Studies of Escherichia coli in Gnotobiotic Pigs III. Evaluation of Orally Administered Specific Antisera*

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SUMMARY

The protective effect of orally administered immune (E. COLI 08:K.:H21) serum was demonstrated in experimentally infected gnotobiotic pigs. The temporary protective effect of the immune serum and the correlation of protection with IN VITRO antibody determinations were discussed. The results indicated that the protective action of the immune serum is apparently not dependent upon the complement-antibody bactericidal system. It was suggested that the most plausible mechanism of action of the immune serum was the inactivation of endotoxin in the lumen of the intestine.

Introduction

The present knowledge of the pathogenesis of *Escherichia coli* caused diarrhea, although incomplete, does indicate that the establishment of large numbers of certain strains of *E. coli* in the anterior portion of the intestinal tract of piglets (24), calves (20), and human beings (5, 28) is of utmost significance.

Although the efficacy of several chemotherapeutic agents has been repeatedly demonstrated, the use of serum as a prophylactic agent has been of questionable value. It has been reported, from studies in colostrum-deprived piglets, that parenteral administration of "normal" swine serum or *gamma* globulin was of little or no value in preventing *E. coli* diarrhea. The same workers reported that oral administration of serum, *gamma* globulin, or colostrum was beneficial (19). It was further suggested that colostrum-deprived piglets were protected for a time directly related to the number of days the serum was administered, i.e., administration for five days was much superior to administration for one or two days. These workers considered the action of the orally administered *gamma* globulin to be possibly the same as described in the classical reports concerning coproantibody associated with vibrionic dysentery (3). Coproantibody has also been studied in bacillary dysentery of human beings (2, 9). Similar findings have been reported from attempts to protect piglets against transmissible gastroenteritis (a virus caused disease of swine) by parenteral and oral administration of immune serum (8).

In view of these reports, one might well have expected favorable results when attempts were made to enhance the protective value of colostrum by immunizing the sows. The results of two controlled experiments, in which sows were immunized with *E. coli* by several different methods, did not reveal enhanced resistance of the piglets to *E. coli* diarrhea (10, 14). It has been suggested that modification of the vaccination schedule may be required to attain benefits from vaccinating sows (27).

The evidence indicates that a system should be developed to evaluate whether orally administered specific antibodies will protect piglets from clinical disease when experimentally infected with "enteropathogenic" *E. coli*. Such a system might be used to establish more effective immunization schedules.

This study was initiated for the primary purpose of establishing whether orally ad-

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ministered antibodies would protect gnotobiotic pigs against the clinical signs of colibacillosis. The source of the antibodies was the serum from gnotobiotic pigs hyperimmunized with the same strain of *E. coli* that was used to produce colibacillosis in gnotobiotic pigs. Gnotobiotic piglets were selected for the reasons previously outlined (12).

Materials and Methods

Experimental animals.—Gnotobiotic piglets were procured and maintained as previously described (12, 15).

Inoculum.—The "Arnold" strain (12) of *E. coli* 08:K.:H21 was used to infect and vaccinate pigs throughout this series of experiments.

Source and preparation of serum for oral administration.—Nonimmune serum was obtained from "germfree" gnotobiotic piglets which were exsanguinated at 6-11 weeks of age.

Immune serum was obtained from gnotobiotic pigs which were orally infected at 4-6 days of age. Thirty to sixty-five days later 1 ml of a living 4 hour brain heart infusion (BHI) broth culture of the same strain was administered intravenously to each pig. Two or three injections were given at 4 day intervals. Approximately 10 days after the final intravenous injection, the pigs were removed from the isolators. The pigs were immediately anaesthetized with ether and exsanguinated.

Aseptic procedures were used in collection and preparation of both immune and nonimmune sera, and in addition, precautions were taken to preserve the complement. The blood was collected in sterile 40 ml centrifuge tubes and allowed to clot for 1-2 hours at room temperature. The tubes were centrifuged at approximately 500 x G for 15 minutes at 4°C. All the serum from a given pig was drawn off and transferred to a sterile flask in an ice bath. Seven ml aliquots were dispensed into screw-cap vials which were immediately frozen and stored at –70°C until immediately prior to use.

All serum used in protection experiments was titered by the bactericidal, indirect hemagglutination, and bacterial agglutination test prior to oral administration to piglets (13).

Evaluation of the effect of oral administration of serum to piglets.—Piglets normally nurse every 1 to 1½ hours during the day and night (1). Piglets in these experiments were fed and observed every 1½ hours during the first 48 hours following oral infection at 4-6 days of age. The control piglets were fed and observed at the same time.

