Two Mannose-resistant Haemagglutinins on Enterotoxigenic *Escherichia coli* of Serotype O6:K15:H16 or H– Isolated from Travellers' and Infantile Diarrhoea

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Enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H– have been shown to cause MRHA of bovine erythrocytes (MRHA_{bov}), a characteristic associated with possession of so-called CFA (CFA/II) which facilitates adhesion to the intestinal mucosa. Using putative anti-CFA/II antisera, raised to whole cell vaccines of MRHA_{bov}⁺ prototype strains of different biotypes with subsequent absorption with their MRHA_{bov}⁺ variants, three immunologically distinct CS antigens were identified on enterotoxigenic strains of this serotype. CS1 antigen was found only on MRHA_{bov}⁺ isolates of biotype A (rhamnose-negative). Specific anti-CS1 antigen serum agglutinated rhamnose-negative strains, gave a single immunoprecipitate in double immunodiffusion against cell surface extracts of these bacteria, and only inhibited the MRHA_{bov} activity of biotype A strains. The presence of CS1 serologically correlated with the presence of a 16.3 kDal polypeptide band upon SDS–PAGE of extracts. CS2 antigen was identified on MRHA_{bov}⁺ strains of biotypes B, C and F (rhamnose-positive). Specific anti-CS2 antigen serum agglutinated only the latter strains, gave a single immunoprecipitate against cell surface extracts of only rhamnose fermenting strains, and only inhibited MRHA_{bov} activity of strains of these biotypes. The presence of CS2 antigen serologically was associated with the presence of a 15.3 kDal polypeptide band in SDS–PAGE patterns of extracts. The CS3 antigen was identified by immunodiffusion tests with extracts of most O6:K15:H16 or H– strains possessing CS1 or CS2 antigen, i.e. independently of biotype, and was associated with the presence of a 14.8 kDal polypeptide band by SDS–PAGE. Anti-CS3 antigen antibodies did not agglutinate nor did they possess haemagglutination inhibition activity. CS3 antigen was also found on an enterotoxigenic isolate series of serotype O8:K40:H9, which was MRHA_{bov}⁺ and lacked the CS1 and CS2 antigens. A number of CS2-only O-serogroup 6 strains were identified. Expression of MRHA_{bov} activity and the production of CS1, CS2 and CS3 antigens were phenotypically suppressed by growth at 18 °C. Loss of ST and also, in most instances, of LT production accompanied loss of MRHA_{bov} activity and loss of production of the CS1, CS2 and CS3 antigens. Thus, the findings demonstrate the presence of two serologically distinct, biotype-associated haemagglutinins with different molecular weight properties on MRHA_{bov}⁺ enterotoxigenic *E. coli* of serotype O6:K15:H16 or H– of human origin and of a third non-haemagglutinating surface-associated antigen common to most strains which may also facilitate adhesion.

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Abbreviations: CFA, colonization factor antigen; CS, coli surface-associated; HD, haemagglutinating dose; HI, haemagglutination inhibition; LT, heat-labile enterotoxin; MRHA, mannose-resistant haemagglutination; MSHA, mannose-sensitive haemagglutination; PBS, phosphate buffered saline; ST, heat-stable enterotoxin; TSB, trypticase soy broth.

INTRODUCTION

In recent years, considerable attention has been devoted to mechanisms of adherence of enterotoxigenic *Escherichia coli* of human origin to human intestinal epithelial cells (Evans *et al.*, 1975; Brinton, 1978; Evans & Evans, 1978; Thorne *et al.*, 1979; Deneke *et al.*, 1980, 1981; Wadström *et al.*, 1980). The MRHA property for certain erythrocyte species has been particularly useful in screening enterotoxigenic *E. coli* of human origin for adherence properties of potential significance in intestinal colonization (Ørskov & Ørskov, 1977; Evans *et al.*, 1977; Evans & Evans, 1978; Freer *et al.*, 1978; Gross *et al.*, 1978; Evans, D. J. *et al.*, 1979; Cravioto *et al.*, 1979*b*; Smyth *et al.*, 1979; Deneke *et al.*, 1981). This has led to the identification on enterotoxigenic *E. coli* of two serologically distinct types of haemagglutinating fimbriae, viz., CFA/I and CFA/II (Evans *et al.*, 1975, 1978, 1979; Evans & Evans, 1978). CFA/I causes MRHA of human, bovine and chicken erythrocytes and is found on enterotoxigenic *E. coli* of serogroups O15, O25, O63, O78, O128 and O153 (Ørskov & Ørskov, 1977; Evans & Evans, 1978; Gross *et al.*, 1978; Cravioto *et al.*, 1979*b*; Evans *et al.*, 1979; Smyth *et al.*, 1979; Wadström *et al.*, 1979; Bäck *et al.*, 1980*b*). CFA/II causes MRHA of bovine and chicken erythrocytes, but not of human erythrocytes and is associated with strains of serogroups O6 and O8, in particular, but also with serogroups O80 and O85 (Ørskov & Ørskov, 1977; Evans & Evans, 1978; Evans, D. J. *et al.*, 1979; Smyth *et al.*, 1979; Wadström *et al.*, 1979).

The present investigation arose from an attempt to produce a specific CFA/II antiserum according to the procedure of Evans & Evans (1978), as in a three-and-a-half year study of tourist diarrhoea among homecoming Swedish residents, enterotoxigenic *E. coli* of serogroups O6 and O8 accounted for approximately 35% of enterotoxigenic isolates (Bäck *et al.*, 1980*a*). In contrast to the findings of Evans & Evans (1978), not all enterotoxigenic isolates of the serotype O6:K15:H16 or H- (Ørskov & Ørskov, 1977) which caused MRHA of bovine erythrocytes (MRHA_{bov}⁺) [i.e. which were putative CFA/II⁺ strains (Evans & Evans, 1978)] reacted with the supposed specific anti-CFA/II serum (Smyth, 1980).

Accordingly, the aims of the present study were (i) to determine whether antigenic differences existed within this serotype of MRHA_{bov}⁺ enterotoxigenic *E. coli*, (ii) to determine the basis for any such antigenic differences detected, (iii) to determine the spectrum of erythrocyte species agglutinated in a mannose-resistant manner by such strains and (iv) to determine whether biological and antigenic differences correlated with the different biotypes within this O:K:H group of enterotoxigenic *E. coli* (Scotland *et al.*, 1977). Two serologically distinct, surface associated haemagglutinins mediating MRHA_{bov} were found, the presence of which correlated with the rhamnose fermentation phenotype of the strains. In addition, a third surface-associated antigen was present which was common to most MRHA_{bov}⁺ enterotoxigenic *E. coli* of serotype O6:K15:H16 or H-.

METHODS

Chemicals. LE agarose was obtained from Marine Colloids, Rockland, Me., U.S.A.; bovine serum albumin fraction V, mucic acid, and phenylmethylsulphonyl fluoride from Sigma; adonitol, dulcitol, raffinose, rhamnose and sorbose from both Merck and Fluka, Buchs, Switzerland; D-mannose from Merck; Dorset egg medium without crystal violet from Oxoid; TSB from BBL; all other chemicals were of analytical grade or best grade commercially available.

