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Lack of association of *Escherichia coli* exhibiting both mannose-resistant haemagglutination and diffuse adherence to HEp-2 cells with acute diarrhoea in children

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SUMMARY

Stool specimens from 631 children with acute diarrhoea and from 277 healthy controls were tested for the presence of non-enteropathogenic, non-enterotoxigenic *Escherichia coli* strains which mediated mannose-resistant haemagglutination of human erythrocytes (MRHA+). Fifty-nine (34·9%) of 169 isolated MRHA+ strains but none of 210 MRHA− strains exhibited diffuse adherence (DA+) to HEp-2 cells. DA+ strains were found in 37 (5·9%) children with diarrhoea and in 22 (7·9%) controls. MRHA+/DA+ strains in comparison to MRHA+/DA− strains significantly less frequently expressed P fimbriae (10·7 *vs.* 73·6%), haemolysin production (12·5 *vs.* 63·2%), and MRHA of other species erythrocytes (21·4 *vs.* 84%).

These data demonstrate that *E. coli* which exhibit the diffuse pattern of adherence to HEp-2 cells also cause MRHA of human erythrocytes. Since these strains were found with similar frequencies in children with and without diarrhoea it seems that DA is not a marker of enteropathogenicity of *E. coli*.

INTRODUCTION

Since the adherence of bacteria to epithelial surfaces is recognized as an important event in the pathogenesis of urinary and intestinal tract infections, much attention is focused on the molecular mechanism of adhesiveness. Fimbrial (P, S fimbriae) and non-fimbrial (AFA, M adhesins) structures and their genetic determinants (*pap*, *sfa*, *afa*, *bma* operons) have been identified in *Escherichia coli* strains isolated from the urine (1, 2). These structures cause mannose-resistant haemagglutination (MRHA), mediate the adherence of bacteria to the epithelial cells *in vitro*, and promote the colonization of urinary tract. The attachment of enterotoxigenic *E. coli* to small bowel enterocytes is mediated by colonization factors which also cause MRHA (3).

Data accumulated in the last few years indicate that the adherence to cultured cells (HEp-2, HeLa) may represent a marker of enteropathogenicity of *E. coli*. The adherence is expressed as localized (LA), diffuse (DA) or aggregative (AA) (4, 5).

Since most of strains with the adhesive ability, particularly those exhibiting DA or AA, do not belong to known intestinal pathogenic strains, they are classified in a new group of potentially diarrhoeagenic *E. coli*, termed enteroadherent *E. coli* (6). It is not clear whether MRHA is an indicator of the adhesive ability of these strains and, furthermore, whether strains displaying these characteristics have an enteropathogenic potential.

The aims of the present study were (*a*) to investigate if a correlation between MRHA and the capacity to adhere to cultured cells exists among faecal *E. coli* and (*b*) to determine the frequency of isolation of strains expressing these traits in patients with and without diarrhoea in an attempt to elucidate their association with acute diarrhoeal disease.

METHODS

Stool samples of 908 children up to 6 years of age were screened for MRHA-mediating *E. coli* during a study on the aetiology of acute diarrhoea, performed in urban and suburban localities of Yugoslavia, from August through November 1986 (7). Six hundred and thirty-one children were patients attending primary care outpatient clinics, and 277 healthy controls were infants visiting well-baby clinics and children in day nursery centres.

Five biochemically identified *E. coli* strains from stool of each child were tested for MRHA with human erythrocytes (3). One isolated MRHA-expressing (MRHA+) strain per person was further assayed for the adherence to HEp-2 tissue culture cells in the presence of 1% D-mannose (8). Two hundred and ten *E. coli* strains which did not exhibit MRHA (MRHA−), isolated from the same number of children in the investigated populations, were also tested for the adhesive ability. The strains included in MRHA+ or MRHA− group neither produced heat-labile and/or heat-stable enterotoxin, nor agglutinated with antisera for classical enteropathogenic *E. coli* (EPEC) (7).

MRHA+ strains were further examined for the presence of other phenotypic traits. MRHA tests with bovine, sheep, pig, rabbit, guinea-pig, chicken, rat, and mouse red blood cells were performed as with human erythrocytes (3). The hydrophobicity was measured by 'salting out' test of Lindahl and co-workers (9). P fimbriae were detected by latex-bead agglutination test (BACH test, Sockerbolaget, Arlöv, Sweden). The production of colicin was investigated by the method of Frederiq (10). Aerobactin-producing strains were detected by slightly modified bioassay of Payne and co-workers (11) (2,2-dipyridyl, 200 μmol/l, served as an iron chelator instead of EDDA). The haemolytic activity was tested by plating the strains onto nutrient agar containing 5% sheep erythrocytes.

The χ^2 test was used for statistical analysis.

