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Salmonella typhimurium Virulence in a Burned-Mouse Model

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Various features of salmonellosis were examined in a burned-mouse model. In this model, which uses an outbred mouse strain, a challenge dose of ca. 100 CFU with any of several strains of *Salmonella typhimurium* caused a fatal infection. A variety of mutated strains attenuated for virulence in *Salmonella*-susceptible parenterally infected mice were also attenuated in the burned-mouse model. When administered as live vaccines injected intraperitoneally the same attenuated strains provided between slight and complete protection against subsequent lethal challenge subcutaneously at the site of a burn. The correspondence of results obtained in the burned-mouse model with those seen in other mouse models coupled with the unique advantages of the burned-mouse model argue for the usefulness of the model in studies of salmonellosis and in testing of strains constructed for use as live vaccines.

Salmonella spp. are a major cause of disease among various mammalian and avian hosts (35). Basic studies of bacteriological (35) and immunological (16) facets of salmonellosis, as well as the assessment of live vaccines for immunization of humans (32) and domestic livestock, e.g., cattle (30), traditionally use parenterally infected mice (9, 25). Conflicting results in such studies can be attributed to the genetically determined differential susceptibility of inbred mice to *Salmonella typhimurium* (27, 28), which frequently necessitates the use of costly inbred strains of mice. Furthermore, the assessment of virulence in parenterally infected mice often entails long periods of animal observation, large numbers of mice when 50% lethal doses are determined, and time-consuming measurements, e.g., viable counts of infected organs.

We noted in a study of flagella as virulence factors of *S. typhimurium* (4, 34) that the results obtained with parenterally administered *S. typhimurium* in C57BL/6J mice (*Salmonella* susceptible) were identical to those obtained in a preliminary unpublished study (M. Carsiotis, R. K. Chan, and I. A. Holder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B78, p. 31) in which outbred mice were used in a burned-mouse model (BMM; 31). This observation prompted us to explore the use of the BMM for the study of several facets of salmonellosis.

We report here the increased susceptibility to *S. typhimurium* following burn injury and the ability of the BMM to detect attenuated virulence in several mutant strains as well as in a strain with an apparent deletion of a hitherto-undetected chromosomal virulence gene. The BMM also proved useful in assessing the immunizing ability of these attenuated strains.

MATERIALS AND METHODS

Bacterial strains and media. The strains of *S. typhimurium* used (Table 1) are, with the exception of strains RIA and SR-11, derivatives either of strain LT2, which is known to produce variable results when tested for mouse virulence, or of three fully virulent strains (for brevity, indicated as FIRN, WRAY, and UCD) which have been used as parents of aromatic-dependent live-vaccine strains (13, 30). Bacteria were grown overnight in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C with shaking and diluted appropriately in saline to provide the challenge dose and to determine viable counts.

Mouse experiments. The mice used were 22- to 24-g female Crl:CF-1 BR non-Swiss, an outbred strain. A brief description of the BMM follows. A partial-thickness nonlethal burn was made on the back of an anesthetized mouse. The challenge dose (0.5 ml) was injected subcutaneously (s.c.) into the burn site. Deaths were recorded over the following 10 days. Burned mice injected s.c. with 0.5 ml of saline at the burn site recovered completely. Normal, i.e., unburned, mice were vaccinated or challenged by intraperitoneal (i.p.) injection.

Genetic methods. The use of phage P22HT12/4 *int-3* for transduction and nutrient agar gelatin for isolation of the motile strain SL488-1 as well as for construction of strains St36 and St38 has been described previously (4). All other mutant alleles to be tested for an effect on behavior in the BMM were introduced by transduction with phage P22HT105/1 *int* grown on LT2 as a donor.

