated in experimentally burned mice (A. Levine and C. Brinton, manuscript in preparation); and (iii) the *Escherichia coli* pilus vaccine whose evaluation in newborn piglets is described in this report. In view of the availability of a suitable model for an *E. coli* diarrheal disease in piglets (16), C. C. Brinton proposed this collaborative investigation and trial of an *E. coli* pilus vaccine. An *E. coli* pilus vaccine for human diarrheal disease had been previously prepared from the human enterotoxigenic strain *E. coli* H10407/S3S for evaluation in human volunteers (Brinton, in press). Although this vaccine was not evaluated for efficacy because of the temporary unavailability of volunteers, the same methods used in its preparation were used to prepare the swine vaccine, and the dosages of pili used for swine immunization were determined from antigenic-

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ity data on the human *E. coli* pilus vaccine.

There are two major steps in the pathogenesis of acute diarrheal disease caused by enterotoxigenic *E. coli*. First, the enterotoxic *E. coli* must establish itself in the small intestine in high numbers (colonization) and, second, it must elaborate enterotoxin that causes the small intestine to secrete fluid and electrolytes. Several lines of evidence indicate that somatic pili on the porcine enterotoxigenic strain 987 facilitate adhesion to and colonization of pig small intestine (12, 14–16; P. Fusco, A. To, S. To, and C. C. Brinton, *Proceedings of the 13th Conference on Cholera*, in press; R. E. Isaacson, R. L. Morgan, H. W. Moon, and C. C. Brinton, *Proceedings of the 13th Conference on Cholera*, in press). The objective of the work reported here was to see if suckling pigs could be immunized against diarrheal disease caused by strain 987 by vaccinating their dams with pili purified from clone 987-5, the piliated phase of this strain.

MATERIALS AND METHODS

Swine. Seventeen gilts were bred when 7.5 months old. These and their offspring were the experimental animals.

***E. coli* strain.** *E. coli* 987 (O9:K103:NM) was isolated from the small intestine of a baby pig with enteric colibacillosis. This strain produces heat-stable but not heat-labile enterotoxin, colonizes porcine ilea with adhesion to villous epithelia, and causes profuse diarrhea in newborn pigs (10, 12, 15). The stock culture of this strain had only a small fraction of piliated cells. A relatively stable, well-piliated clone, designated 987-5, was isolated by identifying a colonial difference between the piliated and nonpiliated phases. This clone was used to grow the large quantities of pili necessary for vaccine preparation.

Preparation of vaccine and vaccination. The purification and properties of 987-5 pili are described in detail elsewhere (P. Fusco, C.-C. To, C.-M. To., and C. C. Brinton, manuscript in preparation). The basic procedure was that of Brinton (6). The purified product was shown by electron microscopy, by dark-field microscopy of purified crystals, and by agglutination in specific antisera to contain strain 987 pili. The product was homogeneous when tested by sodium dodecyl sulfate gel electrophoresis and UV absorption spectra. The purified pilus vaccine was administered in saline (0.85%) containing 0.05% formaldehyde. The placebo consisted of saline (0.85%) and formaldehyde (0.05%). The gilts weighed 122 to 160 kg during the last trimester of pregnancy, when nine were injected with the pilus vaccine (vaccinated group) and eight were injected with the placebo (control group). A 10-ml amount of vaccine (containing 5 mg of protein) or placebo was inoculated subcutaneously in the flank of each gilt 21 to 27 days and again 7 to 13 days before parturition, except that the first two gilts received 8 mg of pilus protein at the first injection. There were no visual or palpable local reactions, nor clinically apparent systemic toxic reactions to any of the vaccinations. The pilus vaccine and the placebo were coded so that people vaccinating the gilts and making clinical and laboratory observation on the pigs did not know which material contained the pili.

During parturition, the gilts were given 20 USP units of oxytocin intramuscularly, and 100 to 200 ml of colostrum was manually drawn from each gilt. Casein was precipitated from colostrum with the addition of renin and calcium chloride in a concentration sufficient to create a firm clot at 42°C. Colostral whey samples were filtered through 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.) and stored at −70°C.

