PASSIVE PROTECTION OF LAMBS AGAINST ENTEROPATHOGENIC *ESCHERICHIA COLI*: ROLE OF ANTIBODIES IN SERUM AND COLOSTRUM OF DAMS VACCINATED WITH K99 ANTIGEN

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DIARRHOEA caused by enterotoxigenic *Escherichia coli* is one of the most common bacterial disorders of the newborn farm animal. The disease involves colonisation of the small intestine by enteropathogenic *E. coli* followed by the production of enterotoxin, which induces diarrhoea (Smith and Jones, 1963). Earlier work at this laboratory (Sojka, 1972, cited by Smith and Linggood, 1972) identified a common antigen, subsequently designated K99 (Ørskov *et al.*, 1975), on the surface of the majority of strains enteropathogenic for calves and lambs. Smith and Linggood (1972) demonstrated that K99 is a plasmid-determined antigen and that removal of the K99 plasmid prevented colonisation and subsequent diarrhoea in calves and lambs.

The neonate is affected by enteropathogenic *E. coli* within the first week of life (Sojka, 1971); in contrast to its behaviour in systemic colibacillosis, the pathogen does not leave the intestinal tract. The presence of colostrum in the gut lumen soon after birth might therefore be important in determining susceptibility to infection; the protective properties of the colostrum would be enhanced if it contained antibodies to relevant antigenic determinants on the pathogen. The K99 antigen has been isolated and examined at this laboratory (Morris, Stevens and Sojka, 1977, 1978*a* and *b*) and lambs passively immunised with colostrum from dams vaccinated with the K99 antigen were successfully protected from diarrhoea after oral challenge with a virulent enteropathogen (Sojka, Wray and Morris 1978). In the present study colostrum and serum from these dams were examined to evaluate a range of serological tests and to gain an insight into the possible mechanism of protection.

MATERIALS AND METHODS

Serum and colostrum. Full details of the vaccination procedure have been described (Sojka *et al.*, 1978). Briefly, 12 pregnant Dorset Horn ewes were given an intramammary injection of partially purified K99 antigen in Freund's incomplete adjuvant followed by a second injection subcutaneously of antigen in phosphate buffer. Six control ewes were treated similarly but with saline in place of antigen. Ewes were bled before each injection and blood samples were taken at parturition. Approximately 20 ml of colostrum were collected from two quarters shortly after parturition and the whey was separated (Logan and Penhale, 1971). Serum and whey were stored at -20°C . Unless stated otherwise, each sample was examined individually. There were no

Received 31 July 1979; accepted 21 Sept. 1979.

significant differences between the results with colostrum samples taken from different quarters of the same animal.

Indirect haemagglutination (IHA) test. Tanned ovine erythrocytes were coated with cell-free K99 antigen (Wray, Morris and Sojka, 1975) and the test was read after incubation at 37°C for 2 h.

Antiglobulin test. The antiglobulin haemagglutination test of Buxton and Thomlinson (1961) was used with rabbit antish sheep gammaglobulin.

Gel-diffusion test. Details of the gel-diffusion test have been reported earlier (Morris *et al.*, 1978a). The gels were examined after incubation at 4°C for 24 h.

Agglutination test. A tube agglutination test (Sojka, 1965) with *E. coli* strain B41 (O101:K99:NM) was used to detect agglutinins to the O101 and K99 antigen; a boiled bacterial suspension was used to detect the O101 antibodies and a live bacterial suspension to detect antibodies to K99.

Haemagglutination-inhibition test. Cell-free K99 antigen was incubated with an equal volume of sample or saline at 37°C for 18 h. The direct haemagglutination titre of the K99 antigen before and after absorption was determined (Morris *et al.*, 1977).

Anti-enterotoxin test. Supernatant fluid from an overnight culture of *E. coli* strain B44 in Heart Infusion Broth (Difco) was used as heat-stable enterotoxin (ST). Samples or saline were incubated with equal volumes of ST at 37°C for 18 h. Absorbed and unabsorbed ST were assayed in 4-day-old suckling mice (Giannella, 1976).

Anti-adhesion test. An overnight culture of *E. coli* strain B44 in Minca broth was washed and resuspended in phosphate-buffered saline to approximately 1×10^9 colony-forming units/ml. Doubling dilutions of pooled colostrum whey from four vaccinated dams or three control dams were incubated with equal volumes of bacteria in microtitration plates and incubated at 37°C for 30 min. A drop from each well was examined for bacterial agglutination by interference microscopy, and dilutions in which agglutination was not observed were mixed with an equal volume of calf brush-border cells (Sellwood *et al.*, 1975). After gentle shaking at 37°C for 60 min. a drop of each suspension was examined by interference microscopy.