The piglets were fed 14 ml of the appropriate diet or diet-serum mixture at 7:30 A.M. on the morning they were to be infected. The last previous feeding had been 75 ml of diet at midnight. At 9:00 A.M. each piglet to be infected was given 1 ml of the inoculum (prepared as previously described (12) orally by means of a glass syringe. The inoculum contained approximately 10^7 living *E. coli* 08:K.:H21. Immediately after the inoculum had been administered, the piglets were fed 14 ml of the appropriate diet or diet-serum mixture. In order to evaluate the effect of the oral administration of immune serum it was necessary to establish several controls. Each experiment was conducted with litter-mates 4-6 days old.

The negative control pigs were gnotobiotic piglets not infected with *E. coli.* These pigs were fed 14 ml diet at each feeding and were used to determine the appearance and responses of "normal" gnotobiotic pigs under the physical conditions of the experiments.

The positive control piglets were gnotobiotic piglets fed 14 ml diet at each feeding and infected orally. These piglets were used to determine the clinical response of gnotobiotic piglets to oral infection with this strain.

The serum control pigs were orally infected and received 7 ml non-immune serum and 7 ml diet at each feeding. These pigs were used to determine whether or not there was any effect on the pigs due to the feeding of serum as a portion of the diet.

The pigs utilized to evaluate the significance of the bactericidal activity of the immune sera were orally infected and were fed 7 ml heated (56°C for 30 minutes) immune serum and 7 ml diet at each feeding. These pigs were used to determine whether the protective effect of the immune serum was dependent upon the presence of heat labile factors. (The bactericidal antibody system for gram negative bacteria is complement dependent.)

The pigs utilized to test the protective value of sera which had bactericidal activity *in vitro,* were fed 7 ml unheated immune serum and 7 ml diet at each feeding. These piglets were orally infected in at-

TABLE I. Summary of Results

Number Pigs	Serum Administered	Number of Pigs	Onset of Diarrhea
14	No serum	12 2	12-24 hours PI 24-36 hours PI
"	Nonimmune serum (7 ml/1½ hr)	4	12-24 hours PI
5	Low titer (Bactericidal 2-64) (7 ml/1½ hr)	5	12-24 hours PI
12[a]	Immune serum (Bactericidal titer 500-1000)	12	40-55 hours PI (12-24 hours after last serum was fed)
7[b]	Immune serum (7 ml/1½ hr)	7	"
3[b]	Heated immune serum[c] (7 ml/1½ hr)	3	"
2[b]	Immune serum (3½ ml/1½ hr)	2	"

[a]The pigs reported in this group are further divided into three subgroups b.
[b]Subdivisions of group a.
[c]Heated (56°C for 30 minutes) immune serum.

tempts to evaluate the effect of the bactericidal properties of the sera.

Some vials were passed out of the isolators immediately after feeding the serum to the pigs. A small amount of serum remained in these vials, and it had the same bactericidal activity in the *in vitro* assay as had been determined before passage into the isolator. This assay was done by a bactericidal test in which the serum served as its own source of complement.

Results

Pigs not fed immune serum.—Fourteen pigs that received no serum were infected between 4 and 6 days of age. They were fed 14 ml of diet every 1½ hours. Twelve of these pigs had diarrhea 12 to 24 hours postinfection while 2 pigs did not have diarrhea until 24 to 36 hours postinfection. Two of these pigs became dehydrated and died within 48 hours following infection. Two pigs were comatose and were euthanatized within 72 hours after infection. The remaining 10 pigs recovered.

Five pigs were fed 7 ml of low titer (bactericidal titer 2-64) serum in 7 ml diet every 1½ hours for 36 hours. All five pigs had diarrhea 12 to 24 hours after oral infection. One pig died 30 hours after infection. The other four recovered.

Four pigs were fed 7 ml non-immune serum in 7 ml diet every 1½ hours until after the onset of diarrhea. Each of these pigs had diarrhea 12 to 24 hours after infection. One pig was comatose and was euthanatized 27 hours after infection. The other three pigs recovered.

Pigs fed immune serum.—Seven ml of immune serum in 7 ml diet was administered orally to seven pigs every 1½ hours for 30 to 36 hours. No diarrhea or other signs of illness were observed during the period when serum was being administered. All seven pigs had diarrhea 12 to 24 hours after the last serum was given. One of these pigs died 52 hours after infection. This was 22 hours after the last serum had been administered. The remaining six pigs recovered or were recovering when euthanatized.

Heated (56°C for 30 minutes), immune serum was fed to three orally infected pigs. Seven ml of this serum in 7 ml diet was fed every 1½ hours for 30 to 36 hours. No diarrhea or other signs of illness were observed during the period when serum was being administered. All three pigs had diarrhea 12 to 24 hours after receiving the last serum. One pig died 55 hours after infection. This was 25 hours after the last serum was administered. The other 2 pigs recovered.

