INFECTION AND IMMUNITY, May 1970, p. 459-463

Vol. 1, No. 5
Printed in U.S.A.

Infection of Cultured Mouse Macrophages with *Shigella flexneri*

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Received for publication 20 November 1969

Virulent, avirulent, and attenuated hybrid strains of *Shigella flexneri* 2a are equally susceptible to phagocytosis by cultured mouse peritoneal macrophages. The virulent strain is highly lethal for the macrophages, whereas the avirulent is not and is killed. The attenuated hybrid strain is intermediate in its lethality. Comparable results were obtained by using virulent and avirulent *S. flexneri* 1b, 3, and 5. Destruction of macrophages occurs shortly after infection, suggesting virulent strains may possess a toxic component. The relationship of the ability to kill macrophages with multiplication of virulent shigellae in mucosal tissue is discussed.

Studies with starved guinea pigs as an experimental model have revealed the sequence of events involved in the pathogenesis of bacillary dysentery (3, 5). Virulent shigellae first penetrate the intestinal epithelial cells and then transmigrate into the lamina propria where they multiply and cause intestinal ulceration. Avirulent strains do not invade mucosal tissue since they are unable to penetrate epithelial cells. Attenuated *Escherichia coli-Shigella flexneri* hybrid strains enter intestinal epithelial cells and reach the lamina propria but do not multiply rapidly and appear to be killed by phagocytes (3).

These observations suggest that resistance to phagocytosis may be an important attribute of virulence in *Shigella*. Also LaBrec and Formal (4) have reported that active phagocytosis of the dysentery bacilli occurs both in the lumen and in the mucosal tissue. The present investigation was initiated to study the infection of cultured mouse macrophages with virulent, avirulent, and attenuated strains of *S. flexneri*. The findings presented here show that all three are equally susceptible to phagocytosis and that the virulent shigellae are the most lethal to the macrophages.

MATERIALS AND METHODS

Bacteria. All strains of *Shigella* were obtained from S. B. Formal of the Walter Reed Army Institute of Research, Washington, D.C. *S. flexneri* 2a, strains M42-43, 2457O, and X-16, were principally used. Strain M42-43 is virulent for guinea pigs and monkeys, 2457O is considered avirulent since it does not invade epithelial cells, and X-16 is an attenuated hybrid strain which had been obtained from mating *S. flexneri* 2a with *E. coli* K-12. All cultures were maintained in the lyophilized state.

Harvesting of macrophages. Peritoneal macrophages were obtained from 8- to 9-week-old adult male CD-I mice supplied by the Charles River Mouse Farms, Boston, Mass. The mice were etherized and exsanguinated by severing the arteries and veins in the left axillary region. Animals were then injected intraperitoneally (IP) with 3 ml of Eagle's Basal Medium containing 10 units of penicillin, 20 μg of streptomycin, and 0.1 mg of heparin per ml. Each animal's abdomen was gently massaged, and the peritoneal washing was removed. Approximately 2.5×10^6 cells were obtained from each mouse.

Cultivation of macrophages. The peritoneal washings were centrifuged at $1,000 \times g$ for 5 min, and the cells were then resuspended in cell culture medium to give a concentration of ca. 5×10^5 cells/ml. The medium consisted of 60% (v/v) Eagle's Basal Medium, 40% (v/v) horse serum and the above antibiotics. The glutamine content of Eagle's Basal Medium was increased to 0.04 M as recommended by Bennett (1). One ml of the cell suspension was added to each Leighton tube and the cultures were incubated at 37 C in an atmosphere of 5% CO_2 in air. After 18 to 24 hr of incubation the culture fluid was replaced with fresh medium.

Infection of macrophage cultures. Lyophilized stock cultures were reconstituted and inoculated into Trypticase Soy Broth (BBL). After 18 hr incubation at 37 C on a rotary shaker, the cultures were centrifuged at $3,000 \times g$ for 10 min. The bacteria were washed once with Hanks Balanced Salt Solution (HBSS) and then resuspended in HBSS to give an optical density of 0.260 at a wavelength of 425 nm in a 1-cm light path in a Coleman Jr. model 6B spectrophotometer. Plate counts showed that such suspensions con-

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tained ca. 9×10^8 shigellae/ml. Dilutions of each bacterial suspension were prepared in the cell culture medium (without antibiotics) for infection.

