Effects of Immune Colostrum on the Expression of a K88 Plasmid Encoded Determinant: Role of Plasmid Stability and Influence of Phenotypic Expression of K88 Fimbriae

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Passaging of the K88-positive *Escherichia coli* strain CN6913 through synthetic medium containing immune colostrum gave rise to large numbers of K88-negative CN6913 variants. These K88-negative variants had all lost a single large plasmid known to encode the K88 genetic determinant. Four other large plasmids harboured by this strain were unaffected. Viable K88-positive and K88-negative variants of CN6913 accumulated at a similar rate in synthetic medium and in medium containing non-immune colostrum. In the presence of immune colostrum, viable cells of the K88-negative variant accumulated faster and to a greater extent in cultures than the K88-positive variant if incubated at 37 °C, which favours the phenotypic expression of K88. However, when similar cultures were incubated at 18 °C, a temperature known to inhibit phenotypic expression of K88, the accumulation of viable cells of the two variants was strictly comparable in all media and no loss of plasmid or increase in K88-negative variants was observed. Cells containing a pBR322-based K88-encoding recombinant plasmid were also eliminated by immune colostrum whereas cells containing pBR322 were not. Plasmids encoding the K99 antigen were not readily eliminated from strains passaged through medium containing immune colostrum. K99-negative variants that were detected still harboured the K99-encoding plasmid.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) can efficiently colonize the small bowel of young domestic animals and humans (Smith & Linggood, 1971; Gaastra & De Graaf, 1982). Adhesion of some ETEC strains to the brush-border epithelial cells present on the gut mucosal surface is mediated by surface associated appendages often referred to as adhesion fimbriae (Dougan & Morrissey, 1985). A number of serologically distinct and species specific adhesion fimbriae have been identified and the genetic determinants for some, but not all, are located on extrachromosomal DNA (Ellwell & Shipley, 1980; Shipley *et al.*, 1978). The K88 antigen associated with porcine ETEC and the K99 antigen associated with porcine and bovine ETEC are normally encoded on large single copy plasmids (Shipley *et al.*, 1978). Acquisition of such plasmids by some non-pathogenic *E. coli* strains enables them to colonize the small bowel in higher than normal numbers (Smith & Linggood, 1971).

Young piglets can be passively protected against diarrhoeal diseases caused by ETEC if they are allowed to suckle colostrum or milk of sows immunized with adhesion fimbriae preparations (Nagy, B. *et al.*, 1978; Nagy, L. K. *et al.*, 1978; Rutter & Jones, 1979). Protection is limited to ETEC strains which express fimbriae contained in the vaccine and does not extend to ETEC which express heterologous non-immunologically crossreactive fimbriae (Morgan *et al.*, 1978). Linggood & Porter (1978) and Linggood *et al.*, (1979) showed that if bacterial strains harbouring wild-type K88 plasmids were passaged serially through media containing colostrum obtained from immune sows they rapidly lost the ability to express surface associated K88 fimbriae. The

authors postulated that factors distinct from anti-K88 antibodies were inducing the loss of K88 expression by interfering with plasmid replication. However, loss of plasmids was not demonstrated and it could be argued that selective pressures acting in the presence of immune colostrum and in favour of spontaneous K88-negative variants in the population could account for the loss of the K88-positive variants. The purpose of the work described in this report was to examine the reasons for loss of expression of K88 fimbriae in cells grown in the presence of immune colostrum.

METHODS

Bacterial strains, plasmids and culture medium. The ETEC strain used in passaging experiments was CN6913 of serotype O8:K87(B)K88ab(L) which harbours a K88 determinant carried on a wild-type K88 plasmid. This strain was isolated from a scouring piglet at Wellcome Research Laboratories, Beckenham, Kent, UK. *E. coli* B44 is an ETEC strain expressing K99 antigen (Morris *et al.*, 1980). *E. coli* strain SN62 was constructed by Dr William Turner at Wellcome Research Laboratories, and harbours a single plasmid (molecular mass 50 MDa) which encodes a K99 determinant. *E. coli* strain B188 has been described previously and is highly resistant to the bactericidal activity of sera (Quackenbush & Falkow, 1979). Plasmids pBR322 and the K88-pBR322 recombinant plasmid pMK005 have been described previously (Kehoe *et al.*, 1981, 1983). All strains were routinely cultured in synthetic minimal medium prepared as described previously (Davis *et al.*, 1980) or on sheep blood agar plates. To test the effect of colostrum or milk on the various *E. coli* strains the synthetic medium was supplemented with 10% (v/v) filter sterilized colostrum or milk. Colostrum of vaccinated and control sows was collected between 12 and 24 h post partum and milk within 7 d. Mammary secretions were centrifuged to eliminate milk fat and were sterilized by sequential filtration through 0.45 then 0.22 µm Millipore filters. Before use they were stored at -40 °C.