Three and one-half ml of immune serum in 11 ml diet was fed every 1½ hours for

24 hours to two orally infected pigs. No illness was noted during the period when serum was being fed. One pig had diarrhea 12 hours after the last serum had been fed. The other pig had diarrhea 21 hours after receiving the last dose of serum. Both pigs recovered.

A summary of the results is reported in Table I.

Discussion

It was effectively demonstrated that complete protection from clinical signs of the disease was provided during the period when immune serum was being administered. No *in vivo* titrations of the immune sera were attempted, but two pigs were completely protected when fed one-half the dosage fed to the other test pigs. These results alone indicated the serum was protective, but an additional demonstration of protection was provided when all of these pigs developed diarrhea 12 to 24 hours after the cessation of serum feeding. These results do not, however, indicate whether the protective value of the immune serum was due to bactericidal activity or not. It may be noted, however, that the dilution factor of the complement in the serum fed to the two pigs at one-half normal dosage (3½ ml/1½ hr.) level appears to indicate that there could have been little bactericidal activity in the lumen of the intestine of these pigs due to complement contributed by the orally administered serum.

The results with heated immune serum (aliquots of the sera from the same source as the immune sera that were fed unheated) indicate that heating the serum did not deplete the protective value of the sera. The same sera were tested in the *in vitro* bactericidal test, and it was demonstrated that all bactericidal activity was lost when the complement source was heated. This appears to indicate that the protective value of orally administered immune serum in colibacillosis is not appreciably dependent upon conventional bactericidal activity.

The results indicate that oral administration of non-immune serum or serum of very low titer does not modify the clinical colibacillosis resulting from experimental infection. This was considered a critical control in the evaluation of immune serum, since it was essential to show whether serum factors unrelated to previous exposure of the serum donor to the antigens would affect the host response to oral infection.

Several factors need to be taken into consideration in the discussion of the mechanism of the protective effect of serum. One may simply state that it functions in the intestine, but this does little to describe the possible mechanisms of activity.

First, it has been shown that little or no antibody is absorbed from the intestinal tract in pigs older than 24 to 36 hours (17, 23, 25). Apparently similar conditions prevail for the absorption of complement from the lumen of the gut (21, 26). In a previous communication related to the current experiments no antibody was detected by the bactericidal or the hemagglutination tests in the days immediately following the oral administration of immune serum to 4- to 6-day old pigs (13). These results indicate that the antibody activity was restricted to the intestinal tract.

Second, the temporary protection provided by the immune serum was depleted within 12 to 24 hours after cessation of administration. This seems to indicate that the activity of the serum was, in all probability, not associated with a cellular mechanism or at least not firmly bound to such a system.

Third, the bacterial agglutination tests with these immune sera revealed that there was little or no agglutinating activity present, even for boiled bacteria. Therefore, the agglutination of bacteria does not appear to be a likely factor in protection by the immune sera.

Fourth, to the authors' knowledge, there has been no demonstration that other substances can replace complement in the serum bactericidal system when applied to gram-negative organisms. The possibility that adequate complement could be available in the intestine of colostrum-deprived pigs of this age seems quite remote. This is especially apparent since the complement level in colostrum-deprived pigs has been shown to be extremely low during the first one or two weeks (21, 26). For these reasons, and especially since no difference at all was noted between pigs fed whole and heated immune serum, it appears that the bactericidal system was not responsible for the protection observed in these experiments.

Fifth, although there is no conclusive proof regarding the mechanism by which colibacillosis of pigs is caused by *E. coli*,

there is evidence to indicate that endotoxin is the most likely substance involved in the initiation of physiological alterations involved in the disease. Diarrhea and vomiting have been observed in piglets shortly after intravenous injection of endotoxins (4, 11). However, no illness has been observed following oral administration of endotoxin or killed bacterial suspensions to pigs (16).

The inactivation or detoxification of endotoxin by immune serum has been demonstrated (6, 7, 18, 22). These experiments have involved parenteral injection of the endotoxin and serum or parenteral injection of the serum treated endotoxin. Parenteral injection into animals, of course, complicates the system considerably, and under these conditions active complement of the host is present as well as the complex cellular defense mechanisms and perhaps even the enzymatic and hormonal systems of the host. All of these factors may well participate in protection against parenterally administered endotoxin.

In the current series of experiments, it has not been proven that endotoxin causes the clinical disease, nor has it been shown precisely what the mechanism of protection by immune serum may be. It has been shown that the protection is provided by serum containing antibody, detectible by bactericidal assay or bacterial hemagglutination, and several mechanisms of antibody activity have been shown to be of very doubtful significance in this particular application. It seems reasonable to postulate, that the most likely mechanism of protection by the immune sera in these experiments is the inactivation of the endotoxin locally in the lumen of the intestine. It also seems possible that this experimental model could be further refined to test this hypothesis.

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