INFECTION AND IMMUNITY, Dec. 1982, p. 948–952
0019-9567/82/120948-05$02.00/0
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Vol. 38, No. 3

Innate Resistance of Mice to *Salmonella typhi* Infection

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Received 1 April 1982/Accepted 18 August 1982

The basis for the natural resistance of mice to *Salmonella typhi* was examined. In contrast to *Salmonella typhimurium*, the virulence of *S. typhi* for mice was independent of the mouse strain and was not affected by inactivation of murine macrophages with silica. However, mice were more susceptible to *S. typhi* when given iron alone or iron and an iron chelator. The results suggest that the failure of *S. typhi* to undergo net growth in murine tissues reflects an inability of the bacterium to multiply rather than rapid killing by resident macrophages.

Salmonella typhi, the agent of human typhoid, fails to cause disease in conventionally raised or germfree outbred mice challenged orally or parenterally with massive doses ($>10^9$ orally; 10^7 to 10^8 parenterally) of bacteria (1–4). Moreover, inbred C57BL/6J and B6D2 are also *S. typhi* resistant (1). However, a lethal *S. typhi* infection can be produced if mice are challenged intraperitoneally (i.p.) with moderate doses ($>10^3$) of mucin-suspended organisms, but deaths are believed to occur from the toxic effects of the endotoxin associated with the rapidly expanding peritoneal population of *S. typhi* (11, 20, 23). The failure of *S. typhi* to undergo net replication in reticuloendothelial system tissues of the murine strains tested to date (1, 4) suggests a genetically conferred intraspecies basis for *S. typhi* resistance. By contrast, mice challenged with *Salmonella typhimurium* or *Salmonella enteritidis* develop a disease similar in its pathogenesis to human typhoid fever (2, 3, 9). Furthermore, inbred strains of mice exhibit a differential susceptibility to *S. typhimurium* infection (16), and three distinct murine genetic loci have been identified which regulate this dose-dependent response to *S. typhimurium* (12). *Ity*, the first *S. typhimurium* response gene discovered, is located on mouse chromosome 1, and two allelic forms of it have been described (14, 18, 19). Mice which carry the Ity^s allele are susceptible to *S. typhimurium* (parenteral 50% lethal dose [LD_{50}] < 20) and display rapid, unrestricted net bacterial growth in their spleens and livers (18, 19). Conversely, Ity^r animals are *S. typhimurium* resistant (parenteral $LD_{50} > 10^3$) and can curtail in vivo *S. typhimurium* multiplication (18, 19). The two other genes, Lps^d and *xid*, are mutant alleles on chromosome 4 and the X-chromosome, respectively, which render mice susceptible to *S. typhimurium* (13, 15). The Lps^d allele also confers endotoxin hyporesponsiveness (24) on mice which are homozygous at that locus (e.g., C3H/HeJ mice), whereas the *xid* allele mediates a selective X-linked B-cell dysfunction (22).

The purpose of this study was to analyze the basis for the intraspecies resistance of mice to *S. typhi*. Since net growth of a bacterium in animal tissues reflects a positive balance between cell division and cell death, the rapid decline in viable counts of *S. typhi* in spleens and livers of intravenously (i.v.) infected mice as observed by Carter and Collins (1) could indicate (i) that the bacteria are rapidly inactivated by resident macrophages within the murine reticuloendothelial cell system, a tenet suggested by Collins and Carter (4); (ii) that the microbes are killed by a substance in the extracellular milieu; (iii) that the organisms multiply poorly, if at all, in murine tissues, possibly because they fail to acquire essential nutrients from host tissues; or (iv) a combination of these factors. Each of these proposals was examined. The findings suggest that *S. typhi* are unable to obtain sufficient levels of iron from the murine host for bacterial growth.

MATERIALS AND METHODS

Mice. Animals used in the investigation included outbred CD-1 (Ity^r genotype) and inbred BALB/c (Ity^s genotype), obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and inbred C3H/HeJ (Lps^d genotype) mice, supplied by Jackson Laboratory, Bar Harbor, Maine. Only female mice, 6 to 8 weeks of age, were used.