Passage of strains in medium with and without colostrum. Inocula for passage experiments were either stored as seed lots in a liquid nitrogen refrigerator or were grown in synthetic minimal medium at 37 °C for 4 to 5 h before inoculation into synthetic minimal medium with or without colostrum. In each case a retrospective viable count was carried out by preparing serial tenfold dilutions of bacteria in phosphate buffered saline (pH 7.4) and plating the dilutions on blood agar plates.

In passage experiments 4.5 ml of synthetic medium and 4.5 ml of synthetic medium supplemented with 10% (v/v) colostrum or milk were inoculated with 0.5 ml of *E. coli* suspension and incubated overnight at 37 °C before 0.5 ml of the culture was transferred in 4.5 ml of identical medium. At the same time blood agar plates were streaked with samples of the culture and the resultant colonies which appeared following overnight incubation at 37 °C were tested by slide agglutination for expression of K88 or K99 fimbrial antigens. These passages were done until no more fimbrial antigens were detectable on any of ten colonies examined or until it became clear that the bacteria were not affected by the treatment. In some experiments, the time and temperature of incubation were varied as indicated. Each culture was sampled immediately after inoculation and then at hourly intervals unless otherwise stated. Viable counts on the samples were made on sheep blood agar plates.

Plasmid DNA preparations and analysis on agarose gels. Strains were screened for the presence of plasmid DNA by the method of Kado & Lui (1981). Plasmid containing samples were analysed on 0.8% (w/v) agarose gels as described previously (Kehoe *et al.*, 1981).

Vaccine and vaccination of swine. The vaccine used to prepare the immune colostrum was described by Nagy *et al.* (1985). It contained K88ab, K99 and 987P fimbrial antigens of *E. coli* prepared from culture supernates of fermenter grown strains. These were absorbed onto alhydrogel (Superfos, Copenhagen) and blended with beta toxoid of *Clostridium perfringens* type C.

Pregnant gilts were vaccinated intramuscularly on two occasions: the first injection was given at around the time of service and the second was given two to four weeks before parturition. Non-vaccinated gilts from the same herd served as controls.

RESULTS

Effect of immune colostrum, immune milk and non-immune colostrum on K88-positive E. coli

K88-positive *E. coli* CN6913 was serially passaged, as described in Methods, through synthetic medium containing 10% (v/v) either immune colostrums (one of the six available), immune milk (one of the two available), non-immune colostrum or in synthetic medium without mammary secretions. The colostrum and milk were prepared from individual sows. It took three passages in one, four passages in four and five passages in the remaining immune colostrum for all ten colonies tested for K88 expression to be negative. All colonies were K88-negative after

seven passages in one of the immune milk samples, whilst in the second immune milk sample and in the non-immune colostrum most of the colonies became rough and auto-agglutinable between the third and eighth passage. However, colonies that remained smooth were K88-positive, as were all colonies tested at the end of the passage in the synthetic medium. Single K88-negative colonies derived after passage in immune colostrum were inoculated into synthetic medium and passaged ten times at 24 h intervals. They all remained K88-negative.

Tests were made to ascertain the minimum time required to obtain ten out of ten K88-negative colonies by passage in immune colostrum. CN6913 was passaged through one of the immune colostrums and subcultured at 18 h intervals. It took 72 h and four subculturings for ten out of ten colonies to be K88-negative. When CN6913 was passaged through the same immune colostrum with subculturing every 3 h it took a total of 30 h for all colonies tested to be K88-negative. With subculturing every 6 h a total time of 30 h was again required to obtain the same result.

Accumulation of viable cells of E. coli CN6913 derivatives in media with and without immune colostrum

When 4.5 ml of synthetic medium containing 10% (v/v) non-immune colostrum was incubated statically at 37 °C for 18 h after inoculation with 10^6 viable cells of either K88-positive *E. coli* CN6913 which had never been passaged through immune colostrum (K88⁺) or with its K88-negative derivative obtained after passage of CN6913 through immune colostrum (K88⁻) or with the K88-positive CN6913 that had remained K88-positive after some passages through immune colostrum (K88⁺⁺) the accumulation of viable cells of all three strains was almost identical (Fig. 1a). The exponential phase of growth of the cultures ceased after about 5–6 h and the number of viable cells remained more or less static over the subsequent 12 h period of incubation. However, when the experiment was repeated in the presence of immune colostrum the accumulation of viable K88-positive cells in the culture was less than that of K88-negative cells (Fig. 1b). This difference was first detected after about 2 h incubation of the culture. After 6–7 h incubation there were 3–4-times more viable K88-negative than K88-positive cells in the appropriate culture and this did not change appreciably over 18 h incubation. The two K88-positive CN6913 derivatives did not differ significantly in their behaviour.

