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Correlation Between Pathogenicity of *Shigella* and Intraperitoneal Survival in Mice

MITSURU NAKAMURA, KATHLEEN E. JACKSON, AND W. R. CROSS
Department of Microbiology, University of Montana, Missoula, Montana 59801

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Phagocytosis of virulent and avirulent strains of *Shigella* and *Escherichia coli* in the mouse peritoneum was studied. A direct correlation between bacterial virulence and resistance to phagocytosis by peritoneal phagocytes was demonstrated. Virulent strains were less readily cleared and were able to multiply to a limited extent within the peritoneal cavity. An epimerase-deficient, rough mutant of *S. flexneri* 2a was highly susceptible to phagocytosis. Restoration of the cell wall structure in these mutants resulted in a significant increase in their resistance to phagocytosis. Susceptibility to phagocytosis in smooth *S. flexneri* was age-dependent. Cells from 16-hr cultures were more resistant to removal from the peritoneum than were cells from 48- and 72-hr cultures.

Certain strains of *Escherichia coli* and *Shigella flexneri* display a difference in pathogenicity for man and laboratory animals (3, 8, 9, 15). Although the reasons for this variance of virulence among strains is not fully understood, Wolberg and DeWitt (16) and Medearis et al. (7) showed that the antigenic structure of *E. coli* is related to the virulence of this organism. Studies by Slopek et al. (14) and Skurski et al. (13) showed that the antigenic structure of *S. flexneri* and other *Enterobacteriaceae* determines their susceptibility to phagocytosis. Yee and Buffenmyer (17) demonstrated that virulent strains of *S. flexneri* are capable of multiplying within cultured macrophages of the mouse, whereas avirulent and attenuated shigellae are rapidly killed after ingestion.

We studied the relationship between the virulence of strains of *E. coli* and *Shigella* for the rabbit eye (W. R. Cross and M. Nakamura, Bacteriol. Proc., p. 75, 1969) and their susceptibility to phagocytosis in vivo. We also studied the role of the complexity of the lipopolysaccharide (LPS) in the cell wall of *S. flexneri* to the susceptibility of the *Shigella* to phagocytosis.

MATERIALS AND METHODS

Experimental animals. Strain CFW mice were used for the phagocytic studies. These mice were obtained from the colony at the University of Montana and given food and water ad libitum. Virulence for the rabbit eye was assayed in New Zealand strain rabbits (1.5 to 2.5 kg).

Bacterial strains. *S. sonnei* and *S. flexneri* 2a were contributed by R. W. Huntington, Kern County General Hospital, Bakersfield, Calif. These strains were isolated from pathological specimens. *S. flexneri* 1690-67, a rough (R) strain with an undefined LPS structure, was obtained from W. H. Ewing, Center for Disease Control (CDC), Atlanta, Ga. *Escherichia-Shigella* hybrid strain FWM1 was contributed by S. B. Formal, Walter Reed Army Institute of Research, Washington, D.C. This strain was produced by mating an *E. coli* Hfr with a strain of *S. flexneri* (F-). The properties of this strain were described in detail by Formal et al. (2). *S. flexneri* 2aR (CDC 8519) is an R mutant that lacks uridine-diphosphate-galactose-4-epimerase and is unable to synthesize galactose from glucose (5). This strain lacks galactose and all sugars distal to it in its LPS. When galactose is supplied exogenously in the culture medium, *S. flexneri* 2aR organisms revert to a smooth form with complete LPS structures (6). *E. coli* B and *E. coli* 026:B6, an enteropathogenic strain, were obtained from the stock culture collection at the University of Montana. Each strain was maintained on Brain Heart Infusion (BHI) agar (Difco, Detroit, Mich.) and periodically checked for purity.

Cultivation procedures. All organisms except *S. flexneri* 2aR were cultured in BHI broth (aerated by shaking) at 37 C. *S. flexneri* 2aR was grown in minimum essential medium (MEM) with Hanks balanced salts under aeration for 24 hr. Galactose (2%) was added to the MEM in order to support the synthesis of a complete LPS by *S. flexneri* 2aR.

