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Effect of Streptomycin Administration on Colonization Resistance to *Salmonella typhimurium* in Mice

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The addition of 5 mg of streptomycin sulfate per ml to the drinking water of Swiss white mice resulted in a 100,000-fold reduction in the 50% implantation dose of streptomycin-resistant *Salmonella typhimurium* for the animals. When streptomycin-treated and untreated mice were challenged orogastrically with 10^3 viable *S. typhimurium* organisms, 100% of the treated and none of the untreated mice excreted the pathogen in their feces. Similarly, translocation of *S. typhimurium* from the intestinal tract to the liver, spleen, and mesentery occurred in 10 of 10 treated mice but in none of the untreated mice 7 days after challenge with 10^3 CFU. Studies of colonization dynamics showed that *S. typhimurium* was present at high population levels in the intestines of streptomycin-treated mice and in detectable levels in the liver, spleen, and mesentery within 72 h after challenge with 10^3, 10^5, or 10^8 organisms. In untreated mice challenged with either 10^3 or 10^5 *S. typhimurium* organisms, the organisms were isolated from ileal and cecal tissues but not from ileal or cecal contents or from extraintestinal tissue 72 h after challenge. When untreated mice were challenged with 10^8 organisms, however, *S. typhimurium* was present in all organs and in intestinal contents. Streptomycin treatment, therefore, facilitated colonization and development of streptomycin-resistant *S. typhimurium* populations in intestines of mice and the subsequent translocation of the organisms from the intestinal tract to other tissues.

Salmonellosis is an economic and health concern of international scope. The problem lies in the wide distribution of salmonellae in nature (3). *Salmonella typhimurium*, which causes the highest incidence of salmonellosis in both human and nonhuman hosts (8), usually causes self-limiting infections in the general populace. Its economic impact, both in manpower loss and on food industries, cannot be overlooked. Furthermore, infection in the compromised host may become complicated and life threatening.

The widespread and indiscriminate use of antibiotics creates problems in the prevention of infection by selecting for antibiotic-resistant organisms that have the potential of producing disease and by reducing the degree of colonization resistance of the host to these organisms. The latter problem was clearly demonstrated by Bohnhoff et al. (5), who showed that oral administration of streptomycin to mice before challenge with *Salmonella enteritidis* resulted in a 100,000-fold increase in susceptibility to infection by this pathogen. Freter (12) demonstrated that oral administration of antibiotics to mice and guinea pigs rendered the animals susceptible to infection with antibiotic-resistant strains of *Shigella flexneri* and *Vibrio cholerae*. These and other investigators have suggested that increased susceptibility to infection after antibiotic administration is due to the disruption of the indigenous flora which normally protects the host against colonization by exogenous bacteria (23). Further evidence for the protective role of the indigenous flora was demonstrated when partial restoration of colonization resistance to *Salmonella* infection was achieved by inoculating streptomycin-treated mice with a fecal suspension from untreated mice before challenge with the pathogen (21). More recently, Barnes et al. (2) showed that the association of chicks with complex mixtures of facultative anaerobes and anaerobes derived from adult birds resulted in resistance to *Salmonella* colonization. Studies done on the route of enteric infection in normal mice by Carter and Collins (7) indicate that the primary sites of *S. enteritidis* invasion are the distal ileum and, possibly, the cecum. Both of these regions support a well-established indigenous flora which, presumably, provides protection against infection with this pathogen.

From studies with animal models and observations of in vitro bacterial interactions, several mechanisms by which indigenous flora protects against infection have been proposed. These are: establishment in the intestine of adverse oxidation reduction potential and pH (16, 20), competition with pathogens for limiting nutrients (13), elaboration of inhibitory substances (e.g., short-chain fatty acids, hydrogen sulfide, and bacteriocins) (13, 16–18), and competition with pathogens for tissue adhesion sites (4, 10). The disruption of the ecological balance between flora components by antibiotic treatment or other means may cause an alteration in the mechanism(s) responsible for colonization resistance.

