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Rough Mutant of *Shigella flexneri* 2a That Penetrates Tissue Culture Cells but Does Not Evoke Keratoconjunctivitis in Guinea Pigs

NOBORU OKAMURA AND RINTARO NAKAYA*

Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Bunkyo-ku, Tokyo, 113, Japan

Received for publication 25 January 1977

A rough mutant, designated 5503-01, has been isolated from a virulent strain of *Shigella flexneri* 2a 5503. Strain 5503-01 produced smooth opaque colonies, whereas its parent strain produced characteristic green-gold translucent ones. Characterization of 5503-01 by agglutination tests, rhamnose content, and sensitivity spectra to "rough-specific" phages revealed that it had lost the specific somatic antigens. When 5503-01 was used to infect HeLa or L cells, it penetrated the cells and multiplied within the cytoplasm. On the other hand, it could not evoke keratoconjunctivitis in guinea pigs. The properties of this strain were remarkably stable against serial passages and preservation for a long period. The presence of a rough mutant with the ability to penetrate tissue culture cells suggests that specific O antigen is not of significance in the early step of the invasive process of shigellosis.

The primary step in the pathogenesis of bacillary dysentery is the penetration of intestinal epithelial cells by the pathogen (10, 16). Strains that lack this capacity are avirulent (10, 16). Once the pathogen gains entrance by penetrating the epithelial cells, it multiplies in the intestinal mucosa and results in acute ulcerative lesions (9, 10). There have been various model systems for enteropathogenicity of dysentery bacilli (2; P. Gemski, Jr., and S. B. Formal, *Microbiology–1975*, p. 165–173, American Society for Microbiology, Washington, D.C., 1975): oral infection of monkeys (6, 10) and starved guinea pigs (9), the guinea pig keratoconjunctivitis test (13, 18), and infection to tissue culture cells (10, 14, 20). Typical virulent strains of dysentery bacilli have been positive in all of the model systems listed above.

Formal et al. (3), using *Escherichia coli* Hfr, established a shigella hybrid strain in which the *E. coli xyl-rha* region was incorporated and showed that the hybrid strain failed to cause overt disease when fed to experimental animals but still retained the ability to penetrate the intestinal mucosa, to cause keratoconjunctivitis in guinea pigs, and to invade HeLa cells. They thus concluded that the overall invasive process of shigellosis could be differentiated into two stages: penetration of the epithelial cell barrier by the pathogen and multiplication of the pathogen within the intestinal wall. However, the ability to evoke keratoconjunctivitis in guinea pigs and to invade the tissue culture cells correlated well among the virulent strains tested (14).

We isolated a virulent rough mutant of *Shigella flexneri* 2a that invaded both HeLa and L cells but could not evoke keratoconjunctivitis in guinea pigs. The present report describes experiments in which the parent and mutant strains were studied. The possibility that the O antigen may not be of significance in the early step of the invasive process of shigellosis is discussed.

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MATERIALS AND METHODS

Bacterial strains. *S. flexneri* 2a 5503 and its derivatives were used. Strain 5503 causes a classical bacillary dysentery when orally administered to rhesus monkeys and causes keratoconjunctivitis in guinea pigs (6). When this strain was stored in cooked-meat medium, a colonial mutant was spontaneously produced, and we named it 5503-01. The parent and mutant strains were maintained in cooked-meat media, and before use in any experiment the strains were checked for purity in colonial morphology. *S. flexneri* 2a 5503-I (16), which is a smooth, avirulent mutant of 5503, and its rough mutant 5503-I-1 were also used as controls.

Media. Luria (L) broth and agar were used for routine cultivation and other experiments. L broth and L agar contained: tryptone, 10 g; yeast extract, 5

g; NaCl, 5 g; glucose, 1 g; distilled water, 1,000 ml (pH 7.0); with and without 10 g of agar, respectively. Penassay broth (Difco) was used for passage of 5503-01. Penassay broth agar was used for the guinea pig keratoconjunctivitis test. Cooked-meat medium (Difco) was used for storage of the strains.

Test for biochemical properties. Auxotrophic characters were tested by using minimal glucose agar medium, which contained, per liter of distilled water: K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $MgCl_2$, 0.05 g; $(NH_4)_2SO_4$, 1 g; and agar, 15 g. When required, amino acids and growth factors were added to the minimal medium at a final concentration of 20 μg/ml. Fermentation characteristics were tested on MacConkey indicator medium, which consisted of MacConkey agar base (Difco) supplemented with an appropriate carbohydrate (1%). For testing indole production, SIM agar (Eiken Kagaku Co., Tokyo) and Kovacs reagent were used.

