The association of haemagglutination and adhesion with lipopolysaccharide of *Shigella dysenteriae* serotype 1

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Summary. In this study the ability of strains of *Shigella dysenteriae* serotype 1 to agglutinate mammalian erythrocytes is attributed to the polysaccharide fraction of bacterial-cell lipopolysaccharide (LPS). LPS obtained from a rough, mutant strain of *S. dysenteriae* serotype 1, lacking the O-antigen polysaccharide side-chain, did not agglutinate erythrocytes, clearly demonstrating a link between O-antigen polysaccharides and haemagglutinating activity (HA). Strains of *S. dysenteriae* serotype 1 adhered well to cultured Henle Intestinal 407 cells, whereas rough strains adhered poorly. Pre-treatment of bacteria with LPS-specific antisera inhibited both HA and binding to cultured human-intestinal cells. The contribution of the polysaccharide side-chain and its associated HA—which appear to facilitate binding to cultured cells—to bacterial attachment to colonocytes and to the pathogenesis of shigellosis *in vivo* needs to be confirmed in animal studies.

Introduction

Shigella dysenteriae serotype 1, the most pathogenic type of *Shigella*, is responsible for major epidemics of bacillary dysentery in developing countries.^{1,2} The ability to invade and multiply within host colonic-epithelial cells is pathognomonic of shigellae.³ Additional factors contributing to virulence may include lipopolysaccharide (LPS), outer-membrane proteins (OMPs) and Shiga toxin, although their precise relative roles are far from clear.

Bacterial adhesion to epithelium of mucosal surfaces is an essential step in the pathogenesis of many diseases, including enteric infections.⁴ This process is mediated by fimbriae, flagella, capsule, glycocalyx, LPS, non-fimbrial adhesin or lectin.^{5,6} The attachment of *Shigella* strains to host cells has been reported; strains of *S. flexneri* serotype 1b adhere to guinea-pig colonic cells⁷ and strains of *S. flexneri* serotype 5 to HeLa cells.⁸ We have shown recently that strains of *S. dysenteriae* serotype 1 and *S. flexneri* agglutinate mammalian erythrocytes, and that haemagglutination (HA) was resistant to D-mannose and sensitive to N-acetylneuraminic acid, N-acetylneuraminlactose, and α_1 -glycoprotein. Fimbriae were observed by electron-microscopy in strains of *S. dysenteriae* serotype 1 that were haemagglutinating.⁹ In the present study we have characterised the haemagglutinin of *S. dysenteriae* serotype 1 and examined its relationship to adherence of bacteria to cells cultured *in vitro*.

Materials and methods

Bacterial strains

Shigella strains included in the study were obtained from the diarrhoeal faeces of patients attending the Clinical Research Centre (CRC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), Dhaka. Strain 14731 of *S. dysenteriae* serotype 1 was used for extraction of cell-surface components, and results were confirmed with three other strains (12588, 3351 and 26406). A non-pathogenic strain, 36000, of *Escherichia coli* of serotype O8:K25 and a rough mutant strain, 60R, of *S. dysenteriae* serotype 1 were obtained from the Centers for Disease Control, Atlanta, GA, USA. Another rough mutant, strain PSD-10, of *S. dysenteriae* serotype 1¹⁰ was obtained from Dr K. Haider (ICDDR, B). Sereny's test¹¹ and Congo-red binding assay¹² were used to confirm the virulence of strains.

Media and storage

Bacteria stored at -70°C were subcultured on trypticase soy (TS) agar and grown in Casamino acid-yeast extract (CYE) broth in the presence of 1 mM calcium chloride for 22 h at 37°C to express HA properties.⁹ Bacteria were grown either in screw-capped test-tubes containing 5 ml of medium for HA expression, or in 500-ml bottles (Gibco Diagnostics, Madison, WI, USA) containing 150 ml of medium for extraction of bacterial components.