These experiments were repeated on at least 10 occasions using different immune colostrums. The accumulation of viable cells of the K88-negative derivative of CN6913 was always greater than that of the K88-positive derivatives, sometimes by a factor of 10 to 1 by the early stationary phase of growth. The difference in viable counts between K88-positive and K88-negative cultures was apparent even as long as 18 h after the initial inoculation of the cultures.

Influence of incubation temperature on the effect of the immune colostrum on K88-positive and K88-negative E. coli

Incubation of CN6913 at 18 °C represses the expression of K88 fimbriae in batch culture. It was, therefore, of interest to test the influence of the lack of phenotypic expression of the K88 determinant on the stability of the determinant following passage in immune colostrum. An immune colostrum was chosen which at a 10% (v/v) concentration in synthetic medium gave rise to ten out of ten K88-negative CN6913 variants within five passages at 6 h intervals. The K88-positive CN6913 and its K88-negative derivative obtained following passage in immune colostrum were used. Each was separately inoculated into synthetic medium containing 10% (v/v) immune colostrum and grown at 18 °C with passaging at 24 h intervals 15 times in all. Samples were taken at each passage by streaking onto blood agar plates which were incubated at 37 °C to promote the expression of K88 fimbriae. Both strains grew at an apparently identical growth rate as judged by the optical densities of the cultures (results not shown). Examination of individual colonies taken from the blood agar plates showed that the cultures remained uncontaminated and the K88-positive CN6913 stably inherited the K88 plasmid determinant and the K88-negative CN6913 derivative remained K88-negative after a total of 360 h incubation. Thus phenotypic expression of the K88 determinant appears to be required for elimination of the K88 determinant from cells passaged in immune colostrum.

Fig. 1. Growth of *E. coli* CN6913 derivatives in synthetic medium. (a) With 10% (v/v) non-immune colostrum; (b) with 10% (v/v) immune colostrum. Δ , CN6913 K88-positive; $\blacksquare$, CN6913 K88-negative derived after passage in medium containing immune colostrum; $\bullet$, CN6913 K88-positive derived after passage in immune colostrum.

Effect of immune colostrum on the plasmids of E. coli strains harbouring the K88 determinant

Strain CN6913 harbours at least five large plasmids (Fig. 2). As shown earlier, passing this K88 positive strain in immune colostrum led to an increase in the proportion of K88-negative cells in the culture. At each passaging stage samples from K88-positive and K88-negative colonies grown on blood agar plates were examined for the presence of plasmid DNA. All colonies which were serologically K88-negative had lost the same plasmid (Fig. 2, track B). This plasmid encodes the K88 determinant (G. Dougan, unpublished result). All the other plasmids appeared unaffected by immune colostrum. To test this phenomenon further, the ability of immune colostrum to eliminate the K88 determinant carried on a different replicon was examined.

pMK005 is a multiple copy pBR322-based K88 recombinant plasmid carrying a 4.2 kbp piece of DNA cloned from a wild-type K88 plasmid (Kehoe *et al.*, 1981, 1983). Cells harbouring pMK005 were transferred to the serum-resistant *E. coli* strain B188 and the strain was passaged through immune colostrum in the usual way. K88-negative variants were detected after seven passages and all colonies were negative after ten passages. Selected colonies were examined for plasmid content and antibiotic resistance. All K88-negative variants examined on agarose gels had lost plasmid pMK005, a fact which was confirmed by testing for the ampicillin resistance marker encoded on the plasmid. In control experiments, pBR322 was not lost when strain B188 harbouring this plasmid was passaged through immune colostrum and pMK005 was not eliminated in medium containing non-immune colostrum.

Behaviour of E. coli strains expressing K99 during passage through immune colostrum

Attempts were made to eliminate K99 expression from two different *E. coli* strains which expressed the K99 fimbrial antigen by passaging the strains through immune colostrum. Strain B44 remained K99-positive even after ten passages through immune colostrum and no plasmids were lost from colonies screened for plasmid DNA. K99-negative colonies could be detected by screening large numbers but they occurred at the same frequency when the strain was passaged in synthetic medium without colostrum. Passaging *E. coli* strain SN62, which harbours a single 50 MDa plasmid encoding the K99 determinant, through immune colostrum resulted in the detection of K99-negative variants by the seventh passage. Some of these colonies regained the ability to express K99 antigen after reculturing on blood agar plates whereas others remained K99-negative even after repeated subculture. These stably K99-negative variants were screened for plasmid DNA and all ten tested still harboured the 50 MDa plasmid. Thus an unknown mechanism was responsible for the loss of K99 expression in these derivatives.