In this investigation, we examined the effects of streptomycin administration on resistance to colonization by *S. typhimurium* present in the intestines of mice.

MATERIALS AND METHODS

Cultures. The parent strain, *S. typhimurium* CR6600 (kindly provided by G. W. Jones, University of Michigan), is a clinical isolate known to be virulent for mice (15). A spontaneous streptomycin-resistant mutant (able to multiply in brain heart infusion [BHI] broth containing 3 mg of streptomycin per ml of broth) was selected by the method of Stocker (described in T. M. Joys, Ph.D. thesis, University of London, London, England, 1961). The resistant strain, designated *S. typhimurium* CR6600(STR-1), was indistinguishable morphologically and biochemically and in growth rate kinetics from the parent strain. Stock cultures were stored at −70°C in 20% (vol/vol) glycerol in BHI broth. Bacteria used in these experiments were grown in BHI broth containing 1 mg of streptomycin per ml for 12 h at 37°C.

Mice. Outbred Swiss white mice (Cox variety; Laboratory Supply Co., Indianapolis, Ind.) were used in all experi-

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TABLE 1. Effect of streptomycin administration on fecal carriage and population levels of *S. typhimurium* in mice

Day after challenge[a]	Untreated		Treated	
	Population[b]	Incidence[c]	Population[d]	Incidence[c]
1	<20	0/10	3.18 ± 1.43	9/10
2	<20	0/10	5.80 ± 1.72	10/10
4	<20	0/10	6.54 ± 1.43	10/10
7	<20	0/10	6.67 ± 0.92	10/10
14	<20	0/10	6.47 ± 1.61	10/10

[a] Challenge dose, 10^3 viable organisms.
[b] Viable organisms per fecal pellet.
[c] Proportion of mice harboring *S. typhimurium*.
[d] Mean $\log_{10}$ viable counts (± standard deviation) per gram (wet weight) of feces.

ments. Animals were housed in groups of five in cages with wire-mesh bottoms and were fed Purina Lab Rodent Diet and water ad libitum. Mice were shown to be free of *Salmonella* infection before experimentation by screening fecal samples for the pathogen by standard bacteriological methods (19). Streptomycin was added at a concentration of 5 mg/ml to drinking water. The antibiotic solution was provided ad libitum 7 days before challenge with *S. typhimurium* and after challenge for the duration of the experiment.

ID_{50} determination. The 50% implantation dose (ID_{50}) of *S. typhimurium* was determined for streptomycin-treated and untreated mice. On day 7 of antibiotic treatment, both the streptomycin-treated and untreated mice were challenged orogastrically with graded numbers of *S. typhimurium* in 0.1 ml of BHI broth introduced directly into the stomach by using a thin plastic feeding tube connected to a tuberculin syringe to ensure safe delivery. On day 3 postchallenge, two fecal pellets were collected from individual mice, and the pellets were emulsified in 1 ml of sterile saline. The emulsion (0.2 ml) was then plated on MacConkey agar containing 1 mg of streptomycin per ml (S-Mac). The presence or absence of *S. typhimurium* was recorded after 24 h of incubation at 37°C. ID_{50}, based on the presence of *S. typhimurium* in the feces, was calculated by the method of Reed and Muench (22).