Characterisation of colostrum antibodies to K99 antigen. Antibodies to K99 from the pooled immune colostrum whey used in the anti-adhesion tests were isolated by affinity chromatography with K99 antigen coupled to Sepharose 4B (Pharmacia). Samples were applied to the column in 100 mM borate buffer containing 500 mM NaCl at pH 8.4 and, after washing in two-column volumes of the same buffer, antibodies were eluted from the gel by 200 mM HCl/glycine buffer containing 500 mM NaCl at pH 2.8. After desalting and concentration the antibodies were identified by gel diffusion against class-specific rabbit antisera to sheep immunoglobulins (Rich Research Co.) Specificity of the antiglobulins was checked by immunoelectrophoresis with whole sheep serum.

RESULTS

Serum antibodies to K99 antigen

Antibodies to K99 antigen were not detected in the pre-vaccination serum samples nor in any of the serum samples from the control group of ewes (table I). The indirect haemagglutination test and the serum-agglutination test with the whole-cell K99 antigen detected antibodies to K99 in the serum of all vaccinated dams when they were bled 23 days after the first vaccination. At this time only three of the 12 vaccinated ewes and none of the control ewes had detectable precipitins to the K99 antigen in their serum. Samples from three vaccinated ewes gave a positive reaction in the antiglobulin test but there was no correlation between the results obtained in the antiglobulin and gel-diffusion tests. At parturition the IHA titres had increased, all the samples from vaccinated ewes but none from the control ewes contained precipitins, and all samples from the vaccinated ewes were positive in the antiglobulin test.

TABLE I
Antibodies in serum from ewes vaccinated with K99 antigen and from control ewes

Class (and number) of ewes	Antibodies to K99: mean (and range) of titre at the stated times in the indicated tests									
	Pre-vaccination*		Before 2nd vaccination				At parturition			
	IHA	IHA	IHA	AGHA	SAT K99	SAT O101	IHA	AGHA	SAT K99	SAT O101
Vaccinated (12)	<2	99 (32-512)	115 (<32-512)	400 (160-640)	300 (<2-640)	307 (128-512)	358 (128-512)	373 (160-640)	336 (80-640)	80 (<2-160)
Control (6)	<2	<2	<2	<2	93 (<2-320)	<2	<2	<2	<2	<2

* Prevaccination samples were examined only by the IHA test.
IHA = Indirect haemagglutination test; AGHA = antiglobulin haemagglutination test; SAT K99 = serum agglutination test against K99 antigen; SAT O101 = serum agglutination test against O101 antigen.

Serum antibodies to O101 antigen

Antibodies to the O101 antigen were not sought in the prevaccination serum samples; they were detected in four of the six control ewes and 10 of the 12 vaccinated ewes 23 days after first vaccination (table I). At parturition agglutinins were still detectable in only four of the six control ewes, but all 12 vaccinated ewes had produced agglutinins.

Colostrum antibodies to K99 and O101 antigens

The indirect haemagglutination, antiglobulin and haemagglutination-inhibition tests did not detect antibodies to the K99 antigen in any of the colostrum samples from the control ewes (table II). Agglutinating activity was detected in these samples when they were examined with the whole-cell K99 antigen and the O101 antigen. High titres to K99 were demonstrated in all the colostrum samples from vaccinated dams by the indirect haemagglutination test and the haemagglutination-inhibition test. Antibodies to K99 were also detected in all these samples by the whole-cell agglutination test. The antiglobulin test detected incomplete antibodies in four samples. Precipitins against the K99 antigen were detected in colostrum from all the vaccinated ewes but not from the control ewes. Agglutinating activity against the O101 antigen was detected in all the samples from vaccinated ewes (table II).

Anti-enterotoxin tests

Groups of eight mice given injection of heat-stable enterotoxin gave a mean gut to body-weight ratio of 0.114 (range 0.108–0.121) to 1; the control preparation of broth produced a ratio of 0.057 (0.054–0.060) to 1 and the heat-stable enterotoxin absorbed with pooled immune colostrum gave a ratio of 0.099 (0.090–0.108).