Serological tests. Overnight bacterial growth from L agar plates was used as an antigen for agglutination tests. The tests were done on a slide glass using 4 and 0.85% NaCl solutions or absorbed type-specific II and group 3,4 antisera (Toshiba Kagaku-Kogyo, Tokyo).

In vitro growth studies. Growth in Penassay broth at 37°C with shaking was measured in an electrophotometer (Coleman, Jr. II) at a wavelength of 550 nm.

Phage sensitivity test. "Rough-specific" phages Felix O, 6SR, C21, T4, and T7 were kindly provided by S. Kanegasaki (Institute of Medical Science, University of Tokyo). These phages were grown on a suitable "rough" strain of *Salmonella typhimurium*. The bacterial strains were spread on agar plates and spotted with drops of phage lysates (approximately 10^{10} plaque-forming units/ml). Bacterial lysis was scored after incubation for 6 to 8 h and overnight at 37°C.

Cell culture assay. HeLa and L cell monolayers were used to study the infectivity of the strains as a simple in vitro system. The cell cultures were maintained in prescription bottles as monolayers grown in Eagle minimal essential medium (Nissui Pharmaceuticals, Tokyo; containing 2 μmol of glutamine and 60 μg of kanamycin per ml) supplemented with 10% calf serum. The cover slip technique as described by LaBrec et al. (10) was used with minor modification to prepare monolayers for penetration experiments. Approximately 24 h before preparing monolayers on cover slips, the culture medium was removed and replaced with antibiotic-free Eagle minimal essential medium (Nissui Pharmaceuticals, Tokyo). Four sterile cover slips (18 by 18 mm) were placed on the bottom of a sterile petri dish covered with sterile silicone rubber. Each cover slip was seeded with approximately 2.4×10^4 HeLa or L cells, and then the dish was 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 cover slip. After incubation for 2 h, the medium was removed from the cover slips, the monolayers were washed twice with phosphate-buffered saline to minimize the extracellular multiplication of the bacteria, and then fresh antibiotic-free minimal essential medium was added. After a further 2 h of incubation, the cover slips were washed twice with phosphate-buffered saline, fixed in methanol, and stained in 2% Giemsa solution for 15 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 20 microscopic fields selected at random.

Guinea pig keratoconjunctivitis test. Details of the guinea pig keratoconjunctivitis test have been described (13, 14, 18). Hartley strain guinea pigs were used; their body weights were approximately 350 g. A few drops of suspensions (0.05 ml containing 10^7 to 10^9 organisms, as indicated in Results) of the test organisms grown on Penassay broth agar were deposited into the 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.

Rhamnose determination. The bacteria were grown overnight on agar. A loopful of bacteria was scraped from the lawn of the agar and suspended in 0.04 N NaOH. The suspension was analyzed for rhamnose by the cysteine-sulfuric acid test (1) and for protein by the method of Lowry et al. (11). Rhamnose was used as the indicator for the presence of the O-repeat unit of lipopolysaccharide.

RESULTS

Isolation and colonial morphology of 5503-01. When *S. flexneri* 2a strain 5503 was grown on an L agar plate from the cooked-meat medium, it was observed that it spontaneously produced a colonial mutant. The colonial appearance of this mutant strain 5503-01 grown on the agar plate was smooth but somewhat granular, suggesting a rough form. In contrast, the colonies of the parent strain showed a green-gold, smooth, translucent form when examined by oblique transmitted light. The colonial appearance of the parent and the mutant is shown in Fig. 1.

Agglutination reactions. The parent strain agglutinated strongly and specifically in absorbed type-specific II and group 3,4 antisera, with rapid formation of large clumps, whereas the mutant strain agglutinated weakly in the absorbed antisera and to the same degree in saline.

Biochemical properties. The characteristics of the parent strain and its rough mutant did not differ from each other in any of the biochemical properties tested, except for the positive indole production in strain 5503-01.

