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Escherichia coli-Associated Porcine Neonatal Diarrhea: Antibacterial Activities of Colostrum from Genetically Susceptible and Resistant Sows

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Antibacterial properties of colostrum from genetically resistant and susceptible sows in a herd in which only the susceptible sows had acquired natural immunity to K88-positive *Escherichia coli* have been investigated. Significant differences in antiadhesive and opsonic activities occurred. Colostrum from susceptible sows inhibited the binding of ^{125}I-labeled K88 antigen to brush borders significantly better than did the colostrum from resistant dams. Colostrum from susceptible dams effected more efficient in vitro opsonic phagocytosis and killing of K88 *E. coli* than did colostrum from resistant dams. Differences in bactericidal properties of colostrum between the two groups of pigs were not significant. Fractionation of colostrum from susceptible dams by gel filtration and ion-exchange chromatography revealed that the fractions rich in immunoglobulin M had the highest opsonic activity, whereas those containing predominantly immunoglobulins G and A were of lower activity.

Neonatal diarrhea is an important disease of pigs. Often the disease can be attributed to enterotoxigenic strains of *Escherichia coli*, many of which possess the surface adhesive antigen K88. Newborn piglets are devoid of protective antibody which is acquired passively from the maternal colostrum and milk.

The secretion of antibodies into milk and colostrum, which has been shown to be protective in experimental infections, may be enhanced by vaccination of sows with killed pathogenic *E. coli* strains either orally (11, 12, 18), parenterally (5, 20, 27, 28), or by both routes (7). The effects of using adhesive *E. coli* antigen preparations in vaccines either alone (15, 21) or combined with heat-killed bacteria (16) have also been investigated.

Further improvement in vaccines will be dependent upon an improved understanding of protective mechanisms; for this reason the class of antibody produced by vaccination has been studied (3), and correlations have been sought between antibody type and protection (7).

The aim of this study was to determine how colostral antibodies could combat bacterial colonization of the small intestine of pigs.

The herd used was one which had been genotyped and was of known genetic susceptibility with regard to infection by K88-positive *E. coli* (8). The susceptible pigs possess receptors for the K88 antigen on their intestinal epithelial cells enabling colonization of the surface of the intestine, whereas resistant pigs lack these receptors and there is only poor colonization. Resistance is inherited at a single locus as an autosomal recessive character inherited in a simple Mendelian way; thus, susceptible piglets can be born to resistant dams. In the diarrhea outbreak described previously (23), most cases of diarrhea occurred in this group of pigs. It was apparent that passive protection of the piglets was inadequate. In contrast, passive protection of piglets born to susceptible dams was adequate, and there was no diarrhea. Genetically resistant piglets were also unaffected since they do not require passive protection from K88-positive *E. coli* infection.

MATERIALS AND METHODS

Colostrum. Colostrum was collected within 6 h of parturition from sows, either genetically susceptible or resistant to K88-positive *E. coli*, which farrowed during a scours outbreak (23). The samples were pooled from as many teats as possible and centrifuged at 80,000 × *g* for 1 h at 4°C. The whey was collected and stored at −20°C.

***E. coli* strains.** The *E. coli* strain G205 [serotype O8:K87(B):K88ac(L)], which had naturally infected the piglets, was used in all experiments (23). Other strains used were W1 (Abbotstown) [serotype O149:K91(B), K88ac(L):H10], J2 (a K88$^-$ mutant of this strain), and 987P$^+$ [serotype O9:K103(A)], also isolated during the outbreak of diarrhea. Overnight broth cultures of the strains were centrifuged at 800 × *g* for 10 min. The bacteria were washed twice with sterile physiological saline and suspended to a final

concentration of 5×10^8 colony-forming units per ml.

PMNs. Polymorphonuclear leucocytes (PMNs) were prepared from the blood of 5- to 6-week-old genetically resistant pigs (4). The lysis of erythrocytes by the addition of sterile distilled water for 30 s was repeated after the initial sedimentation of the cells. The PMNs were incubated at 37°C for 1 h with gentle rolling on a rotary mixer to remove any cytophilic antibody. They were centrifuged at $120 \times g$ for 10 min, washed twice in 20 volumes of Hanks buffer (pH 6.8), and resuspended to a final concentration of 7.5×10^6 cells per ml.