HA assays

These were performed on slides,⁹ or in U-bottomed microtitration plates (Cooke, Alexandria, VA, USA) with erythrocytes 0.5% v/v. Equal volumes (30 µl) of doubling dilutions of bacterial components and guinea-pig erythrocytes were mixed, and plates were sealed and incubated for 1 h at room temperature with shaking (160 rpm) on a rotary shaker. Results were recorded after 30 min and scored as positive, when the erythrocyte suspension was distributed over the bottom of the wells, or as negative when a red button was clearly visible. Titres were recorded as the arithmetic mean of the minimum concentration of material at which HA was clearly visible. HA was tested with guinea-pig, human (types A, B, O), monkey, rabbit and sheep erythrocytes.⁹ However, in most assays guinea-pig and human (type O) erythrocytes only were used.

Inhibition of HA

Inhibition of HA was tested with *N*-acetylneuraminic acid (Sigma) 1% w/v, fetuin (from fetal calf serum, Sigma) 1% w/v and other carbohydrates.⁹ Inhibition by LPS-specific rabbit antibody was tested after incubation with test material for 1 h at 37°C.

Extraction and purification of bacterial components

To determine whether in strains of *S. dysenteriae* serotype 1 HA was mediated by protein, extraction methods used for *E. coli* were utilised.^{13,14} For isolation of haemagglutinin, OMPs were also prepared by either a lysozyme and EDTA-extraction procedure,¹⁵ or a simple buffer-extraction technique.¹⁶ LPS was extracted with hot phenol-water,¹⁷ and purified by ultracentrifugation at 100 000*g* for 4 h. Nucleic-acid contaminants in LPS (5–10 mg/ml) were removed by treatment with DNAase and RNAase (Sigma) 20 µg/ml, and protein contaminants by treatment with proteinase K (Sigma) 100 µg/ml. LPS was collected by centrifugation at 100 000*g* for 4 h.

Delipidation of LPS

LPS from strains of *S. dysenteriae* serotype 1 was delipidated¹⁸ by refluxing in acetic acid 1% v/v. The aqueous layer was lyophilised, resuspended in water and centrifuged at 100 000*g* for 4 h to remove unhydrolysed LPS. After lyophilisation of supernate, polysaccharide was chromatographed on BioGel P-10. Eluates were collected and analysed by the phenol-sulphuric acid test.¹⁹ Polysaccharide fractions were pooled, dialysed and lyophilised.

Cell debris from bacteria grown in CYE medium was removed by centrifugation at 8000*g* for 30 min. The clear culture supernate was collected, passed through a membrane filter (0.45 µm, Millipore), dialysed for 24 h against deionised water and lyophil-

ised. Culture supernates prepared in this manner are referred to as "CS" hereafter. Uninoculated culture medium, treated as above, was used as control.

Components used in HA assays described below were freeze-dried and reconstituted in PBS. Protein estimation was by the dye-binding method,²⁰ and carbohydrate content in LPS was determined by the phenol-sulphuric acid method.¹⁹ Purified LPS and other LPS-containing fractions were quantified on the basis of carbohydrate content rather than dry weight. To confirm further the presence of LPS, the content of 2-keto-3-deoxy-octulonate (KDO), a core sugar in LPS, was estimated.²¹ LPS was also detected in fractions by the Chromogenic Limulus Amoebocyte Lysate (LAL) method (Whittaker Bioproducts Inc., Walkersville, MD, USA) with LPS from *E. coli* serotype O55:B5 used as standard.²² Sialic-acid content in materials was assayed²³ with *N*-acetyl neuraminic acid as standard.

Gel filtration

Culture supernates were fractionated on columns (74 × 2 cm) of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The elution volume was 108 ml for Blue Dextran (Pharmacia) and 118 ml for LPS from strains of *S. dysenteriae* serotype 1. Fractions were assayed for protein (OD at 280 nm) and for carbohydrate content.¹⁹ Fractions were pooled, lyophilised and tested for HA activity.

Enzyme treatment

Suspensions of *S. dysenteriae* serotype 1 (1 × 10¹⁰ cfu) in PBS (0.5 ml) were treated with trypsin (Flow Laboratories, Herts) 50 µg or proteinase K 100 µg and incubated for 4 h at 37°C. Culture supernates and LPS were treated similarly.

Guinea-pig erythrocytes (50% v/v) were treated with 100–1000 milli-units of neuraminidase from *Clostridium perfringens* (Sigma) and incubated, as described above, before testing in HA assays.

Antibodies

New Zealand white rabbits were immunised by two subcutaneous doses of CS (100 µg) mixed with Freund's complete adjuvant, given at a 10-day interval, followed by a third dose (150 µg), given 15 days later. Serum was collected 1 week after the last injection and stored at –20°C. The rabbit-antibody titre to LPS of *S. dysenteriae* serotype 1 (anti-CS) was determined by ELISA² with LPS purified from *S. dysenteriae* serotype 1 (25 µg/ml) used as source of antigen; the titre was 2.56 × 10⁴. For absorption studies, serum (1 ml) was incubated with LPS of *S. dysenteriae* serotype 1 (3 mg) for 1 h at 37°C and then overnight at 4°C. After incubation, the absorbed serum was centrifuged at 35 000*g*. Convalescent sera, collected 21 days after

onset of infection in three adult patients infected with *S. dysenteriae* serotype 1, were also included in the study.

Crossed immuno-electrophoresis and double immunodiffusion

Crossed immuno-electrophoresis²⁴ was performed on glass plates (5 × 5 cm) with agarose 1% w/v in Tricine buffer (pH 8.7). Double immunodiffusion was performed according to the method of Ouchterlony²⁵ on glass microscope slides.

Bacterial adhesion to cultured epithelial cells

Henle Intestinal 407 cells (Int. 407) (Flow Laboratories) were maintained in Basal Eagle's Medium (Flow) supplemented with newborn calf serum (Flow) 15% v/v and 2 mM glutamine. Cells were seeded (1×10^5) in culture vials (Kimble, Toledo, OH, USA) containing cover slips (12-mm diameter). After 18 h, cells were inoculated with bacterial suspensions (100 µl) containing 1×10^9 cfu and centrifuged at 500g for 10 min at 4°C. The vials were incubated for a further 20 min at 37°C in air + CO₂ 5% and washed ($\times 10$) by aspiration with PBS to remove non-adherent bacteria. Cover slips were fixed in methanol, stained with Giemsa and examined by oil-immersion microscopy ($\times 1000$). Adhesion and invasion were also confirmed by performing quantitative bacterial counts.²⁶ Both smooth virulent (Congo-red positive) and rough mutant strains of *S. dysenteriae* serotype 1 were examined in adhesion assays (table II). The effect of serum, bacterial components, carbohydrates, proteins, and enzymes on the adhesion of bacteria to Int. 407 cells was determined (table III). For each assay bacteria adhering to 10 infected cells were counted and the number of bacteria adhering per cell was calculated. The mean and SEM of bacteria adhering per cell from five separate experiments were calculated. Extent of adhesion under different conditions was expressed as the percentage of binding compared to that of the smooth, virulent control strain 14731. Student's *t* test was used to calculate differences in adhesion due to the effect of various pre-treatments; *p* values > 0.05 were considered to be not significant.

Bacterial adhesion to immobilised mucin

Human mucin (extracted from a human colon at necropsy) was fractionated (20 mg/ml) on a column (2.2 × 36 cm) of Sepharose 4B. Fractions eluting in the region of Blue Dextran (60 ml) were dialysed against PBS and lyophilised. For immobilisation purposes, 0.5 ml of human mucin (1.5 mg/ml) was immobilised²⁷ on culture vials containing cover slips. Adhesion assays with bacteria were performed as described above and adhesion was expressed as the number of adherent bacteria per mm².

Results

Extraction and purification of haemagglutinin

The OMPs and cell-surface proteins of strain 14731 of *S. dysenteriae* serotype 1, prepared by different methods, did not agglutinate erythrocytes in protein concentrations of ≤ 10 mg/ml, either on slides or in microtitration plates. Treatment of haemagglutinating bacteria with trypsin or proteinase K had no effect on HA nor did pre-treatment of erythrocytes with neuraminidase.

Culture supernates of strain 14731 of *S. dysenteriae* serotype 1 caused HA at concentrations of ≥ 52.6 µg/ml (table I). Heating of CS at 100°C for up to 30 min or treatment with trypsin or proteinase K had no effect on HA. When CS was fractionated through a column of Sepharose 4B, HA (30.6 µg/ml) was detected in materials eluting (Pool-1, fractions 90–115 ml) at a volume similar to the region at which LPS (118 ml) from *S. dysenteriae* serotype 1 was eluted. Subsequent fractions (230–380) did not contain HA activity. Pool-1 contained a high concentration of carbohydrates (1.05 mg/ml) but a relatively low concentration of protein (0.126 mg/ml). Pool-1 showed a reaction of identity with LPS when analysed by double immunodiffusion by Ouchterlony's method against rabbit anti-CS serum. CS, Pool-1 and LPS showed similar precipitin lines when analysed against rabbit anti-CS serum by crossed immuno-electrophoresis. Pool-1 gave a positive reaction with thiobarbituric acid confirming the presence of KDO and was positive in the LAL assay indicating the presence of endotoxin (at ≤ 50 pg/ml). Heat treatment or treatment with proteolytic

Table I. Haemagglutinating activity (HA) of bacterial components of *S. dysenteriae* serotype 1 strain 14731 and LPS of other strains

Bacterial component	HA	Minimum concentration required for HA (µg/ml)*
Culture supernate	+	52.6 (SEM 3)
Sepharose 4B eluate (Pool-1)	+	30.6 (SEM 1.8)
LPS (from <i>S. dysenteriae</i> serotype 1)	+	18.58 (SEM 1.01)
Polysaccharides (from delipidation of LPS)	+	3.98 (SEM 0.152)
LPS (from strain 36000 of <i>E. coli</i> serotype O8:K25)	—†	...
LPS (from a strain of <i>E. coli</i> serotype O55:B5)	—	...
LPS (from strain 6577 of <i>S. sonnei</i> form 1)	—	...
LPS (from strain 33744 of <i>S. boydii</i> serotypes 1–6)	—	...
LPS (from rough mutant strain PSDIO)	—	...
LPS (from rough mutant strain 60R)	—	...

* All materials were quantified on the basis of carbohydrate content and results shown are the mean (and SEM) of three experiments.

† Components negative for HA were tested up to a concentration of 10 mg/ml.

enzymes did not affect HA activity, results confirming that Pool-1 contained LPS.

Purified LPS obtained from strain 14731 of *S. dysenteriae* serotype 1 agglutinated erythrocytes (table I). Treatment with heat or enzymes did not decrease activity. HA activity of CS, Pool-1 and LPS (at $\leq 80 \mu\text{g/ml}$) was completely inhibited by rabbit anti-CS serum (at a dilution of 1 in 100 in PBS). However, serum absorbed with LPS did not inhibit the HA of these preparations, results confirming that HA in cell-free cultures of *S. dysenteriae* serotype 1 was associated with LPS. Similar results were obtained with CS and LPS derived from haemagglutinating strains 12588, 3351 and 26406 of *S. dysenteriae* serotype 1.