Challenge. At birth, pigs were separated from the gilts for 2 to 6 h, until all were born. They were then weighed and returned to the gilts for 30 min and allowed to suckle. After this initial colostrum intake, each pig was inoculated intragastrically with strain 987. The stock inoculum was kept frozen at −70°C in 10% glycerol and contained 5.4×10^8 viable bacteria per ml. Immediately before the inoculation of each litter, one vial containing 1 ml of the frozen inocula was thawed, and 0.25 ml of it was diluted in 15 ml of cold Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.). One milliliter of this Trypticase soy broth dilution, containing about 9×10^6 viable bacteria, was added to an additional 10 ml of cold Trypticase soy broth and inoculated into each pig intragastrically via the stomach tube. This dose was chosen because preliminary experiments indicated it was an approximate 50% lethal dose.

Observations on pigs. Pigs were weighed individually, and their clinical status was recorded at challenge, at 16 h postchallenge, and again at 2, 3, 4, 5, and 6 days postchallenge. One pig from each litter was selected 16 h postchallenge and killed. The intent was to select the most severely affected pig from each litter. Thus, when weight loss occurred, the pig that lost the most weight was selected. When none of the pigs in a litter lost weight, the pig that gained the least weight was selected. A 10-cm segment of ileum was removed from each pig selected, and the degree of adhesion (association index) and the number of viable strain 987 bacteria were determined as described earlier (16), except that efforts were made to determine the association index even of those segments with $<10^8$ *E. coli*. All pigs that died during the experiment were examined postmortem, and only those with gross lesions compatible with enteric colibacillosis (13) were included in the results. Two pigs died of disease other than colibacillosis: one, from a vaccinated gilt, died of starvation; the other, from a nonvaccinated gilt, died of bleeding from the umbilical cord. Neither of these pigs had diarrhea.

Serology. The pilus antibody response of the dams to immunization with strain 987 pilus vaccine is described in detail in another paper in this series (Brinton, manuscript in preparation). Agglutination of piliated bacteria was used as the assay, and both serum and colostrum were tested. The method was standardized with antisera to purified pili. The preparation and use of absorbed antiserum to identify strain 987 pili on *E. coli* recovered from the pigs was as described pre-

viously (16). Colostrum from gilts was tested for antibodies to the O and polysaccharide K antigens of strain 987 by the tube agglutination method of Edwards and Ewing (11). The K−P− and K+P− mutants of strain 987 (12) were used as O9 and K103 antigens, respectively, in these tests.

RESULTS

Intestinal colonization, adhesion of bacteria to intestinal villi, death, and incidence and duration of diarrhea were significantly less, whereas weight gain was significantly more in the pigs from vaccinated gilts than in the pigs from the nonvaccinated gilts (control) (Table 1; Fig. 3).

Intestinal colonization. There were significantly fewer viable bacteria of strain 987 per 10 cm of ileum of pigs from the vaccinated group (10^6 to 10^9) than from the control group (10^6 to 10^{11}) (Table 1). Also, small, translucent, richly piliated (P^+) colonies of strain 987, agglutinable in antipilus serum, were present in a high proportion (38 to 99% of the total) of the strain 987 population recovered from the ilea of three pigs tested from the control group. In contrast, no P^+ colonies were found in ileal isolates of strain 987 from the three pigs of the vaccine group so tested.

Association index. The association index was used to express the degree of adhesion of the challenge strain to the ileal epithelium. In fluorescent antibody-stained ileal sections from pigs in the vaccine group, the challenge bacteria were either too few to identify or were predominantly in the luminal area without any tendency to adhere to the intestinal epithelia (Fig. 1). Thus, all pigs of this group had low association indexes (Table 1).

In contrast, four of eight pigs from the control group had high association indexes; that is, adherent bacteria of strain 987 covered the intestinal villi as a layer (Fig. 2; Table 1). The difference in association indexes between the two groups was significant ($P < 0.05$).

Death losses. None of the pigs in the vaccine group died of enteric colibacillosis, but 30% of the pigs in the control group died of colibacillosis during the first 6 days of life. Most deaths occurred on day 2 or 3.