Streptomycin concentration in cecal contents. A sample of cecal contents from each antibiotic-treated mouse was diluted sixfold (vol/wt) in 0.1 M phosphate buffer (pH 8.0). After thorough mixing, the diluted contents were centrifuged to pack the solid material, and the supernatant was passed through a membrane filter (pore size, 0.45 μm; Gelman Instrument Co., Ann Arbor, Mich.). An overnight culture of *Staphylococcus aureus* ATCC 25923 was adjusted to a 0.5 McFarland standard density, and 3.0 ml was mixed with 30 ml of molten antibiotic medium 5 (Difco Laboratories, Detroit, Mich.) in a petri dish. After solidification, wells (diameter, 4 mm) were punched into the agar medium and filled with 20 μl of cecal filtrate from an antibiotic-treated mouse, 20 μl of cecal filtrate from an untreated mouse (which served as a negative control), or 20 μl of standard solutions of streptomycin. After overnight incubation at 37°C, inhibition zone sizes around the wells were measured. The streptomycin concentration in the cecal filtrate was quantitated by reference to the sizes of inhibition zones produced by the streptomycin standards. Streptomycin concentration was reported as milligrams per gram (wet weight) of cecal contents.

Incidence and fecal population level. Streptomycin-treated and untreated mice were challenged with 10^3 CFU of *S. typhimurium*, administered orogastrically. Fresh fecal pellets from individual animals were collected at 1, 2, 4, 7, and 14 days after challenge. The fecal pellets were emulsified in 2 ml of phosphate-buffered saline (PBS), and the suspension was serially diluted. The appropriate dilutions (0.1 ml) were plated on S-Mac agar, and plate counts were recorded as the number of CFU of *S. typhimurium* per gram (wet weight) of feces.

Translocation from the intestine. Mice were sacrificed by cervical dislocation 7 days after challenge with *S. typhimurium*. The spleen, mesentery, cecum, and ileum were aseptically removed. The samples were homogenized in 9 volumes (vol/wt) of sterile PBS by using a motor-driven Teflon homogenizer. The homogenates were serially diluted in PBS, and 0.1 ml of the appropriate dilutions were plated on S-Mac. Bacterial counts were reported as CFU per gram (wet weight) of sample.

Dynamics of colonization. Groups of five streptomycin-treated and untreated mice were challenged with 10^3, 10^5, or 10^8 viable *S. typhimurium* organisms. At 2, 12, 24, 48, and 72 h after challenge, a mouse from each group was sacrificed, and the various organs were examined for the presence of *S. typhimurium*. The liver, spleen, and mesentery were processed as previously described. The ileum and cecum were opened longitudinally, and the contents were separated from the tissue for determination of *S. typhimurium* counts. The tissues were washed in 15 changes of PBS. Each wash was done by gently inverting the tube containing the tissue in 10 ml of PBS 10 times. The washed tissues were weighed, homogenized in 2 ml of PBS, serially diluted, and plated on S-Mac agar for *S. typhimurium* counts. Bacterial counts were recorded as CFU per gram (wet weight) of tissue. The experiments were repeated four times.

RESULTS

ID_{50}. The effect of streptomycin administration on the susceptibility of mice to implantation with *S. typhimurium* was determined. The ID_{50} for untreated mice was 10^5 CFU. The administration of streptomycin for 7 days before challenge with *S. typhimurium* reduced the ID_{50} to approximately 1 CFU, an increase in susceptibility of 100,000-fold. Streptomycin was detected in all of the antibiotic-treated mice at a mean concentration of 1.65 mg/g (wet weight) of cecal contents.

Incidence and fecal population level. The incidence of fecal carriage and population levels of *S. typhimurium* in the feces of streptomycin-treated and untreated mice, challenged with 10^3 CFU of the pathogen, were monitored for 2 weeks (Table 1). The pathogen was detected in 90% of the fecal samples from streptomycin-treated mice on day 1 postchallenge and in 100% of the samples during the remainder of the experimental period. The geometric mean of approximately 1.5×10^3 CFU/g (wet weight) of feces on day 1 after challenge increased to 10^6 on day 4 postchallenge and remained at this population level for the duration of the experimental period. By contrast, *S. typhimurium* was not detected in any of the fecal samples from untreated mice.

Translocation study. The ability of *S. typhimurium* to cause systemic infection was examined next. Streptomycin-treated and untreated mice were challenged with approximately 10^3 CFU of *S. typhimurium*. The population levels of *S. typhimurium* in the cecum, ileum, liver, spleen, and mesentery were determined 7 days after challenge (Table 2). Although *S. typhimurium* was detected in the organs of all treated mice, none of the untreated mice harbored detectable levels of the organism.