Fig. 2. Plasmid content of *E. coli* CN6913 derivatives. Track A, K88-positive variant grown on synthetic medium without colostrum; track B, K88-negative variant derived after passage through medium containing immune colostrum; track C, K88-positive variant derived after passage through medium containing immune colostrum. Samples were electrophoresed on a 0.8% (w/v) agarose gel and stained in ethidium bromide solution. The DNA was visualized using a 300 nm UV-light source. Arrow indicates position of missing plasmid in track B.

DISCUSSION

Serial passaging of the K88-positive *E. coli* strain CN6913 through medium containing immune colostrum resulted in the disappearance of K88-positive bacteria from the population, thus confirming the initial observations of Linggood & Porter (1978) and Linggood *et al.* (1979). The vaccine used in this study differed from that used in the original studies indicating that the property of K88 elimination was not unique to their vaccine. The active component of the vaccine is confirmed as the K88 antigen since colostrum collected from non-immunized animals or animals immunized with other vaccines failed to eliminate expression of K88. However, the efficiency by which different immune colostrum and milk batches eliminated K88-producing cells did show significant differences. Linggood *et al.* (1979) proposed that immune colostrum contained a factor that was actively curing plasmids encoding the K88 determinant from the bacteria and that a heat-stable component, distinct from anti-K88 antibodies, was responsible for the curing. Another possible explanation for the loss of cells expressing K88 from the population is that immune colostrum actively selects for cells that have spontaneously lost the K88 plasmid. Such cells appear at a low frequency in normal cultures. This latter mechanism is favoured by several observations reported in this paper. The accumulation in cultures of cells expressing K88 fimbriae was severely retarded, compared to similar cells which had lost the ability to express K88, in medium containing immune colostrum incubated at 37 °C. However, the accumulation of viable cells of these two types was indistinguishable in synthetic medium with or without non-immune colostrum. Thus medium containing immune colostrum would clearly favour accumulation of viable cells which no longer expressed K88. This effect was not manifested when the cultures were grown at 18 °C, conditions which phenotypically suppress

the expression of K88 fimbria. The mechanism by which the accumulation rate of K88-producing cells is apparently inhibited *in vitro* was not investigated and remains unknown.

Analysis of the plasmid content of K88-positive and K88-negative CN6913 variants showed that all K88-negative variants obtained following passage through medium containing immune colostrum had lost a single large plasmid known to encode the K88 determinant. This confirms that plasmid elimination and not some other unknown mechanism involving K88 gene expression is responsible for loss of K88 expression. The stability of the other four large plasmids harboured by CN6913 was not affected in immune colostrum suggesting that non-specific plasmid curing effects were not responsible for loss of the K88 plasmid. Thus it is unlikely that direct interference with plasmid replication is responsible for the elimination of the K88 plasmid. The availability of pMK005, a pBR322-based recombinant plasmid, enabled us to test this further. pBR322-based replicons exist in the bacterial cell in multiple copies, unlike wild-type K88 plasmids, and replicate using a different origin of replication and replication mechanism. pMK005 was readily eliminated from the population by immune colostrum whereas pBR322 was not. The only extra DNA carried by pMK005 compared to pBR322 encodes the K88 determinant, again suggesting that the K88 elimination mechanism is targeted against K88 fimbriae. *E. coli* strains harbouring pBR322-based plasmids spontaneously give rise to plasmid-free cells at a readily detectable frequency (Summers & Sherratt, 1984).

Expression of K99 was not as readily eliminated from populations of cells expressing this antigen, after passing through medium containing immune colostrum. The reason for this is not clear although it could involve the mechanisms by which K99 expression is regulated by the cell. Expression of K99 is more sensitive to medium composition than expression of K88. For example, K99 expression is readily repressed by the amino acid alanine (De Graaf *et al.*, 1980). Thus phenotypic suppression of K99 antigen may be important in stabilizing the K99 determinant. However, further investigations are required before this observation can be explained.

These results suggest that there is a strong selection against bacteria expressing the K88 antigen in populations of animals immunized with K88-containing vaccines. A number of serological variants of K88 have been identified and it has been proposed that these have been selected for in K88 immune animals (Gaastra & De Graaf, 1982). Spontaneous loss of the K88 plasmid determinant along with serological variation may be methods by which *E. coli* copes with the immunological pressures acting in the immune animal. The importance of plasmid elimination from bacteria growing *in vivo* as a means of avoiding host immunological defences should be further investigated.

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