Diarrhea. Sixteen hours postchallenge, 56% of the pigs from the vaccine group had diarrhea, whereas 72% of the pigs from the control group had diarrhea (Fig. 3). From that time, the number of pigs with diarrhea sharply decreased in the vaccine group, and all of these pigs were normal by 5 days postchallenge. In contrast, the number of pigs in the control group with diar-

TABLE 1. *Response of pigs, suckling vaccinated[a] or control gilts, to challenge with enterotoxigenic E. coli 987*

Group	Dam no.	16 h after challenge		Pigs 6 days after challenge		
		Log_{10} *E. coli*/10 c m of ileum	Association index	No. dead/total	No. with diarrhea/total	Wt gain (g/h)
Vaccinated	1	8.6	1.0	0/8	0/8	7.8
	2	8.1	1.7	0/7	0/7	8.9
	5	8.4	1.0	0/11	0/11	8.6
	6	8.9	1.0	0/8	0/8	6.5
	10	6.0	1.0	0/11	0/11	4.5
	11	7.9	1.0	0/6	0/6	5.0
	12	6.0	1.0	0/3	0/3	4.4
	15	6.0	2.5	0/7	0/7	7.2
	16	6.0	2.0	0/8	0/8	7.3
		7.3[b]	1.4[b]	0/69[c]	0/69[c]	6.7[b]
Control	3	8.7	1.0	0/7	0/7	8.8
	4	10.2	5.0	7/10	3/3	1.3
	7	9.8	1.0	1/6	3/5	6.3
	8	8.9	4.2	6/11	1/5	1.3
	9	10.8	5.0	1/3	2/2	0.3
	13	10.4	4.2	2/8	0/6	1.2
	14	8.2	1.0	0/7	2/7	5.8
	17	6.0	1.0	0/4	0/4	8.9
		9.1[b]	2.8[b]	17/56[c]	11/39[c]	4.2[b]
t Test		$P < 0.05$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.05$

[a] Vaccinated with purified pili of *E. coli* 987.
[b] Mean.
[c] Totals.

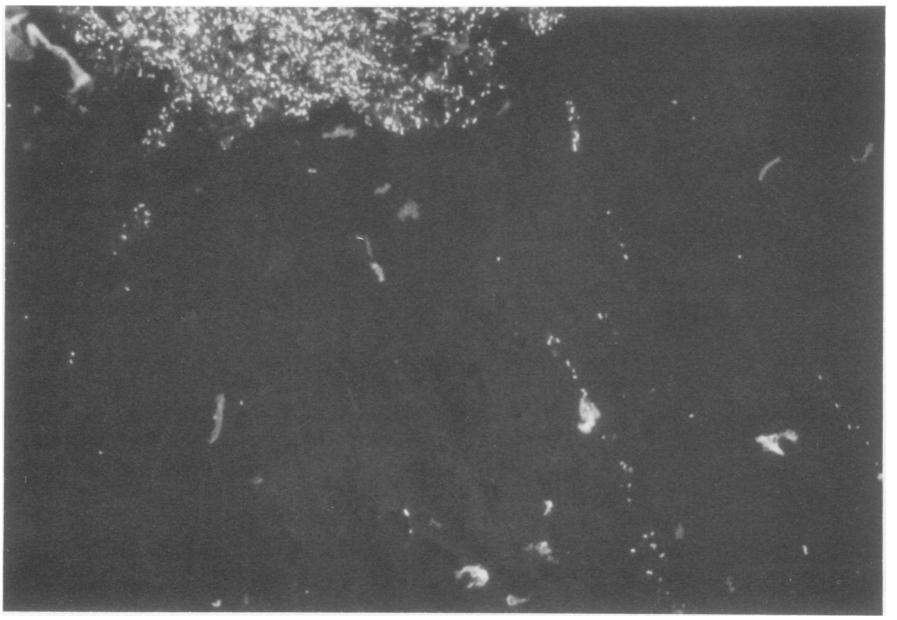

FIG. 1. *Ileum of a pig from a vaccinated gift. Large mass of E. coli 987 in the lumen, 16 h after challenge. Fluorescent antibody-stained frozen section; association index, 1.7. ×27.*