RESULTS AND DISCUSSION

Virulence of *S. typhimurium* in the BMM. Previous studies of mouse typhoid used a number of strains of *S. typhimurium*, including wild-type strain LT2. The extensive information of its physiology and genetic manipulativeness (24) make it an ideal parental strain in which to study the effect of genetic modifications on virulence. Published reports differ as to the degree of virulence in mice of parenterally injected LT2 (12, 14); a 10^4-fold difference was found in the 50% lethal doses of two isolates of LT2 in the same mouse strain (12). These variations were probably due to the previous history of the maintenance of the strain or undetected mutations which affected virulence (3, 33). In an exploratory experiment on the possible effect of the presence or absence of flagella on the virulence of *S. typhimurium* in the BMM, the strain of LT2 used did not kill when 3×10^6 CFU were injected i.p. into normal, outbred mice, but 350 CFU of the same strain killed when injected s.c. at the site of the

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TABLE 1. *S. typhimurium* strains

Strain	Description	Source and/or reference(s)
LT2	Prototroph	J. C. Loper; 24
LT2-22	Spontaneous deletion in LT2; Δ*hisBH22*	J. C. Loper; 11
ST1	Prototroph selected for good serine taxis from the LT2 strain of B. Ames	1
SL488	Spontaneous deletion in LT2; Δ*flgABCDE25* (formerly *flaF25*)	17, 34
SL488-1	Motile transductant of SL488	This study
RIA	Mouse nonvirulent	29
SR-11	Mouse virulent	3, 12, 29
SL3201	Mouse virulent; ColE1-30; *leu-1051 cysI1173 hisC527*(Am); FIRN biotype	13
St36	Δ*flgABCDE25 zcd-907*::Tn*10* derivative of SL3201	4
St38	*flg*$^+$ *zcd-907*::Tn*10* derivative of SL3201	4
SL1344	Derived from prototrophic S2337/65; WRAY, mouse and calf virulent; *hisG46 aro*$^+$	13, 30
SL3261	Δ*aroA* derivative of SL1344	13, 30
SL1420	Prototroph; calf virulent; UCD, identical to UCD 108-11	30
SL1479	Derived from SL1420; *aroA hisC527*(Am)	30
SL5327[a]	Δ*purHD343* derivative of SL1344	B. A. D. Stocker
SL5328[a]	Derived from SL1344; *purHD*$^+$	B. A. D. Stocker
SL5329[b]	Derived from SL1344; *hisG46 galE503 bio*::Tn*10*	B. A. D. Stocker

[a] SL5327 and SL5328 are sister *met*$^+$ transductants isolated by treatment of a *metA*::Tn*10* derivative of SL1344 with phage P22HT105/1 *int* grown on LT2 Δ*purHD343*.
[b] *galE503* was introduced by cotransduction with *bio*::Tn*10*.

standard burn (Table 2). Four other strains of *S. typhimurium*, two considered virulent (SR-11 and SL3201; 3, 12, 13, 29) and one considered avirulent (RIA; 29) in other mouse models, as well as ST1, an LT2-derived variant (1), were all virulent in the BMM (Table 3). The potentiation of virulence by the burn injury parallels the results obtained with normal and burned mice infected with *Pseudomonas aeruginosa* (31). A similar potentiation was shown previously not to occur upon infection of burned mice with three gram-negative bacteria, a gram-positive bacterium, or *Candida albicans* (31). Recently, however, potentiation has been reported for *Klebsiella pneumoniae* and *C. albicans* in burned mice (7, 23).

The relatively modest challenge dose required to produce a lethal infection suggested that the BMM could be useful in studies of salmonellosis. Therefore, we tested whether results obtained in the BMM were concordant with those obtained with traditional mouse models.

Virulence of mutant strains in the BMM. A number of mutations attenuate the virulence of *Salmonella* spp. in parenterally injected mice, whereas others are without effect. Among the former are certain mutations in purine biosynthesis (2, 20, 26), *aroA* mutations (13), and *galE* mutations (10); the latter include amino acid auxotrophy mutations (12, 33). We therefore tested the effect of these mutations on virulence in the BMM.

TABLE 2. Increased susceptibility to *S. typhimurium* LT2 following burn injury

Mice	Challenge dose (CFU)	No. dead (days to death)[a]
Normal[b]	3.5×10^4	0
	3.5×10^5	0
	3.5×10^6	0
	3.5×10^7	2 (4, 4)
	3.5×10^8	2 (4, 4)
Burned[c]	3.5×10^2	2 (4, 4)

[a] Two mice were tested per dose.
[b] Unburned mice were injected i.p.
[c] Burned mice were injected s.c. at the burn site.