Phagocytic studies. A modification of the method of Cohn (1) was used for studies of phagocytic activity. Mice were injected intraperitoneally (ip) with 5×10^6 washed, viable bacteria (approximately 0.1 LD_{50}) suspended in sterile saline. At various intervals (30 min to 24 hr) after injection the mice were sacrificed by cervical dislocation. The peritoneal cavity was washed by injecting 5.0 ml of sterile saline, and the wash fluid was recovered with a syringe. Samples (0.1 ml) of the wash fluid were plated in triplicate on BHI agar. The plates were incubated for 18 hr at 37 C and the number

TABLE 1. *Rate of clearance of keratoconjunctivitis-producing and keratoconjunctivitis-negative strains of Shigella and Escherichia coli from the mouse peritoneum*

Strain	Production of keratoconjunctivitis	Viable cells recovered from the peritoneum[a]					
		0.5 hr	3 hr	6 hr	9 hr	12 hr	24 hr
S. flexneri 2a	+	3×10^6	2×10^5	5×10^4	2×10^3	9×10^4	4×10^3
Escherichia-Shigella hybrid FWM1	+	3×10^6	1×10^5	3×10^6	3×10^5	6×10^4	2×10^3
S. sonnei	+	1×10^6	1×10^6	2×10^5	9×10^4	7×10^2	2×10^2
S. flexneri 1690–67	−	7×10^3	2×10^3	2×10^2	5×10^2	9×10^2	5×10^1
E. coli 026:B6	±	2×10^6	4×10^4	3×10^4	4×10^4	2×10^3	3×10^4
E. coli B	−	3×10^4	3×10^3	2×10^2	1×10^2	6×10^1	1×10^2

[a] At time equal to 0, 5×10^6 viable bacteria were injected intraperitoneally.

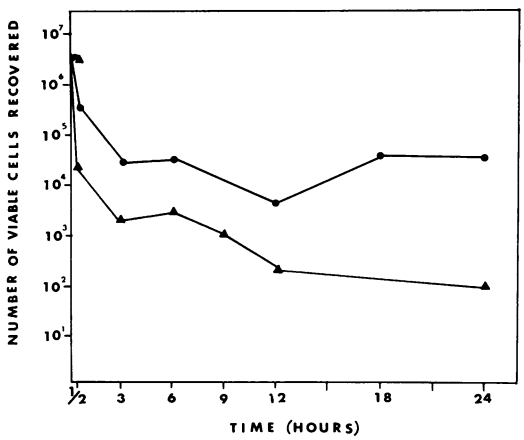

FIG. 1. *Clearance of Shigella flexneri 2aR with complete and incomplete lipopolysaccharide from the mouse peritoneum. Symbols: ▲, Shigella grown in glucose (incomplete LPS) and injected with glucose; ●, Shigella grown in galactose (complete LPS) and injected with galactose.*

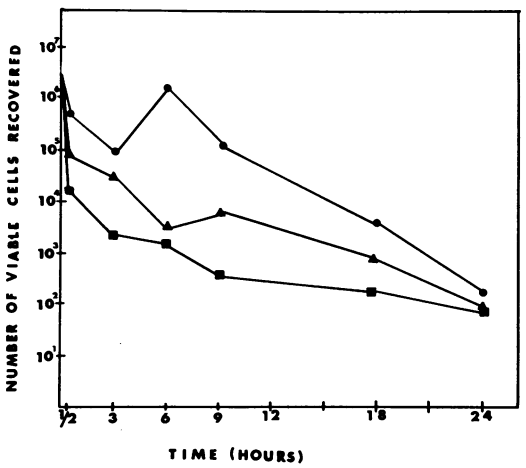

FIG. 2. *Clearance of Escherichia-Shigella hybrid grown for various periods of time from the mouse peritoneum during 24 hr after challenge. Symbols: ●, 16-hr cultures; ▲, 48-hr cultures; ■, 72-hr cultures.*

of viable organisms in the peritoneal cavity was determined by multiplying the number of organisms per ml (obtained from the plate counts) by 5 ml (the volume of the wash fluid). No distinction between intracellular (ingested organisms) and extracellular bacteria was made. Ten mice were used for each time-interval employed.

We are aware of the possibilities that other factors besides phagocytosis may be involved relative to the number of bacteria that can be washed from the peritoneum of the mice. Since the peritoneum is not free of blood plasma and the humoral defenses, some of the bactericidal activity may have been due to mechanisms other than phagocytosis. However, based on past experience, the assumption was made that bactericidal action was phagocytic.

Assay of virulence. Rabbit eyes were inoculated with 1×10^8 to 3×10^8 bacteria of each strain by dripping the bacteria onto the surface of the conjunctiva. Virulent strains of *Shigella* cause keratoconjunctivitis, but avirulent strains do not (2, 12; W. R. Cross and M. Nakamura, J. Infec. Dis., *in press*). Each rabbit was observed for a positive eye reaction for 5 days after inoculation.