Infection to tissue culture cells. The ability of the parent and the mutant strains to penetrate HeLa or L cells was tested by the proce-

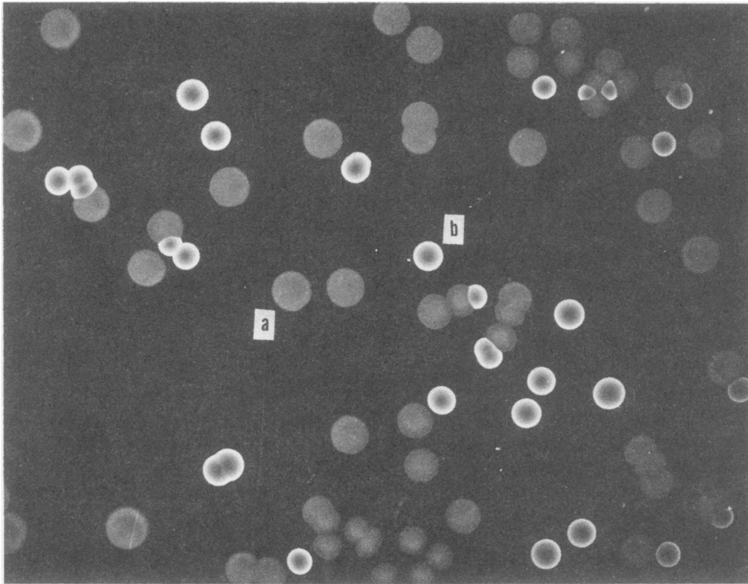

FIG. 1. *Colonies of S. flexneri 2a 5503 (a) and its rough mutant 5503-01 (b) grown on an L agar plate overnight at 37°C. Observed by oblique transmitted light. ×4.*

dures described in Materials and Methods. Smooth, avirulent strain 5503-I and its rough mutant 5503-I-1 were also used as negative controls. When the organisms of 5503-01 were incubated with monolayers of HeLa or L cells, they invaded the cells and multiplied within the cytoplasm. Occasionally, the cytoplasmic space of invaded cells contained a greater number of shigella bacilli, as compared with that infected with the parent strain. Calculating the percentage of infected cells, the mutant strain showed a high infectivity to HeLa cells (25.6%) as compared with the parent strain (3.6%) (Table 1). The percentages of infected cells with 5503-01, which had been preserved in L broth for 3 weeks at room temperature, and with 5503-01, which had been passaged 10 times through Penassay broth and preserved in cooked-meat medium for 2 years, were 24.5 and 33.9%, respectively (Table 1). These experiments suggest that 5503-01 is remarkably stable on passage and storage. Smooth, avirulent strain 5503-I and its rough mutant 5503-I-1 did not adhere to the cells and existed only extracellularly. In addition, strain 5503-01 was relatively cytotoxic when the inoculum was large (10^8 bacteria per cover slip, i.e., at a multiplicity of infection of 4×10^3). Similar results were observed with *S. typhimurium* by Giannella et al. (5) and by Kihlström and Edebo (8).

Rhamnose content. The rhamnose content of the organisms was determined as the indicator for the presence of the O-repeat unit of lipopolysaccharide. Mutant strain 5503-1 showed a significant decrease in rhamnose content as compared with the parent strain (Table 2). Smooth, avirulent strain 5503-01 was also measured for rhamnose content and was found to contain as much rhamnose as the parent strain. These results suggest that strain 5503-01 has lost specific O antigens.

Sensitivity to rough-specific phages. Phages have been used for characterization and classification of R mutants (12, 19). To determine the R chemotype, the sensitivity of the strains to rough-specific phages was tested (Table 3). The parent strain was resistant to all of these phages, whereas the mutant strain was sensi-

TABLE 1. *Infectivity of S. flexneri strains to HeLa cells*

Strain	Cell infection rate (%)[a]
5503	3.6
5503-I	0
5503-I-1	0
5503-01	25.6
5503-01 (preserved)[b]	24.5
5503-01-10[c]	33.9

[a] Percentage of the number of infected cells containing 10 or more bacteria in 20 microscopic fields selected at random.

[b] Strain 5503-01 cultured in L broth and preserved for 3 weeks at room temperature.

[c] Strain 5503-01 passaged 10 times through Penassay broth and preserved in cooked-meat medium for 2 years.

TABLE 2. *Rhamnose content of whole cells of the smooth and rough strains of S. flexneri 2a*

Strain	Rhamnose content (μg/mg of protein)
5503	31.4
5503-I	40.1
5503-01	5.4

tive to C21, T4, and T7. These results suggest that this strain would be group Rc or Rd_1 (21).

Guinea pig keratoconjunctivitis test. The mutant strain was further characterized by its ability to evoke keratoconjunctivitis in guinea pigs. Approximately 10^7, 10^8, and 10^9 cells of the parent and mutant strains were dropped into the conjunctival sacs of guinea pigs (three guinea pigs for each bacterial dose). Within 48 h, all the animals receiving the parent strain exhibited keratoconjunctivitis, whereas all of those receiving the mutant strain remained normal. Further experiments were conducted using a total of 50 guinea pigs, which were infected with 10^9 organisms of the mutant strain, and all proved to be negative.

