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HeLa Cell Invasiveness and O Antigen of *Shigella flexneri* as Separate and Prerequisite Attributes of Virulence to Evoke Keratoconjunctivitis in Guinea Pigs

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Many rough mutants selected from isogenic smooth virulent and avirulent strains of *Shigella flexneri* were examined for virulence, using tissue culture infection and Sereny tests. Many of the rough mutants isolated from a virulent smooth strain were capable of penetrating tissue culture cells but incapable of producing a positive Sereny test. In contrast, we could not obtain from smooth avirulent strains any rough mutants capable of penetrating HeLa cells. Chemical analysis of lipopolysaccharide of some representative rough strains showed several patterns of sugar composition with a range of from Ra to Re chemotypes. There was no correlation between HeLa cell invasiveness and chemotypes of lipopolysaccharides, thus indicating little significance of oligosaccharides of the rough core as well as O antigens in the ability of *S. flexneri* to penetrate HeLa cells. When these invasive rough strains were given O antigen genes from a smooth avirulent *Shigella* Hfr strain, most of the transconjugants that expressed O antigens regained the ability to evoke keratoconjunctivitis in guinea pigs. We also examined the chromosomal loci of HeLa cell invasion by transferring carbohydrate fermentation genes of *Escherichia coli* K-12 Hfr and found two chromosomal loci, the *rha* and *lac-gal* regions, which control the ability to penetrate HeLa cells. These results suggested that O antigens and ability to penetrate tissue culture cells are independent and prerequisite attributes of virulence in *Shigella flexneri* to evoke keratoconjunctivitis in guinea pigs.

The primary step in the pathogenesis of bacillary dysentery is the penetration of intestinal epithelial cells by the pathogen (12, 17). Strains that lack this capacity are avirulent (12, 17). Two of the several experimental model systems to assess the virulence of *Shigella* spp., that is, keratoconjunctivitis of guinea pigs (Sereny test) (21) and penetration of tissue culture cells (22), have been considered to be two different ways to measure the same property (5, 8, 12). We demonstrated, however, the disassociation between these tests in our previous study, where a rough mutant was obtained from a smooth virulent strain of *Shigella flexneri* 2a that penetrates tissue culture cells but does not evoke keratoconjunctivitis in guinea pigs (18). In that paper, we suggested that the presence of such a mutant might indicate that O-specific antigen is not required for penetration of tissue culture cells. Since we neither analyzed the complete sugar composition of lipopolysaccharide (LPS) of that mutant nor went a step further to clarify the mechanism of penetration by a rough strain in comparison to that by a smooth strain, it remained to be determined whether the mutant has on its surface a small quantity of O antigen that is essential for penetration of tissue culture cells and whether the mechanism of penetration of tissue culture cells by a rough strain is different from that by virulent smooth strains and, thus, not related to virulence.

In the present paper, to answer these questions, we isolated many rough mutants from isogenic smooth virulent and avirulent strains of *S. flexneri* and grouped them into several sensitivity patterns to LPS phages. We further examined the sugar composition of LPS of the representative strains in these groups and assessed the virulence by two experimental model systems described above. We also examined the virulence of clinical isolates and their rough mutants. The results led us to conclude that oligosaccharides of both O antigens and rough cores of LPS have nothing to do with the ability to penetrate tissue culture cells. Genetic experiments were also done to determine whether the

TABLE 1. Bacterial strains

Species	Strain designation	Serotype	Relevant characteristics	Virulence		Source
				HeLa cell invasion	Sereny test	
S. flexneri	5503	2a	*nic* Str^r	+	+	This laboratory
	5503-I	2a	*nic* Str^r	−	−	Mutant of 5503
	RN3	Y	*nic* Str^r	+	+	Mutant of 5503
	Rm3-58	Y	*nic* Str^r	−	−	Mutant of RN3
	5503-01[a]	R	*nic* Str^r F$^-$	+	−	Mutant of 5503
	Rm3-89	R	*nic his* Str^r F$^-$	+	−	This work
	Rm3-8	R	*nic his* Str^r F$^-$	−	−	This work
	Rm3-25	R	*nic his* Str^r F$^-$	−	−	This work
	FH1049	Y	*nic lac*$^+$ Hfr	−	−	This work
	Clinical isolates (46 strains)	2a				A. Nakamura
E. coli	K-12 HfrH	R	*thi* Hfr	−	ND[b]	M. Yoshikawa
	K-12 W1895	R	*met* Hfr	−	ND	M. Yoshikawa
	K-12 P4X-6	R	*met* Hfr	−	ND	M. Yoshikawa

[a] In genetic experiments, mutants (*gal* or *his gal*) were selected from strain 5503-01 and used as recipients.
[b] ND, Not done.

mechanism of penetration of tissue culture cells by rough strains is identical to that by smooth virulent strains, and the results showed this to be the case.