TABLE 2. Influence of streptomycin treatment on the susceptibility of *S. typhimurium* in mice

Organ[a]	Untreated		Treated	
	Population[b]	Incidence[c]	Population[d]	Incidence[c]
Cecum	$<10^2$	0/10	8.50 ± 0.78	10/10
Ileum	$<10^2$	0/10	5.99 ± 1.66	10/10
Liver	$<10^2$	0/10	4.67 ± 1.16	10/10
Spleen	$<10^2$	0/10	5.36 ± 1.13	10/10
Mesentery	$<10^2$	0/10	5.52 ± 0.77	10/10

[a] Challenge dose; 10^3 viable organisms.
[b] Viable organisms per gram of sample.
[c] Proportion of mice harboring *S. typhimurium*.
[d] Mean $\log_{10}$ viable counts (± standard deviation) per gram (wet weight) of sample.

Dynamics of colonization. The dynamics of *S. typhimurium* infection in streptomycin-treated and untreated mice was examined with inocula of 10^3, 10^5, and 10^8 viable cells. The population levels of *S. typhimurium* in the intestinal and extraintestinal organs were determined for periods up to 72 h after challenge. The results of the experiment with 10^3 CFU of *S. typhimurium* are illustrated in Fig. 1. There was a gradual increase in *S. typhimurium* counts in various organs of streptomycin-treated mice over a 72-h period. High population levels were reached, especially in the cecal contents, by 72 h after challenge (Fig. 1a). The incidence of translocation was 50% in these animals. On the other hand, *S. typhimurium* was not detected in the intestinal contents of the untreated mice from 12 to 72 h after challenge (Fig. 1b), although small numbers of the organism persisted in the cecal and ileal tissues up to 72 h after challenge. *S. typhimurium* was detected in the liver of one of these mice at 12 h after challenge and in the spleen of another mouse at 72 h after challenge. When the challenge dose was increased to 10^5 CFU (Fig. 2), about a 100-fold drop in the *S. typhimurium* count was observed in the ileal tissue and contents 12 h after challenge of both streptomycin-treated and untreated animals. This decrease was temporary, however, as an increase in viable *S. typhimurium* count subsequently occurred. *S. typhimurium* was present in the extraintestinal organs of 50% of streptomycin-treated animals 2 h after challenge. By 72 h, the incidence increased to 100%. In contrast, with the exception of one mouse which harbored *S. typhimurium* in the liver at 48 h after challenge, the pathogen was not detected in the extraintestinal organs of untreated mice (Fig. 2b). When the challenge dose was increased to 10^8 organisms (Fig. 3), translocation of *S. typhimurium* into the extraintestinal tissue occurred within 2 h after challenge of both treated and untreated mice. The pathogen was not detected in the liver, spleen, or mesentery 12 h after challenge of untreated mice but was present again at 24 h. Its population increased thereafter for the duration of the experiment (Fig. 3b).

The colonization dynamics of *S. typhimurium* in the cecum of untreated mice was different than that observed in streptomycin-treated mice (cf. Fig. 3a and b). However, the colonization pattern in the ileum of the untreated mice was similar to that observed in the treated mice, with an initial reduction in the *S. typhimurium* population, followed by an increase. The population of *S. typhimurium* in the cecum of untreated mice remained low after the initial decrease but persisted at a high level in streptomycin-treated animals.