Growth studies. To determine whether the high percentage of infection to HeLa cells by the mutant strain is a function of generation time of the organisms, in vitro growth studies were conducted. The in vitro growth rates of the parent and mutant strains were compared in Penassay broth. The results showed that strain 5503-01 grew slightly more rapidly in the exponential phase than the parent, but this difference does not appear to be significant.

DISCUSSION

These studies revealed that the strain we isolated, 5503-01, is a rough mutant, which penetrates tissue culture cells but does not evoke keratoconjunctivitis in guinea pigs. As originally demonstrated by LaBrec et al. (10) and subsequently confirmed by Ogawa et al. (16) and Nakamura (14), ability both to evoke keratoconjunctivitis in guinea pigs and to invade the tissue culture cells correlated well among the virulent strains tested. Since the model system of tissue culture infection is considered to be a simple in vitro system for testing the virulence of shigella organisms, strain 5503-01 may retain some functions indispensable for the early step of the invasive process, although it does not evoke keratoconjunctivitis in guinea pigs. We are now investigating the reasons why this strain does not produce keratoconjunctivitis in guinea pigs: whether it is due to the inability of this strain to multiply within the epithelial cells, despite its ability to penetrate the cells, or to the inability to penetrate the epithelial cells owing to humoral factors such as lysozyme or immunoglobulins contained in tears and conjunctival epithelium (17).

TABLE 3. *Phage response of S. flexneri 2a 5503 and its rough mutant 5503-01* [a]

Bacterial strain	Response to phage				
	Felix O	6SR	C21	T4	T7
5503	R	R	R	R	R
5503-01	R	R	S	S	S

[a] R, Resistant; S, sensitive.

Strain 5503-01 was proved to be a rough mutant by means of the slide agglutination test, determination of rhamnose content of the whole cells, and a sensitivity test to rough-specific phages. On infecting tissue culture cells and calculating the percentage of infected cells, the rough mutant showed a remarkably high infectivity to HeLa cells as compared with the parent strain. Giannella et al. (5) and Kihlström and Edebo (8) obtained similar results in the experiments with *S. typhimurium*; i.e., rough strains of *S. typhimurium* could penetrate HeLa cells and rabbit ileal loop mucosa in the same way as their parent strains (5), and the rough strain was found to have a greater tendency to interact than the smooth strain (8), although Kihlström and Edebo did not distinguish between bacteria attached to the HeLa cell surface and bacteria that had become internalized (8). Thus, these findings raise the question of whether "penetration" is due to nonspecific adhesion and internalization. We isolated a number of rough mutants from virulent and avirulent strains and found that all of the rough mutants derived from avirulent strain 5503-I did not attach to HeLa cells, whereas the majority of the rough mutants derived from the virulent strain associated with HeLa cells, but a few strains did not (Okamura and Nakaya, in preparation). These results rule out the possibility that rough strains may nonspecifically attach to and be internalized by HeLa or L cells. Ogawa et al. observed cinemicrographically that a smooth virulent shigella strain exhibits a vigorous movement in the cells (15). On cinemicrographic observation, strain 5503-01 attaches to and invades the primary cell cultures of the intestinal epithelium of newborn mice and multiplies in the cytoplasm, but moves less vigorously in the cells (T. Asaka, personal communication). In addition, pili, which are considered to play an important role in bacterial adhesion to mucosal surfaces, could not be detected in either the

parent or the mutant strains used in the present study by electron microscopic observation (data not shown). We still cannot exclude the possibility of strain 5503-01 being in a partial rough state. However, since the percentage of infected cells is higher with this strain than with its parent, and taking into account the facts described above, specific O antigens may not be of significance in the early step of the invasive process of shigellosis. Gemski et al. (4) reported that *S. flexneri* hybrids with the *E. coli* O-25 antigen conserved virulence, whereas the hybrids with O antigen 8 failed to evoke keratoconjunctivitis in guinea pigs. On the basis of their data, they suggested that the chemical composition and structure of the O-repeat unit may represent one of the determining factors for bacterial penetration of mucosal epithelial cells. However, since they used the model systems of keratoconjunctivitis as well as histology of the infected intestine of guinea pigs to assess the virulence of the hybrid strains, there is the possibility that the presence of O antigens plays a role either in the multiplication of the pathogen after penetration of the epithelial cells (7) or in the defense against the humoral factors of the host before penetration.

