Demonstration by immuno-electronmicroscopy of antigenic heterogeneity among P fimbriae of strains of *Escherichia coli*

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Summary. The identification of P fimbriae on urinary strains of *Escherichia coli* isolated from urine was made (i) by observation of the patterns of mannose-resistant and eluting haemagglutination of erythrocytes of seven animal species (and including those of human $\bar{p}$ phenotype), and (ii) by haemagglutination-inhibition tests with hydatid-cyst fluid known to contain an analogue of P-fimbrial receptor. In tests with five different, pure P-fimbrial antisera prepared in rabbits, agglutinin titres of 37 P-fimbriate strains revealed differences in their reactivity; immuno-electronmicroscopy studies with the same five antisera showed that P-fimbriate strains were markedly different in the extent to which their P fimbriae were coated with antibody. The antigenic heterogeneity observed among P fimbriae is discussed with regard to the development of P-fimbrial vaccines.

Introduction

The ability of *Escherichia coli* to adhere to host epithelium, as observed, for example, in pyelonephritis and diarrhoea in man, is a significant determinant of pathogenicity (Svanborg Edén *et al.*, 1976; Gaastra and de Graaf, 1982). Adherence of bacteria to specific host-cell receptors is often associated with proteinaceous fimbriae (Duguid *et al.*, 1955), of which two kinds are commonly present in urinary isolates of *E. coli*: (i) type-1 fimbriae are associated with mannose-sensitive haemagglutination (Duguid and Old, 1980) and bind to uromucoid, Tamm-Horsfall protein (Ørskov *et al.*, 1980), but have no clear association with bacterial virulence in man; (ii) P fimbriae are associated with adhesion to P blood-group-specific glycosphingolipids on human uroepithelium and with virulence in upper urinary-tract infection in man (Svanborg Edén and Hansson, 1978; Korhonen *et al.*, 1982; Rhen *et al.*, 1983).

Previous studies have indicated the existence of antigenic heterogeneity among P fimbriae (Korhonen *et al.*, 1982; Hanley *et al.*, 1985; Parry and Rooke, 1985) and in this paper we further describe antigenic heterogeneity among P fimbriae and antigenic relationships among five P-fimbriate strains used for immunisation.

Materials and methods

Bacteria

Thirty-eight epidemiologically unrelated strains of *E. coli* isolated from urine samples were examined. These included: four strains from the National Collection of Type Cultures (NCTC), London; six from Professor W. Brumfitt, The Royal Free Hospital, London; three from Dr G. Källénus, Department of Bacteriology, National Bacteriological Laboratory, Stockholm, Sweden; four from Dr I. Ørskov, International Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, Denmark; one from Dr T. Korhonen, Department of General Microbiology, University of Helsinki, Helsinki, Finland; and 20 strains which had been isolated in significant numbers ($\geq 10^5$ /ml) from freshly voided urine specimens examined in this department in the course of routine diagnostic bacteriology. The collection included eight reference strains of known fimbrial-antigen type: C1212/77 (F7⁺); C1254/77 (F8⁺); 3669 (F9⁺); C1976/76 (F11⁺); C1979/79 (F12⁺); JR1 (P⁺); ER2 (P⁺); and SS10 (X⁺, i.e., of unknown receptor specificity, Dr G. Källénus, personal communication).

Culture media

Nutrient Agar (NA) and Nutrient Broth No. 2 (NB) were from Oxoid; phosphate-buffered broth (PBB) was NB with KH₂PO₄ 0.36% w/v and Na₂HPO₄ 0.64% w/v pH 7.0. Phosphate-buffered agar (PBA) was PBB with Davis New Zealand Agar 1.5% w/v. Broths were used in 10 ml amounts in cotton-wool-stoppered test tubes and

agar media in 20 ml amounts in 90 mm plastic petri dishes.