Bacteria. Pertinent characteristics and sources of the *Escherichia coli* strains used in this study are given in Table 1. Strains were preserved in TSB containing 15% (w/v) glycerol at -70°C and Dorset egg medium at 4°C.

Biotyping. The scheme developed by Scotland *et al.* (1977) was used. Carbohydrate breakdown tests on adonitol, dulcitol, raffinose, rhamnose and sorbose were performed in heart infusion broth containing 1% (w/v) of the saccharide with bromocresol purple as indicator. The mucate test was prepared according to Edwards & Ewing (1972) with bromothymol blue as indicator.

Serotyping. O and K antigen determinations of strains supplied by E. Bäck and of Ethiopian isolates (Table 1) had been previously performed by B. Kaijser, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden. Strains C922a, B303dEt and B34212j were O:K:H serotyped by F. & I. Ørskov, Collaborative Centre for Reference and Research on *Escherichia* and *Klebsiella*, Statens Seruminstitut, Copenhagen, Denmark. The serotypes of strains from M. Gurwith (Table 1) were accepted as supplied. H antigen determinations were performed by agglutination using an anti-H16 antiserum obtained by absorption of a polyvalent O6:K15:H16

Table 1. *Characteristics of Escherichia coli strains*

Strain/Year of isolation*	Serotype	Enterotoxin production	MRHA _{bov} †	Origin‡	Country of origin	Source§	Reference
[C921a, b, d/77	O6 : K15 : H16	LT*ST+	+	TA	Spain	EB	Bäck <i>et al.</i> (1980a) Smyth <i>et al.</i> (1979)
C921f/77	O6 : K13 : H+	LT*ST—	—	TA	Spain	EB	
[C922a, b, e, f/77	O6 : K15 : H16	LT*ST+	+	TA	Spain	EB	
C922c/77	O6 : K15 : H16	LT*ST—	—	TA	Spain	EB	
C91a, b, c, d, e, f/78	O6 : K15 : H16	LT*ST+	+	TA	France	EB	Bäck <i>et al.</i> (1980a)
C407a/78	O6 : K15 : H16	LT*ST+	+	TA	Spain	EB	
C470e/78	O6 : K15 : H—	LT*ST+	+	TC	Tunisia	EB	Bäck <i>et al.</i> (1980a)
C666c/78	O6 : K15 : H16	LT*ST+	+	TA	Greece	EB	Bäck <i>et al.</i> (1980a)
C728b/78	O6 : K15 : H16	LT*ST+	+	TA	The Gambia	EB	Bäck <i>et al.</i> (1980a)
C735c/78	O6 : K15 : H16	LT*ST+	+	TA	Tunisia	EB	Bäck <i>et al.</i> (1980a)
C806c/78	O6 : K15 : H16	LT*ST+	+	TA	France	EB	Bäck <i>et al.</i> (1980a)
C846e/78	O6 : K15 : H16	LT*ST+	+	TA	Sri Lanka	EB	Bäck <i>et al.</i> (1980a)
E90a/79	O6 : K15 : H16	LT*ST+	+	TA	India	EB	Bäck <i>et al.</i> (1980a)
E583a/79	O6 : K15 : H16	LT*ST+	+	TA	Cuba	EB	Bäck <i>et al.</i> (1980a)
E596a/79	O6 : K15 : H16	LT*ST+	—	TA	Peru	EB	Bäck <i>et al.</i> (1980a)
E628b/79	O6 : K15 : H16	LT*ST+	+	TC	Mozambique	EB	Bäck <i>et al.</i> (1980a)
E661a/79	O6 : K15 : H16	LT*ST+	+	TA	USSR	EB	Bäck <i>et al.</i> (1980a)
B303dEt/77	O6 : K15 : H16	LT*ST+	+	I	Ethiopia	RM	Bäck <i>et al.</i> (1980b) Smyth <i>et al.</i> (1979)
[34318a, c, e, f/78	O6 : K15 : H16	LT*ST+	+	I	Ethiopia	GS	
3699/78							Stintzing <i>et al.</i> (1982)
[34334f/78	O6 : K15 : H—	LT*ST+	+	I	Ethiopia	GS	Stintzing <i>et al.</i> (1982)
E3772/78							
[E3773/78	O6 : K15 : H16	LT*ST+	+	I	Ethiopia	GS	Stintzing <i>et al.</i> (1982)
34413b/78							
4071/78	O6 : K15 : H16	LT*ST+	+	I	Ethiopia	GS	Stintzing <i>et al.</i> (1982)
342(E4904)/76	O6 : H16	LT*ST+	+	SIDS	Canada	MG	Brunton <i>et al.</i> (1980)
344(E4833)/76	O6 : H16	LT*ST+	+	SIDS	Canada	MG	Brunton <i>et al.</i> (1980)
B34212b, c, h, j/78	O8 : K40 : H9	LT*ST+	—	I	Ethiopia	GS	Stintzing <i>et al.</i> (1981)
H10407	O78 : H11 : CFA/I+	LT*ST+	+	A	Bangladesh	DE	Evans <i>et al.</i> (1975)
H10407-P	O78 : H11 : CFA/I—	LT*ST—	—	LS		DE	Evans <i>et al.</i> (1975)
NCTC 8623	O125 : K70 : H19	LT—ST—	—	G	England	JHF	Sweeney & Freer (1979)
EC 25/06/75	O6 : K2a2c : H1	LT—ST—	+	UTI	Sweden	RM	—
C921b-1¶	O6 : K15 : H16 or H— or O6 : H16	LT*ST+	+	LS		CJS	Wadström <i>et al.</i> (1979) Wadström <i>et al.</i> (1980a, b)
C922a-1¶							
C922b-9, -13, -20							
342-15 _x -17							
E583a-7							

(continued)

Table 1 (*continued*)

Strain/Year of isolation*	Serotype	Enterotoxin production	MRHA _{bov} †	Origin‡	Country of origin	Source§	Reference
C921b-2¶ C922a-2¶ C922b-6 B303d-1,-5,-13,-20 342-46,-59 C91f-6,-29,-31 34334f-50 C922b-19,-22 E583a-14	O6:K15:H16 or O6:H16 O6:K15:H16	LT-ST- LT+ST-	- -	LS LS		CJS CJS	Wadström <i>et al.</i> (1979) Wadström <i>et al.</i> (1980a,b) —

* Designations a, b, c etc. indicate single colony isolates from the same patient upon primary culture of stool specimens on Endo agar; square bracketed strains indicate strains from same patient; numbers after strain designation, e.g. C922a-1, indicate single colony substrains from parent strain elsewhere in Table.