MATERIALS AND METHODS

Bacterial strains. The characteristics of strains employed in this study are summarized in Table 1. *S. flexneri* Y FH1049, a donor strain employed in genetic crosses with *S. flexneri* rough strains, was derived from *S. flexneri* Y FH10 (kindly provided by P. Gemski, Walter Reed Army Institute of Research) by the method of F-linked terminal-marker selection. *Escherichia coli* K-12 P4X-6, an Hfr strain which transfers as its terminal marker the lactose utilization genes linked to the F plasmid, was mated with *S. flexneri* Y FH10, and Lac$^+$ recombinants were selected. One of the recombinants, strain FH1049, was found to be stable for the Lac$^+$ character and efficiently transferred chromosomal genes in crosses with *E. coli* recipients with a polarity similar to that of P4X-6. Clinical isolates of *S. flexneri* 2a that had been isolated from dysentery patients in Southeast Asia, Australia, and South Pacific countries were kindly provided by A. Nakamura of the National Institute of Health, Japan.

Phages. Six phages, Felix O, 6SR, C21, T4, T7, and Sf6, were used. These phages have the specific receptor sites on the carbohydrates of the LPS of the outer membrane (14). Phage Sf6, which was kindly provided by P. Gemski, has its receptor site on the *S. flexneri* 3,4 group antigens (10). A clear plaque mutant of phage Sf6 was obtained by UV light mutagenesis and was used for the isolation of rough mutants from *S. flexneri* Y RN3 and Rm3-58.

Media. Luria (L) broth and agar and Penassay broth agar (Difco Laboratories) were used for routine cultivation and other experiments. Cooked-meat medium (Difco Laboratories) was used for storage of the strains. For the preparation of LPS, nutrient broth was used to cultivate large batches of bacterial strains. Minimal medium, used in genetic experiments for selecting and scoring recombinant hybrids, contained, per liter of distilled water: K_2HPO_4, 1.05 g; KH_2PO_4, 0.45 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2$, 0.005 g; and agar, 10 g. Nicotinic acid was supplemented at a concentration of 2 μg/ml. When required, amino acids and growth factors were added to minimal medium at a final concentration of 20 μg/ml. Streptomycin, employed as a counterselective agent against male cells in some of the matings, was added to minimal medium at a final concentration of 100 μg/ml. Sugars were dissolved in water, sterilized, and added to a medium to a concentration of 1%. Fermentation characters were scored on MacConkey indicator medium, which consisted of MacConkey agar base (Difco Laboratories) supplemented with appropriate carbohydrate (1%).

Phage sensitivity test. Phages were grown on suitable strains of rough *Salmonella typhimurium* (chemotype: semirough, Ra, and Rc), *S. flexneri* Y FH10, and *E. coli* B. The bacterial cultures were spread on L agar plates and spotted with drops of phage lysates (approximately 10^9 to 10^{10} PFU/ml). Bacterial lysis was scored after incubation for 6 to 8 h and overnight at 37°C.

Serological tests. Overnight bacterial growth from L agar plates was used as an antigen for agglutination tests. The tests were done on a glass slide, using 4 and 0.85% NaCl solutions or absorbed type-specific II and group 3,4 antisera against *S. flexneri* (Toshiba Kagaku-Kogyo). Agglutination tests in test tubes were also performed, using rabbit antiserum against *S. flexneri* Y RN3.