Conditions of culture

For production of type-1 fimbriae, each strain was cultured serially six times at 3-day intervals in NB incubated statically in air at 37°C, or six times at 7-day intervals at ambient temperature (c. 20°C) (Duguid *et al.*, 1955; Old, 1985). The bacteria were concentrated, by centrifugation and resuspension in saline, to c. 10^{10} cfu/ml. For the production of mannose-resistant and eluting haemagglutinins (MRE-HAs), strains were grown at 37°C on PBA for 24 h or in PBB for 48 h (Duguid *et al.*, 1979; Crichton, 1980). Bacteria from PBA cultures were resuspended in saline to c. 10^{12} cfu/ml; those from PBB cultures were concentrated as for NB cultures.

Haemagglutination tests

Bacterial suspensions were tested, in the presence or absence of α -methyl-D-mannoside on rocked tiles at 4°C and at ambient temperature, for agglutination of the erythrocytes of species of: fowl (F); guinea pig (G); horse (H); man, group O of P₁ or P₂ phenotypes (M); ox (O); pig (P); sheep (S); and the rare human $\bar{p}$ phenotype (Old and Crichton, 1986). Cultures producing mannose-sensitive haemagglutinin (MS-HA) agglutinated guinea-pig erythrocytes at both low and high temperatures in the absence of α -methyl-D-mannoside; cultures producing MRE-HAs agglutinated erythrocytes in the presence and absence of α -methyl-D-mannoside but did so better at 4°C than at 20°C and they also eluted from the erythrocytes when the test mixture was warmed to c. 50°C. Several patterns of MRE-HA have been reported (Duguid *et al.*, 1979); thus, MRE-HA reacting with erythrocytes of man, pig and sheep has been designated as pattern MPS (Crichton and Old, 1980). Strains that agglutinated in MRE fashion human P₁ or P₂ erythrocytes but not those of the $\bar{p}$ phenotype were P-fimbriate (Källénus *et al.*, 1981; Väisänen *et al.*, 1981). P-fimbriate strains were also identified in haemagglutination-inhibition tests with hydatid-cyst fluid (HCF) from sheep or tissue cultures infected with *Echinococcus granulosus*. HCF contains P₁ substance, a natural analogue of the glycoprotein receptor of P fimbriae. Rocked-tile tests were carried out as before except that bacterial suspensions, tested near and at dilutions of 1 in 5 and 1 in 10, were shaken at 4°C for 30 min before addition of erythrocytes (Parry *et al.*, 1984). Inhibition of MRE-HA of human erythrocytes of P₁ or P₂ phenotypes by HCF indicated the presence of P fimbriae.

Preparation of pure P-fimbrial antisera

Antisera were raised in rabbits by the methods of Adegbola and Old (1982) against five P-fimbriate strains of *E. coli*: JR1 (serogroup O4); ER2 (O4); 45700/77 (O1); 61675/77 (O75); and NCTC 5183 (O1). Unwanted O, H,

K and other non-P fimbrial antibodies were removed by repeated absorptions with packed bacterial cells from nutrient-agar and nutrient-broth cultures incubated at 18°C.

Agglutination tests

Each strain was examined in agglutination tests with five P-fimbrial antisera following the methods of Gillies and Duguid (1958).

Electronmicroscopy

Portions of bacterial cultures sampled before testing for HA activity were negatively stained with uranyl acetate (pH 4.6) 0.3% w/v as described before (Adegbola and Old, 1982). Grids were examined with a Jeol 100CX electronmicroscope; micrographs were taken of different kinds of fimbriae seen in different cultures.

Cultures were also negatively stained as above, after treatment with P-fimbrial antiserum used at a dilution of 1 in 10. Coating of fimbriae with antibody was estimated by electronmicroscopy and graded arbitrarily from 0 to + + + + (Adegbola and Old, 1982).