As indicated by many investigators, a virulent shigella strain frequently loses virulence during its passage and preservation in artificial media, but the rough mutant we isolated is remarkably stable, as described above. We have obtained several rough mutants from various virulent strains of *S. flexneri* isolated from patients with clinical shigellosis that possess the ability to penetrate tissue culture cells, and we are now investigating the characteristics of these strains (T. Nagai, N. Okamura, K. Ishizu, and R. Nakaya, in preparation). These strains should be useful experimental materials for the study of invasive mechanisms of shigella bacilli.

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LITERATURE CITED

1. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methylpentoses and a spectrophotometric micro-method for their determination. J. Biol. Chem. 175:595–603.
2. Formal, S. B., H. L. DuPont, R. Hornick, M. J. Snyder, J. Libonati, and E. H. LaBrec. 1971. Experimental models in the investigation of the virulence of dysentery bacilli and *Escherichia coli*. Ann. N.Y. Acad. Sci. 176:190–196.
3. Formal, S. B., E. H. LaBrec, T. H. Kent, and S. Falkow. 1965. Abortive intestinal infection with an *Escherichia coli-Shigella flexneri* hybrid strain. J. Bacteriol. 89:1374–1382.
4. Gemski, P., Jr., D. G. Sheahan, O. Washington, and S. B. Formal. 1972. Virulence of *Shigella flexneri* hybrids expressing *Escherichia coli* somatic antigens. Infect. Immun. 6:104–111.
5. Giannella, R. A., O. Washington, P. Gemski, and S. B. Formal. 1973. Invasion of HeLa cells by *Salmonella typhimurium*: a model for study of invasiveness of *Salmonella*. J. Infect. Dis. 128:69–75.
6. Honjo, S., M. Takasaka, T. Fujiwara, M. Nakagawa, M. Ando, H. Ogawa, R. Takahashi, and K. Imaizumi. 1964. Shigellosis in cynomologus monkeys (Macaca irus). II. Experimental infection with *Shigella flexneri* 2a with special references to clinical and bacteriological findings. Jpn. J. Med. Sci. Biol. 17:307–319.
7. Kétyi, I. 1974. Role in virulence of *Shigella flexneri* antigens derived from lysogenic conversion. Infect. Immun. 9:931–933.
8. Kihlström, E., and L. Edebo. 1976. Association of viable and inactivated *Salmonella typhimurium* 395 MS and MR 10 with HeLa cells. Infect. Immun. 14:851–857.
9. LaBrec, E. H., and S. B. Formal. 1961. Experimental Shigella infections. IV. Fluorescent antibody studies of an infection in guinea pigs. J. Immunol. 87:562–572.
10. LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal. 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. 88:1503–1518.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
12. Lüderitz, O., C. Galanos, H. J. Risse, E. Ruchmann, S. Schlecht, G. Schmidt, H. Schulte-Holthausen, R. Wheat, O. Westphal, and J. Schlosshardt. 1966. Structural relationships of *Salmonella* O and R antigens. Ann. N.Y. Acad. Sci. 133:349–374.
13. Mackel, D. C., L. F. Langley, and L. A. Venice. 1961. The use of the guinea pig conjunctivae as an experimental model for the study of virulence of *Shigella* organisms. Am. J. Hyg. 73:219–223.
14. Nakamura, A. 1967. Virulence of Shigella isolated from healthy carriers. Jpn. J. Med. Sci. Biol. 20:213–223.
15. Ogawa, H., A. Nakamura, and R. Nakaya. 1968. Cinemicrographic study of tissue cell cultures infected with *Shigella flexneri*. Jpn. J. Med. Sci. Biol. 21:259–273.
16. Ogawa, H., A. Nakamura, R. Nakaya, K. Mise, S. Honjo, M. Takasaka, T. Fujiwara, and K. Imaizumi. 1967. Virulence and epithelial cell invasiveness of dysentery bacilli. Jpn. J. Med. Sci. Biol. 20:315–328.
17. Reed, W. P., and A. H. Cushing. 1975. Role of immunoglobulins in protection against shigella-induced keratoconjunctivitis. Infect. Immun. 11:1265–1268.
18. Serény, B. 1957. Experimental keratoconjunctivitis shigellosa. Acta Microbiol. Acad. Sci. Hung. 4:367–376.
19. Stocker, B. A. D., R. G. Wilkinson, and P. H. Mäkelä. 1966. Genetic aspects of biosynthesis and structure of *Salmonella* somatic polysaccharide. Ann. N.Y. Acad. Sci. 133:334–348.
20. Watkins, H. M. S. 1960. Some attributes of virulence in *Shigella*. Ann. N.Y. Acad. Sci. 88:1167–1186.
21. Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker. 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. 70:527–554.