† Mannose-resistant haemagglutination of bovine erythrocytes; reactions determined by author and cited by Bäck *et al.* (1980a,b), Stintzing *et al.* (1981, 1982), Smyth *et al.* (1979) and Wadström *et al.* (1979).

‡ TA, travellers' diarrhoea, adult; TC, travellers' diarrhoea, child; I, infantile diarrhoea; G, gastroenteritis; A, adult cholera-like diarrhoea; SIDS, sudden infant death syndrome; LS, laboratory substrain from single colony; UTI, urinary tract infection.

§ EB, Erik Bäck, Regionsjukhuset, Örebro, Sweden; RM, Roland Möllby, National Bacteriological Laboratory, Stockholm, Sweden; GS, Gudmund Stintzing, National Bacteriological Laboratory, Stockholm, Sweden; MG, Marc Gurwith, Division of Infectious Diseases, Wadsworth Veterans' Hospital, Los Angeles, Calif., U.S.A.; JHF, John H. Freer, Department of Microbiology, University of Glasgow, Glasgow, Scotland; DE, Dolores and Doyle Evans, University of Texas Medical School at Houston, Houston, Tex., U.S.A.

¶ Strain designations in parentheses are the Colindale numbers assigned to the strains originally received from M. Gurwith (Scotland *et al.*, 1977).

¶ Only strains indicated used in literature cited.

antisera, kindly supplied by F. & I. Ørskov, with live and autoclaved cells of MRHA_{bov}⁺ enterotoxigenic *E. coli* of serotype O6:K15:H-.

Enterotoxin tests. The enterotoxigenic strains in Table 1 had been shown to produce LT using the Y1 adrenal cell test (Söderlind & Möllby, 1978; Bäck *et al.*, 1979, 1980a, b; Stintzing *et al.*, 1981, 1982; Brunton *et al.*, 1980) or using the Chinese hamster ovary cell test (Guerrant *et al.*, 1974). Production of ST was monitored by the infant mouse test according to Giannella (1976), with an intestinal weight to remaining carcass weight ratio of ≥ 0.090 being considered as positive (Olsson & Söderlind, 1980). Determination of enterotoxin production by MRHA_{bov}⁺ substrains and MRHA_{bov}⁻ variants (Table 1) and repetition of tests on particular strains was performed by these procedures. The author thanks E. Olsson, Department of Bacteriology and Epizootiology, Biomedical Centre, Uppsala and G. Sigstam, Department of Bacteriology, Karolinska Institute, Stockholm, Sweden for performing these ST and LT tests, respectively.

Haemagglutination. Strains were routinely grown on modified CFA agar medium (Evans *et al.*, 1977) at 37 °C for 18 h. Bacteria were suspended directly from CFA agar in 0.15 M-NaCl using cotton swabs to obtain homogeneous suspensions. The growth from half of a CFA agar plate (90 mm) when suspended in 1 ml NaCl yielded about 10^{10} bacteria ml⁻¹. All erythrocytes were washed three times with 0.15 M-NaCl and suspended to 3% (v/v) with respect to initial blood volume. The following erythrocyte species were tested: cat, chicken, cow, dog, guinea-pig, hen, horse, human Group A, mouse, pig, rabbit and sheep.

Haemagglutination tests were performed by mixing one drop each of bacterial suspension, erythrocyte suspension and 0.1 M-D-mannose in 0.15 M-NaCl or 0.15 M-NaCl. Glass cavity slides or glass tiles with oval or round depressions were rocked gently with a swirling action at 20 °C–22 °C. Strong agglutination reactions occurred within 30 s. Mixtures were subsequently incubated at 4 °C for up to 20 min before another reading was made.

In addition, strains were sequentially subcultured three times at 48 to 72 h intervals in 10 ml TSB in test tubes with incubation without shaking at 37 °C (Nowotarska & Mulczyk, 1977; Duguid & Old, 1980). Cells were sedimented by centrifugation (3250 g, 10 min, 20 °C) and suspended gently in 1 ml of 0.15 M-NaCl for haemagglutination tests in the presence and absence of D-mannose.

Titration of haemagglutinating activity was performed using doubling dilutions of bacterial suspensions in 50 µl of 0.15 M-NaCl to which 50 µl each of 0.1 M-D-mannose and erythrocyte suspension was added. One HD of bacteria was defined as the amount giving complete or virtually complete haemagglutination of bovine erythrocytes in 5 min at 20 °C.

Selection of MRHA_{bov} variants. This has been detailed previously (Wadström *et al.*, 1979). Briefly, colonies were subcultured from modified CFA agar plates to fresh CFA agar plates (four per plate), incubated at 37 °C for 18 h, and bacterial suspensions tested for the MRHA_{bov} property. To select variants in Table 1, any number of colonies from 5 to 1000 had to be tested to find at least one MRHA_{bov} variant for any particular strain. MRHA_{bov} variants were checked for biotype and O6:H16 serotype.

Preparation of antisera against cell surface-associated antigens of MRHA_{bov} strains. The procedure and schedule of Evans *et al.* (1975) for production of anti-CFA/I and anti-CFA/II sera (Evans & Evans, 1978) was followed using formalin-treated vaccines of strains C922a-1, B303dEt, C91f and 34334f (Table 1). Two rabbits each were used with the first two named strains and one rabbit each with the latter two strains. In each instance, an antiserum pool was absorbed with live and autoclaved cells of a MRHA_{bov} variant or substrain of the same parent culture, viz., strains C922a-2, B303d-5, C91f-6 and 34334f-50, respectively (Table 1).

The immunoglobulin fractions were partially purified from the absorbed sera by ammonium sulphate precipitation, followed by sequential dialysis against acetate buffer and distilled water according to Harboe & Ingild (1973). Precipitate was removed by centrifugation before final dialysis against 0.1 M-NaCl containing 15 mM-Na₂N₃ as preservative. Immunoglobulins were concentrated threefold with respect to serum volume (used undiluted in immunodiffusion tests) and stored at 4 °C. Antisera termed anti-C922a-1, anti-B303dEt, anti-C91f and anti-34334f refer to the partially purified immunoglobulin preparations from absorbed sera.

Anti-CFA/I serum was prepared in an identical manner using strain H-10407 as the vaccine and H-10407-P for absorption (Evans *et al.*, 1975; Smyth *et al.*, 1979; Wadström *et al.*, 1979). Specific antisera against CS antigens designated CS1, CS2, and CS3 (see Results) were obtained by cross-absorption of sera. Anti-CS3 serum was obtained by absorption of anti-34334f serum with strain C91f, and anti-CS1 and anti-CS2 sera, by absorption of anti-C922a-1 and anti-34334f sera, respectively, with strain B34212j. In addition, anti-C91f serum served as an anti-CS2 serum by its nature (see Fig. 1 and Results).

Agglutination. Bacterial suspensions (about 10⁹ bacteria ml⁻¹ of 0.15 M-NaCl) prepared from overnight growth on CFA agar plates were tested by slide agglutination procedures using saline or prevaccination rabbit serum for controls.