The medium was removed from 2- to 3-day-old macrophage cultures and was replaced with the appropriate dilution of bacteria. Four cultures were infected with each dilution. After 2 hr at 37 C, the bacteria were removed, and each tube was washed twice with HBSS before adding fresh medium containing antibiotics and reincubated. Unless noted otherwise, the number of surviving macrophages was determined at 1 day postinfection. Cultures of uninfected macrophages served as controls. The percentage decrease in the number of macrophages was calculated using the formula $[(N_0 - N_i) - (N_0 - N_u)/N_0] \times 100$, where N_0 = number before infection, N_i = number in infected cultures, and N_u = number in uninfected cultures. The decrease in uninfected cultures ranged from 0 to 25%.

Enumeration of cultured macrophages. A modification of the nuclei count procedure of Sanford et al. (8) was used. After removal of the medium, each macrophage culture was washed twice with HBSS. The macrophages then were incubated at 37 C in the presence of 1.0 ml of 0.1 M citric acid to release the nuclei. The nuclei were stained by the addition of 1.0 ml of 0.1% (w/v) crystal violet in 0.1 M citric acid and counted in a hemacytometer.

Phagocytic index. When phagocytic indexes were to be determined, Leighton tubes containing cover slips (9 by 22 mm) were used for the cultivation of macrophages. At the appropriate time interval after infection, duplicate cover slips were removed, and cells were fixed in absolute methanol and stained with Giemsa solution. One hundred macrophages per cover slip were counted.

RESULTS

Preliminary experiments were done by infecting cultured mouse macrophages with strain M42-43. At a multiplicity of infection (MOI) of 10 shigellae:1 macrophage, the number of macrophages remaining attached to the tubes at 1 day postinfection was reduced ca. 40%. The culture fluid of infected tubes contained appreciably fewer intact macrophages than that of uninfected tubes, 1.2×10^8 and 9.0×10^8/tube, respectively. This latter observation showed that infection with the virulent dysentery bacilli resulted in lysis, and not merely detachment, of the peritoneal cells. A greater degree of lethality was obtained when infection was carried out in the presence of 40%, rather than 2%, horse serum. The former serum concentration was used in the experiments described below.

Macrophage cultures were infected with various concentrations of the three strains of *S. flexneri* 2a. The number of surviving cells was determined at 1 day postinfection. The virulent strain M42-43 had the greatest lethal effect (Table 1). The avirulent strain, 2457O, exerted killing action only at high concentrations, and the attenuated hybrid strain, X-16, was intermediate in its degree of lethality. Incubation of infected cultures for longer periods (2 to 5 days) did not result in further destruction of the peritoneal cells by the shigellae. Replicate experiments showed that the decrease in macrophages after infection with strain M42-43 ranged from 25 to 50% with a MOI of 5:1 and 40 to 65% with 10:1.

Cover slip preparations were removed at 2 hr after the addition of shigellae and examined to determine whether there was any difference in the degree of phagocytosis of the three strains. It was found that the phagocytic indexes and the number of bacteria ingested were similar among the three cultures (Table 2). Most of the intracellular bacteria were enclosed in vacuoles.

Intracellular bacteria were present 24 and 48 hr after infection (Table 2). Since it was not known whether these bacteria were alive, viable counts were performed on infected cultures. At various time intervals, the macrophages were washed with HBSS and then lysed by treatment

TABLE 1. *Survival of cultured mouse macrophages infected with virulent, avirulent, and attenuated Shigella flexneri 2a*

Strain	Multiplicity of infection[a]				
	5:1	10:1	25:1	50:1	100:1
M42-43	7.5[b]	5.0	3.0	0.1	0.1
2457O	11.7	10.8	11.3	9.2	9.5
X-16	10.3	8.2	6.4	3.0	0.6

[a] Ratio of bacteria to macrophage.

[b] Average count ($\times 10^4$) of four tubes of surviving macrophages 1 day postinfection. Average count before infection was 15.2, and that of uninfected cultures 1 day postinfection was 12.1.

TABLE 2. *Phagocytosis of virulent, avirulent, and attenuated S. flexneri 2a by cultured mouse macrophages*[a]

Strain	Phagocytic index (%) at end of			Bacteria in macrophages at end of[b]		
	2 hr	24 hr	48 hr	2 hr	24 hr	48 hr
M42-43	46	18	24	209	172	128
2457O	46	22	28	194	177	126
X-16	52	18	22	212	182	169

[a] Multiplicity of infection was 5:1.