DISCUSSION

The increased susceptibility to *S. typhimurium* infection after streptomycin administration was clearly demonstrated

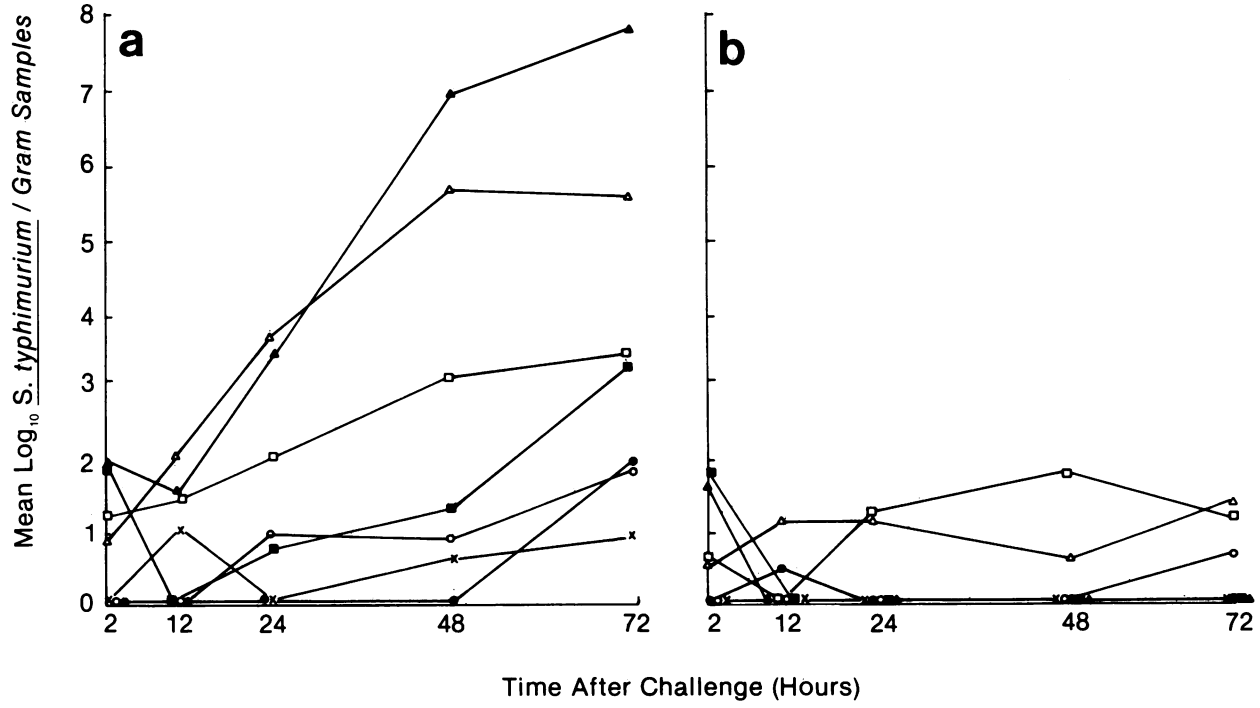

FIG. 1. Colonization dynamics of *S. typhimurium* in mice challenged orogastrically with 10^3 CFU. (a) Streptomycin-treated mice; (b) untreated mice. Each point represents the mean $\log_{10}$ CFU per gram of sample from four mice. Symbols: ●, liver; ○, spleen; ×, mesentery; ▲, cecal contents; △, cecal tissue; ■, ileal contents; □, ileal tissue.

