ADHESION OF FIMBRIATE *ESCHERICHIA COLI* TO BOVINE MAMMARY-GLAND EPITHELIAL CELLS *IN VITRO*

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PLATE VI

THE possible significance of bacterial attachment to epithelial cell surfaces as an initial stage in the establishment of infection has attracted increasing attention in recent years. The K88 and K99 surface antigens of *Escherichia coli* have been found to mediate specific attachment of the organism to brush-border cells of the piglet (Jones and Rutter, 1972) and calf (Burrows, Sellwood and Gibbons, 1976). Furthermore, the role of fimbriae (pili) in epithelial-cell binding of group-A streptococci (Beachey and Ofek, 1976) and gonococci (Ward and Watt, 1972; Swanson, 1973) has been studied.

It has been postulated that the ability of a micro-organism to attach to bovine mammary-gland epithelium might be of significance in the pathogenesis of mammary infections of the lactating dairy cow, in which bacteria have to withstand flushing out during milking (Reiter and Bramley, 1975). Frost (1975) and Frost, Wanasinghe and Woolcock (1977) reported that *Streptococcus agalactiae* and *Staphylococcus aureus* could adhere to isolated ductular cells whilst *E. coli* could not. In addition, no evidence of adhesion was found when K88-positive and -negative strains of *E. coli* were used to infect the mammary glands of the mouse and the cow (Anderson, Burrows and Bramley, 1977). However, it was known that most mastitis isolates of *E. coli* were at least genotypically fimbriate and that fimbriae could mediate attachment of *E. coli* and *Salmonella* and *Shigella* spp. to epithelial cells (Duguid and Gillies, 1957; Ofek, Mirelman and Sharon, 1977). Furthermore it has been reported that a fimbriate strain of *Salmonella typhimurium* infected mice more readily by the oral route than did a non-fimbriate mutant of the same strain (Duguid, Darekar and Wheeler, 1976).

The present paper reports investigations on the role of fimbriae in the adhesion of *E. coli* to mammary-gland epithelial cells *in vitro*. An in-vitro test of adhesion to cryostat-prepared tissue sections is described.

MATERIALS AND METHODS

Organisms

E. coli strain P4 (O32 : K ?-NCDO 2070) was isolated from a clinical case of mastitis and shown to be virulent for the lactating cow (Bramley, 1976); fimbriae were demonstrated by

Received 27 July 1977; accepted 5 Sept. 1977.

electronmicroscopy and by the ability of the organism to produce mannose-sensitive haemagglutination of a freshly prepared suspension of red blood cells (3%) from guinea-pigs (GPRBC). The strain retains fimbriae even when subcultured on agar.

E. coli strain E4 (O86 : K61 : H8) was isolated from a human patient with infantile enteritis and produces fimbriae only when subcultured serially in broth. It is sensitive to the bactericidal activity of normal bovine serum and is avirulent for the lactating udder.

Growth of bacteria for the adhesion test

Organisms were maintained on nutrient agar (NA) slopes at 4°C and were also subcultured daily in trypticase soy broth (TSB) to enhance their production of fimbriae. Test organisms were subcultured in 5 ml of Lemco Broth (LB), the inoculum being taken either from a NA slope or TSB culture, depending upon the state of fimbriation required. The LB culture was incubated for 16–18 h at 37°C; the organisms were then washed three times in 0.01M phosphate-buffered saline (PBS) and diluted 1 in 10 in PBS. This diluted suspension contained approximately 10^8 colony-forming units (c.f.u.) per ml (range 0.4×10^8 to 2.0×10^8) and was used in the adhesion test.

Preparation of epithelial cell suspensions

Barren, lactating cows from the Institute herd, known to be free from mammary infection, were used. After the animals were killed, the teats were thoroughly cleaned and then removed, bearing with them a portion of the lactiferous sinus and lower mammary gland. This tissue was kept on ice and transported to the laboratory where the teat exterior was cleaned with 70% ethanol and the teat and lactiferous sinus were separated. The teat sinus (TS), lactiferous sinus (LS) and larger lactiferous ducts were exposed by dissection and washed with 75–100 ml of sterile PBS. The cells from the three areas of epithelium were then gently detached by means of a test-tube brush and suspended in 5 ml of PBS. Cells from the TS and LS were washed twice in PBS at 4°C and in most experiments the suspensions from the quarters of the udder were pooled to give a bulk teat- or lactiferous-sinus cell suspension. In the later experiments the teat- and lactiferous-sinus cell suspensions were bulked to give a single suspension. These suspensions were used in the test described below and they contained approximately 10^6 cells per ml (range 0.5×10^6 to 5×10^6).