Agglutination titres of antisera were determined by serial doubling dilutions of antisera (50 µl) in PBS containing 0.02% (w/v) bovine serum albumin (Evans & Evans, 1978) using 96-well, U-shaped microtitre plates (Linbro Chemical Co., New Haven, Conn., U.S.A.). Suspensions of CFA agar-grown bacteria were adjusted to an A₆₀₀ = 2.0 in a 10 mm lightpath (Evans & Evans, 1978). Portions (50 µl) of the bacterial suspensions were added to the antiserum-containing wells, the plates sealed and the mixtures incubated at 37 °C for 2 h. The agglutinating titre was read after incubation overnight at 4 °C. The titre was defined as the dilution of antiserum giving 50–100% agglutination of the bacteria. Titres of immunoglobulin preparations from absorbed antisera were all expressed relative to serum volume.

Haemagglutination inhibition. Antisera (25 µl) were diluted in doubling dilution series in PBS with 0.02% bovine serum albumin in wells of glass tiles. To each well, 25 µl of bacterial suspension was added. The bacterial suspensions had been adjusted to contain 8 to 16 HD of bacteria per sample, i.e. the bacterial suspensions had different cell densities (A₆₀₀ values). In HI tests most authors use 4 HD; the higher dose was chosen herein as it gave clearcut haemagglutination reactions with different strains, especially those fermenting rhamnose (see Results). After incubation of the mixtures for 1 h at 37 °C, they were allowed to cool for 10 min to room temperature (18 °C to 20 °C). To each well 25 µl 0.1 M-D-mannose and 3% bovine erythrocyte suspension was added. The dilution of antiserum showing inhibition of haemagglutination after incubation at 20 °C for 5 min followed by 20 min at 4 °C was taken as the HI titre.

Preparation of extracts containing cell surface-associated antigens. A method similar to that first used to isolate K88 antigen (Stirm *et al.*, 1967) and which has subsequently been used in the isolation of K99 antigen (Morris *et al.*, 1977), CFA/I (Freer *et al.*, 1978) and F7 antigen (Ørskov *et al.*, 1980) was employed. Bacteria were harvested from five CFA agar plates in 2 ml 0.15 M-NaCl, pH 7.0 using cotton-tipped swabs. The suspensions were heated at 60 °C for 20 min and the bacteria sedimented by centrifugation (27 000 g, 10 min, 4 °C). Supernatant fractions were filtered through membrane filters (0.65 µm pore diam., Millipore). Extracts were stored as 50 or 100 µl portions at -70 °C until used in immunodiffusion or SDS-PAGE studies (see below). After thawing once, any remaining extract was discarded. In certain control experiments (see Results), extracts were prepared by shearing bacteria in a homogenizer (6 × 1 min, 20 000 rev. min⁻¹ on ice) with subsequent processing as heat-released extracts. To control for proteolysis, heat extracts were prepared from saline suspensions of bacteria in the presence and absence of 1 mM-phenylmethylsulphonyl fluoride using the same batches of cells.

Double immunodiffusion. Tests were performed in 1% (w/v) agarose gels in sodium barbital-barbital buffer containing Na₂N₃ (I = 0.02, pH 8.6) (Weeke, 1973). Glass slides (50 × 50 mm) were lightly coated with agarose. Gels were cast on a levelling table to give a volume-to-surface ratio of 0.14 ml agarose cm⁻². Wells of 2.5 mm diam. were routinely used with an intercentre distance of 7.5 mm. In the absorption experiments (see Results), 3 or 4 mm diam. wells were used for antiserum or antigen or both. Wells were charged with 10 µl (20 µl with larger wells) of

antigen preparation or antiserum and the slides incubated at 20 °C to 22 °C for 48 h. Drawings were made of immunoprecipitates before gels were washed, dried, and stained with Coomassie brilliant blue according to Weeke (1973). Immunoprecipitates and reactions of identity or non-identity which were not visible in the unstained gels were added to the original diagrams in a distinguishing colour.

Preparation of immunoprecipitates for immunization. Heat-released extracts of strain C922a-1 were diffused against anti-C922a-1 serum to obtain CS1 immunoprecipitates, and heat extracts of strain B34212j, against anti-B303dEt serum to obtain CS3 immunoprecipitates. Gels were washed with 0.1 M-NaCl and pressed between washes according to Weeke (1973). Immunoprecipitates were excised after washing for 48 h using a 1.5 mm diam. gel punch. Gel discs from two 9-well immunoplates were pooled for each immunogen into 2.0 ml of 0.15 M-NaCl and the mixtures homogenized using glass tissue grinders until the agarose was visibly dispersed. Immunogens were stored at -20 °C until used as small samples. Preparations were only used when subsequent staining of immunoplates provided evidence of precision in excision. Rabbits received the immunogens in Freund's incomplete adjuvant as five equal doses: day 0, intracutaneously at multiple sites on the back; days 7, 14, 21 and 40, intramuscularly.

Antisera were absorbed with strain C922a-2 (live and autoclaved cells) and with strain B34212j (autoclaved cells only), respectively. The specificities of these antisera were checked by immunodiffusion tests and agglutination tests before use. The anti-CS1 and anti-CS3 sera obtained in this manner behaved identically to those obtained by cross-absorption (see above).

SDS-PAGE. For SDS-PAGE, samples adjusted to a concentration of 0.5 mg protein ml⁻¹ were suspended in 1% (w/v) SDS, 2% (w/v) mercaptoethanol, 20 mM-Tris, 8 mM-sodium phosphate and 5% (w/v) glycerol, pH 7.8. Preparations were boiled for 5 min immediately prior to application to the polyacrylamide gel slabs.

The discontinuous SDS gel system was essentially that of Laemmli (1971) with slight modifications. The separation gel (0.5 mm thick, 0.1% SDS, 15% w/v polyacrylamide) had a pH of 8.9. The stacking gel (pH 7.8) comprised 5% (w/v) acrylamide, 62.5 mM-Tris, 56 mM-sodium phosphate and 0.1% (w/v) SDS. The electrode buffer contained 29 mM-Tris, 220 mM-glycine and 0.1% SDS, pH 8.6. After electrophoresis, the gels were stained with Coomassie brilliant blue according to Fairbanks *et al.* (1971), mounted on filter paper, and dried under vacuum. Molecular weight standards were bovine serum albumin (68 kDal), ovalbumin (45 kDal), carbonic anhydrase (29 kDal), soybean trypsin inhibitor (21 kDal), myoglobin (17.8 kDal) and cytochrome *c* (12.4 kDal).