Pre-treatment of LPS, CS and Pool-1 with *N*-acetyl neuraminic acid or fetuin resulted in loss of HA activity. Polysaccharides obtained by delipidation of LPS showed highest HA activity (table I). LPS obtained from *S. sonnei*, *S. boydii*, rough mutant strains (PSD-10, 60R) of *S. dysenteriae* serotype 1, *E. coli* serotype O55:B5 or a non-pathogenic strain of *E. coli* serotype O8:K25 did not show HA activity (table I).

Bacterial cultures, PBS extracts, CS and LPS did not contain sialic acids when tested by the resorcinol-HCl method.²³

Adhesion to Int.407 cells

When incubated with Int.407 cells for 20 min at 37°C, haemagglutinating strains of *S. dysenteriae* serotype 1 showed adherence (table II) (fig. 1b). When incubation of bacteria with Int.407 cells was allowed

to progress longer, cell invasion occurred (fig. 1c). Int.407 cells, grown for 18 h on coverslips, were better suited for this assay than cells grown for ≥ 40 h. Smooth (Congo-red positive) virulent strains adhered to Henle 407 cells; in contrast, rough mutant strains of *S. dysenteriae* serotype 1 bound poorly (table II).

Binding of strains of *S. dysenteriae* serotype 1 to Int.407 cells was completely inhibited by pre-treatment of cells with homologous LPS (table III) at 0.18 mg/ml. CS at a similar concentration inhibited c. 60% of adherence. Human convalescent sera and rabbit anti-CS serum also inhibited, to some extent, bacterial adhesion. When Int.407 cells were treated with wheat-germ agglutinin, binding of bacteria was reduced by 84%. Neuraminidase treatment of intestinal cells also reduced considerably the numbers of bound bacteria. However, the presence of human mucin increased binding of bacteria to Int.407 cells (174%). Thus, various pre-treatments (table III) significantly affected the adhesion of *S. dysenteriae* serotype 1 to Int.407 cells (*p* values, ≤ 0.01).

Table II. Adhesion to Int.407 cells by strain 14731 of *S. dysenteriae* serotype 1 and by rough mutant strains

Strain no.	Number (SEM) of adherent bacteria/cell*	Adhesion (percentage of control)	<i>p</i> value
14731 (control)	75.8 (7.05)	100	
Rough mutant PSD-10	12.8 (2.59)	16.9	<0.01
Rough mutant 60R	15.2 (3.84)	20.0	<0.01

* Results are expressed as mean (and SEM) of five experiments.

Fig. 1. Uninoculated (control) cells of Int.407 (a); and, after incubation with strain 14731 of *S. dysenteriae* serotype 1, showing adhesion after 20 min (b) and invasion after 3 h (c); binding to immobilised mucin is also shown (d).

Table III. Effect of various pre-treatment regimens on adhesion of strain 14731 of *S. dysenteriae* serotype 1 to Int. 407 cells

Pre-treatment	Concentration (/ml)	Adhesion (percentage of control)*	Difference from control (p value)
Neuraminidase	100 units	20.0	<0.01
Wheat-germ agglutinin	0.05 µg	16.1	<0.01
Mucin (human)	0.05 µg	174.6	<0.01
LPS (<i>S. dysenteriae</i> serotype 1)	0.18 µg	0.0	<0.01
Culture supernate	0.18 µg	40.89	0.01
Rabbit (anti-CS) serum*		33.4	<0.01
Convalescent serum from patient 1*		18.46	<0.01
patient 2		25.1	<0.01
patient 3		37.9	<0.01

* All sera were tested at a dilution of 1 in 50 in PBS.

Binding to mucin

Strains of *S. dysenteriae* serotype 1 also bound to immobilised human mucin (average of $1.2 \times 10^4/\text{mm}^2$) (fig. 1d). Binding of bacteria to Int.407 cells in the presence of mucin could not be inhibited by any of the above conditions.