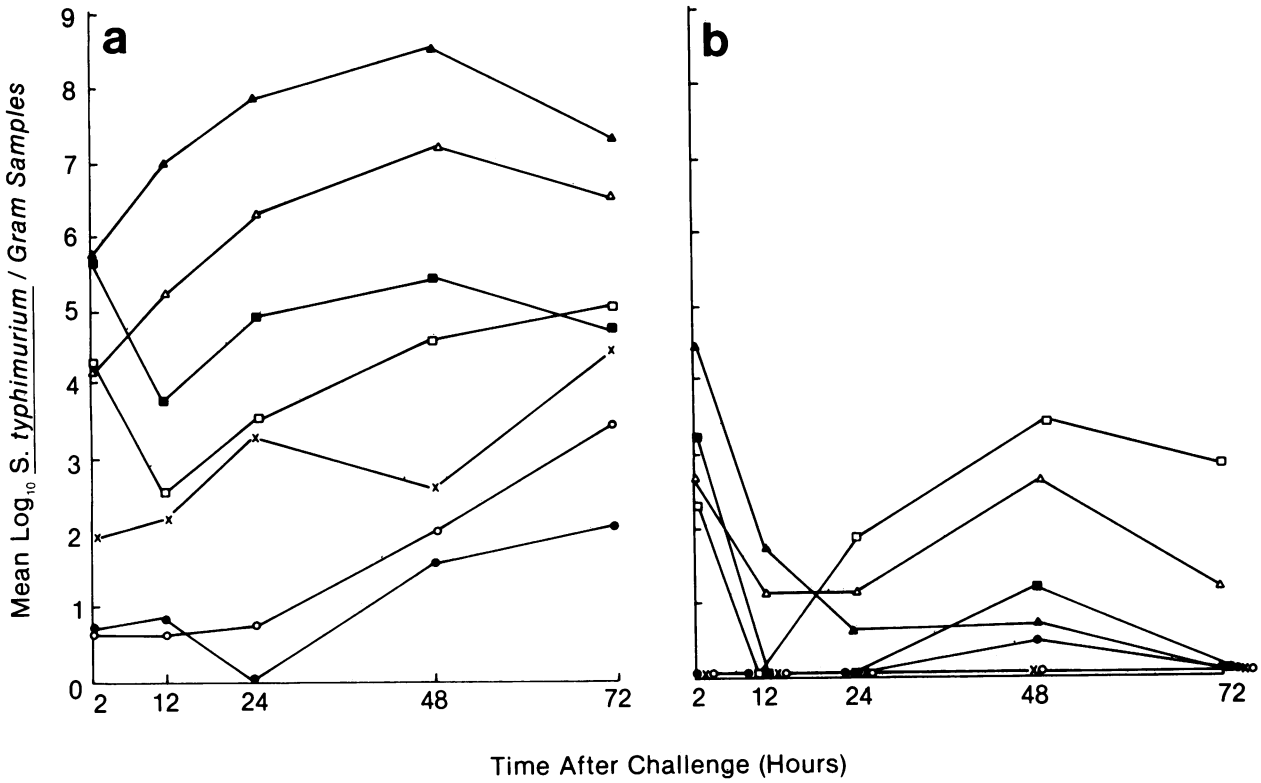

FIG. 2. Colonization dynamics of *S. typhimurium* in mice challenged orogastrically with 10^5 CFU. (a) Streptomycin-treated mice; (b) untreated mice. Each point represents the mean $\log_{10}$ CFU per gram of sample from four mice. Symbols: ●, liver; ○, spleen; ×, mesentery; ▲, cecal contents; △, cecal tissue; ■, ileal contents; □, ileal tissue.