The virtually complete loss of virulence, as measured by i.p. injection of genetically susceptible mice, caused by the *aroA* mutation (13) was also seen when *aroA* derivatives of two virulent strains were tested in the BMM (Table 4, experiment 1). Challenge doses 100-fold higher than that constituting a lethal challenge for the virulent parental strains produced no deaths; doses 10,000-fold higher caused deaths of only a minority of the challenged mice. Attenuation by purine auxotrophy (2, 20, 26) was also evident in the BMM (Table 4, experiment 2). Strain SL5327, a Δ*purHD343* derivative of the virulent parental strain, had greatly reduced virulence in the burned mice (none of five dead from a dose of 10^3 CFU and two of five dead from a dose of 10^6 CFU). In the experiments of McFarland and Stocker (20) the corresponding derivative of a mouse-virulent strain of *S. dublin* showed only a partial loss of virulence. Perhaps the greater attenuation observed in the BMM for the one purine auxotroph tested resulted from the use of outbred mice in our experiments instead of the *Salmonella*-susceptible inbred mice used by McFarland and Stocker (20).

Mutants of *S. typhimurium* carrying *galE* and lacking UDP galactose-epimerase have greatly reduced virulence in mice, as tested by i.p. injection (35). The virulent WRAY strain of *S. typhimurium*, given the nonleaky *galE503* mutation by transduction, had greatly reduced virulence in the BMM; 10^3 CFU caused no deaths in five mice, and three of five mice survived 10^6 CFU (Table 4, experiment 3). The failure of injection at the site of a burn to restore virulence to a *galE* mutant of *S. typhimurium* contrasts with the effect of cyclo-

TABLE 3. Virulence of various *S. typhimurium* strains in the BMM

Strain[a]	No. dead (days to death)[b]
LT2	5 (2, 2, 3, 5, 5)
SR-11	5 (2, 2, 2, 2, 2)
SL3201	5 (2, 2, 2, 3, 5)
RIA	5 (3, 4, 5, 5, 6)
ST1	5 (2, 3, 3, 3, 4)

[a] Burned mice were injected s.c. at the burn site with ca. 10^2 CFU.
[b] Five mice were tested per strain.

TABLE 4. Attenuation of virulence in mutated strains the BMM

Expt	Strain	Relevant genotype	Challenge dose (CFU)[a]	No. dead (days to death)[b]
1	SL1344	*aro*$^+$	10^2	5 (2, 3, 3, 4, 4)
	SL3261	Δ*aroA*	10^4	0
			10^6	2 (2, 3)
	SL1420	*aro*$^+$	10^2	5 (2, 2, 3, 4, 4)
	SL1479	Δ*aroA*	10^4	0
			10^6	0
2	SL5328	*pur*$^+$	10^3	5 (2, 2, 2, 2)
	SL5327	Δ*purHD343*	10^3	0
			10^6	2 (2, 4)
3	SL5329	*galE503*	10^3	0
			10^6	2 (2, 5)
4	LT2-22	Δ*hisBH22*	10^2	5 (2, 3, 3, 4, 5)

[a] Injected s.c. at the burn site.
[b] Five mice were tested per dose.

TABLE 5. Attenuation of virulence in Δ*flgABCDE25* strains

Expt	Strain	Relevant genotype	Challenge dose (CFU)[a]	No. dead (days to death)[b]
1	SL488	Δ*flgABCDE25*	10^4	1 (5)
			10^6	1 (5)
	SL488-1	*flg*$^+$	10^2	5 (2, 2, 3, 3, 3)
			10^4	5 (2, 2, 3, 3, 4)
2	St36	Δ*flgABCDE25* *zcd-907*::Tn*10*	10^4	1 (10)
	St38	*flg*$^+$*zcd-907*::Tn*10*	10^2	5 (2, 2, 2, 3, 3)
			10^4	5 (2, 2, 2, 2, 3)

[a] Injected s.c. at the burn site.
[b] Five mice were tested per dose.

phosphamide reported by Morris et al. (22), who used a different *galE* strain of *S. typhimurium*; nearly all of a group of mice given cyclophosphamide died from multiplication of a normally harmless i.p. inoculum of the *galE* strain. The mechanism of the reduction of mouse virulence by the *galE* mutation is not known; the recent observation of nonreduced virulence for volunteers of a strain of *S. typhi* with a *galE* deletion and lacking Vi antigen was surprising (15). We therefore refrain from speculation on the different effects of injection at a burn site and of cyclophosphamide injection on the behavior of *galE* mutants.