RESULTS

MRHA+ *E. coli* were isolated from stools of 169 (18·6%) of 908 children. Fifty-nine (34·9%) of these but none of 210 MRHA− strains expressed DA to HEp-2 cells (Table 1). In all instances DA was easily recognized: more than 20 bacteria were attached to 50–80% of cells. Other types of adherence (LA or AA) were not

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Table 1. *Isolation of* Escherichia coli *exhibiting mannose-resistant haemagglutination and diffuse adherence to HEp-2 cells in children with diarrhoea (D) and in healthy controls (C)*

	No. (%) of strains	No. (%) of isolation	
		D	C
	MRHA+*	(n = 631)	(n = 277)
Tested for adherence	169 (100)	118 (18·7)	51 (18·4)
Exhibited diffuse adherence	59 (34·9)	37 (5·9)	22 (7·9)
Did not exhibit adherence	110 (65·1)	81 (12·8)	29 (10·5)
	MRHA−		
Tested for adherence	210 (100)	137 (21·7)	73 (26·3)
Exhibited adherence	0		

* Non-enteropathogenic, non-enterotoxigenic *E. coli* which exhibited or not mannose-resistant haemagglutination (MRHA+ and MRHA−, respectively).

detected, either in MRHA+ or in MRHA− group of *E. coli*. MRHA+/DA+, MRHA+/DA−, and MRHA− strains were isolated with similar frequencies in children with and without diarrhoea.

Of the total number of isolated MRHA+ strains, 162 were further analysed for other phenotypic characteristics (seven were excluded because of the loss of MRHA activity during storage). The expression of P fimbriae, haemolysis or MRHA of other species erythrocytes was significantly more often encountered among MRHA+/DA− strains than among the strains which exhibited DA (Table 2). The difference in the detection of aerobactin or colicin production was not statistically significant when comparing these two groups of strains. Only two strains (from MRHA+/DA− group) showed hydrophobicity, i.e. they aggregated in 0·05 M ammonium sulphate.

Of 101 strains, which also mediated MRHA of other red-blood cell species, 90 (6 in MRHA+/DA+ and 84 in MRHA+/DA− group) agglutinated sheep and pig erythrocytes (5 strains from MRHA+/DA− group also agglutinated rabbit erythrocytes), 5 expressed MRHA of pig erythrocytes only (all were from MRHA+/DA− group), and 6 mediated MRHA of rabbit erythrocytes only (all were from MRHA+/DA+ group). MRHA of human erythrocytes was not restricted to group A but was also expressed with other blood groups (B, O and AB); it varied from weak (1+) to strong (4+) among the investigated strains, occasionally showing the phenomenon of elution. MRHA with sheep erythrocytes was always of eluting character.

P fimbriae were detected by latex-bead agglutination test on 84 (51·8%) of 162 MRHA+ strains; all of them also agglutinated sheep and pig erythrocytes. Of 84 P-fimbriated strains, 67 (79·8%) synthesized haemolysin in comparison to only 7 (9%) of 78 strains without detected P fimbriae ($P < 0·0005$). Aerobactin-producing strains were found to possess P fimbriae significantly more often (74 of 127 or 58·3%) than aerobactin-negative strains (10 of 35 or 28·6%) ($P < 0·05$). Haemolysin synthesis was not associated with aerobactin production; 47·2

Table 2. *Phenotypic characteristics of MRHA-causing* faecal* E. coli *and their isolation rates in children with diarrhoea (D) and in healthy controls (C)*

Characteristic	No. (%) of strains with indicated characteristic				No. (%) of isolation	
	All MRHA ($n = 162$)	MRHA/DA+† ($n = 56$)	MRHA/DA− ($n = 106$)	χ^2 test‡	D ($n = 631$)	C ($n = 277$)
P fimbriae	84 (51·8)	6 (10·7)	78(73·6)	$P < 0·0005$	62 (9·8)	22 (7·9)
Haemolysin	74 (45·7)	7 (12·5)	67 (63·2)	$P < 0·0005$	56 (8·9)	18 (6·5)
MRHA of other species erythrocytes	101 (62·3)	12 (21·4)	89 (84·0)	$P < 0·0005$	75 (11·9)	26 (9·4)
Colicin	42 (25·9)	10 (17·8)	32 (30·2)	N.S.	31 (4·9)	11 (4·0)
Aerobactin	127 (78·4)	48 (85·7)	79 (74·5)	N.S.	91 (14·4)	36 (13·0)
Hydrophobicity	2 (1·2)	0	2 (1·9)	N.S.	2 (0·3)	0

* Non-enteropathogenic, non-enterotoxigenic *E. coli* which caused mannose-resistant haemagglutination of human erythrocytes.

† Strains which exhibited or not diffuse adherence to HEp-2 cells (DA+ and DA−, respectively).

‡ MRHA/DA+ compared with MRHA/DA− strains (N.S., not significant).

and 40% of aerobactin-positive and aerobactin-negative strains synthesized haemolysin, respectively.