Bacteria. Animals were infected with either the Quailes strain of *S. typhi* (*S. typhi* QS), a strain previously used as the challenge organism in the evaluation of the efficacy of several typhoid vaccines (6), or *S. typhimurium* TML, a strain originally isolated from a patient with salmonellosis (5). Both orga-

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TABLE 1. Virulence of *S. typhimurium* TML and *S. typhi* QS for mice of different genotypes

Mouse strain	*S. typhimurium* genotype	Treatment	Route of bacterial challenge	LD_{50}[a]	
				S. typhimurium	*S. typhi*
C3H/HeJ	*Lps*^{d}	Diluent	i.p.	<2	3×10^8
		Diluent	i.v.	<2	3×10^8
		Silica	i.v.	ND[b]	3×10^8
		Iron	i.p.	ND	2×10^{7}*
		Iron plus DHB[c]	i.p.	ND	4×10^{5}*
BALB/c	*Ity*^{s}	Diluent	i.p.	<2	3×10^8
		Diluent	i.v.	3	3×10^8
		Silica	i.v.	ND	3×10^8
		Iron	i.p.	ND	4×10^{7}*
		Iron plus DHB	i.p.	ND	1×10^{6}*
CD-1[d]	*Ity*^{r}	Diluent	i.p.	6×10^4	3×10^8
		Diluent	i.v.	4×10^5	3×10^8
		Silica	i.v.	<10*	3×10^8
		Iron	i.p.	7×10^{1}*	3×10^8
		Iron plus DHB	i.p.	<2*	2×10^{6}*

[a] The LD_{50} was estimated by the Miller-Tainter method (10) from 28 days of cumulative mortality data. Asterisks indicate that the values were statistically significantly different (95% confidence limits did not overlap) from the appropriate diluent-treated controls.

[b] ND, Not done.

[c] DHB behaves as an iron chelator for *S. typhimurium* (7). The LD_{50} of *S. typhi* for all strains of mice given DHB without added iron was 3×10^8.

[d] The genotype of these outbred mice has not been confirmed by backcross linkage analysis, but the animals are phenotypically *Ity*^{r}, i.e., they control early net *S. typhimurium* replication in their spleens and livers.

nisms were grown overnight at 37°C in 50-ml shake cultures of Penassay broth (Difco Laboratories, Detroit, Mich.).

Lethal dose studies. Overnight cultures (~2×10^9 organisms per ml) of the bacteria were serially diluted in sterile saline (0.85% NaCl). The actual number of viable organisms in the challenge inoculum was assessed by plate counts on tryptic soy agar (Difco Laboratories). Groups of five mice were infected i.p. or i.v. with graded doses of bacteria suspended in 0.5 ml of saline. In some studies, mice were treated with different substances before infection. These materials included: (i) silica (0.17 mg per g of mouse) prepared as previously described (16) and given i.v. 24 h before i.v. bacterial challenge; (ii) 0.32 mg of Fe^{3+} per g of mouse given i.p. (as $FeCl_3$ in 10^{-4} N HCl) in solution 4 h before i.p. salmonella injection; (iii) 2,3-dihydroxybenzoic acid (DHB; Aldrich Chemical Co., Milwaukee, Wis.) (0.5 mg in sterile 0.85% NaCl [saline]) given i.p. four times per day on day 1, three times on the day of bacterial challenge and iron administration, and twice daily thereafter for 10 days; (iv) human serum (0.3 ml) given i.p. once daily on days 1 through 10 of infection; (v) a lysate of human peripheral leukocytes (kindly supplied by William Biddison, National Institutes of Health) prepared by a 30-s sonic oscillation of a saline suspension with 2.6×10^6 cells per ml and given i.p. once daily in 0.2-ml aliquots on days −1 through +2 of infection. None of these treatments were toxic for uninfected control mice. The LD_{50} of *S. typhi* or *S. typhimurium* was estimated graphically by the method of Miller and Tainter (10) from 28 days of cumulative mortality data. This mathematical procedure was selected so that 95% confidence limits on the LD_{50} could be determined.

RESULTS AND DISCUSSION

Two approaches were used to test whether *S. typhi* fails to cause progressive systemic disease in mice because the microbe is promptly killed by resident reticuloendothelial system macrophages. First, mice (*Lps*^{d} and *Ity*^{s}) which are unable to prevent rapid replication of *S. typhimurium* in their splenic and hepatic tissues were tested for *S. typhi* susceptibility after i.v. and i.p. challenge. Since the *S. typhimurium* susceptibility of both *Lps*^{d} and *Ity*^{s} animals appears to involve defective macrophage containment of *S. typhimurium* growth, albeit by distinct mechanisms (12, 13), it was reasoned that such deficiencies might enhance the sensitivity of these mice to *S. typhi*. However, neither *Lps*^{d} nor *Ity*^{s} mice were susceptible to *S. typhi* (Table 1). The *S. typhi* resistance of BALB/c mice (*Ity*^{s}) supports the finding of Carter and Collins that *Ity*^{s} mice of the C57BL/6J strain are refractory to *S. typhi* challenge (1). A second method used to test the involvement of macrophages in murine resistance to *S. typhi* was to treat mice with silica, an agent which is toxic for macrophages (16). As previously demonstrated with *S. typhimurium*-resistant (CBA/N × DBA/2N)F_1 female mice (16), this procedure rendered outbred CD-1 mice both *S. typhimurium* sensitive (Table 1) and unable to contain splenic growth of the organism (Fig. 1). Administration of silica did not alter the *S. typhi* resistance of *Lps*^{d}, *Ity*^{s}, or