Amino acid requirements do not, in general, affect the mouse virulence of *Salmonella* spp., as tested by the parenteral route in normal mice (12, 23). Correspondingly, we found that strain SL3201, a *his cys leu* mutant, and strains SL1344 and LT2-22, both *his* mutants, were fully virulent in the BMM, with no survivors of a 10^2-CFU challenge (Tables 3 and 4).

Attenuation of virulence by the Δ*flgABCDE25* mutation. The role of functional flagella in the virulence of *P. aeruginosa*, demonstrated by use of the BMM (6, 21), prompted a similar study with an isogenic pair of strains of *S. typhimurium*, SL488 and SL488-1. The LT2-derived strain SL488 contained the mutation originally designated *flaF25* (17), which rendered it nonflagellated. This mutation is now designated Δ*flgABCDE25* on the basis of a recent report of its multicistronic nature (19) and the revised nomenclature for flagellar genes (18). We shall refer to it as Δ*flg-25*. Strain SL488-1, an isogenic flagellated motile derivative of SL488, was prepared by transduction, and both strains were tested for virulence in the BMM. The nonflagellated strain, SL488, was markedly less virulent than SL488-1, its isogenic flagellated motile partner (Table 5, experiment 1). We could not use this pair of isogenic strains to compare the effect of the Δ*flg-25* mutation on virulence in the BMM with its effect in parenterally infected C57BL/6J mice, since C57BL/6J mice are highly resistant to infection by the parent strain of SL488, strain LT2. We therefore used standard genetic procedures (4) to derive an isogenic pair of strains, St36 (Δ*flg-25*, nonflagellated) and St38 (*flg*$^+$, flagellated), from another strain, SL3201. Since the parental strain SL3201 is virulent both in the i.p.-infected C57BL/6J mouse model (4, 34) and in the BMM (Table 3), we could compare the effect of the Δ*flg-25* mutation on virulence in the two models. We found that St36, the nonflagellated Δ*flg-25*-bearing strain, was considerably less virulent in the BMM than St38, its isogenic flagellated motile *flg*$^+$ partner (Table 5, experiment 2). This result agreed with our previous result (4) that the Δ*flg-25* mutation caused a marked decrease in virulence in parenterally or orally infected C57BL/6J mice. Thus, the attenuating effect of the Δ*flg-25* mutation was equally discernible in the two mouse models.

We recently reported (M. Carsiotis, B. A. D. Stocker, I. A. Holder, D. Weinstein, and A. D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B169, p. 53) that the loss of virulence in strains bearing the Δ*flg-25* mutation was not due to the nonmotile, nonflagellate character of such strains. Instead, we hypothesized that the attenuation observed in both the BMM and parenterally injected C57BL/6J mice was due to the deletion of a chromosomal virulence gene linked to the *flg* gene cluster. Experiments are in progress to test this hypothesis.

Use of the BMM to test live-vaccine strains. The administration of attenuated strains of *Salmonella* spp. as live vaccines is considered to confer much better protection against experimental or natural *Salmonella* disease than is injection of killed-bacterium vaccines, even in multiple doses (5, 8, 16). In recent years strains of *S. typhi*, *S. typhimurium*, and *S. dublin* attenuated by different mutations have been developed as candidate live-vaccine strains (8, 13, 14, 30). For practical reasons, including economy, the extent of attenuation and of safety of such live-vaccine strains has to be tested, at least in the first instance, by administration to mice, commonly by i.p. injection. Because of the incomplete and variable susceptibility of outbred mice to *Salmonella* infection, inbred *Salmonella*-susceptible mice are commonly used for this purpose. To learn whether the BMM could serve as a guide to the efficacy of attenuated strains as live vaccines, we vaccinated groups of normal mice with live bacteria of an attenuated strain of *S. typhimurium*. We chose five strains shown to be attenuated in the BMM. Three weeks after the single i.p. vaccination, the mice were burned and challenged with a 10^3- or 10^4-fold-higher-than-lethal dose of one or another of several strains shown to be virulent in the BMM. