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Chemotherapy of Mice Experimentally Infected with *Shigella flexneri*

N. K. DAS AND R. E. CHAMBERLAIN

Research and Development Department, The Norwich Pharmacal Company, Norwich, New York 13815

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A chronic infection with *Shigella flexneri* 2a has been established in mice for the evaluation of compounds for therapeutic potential. Evidence of infection is indicated by prolonged symptomless excretion in the feces and by positive isolation of organisms from different segments of the intestinal tract and from mesenteric lymph nodes. Serum antibody titer reaches a maximum after 9 days of infection and remains at a low level until 32 days postinfection. In this model, five drugs used in human shigellosis were evaluated for efficacy. Ampicillin was found to be the most active followed by oxytetracycline and kanamycin. Neomycin and colistin were the least active in this system.

Since the report by Freter (6) of an experimental *Shigella* infection in mice, other investigators (1, 16) have used this model in pathogenicity studies. To our knowledge, however, there is no report that this system has been used for in vivo chemotherapy studies. We have adapted the model for use in the routine evaluation of potential chemotherapeutic agents.

MATERIALS AND METHODS

Animals. Four-week-old (18 to 20 g) male Swiss Webster mice were used throughout.

Bacterial culture. A lyophilized culture of *S. flexneri* 2a obtained from S. B. Formal, Walter Reed Army Institute of Research, Washington, D.C., was used throughout these experiments. This strain was made resistant to streptomycin (>4,000 μg/ml) by the gradient plate technique (21). Resistant mutants were isolated, observed under oblique illumination for typical coloration (2, 11), and checked for colonial morphology and virulence (2, 19). To avoid loss of virulence, a large batch of streptomycin-resistant organisms was grown on gradient plates containing streptomycin (1,000 μg/ml), harvested in 20% skim milk (Difco), and lyophilized in 0.2-ml quantities in small ampoules. The ampoules were stored at 4 C, and a new ampoule was used for each experiment. To prepare the bacterial suspension for in vivo use, the contents from an ampoule were suspended in 1 ml of Brain Heart Infusion (BHI) broth (Difco) and several gradient plates were spread with 0.2 ml of the suspension. After 24 hr of incubation at 37 C, growth from the plates was harvested in BHI broth and the turbidity was adjusted to give 62% light transmission at 440 nm with a Bausch & Lomb Spectronic-20. The suspension was further diluted in broth so that 0.5 ml would contain approximately 10^8 viable organisms as determined by plate count.

Oral infection of mice. Normal animals were transferred to sterilized metal boxes (five in each) containing sterilized sawdust. The animals were supplied with sterilized Purina Laboratory Chow and drinking water containing neomycin (4 mg/ml) and bacitracin (260 units/ml) ad lib for a period of 4 days to reduce the normal enteric flora. After this, each mouse was challenged perorally, via feeding needle, with approximately 10^8 viable cells. At the same time, the neomycin in the drinking water was replaced with streptomycin (1 mg/ml) and the bacitracin concentration was decreased to 25 units/ml. This combination was supplied ad lib for the remainder of the test period.

Bacteriological analysis. At different time periods after infection, feces (250 mg) from each group of five mice were collected aseptically, pooled, and homogenized thoroughly in 2.25 ml of sterile saline. Further 10-fold dilutions were prepared, as required, in saline and 0.1 ml of each was spread on BHI agar (Difco) containing streptomycin (200 μg/ml) and on Xylose Lysine Desoxycholate (XLD) agar (BBL) plates. After 24 hr of incubation at 37 C, colonies were counted and examined under oblique illumination to determine similarity to the challenge organism. At least five colonies were selected at random from each plate and examined by slide agglutination with *Shigella* type-specific antiserum. To determine the extent of gut colonization, groups of five mice were sacrificed on the 2nd, 4th, 9th, 12th, 16th, 20th, 24th, and 32nd day of infection and the entire intestinal tract was removed aseptically. Sections of duodenum, jejunum, ileum, caecum, and proximal and distal colon including contents were removed, weighed, ground with sterile sand and saline, and the number of *Shigella* present was determined by plating on both BHI and XLD agar. The gall bladder and mesenteric lymph nodes were collected aseptically from each mouse and cultured in 5 ml of Gram negative (GN) broth (Difco). The cultures were incubated for 24

to 48 hr at 37 C, and the organisms from tubes showing growth were identified as before, after subculturing on BHI agar plates.