RESULTS

Initial observations

Anti-C922a-1 serum was initially tested in the autumn of 1978 as a putative anti-CFA/II serum against the isolate series C921a, b, d, C922a, b, f, C91a-f and strain B303dEt (Table 1), and seven Ethiopian isolates and one East German strain of enterotoxigenic *E. coli* not included in the present study but also of serotype O6:K15:H16 or O6:K15, which together comprised the 21 MRHA_{bov}⁺ strains from 10 patients included in the study of Smyth *et al.* (1979). All except strains B303dEt and C91a-f were agglutinated by anti-922a-1 serum. Using the biotyping scheme of Scotland *et al.* (1977), the agglutinable strains were of biotype A, whereas strains B303dEt and C91a-f were of biotypes B and C, respectively. Accordingly, strains B303dEt, C91f and 34334f, a non-agglutinable MRHA_{bov}⁺ strain of biotype F, were chosen as representative MRHA_{bov}⁺ strains of biotypes other than biotype A from which to select MRHA_{bov}⁻ variants and were used for production and absorption of antisera, respectively.

Agglutination and double immunodiffusion tests with prototype MRHA_{bov}⁺ strains of biotypes A, B, C and F and their MRHA_{bov}⁻ variants

The absorbed antisera were titrated against the homologous and heterologous MRHA_{bov}⁺ strains and their MRHA_{bov}⁻ variants (Table 2). The anti-C922a-1 serum agglutinated only the homologous strain. The anti-B303dEt, anti-C91f and anti-34334f sera did not agglutinate strain C922a-1, but each gave equivalent titres against the homologous and the other two heterologous MRHA_{bov}⁺ strains. The MRHA_{bov}⁻ variants of these prototype strains were not agglutinated to any significant extent by any of these four antisera. Heated bacterial suspensions (121 °C, 2 h) were not agglutinated by any of the antisera. Neither was any of these strains agglutinated by the anti-CFA/II serum.

Immunodiffusion tests with heat-released extracts gave the homologous and heterologous reactions shown in Fig. 1. Three distinct immunoprecipitates were recognized. The antigens

Table 2. Agglutination titres of absorbed antisera against prototype MRHA⁺_{bov} strains of enterotoxigenic *Escherichia coli* of O-serogroup 6 and their MRHA⁻_{bov} variants

Strain	MRHA _{bov}	Biotype*	Agglutinating antibody titre of antisera to			
			C922a-1	B303dEt	C91f	34334f
C922a-1	+	A	1:32	<1:2	<1:2	<1:2
C922a-2	-	A	<1:2	1:2	<1:2	<1:2
B303dEt	+	B	<1:2	1:128	1:128	1:1024
B303d-5	-	B	<1:2	1:2	<1:2	<1:2
C91f	+	C	<1:2	1:128	1:128	1:1024
C91f-6	-	C	<1:2	1:2	<1:2	<1:2
34334f	+	F	<1:2	1:64	1:128	1:1024
34334f-50	-	F	<1:2	<1:2	<1:2	<1:2

* According to Scotland *et al.* (1977); biotype A is rhamnose-negative and biotypes B, C and F are rhamnose-positive

giving rise to these immunoprecipitates were designated CS antigens, viz., CS1, CS2 and CS3 (Fig. 1). CS1 antigen was specific to strain C922a-1 and precipitins to it were only found in the homologous antiserum. CS2 antigen was detected in extracts of strains B303dEt, C91f and 34334f using any of the antisera to these three strains, but not in extracts of strain C922a-1. Antigen CS3 was present in extracts of strains C922a-1, B303dEt and 34334f, despite the lack of cross-agglutination of strain C922a-1 with antisera to either of the other two strains and vice versa (Table 2). The precipitins against CS3 were of lower titre in the anti-C922a-1 serum than in the anti-B303dEt and anti-34334f sera (in Fig. 1 these antisera are used at equivalent concentration). Anti-C91f serum lacked precipitins for the CS3 antigen and extracts of strain C91f were negative for antigen CS3. No immunoprecipitates were obtained between extracts of these four strains and anti-CFA/I serum. Extracts of the four MRHA⁻_{bov} variants yielded no immunoprecipitates with any of these five antisera (Fig. 1). By appropriate immunodiffusion tests, CS1, CS2 and CS3 showed reactions of non-identity (see Fig. 1*b* and *d*; other data not shown).

Extracts of the four prototype MRHA⁺_{bov} strains prepared by shearing of cells but otherwise processed identically to heat-released extracts gave rise to immunoprecipitation patterns indistinguishable from those obtained in Fig. 1 and gave reactions of identity with the standard extracts. As several proteases and peptidases have been described to be membrane-associated in *E. coli* or to be present in the periplasm (e.g. Lazdunski *et al.*, 1975; Paucaud *et al.*, 1976), heat-released extracts of strains were prepared in the presence of PMSF. These PMSF extracts of the prototype strains gave identical immunodiffusion patterns to those obtained with the standard heat-released extracts (Fig. 1). Suspensions of bacteria in saline containing PMSF retained the same agglutinability as those in saline alone (cf. Table 2).

In a previous study (Smyth *et al.*, 1979), it had been shown that growth of MRHA⁺_{bov} enterotoxigenic *E. coli* of serotype O6:K15 at 18 °C on CFA agar suppressed expression of the MRHA_{bov} characteristic. Suspensions of 18 °C-grown bacteria (48 h) of the prototype strains were MRHA⁻_{bov} and were not agglutinated by any of the four absorbed antisera. Heat-released extracts of these 18 °C-grown bacteria did not contain CS1, CS2 and CS3 antigens by double immunodiffusion tests. Upon subculture of the 18 °C-grown bacteria to fresh CFA agar plates and growth at 37 °C for 18 h, the three properties were regained.

Cross-absorption studies

Samples of anti-C922a-1 and anti-34334f sera were absorbed with strains C922b-13 (a single colony substrain identical in all respects to strain C922a-1) C91f and 34334f. Absorption of anti-C922a-1 serum with strain C922b-13 abolished agglutinating activity towards the biotype A strains C922a-1 and C922b-13, whereas absorption with strain C91f possessing only the CS2 antigen or with strain 34334f possessing both CS2 and CS3 antigens did not affect the agglutinating titre towards the biotype A strains. Conversely, absorption of anti-34334f with

Fig. 1. Double immunodiffusion analysis of heat-released extracts (60 °C, 20 min) of prototype MRHA⁺_{bov} enterotoxigenic *E. coli* of serotype O6:K15:H16 or H- and of their MRHA⁻_{bov} variants. Peripheral wells of immunoplates (a), (b), (c) and (d) were loaded with heat-released extracts of strains C922a-1(a), B303dEt (b), C91f (c), 34334f (d), C922a-2 (e), B303d-5 (f), C91f-6 (g) and 34334f-50 (h), 10 µl portions being used in each instance. Centre wells were charged with 10 µl anti-C922a-1 (a), anti-B303dEt (b), anti-C91f (c) and anti-34334f (d) antisera. Immunoplates were stained with Coomassie brilliant blue after 48 h at 20 °C. Immunoprecipitates corresponding to CS antigens CS1, CS2 and CS3 are indicated.

strain C922b-13 possessing antigens CS1 and CS3 had no effect on its agglutinating titre towards strains C91f and 34334f, whereas absorption with either of the latter strains removed agglutinating antibodies for these strains.