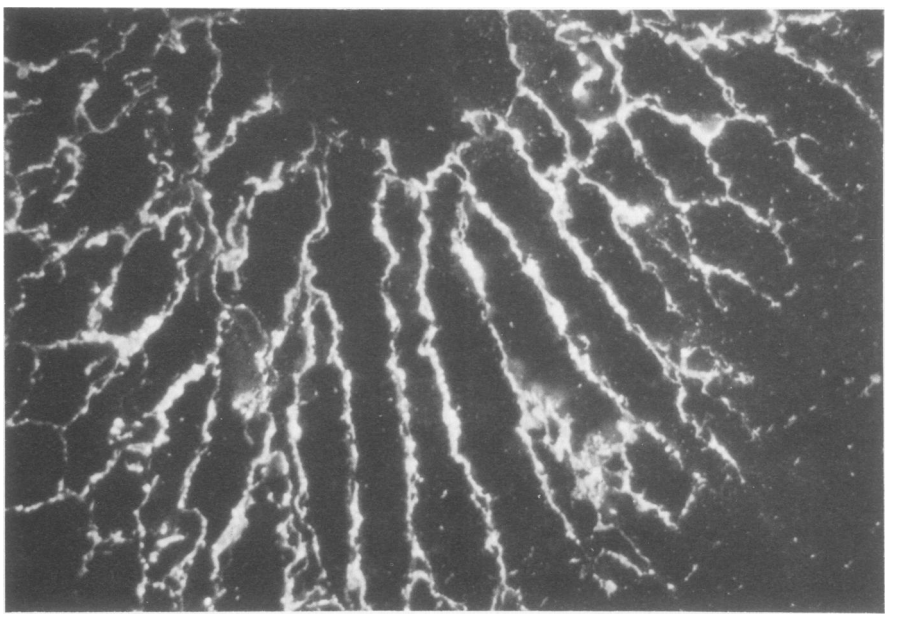

FIG. 2. *Ileal section of a pig from a control gift. The E. coli adhere to and uniformly cover the villi, 16 h after challenge. Fluorescent antibody-stained frozen section; association index, 5.0. ×27.*

rhea decreased only after day 3 postchallenge, and 28% of the surviving pigs still had diarrhea by day 6 (Table 1; Fig. 3). The most striking difference between vaccine and control groups developed at day 3 postchallenge.

Weight gain. The mean growth rate to 6 days of the litters from the vaccine group was 6.7 g/h but was significantly less (4.2 g/h; $P < 0.05$) for those in the control group which survived to 6 days.

Litter-related resistance. There was significant ($P < 0.005$) litter to litter variation in death

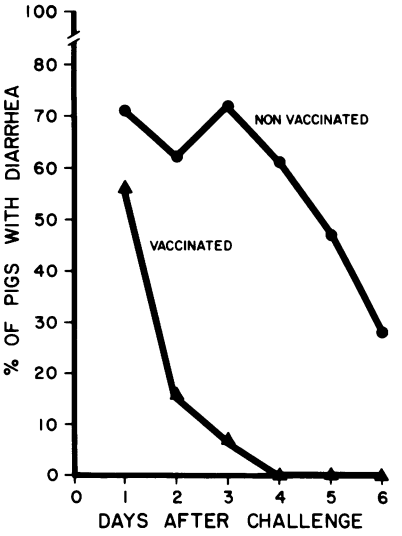

FIG. 3. *Percentage of pigs with diarrhea born to and suckled by vaccinated gilts compared with those of nonvaccinated gilts during the first 6 days of life.*

loss within the control group (Table 1). Four litters (those of dams 3, 7, 14, and 17) lost a total of one pig only, but the other four litters lost 16 pigs. At 16 h postexposure, pigs from dams 3, 7, 14, and 17 also had lower numbers of *E. coli* in their ilea and lower association indexes than pigs from the other litters in the control group (Table 1).

