ROLE OF HEAT LABILE ANTIGENS OF *SHIGELLA FLEXNERI* IN HELA CELL INVASION

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SUMMARY. In studies of the role of surface antigens of *Shigella flexneri* in HeLa cell invasion, three antisera were employed to block the invasion. Antisera against live (ALS) and boiled (ABS) *S. flexneri* blocked invasion very effectively. Reduction in the numbers of intracellular shigellae was always accompanied by reduction in the number adherent to the cells, indicating the importance of adhesion in the invasive process. Anti-live absorbed antiserum (ALAS) prepared by exhaustive absorption of ALS with boiled *S. flexneri* blocked adhesion and invasion at dilutions of 20 or 50; the efficiency of the absorption was indicated by absence of agglutinating and anti-lipopolysaccharide (LPS) antibodies. *S. flexneri* LPS did not block adhesion and invasion even at a concentration of 1.0 mg/ml. Hence it was concluded that heat-labile surface antigens are important in adhesion and invasion of HeLa cells by *S. flexneri*. Antiserum against heat stable antigen (ABS) probably blocks adhesion by steric hindrance.

INTRODUCTION

Epithelial cell invasion by virulent shigellae is an important step in the pathogenesis of bacillary dysentery (LaBrec *et al.*, 1964). Though the process of internalisation of the shigellae into the epithelial cell has been referred to as invasion, it appears to be due to active endocytosis by the epithelial cell (Hale, Morris and Bonventre, 1979). In this paper, we report studies on antigenic interactions between the shigellae and the epithelial cell occurring prior to invasion.

MATERIAL AND METHODS

Bacterial strain. A strain of *Shigella flexneri* isolated from a case of bacillary dysentery was used. Since shigellae are known to lose invasiveness after repeated passage on artificial media (Mackel, Langley and Venice, 1961; Okamura and Nakaya, 1977), the strain was lyophilised immediately after isolation and identification. The lyophilised culture was stored at 4°C. On each test occasion the HeLa cell invasion assay was performed with a freshly revived culture, so that results would be comparable. A laboratory isolate of *Escherichia coli* was used as a control organism.

Source and maintenance of HeLa cell line. The HeLa cell line was obtained from the National Institute of Virology, Pune, India. The cell cultures were maintained routinely as monolayers in Dulbecco's modified Eagle's medium with newborn calf serum 15%, penicillin G 100 U/ml, streptomycin 100 µg/ml and amphotericin B 2.5 µg/ml.

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Antisera. Hyperimmune rabbit antisera were raised as described by Edwards and Ewing (1972). Rabbits received intravenously (i.v.) 0.5, 1.0, 2.0 and 3.0 ml of an appropriate suspension of *S. flexneri* at intervals of 3–4 days and were bled 1 week after the last injection.

Anti-live S. flexneri antiserum (ALS) was prepared by immunisation with live cells of *S. flexneri*. Because these were quite toxic to rabbits, two additional initial immunisations were given with 0.25 ml of a suspension containing 10^9 organisms/ml of formalinised *S. flexneri*.

Anti-boiled S. flexneri antiserum (ABS) was raised by immunisation with a suspension of boiled cells of *S. flexneri* (10^9 /ml) prepared as described by Edwards and Ewing (1972).

Anti-live S. flexneri absorbed antiserum (ALAS) was ALS absorbed with washed and boiled cells of *S. flexneri* until all anti-LPS activity was absorbed.

Assay of antibody activity. *Whole bacterial cell agglutination titre* was determined with a suspension of boiled *S. flexneri*, by the method of Williams and Chase (1977). *Anti-LPS titre* was determined by microtitre passive haemagglutination of sheep red cells sensitised with *S. flexneri* LPS as described by Kwapinski (1972).