The isolation rates of strains with various phenotypic characteristics in children with and without diarrhoea are shown in Table 2. No one of these characteristics was found significantly more frequently among strains isolated from diarrhoeal than from control group of children. Strains expressing P fimbriae, haemolysin, and aerobactin *en bloc* were isolated from 43 (6·8%) children with diarrhoea and from 14 (5%) healthy controls.

DISCUSSION

Adherence to epithelial cells is frequently associated with MRHA of human erythrocytes among uropathogenic *E. coli*. These isolates possess various adhesins of which P fimbriae recognize a specific (Gal-Gal) receptor on erythrocytes, which is a part of P antigen, whereas X adhesin is P antigen-independent, i.e. it also mediates agglutination of erythrocytes lacking P antigen ($\bar{p}$ erythrocytes) (12, 13). The correlation between MRHA and adhesiveness has not been investigated to such an extent with faecal *E. coli* as with uropathogenic strains. It has been shown (14, 15) that two *E. coli* strains, isolated from severe infantile diarrhoea, adhered to and penetrated HEp-2 cells and also mediated MRHA of human erythrocytes. Tavendale and Old (16) reported on a few faecal strains which agglutinated only human erythrocytes among 14 red-cell species tested and adhered well to HEp-2 cells; the pattern of adherence was not precisely determined. This study suggests a strong association of diffuse type of adherence to HEp-2 cells with MRHA of human erythrocytes. Four previously reported DA-exhibiting EPEC isolates (7) also mediated MRHA. Furthermore, the loss of DA in three *E. coli* strains during storage was accompanied with the loss of MRHA activity. Some authors reported on a few DA-expressing strains which failed to cause MRHA (17–19). In contrast, there is no correlation between localized type of adherence and MRHA since none of our 19 EPEC strains (7) or 121 EPEC strains studied by Scaletsky and colleagues (4), which exhibited LA, mediated MRHA. Vial and co-workers (20) reported that 47·5% of strains displaying AA agglutinated human and/or bovine erythrocytes.

P fimbriae and haemolysin synthesis are often expressed *en bloc* in uropathogenic strains as a consequence of close linkage of their genetic determinants (21–23). In these isolates the association of aerobactin-production and P fimbriae-expression was also found (21, 24). Our results are in accordance with these findings and indicate that they could be extended to faecal *E. coli*. We could not confirm the observation that (pyelonephritogenic) strains expressing MRHA, haemolysis and P fimbriae are frequently associated with O antigens O 1, O 4, O 6, O 16, O 18, O 25, O 50, and O 75 (12, 22), since with the available antisera (for all antigens mentioned above, except O 16 and O 50) the serogroup of only 6 of all our intestinal MRHA+ strains was determined (5 belonged to O 6 and 1 to O 4 serogroup).

Further examination of our MRHA+ isolates showed a marked difference between the MRHA+/DA− and MRHA+/DA+ groups of *E. coli*: a high proportion of MRHA+/DA− strains possessed P fimbriae, synthesized hae-

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molysin and mediated MRHA of other species erythrocytes, whereas the majority of MRHA+/DA+ strains was without P fimbriae, was non-haemolytic and expressed MRHA of human erythrocytes only. According to the criteria proposed by Archambaud and colleagues (1) *E. coli* strains which agglutinate human erythrocytes only and adhere well to HEp-2 cells possess afimbrial (AFA) adhesin, although Bilge and co-workers (25) found that a fimbrial adhesin (F1845) mediated these traits in one intestinal isolate. The detection of P fimbriae on several of our MRHA+/DA+ strains implies that more than one adhesin may be present on the same bacterial cell; by DNA-probe technique various operons coding for these structures have been found within a single strain (1, 2).

Although P fimbriae, haemolysin, and aerobactin production are discriminating pathogenic properties of *E. coli* strains causing urinary tract infections, it appears that these phenotypic traits do not contribute to enteropathogenicity since we did not find a significantly higher incidence of these properties in strains from diarrhoeal cases than from healthy controls, even when all of them were expressed within a single strain. The same observation could be extended to strains possessing adhesin which mediates DA. Mathewson and co-workers (6, 26) found the association of strains exhibiting DA with acute diarrhoea and suggested their role in the aetiology of this disease in travellers and young children. Subsequent investigations in Thailand (27), Chile (28), Mexico (29), and Brazil (30) did not confirm their findings. Assuming that *E. coli* which express DA also mediate MRHA of human erythrocytes, as our data indicate, we detected most of the individuals colonized with these agents; the isolation rates were similar in children with and without diarrhoea.

In conclusion, the results obtained in this study indicate that diffuse type of adherence to HEp-2 cells is strikingly correlated with MRHA of human erythrocytes. Furthermore, it seems that DA is not a useful marker in distinguishing diarrhoeagenic from non-diarrhoeagenic *E. coli*.

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