Antibodies in colostral whey. Colostrum from all gilts had antibody to the O9 antigen of strain 987. The titers of vaccinated (range, 1:80 to 1:640; mean, 1:258) and control gilts (range, 1:20 to 1:640; mean, 1:262) were similar. Antibodies to the K103 antigen of strain 987 were not detected in colostrum from any of the gilts even when undiluted whey was tested. Colostrum from vaccinated gilts had high antibody titers to 987 pili (range, 1:2560 to 1:10240; mean, 1:4836) in contrast to the low titers in colostrum from controls (range, 1:2 to 1:40; mean, 1:10).

DISCUSSION

The differences between the vaccine and control groups in the incidence of death and diarrhea, as well as in weight gain, indicate that vaccination of gilts with purified pili from *E. coli* 987 provided passive protection for their pigs against the challenge with the parent strain. The comparatively low association indexes and viable numbers of *E. coli* in the ilea of pigs from the vaccine group demonstrate that strain 987 did not attach or attain large numbers in the ilea of the protected pigs. The most likely explanation for the latter is that the protection was due to antipilus antibodies which probably prevented adhesion of strain 987 to the mucosa. Because pigs do not acquire passive immunity in utero, the antibodies were probably transmitted to the pigs via colostrum of the vaccinated gilts. The high proportion of P^+ (small, piliated) colonies in the ileal isolates of control pigs in contrast to those from the vaccinated pigs is also consistent with the above explanation. It is not likely that the vaccine contained some immunizing antigen(s) other than pili which accounted for protection in the vaccine group. The purified pilus preparation appeared to be homogeneous. Colostral antibody titers to O and polysaccharide K antigens of strain 987 were similar in vaccinated and control gilts. Strain 987 produces heat-stable but not heat-labile enterotoxin. Heat-stable enterotoxin is not antigenic, nor is it neutralized by antibody to heat-labile enterotoxin. Thus, anti-enterotoxic immunity was not likely, and protection appeared to be antibacterial (Table 1). Furthermore, in another experiment (Isaacson et al., in press), vaccination with pili (purified from strain 987 by the method used here) immunized against challenge with a different strain of enterotoxigenic *E. coli*. The latter strain also had *E. coli* 987 pili but had different O and polysaccharide K antigens than strain 987.

The litters of some control gilts were also relatively resistant to strain 987. This resistance could be the consequence of naturally acquired antibodies against this strain (9) or some other form of resistance to colonization (19). It is unlikely that the differences between pigs from vaccinated and control litters (Table 1) were the result of chance assignment of only resistant animals to the vaccine group. For example, if one-half of the 17 litters were naturally resistant, then the chance that nine of nine litters randomly assigned to the vaccine group would be resistant is 1 in 500. Furthermore, this experiment was recently replicated, with gilts from another herd, and again in that replication immunity was correlated with vaccination of dams (Isaacson et al., in press).

The data are consistent with the hypothesis that pili of strain 987 act as virulence factors by facilitating adhesion to intestinal epithelia. Pregnant gilts vaccinated with K99 antigen also protected their suckling pigs against challenge with $K99^+$ enterotoxigenic *E. coli* (Isaacson et al., in press), and those vaccinated with K88 antigen protected their suckling pigs against challenge with $K88^+$ enterotoxigenic *E. coli* (17, 18). Antigens K99 and K88 are also pili- or pilus-like. Vaccination of dams with pili appears to be a safe and effective method for immunizing against diarrheal disease caused by enterotoxigenic *E. coli* in suckling neonates.

These results provide additional confirmation of the validity of the direct approach to bacterial disease control with purified pilus vaccines (C. C. Brinton, in press). The pili of *E. coli* H10407/S3S belong to the type 1 pilus family, which includes pili of the laboratory strains *E. coli* K-12 and *E. coli* B as well as a rabbit diarrheogenic strain *E. coli* RDEC-1. The pili of the porcine strain *E. coli* 987 belong to a separate class unrelated or distantly related to the type 1 pilus family (P. Fusco et al., in press). An evaluation of an *E. coli* H10407/S3S pilus vaccine for human diarrheal diseases is planned as part of the program of the Pittsburgh laboratory.

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