Adhesion of *Shigella dysenteriae* type 1 and *Shigella flexneri* to guinea-pig colonic epithelial cells *in vitro*

B. GUHATHAKURTA, D. SASMAL and A. DATTA

National Institute of Cholera and Enteric Diseases, Calcutta 700 010, India

Summary. Adhesion of bacteria to guinea-pig colonic epithelial cells *in vitro* was inhibited by fucose with all the four strains tested (two of *Shigella dysenteriae* type 1 and two of *S. flexneri*). N-acetyl neuraminic acid and N-acetyl mannosamine also caused inhibition, suggesting a multiplicity of receptors on the epithelial cell. Congo-red binding of the strains correlated with their adhesive ability, whereas haemagglutination of rabbit erythrocytes by the bacteria did not.

Introduction

In shigellosis, bacterial invasion of colonic epithelial cells (CECs) and the production of toxin are the most significant events responsible for the clinical manifestation of the disease. Adhesion of the bacteria to the epithelial cells before internalisation is, therefore, a pre-requisite. Attachment of bacteria to mammalian cells is often mediated through sugar-lectin interaction; and the adhesion molecule may be a haemagglutinin also. We have observed haemagglutination by *Shigella dysenteriae* type 1 and *S. flexneri* previously.¹

The objective of this study was to observe adhesion of isolates from patients to guinea-pig CECs *in vitro* under different conditions, and to investigate surface properties of the bacteria—hydrophobicity, Congo-red binding and haemagglutination—which may relate to such adhesion.

Materials and methods

Bacterial strains and growth

Two isolates of *S. dysenteriae* type 1 (strains 1609 and PB60) and two of *S. flexneri* (strains IDBM330 and ND22) from clinical cases of human shigellosis were grown for 18 h at 37°C in colonising factor (CF) broth or on CF agar.²

Haemagglutination

Bacteria grown on CF agar were suspended in Tris-buffered Krebs Ringer (KRT) solution³ at a con-

centration of $c. 10^{11}$ cfu/ml, and 25 μ l of two-fold dilutions were mixed with 25 μ l of rabbit erythrocytes (3% suspension) in polyvinyl microtitration plates. After incubation at 22°C for 1 h, haemagglutinin (HA) activity was recorded as the highest dilution of bacterial suspension giving strong haemagglutination.

Salt aggregation test (SAT)

Bacterial suspensions in physiological saline were mixed with different concentrations of ammonium sulphate (0.5–3.0 M) in 0.02 M sodium phosphate buffer, pH 6.8, on glass slides, and examined after 2 min. The lowest concentration of ammonium sulphate that produced bacterial clumps (SAT value) was recorded to show the relative cell-surface hydrophobicity, lower SAT values corresponding to greater cell-surface hydrophobicity.⁴

Congo-red binding to bacteria

CF broth cultures were centrifuged, washed in phosphate-buffered saline (PBS), and re-centrifuged; then 3×10^{10} bacteria were incubated for 10 min at room temperature in 3 ml of PBS containing 50 μ g of Congo red. The bacteria were removed by centrifugation, and the Congo red remaining in the supernate was determined⁴ by measuring the absorbance at 480 nm.

Colonic epithelial cells (CECs) of guinea-pig

Preparation of cell suspension. CECs were prepared by the method of Dean and Isaacson.⁵ The colon was excised from a 500-g adult guinea-pig. The external and internal surfaces were rinsed with cold 0.1 M PBS, pH 7.5, and kept in PBS at 4°C for 30 min. The intestine was slit open and cleaned of any mucus and

excreta, and the tissue was washed in PBS. Epithelium was removed by scraping with the blunt edge of a scalpel blade. Epithelial cells were collected by centrifugation at 200 *g* for 10 min, washed three times in PBS and resuspended in PBS at a concentration of 10⁹ CECs/ml. Cell density was determined by use of a haemocytometer and a Leitz Dialux 22 phase-contrast microscope. All the manipulations were performed at 4°C.