HeLa cell invasion assay. A modified assay of LaBrec *et al.* (1964) was done with confluent monolayers in 35 mm disposable plastic petri dishes incubated at 37°C in air + CO₂ 5% in a humidified chamber: 3 h before the assay, the culture medium containing antibiotics was replaced with 2 ml of antibiotic-free Dulbecco's medium and incubation was continued for 3 h. Then the medium was replaced by 1 ml of Dulbecco's medium containing the requisite number of shigellae. A volume of the bacterial inoculum was diluted and plated out to compute the number of viable organisms deposited on the monolayers. The challenged monolayers were further incubated as before for 3 h and then washed six times with 0.15 M phosphate-buffered saline (PBS) at pH 7.2. To one set of monolayers, Dulbecco's medium containing gentamicin 50 µg/ml was added to kill extracellular bacteria. A parallel set was given Dulbecco's medium without any antibiotic, to provide an estimate of adherent extracellular bacteria along with intracellular bacteria. After incubation for 6 h at 37°C, the monolayers were treated with trypsin 0.25% in Hanks' balanced salt solution (BSS). The cell suspensions were transferred to siliconised centrifuge tubes, and washed three times with Hanks' BSS containing gelatin 0.1%. After the last wash, the cell pellet was suspended in distilled water containing bovine serum albumin 0.1%. It was rapidly freeze-thawed three times to lyse the cells and to liberate intracellular bacteria. The lysate was then diluted and plated out to compute the viable bacteria by counting colony forming units (cfu).

Statistical analysis. The data were analysed by the Student t test (Bancroft, 1957).

RESULTS

Characterisation of the antisera

The whole cell agglutination and anti-LPS titres of the three antisera are shown in table I. The anti-LPS titre of the serum prepared against boiled bacteria (ABS) was

TABLE I
Details of the antisera used

Antiserum*	Whole cell agglutination titre	Anti-LPS titre
ALS	320	1024
ABS	320	20480
ALAS	nil	nil

* See *Methods*.

TABLE II

Results of a representative experiment with the HeLa cell invasion assay

Challenge organism (10^9 /monolayer)	Mean count* (cfu/plate) of	
	intracellular organisms†	total intracellular and extracellular organisms‡
<i>Shigella flexneri</i>	$1.65 (\pm 0.176) \times 10^6$	$9.0 (\pm 0.132) \times 10^6$
<i>Escherichia coli</i>	$4.07 (\pm 0.01) \times 10^3$	$5.73 (\pm 0.63) \times 10^8$

* $\pm$ Standard error of the mean.

† cfu/monolayer in the presence of gentamicin.

‡ cfu/monolayer in medium without gentamicin.

much higher than that of the anti-live serum (ALS). The anti-live absorbed serum (ALAS) was free of agglutinating and anti-LPS antibodies.

Quantitative aspects of HeLa cell invasion assay

Results of a representative experiment are shown in table II. Confluent HeLa cell monolayers in 35 mm plastic petri dishes were incubated with 10^9 cells of *S. flexneri*. After 3 h the plates were washed with PBS and two parallel sets of monolayers were established (see *Methods*).

The mean bacterial count per plate in the presence of gentamicin was 1.65×10^6 . This number is an estimate of the intracellular bacteria since gentamicin cannot penetrate the live HeLa cells. The mean bacterial count per plate in the absence of gentamicin was 9.0×10^6 and this indicates the total number of intracellular and extracellular adherent shigellae. It is clear that more than 80% of the shigellae were extracellular and adherent to the monolayers and could not be dislodged by washing. However, it is significant that as many as 18.3% of the shigellae were intracellular. Strains of normal colonic *Escherichia coli* when similarly tested on HeLa cell monolayers yielded negligible numbers of organisms in the presence of gentamicin. Results of one representative experiment are shown (table II) in which only 0.0007% of the total *E. coli* organisms survived on the monolayers in the presence of gentamicin. The difference between the estimates for intracellular shigellae and *E. coli* was highly significant ($p < 0.005$).

Blockade of adhesion and invasion by the antisera

HeLa cell monolayers were incubated with *c.* 10^7 cells of *S. flexneri* suspended in 2.0 ml of Dulbecco's medium containing heat-inactivated antisera or normal rabbit serum (NRS). The results given in table III show that ALS and ABS reduced the number of intracellular organisms by *c.* 31–89% at a dilution of 200. The total numbers of organisms, i.e. both intracellular and extracellular adherent organisms, were also reduced in the presence of the two antisera by *c.* 46–89%. Since >90% of the total

TABLE III

Effect of the test antisera on adhesion and invasion of HeLa cells by S. flexneri