Opsonic phagocytosis and bactericidal assays. Bacterial killing in the presence of colostrum, with or without PMNs, was performed by a modification of the method of Solberg (26). PMN suspension (0.2 ml) and 0.1 ml of bacterial suspension (diluted to give 5.0×10^6 colony-forming units per ml) were added to 0.3 ml of colostrum (diluted 1/10 in saline) in small plastic capped vials. The vials were mixed by rolling at 120 rpm for 2 h at 37°C. Serial 10-fold dilutions were made in saline, and 3 0.02-ml volumes of each dilution were dropped onto dried blood agar plates. Viable counts were estimated from the mean number of colonies per drop (13). In bactericidal experiments precolostral piglet serum was added instead of the PMN suspension.

Adhesion inhibition test. The ability of colostrum to inhibit the adhesion of K88 antigen to intestinal epithelial cell brush borders was performed by the method of Sellwood (24). Radioiodinated K88 antigen (10 μl) was mixed with 80 μl of phosphate-buffered saline (PBS) (pH 7.3) containing 0.1% (wt/vol) bovine serum albumin and 10 μl of colostrum (diluted 1/10 in PBS-bovine serum albumin) in small plastic centrifuge tubes and allowed to stand at room temperature for 1 h. Pig brush borders pretreated with Formalin (100 μl) were added (24), mixed thoroughly, and allowed to stand for 1 h. The tubes were centrifuged at $10,000 \times g$ for 30 s, the pellet was washed three times with PBS-bovine serum albumin, and the amount of bound ^{125}I-labeled K88 antigen determined.

Serological tests. Agglutination tests were performed in microtiter plates (20). Passive hemagglutination titers for the O8 antigen of *E. coli* strain G205 were determined in microtiter plates by using 3% sensitized sheep erythrocytes (25).

Fractionation of colostrum. Colostrum was collected from a homozygous dominant (susceptible) sow (8) immediately postpartum, and the whey was fractionated as follows. (i) Whey (5 ml) was dialyzed against 0.05 M PBS (pH 7.3) and applied to a column (1.6 by 90 cm) of Bio-Gel A 0.5 M (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with PBS at 4°C. Fractions (3 ml) were collected during elution with PBS at a flow rate of 10 ml/h. Each fraction was tested for the presence of opsonins by the in vitro phagocytosis assay. (ii) Whey (10 ml) was equilibrated with 0.01 M NaH_2PO_4 (pH 7.6) and applied to a column (2.5 by 50 cm) of DE52 Cellulose (Whatman, Maidstone, England). Stepwise elution of colostrum components with a series of phosphate buffers was employed (17). The eluted fractions were pooled, concentrated to 10 ml by pressure ultrafiltration through a PM 10 membrane having a cutoff point of 10,000 daltons (Amicon, High Wycombe, England), dialyzed against PBS, and assayed for opsonic activity.

RESULTS

The agglutination titers, passive hemagglutination titers, and the antiadhesive ability of the colostrum samples are shown in Table 1. Antibodies to the O8 antigen (determined by the passive hemagglutination assay) and agglutinins were present in the colostrum from the susceptible and resistant sows, and there was a small but significant difference between the two groups in both cases. Agglutination titers to the K88-positive *E. coli* strain, W1, and the K88-negative mutant, J2, suggest that the agglutinins in the colostrum are directed towards the K88 antigen.

Colostrum from susceptible sows inhibited binding of ^{125}I-labeled K88 antigen to brush borders significantly better ($P < 0.001$) than colostrum from resistant sows. The inhibition was never complete and varied between 32.4 and 54.1% in the susceptible group and between −9.3 and 34.4% in the resistant group. Higher concentrations of colostrum could not be used as precipitation of ^{125}I-labeled K88 by antibody occurred.

Bactericidal activity of the two groups of samples is shown in Fig. 1. There were only two samples which promoted bacterial killing by colostrum alone. The remaining samples either were bacteriostatic or in most cases supported multiplication of the organisms. The addition of complement in the form of fresh precolostral piglet serum enabled substantial killing in 9 of 14 samples. Bacterial stasis or growth occurred in the remaining five samples. The colostrum of five of seven susceptible sows and four of seven resistant sows was effective in complement-dependent killing of bacteria.

Results of opsonic phagocytosis of the bacterial strain G205 are shown in Fig. 2. Six of seven samples from susceptible sows has opsonins important for the killing of *E. coli* strains G205 by PMNs, whereas only two samples from resistant sows had opsonins which were only minimally effective in promoting killing, and in most cases (five of seven) there was bacterial growth. When any of the colostrum samples was used, there was no killing by PMNs of *E. coli* strain 987P$^+$, the strain which had also been associated with the diarrheal outbreak.