Assay of shigella adhesion. Quantitation of the adhesion of radiolabelled shigella to CECs was performed essentially by the method of Sugarman and Donta.⁶ Bacteria were grown in 20 ml of CF broth containing 1 µCi of C¹⁴ leucine for 18 h at 37°C with shaking; and 200 µl of a bacterial suspension (10¹¹ cfu/ml) in KRT, washed three times in KRT, was incubated with an equal volume of CECs (10⁹/ml) in a shaking water bath (60 horizontal strokes/min) for 1 h at 37°C. After incubation, the mixture was filtered through a membrane filter of pore size 8 µm (Millipore Corporation) fitted in a disc filter holder attached to a glass syringe. The filters were washed with 15 ml of KRT to remove non-adhering bacteria and then dried for 18 h at 37°C. Dry filters were then placed in Ready-Solv[®] HP (Beckman Instrument Inc., USA) and radioactivity (dpm) of the adherent bacteria retained by the filter was assessed with a Beckman liquid scintillation counter LS1801. Adherence was expressed as a percentage of the radioactive count (dpm) of bacteria in the incubation-mixture, that was found in the radioactive count (dpm) of bacteria adhering to epithelial cells retained on the 8-µm filter.

To observe the effect of monosaccharides, glycoproteins or enzymes on the adhesion, either the epithelial cells or the bacteria were treated with 200 µl of reagent in KRT at 37°C for 1 h on a shaker water bath; concentrations of the reagents (/ml) were glucose 10 mg, mannose 10 mg, fucose 10 mg, N-acetyl neuraminic acid 10 mg, N-acetyl glucosamine 10 mg, N-acetyl mannosamine 10 mg, fetuin 200 µg, thyroglobulin 200 µg, trypsin 10 mg, protease 10 mg, neuraminidase 5 units. The treated cells or bacteria were then washed and resuspended in KRT for adherence tests.

Appropriate controls without epithelial cells were

included to measure background bacterial adherence to the filter alone, in the presence or absence of test reagents. Such background adherence never exceeded 0.025 % of the test value (100 dpm).

Results

Table I shows that all four strains adhered to guinea-pig CECs *in vitro*, though the strains of *S. flexneri* (especially strain ND22) adhered less well than those of *S. dysenteriae* type 1. The high SAT values indicate that all the strains had a similar but low degree of hydrophobicity. Neither the SAT value nor the HA titre was related to the degree of adhesion; but, with these four strains, Congo-red binding showed some correlation with adhesive ability.

When bacteria were treated with fucose before mixing with CECs, a marked decrease in adherence was observed with all four strains (table II). After pre-treatment with N-acetyl mannosamine and thyroglobulin, only three strains showed marked inhibition of adherence. The inhibitory effect of N-acetyl neuraminic acid was very weak with two strains but very strong with the other two (one of each species). Adherence of strain ND22 was inhibited only by fucose. Other monosaccharides and glycoproteins (glucose, mannose, N-acetyl glucosamine and fetuin) had no effect on any strain. As with N-acetyl neuraminic acid, pre-treatment with the three enzymes (neuraminidase, protease and trypsin) affected one strain of each species more strongly than the other.

Pre-treatment of the CECs with the same monosaccharides, glycoproteins and enzymes had no effect on the adhesion of any of the bacterial strains.

Discussion

Our observations suggest that, as with many other enteric bacteria, the adherence of *S. dysenteriae* type 1 and *S. flexneri* to guinea-pig CECs is mediated by an adhesin which binds to the receptor on the epithelial surface and involves sugar moieties. Fucose is probably the monosaccharide mainly involved in the

Table I. Correlation in four *Shigella* strains between CEC-adherence, haem-agglutination, Congo-red binding and salt aggregation

Species	Strain no.	Mean (SD) percentage adherence* to CECs	HA titre	Congo-red binding (µg/10 ¹⁰ bacteria)	SAT†
<i>S. dysenteriae</i> type 1	1609	40.1 (2.2)	512	6.7	2.5
	PB60	31.3 (1.1)	0	6.7	2.5
<i>S. flexneri</i>	IDBM330	23.2 (1.3)	128	5.9	2.5
	ND22	13.0 (1.5)	0	4.9	2.5

* From five experiments.