Removal of precipitins towards CS1 and CS2 from anti-C922a-1 and anti-34334f sera, respectively, correlated with the loss of agglutinating antibodies. Anti-34334f serum absorbed with strain C91f, i.e. specific anti-CS3 serum, did not agglutinate any of these four test strains. The cross-absorptions with strains C922b-13 and 34334f removed anti-CS3 precipitins only.

Absorption of these two antisera with *E. coli* strain NCTC 8623, grown as unshaken TSB cultures to provide abundant type 1 fimbriae production (Nowotarska & Mulczyk, 1977; Sweeney & Freer, 1979), neither affected the agglutinating titre of these antisera nor removed

precipitins for CS1, CS2 and CS3 antigens. Likewise, absorption of these two antisera with the prototype CFA/I⁺ strain H-10407 removed neither agglutinins nor precipitins.

Isolate series B34212b, c, h and j/78

By a fortuitous accident, extracts of the MRHA_{bov}⁻ enterotoxigenic *E. coli* strain B34212j of serotype O8 : K40 : H9 (Table 1), obtained by both shearing and heat-release, were examined in immunodiffusion tests against anti-C922a-1, anti-B303dEt, anti-C91f and anti-34334f sera. Although none of these sera agglutinated strain B34212j, an immunoprecipitate showing a reaction of identity with that of CS3 antigen was observed. Identical findings were obtained with the other substrains of the B34212 isolate series. Moreover, absorption of anti-C922a-1 and anti-34334f sera with strain B34212j absorbed out anti-CS3 antibodies resulting in specific anti-CS1 and anti-CS2 sera, which retained the ability to agglutinate strain C922a-1 and strains B303dEt, C91f and 34334f, respectively. The anti-CS1 and anti-CS2 sera neither agglutinated the MRHA_{bov}⁻ variants of the prototype strains nor reacted with extracts of the MRHA_{bov}⁻ variants by immunodiffusion.

Haemagglutinin identity

In order to determine the relationship between the antigens detected in immunodiffusion tests and the haemagglutinin(s) on the four prototype strains, antisera specific for CS1, CS2 and CS3 antigens were employed. Anti-CS1 and anti-CS3 sera were obtained by cross-absorption and by immunization with immunoprecipitates; one anti-CS2 serum was obtained by cross-absorption and the other was the anti-C91f serum (see above and Methods). The two anti-CS1 sera inhibited only the MRHA_{bov}⁺ activity of the biotype A strain C922a-1. The two anti-CS2 sera caused inhibition only of the MRHA_{bov}⁺ reactions of strains known to possess this antigen, i.e. strains B303dEt, C91f and 34334f. The two anti-CS3 sera lacked haemagglutination inhibition activity against all four prototype strains.

SDS-PAGE of extracts of MRHA_{bov}⁺ prototype strains

Examination of the heat-released extracts of the prototype strains by SDS-PAGE revealed the presence of three characteristic polypeptide bands which were absent from the profiles of their MRHA_{bov}⁻ variants, as exemplified in Fig. 2. One band of 14.8 kDal was common to strains C922a-1, B303dEt and 34334f, but lacking in the SDS-PAGE profile of strain C91f. The other two bands showed a biotype correlation, namely, that of 16.3 kDal was only present in extracts of C922a-1, whereas that of 15.3 kDal was only present in the profiles of the prototype strains of biotypes B, C and F. The SDS-PAGE polypeptide band profiles of extracts obtained by shearing bacteria differed only in the intensity of bands other than those specific for MRHA_{bov}⁺ prototype strains. Extracts of isolates B34212b, c, h and j showed a strong polypeptide band corresponding to the 14.8 kDal component of the serogroup O6 prototype strains (data not shown).

The polypeptide band patterns of heat-released extracts of the prototype CFA/I⁺ and CFA/I⁻ strains of Evans *et al.* (1975) are representative of those obtained for each of seven other paired CFA/I⁺ and CFA/I⁻ strains selected by the author (strains used in Wadström *et al.*, 1979) (Fig. 2, lanes 5 and 6). Each extract from the CFA/I⁺ member of the paired strains showed a major polypeptide band of 13.8 kDal which was lacking in the polypeptide band profiles of extracts of the CFA/I⁻ variants. Heat-released extracts of strain NCTC 8623, grown under conditions allowing good expression of common type fimbriae, showed no polypeptide bands corresponding to any of those designated above (Fig. 2, lane 7). It was not established whether common type fimbriae were released by heat treatment or, as would be expected, they failed to solubilize under the present experimental conditions (McMichael & Ou, 1979).

Correlation of CS antigens with polypeptide bands in SDS-PAGE

The correlation of the serological presence of the CS1, CS2 and CS3 antigens with biotype and the presence of characteristic polypeptide bands in SDS-PAGE profiles was tested using individual strains, multiple isolates from individual patients and single colony substrains (Table

Fig. 2. SDS-PAGE of heat-released extracts of haemagglutinin-positive and haemagglutinin-negative pairs of enterotoxigenic *E. coli*. Lane 1, strain C922a-1, MRHA⁺_{bov} CS1⁺ CS3⁺; lane 2, strain C922a-2, MRHA⁻_{bov} CS1⁻ CS2⁻; lane 3, strain B303dEt, MRHA⁺_{bov} CS2⁺ CS3⁺; lane 4, strain B303d-5, MRHA⁻_{bov} CS2⁻ CS3⁻; lane 5, strain H-10407, MRHA⁺_{human} CFA/I⁺; lane 6, strain H-10407-P, MRHA⁻_{human} CFA/I⁻; lane 7, strain NCTC 8623, broth-grown cells expressing type 1 fimbriae, MSHA⁺_{guinea-pig}. The protein load to each lane was 10 µg.

3). Twelve MRHA⁺_{bov} strains of biotype A and seven additional multiple isolates from three patients were each agglutinated by anti-CS1 serum, possessed the CS1 and CS3 antigens, and revealed the 16.3 kDal and 14.8 kDal components in SDS-PAGE. Using four MRHA⁻_{bov} substrains, loss of CS1 and CS3 serologically was accompanied by the absence of these polypeptide bands in SDS-PAGE.