Anti-adhesion tests

Bacteria absorbed with saline readily attached to brush-border cells pre-

TABLE II
Antibodies in colostrum from ewes vaccinated with K99 antigen and from control ewes

Class (and number) of ewes	Antibodies to K99: mean (and range) of titre				
	IHA	AGHA	SAT K99	SAT O101	HAI
Vaccinated (12)	2048 (512–4096)	11302 (512–33768)	640 (320–1280)	334 (80–1280)	1024
Control (6)	2	2	60 (20–160)	80 (2–320)	2

HAI = Haemagglutination inhibition; other footnotes as in table I.

pared from calf intestine. Pooled colostrum whey from non-vaccinated ewes prevented adhesion at dilutions of 1 in 80 or less, but at these dilutions microscopic agglutination occurred. The pooled whey sample from the vaccinated group did not agglutinate bacteria at dilutions greater than 1 in 640 but adhesion of bacteria to brush-border cells was inhibited at dilutions up to 1 in 2560.

Characterisation of colostrum antibodies to K99

Antibodies to K99 isolated by affinity chromatography gave a single line of precipitation in agar gels on diffusion against rabbit antiserum IgG serum. Precipitin lines were not observed when the K99 antibodies were tested against antiserum to sheep IgM or IgA.

DISCUSSION

Earlier work at this laboratory (Sojka *et al.*, 1978) showed that lambs passively immunised with colostrum from dams vaccinated with the K99 antigen were successfully protected from diarrhoea after oral challenge with a virulent strain of enteropathogenic *E. coli*. The present study showed that vaccination produced high levels of serum antibody to K99 antigen in all of the dams. The indirect haemagglutination test and whole-cell K99 agglutination test were the most reliable of the tests for detecting serum antibody to K99 but at parturition, serum antibodies could be detected by all the tests used. It was evident that serum agglutinins to the somatic antigen O101 were present in both sets of ewes but because the titres remained constant it is unlikely that the vaccine contained significant amounts of this antigen.

The agglutinating activity in the colostrum of vaccinated and non-vaccinated dams demonstrated by the whole-cell K99 antigen was not unexpected. Rutter *et al.*, (1976) found similar activity with K88-positive bacteria and normal sow colostrum which they attributed to glycoproteins and oligosaccharides. Reiter and Brown (1976) reported that bovine milk globules might be capable of binding directly to the K88 or K99 antigen on the bacterium. Nevertheless, the agglutination titres of the colostrum from non-vaccinated ewes were lower than those of the colostrum from vaccinated ewes and agglutinating antibody to K99 in the latter probably supplemented the non-specific agglutinins. Colostrum antibodies could be demonstrated most reliably by the gel-diffusion and indirect haemagglutination tests, which clearly showed that vaccination produced antibodies to K99 in the colostrum. Characterisation of these antibodies showed that the anti-K99 activity was IgG. The predominant immunoglobulin in sheep colostrum is IgG₁ (Mackenzie and Lascelles, 1968), which is thought to be derived mainly from the serum (Sullivan *et al.*, 1969). If this is correct, the intramammary route of vaccination may not be necessary.

The ewes were immunised with K99 antigen isolated from *E. coli* strain B41 (O101:K99) and their lambs were challenged with strain B44 (O9:K30, K99).

Thus K99 was the only antigen known to be common to both strains and it was therefore concluded that the K99 antibodies in the colostrum contributed significantly to the protection of lambs from vaccinated dams. Heat-stable enterotoxin produced *in vitro* was not neutralised by colostrum from vaccinated dams; such colostrum, unlike normal colostrum, did inhibit the direct haemagglutinating activity of cell-free K99. Furthermore, pooled immune colostrum prevented in-vitro adhesion of bacteria to calf intestinal brush borders. This is consistent with the opinion that passive protection conferred by colostrum from vaccinated dams was not due to antitoxic activity but to its property of reducing the adhesion of the enteropathogen to the gut. Bacterial association with the mucosa is a complex process, but antibodies elicited by the K99 vaccine might coat the bacterial "adhesins" *in vivo* and thereby reduce the attachment of the organism to the intestinal epithelium. In the lumen, mechanisms such as peristalsis and villous motility would prevent rapid proliferation of the enteropathogen and permit control by non-specific bactericidal factors in the colostrum.

SUMMARY

Lambs from suckling ewes vaccinated with the K99 antigen were resistant to challenge with K99-positive enteropathogenic *Escherichia coli*. Serum and colostrum from these ewes were compared with samples from control ewes to establish methods for monitoring vaccination and to determine the mechanism of protection.

Vaccination stimulated production of K99 antibodies. These could be detected by an indirect haemagglutination test and a haemagglutination-inhibition test. Antiglobulin and gel-diffusion tests were less reliable. Experiments with brush-border cells from calf intestine showed that the antibodies were associated with anti-adhesive activity. The antibodies were predominantly IgG and did not neutralise the activity of heat-stable enterotoxin. It was concluded that neutralisation of the adhesive properties of the K99-positive *E. coli* by colostral antibodies significantly contributed to the resistance of the lambs from vaccinated ewes.

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