Results

Haemagglutinins

Thirty-two (84%) of the 38 strains produced MS-HA and all strains produced MRE-HA. The pattern of MRE-HA activity of any strain with the different species of erythrocytes was generally reproducible in identical tests performed on different occasions. Twenty-six (68%) of the 38 strains showed the same MRE-HA pattern regardless of whether they had been grown on PBA or in PBB: pattern MPS (produced by 20 strains) or variants of that pattern, e.g., GMPS (3 strains) and GHMOPS (2 strains); pattern FGMOP was produced by the X-HA⁺ reference strain SS10. Each of the remaining 12 strains produced MRE-HA of pattern MPS or its broader-spectrum variants but showed growth medium-dependent differences in the full spectrum of erythrocyte species agglutinated. Thus, eight strains showed a broader spectrum of haemagglutinating activity when grown in PBB than on PBA; the other four strains showed a narrower spectrum of haemagglutinating activity when grown in PBB than on PBA.

Altogether, 37 of the strains gave an MRE-HA pattern that was MPS or gave other patterns of broader spectrum that included MPS; they agglutinated human erythrocytes but not those of the $\bar{p}$ phenotype; and their haemagglutination of human P₁ or P₂ erythrocytes was inhibited by HCF. Thus,

these 37 strains (i.e., other than strain SS10) were P-fimbriate.

Fimbrial agglutinins

In tube agglutination tests with five pure P-fimbrial antisera (PFAS), none of the strains cross-reacted to the same degree with all five antisera. For example, in tests with PFAS raised against *E. coli* strain JR1, the homologous strain and two others gave a high titre (5120) of agglutination but reacted to moderate or low titres only with the other four PFAS. Nine strains gave moderate titres (1280–2560) and ten strains gave low titres (80–640) in tests with PFAS “anti-JR1”. In tests with the other four PFAS, homologous titres were shown by: two strains with PFAS “anti-ER2”; four strains with PFAS “anti-45700/77”; five strains with PFAS “anti-61675/77”; and seven strains with PFAS “anti-NCTC 5183”. Other strains showed moderate or low agglutination titres with the antisera or they were unreactive with them. Thus, strains showed wide variations in their cross-reactivities.

The affinity of fimbrial antibodies for fimbriae, first demonstrated by Gillies and Duguid (1958), has been successfully employed to distinguish antigenically different fimbriae on the same bacterium (Adegbola and Old, 1983) and morphologically indistinguishable types of fimbriae or fibrillae on the same bacterium (Smyth, 1982). Incubation of suspensions of the 38 bacterial strains with each of the five different PFAS, followed thereafter by negative staining for immuno-electronmicroscopy, revealed cross-reacting P fimbriae thickened by irregular deposits of antibody along their edges; other fimbriae on the same strain, sometimes on the same bacterium (see fig. 1) were not coated. The results of antibody-coating tests done on the 38 strains are summarised (table I). Different P-fimbriate strains exhibited marked differences in the degree of coating with these five PFAS (figs. 2–6), differences corresponding to those revealed by the agglutination tests.

P-fimbrial antigens

Serological cross-reactions among the five P-fimbriate immunising strains were demonstrated by slide agglutination and immuno-electronmicroscopy tests; the degree of activity was almost identical by both test methods. Two of the PFAS (“anti-JR1” and “anti-45700/77”) showed almost identical agglutination and coating reactions in tests with the other immunising strains but the

Table I. Antibody coating of fimbriae of 38 fimbriate strains of *E. coli* in immuno-electronmicroscopy tests with absorbed P-fimbrial antisera

Number of strains showing fimbrial coating	Coating* with absorbed P-fimbrial antiserum raised against <i>E. coli</i> strain no.				
	JR1	ER2	45700	61675	5183
4	+	+	+	+	+
1	+	+	+	+	–
5	+	+	+	–	+
1	+	+	+	–	–
2	+	+	–	+	+
4	+	+	–	–	+
2	+	+	–	–	–
1	+	–	+	–	+
1	+	–	–	+	–
1	+	–	–	–	–
5	–	+	+	+	+
2	–	+	+	+	–
1	–	+	+	–	+
1	–	+	–	+	+
2	–	+	–	+	–
1	–	–	+	–	–
4†	–	–	–	–	–

* Coating of fimbriae was: + = present (to any degree); – = absent.