[b] Intracellular bacteria per 100 infected macrophages.

with 1.5 ml of 0.5% (w/v) sodium deoxycholate in HBSS at 37 C for 10 to 15 min. This treatment was not lethal for the dysentery bacilli (Table 3). The lysates were then plated out on Trypticase Soy Agar (BBL). The results showed there was a marked decrease in the number of viable intracellular bacteria at 1 day postinfection (Table 4). With the exception of experiment 1, essentially all shigellae were killed by the macrophages after 4 days postinfection.

The interpretation of these findings on the cultures infected with strain M42-43 will be discussed later. Two explanations could be given for the observed bactericidal action in the cultures infected with strain 2457O: the macrophages either had killed the avirulent shigellae or had taken up bactericidal amounts of streptomycin. Streptomycin sensitivity tests by the tube dilution method showed that 2.5 μg of streptomycin per ml was bacteriostatic for both strains in macrophage culture medium and 5.0 μg/ml was bactericidal. An inoculum of 3×10^5 colony-forming units (CFU)/ml was used. We therefore infected macrophages with strain 2457O and maintained them in antibiotic-free medium. The number of viable intracellular shigellae was determined at the end of 2 and 8 hr postinfection. For the latter period, the culture medium was changed every 1 hr to minimize the number of extracellular dysentery bacilli. During the 6-hr interval, approximately a 2 log decrease in viable intracellular bacteria occurred, 1.2×10^5 CFU/culture at 2 hr and 9.4×10^2 at 8 hr. Thus, at least a part, if not all, of the decrease in intracellular avirulent shigellae shown in Table 4 could be

TABLE 3. *Survival of virulent and avirulent Shigella flexneri 2a in sodium deoxycholate*

Strain	CFU/ml[a]	
	HBSS[b]	DOC[c]
M42-43	3.0×10^5	2.8×10^5
2457O	2.1×10^5	2.4×10^5

[a] Colony-forming units after 30 min of incubation at 37 C. The count before incubation was 2.8×10^5 for strain M42-43 and 2.6×10^5 for strain 2457O.

[b] Hanks Balanced Salt Solution.

[c] Sodium deoxycholate, 0.5% (w/v), in HBSS.

TABLE 4. *Intracellular survival of virulent and avirulent Shigella flexneri 2a in cultured mouse macrophages*[a]

Expt	Strain	Viable counts per tube at end of[b]				
		2 hr	1 day	2 days	4 days	7 days
1	M42-43	290,000	250	75	0	0
	2457O	245,000	TNTC[c]	2,000	50	0
2	M42-43	400,000	24	0	0	0
	2457O	750,000	307	118	0	1
3	M42-43	140,000	45	0	0	0
	2457O	220,000	150	24	0	0

[a] Multiplicity of infection was 5:1.

[b] Values represent average of duplicate cultures which were assayed for each time interval. Values for 2 hr were calculated from colony counts performed on dilutions of two 0.1-ml samples of each lysed macrophage suspension. For remaining time periods, 10 0.1-ml samples per tube were plated out.

[c] Too numerous to count.

TABLE 5. *Macrophage survival at various times after infection with virulent and avirulent Shigella flexneri 2a*

Time	Multiplicity of infection				
	5:1		10:1		
	Uninfected	M42-43	Uninfected	M42-43	2457O
hr					
0	29.4[a]	31.9	20.1	20.8	23.0
3	30.5	29.7	18.9	11.5	19.3
4	31.0	26.8	19.9	11.8	18.4
5	27.2	17.5	20.8	12.0	19.6
6	25.2	13.9	ND[b]	ND	ND
8	25.6	16.0	19.5	9.2	21.2
24	24.5	15.1	20.0	8.8	18.1

[a] Average count ($\times 10^4$) of four tubes of surviving macrophages at 1 day postinfection.

[b] Not done.

TABLE 6. *Effect of virulent and avirulent strains of various Shigella flexneri serotypes on cultured mouse macrophages*[a]

Strain	Serotype	Per cent decrease in macrophages	
		Expt 1	Expt 2
Virulent			
M42-43	2a	41	61
M25-8	1b	40	52
M90T	5	44	65
Avirulent			
2457O	2a	9	0
2381O	1b	8	5
M90TX	5	12	6

[a] Multiplicity of infection was 10:1.

attributed to the bactericidal action of the macrophages.

The number of surviving macrophages was determined at various time intervals up to 1 day postinfection. The destruction of peritoneal cells by strain M42-43 occurred rapidly (Table 5). At an MOI of 5:1, a significant decrease in the numbers of macrophages was detected 5 hr after infection and maximum destruction was observed after 6 hr. Replicate experiments indicated that the range of the two periods was 4 to 5 and 6 to 8 hr, respectively. At an MOI of 10:1, cell destruction was observed at the end of 3 hr postinfection. As had been observed previously, strain 2457O was not lethal for the macrophages.