Test no.	Inoculum per monolayer (10^7 organisms)	Serum (and test dilution)	Mean count* (10^6 cfu/plate)		Percentage reduction of intracellular organisms	Percentage inhibition of adhesion
			intracellular organisms	total intracellular and extracellular organisms		
1	1.03	NRS (200)	0.392 (± 0.03)	8.8 (± 0.09)	57.9	62.2
		ALS (200)	0.165 (± 0.003)	3.32 (± 0.17)		
2	1.57	NRS (200)	0.289 (± 0.01)	7.58 (± 0.19)	89.23	89.3
		ALS (200)	0.031 (± 0.001)	0.81 (± 0.03)		
3	1.59	NRS (200)	0.42 (± 0.01)	9.57 (± 0.1)	31.0	46.4
		ABS (200)	0.293 (± 0.01)	5.13 (± 0.1)		
4	1.88	NRS (200)	ND	54.7 (± 0.7)	...	6.5
		ALAS (200)	ND	51.1 (± 0.6)		
5	1.7	NRS (50)	ND	51.6 (± 0.19)	...	65.9
		ALAS (50)	ND	17.6 (± 0.24)		
6	1.1	NRS (20)	1.16 (± 0.01)	32.3 (± 0.2)	73.0	72.0
		ALAS (20)	0.313 (± 0.02)	8.9 (± 0.07)		

NRS = Normal Rabbit Serum; ABS, ALS, ALAS—see *Methods*; ND = not done. * See table II.

organisms on the monolayers are extracellular adherent bacteria, the reduction in their numbers clearly indicates inhibition of adhesion of the shigellae to the monolayers by the two antisera. It could be argued that the observed effect was due to clumping of bacteria in the presence of the antisera. This can be ruled out because no bacterial agglutination was observed when *S. flexneri* were mixed at a concentration of 10^7 cells/ml with the antisera at a dilution of 200.

The ALAS did not block adhesion to the monolayers at a dilution of 200 but did so at dilutions of 50 and 20 (table III). Its ability to reduce the numbers of intracellular shigellae was also tested and confirmed (table III). The results were significant, *p* values ranging from <0.05 to <0.005 .

Effect of inoculum size on inhibition of adhesion and invasion by ABS and ALS

A series of HeLa cell monolayers were incubated in the presence of heat-inactivated ABS and ALS at a dilution of 200 with graded numbers of *S. flexneri*. The antisera

TABLE IV

Relationship between inoculum size and blockade of adhesion and invasion by ALS and ABS

Test no.	Inoculum per monolayer	Serum (and test dilution)	Mean count* (10 ⁶ cfu/plate)		Percentage reduction in intracellular organisms	Percentage inhibition of adhesion
			intracellular organisms	total intracellular and extracellular organisms		
1	1.59 × 10 ⁷	NRS (200)	0.42 (±0.007)	9.57 (±0.146)	31.0 (p < 0.01)	46.4 (p < 0.01)
		ABS (200)	0.293 (±0.001)	5.13 (±0.088)		
2	1.57 × 10 ⁷	NRS (200)	0.289 (±0.01)	7.58 (±0.192)	89.24 (p < 0.005)	89.31 (p < 0.005)
		ALS (200)	0.031 (±0.001)	0.81 (±0.028)		
3	2.0 × 10 ⁸	NRS (200)	2.37 (±0.298)	43.9 (±1.88)	17.7 (NS)	No reduction
		ALS (200)	1.963 (±0.043)	48.8 (±2.86)		
4	2.05 × 10 ⁹	NRS (200)	6.47 (±0.16)	34.8 (±1.11)	No reduction	No reduction
		ABS (200)	9.75 (±0.382)	57.2 (±1.51)		

NS = not significant; NRS = Normal Rabbit Serum; ABS, ALS—see *Methods*. * See table II.