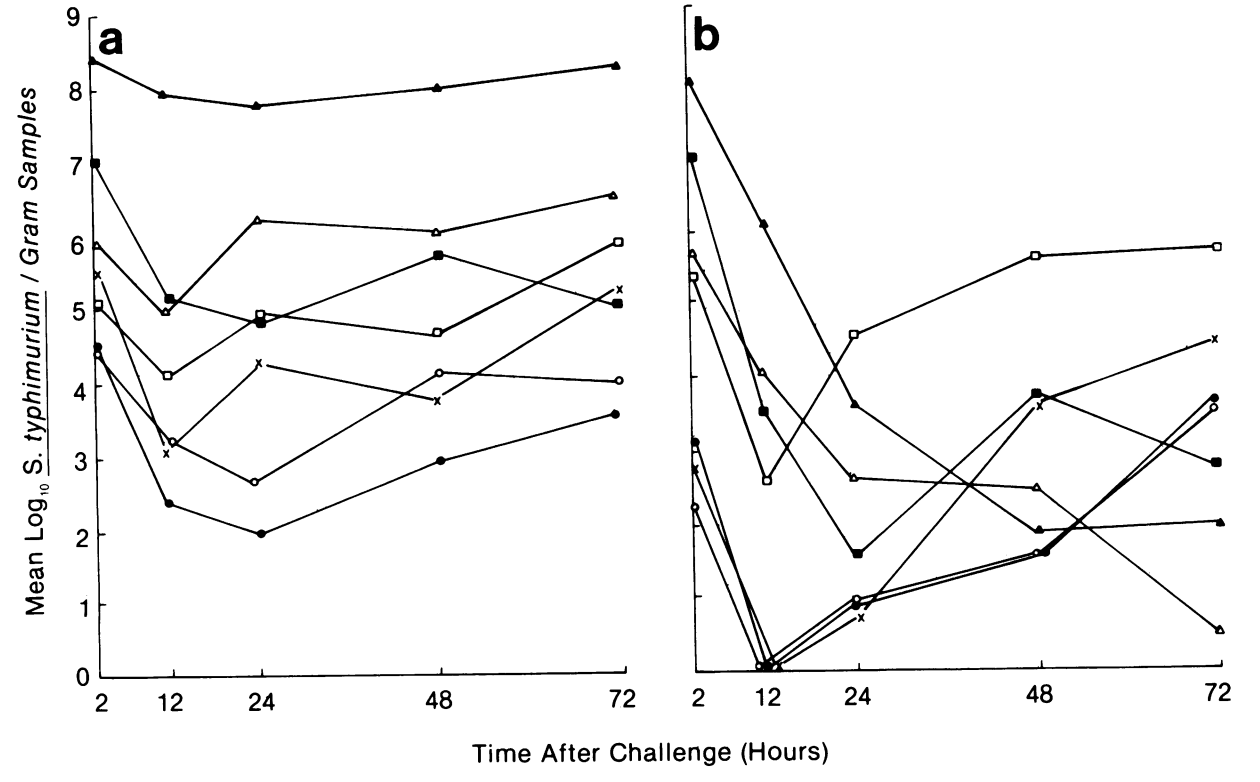

FIG. 3. Colonization dynamics of *S. typhimurium* in mice challenged orogastrically with 10^8 CFU. (a) Streptomycin-treated mice; (b) untreated mice. Each point represents the mean $\log_{10}$ CFU per gram of sample from four mice. Symbols: ●, liver; ○, spleen; ×, mesentery; ▲, cecal contents; △, cecal tissue; ■, ileal contents; □, ileal tissue.

in this study and agrees with the findings of early investigators (5). Several hypotheses have been proposed to explain the mechanism(s) by which exogenous microorganisms are removed from the ecosystem of the gastrointestinal tract. The results of our study indicate that *S. typhimurium* is able to multiply more rapidly in the gastrointestinal tracts of streptomycin-treated mice than in those of untreated mice. Although the pathogen was found to be attached to the intestinal tissue of 50% of untreated mice 72 h after challenge with 10^3 CFU of *S. typhimurium* (Fig. 1b), it was not detected in the intestinal contents of these animals, suggesting rapid elimination of the unattached organisms. In contrast, *S. typhimurium* gradually increased in numbers in the intestinal contents of streptomycin-treated mice, with a subsequent increase in the numbers of attached bacteria. Presumably, the pathogen is eliminated from the intestinal tract of untreated animals because of its inability to multiply at a rate exceeding the washout rate (1, 11, 14). This is not the case with streptomycin-treated mice, however, in which the multiplication rate of *S. typhimurium* exceeds its washout rate.

During the course of infection, a temporary drop in *S. typhimurium* counts in the ileal tissues of both streptomycin-treated and untreated mice was observed at 12 h postchallenge (Fig. 2 and 3). This decrease in counts may be a reflection of a required period of adaptation by the pathogen to different environmental conditions. Upon adaptation, the bacteria multiply rapidly, hence, the observed increase in population counts.