Cell culture assay. HeLa and L cell monolayers were used to study the infectivity of the strains as an in vitro

system. The cell cultures were maintained in prescription bottles as monolayers grown in Eagle minimal essential medium (Nissui Pharmaceuticals; containing 2 μmol of glutamine and 60 μg of kanamycin per ml) supplemented with 10% calf serum. Lab-Tek eight-chamber slides (Miles Laboratories, Inc.) were employed to prepare monolayers for penetration experiments. Approximately 24 h before preparing monolayers on chamber slides, the culture medium was removed and replaced with antibiotic-free Eagle minimal essential medium (Nissui Pharmaceuticals). Each chamber was seeded with approximately 2.5×10^4 cells, and the chamber slides were placed in a 37°C, 5% CO_2 incubator for 24 h. The monolayers were infected by addition of bacteria suspended in antibiotic-free Eagle minimal essential medium to give a final concentration of approximately 10^7 or 10^8 bacteria per chamber. After incubation for 2 h, the medium was removed from the chambers, the monolayers were washed twice with phosphate-buffered saline to minimize the extracellular multiplication of the bacteria, and then fresh minimal essential medium containing kanamycin was added. Kanamycin affects only extracellular bacteria (R. Nakaya and N. Okamura, Proceedings of the International Symposium on Bacterial Diarrheal Disease, in press). After a further 3 h of incubation, the chambers were washed twice with phosphate-buffered saline, fixed in methanol, and stained in 2% Giemsa solution for 30 min. The proportion of infected cells was determined by counting the total number of cells and the number of cells containing 10 or more bacteria in 15 microscopic fields selected at random.

Sereny test. Details of the Sereny test have been described (21). Hartley strain guinea pigs were used; their body weights were approximately 300 g. A few drops of suspensions (0.05 ml containing 10^9 organisms) of the test organisms grown on Penassay broth agar were deposited into conjunctival sacs of guinea pigs, and the animals were observed daily for 72 h. A reaction was considered to be positive when the cornea became opaque and conjunctivitis developed.

Preparation of LPS. LPS was isolated from the lyophilized or acetone-dried cells by the phenol-water method of Westphal et al. (24) or phenol-chloroform-petroleum ether method of Galanos et al. (9) and purified by repeated ultracentrifugation (105,000 × *g*) and treatment with RNase (20 μg/ml in 25 mM Tris-hydrochloride buffer, pH 7.4) (4). Neither ribose nor deoxyribose, both sugar constituents of nucleic acids, was detected in the preparation of LPS.

Determination of sugar composition of LPS. Neutral sugars were determined after hydrolysis in 2 N trifluoroacetic acid at 120°C for 1 h by gas-liquid chromatography as alditol acetates by the method of Laine et al. (13) (column: 3% ECNSS-M coated on Chromosorb W [Gasukuro Kogyo Co.], 3 mm by 2 m), using xylose as an internal standard. For amino sugar determination, LPS was hydrolized in 4 N HCl at 100°C for 8 h, and the hydrolysates were fractionated on a Dowex 50 (H^+) column by the method of Wheat (25). The amino sugar fraction thus separated was N acetylated with acetic anhydride and $NaHCO_3$ by the method of Lüderitz et al. (15), reduced with $NaBH_4$, and then peracetylated by the method of Laine et al. (13). Amino sugars were then determined as *N*-acetylalditol acetates by gas-liquid chromatography (column: Tabsorb [Regis Chemical Co.], 3 mm by 2 m), using mannose as an internal standard. The identities of the neutral and amino sugars were based on retention time values and mass spectra, compared with those of the derivatives of authentic standards. Gas-liquid chromatography/mass spectrometry was performed on an LKB 9000S instrument (LKB Instruments Inc.) with the same column as described above. Heptose was determined either by the method of Osborn (19) or by gas-liquid chromatography. Standard L-glycero- and D-glycero-D-mannoheptose was prepared as described by Bagdian et al. (2). KDO (2-keto-3-deoxyoctonic acid) was estimated by the method of Weissbach (23). KDO purchased from Sigma Chemical Co. was used as a standard. KDO was also determined by gas-liquid chromatography as alditol acetate, using the Tabsorb column, as were the alditol acetate derivatives of amino sugars.

Mating procedure. Donor and recipient strains were grown overnight at 37°C in Penassay broth. Overnight cultures were grown to the exponential phase of growth, and both donor and recipient strains were mixed with a volume ratio of 1 part donor culture to 4 parts recipient culture. The mixtures were then incubated at 37°C for 20 h with gentle agitation. Overnight mixtures were centrifuged, suspended in saline, and spread on selective minimal media. After incubation at 37°C for 2 days, recombinant colonies were picked up and purified by streaking on the original selective medium.

RESULTS

Isolation of rough mutants from smooth virulent and avirulent *S. flexneri* strains. By selection with a clear plaque mutant of phage Sf6, 144 strains were isolated from *S. flexneri* Y RN3 and were designated Rm3 strains. In the same manner, we isolated 82 strains from Rm3-58. Strain Rm3-58 is a spontaneous isolate from RN3. This strain had the same sensitivity pattern to phages as the parent strain RN3 and strongly agglutinated against group 3,4 antiserum. To isolate rough strains from *S. flexneri* 2a 5503-I, we cultivated this strain in cooked-meat medium and preserved it at room temperature for several days. The culture was then cultivated in Penassay broth and spread onto L agar plates. We picked up 130 opaque colonies, which was suspected as rough.

Phage sensitivity pattern of rough strains isolated from smooth *S. flexneri*. We examined the rough strains for sensitivity to LPS phages. Phage sensitivity tests were performed on 144 strains from RN3, 82 strains from Rm3-58, and 130 strains from 5503-I. Several phage sensitivity patterns were observed (Table 2). Although Table 2 shows sensitivity patterns for only 41 strains isolated from RN3, other isolates from RN3, Rm3-58, and 5503-I had similar sensitivity patterns to LPS phages. Most of the strains except those sensitive to Sf6 did not agglutinate strongly with specific antisera against type and group antigens. A small proportion of isolates

TABLE 2. Virulence and sensitivity pattern to LPS phages of Rm3 strains isolated from *S. flexneri* Y RN3

Strain	Sensitivity to phage[a]:						Sereny test[b]	HeLa cell invasion[b]	Cell infection rate (mean ± SD)[c]
	Sf6	Felix O	6SR	C21	T4	T7			
5503	−	−	−	−	−	−	+	+	3.6
RN3	+	−	−	−	+	−	+	+	3.1
Rm3	+	−	−	−	+	−	5/6	5/6	12.5 ± 1.5
	+	−	−	−	+	+	1/3	2/3	14.1 ± 1.9
	−	−	+	−	+	+	0/5	5/5	12.4 ± 10.9
	−	−	−	+	+	−	0/6	5/6	13.2 ± 9.0
	−	+	+	−	+	+	0/4	2/4	4.5 ± 0
	−	+	+	+	+	+	0/5	3/5	17.5 ± 13.8
	−	−	−	−	+	+	0/6	4/6	10.7 ± 11.7
	Other patterns						1/6	6/6	13.0 ± 9.1

[a] +, Sensitive; −, resistant.
[b] Positive/number of strains tested.
[c] Percentage of the number of infected cells containing 10 or more bacteria in 15 microscopic fields selected at random.

included smooth strains which could not be differentiated from original smooth strains as assessed by the slide agglutination test and sensitivity to LPS phages. As shown in Table 2, we considered that strains sensitive only to phages Sf6 and T4 are smooth (and thus are clones of the original RN3 strain because they had the same phage sensitivity pattern as RN3) and had as much agglutination titer to antiserum against RN3 as did RN3.

Virulence of rough strains isolated from isogenic *S. flexneri* strain. We chose a total of 41 out of 144 strains from RN3 and examined them for virulence. Almost all of the 41 strains, except the strains considered to be smooth which were sensitive to Sf6 and T4, failed to produce a positive Sereny test, although the majority of these strains penetrated the HeLa cells. As a control experiment, we also examined the ability to penetrate HeLa cells on 82 strains isolated from strain Rm3-58 and on 130 strains isolated from 5503-I. None of them penetrated the HeLa cells. Cell infection rates of invasive strains were calculated, and it was found that the distribution of the cell infection rates was not uniform even among the strains that showed the same phage sensitivity pattern (Table 2). Preliminary experiments showed that only 3 out of 144 Rm3 strains were incapable of penetrating HeLa cells. However, upon passage and preservation, several other strains changed to become noninvasive to HeLa cells. Two strains with other than the phage sensitivity pattern of the parent strain (Rm3-36 and Rm3-120, sensitive to phages Sf6, T4, T7, and Sf6, C21, T4, T7, respectively) produced a positive Sereny test (Table 2).