In-vitro adhesion test methods

Suspension test. Equal volumes (1 or 2 ml) of bacterial and epithelial-cell suspensions were mixed and incubated at 37°C for 1 h on a roller rotating at 45 r.p.m. The samples were then washed three times with PBS at 4°C, slow centrifugation (120 g) being used to minimise cell damage and improve the separation of bacteria and epithelial cells. After the final wash, the samples were resuspended in PBS to half the original volume and 0.01-ml amounts were spread in duplicate over 1-cm² areas etched on glass slides. The films were air-dried, fixed with methanol and stained with polychrome methylene blue for 5 min. The numbers of epithelial cells per ml of the initial suspension, the numbers of bacteria adhering to 50 or 100 cells, and the percentage of cells showing adherent bacteria were measured. Fields were counted over the film at random except that thick clumps of cells were avoided. In each test a control suspension with no added bacteria was examined to ensure that no contaminating bacteria were visible.

Slide test. Teat sinus tissue was removed from killed lactating cows of the Institute herd and suitably-sized pieces were snap frozen in isopentane cooled with liquid nitrogen. Thick cryostat sections (6 µm) were cut and picked off the knife with a glass slide at room temperature. The section-bearing slides were stored at 4°C until required.

In the adhesion test, 20 ml of a washed suspension of *E. coli* in PBS (approximately 10^9 c.f.u. per ml) and a section-bearing slide were placed in a rotating plastic container and incubated at 37°C for 1 h at 45 r.p.m. The slide was then removed, rinsed thoroughly with PBS and fixed in 10% formalin in ethyl alcohol. Sections were stained with polychrome methylene blue for 5 min. and then dehydrated and mounted. A control slide incubated with sterile PBS was similarly processed.

Measurement of haemagglutinating (HA) activity of E. coli. A second 5-ml LB culture was incubated in parallel with the adhesion test culture at 37°C for 16–18 h. The cultures were centrifuged and the supernates discarded, leaving a small volume (<0.5 ml) in which the deposit was resuspended. These suspensions were then tested for their ability to produce mannose-sensitive haemagglutination of a 3% suspension of GPRBC (Duguid and Gillies, 1957).

Relative HA potencies were measured by resuspending the bacterial pellet in 0.5 ml of sterile 0.85% saline and preparing doubling dilutions up to 1 in 64. These were tested for their ability to haemagglutinate GPRBC suspension. The viable count of the bacterial suspension and the optical density at 650 nm of a 10-fold dilution in saline were measured.

RESULTS

Tests of adhesion of fimbriate and non-fimbriate Escherichia coli to mammary-gland epithelial cells in vitro

Adhesion tests were done in duplicate and a fresh cell suspension from a different cow was used for each. The numbers of bacteria adhering to the cells and the percentage of cells showing adherent bacteria are detailed in table 1.

A non-fimbriate suspension of strain E4 grown in LB seeded from a NA slope showed no HA activity for GPRBC and adhered poorly to the epithelial cells (fig. 1). Fimbriate suspensions of strains E4 and P4 grown from broth-culture inocula showed mannose-sensitive haemagglutination of GPRBC and striking adhesion to the epithelial cells (fig. 2). The variation in degree of adhesion detected in the first and second tests with strain E4 probably reflects differences in the state of fimbriation of the cultures used. The results obtained with the teat and lactiferous-sinus cell suspensions were similar.

Relationship between adhesion to epithelial cells and the degree of fimbriation

Seven broth cultures of *E. coli* strain E4 were prepared in such a manner that, at the time of testing, they had been subcultured daily in LB for 1, 2, 5, 8, 11, 14

TABLE I

Adhesion of Escherichia coli strain P4 and fimbriate and non-fimbriate cultures of E. coli strain E4 to mammary-gland epithelial cells in vitro