The results of opsonic phagocytosis of *E. coli* strain G205 using fractions of colostrum obtained by gel filtration through Bio-Gel A 0.5 M are shown in Fig. 3. There was efficient killing of bacteria when the high-molecular-weight fractions were used. These fractions were shown to contain immunoglobulin M (IgM) and IgA. The lower-molecular-weight fractions containing IgG had lower activity. The immunoglobulins were identified by immunodiffusion with antisera specific for the Fc fragment of each immunoglobulin

TABLE 1. Antibody titers[a] and K88 antigen binding inhibition levels in colostrum of susceptible and resistant sows

Susceptible sows						Resistant sows					
Sow no.	Agglutinin titer		PHA[b] titer	K88 binding (% inhibition)	Sow no.	Agglutinin titer			PHA titer	K88 binding (% inhibition)
		W1	J2			G205	W1	J2		
1	256	32	4	320	54.1	8	256	8	4	160	4.6
2	256	64	2	640	37.2	9	128	32	16	320	34.4
3	512	128	2	320	45.5	10	128	32	4	160	−2.2
4	256	128	4	320	37.1	11	64	64	2	160	4.1
5	512	128	4	640	32.4	12	256	128	4	160	−9.3
6	128	128	2	320	46.1	13	128	128	4	320	13.3
7	256	128	4	160	40.5	14	64	32	2	320	25.0
Mean log titer ± SEM	2.45 ± 0.09	1.98 ± 0.09	0.59 ± 0.11	2.55 ± 0.08	41.84[c] ± 2.75		2.11 ± 0.08	1.63 ± 0.16	0.60 ± 0.11	2.33 ± 0.06	9.98[c] ± 5.81
P	<0.02	<0.01	NS[d]	<0.1	<0.001						

[a] Titers are given as reciprocal titers.
[b] PHA, Passive hemagglutination assay, O8 antigen of *E. coli* strain G205.
[c] Arithmetic mean ± SEM.
[d] NS, Not significant.

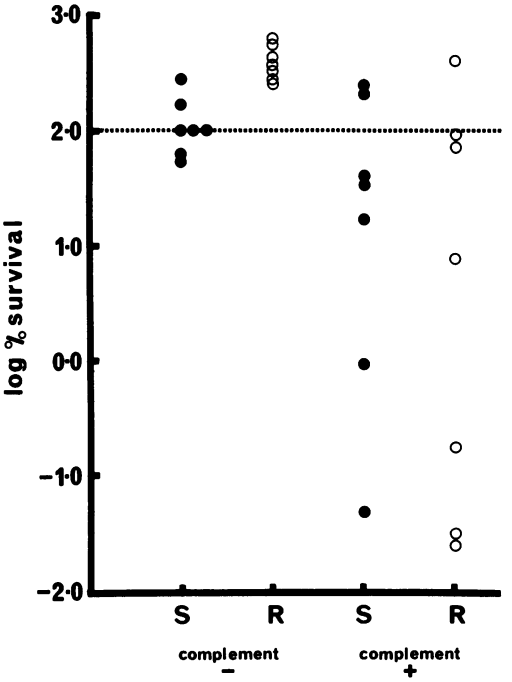

FIG. 1. Bactericidal activity of colostrum from susceptible (S) and resistant (R) sows on *E. coli* strain G205. Broken line indicates position of 100% survival.

species (Miles Laboratories, Slough, England). The high-molecular-weight fraction was rechromatographed on Bio-Gel A 1.5 M (exclusion limit 1.5×10^6 daltons). The first shoulder of the peak contained predominantly IgM, and most of the opsonic activity was associated with this first fraction.

Ion-exchange chromatography of colostral whey on DEAE-cellulose resulted in five fractions; the opsonic activity of each pooled fraction is shown in Fig. 4.

Fractions 1, 2, and 3 contained IgG; fraction 4 contained IgA with minor amounts of IgG, and fraction 5 contained IgM with minimal contamination by IgA. Fractions 1, 2, and 3 all possessed some opsonic activity (bacterial survival, 46 to 52%). Fraction 4 was of low activity (88.5% survival), but fraction 5 was the most active (3.3% survival).