TABLE V

Effect of LPS on adhesion and invasion of HeLa cells by S. flexneri

Test no.	Inoculum per monolayer (10 ⁷ organisms)	LPS	Mean count* (10 ⁶ cfu/plate)		Percentage reduction in intracellular organisms	Percentage inhibition of adhesion
			intracellular organisms	total intracellular and extracellular organisms		
1	0.94	<i>E. coli</i> (1 mg/ml)	0.77 (±0.02)	16.0 (±1.2)	2.99 (NS)	6.25 (NS)
		<i>S. flexneri</i> (1 mg/ml)	0.747 (±0.016)	15.0 (±0.75)		
2	1.56	none	ND	36.0 (±0.19)	...	No reduction
		<i>E. coli</i> (1 mg/ml)	ND	38.4 (±0.21)		
		<i>S. flexneri</i> (1 mg/ml)	ND	35.1 (±0.2)		
3	5.12	none	ND	92.0 (±0.73)	...	4.9 (NS)
		<i>S. flexneri</i> (100 µg/ml)	ND	87.5 (±0.284)		

ND = not done; NS = not significant. * See table II.

that could block adhesion and invasion effectively when the inoculum contained *c.* 10^7 shigellae could not do so when the inoculum size was raised to *c.* 2×10^8 cells (table IV).

Effect of homologous LPS on adhesion and invasion of HeLa cells by S. flexneri

HeLa cell monolayers were incubated with 1.56×10^7 and 5.12×10^7 cells of the test strain of *S. flexneri* in the presence of its LPS at concentrations of 100 µg/ml and 1.0 mg/ml. As controls, similar monolayers were set up with *E. coli* LPS at 1.0 mg/ml and with Dulbecco's medium containing no LPS. LPS did not block adhesion or invasion (table V).

DISCUSSION

These blockade studies were designed as a simple attempt to categorise the antigens of *S. flexneri* involved in epithelial cell invasion. Since ALS was raised against live *S. flexneri*, it is expected to contain antibodies against multiple surface components, some or all of which may be involved in the process of invasion. ABS is an antiserum against heat-stable antigens. ALAS was prepared by absorbing ALS exhaustively to remove antibodies against heat-stable surface antigens. The completeness of the absorption is illustrated by the fall of the anti-LPS titre to zero. Hence for practical purposes, and with some obvious reservations, ALAS can be considered as an antiserum against heat-labile antigens.

ALS and ABS reduced the total number of intracellular shigellae by *c.* 31–89%. This reduction was always accompanied by reduction of the same order in the numbers of shigellae adherent to the monolayers. This indicates that the reduction in invasion is a direct result of reduction in the adhesion of the shigellae to the monolayers. Control experiments at this point seemed to exclude agglutination and clumping of bacteria by the test sera as an alternative explanation for these findings.

Because ABS blocked adhesion and invasion, it was expected that ALS would lose its blocking activity after absorption with boiled cells. Surprisingly, ALAS also blocked adhesion and invasion at dilutions of 20 and 50 though not at 200. This strongly suggests that the adhesion of shigellae to the HeLa cells is mediated by heat-labile surface antigens.

There are two possible explanations for these apparently contradictory results: (1) both heat-labile and heat-stable surface antigens of *S. flexneri* may participate in adhesion; or (2) the adhesion may be mediated by heat-labile surface antigens whilst the observed blockade by ABS could reflect steric hindrance by heat-stable surface antigens that have no role in the adhesion of shigellae to HeLa cells. Because the predominant heat-stable surface antigen is LPS, its role in adhesion was investigated and ruled out.

The importance of heat-labile surface antigens in shigella adhesion and invasion has been demonstrated in another system by Adamus *et al.* (1980). Guinea pigs and rabbits immunised with outer membrane proteins (OMP) of *S. flexneri* were protected against experimentally induced keratoconjunctivitis whereas those immunised with heat-killed *S. flexneri* were not.

In similar studies to ours, Hale and Bonventre (1979) found that antiserum against boiled cells did not block invasion of Henle intestinal epithelial cells by shigellae.

These results clearly contradict ours. It is likely that this is an inoculum effect; at the bacterial concentrations used by Hale and Bonventre, the blocking activity of the antiserum is likely to be saturated. When the size of the inoculum per monolayer was increased, the blocking activity of ABS was not observed in our studies.

We conclude from our present evidence that adhesion is an important step in the process of invasion of epithelial cells by shigellae and it is likely to be achieved through heat-labile surface antigens such as proteins. Heat-stable surface antigens such as LPS do not seem to be important in this phase of invasion.

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