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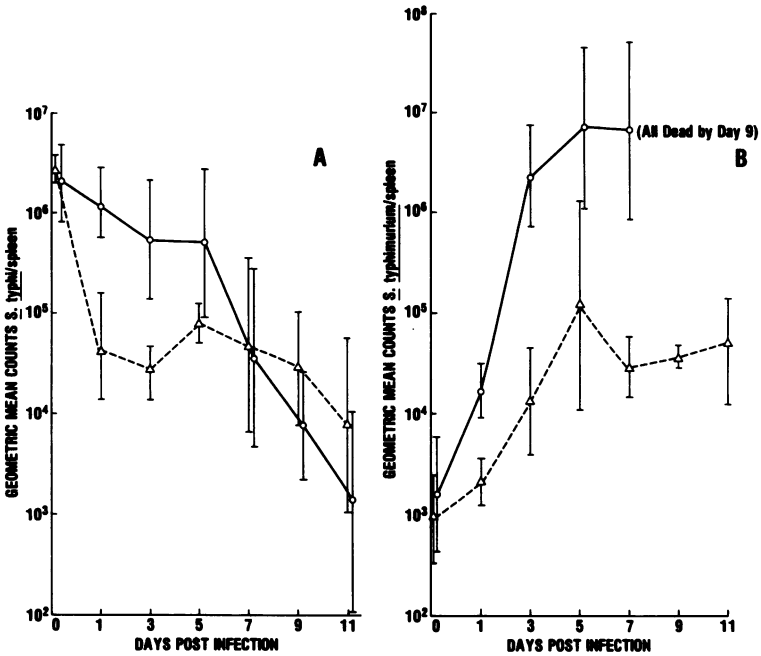

FIG. 1. Geometric mean counts (± two standard errors) of *S. typhi* (A) or *S. typhimurium* (B) in silica-treated mice. Groups of 45 CD-1 mice were given 4.5 mg of silica (○) or diluent (△) 24 h before i.v. challenge with 10^4 *S. typhimurium* or 10^8 *S. typhi*. At various intervals thereafter, six animals per group were sacrificed, and the salmonellae in their spleens were enumerated by plate counts. Statistically significant differences ($P < 0.05$ by Student's nonpaired *t* test) between the diluent- and silica-treated groups occurred at 1 and 3 days in (A) and at day 1 and thereafter in (B).

CD-1 mice (Table 1), nor did the substance prevent the clearance of *S. typhi* from spleens of CD-1 mice infected i.v. (Fig. 1). The results of these experiments suggest that the resistance of mice to *S. typhi* is not solely a consequence of a macrophage-dependent microbicidal mechanism.

To test the possibility that *S. typhi* is killed extracellularly by a humoral substance, the replication of the bacterium in fresh CD-1 and C3H/HeJ mouse sera was compared with that of *S. typhimurium*. Samples (30 μl) of saline-suspended bacteria at 4×10^5 organisms per ml were mixed with 0.27 ml of the CD-1 serum or 0.27 ml of the C3H/HeJ serum or saline and incubated at 37°C, and aliquots were taken for colony counts at various intervals. Neither microbe replicated in saline, but both strains multiplied equally well in mouse sera (Table 2). Thus, *S. typhi* is probably not inactivated extracellularly in the murine host if fresh serum is representative of that environment.

Lastly, the hypothesis that *S. typhi* cannot multiply within murine tissues because the environment is nutritionally inadequate was examined. Since the relationship between the capacity of a microbe to obtain iron in vivo and its virulence is well documented (17), the effect of iron administration on the LD_{50} of *S. typhi* for mice of different genotypes was determined.

TABLE 2. Growth of *S. typhimurium* TML and *S. typhi* QS in fresh normal mouse sera

Organism	Bacteria suspended in	Log_{10} geometric mean viable counts/ml ± 2 SE at time[a]			
		0 h	2 h	4 h	24 h
S. typhimurium	CD-1 serum	4.55 ± 0.18	4.75 ± 0.35	6.55 ± 0.18	9.20 ± 0.25
	C3H/HeJ serum	4.78 ± 0.14	5.43 ± 0.14	6.71 ± 0.32	9.41 ± 0.09
	Saline	4.64 ± 0.29	4.37 ± 0.17	4.36 ± 0.16	5.10 ± 0.5
S. typhi	CD-1 serum	4.30 ± 0	4.53 ± 0.29	5.73 ± 0.37	9.43 ± 0.73
	C3H/HeJ serum	4.58 ± 0.21	5.49 ± 0.13	6.04 ± 0.18	9.91 ± 0.08
	Saline	4.47 ± 0.17	4.47 ± 0.17	4.49 ± 0.10	4.35 ± 0.19

[a] Calculated from triplicate samples.