Discussion

The results show that HA activity of *S. dysenteriae* serotype 1 is associated with LPS present in culture supernates and with LPS purified from the bacteria. Proteins were not linked with LPS in this interaction because heat and proteases did not affect the HA activity of purified LPS or components separated from culture supernate. The observations that rabbit serum raised against CS inhibited this activity, and that absorption of LPS antibodies removed this inhibition, further confirmed the role of LPS in the agglutination of erythrocytes.

The agglutination of erythrocytes by LPS from *S. dysenteriae* serotype 1 appeared to be highly specific and possibly involved the O-antigenic carbohydrate side-chains. Polysaccharides separated from the lipid A component of LPS also agglutinated erythrocytes. It was not possible to test lipid A for HA because of its insolubility in aqueous solutions, although this component is known to have affinity for cell membranes.²⁸ LPS extracted from rough mutant strains of *S. dysenteriae* serotype 1, that are deficient in polysaccharide side-chains, did not show agglutinating activity. Rough strains also exhibited poor adherence to Int.407 cells.

LPS derived from smooth strains inhibited adhesion of *S. dysenteriae* serotype 1 to Int.407 cells. Izhar *et al.*⁷ have shown that LPS from *S. flexneri* serotype 1b

inhibited binding of homologous strains to guinea-pig colonic cells, suggesting that bacteria adhere *via* LPS to a glycoprotein receptor on host tissue. Our results also indicate that the heat- and protease-resistant adhesin (for both erythrocytes and cultured epithelial cells) is associated with LPS. It has been shown before that only smooth strains of *Shigella*^{3,29} are virulent, although an exact function was not assigned to LPS. However, in *Salmonella typhi*, it has been shown that smooth LPS is necessary for adhesion and invasion.²⁶ LPS obtained from strains of *S. sonnei* form 1, *S. boydii* serotypes 1–6 and from *E. coli* serotypes O8:K25 or O55:B5, under the same culture conditions, did not agglutinate erythrocytes, a finding pointing perhaps to the specificity of LPS required for such activity.

HA activity of *S. dysenteriae* serotype 1 is sensitive to sialic acids and the haemagglutination ability of LPS is also sensitive to sialic acid or to fetuin obtained from fetal calf serum, a sialic acid-containing (5.7% NANA) protein. This suggests that LPS binds to sialic acid-containing receptors on erythrocytes. The binding of protein adhesins to erythrocytes is mediated *via* receptors containing specific carbohydrates, including sialic acids which may, or may not, be sensitive to sialinidases such as neuraminidase.^{13,30–32} We have demonstrated that treatment of erythrocytes with neuraminidase from *C. perfringens* did not decrease the affinity of the haemagglutinin to erythrocytes, whereas similar treatment of cultured intestinal cells resulted in a marked decrease in binding of bacteria, as has been reported in other interactions. The reason for the resistance of erythrocytes to treatment with neuraminidase is not clear, but indicates perhaps that the enzyme has no effect on LPS-binding site. Other proteolytic and hydrolytic enzymes will have to be tested to characterise further the haemagglutinin-binding site on the erythrocyte surface.

Although the association of adhesins with LPS is less common than with protein, it has been reported before. Other bacteria, the adhesins of which were shown to be LPS-associated, include *Campylobacter jejuni*,⁶ *Vibrio cholerae*,³³ *S. typhi*,²⁶ and *E. coli* F-18.³⁴ We conclude that strains of *S. dysenteriae* serotype 1 have an adhesive property which is associated with O-antigenic polysaccharide. This study and those of Izhar *et al.*⁷ and Pal and Hale⁸ highlight the role of adhesins in the interaction of strains of *S. flexneri* and *S. dysenteriae* serotype 1 with host-cell surfaces. The significance of bacterial adhesion in the pathogenesis of shigellosis needs to be clarified *in vivo*; it is possible that high titres of serospecific LPS antibody, that can be demonstrated in patients recovering from *S. dysenteriae* serotype 1 and *S. flexneri* infections,² play a major role in protection against species- and serotype-specific re-infection by preventing bacterial adhesion.

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