Histopathological examination. Sections from the duodenum, jejunum, ileum, caecum, and colon were removed at different time intervals from antibiotic-treated uninfected and antibiotic-treated infected animals and placed in 10% Formalin buffer solution. Transverse sections from each of the above areas were stained with hematoxylin-eosin.

Serum antibody. Antibody levels in sera were determined by the indirect bacterial hemagglutination technique (7, 10). After infection, blood was collected from the axillary vein of five anaesthetized mice and the pooled serum was titrated against sheep Formocells (Difco) sensitized with the "O" antigen of a steamed suspension of the infecting organism. The initial serum dilution was 1:5 and the days of testing were the same as for bacterial sampling of the intestinal tract.

Administration of test compounds. Groups of five mice were used for each dosage level of each test compound. Therapy was initiated 48 hr after infection when bacteriological examination of the feces showed that infection was well established. Test compounds were suspended in 0.5% carboxymethyl cellulose or in water and administered orally in 0.5-ml volumes with a feeding needle. The total daily dose (milligrams/kilogram) was divided equally and given at 8:30 AM, 1:00 PM, and 4:30 PM for a period of 10 days.

RESULTS

Persistence of Shigella in the intestinal tract. To establish infection in mice with this *Shigella* strain, it was necessary to administer a minimum of 10^8 viable cells. To maintain the infection, it was also necessary to include antibiotics in the drinking water. Under these conditions (Table 1), 10^8 organisms per g of feces could be recovered for a period of at least 4 weeks. It was also observed that, although active multiplication was evidenced by the recovery of relatively large numbers of viable organisms, at no time were any signs of illness noted. This finding coincides with previous observations (1, 16).

Isolation of Shigella from intestinal tract and other organs. The results of these experiments (Fig. 1) indicate that, within 2 days of peroral inoculation, the organisms could be found throughout the intestinal tract, greater numbers being obtained from the caecum and proximal and distal colon. By day 4, the number of organisms recovered from the caecum and colon reached levels the same as or slightly higher than the challenge dose of 10^8 and this level was maintained until about 16 days postinfection. During this period, however, there was a con-

TABLE 1. *Duration of Shigella excretion in the feces of infected mice*[a]

Time after infection	Viable count per g of feces[b]
days	
2	2.5×10^8
5	5.5×10^8
9	8.0×10^8
12	9.8×10^8
16	6.8×10^8
20	6.7×10^8
25	3.2×10^8
30	2.1×10^8

[a] Animals were fed approximately 10^8 cells.
[b] Geometric mean of results in five experiments.

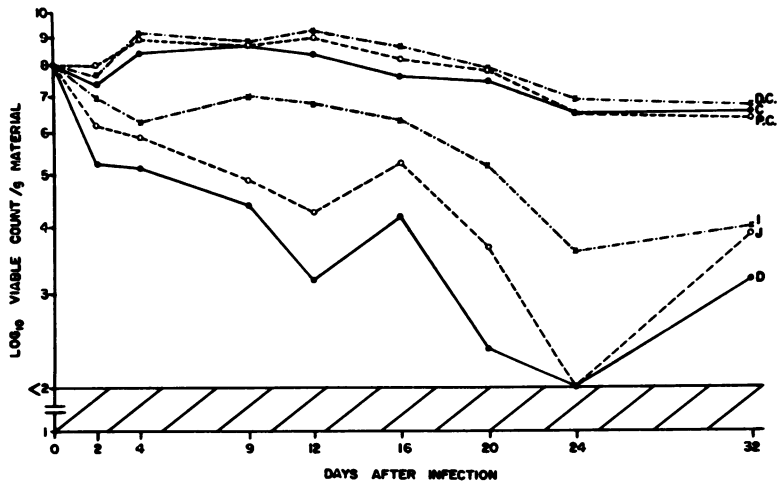

FIG. 1. *Distribution of Shigella flexneri 2a in the intestinal tract of mice after oral administration of 10^8 organisms. Abbreviations: D, duodenum; J, jejunum; I, ileum; C, caecum; PC, proximal colon; DC, distal colon. Each point represents the geometric mean of viable counts from five mice.*