In the case of strains of biotypes B, C, and F, six MRHA⁺_{bov} strains and a single colony substrain were agglutinated by anti-CS2 serum, possessed the CS2 and CS3 antigens and showed

Table 3. *Biotype, agglutinability, immunodiffusion and SDS-PAGE data relating to MRHA_{bov}⁺ enterotoxigenic E. coli of serotype O6 : K15 : H16 or H-, MRHA_{bov}⁻ variants and other E. coli*

Strain	Biotype*	Agglutinability with†		Presence of antigens by immunodiffusion tests†			Presence of indicated polypeptide by SDS-PAGE‡		
		Anti-CS1 factor serum	Anti-CS2 factor serum	CS1	CS2	CS3	16·3 kDal	15·3 kDal	14·8 kDal
C921a, b, d	A	+	-	+	-	+	+	-	+
C922b, e, f									
C407a, C666c									
C735c, E90a									
E628b, E661a									
34318a, c, e, f									
34413b, 3699									
4071	A	+	-	+	-	+	NT	NT	NT
C921b-1									
C922b-9, -13, -20									
C921b-2	§ A	-	-	-	-	-	-	-	-
C922b-6, -19, -22									
342, 344	B	-	+	-	+	+	-	+	+
C728b, C846e									
B303d-1, -13, -20§	B	-	-	-	-	-	NT	NT	NT
342-15, -17	B	-	+	-	+	+	NT	NT	NT
342-46, -59§	B	-	-	-	-	-	-	-	-
C91a, b, c, d, e	C	-	+	-	+	-	-	+	-
C806c									
E583a, E583a-7	§ C	-	+	-	+	+	-	+	+
C91f-29, -31									
E583a-14									
E596a§	C	-	-	-	-	-	-	-	-
C922c§	D	-	-	-	-	-	-	-	-
C470e	F	-	+	-	+	+	-	+	+
E3772, E3773	F	-	+	-	+	-	-	+	-
NCTC 8623	NA	-	-	-	-	-	-	-	-
C921f									
EC 25/06/75									
H10407, H10407-P									

NT, Not tested. NA, Not applicable, non-O6 : K16 : H16 or H- strains.

* According to the biotyping scheme of Scotland *et al.* (1977).

† CS antigens defined in Fig. 1.

‡ See Fig. 2.

§ MRHA_{bov}⁻ strains.

the presence of the 15·3 and 14·8 kDal polypeptide bands in extracts. In contrast, eight MRHA_{bov}⁺ isolates from three patients which were agglutinated by anti-CS2 serum demonstrated only the CS2 antigen and the 15·3 kDal polypeptide band in SDS-PAGE profiles of extracts. Five MRHA_{bov}⁻ single colony substrains of these biotypes exhibited concomitant loss of agglutinability, CS2 (and CS3) antigen(s) and the 15·3 kDal (and 14·8 kDal) polypeptide band(s). In addition, two MRHA_{bov}⁻ enterotoxigenic strains of biotypes C and D and one enterotoxigenic strain of serotype O6 : K13 and one non-enterotoxigenic strain of serotype O6 : K2 : H1 were serologically negative for CS1, CS2 and CS3 and lacked the characteristic polypeptide bands in SDS-PAGE.

Thus, in each case, the immunologically identified CS1, CS2 and CS3 antigens correlated with the 16·3, 15·3 and 14·8 kDal polypeptide bands, respectively.

Haemagglutination profiles

In view of the apparent association of CS1 antigen with biotype A and of CS2 antigen with biotypes B, C and F, the MRHA profiles of these enterotoxigenic *E. coli* were examined to

determine whether or not differences might exist between the haemagglutinins on the different biotypes. Eleven species of erythrocyte other than bovine were tested.

As previously described (Evans & Evans, 1978; Smyth *et al.*, 1979; Wadström *et al.*, 1979), all MRHA_{bov}⁺ strains failed to agglutinate human erythrocytes. Eight other species of erythrocyte (cat, dog, guinea-pig, horse, mouse, pig, rabbit and sheep) were likewise not agglutinated in the presence of mannose. Chicken erythrocytes from 6 week old broiler fowl were agglutinated (Evans, D. J. *et al.*, 1979) irrespective of the biotype. Biotype A strains failed to agglutinate hen erythrocytes (White Leghorn breed) in the presence of mannose, whereas the strains of biotypes B, C and F caused rapid agglutination of this blood type. The latter strains tended to agglutinate hen erythrocytes more rapidly and readily than bovine erythrocytes. Strain B34212j did not agglutinate any of these test bloods.

No strain grown on CFA agar medium haemagglutinated guinea-pig erythrocytes in the absence of mannose. Only strains C91a-f, C806c and C922c, when grown and passaged in TSB without shaking, demonstrated MSHA of guinea-pig and chicken or hen erythrocytes, indicating the presence of type 1 fimbriae (Nowotarska & Mulczyk, 1977; Sweeney & Freer, 1979; Duguid & Old, 1980).

None of the MRHA_{bov}⁻ variants grown on CFA agar agglutinated any of the test bloods in the presence of mannose. However, TSB-grown cells of the MRHA_{bov}⁻ variants of strain C91f gave MSHA_{guinea-pig} and MSHA_{hen}, like their parent strain.

Loss of enterotoxigenicity in relation to loss of antigens CS1, CS2 and CS3

Of the 16 MRHA_{bov}⁻ variants studied, 13 lost the ability to produce both LT and ST (Table 1). The remaining three retained LT production. Thus, loss of ST with or without concomitant loss of LT production accompanied loss of the MRHA_{bov}⁻ property and of the CS1, CS2 and CS3 antigens.

DISCUSSION

Enterotoxigenic *E. coli* of serogroups O6 and O8 of human origin have been previously shown to possess the MRHA_{bov} characteristic (Ørskov & Ørskov, 1977; Evans & Evans, 1978; Smyth *et al.*, 1979). Moreover, this haemagglutinating ability has been taken to be indicative of or synonymous with the presence of CFA/II, a fimbriate adhesin on the cell surface of such strains (Evans & Evans, 1978; Bäck *et al.*, 1980a, b; Levine *et al.*, 1980). Although there are differences of opinion at present as to the number of different biotypes within enterotoxigenic *E. coli* of serotype O6:K15:H16 or H- (Ørskov & Ørskov, 1977; Scotland *et al.*, 1977; Merson *et al.*, 1979), five biotypes were recorded herein, only four of these being associated with MRHA_{bov}⁺ strains. The strain C922c of biotype D was MRHA_{bov}⁻. The presence of CS1 antigen or of CS2 antigen in these bacteria correlated with fermentation of rhamnose, namely, biotype A strains possessed CS1 antigen and were rhamnose-negative, whereas rhamnose-positive strains (biotypes B, C and F) possessed the CS2 antigen. Moreover, antisera specific for CS1 and CS2 only neutralized the MRHA_{bov} reactions of rhamnose-negative and rhamnose-positive strains, respectively. Rhamnose-positive and rhamnose-negative strains were also found to differ with respect to their abilities to agglutinate hen erythrocytes. Thus, these closely related enterotoxigenic *E. coli* of identical O:K:H serotype may possess one or other of two antigenically distinct mannose-resistant haemagglutinins with slightly differing biological properties.

It is not possible to directly identify CS1, CS2 or CS3 antigen with the common antigen detected by Evans & Evans (1978) in extracts of enterotoxigenic *E. coli* of serogroups O6 and O8 by using immunodiffusion tests without access to their prototype strain PB-176 and their reference CFA/II antiserum *sensu stricto*. However, strain PB-176 is of biotype A, i.e. rhamnose-negative (F. & I. Ørskov, WHO Collaborative Centre for Research and Reference on *Escherichia coli*, Copenhagen, Denmark, personal communication). Moreover, strains C922a-1 and C922a-2, the prototype biotype A strains herein of MRHA_{bov}⁺ and MRHA_{bov}⁻ character, have been typed as CFA/II⁺ and CFA/II⁻, respectively (tests kindly performed by D. G. and D.