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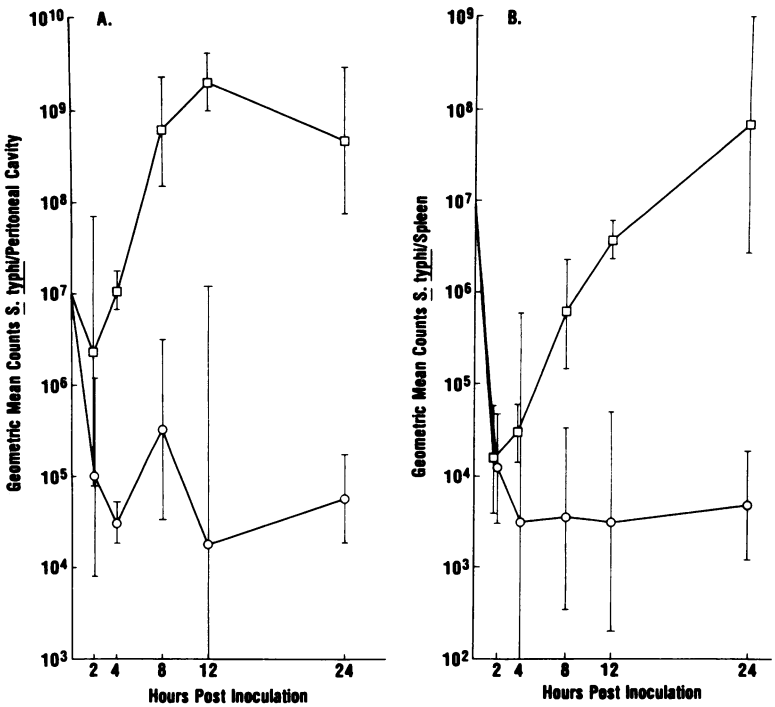

FIG. 2. Geometric mean counts (± two standard errors) of *S. typhi* in the peritoneum (A) or spleen (B) of saline-treated (○) or DHB- and iron-treated (□) C3H/HeJ mice after i.p. challenge with 10^7 bacteria. Mice in the DHB-plus-iron group were given four i.p. doses of DHB (0.5 mg each) at 2-h intervals the day before infection followed by two i.p. doses of DHB and one i.p. dose of iron (0.32 mg) on the day of bacterial challenge. *S. typhi* was administered 2 h after iron inoculation of the animals. Saline-treated mice received an equal number of inoculations. At various intervals after *S. typhi* infection, three animals per group were sacrificed, the contents of their peritoneal cavities were obtained by saline lavage, and their spleens were washed with saline and homogenized. Viable bacteria in the peritoneal lavages and spleen cell suspensions were enumerated by plate counts.

Although such treatment dramatically enhanced the virulence of *S. typhimurium* for CD-1 mice (Table 1), a finding consistent with the observations of other investigators (7, 8, 21), the LD_{50} of *S. typhi* was not altered for CD-1 mice and was only slightly (albeit statistically significantly) reduced for C3H/HeJ or BALB/c animals. However, combined treatment of mice with a single dose of iron and multiple doses of DHB, a substance reported to act as an iron chelator for *S. typhimurium* (7), permitted systemic replication of *S. typhi* (Fig. 2) and decreased the LD_{50} of *S. typhi* 150-fold for CD-1 mice, 300-fold for BALB/c mice, and 750-fold for C3H/HeJ animals (Table 1). The regimen did not kill uninfected control mice. Further attempts to increase the virulence of *S. typhi* for CD-1 mice by administration of human serum or a leukocyte extract were not successful (LD_{50} remained 3×10^8). The rationale for these latter experiments was that nutrients necessary for the growth of *S. typhi* must be available either in human cells or serum since *S. typhi* can replicate in the human host. Overall, the data suggest that the murine environment is inadequate to support multiplication of *S. typhi* and implicate iron as at least one nutrient in insufficient supply to the microbe. That other nutritional deficiencies may also exist is indicated by the failure to render mice highly susceptible to *S. typhi* even when the animals were treated with both iron and a microbial iron chelator.

ACKNOWLEDGMENTS

This work was supported by the Uniformed Services University of the Health Sciences grant no. R07313.

I thank Thomas Lively for his excellent technical assistance and Bruce Stocker (Department of Medical Microbiology, Stanford University) and Harry Smith (Department of Microbiology, University of Birmingham, Birmingham, England) for their helpful discussions.

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