Sugar composition of LPS. It was conceivable that such phage sensitivity patterns as we found above reflect the various states of LPS existing in the outer membrane of the *Shigella* strains. We chose among isogenic strains several strains as representative strains of each phage sensitivity group and examined the sugar composition of their LPS. Table 3 shows the sugar composition of LPS of each representative strain. Several chemotypes of sugar composition were observed. Strain 5503 had naturally much rhamnose content, since rhamnose is one of the sugar constituents of O-repeating units, whereas strain RN3 did not have so much rhamnose content as that of the parent strain 5503 (Table 3), suggesting that the latter has shorter O-antigenic repeating units than does the parent strain. Our data of the chemical analysis of LPS fundamentally agreed with those described by Johnston et al. (11), although we found a strain (Rm3-89) that could not be classified exactly into one of the chemotypes proposed by Lüderitz et al. (16), suggesting that this strain has microheterogeneous chains of oligosaccharides. We also found a heptoseless mutant (Rm3-109) that is still invasive to HeLa cells (Table 3). The presence of such a mutant suggested that oligosaccharides of the rough core are not responsible for HeLa cell penetration. The phenol-chloroform-petroleum ether method of Galanos et al. (9) was also employed for the preparation of LPS from Rm3-89 and Rm3-109, and the quantitative sugar composition of LPS extracted by this method was similar to those of LPS extracted by the phenol-water method of Westphal et al. (24). Thus, we confirmed that various sensitivity patterns to LPS phages in *S. flexneri* reflect the various states of the LPS structure existing in the outer membrane. A more detailed relationship between the sensitivity pattern to phages and the chemical structure of LPS is under further investigation.

Virulence and sensitivity patterns to LPS phages of S and R forms of clinical isolates of *S.*

TABLE 3. Quantitative analysis of sugar components of some representative *S. flexneri* S and R LPS

Strain	Sensitivity pattern to LPS phages[b]	Sugar composition of LPS[a]						Tentative chemotype[c]
		Rha	Gal	Glc	GlcN	Hep	KDO	
5503	Resistant	33.1 (44.8)	0.9 (1.1)	16.0 (19.7)	12.7 (13.1)	3.7 (3.9)	2.3 (2.0)	S
RN3	Sf6,T4	6.0 (3.8)	1.7 (1.0)	8.0 (4.6)	9.3 (4.4)	8.4 (4.1)	5.0 (2.0)	S
Rm3-62	6SR,T4,T7	—[d]	2.1 (0.9)	10.7 (4.5)	6.2 (2.2)	12.0 (4.4)	6.7 (2.0)	Ra
Rm3-6	T4,T7	—	2.0 (0.9)	5.4 (2.4)	4.2 (1.6)	16.4 (6.2)	6.4 (2.0)	Rb
Rm3-122	T4	—	—	5.9 (2.5)	4.1 (1.4)	15.4 (5.6)	6.7 (2.0)	Rc
RT2	C21,T4,T7	—	—	5.9 (2.7)	4.2 (1.6)	17.5 (6.9)	6.2 (2.0)	Rc
Rm3-89	T4	—	—	0.3 (0.1)	7.2 (1.1)	1.9 (0.3)	16.2 (2.0)	Rc-Re
Rm3-109	Resistant	—	—	—	7.7 (1.1)	—	15.9 (2.0)	Re

[a] Percentage of sugar in LPS. Values in parentheses are molar ratios of sugar components, where KDO was taken as 2.0. Rha, rhamnose; Gal, galactose; Glc, glucose; GlcN, glucosamine; Hep, aldoheptose; KDO, 2-keto-3-deoxyoctonic acid.

[b] Phages to which these strains were sensitive.

[c] According to the classification of Lüderitz et al. (16).

[d] —, The sugar was not detected on chromatographic and quantitative analyses.

***flexneri* 2a.** We also examined whether those phenomena described above are common among clinical isolates of *S. flexneri*. We used 46 strains of clinical isolates of *S. flexneri* 2a which were isolated from patients with dysentery. These strains were preserved in cooked medium. When these strains were restreaked on L agar plates, 30 out of 46 strains segregated into opaque colonies that were much different from typical colonies of green-gold transparent colonial morphology (Table 4). We picked up one colony from each of those strains, employed a sensitivity test to LPS phages and a slide agglutination test, and also assessed the virulence. Transparent colonies obtained were either resistant or sensitive only to T4 and strongly agglutinated with group- and type-specific antisera, indicating that they were smooth strains,

TABLE 4. Sensitivity patterns to LPS phages and virulence of S and R forms of clinical isolates of *S. flexneri* 2a

Colonial form	Phage sensitivity[a]	No. of strains	No. with positive Sereny test	No. with L cell invasion
Transparent (S)	Resistant	30	7	7
	T4	16	4	4
Opaque (R)	T4,T7	11	0	2
	Felix O,C21,T4,T7	8	1	4
	Felix O,T4,T7	2	0	0
	C21,T4,T7	2	0	0
	6SR,T4,T7	2	0	1
	Others	5	0	2

[a] Phages to which these strains were sensitive.

TABLE 5. Transfer of O antigens to rough *Shigella* strains by *S. flexneri* Y Hfr[a]

Recipient strain	No. of *his*$^+$ hybrids tested	O antigen		Virulence[b]	
		Group	Type II	Sereny test	HeLa cell invasion
5503-01	36	14	14	4/12	4/12
Rm3-89	15	11	0	10/11	10/11
Rm3-8	64	55	0	0/10	0/10
Rm3-25	64	58	0	0/10	0/10

[a] Conjugation between avirulent *S. flexneri* Y FH1049 (Hfr) and rough strains which were made *his* was conducted, and *his*$^+$ hybrids were selected. Streptomycin was used as a counterselective agent against male cells.

[b] Positive number of smooth hybrid strains tested.

whereas opaque colonial strains showed various phage sensitivity patterns and did not agglutinate with group- and type-specific antisera. As for virulence, similar results were obtained as for the isogenic *Shigella* strains described above. Although most of the smooth strains lost virulence, there was complete correlation between the positive Sereny test and ability to invade HeLa cells. On the other hand, none but one out of nine rough strains which had the ability to invade L cells produced a positive Sereny test (Table 4).

Genetic transfer of *S. flexneri* O antigens to rough *Shigella* strains. We then attempted to prove that penetration of HeLa cells by rough *Shigella* strains is an attribute of virulence and hence is identical to that by smooth *Shigella* strains. Formal et al. (6) previously reported that genes controlling the synthesis of *S. flexneri* group antigen are linked to the *his* locus and that expression of the type-specific antigen II (linked to the *pro* locus) depends on the presence of the group antigen. Likewise, we obtained histidine auxotrophs from several rough invasive and noninvasive strains and conducted matings between the avirulent smooth Hfr strain FH1049 and those auxotrophs with *his* as a selective marker. As shown in Table 5, most of the *his* transconjugants exhibited group antigens. Moreover, strain 5503-01, which was obtained from *S. flexneri* 2a, showed that upon transfer of *his* genes they expressed not only group antigens but also type II antigens. When rough invasive strains were transferred O antigen genes and thus became smooth, they gained the ability to produce keratoconjunctivitis in guinea pigs. However, in the case of rough noninvasive strains, they still did not give a positive Sereny test (Table 5).

Intergeneric matings between a rough invasive *Shigella* strain and *E. coli* K-12 Hfr strains. By means of intergeneric conjugation between *E. coli* K-12 Hfr and an *S. flexneri* 2a virulent recipient, Formal et al. (7) established a chromosomal locus on the genome of *S. flexneri* 2a which is necessary for producing a positive Sereny test. The locus which they termed *kcpA* is positioned between the *lac* and *gal* chromosomal markers and is cotransducible with the *purE* allele (7). In this context, to find the approximate locus (or loci) on the genome of an invasive rough strain which is necessary for the ability to penetrate HeLa cells, we employed similar intergeneric matings between *S. flexneri* 5503-01 and *E. coli* K-12 Hfr strains as Formal et al. did previously (7). We obtained a *gal* mutant from 5503-01 and used this strain as a recipient. This mutant strain also had the ability to penetrate HeLa cells. This recipient was mated with the various *E. coli* Hfr strains, and selections were made for the following carbohydrate utilization markers of the donors: *lac*$^+$, *ara*$^+$, *rha*$^+$, *xyl*$^+$, *fuc*$^+$, and *gal*$^+$. After 2 days of incubation, hybrid clones were purified by streaking on the original selective medium and were scored for the acquisition of nonselected markers and for their capacity to penetrate HeLa cells. As shown in Table 6, many hybrids which had inherited either *rha* or *lac-gal* chromosomal