† Expressed as minimal molar concentration of ammonium sulphate that caused bacterial clumping.

Table II. Effect, on bacterial adherence to CECs, of pre-treatment of bacteria with monosaccharides, glycoproteins and enzymes

Species	Strain no.	Mean (SD) relative adherence* after treatment with						
		L-fucose	N-acetyl neuraminic acid	N-acetyl mannosamine	thyroglobulin	neuraminidase	protease	trypsin
<i>S. dysenteriae</i> type 1	1609	55.0 (2.5)	84.8 (2.1)	48.0 (2.7)	48.5 (2.5)	91.1 (2.4)	72.4 (2.1)	79.2 (1.9)
	PB60	62.4 (2.2)	35.5 (1.2)	60.6 (1.3)	48.3 (2.3)	51.9 (2.9)	54.5 (1.3)	56.8 (2.7)
<i>S. flexneri</i>	IDBM330	66.5 (2.1)	43.6 (2.6)	60.0 (2.6)	60.8 (1.6)	55.0 (2.3)	68.8 (1.9)	47.0 (1.8)
	ND22	39.6 (3.0)	95.0 (1.7)	78.6 (2.1)	75.1 (2.3)	78.4 (1.8)	75.3 (2.6)	70.2 (2.2)

* Relative adherence is expressed as a percentage of the adherence of untreated bacteria. The results are recorded as the mean (SD) of five experiments.

adherence of both *Shigella* spp. However, N-acetyl neuraminic acid and N-acetyl mannosamine were effective inhibitors with some of the strains only; it is possible that more than one adhesin molecule, with different sugar specificities in different strains, are involved in the adherence process. Izhar *et al.*⁷ have suggested fucose to be the important monosaccharide in the adherence of *S. flexneri* to guinea-pig intestinal cells, although they obtained the effect by sugar-pre-treatment of the epithelial cells rather than the bacteria. A fucose-sensitive adhesin has been suggested for *Vibrio cholerae* also³. Unlike the adhesins of

Escherichia coli and *V. cholerae*, the adhesin of *S. dysenteriae* type 1 and *S. flexneri* probably is not an HA because adhesion occurred with strains showing no haemagglutination.

Qadri *et al.*⁴ observed that Congo-red binding was related to the virulence of *Shigella* spp. Our observation that Congo-red uptake correlated with the adhesiveness of strains supports their contention.

The inhibitory effect of pre-treatment with enzymes, on adhesion of bacteria to CECs, may possibly be due to alteration of the binding sites or hydrophobicity and net surface charge of the bacteria.

References

1. Guhathakurta B, Sasmal D, Sen D, Datta A. Haemagglutination of *Shigella dysenteriae* I and *Shigella flexneri*. *Med Sci Res* 1988; **16**: 77-78.
2. Evans DJ, Evans DG, DuPont HL. Hemagglutination patterns of enterotoxigenic and enteropathogenic *Escherichia coli* determined with human, bovine, chicken, and guinea-pig erythrocytes in the presence and absence of mannose. *Infect Immun* 1979; **23**: 336-346.
3. Jones GW, Abrams GD, Freter R. Adhesive properties of *Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and hemagglutinating activity. *Infect Immun* 1976; **14**: 232-239.
4. Qadri F, Hossain SA, Čiznár I *et al.* Congo red binding and salt aggregation as indicators of virulence in *Shigella* species. *J Clin Microbiol* 1988; **26**: 1343-1348.
5. Dean EA, Isaacson RE. In vitro adhesion of piliated *Escherichia coli* to small intestinal villous epithelial cells from rabbits and the identification of a soluble 987P pilus receptor-containing fraction. *Infect Immun* 1982; **36**: 1192-1198.
6. Sugarman B, Donta ST. Adherence of Enterobacteriaceae to human buccal cells. *J Med Microbiol* 1979; **12**: 373-378.
7. Izhar M, Nuchamowitz Y, Mirelman D. Adherence of *Shigella flexneri* to guinea pig intestinal cells is mediated by a mucosal adhesin. *Infect Immun* 1982; **35**: 1110-1118.