RESULTS

Bacterial virulence and susceptibility to phagocytosis. Table 1 shows the virulence of various *Shigella* and *E. coli* for the rabbit eye and the recovery of these strains from the mouse peritoneum. It can be seen that a direct correlation exists between the ability of a strain to produce a positive eye reaction and its survival within the peritoneal cavity. Virulent strains that gave positive eye reactions survived within the peritoneum in numbers significantly higher than did the avirulent strains that gave negative eye reactions. At 30 min, 24 to 70% of the inocula of virulent organisms could be recovered, whereas less than 0.006% of the avirulent inocula could be recovered. Rough *S. flexneri* 1690–67 and *E. coli* B

were most rapidly inactivated within the peritoneal cavity.

Effect of LPS structure on susceptibility to phagocytosis. Figure 1 shows the clearance of *S. flexneri* 2aR organisms after their injection into the peritoneal cavity. When galactose was not present and an incomplete LPS was formed, *S. flexneri* 2aR was rapidly removed from the peritoneum. Thirty minutes after inoculation, less than 0.01% of the rough shigellae could be recovered. On the other hand, *S. flexneri* 2aR organisms with a complete LPS survived within the peritoneum in higher numbers than did the rough cells. In addition, the galactose-grown organisms with a complete LPS were able to multiply limitedly within the peritoneum during a period of 12 to 18 hr, whereas the rough cells did not (Fig. 1).

Effect of age on susceptibility to phagocytosis. The rate of phagocytosis of *Escherichia-Shigella* hybrid FWM1 grown for various lengths of time is shown in Fig. 2. Susceptibility to phagocytosis is directly related to the age of the cells employed. Sixteen-hour-old cells were slowly cleared, and multiplied extensively within the peritoneum during the period 3 to 6 hr after injection. Cultures of the hybrid grown for 48 and 72 hr were readily removed from the peritoneum and did not multiply during the 24-hr period after inoculation of the mice (the duration of the experiment).

DISCUSSION

The results indicate that there is a direct correlation between resistance to phagocytosis in vivo and ability of *Shigella* and *E. coli* to infect the rabbit eye. Yee and Buffenmyer (17) showed that virulent, attenuated, and avirulent *S. flexneri* are all equally susceptible to phagocytosis in vitro; however, only virulent organisms are capable of multiplying within the macrophages. Our results suggest that a difference in susceptibility to ingestion may exist between virulent and avirulent organisms. This concept is supported by the fact that less than 0.006% of the avirulent inocula was recoverable at 30 min, whereas 24 to 70% of the virulent inocula was recoverable at this time. However, it is possible that all strains were ingested with equal efficiency, but only the avirulent organisms were immediately killed by the phagocytes.

It has been shown that a correlation between virulence and survival in vivo existed for strains of *E. coli* (8, 11), *Staphylococcus* (1), and plague bacillus (4). Medearis and Kenny (8) showed that a mouse virulent strain of *E. coli* resisted phagocytosis by macrophages in vivo and by polymorphonuclear leukocytes in vitro. Our studies add to the evidence that the interaction of the host's phagocytes with pathogenic bacteria, namely, *Shigella*, plays an important role in the pathogenesis of shigellosis.

We have shown that survival of a strain of *S. flexneri* within the peritoneum is determined by the complexity of its LPS. Employing similar mutants of *E. coli*, Medearis et al. (7) reported that resistance to phagocytosis is dependent upon the complexity of the LPS. Slopek et al. (14) showed for a variety of *Enterobacteriaceae* that susceptibility to phagocytosis increases as various somatic antigens are destroyed by heat. Wolberg and DeWitt (16) postulated that opsonins, O antigen, and K antigen play integral roles in determining the degree of bacterial virulence and host resistance to infection. Although LPS complexity is related to virulence, it is not the sole determinant, as *S. flexneri* 2aR cells with complete or incomplete LPS structures are not capable of infection of the rabbit eye (W. R. Cross and M. Nakamura, J. Infec. Dis., *in press*).

The relationship of age of culture and resistance to phagocytosis is difficult to explain at this time. However, our findings that aged shigellae are more susceptible to phagocytosis may help to explain the observations of Séreny (12), who reported that *S. flexneri* organisms cultured more than 24 hr are not virulent for the guinea pig eye. Roantree (10) suggested that a possible reduction in the number of repeating saccharide units in the LPS of *Salmonella* may result in an avirulent smooth organism. The possibility that continued culture may affect the number of saccharide units in the LPS of *Shigella*, its resistance to phagocytosis, and consequently its virulence is currently under investigation.

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