siderable decrease in the number of organisms recovered from the small intestine; the largest reduction was noted in the duodenum followed by the jejunum and ileum. Further reduction in recoverable organisms was observed in the small intestine by day 20 through day 24, following which an increase in viable count was noted in both duodenum and jejunum after 32 days of infection. Although there was a slight decrease in the number of organisms obtained from the caecum and colon, at no time was the viable count decreased to less than 1/100 the challenge dose.

Shigella were routinely isolated from the mesenteric lymph nodes of most animals up to 12 days after infection but not thereafter. In only one case was the organism recovered from the gall bladder and never from the heart, liver, or spleen.

Serum antibody level. As illustrated in Fig. 2, the peak antibody titer (1:40) was obtained about 9 days after infection. The titer decreased rapidly to 1:5 by day 12, after which it fluctuated between 1:5 and 1:20 during the remainder of the test period.

Histological findings. Preliminary histological examination of the intestinal wall of *Shigella* carrier mice revealed the presence of organisms on the mucosal surface of the small intestine, caecum, and colon. However, penetration of organisms into the lamina propria, glands (crypts), and muscularis was noted only in the caecum. A marked infiltration of inflammatory cells was also noted at these sites. No histological changes were noted in the intestinal sections from antibiotic-treated uninfected mice.

Chemotherapy in Shigella-infected mice. The test compounds were selected on the basis of their use in human shigellosis (12). The results

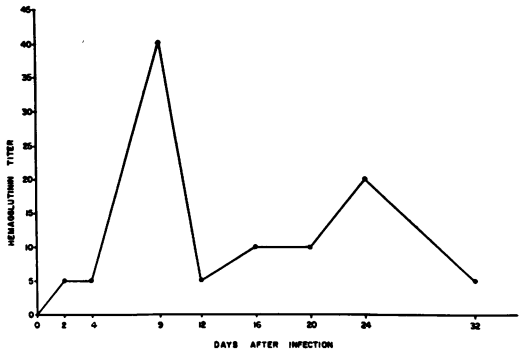

FIG. 2. *Serum antibody level in mice after Shigella flexneri 2a infection.*

TABLE 2. *Effect of oral therapy on counts of viable Shigella in feces of infected mice*

Drugs	Daily dose[a] (mg/kg)	Viable count per g of feces[b]			
		0[c]	4	7	10
Ampicillin	12.5	2.8×10^8	2.5×10^8	7.5×10^7	2.2×10^8
	25.0	5.0×10^8	4.3×10^3	1.2×10^3	2.0×10^3
	50.0	4.4×10^8	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Colistin	300	4.6×10^8	3.0×10^8	7.5×10^8	3.3×10^8
	400	3.1×10^8	4.5×10^4	2.0×10^4	2.5×10^5
	500	6.0×10^8	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.8×10^4
Kanamycin	300	4.5×10^8	4.0×10^3	1.7×10^3	5.4×10^4
	400	2.3×10^8	1.0×10^2	3.0×10^2	1.6×10^2
	500	3.9×10^8	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Neomycin	300	3.2×10^8	1.2×10^7	7.9×10^4	1.3×10^4
	400	2.8×10^8	1.1×10^4	2.1×10^3	7.1×10^3
	500	2.8×10^8	2.5×10^3	$<1.0 \times 10^2$	1.5×10^2
Oxytetracycline	50	2.5×10^8	2.8×10^6	4.4×10^4	3.2×10^6
	100	1.4×10^8	1.8×10^6	1.4×10^5	6.0×10^3
	200	3.5×10^8	6.4×10^5	7.0×10^3	$<1.0 \times 10^2$
Control	None	2.0×10^8	3.6×10^8	3.1×10^8	1.6×10^8

[a] Divided into three equal doses and given orally at 8:30 AM, 1:00 PM, and 4:30 PM beginning 48 hr after infection.

[b] Geometric mean of results in five experiments.