TABLE 6. Ability of *S. flexneri* 5503-01 hybrids derived from matings with *E. coli* K-12 Hfr strains to penetrate HeLa cells

E. coli K-12 donor	Selection[a]	Hybrid class[b]			HeLa cell invasion[c]
		lac	*gal*	*rha*	
Expt 1					
W1895	*rha*$^{+d}$	−	−	+	0/5
	lac$^+$	+	−	−	23/25
	fuc$^+$	−	−	−	11/11
		−	+	−	1/3
	ara$^+$	+	−	−	1/1
		−	+	−	2/3
	gal$^+$	−	+	−	5/5
P4X-6	*fuc*$^+$	−	−	−	3/3
		−	+	−	1/2
Expt 2					
HfrH	*lac*$^+$	+	−	−	9/13
		+	+	−	1/12
	gal$^+$	−	+	−	2/4
P4X-6	*rha*$^{+d}$	−	−	+	0/10
	xyl^{+d}	−	−	−	5/5

[a] *rha*, Rhamnose; *lac*, lactose; *fuc*, fucose; *ara*, arabinose; *gal*, galactose; *xyl*, xylose.

[b] For simplicity and convenience of correlating the results of HeLa cell invasion tests, hybrids are classified on the basis of their incorporation of the unselected markers *lac*, *gal*, and *rha*.

[c] No. of invasive hybrids/no. of hybrids tested.

[d] Unselected markers were negative except for one *lac*$^+$ *ara*$^+$ *rha*$^+$ hybrid in experiment 1.

markers of *E. coli*, regardless of the Hfr used, lost also the capacity to penetrate HeLa cells.

DISCUSSION

In the present study, we isolated many rough mutants from smooth virulent and avirulent strains and observed that rough isolates from virulent strains are usually invasive and that rough isolates from avirulent strains are noninvasive (Table 2). We used two different techniques to isolate rough strains from smooth strains, but the results were the same; that is, whether the selection was made or not to isolate rough strains, any rough mutants capable of penetrating HeLa cells could not be obtained from smooth avirulent strains. These results suggested that penetration of HeLa cells by rough strains was not a mere attachment and a subsequent internalization by HeLa cells, simply because they became rough, and that mutation from invasiveness to noninvasiveness and S-R mutation occurred independently.

We then wondered whether these rough strains really lacked O-specific side chains or had in their LPS moiety trace amounts of O-specific side chains (so-called partial rough state) which might be essential for penetration of HeLa cells. We also wanted to know whether some of the sugars in rough cores of *Shigella* LPS are responsible for penetration. As shown in Table 3, the rough strains we isolated did not have O-specific side chains in their LPS since rhamnose, a constituent of O-specific side chains, was not detected in their LPS. We also analyzed the sugar composition of representative strains of each phage sensitivity group (Table 3). Although we know very little about the structure of the polyheptose phosphate core of *S. flexneri*, we could tentatively determine the chemotype of each representative strain according to the classification proposed by Lüderitz et al. (16) and compare it with the phage sensitivity pattern. We found a strain which did not have heptoses in their LPS and thus belonged to the Re chemotype (Table 3). Since this strain had the ability to penetrate HeLa cells, we concluded that oligosaccharides of the rough core have nothing to do with their ability to penetrate HeLa cells. The chemical nature of the heptoseless mutant we obtained in this study will be published elsewhere. We are also investigating the LPS sugar composition of two apparently rough strains that were found to produce positive Sereny tests.

Similar experiments with clinical isolates led us to further conclude that such a phenomenon described above occurred commonly among many wild-type strains of *S. flexneri* (Table 4).