† Including the X-fimbriate strain SS10.

other PFAS gave quite distinct patterns of reactivity (table II). Two-way cross-reactivity existed between PFAS and P fimbriae as follows: JR1 and ER2, 45700/77, and NCTC 5183; ER2 and 61675/77, NCTC 5183, and 45700/77; 45700/77 and NCTC 5183. One-way cross-reactivity existed between P-antiserum (NCTC 5183) and P fimbriae (61675/77). There was no activity between PFAS and P fimbriae of JR1 and 61675/77, 45700/77, and 61675/

Fig. 1. P-fimbriate bacillus from a PBB culture of *E. coli* strain JR1 grown at 37°C for 48 h showing interaction with P-fimbrial antiserum (PFAS) “anti-ER2”; P fimbriae coated (large arrow); other fimbriae uncoated (small arrows). Bar = 200 nm.

Figs. 2-4. Interaction of three P-fimbriate strains of *E. coli* with PFAS "anti-JR1": (2) strain P25 heavily coated, ++++; (3) strain NCTC5183 weakly coated, ++; (4) strain C1979/79 very weakly coated, +. Bar = 200 nm.

77 (table II). Because it was clear from the wide variation in their cross-reactivities that P fimbriae exhibited a marked degree of antigenic heterogeneity, cross-absorption studies were done on the five PFAS to determine any antigenic similarity between the various P-fimbrial types of the vaccine

strains. Residual activity after absorption was determined by slide agglutination and, if present, was considered to be due to a distinct antigenic grouping (Parry *et al.*, 1982; Parry and Rooke, 1985). Analysis of cross-reactions demonstrated four distinct P-antigenic determinants which occurred in different combinations of two in four of the immunising strains; one of these strains (61675/77) shared each of its two P determinants with other strains; the fifth strain (JR1) possessed one such determinant present also in three other strains (for details, see Yakubu, 1986).

Discussion

A high correlation between HA activity and the presence of fimbriae on extraintestinal isolates of *E. coli* was demonstrated by Duguid *et al.* (1979) who showed that MS-HA activity was associated with the presence of type-1 fimbriae and that MRE-HAs of different erythrocyte specificities were generally associated with the presence of different kinds of MRE fimbriae. In the present study, the HA test was essentially that of Duguid *et al.* (1979), one known to be suitable for the detection of fimbrial adhesins on uropathogenic strains of *E. coli* (Parry *et al.*, 1982; Old, 1985).

Though the pattern of MRE-HA activity of any strain was reproducible in tests made on different occasions, a minority (12 of 38) of strains sometimes showed differences in the spectrum of erythrocyte species agglutinated; these differences seemed to depend on the medium used for cultivation. It is important, therefore, that a range of culture media should be employed to detect the maximal expression of MRE-HA activity of all strains of *E. coli*. Caution is even more relevant for those P-fimbriate strains of *E. coli* that do not agglutinate human erythrocytes when grown on media traditionally chosen for MRE-HA production (Gander *et al.*, 1985; Yakubu, 1986). The susceptibility of different erythrocytes to agglutination by our P-fimbriate strains correlated roughly with their content of globoseries-glycolipid receptor. The erythrocytes of man, pig, sheep, pigeon, fowl, goat and dog, for example, contain the globoside receptor (Leffler and Svanborg Edén, 1980); again, a species of the globoseries is found as a minor component (globotriaosylceramide) in erythrocytes of rabbit and ox. Thus, although it is clear that identity of MRE-HA is not synonymous with identity of fimbrial type (Old, 1985), the combination of examination of MRE-HA pattern and inhibition of that MRE-HA activity by receptor analogue identified 37 of our 38

Figs. 5–6. P-fimbriae strain JR1 of *E. coli* showing interactions with different P-fimbrial antisera: (5) heavily coated, ++++ with PFAS “anti-ER2”; (6) weakly coated, ++, with PFAS “anti-5183”. Bar = 200 nm.

strains as P-fimbriae, as it had done before with other strains (Old and Crichton, 1986).