Strain and fimbrial state	Haemagglutination of 3% GPRBC	Viable count of organisms (as 10 ⁸ c.f.u.) per ml suspension	Number of epithelial cells (10 ⁶) per ml of		Number of <i>E. coli</i> adhering to 50 cells in		Percentage of 50 cells in the stated suspension showing adherent <i>E. coli</i>	
			TS	LS	TS	LS	TS	LS
<i>E. coli</i> P4 (fimbriate)	+	0.78	0.72	0.95	466	408	82	78
	+	1.2	0.56	1.2	336	227	76	68
<i>E. coli</i> E4 (fimbriate)	+	0.91	0.72	0.95	96	179	50	62
	+	1.2	0.56	1.2	425	491	84	84
<i>E. coli</i> E4 (non-fimbriate)	—	0.52	0.72	0.95	33	11	38	14
	—	0.77	0.56	1.2	9	8	14	10

GPRBC = A 3% suspension of guinea-pig red blood cells; TS = teat-sinus cell suspension; LS = lactiferous-sinus cell suspension.

or 17 successive days to produce cultures with graded degrees of fimbriation. The ability of suspensions prepared from these cultures to haemagglutinate GPRBC and to adhere to pooled teat- and lactiferous-sinus cell suspensions was then measured.

Details of growth and HA activity, and the adhesion test result may be seen in table II.

An increase in HA activity was associated with higher viable counts and an increase in optical density of the suspensions. The consequence of this was that the ratio of *E. coli* organisms to epithelial cells in the test mixtures increased with increasing HA activity. To counteract this bias, the haemagglutination titre has been related either to the viable count (coefficient 1) or to the optical density (coefficient 2). With either method of assessment, an increasing trend in HA activity was associated with an increase both in the numbers of *E. coli* adhering to 100 cells and in the percentage of cells showing adherent bacteria.

The effect of 2% D-mannose, skim and whole milk on the adhesion of fimbriate E. coli to epithelial cells

The ability of fimbriate organisms of *E. coli* strain P4 to adhere to isolated epithelial cells (pooled from the teat and lactiferous sinuses) was measured when the cells were incubated in (i) PBS, (ii) PBS+2% D-mannose, (iii) sterile skim milk, or (iv) aseptically-collected whole milk with a total bacterial count of <10 per ml, and a coli-aerogenes count of <1 per ml. After incubation the routine washing procedure with PBS was used.

The adhesion data are given in table III. Skim milk and PBS suspensions gave similar adhesion values. Striking reductions in the numbers of adherent *E. coli* were detected when either 2% D-mannose in PBS or whole milk was used as the suspending medium (11.5% and 48.5% of the PBS values respectively).

TABLE II

Haemagglutinating (HA) activity for guinea-pig erythrocytes and adhesion to mammary-gland epithelial cells of bacterial suspensions prepared from broth cultures of Escherichia coli strain E4 after daily subculture for different periods

Period of days during which <i>E. coli</i> E4 had undergone daily subculture in broth	(a) Viable count of organisms (as 10^9 c.f.u.) per ml of HA suspension	(b) Optical density at 650 nm of 1 in 10 dilution of HA suspension	(c) Haemagglutination titre	Haemagglutination coefficient		Number of <i>E. coli</i> * adhering to 100 cells	Percentage of cells showing adherent <i>E. coli</i>
				1 (c) : (a)	2 (c) : (b)		
1	4.5	0.75	...	...	...	19	15
2	2.5	0.65	...	...	...	40	26
5	6.5	0.90	8	1.23	8.9	455	76
8	7.0	0.90	4†	0.57	4.45	211	47
11	6.5	1.0	8†	1.23	8.0	415	68
14	7.5	1.0	8†	1.07	8.0	594	85
17	13.5	1.1	16†	1.28	14.5	982	78

* Range of viable *E. coli* per ml in adhesion test suspensions was 0.7×10^8 to 1.4×10^8 c.f.u. per ml.

† Trace reaction at next doubling solution.

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FIG. 1.—Epithelial cells after incubation with non-fimbriate *Escherichia coli* strain E4. Polychrome methylene blue (MB). $\times 1300$.

FIG. 2.—Epithelial cells showing adherent bacteria after incubation with fimbriate *Escherichia coli* strain E4. MB. $\times 1300$.

FIG. 3.—Section of teat sinus wall showing adherent bacteria after incubation with fimbriate *Escherichia coli* strain P4. MB. $\times 500$.

FIG. 4.—Section of teat sinus wall after incubation with non-fimbriate *Escherichia coli* strain E4. MB. $\times 500$.