DISCUSSION

Passive protection against bacterial diarrhea in neonates relies on mechanisms which either prevent the colonization of the intestine by bacteria or neutralize the effect of enterotoxins. Colonization may be controlled by bactericidal or bacteriostatic mechanisms or by interfering with adherence of the organisms to the gut mucosa. This paper is concerned with the relative importance of these mechanisms.

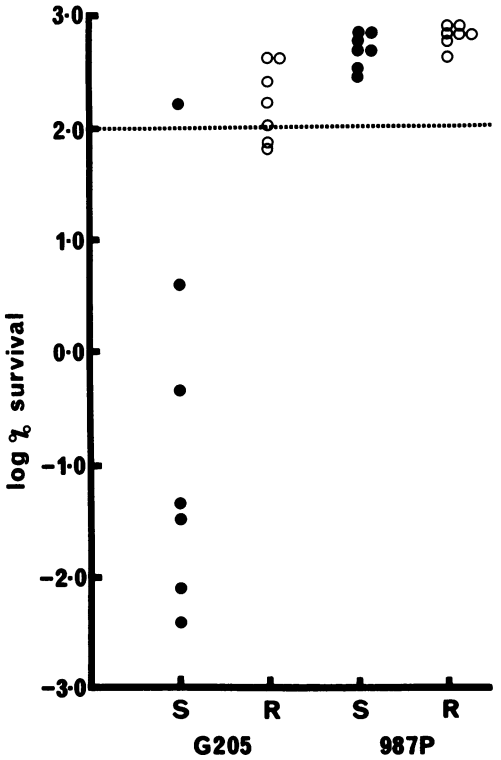

FIG. 2. Opsonic phagocytosis of *E. coli* strains G205 and 987P$^+$ by colostrum from susceptible (S) and resistant (R) sows. Broken line indicates position of 100% survival.

It is interesting that the agglutination titers and passive hemagglutination titers in colostrum were relatively low when compared with titers often reported in vaccination studies (5, 20). This may reflect differences in the route(s) of administration of the antigen(s) used in vaccination compared with a herd which has apparently built up immunity quite naturally. Porcine colostral antibody is mainly a serum transudate, and it is not surprising that antibody titers can be high after parenteral injection of *E. coli* antigens. Natural immunity is probably produced by the oral route alone which may not lead to colostrum with high agglutination titers.

Bactericidal activity was absent from all but two samples, which promoted minimal killing, unless complement was added to the assay. This led to the efficient killing of bacteria in some cases; however, there was no correlation between bactericidal activity and the genotype of the sow, incidence of diarrhea, or piglet mortality. Only susceptible piglets of resistant dams had diarrhea with high mortality in the natural disease outbreak (23). This suggests that complement-dependent killing of bacteria by colostrum is not a major factor in the protective function in the gut. Other studies of vaccinated and nonvaccinated sows have indicated that agglutinating antibodies and bactericidal activities are not of primary importance in the protective mechanism (10, 20); the results presented here are consistent with this hypothesis.

Antiadhesive effects of the colostrum were significantly different between the two groups of pigs ($P < 0.001$), which indicates that antiadherence mechanisms may play an important role in host defences. However, the differences although significant were not as great as might have been expected. This may have been because the assay used cannot truly represent the events in vivo. Antiadherence effects of colostrum and milk have previously been reported

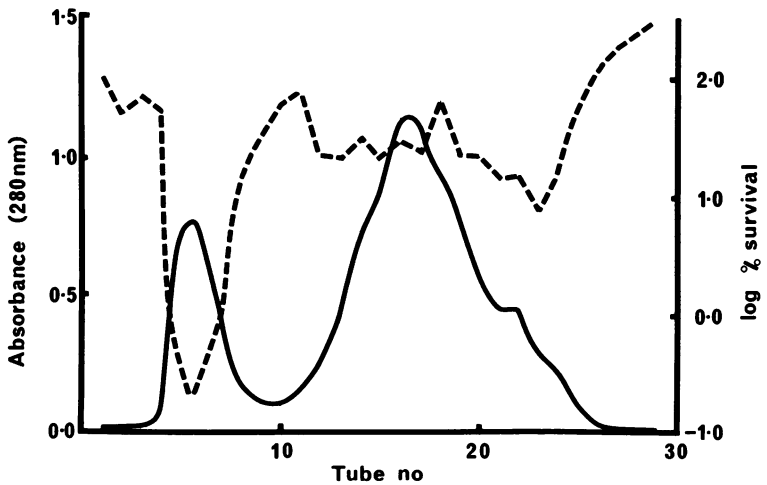

FIG. 3. Fractionation of colostrum from a susceptible sow by gel filtration on Bio-Gel A 0.5 M and the opsonic activity of each fraction against *E. coli* strain G205; (———) absorbance at 280 nm; (----), opsonic activity).