Infection of macrophage cultures with other virulent and avirulent *S. flexneri* serotypes showed that only virulent strains were highly lethal. The results obtained with serotypes 1b and 5 are given in Table 6. In a separate experiment, a 57% decrease in the number of macrophages occurred in cultures infected with a virulent strain (J17/B) of serotype 3. In contrast, no significant reduction, 2%, was observed with an avirulent strain (3-5O).

DISCUSSION

These studies show that virulent strains of *S. flexneri* are appreciably more lethal for cultured mouse macrophages than attenuated and avirulent ones. Shigellae which do not destroy the macrophages are killed by these phagocytes. This lethal activity for macrophages suggests a possible mechanism by which the virulent shigellae can survive phagocytosis, be released, and then multiply in the lamina propria. Attenuated strains, being less cytotoxic, may be destroyed by phagocytes in mucosal tissue. In conjunction with these data, it should be pointed out that the dysentery bacilli are phagocytosed by both neutrophils and macrophages in mucosal tissue (10). Studies with *Pasteurella pestis*, *Brucella abortus*, and *Staphylococcus aureus* have shown that neutrophils are more bactericidal than monocytes (2, 6, 9). Thus, work needs to be done on the interaction of *S. flexneri* and neutrophils to determine the importance of the above lethal activity in *Shigella* pathogenesis.

The phagocytic index of infected macrophage cultures decreases between 2 and 24 hr postinfection (Table 2). This reduction in cultures infected with strain M42-43 probably reflects macrophage destruction with release of the bacteria into the antibiotic-containing medium. It is not known why all macrophages ingesting this strain are not killed. Some of the phagocytes may be resistant or avirulent mutants may be present, or both conditions may exist. The decrease in the phagocytic index of cultures infected with strain 2457O probably is a result of bactericidal and lytic action of the macrophages.

Viable bacterial counts of infected cultures show that most of the intracellular shigellae detected on stained cover slips at 24 and 48 hr postinfection were dead (Table 4). The results also demonstrate that all intracellular dysentery bacilli may be killed within 4 days postinfection. These findings indicate that *S. flexneri* cells which are not lethal for macrophages are killed by the phagocytes.

The rapidity by which the macrophages are killed suggests that virulent *S. flexneri* contains a toxic substance. It is unlikely that the lethality is due to endotoxin since this lipopolysaccharide is also present in avirulent strains. Studies to detect the presence of a *Shigella* cytotoxin are in progress.

Presently, the ability to invade cultured epithelial cells is the main in vitro criterion for the presumptive detection of virulent shigellae (5, 7, 11). We propose that the mouse macrophage system described herein also can be used. The observation that the X-16 hybrid strain is less lethal than virulent strains suggests virulent and attenuated strains may be differentiated by this cell culture system.

ACKNOWLEDGMENTS

We thank Marietta Sisca for her technical assistance.

This investigation was supported by the Public Health Service grant FRO5451-06 from the Division of Research Facilities and Resources of the National Institutes of Health and by the U.S. Army Medical Research and Development Command, Department of the Army, under research contract no. DADA-17-67-C-7157 under the sponsorship of the Commission on Enteric Infections of the Armed Forces Epidemiological Board.

LITERATURE CITED

1. Bennett, B. 1966. Isolation and cultivation *in vitro* of macrophages from various sources in the mouse. Amer. J. Pathol. 48:165–181.
2. Cavanaugh, D. C., and R. Randall. 1959. The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-born plague. J. Immunol. 83:348–363.
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. 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.
5. 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.

6. Mackaness, G. B. 1960. The phagocytosis and inactivation of staphylococci by macrophages of normal rabbits. J. Exp. Med. **112**:35–53.
7. 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. Jap. J. Med. Sci. Biol. **20**:315–328.
8. Sanford, K. K., W. R. Earle, V. J. Evans, H. K. Waltz, and J. E. Shannon. 1951. The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. **11**:773–795.
9. Smith, H. 1968. Biochemical challenge of microbial pathogenicity. Bacteriol. Rev. **32**:164–184.
10. Takeuchi, A., S. B. Formal, and H. Sprinz. 1968. Experimental acute colitis in the Rhesus monkey following peroral infection with Shigella flexneri. Amer. J. Pathol. **52**:503–529.
11. Watkins, H. M. S. 1960. Some attributes of virulence in Shigella. Ann. N.Y. Acad. Sci. **88**:1167–1168.