TABLE III

*Effect of 2% D-mannose, skim and whole milk on the adhesion of E. coli P4 to mammary-gland epithelial cells**

Suspending medium	Number of <i>E. coli</i> † adhering to 100 cells	Percentage of cells showing adherent <i>E. coli</i>
PBS	398	75
PBS + 2% D-mannose	46	40
Skim milk	370	66
Whole milk	192	36

* The test bacterial suspension contained 10^8 c.f.u. per ml, and the test epithelial-cell suspension contained 2.4×10^6 cells per ml.

† Mean of three counts.

Adhesion studies with sections of bovine teat-sinus tissue

The attachment of fimbriate and non-fimbriate cultures of *E. coli* strain E4 and of fimbriate *E. coli* strain P4 was examined. Large numbers of adherent bacilli were found when fimbriate cultures of *E. coli* P4 and E4 were used, but few adherent organisms were found with a non-fimbriate culture of *E. coli* E4 (figs. 3 and 4). The adhesion seen was not specific for epithelial cells, and bacilli occurred in rather greater numbers on the subepithelial collagen layer. The numbers of adherent bacteria were greatly reduced when whole milk or 2% D-mannose was used as suspending medium.

DISCUSSION

These results contrast sharply with those previously reported by Frost (1975) who detected little or no adhesion of *E. coli*. He used *E. coli* isolated from faeces, but Frost *et al.* (1977) reported that they were unable to detect adhesion when *E. coli* strains associated with bovine mastitis were used. We used one such strain and one human strain of *E. coli* and found that both adhered to epithelial cells from the teat or lactiferous sinuses. Adhesion was associated with the ability of the organisms to agglutinate a suspension of guinea-pig erythrocytes. Haemagglutination and adhesion could be prevented by the addition of 2% D-mannose, and this indicated that both effects were mediated by bacterial fimbriae (Duguid and Gillies, 1957) and distinct from other haemagglutination mechanisms that may also be associated with adhesion of Gram-negative bacteria (Burrows *et al.* 1976; Jones, Abrams and Freter, 1976).

If the organisms used by Frost were phenotypically non-fimbriate, little or no adhesion would have been detected in his tests. Our observation that whole milk reduced adhesion *in vitro* is important and may indicate why adhesion of *E. coli* has not been demonstrated *in vivo* (Frost, 1975; Anderson *et al.* 1977). The mechanism of inhibition of *E. coli* adhesion in milk is unclear. As adhesion occurred readily in skim milk, the inhibition observed with whole

milk is possibly attributable to the adhesion of *E. coli* to milk fat-globule membranes. It has been shown that milk fat can inhibit the haemagglutination of red blood cells by K88 and K99 adhesin (Reiter and Brown, 1976).

The use of sections of teat-sinus tissue allowed adherent and non-adherent *E. coli* suspensions to be distinguished, but it did not easily allow a quantitative assessment of adhesion. In the present study the adhesion observed was relatively non-selective in that bacteria were seen adhering to tissue over the whole section, particularly to collagen, and not solely to cells lining the sinus. On the other hand, the technique has been satisfactorily used to demonstrate the specific attachment of K88-positive strains of *E. coli* to porcine intestine (Turvey; unpublished).

These results indicate that care should be taken in relating results of *in vitro* tests with isolated cells to pathogenic mechanisms *in vivo*; for example it is clear that suspending fluids such as PBS may allow non-physiological mechanisms to operate. Moreover the growth conditions for the organism may influence the outcome of the test; this was shown by the fimbrial *E. coli* model in the present study, the gonococcal model of Novotny *et al.* (1977) and the non-fimbrial attachment study of Jones *et al.* (1976) with *Vibrio cholerae*.

SUMMARY

The adhesion of a bovine and a human isolate of *Escherichia coli* to epithelial cells from the teat and lactiferous sinuses of the udder was examined. Adhesion was detected with bacterial suspensions that produced mannose-sensitive agglutination of guinea-pig red cells. Adhesion to epithelial cells could be inhibited by mannose and the degree of adhesion occurring with a suspension correlated with its haemagglutinating activity. This demonstrated that fimbriae were responsible for the adhesion. The observation that whole milk inhibited attachment of *E. coli* to cells *in vitro* indicates that such attachment may not occur *in vivo* in the lactating cow.

We thank Miss E. M. Hogben for her technical assistance and B. E. Brooker for his helpful advice and co-operation. The willingness of the staff of Alf Meade Ltd (Reading) to co-operate during the slaughter of animals is appreciated.

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