[c] Duration of therapy (days).

of these experiments are summarized in Table 2. Ampicillin was the most effective drug in this series. At 50 mg per kg per day, it almost eliminated the organism after 4 days of therapy. However, at 25 mg/kg daily it produced only a 100,000-fold reduction in the viable fecal count during 10 days of therapy. No reduction in cell count was observed at 12.5 mg per kg per day.

Under the same conditions, feeding of oxytetracycline at a daily dose of 200 mg/kg for a period of 7 to 10 days was necessary to achieve results similar to ampicillin at 50 mg per kg per day. Reducing the daily dosage to 100 and 50 mg/kg was found to be less effective.

Of the three nonabsorbable drugs tested, administration of kanamycin at either 400 or 500 mg per kg per day resulted in almost complete elimination of fecal organisms, whereas there was only a 10,000-fold reduction in viable count at 300 mg/kg daily. Neomycin or colistin failed to eradicate the organisms even at the highest level (500 mg per kg per day) tested.

It was also noted that, although some of the drugs reduced the viable count to $<10^2$, none was capable of completely eradicating the organism; the count always increased after cessation of therapy.

In vitro tests were performed with the streptomycin-bacitracin mixture used to supress the normal flora and a wide range of concentrations of each of the antibiotics listed in Table 2. The results indicated that the antibiotic mixture had no detectable effect on the therapeutic activity of the antibiotics in this system.

DISCUSSION

The results of our experiments confirm the findings of others (1, 6, 16) that reproducible *Shigella* infections can be established in the mouse intestinal tract after elimination of the normal enteric flora. This infection can be maintained at a uniform level for at least 1 month. However, there are several critical factors in the establishment of this infection which must be emphasized. First, it is necessary to maintain the virulence of the organism before infecting the mice. Loss of virulence results in failure to maintain the infection at a uniform level. The second involves the continuous supply of streptomycin and bacitracin in the drinking water. Ancillary experiments indicated that, within a few days after removal of streptomycin and bacitracin, the normal flora returns to pretreatment levels and *Shigella* disappears. Third, it is essential to keep the animals in sterilized metal boxes, containing sterilized sawdust, which are changed every other day to minimize contamination. It is also necessary to provide the animals with sterilized food throughout the experiment.

Although the infected mice never showed any gross signs of illness, repeated isolations of organisms from the feces, mesenteric lymph nodes, and various sections of the intestinal tract indicated that a chronic infection had been established. Similar symptomless chronic excretion of *Shigella* organisms is not uncommon in man (3).

Havlik et al. (10) found that in human shigellosis significant serum antibody titers appeared within 6 to 8 days after the onset of symptoms and titers remained elevated for several weeks. Similarly Haltalin and co-workers (7) reported an early appearance of hemagglutinins which reached peak titer on the 5th to 14th day of illness based on the clinical history of diarrhea. In the mouse *Shigella* model, a similar temporal increase and decrease in serum antibody titer were observed.

If it can be assumed that the rapidity of disappearance of the organism from the feces is a rough index of the effectiveness of therapy in *Shigella*-infected mice, it is evident from the results obtained that drugs which are at least partially absorbed from the intestinal tract are more effective than the socalled nonabsorbable drugs. This concept is supported by similar results reported recently in human shigellosis (8). Ampicillin, considered by some clinicians to be the drug of first choice (8, 12, 15), was found to be active at a dose similar to that used in humans (9). Oxytetracycline also was effective but required a higher daily dose (200 mg/kg) and a longer treatment regimen (10 days) to bring about a comparable reduction in the number of organisms. Much larger doses of the nonabsorbable drugs kanamycin, neomycin, and colistin were required to produce reductions in viable count equivalent to those caused by ampicillin or oxytetracycline. This may reflect the variability in results occasionally reported in humans (4, 5, 13, 17, 18, 20).

The reason for the lower activity of the nonabsorbable drugs in the mouse is speculative. Since it was possible to recover organisms from the mesenteric lymph nodes of the infected mice and also to locate organisms in the lamina propria of the intestinal wall, it is quite possible that organisms located in these sites are protected from the nonabsorbable drugs by the bowel mucosa.

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