Our data suggest that streptomycin treatment decreases the washout rate of *S. typhimurium* from the ileum of mice. Larger populations of *S. typhimurium* in the ileal tissue and contents of streptomycin-treated mice than in untreated mice (Fig. 2) at 2 h after challenge with 10^5 CFU support this hypothesis. A higher washout rate would reduce the amount of contact between the pathogen and the attachment site, resulting in fewer tissue-associated *S. typhimurium* organisms. Similar population levels of the pathogen in ileal contents and tissue of treated and untreated mice (Fig. 3) at 2 h after challenge with 10^8 organisms indicate that a very high challenge dose may offset the higher washout rate in the untreated mice, so that a considerable amount of contact occurs between the bacteria and the adhesion sites. It is also possible, however, that the greater *S. typhimurium* population in the ileum of the treated mice is a reflection of an increased facility of the pathogen to attach to ileal tissue sites in the absence of competing flora components. When large challenge doses of *S. typhimurium* (10^8) are used, tissue sites in treated as well as untreated mice are occupied because the pathogen overwhelms the competitive advantage of indigenous bacteria by sheer numbers. However, the strikingly greater *S. typhimurium* populations in the ceca of streptomycin-treated mice, regardless of challenge dose, indicate that the inhibitory environment existing in the contents of untreated animals has been moderated by antibiotic administration, resulting in an increased multiplication rate.

The initial event in the infectious process produced by pathogenic bacteria is the attachment of the microorganism to the mucosal surface (14). Several investigators have proposed that competition for adhesion sites plays a role in colonization resistance. Bibel et al. (4) have shown that prior adherence to an epithelial cell by one bacterium may interfere with the colonization of bacteria that subsequently enter the area. Davidson and Hirsh (10) have used K-88 antigens for in vivo bacterial competition with enteropathogenic *Escherichia coli*. It is probable that competition for attachment sites is involved in the exclusion of some nonindigenous bacteria by intestinal flora components. Our data, however, indicate that although competition for attachment sites may play a role in colonization resistance against *S. typhimurium*, it does not appear to be the major mechanism involved.

A more plausible explanation for the difference in susceptibility to *S. typhimurium* infection between streptomycin-treated and untreated mice is the apparent environmental changes that occur in intestinal contents when the animals are treated with streptomycin. Presumably, disruption of flora components by the antibiotic results in an environment less hostile for *S. typhimurium*, resulting in a multiplication rate which exceeds the washout rate. It is conceivable that the hostile environmental conditions are the consequence of the activity of inhibitory factors in intestinal content (6, 13, 16–18, 20), which moderates the growth of the pathogen, to allow for its removal by intestinal motility. We are currently examining the effect of streptomycin administration on flora composition and environmental conditions in mouse intestines to resolve this question.

S. typhimurium is an invasive organism that produces systemic infections in mice. Our results show that when a large challenge dose (10^8 CFU) is used, translocation of the pathogen from the intestinal tract occurs in untreated as well as treated mice within 2 h after challenge (Fig. 3b). The translocation of the pathogen in the untreated mice early during the course of infection may have allowed for its survival and subsequent reinfection of the gut by reseeding (9). On the other hand, when a small inocula (10^3 CFU) of *S. typhimurium* is used, the pathogen is found in the extraintestinal organs of treated (Fig. 1a) but not untreated mice (Fig. 1b). This appears to be the consequence of the retention of *S. typhimurium* in the intestinal contents of the treated mice (cf. Fig. 2a and b). The inability of the pathogen to multiply rapidly enough in the intestinal contents of untreated mice appears to be the key factor responsible for resistance against infection with *S. typhimurium*. The mechanisms responsible for colonization resistance are unknown. Clearly, additional studies need to be done in an effort to identify the protective factors in intestines.

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