J. Evans, Jr, University of Texas Medical School at Houston, Houston, Tex., U.S.A.). Thus, the likely candidate is CS1 or CS3. If all the enterotoxigenic O6 : H16 strains examined by Evans & Evans (1978) were of biotype A, then the common immunoprecipitate might have been due to either antigen. If they were not all of biotype A, CS1 would seem improbable. Moreover, the CS3 antigen was the only antigen common to most of the enterotoxigenic MRHA_{bov}⁺, O-serogroup 6 strains in the present investigation and was also found on an enterotoxigenic O-serogroup 8 series of isolates, which is consistent with the description of Evans & Evans (1978) for CFA/II antigen detected in surface extracts of their strains. Finally, although the property of MRHA_{bov} correlated with tissue adhesiveness and the presence of fimbriae on PB-176, Evans & Evans (1978) did not establish that the precipitating antibodies inhibited haemagglutination or tissue adhesiveness or bound to the fimbriae, i.e. that their antigen detected by immunodiffusion was synonymous with the haemagglutinin, the tissue adhesin or fimbrial in nature.

The enterotoxigenic MRHA_{bov}⁺ strain of serotype O6 : H16, i.e. E1392/75, used by Cravioto *et al.* (1979a) to raise a putative anti-CFA/II antiserum was also of biotype A (Scotland *et al.*, 1977). Moreover, the immunoprecipitate obtained with surface extracts of their strain was stated to show identity with that obtained with extracts of PB-176 using antiserum absorbed in an identical manner to that used both herein and by Evans & Evans (1978). Whether these precipitating antibodies had HI activity was not investigated. Based on the present findings, Cravioto *et al.* (1979a) may have been observing CS1 or CS3 antigen in immunodiffusion.

One other group has reported on the occurrence of adhesins on enterotoxigenic *E. coli* of O-serogroup 6 (Deneke *et al.*, 1980, 1981; Thorne *et al.*, 1979). Interestingly their strains B2C and TD219c1 (both of serotype O6 : H16) possessed serologically distinct fimbriae corresponding to those found on prototype strains M9800-5 and D563 of unknown serotype (Deneke *et al.*, 1980, 1981). A third strain of serotype O6 : H16 (TD462c1) reacted with the antisera prepared to fimbriae from both M9800-5 and D563. These authors' observations are complicated by their apparent requirement to cross-link fimbriae with the bifunctional reagent *N,N'*-*p*-phenylenedimaleimide in order to obtain immunoprecipitation in immunodiffusion tests. Furthermore, these authors did not report on the ability of their antisera to inhibit haemagglutination nor on the biotypes of their strains. Thus, unfortunately, it is not possible to directly compare their findings with those presented here.

What are the natures of the CS1, CS2 and CS3 antigens? Antigens CS1, CS2 and CS3 were each associated with characteristic polypeptide bands in SDS-PAGE which were readily distinguishable from minor bands, probably contributed by sloughed off envelope material during preparation of extracts, by the comparative use of extracts of MRHA_{bov}⁺ variants and of strains characterized herein as possessing only one of these antigens (see Table 3). Moreover, CFA agar grown cells of MRHA_{bov}⁺ bacteria of serotype O6 : K15 : H16 or H- are heavily fimbriate, whereas their MRHA_{bov}⁻ variants are not (J. H. Freer, A. Ellis & C. J. Smyth, unpublished results). Recently, Jann *et al.* (1981) examined fimbriae from *E. coli* of different origin, including strain 20174 of serotype O6 : H- which was stated to be CFA/II⁺ on the basis of being MRHA_{bov}⁺. This strain is of biotype B, rhamnose-positive (C. J. Smyth, unpublished data). Using purified preparations of fimbriae of strain 20174, two distinct polypeptide bands of unstated molecular weight were obtained with SDS-PAGE (Jann *et al.*, 1981). Deneke *et al.* (1980) also reported a similar finding with a preparation of fimbriae from *E. coli*. Heat extraction of bacterial suspensions as used herein has been demonstrated to yield preparations of the fimbrial adhesins CFA/I (Freer *et al.*, 1978) and the F7 antigen (Ørskov *et al.*, 1980). Thus, although the present apparent molecular weights of observed polypeptide bands with SDS-PAGE can not be readily compared with data from the studies of Jann *et al.* (1981) and of Deneke *et al.* (1980), the molecular weights of the polypeptide bands associated with the presence of CS1, CS2 and CS3 in extracts of MRHA_{bov}⁺ strains are not inconsistent with reported molecular weights for fimbrial subunit proteins (Brinton, 1978; Deneke *et al.*, 1980; Wevers *et al.*, 1980; Jann *et al.*, 1981). One interpretation of the SDS-PAGE data has been that two different subunits may exist in a single fimbrial structure (Deneke *et al.*, 1980; Jann *et al.*, 1981). However, the serological data of the present report would not be consistent with this view in the case of the CS antigens as neither heat-release nor shearing is likely to yield fimbrial subunits. In

contrast, that more than one type of fimbria or adhesin may be present on *E. coli* is not without precedent (e.g. Morris *et al.*, 1980; Ørskov *et al.*, 1980).

Although the CS3 antigen appears to be non-haemagglutinating, this does not exclude the possibility that it could have adhesive significance *in vivo*. For example, the 987P antigen found on certain porcine enterotoxigenic *E. coli* lacks haemagglutinating activity (Isaacson & Richter, 1981). Tests with ten of the erythrocyte species used herein and the prototype strain 987 have also proved negative in this laboratory (C. J. Smyth, unpublished data). Moreover, strain B34212 (CS3 only) has been shown to adhere to human intestinal epithelial cells (Wadström *et al.*, 1980). However, just as all fimbriae are not haemagglutinating (Duguid & Old, 1980; Isaacson & Richter, 1981), it is also true that not all fimbriae are adhesive and that not all adhesins are fimbrial (Duguid & Old, 1980). Nonetheless, CS3 antigen neither appears to be an artefact of preparation nor a product of partial proteolysis of CS1 and CS2 antigens.

In summary, in contrast to enterotoxigenic *E. coli* bearing the haemagglutinin CFA/I, strains producing so-called CFA/II or putatively identified as CFA/II⁺ on the basis of the possession of the MRHA_{bov} property, appear to produce more than one type of adhesin. There is a need to resolve the comparative descriptions of adhesins on enterotoxigenic *E. coli* of serogroup O6 of human origin before more extensive genetic analysis of such strains is undertaken. It is also to be recommended that future investigators report the biotype of strains according to the scheme of Scotland *et al.* (1977) but especially the results of the differential rhamnose test to enable better comparison of findings between research groups.

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