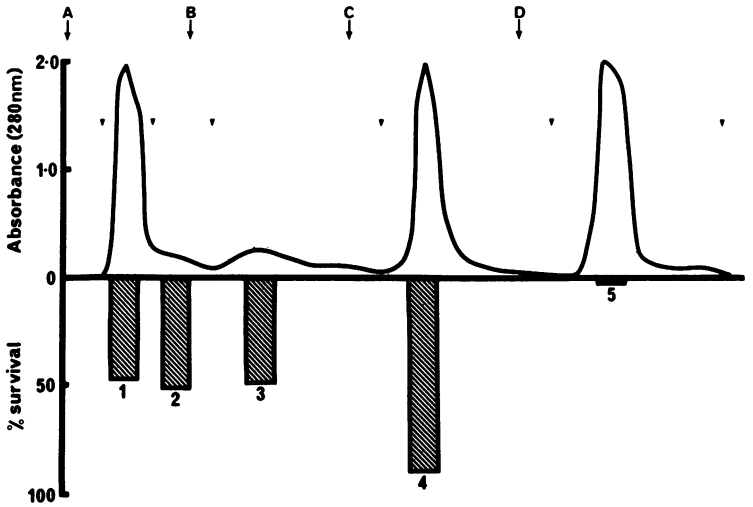

FIG. 4. Opsonic phagocytosis activity of pooled fractions of colostrum from a susceptible sow fractionated by anion-exchange chromatography on DEAE cellulose (DE52) with stepwise elution. Buffers: A, 0.01 M NaH_2PO_4 (pH 7.6); B, 0.02 M NaH_2PO_4 (pH 6.3); C, 0.05 M NaH_2PO_4, (pH 4.5); D, 0.3 M NaH_2PO_4, (pH 4.5). Fractions collected are labeled 1 through 5; pooled for each fraction.

(10, 16, 22). In these studies (10) there was high activity in colostrum which decreased during the transition to milk and diminished to very low levels 7 days postpartum.

The opsonic activities of the colostrums present an interesting concept related to the whole strategy of protection in relation to enteric infection. The significant difference in opsonisation between the two groups ($P < 0.001$) suggests that this is an important mechanism provided that it functions within the lumen of the intestine. Migration of neutrophils into the lumen of the intestine must occur for this mechanism to be important in the control of the disease. An active emigration of large numbers of PMNs across the epithelium of the small intestine into the lumen in certain circumstances can occur (2). It has also been demonstrated that phagocytosis of *E. coli* occurred within ligated gut loops of both parenterally immunized and nonimmunized pigs, but was significantly increased in the immunized group (1). Also, Moon, (14) has observed phagocytosis of bacteria on the absorptive cell surfaces of the small intestine of pigs after enteropathogenic *E. coli* infection.

It would, therefore, appear that the various components of a phagocytic mechanism, namely, PMNs and opsonins are available for this function to operate in vivo. However, newborn piglets are immunologically incompetent, and the question which remains is whether emigration of neutrophils across intestinal epithelium can occur in these pigs.

The apparent lack of killing of the $987P^+$ strain by PMNs in the presence of colostrum may suggest a phagocytosis-inhibitory effect similar to that reported for an *E. coli* capsular antigen (9). If this mechanism of killing is important in vivo, then some capsular strains of *E. coli* could be a far greater threat to the neonate defences.

The finding that the opsonin for the K88-positive *E. coli* strain appears to be predominantly of the IgM class is of interest, although IgG also appears to function similarly but with lower efficiency. Chidlow and Porter (6) showed that the peak antibody response to oral immunization with *E. coli* polysaccharide antigens followed by a single parenteral dose was of the IgM class of immunoglobulins; also, this vaccination protocol correlated well with protection (7). In contrast, a double parenteral injection program led to a response which was predominantly of the IgG class and protection was not as effective. The IgM class of immunoglobulin has also been shown to be an extremely good opsonin for PMNs in rabbits, ~1,000 times more efficient than IgG (19). Although IgM may account for only ~4% of the total colostral immunoglobulins, its high efficiency could make it an important factor in the host defence mechanisms in neonatal enteric infection.

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