Agglutination titres of the 38 strains of *E. coli* with five absorbed P-fimbrial antisera showed wide variations in cross-reactivities suggesting a considerable degree of antigenic heterogeneity of P fimbriae, an observation supported by results from cross-absorption studies. In another study of invasive strains of *E. coli*, Hanley *et al.* (1985) also showed different antigenic variants of P fimbriae, despite their N-terminal amino-acid sequence homology as determined by immunochemical methods, and concluded that the receptor-binding domains, probably identical in all P-fimbriae strains, are immunorecessive. Again, a recent immunoprecipitation analysis of P fimbriae of *E. coli* strains has shown that P fimbriae on strains of serogroup O2 were serologically different from those on strains of serogroups O4 and O6 (Pere *et al.*, 1986). Our findings demonstrated that immunising strains of serogroups O1, O4 and O75, each

with its own quite distinct P-antigenic make-up, possessed common P-fimbrial determinants. These results emphasise again the serological complexity of P fimbriae in *E. coli*.

Antigenic variation is an important attribute of many pathogenic micro-organisms that enables them to evade host defences. Because fimbriae, as well as being important virulence factors, are excellent immunogens that readily interact with specific host defences, their production is under certain constraints (Klemm, 1985). Thus, fimbriae bacteria would be expected to respond to selective pressures by means of antigenic variation. Those parts of the fimbriae not contributing to the interaction of subunits or serving as binding sites would engage in substantial amounts of amino-acid substitutions to promote maximal immunological heterogeneity. These, presumably hypervariable, regions appear in K88 antigenic variants which, in common with P fimbriae, show variations involving “in-frame” insertions and deletions as well as

Table II. Antigenic cross-reactions among P adhesins of five different strains of *E. coli*

Strain no.	Receptor specificity of adhesins	Agglutination and fimbrial coating* in tests with absorbed P-fimbrial antiserum raised against strain no.				
		JR1	ER2	45700	61675	5183
JR1	P and MS	++++	++++	++++	0	++
ER2	P and MS	++++	++++	+++	++	+++
45700	P	++++	+†	++++	0	++
61675	P and MS	0	++++	0	++++	++
5183	P	++	+	+	0	++++

* Agglutination and coating of fimbriae were: ++++ = strong; +++ = moderate; ++ = weak; + = very weak; and 0 = not detected (i.e., agglutination titre was <40).

† Agglutination, however, was 0.

single-base changes in specific regions of the structural gene (Hagblom *et al.*, 1985), mechanisms also available to *Neisseria gonorrhoeae* for variation in its fimbrial proteins (Sparling *et al.*, 1986).

The importance of P fimbriae as virulence factors in strains of *E. coli* responsible for acute, non-obstructive pyelonephritis has been confirmed (Domingue *et al.*, 1985). Recognition of their role in infection has focussed much interest on the use of P fimbriae in vaccines against pyelonephritis in man. Vaccination of monkeys with purified P fimbriae offered protection against experimentally induced urinary-tract infections caused by heterologous P-fimbriate strains of *E. coli* (Svenson *et al.*, 1984). Despite the demonstrable antigenic heterogeneity among P fimbriae, we have shown, nevertheless, that some homology exists. Thus, it is theoretically possible that a vaccine based on a few representative kinds of P fimbriae might be effective against most uropathogenic strains of *E. coli* (Klemm, 1985); even then, its use would still be limited. Essential for the development of any such vaccine is the detection of a kind of P fimbria, or even a P-fimbrial fragment, that is present in most

strains, is immunogenic and shares functional and binding activities. If, however, the bacterial strategy used for P-fimbrial antigenic variation is similar to that in *N. gonorrhoeae* (Sparling *et al.*, 1986) then efforts aimed at using whole fimbriin protein for vaccine production might well be unprofitable because that fragment (the receptor-binding region) common to all strains is poorly immunogenic and unlikely to prime the host-immune system. Furthermore, the strain's ability to change its fimbrial antigenicity during infection enables it to be in advance of any host immunity stimulated by a static vaccine. The significance of antigenic heterogeneity of P fimbriae, as shown in this and other studies, will be appreciated fully only when the genetic mechanism of antigenic variation is completely understood. In the meantime it seems unlikely that a suitable vaccine against this important virulence factor can be developed easily.

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