We then attempted by genetic means to prove that ability to penetrate HeLa cells even in invasive rough strains is one of the attributes of virulence in *S. flexneri* strains. In experiments where mating between avirulent *S. flexneri* Y Hfr and rough *S. flexneri* strains were conducted and selection was made with a *his*$^+$ marker, most of the *his*$^+$ hybrids expressed group antigens, and, as we expected, many hybrid strains from invasive rough recipients regained the ability to produce a positive Sereny test, whereas all of the hybrid strains from noninvasive rough recipients still failed to produce a positive Sereny test (Table 5). These results showed us that if a rough strain has the ability to penetrate HeLa cells, it can produce a positive Sereny test once introduced the O antigen. We used as a recipient several rough strains with different sensitivity patterns to LPS phages, and we do not know yet which locus or loci of the chromosome responsible for LPS synthesis in these strains were affected. In fact, as for a few rough recipient strains, we could not obtain smooth hybrids when *his*$^+$ was used as a selective marker. However, it did not seem to matter so far as the smooth hybrids were concerned since we isolated noninvasive strains as well as invasive strains during the process of obtaining *his* mutants from an invasive rough strain. These noninvasive strains, after mating with Hfr, expressed O antigens but failed to produce positive Sereny tests. Recently, a large plasmid has been found to be involved in the invasive ability of *S. flexneri* (20). In our experiments, however, the recipient strains as well as the isogenic smooth strains 5503 and 5503-I had a large plasmid, whereas the nonisogenic donor strain did not have a large plasmid (unpublished data). Thus, we could rule out the possibility that the plasmid in the donor had any positive role on hybrids regaining the ability to produce a positive Sereny test.

Another line of genetic experiments revealed that there are at least two chromosomal loci, the *rha* and *lac-gal* regions, responsible for penetration of HeLa cells (Table 6). These results partly coincided with the study by Formal et al. (7), in which they established between *lac* and *gal* chromosomal markers a locus which is necessary for the ability to penetrate epithelial cells as measured by the Sereny test. Our results again confirmed that ability of rough strains to penetrate HeLa cells is one of the attributes of virulence of *S. flexneri* to evoke keratoconjunctivitis. Since the recipient had a large plasmid (unpublished data) which was supposed to be the one observed by Sansonneti et al. (20), we must discuss two possibilities which might have affected the virulence plasmid in the recipient strain during the mating: (i) the F plasmid entered the recipient cell and somehow put a large

plasmid out of the recipient cell; (ii) fragments of an *E. coli* chromosome recombined with a large plasmid. It is unlikely, however, that these two events were involved in the loss of invasiveness among *rha*$^+$ and *lac*$^+$-*gal*$^+$ recombinants because these recombinants retained a large plasmid in the cell which exhibited the same electrophoretic mobility as that of the recipient strain (unpublished data). Our results can be compared with those of Falkow et al. (3) and Formal et al. (8). Although Falkow et al. (3) reported that the *rha*$^+$-*xyl*$^+$ region is essential for virulence as assessed by oral infection of starved guinea pigs, Formal et al. (8) showed that a *rha*$^+$ strain still had ability both to produce Sereny tests and to invade HeLa cells. Inspection of results done by Formal et al. (8) suggests that the *rha*$^+$ strain they isolated incorporated a considerable amount of *E. coli* K-12 chromosome (*ara*$^+$ *rha*$^+$ *xyl*$^+$ *mal*$^+$) so that the gene(s) responsible for epithelial cell penetration might not have been affected, and genes other than *rha*$^+$ might have been responsible for the abortive intestinal infection in guinea pigs. Since they described that the colonial appearance of that strain is granular in spite of its similar agglutinin titer to that of the parent *Shigella* strain, analysis of its LPS would be of great interest.

It is also evident from our studies that O antigens were necessary for evoking keratoconjunctivitis, although our results suggested that full expression of O-repeating units is not necessarily required. The minimum quantity of O-antigenic repeating units required for a positive Sereny test remains to be determined.

Little is known yet about the substances responsible for epithelial cell penetration, although Adamus et al. (1) reported that immunization with outer membrane proteins of *Shigella* species protected nonspecifically against keratoconjunctivitis. In this respect, a study in an aim of finding the differences between virulent and avirulent cells of *S. flexneri* on the basis of the outer membrane constituents including protein is now under investigation in our laboratory.

In summary, our present paper confirmed that O antigens and ability of *S. flexneri* to penetrate HeLa cells are separate and prerequisite attributes of virulence responsible for evoking keratoconjunctivitis in guinea pigs and further demonstrated that at least two chromosomal genes are necessary for *S. flexneri* to express the capacity to penetrate HeLa cells.

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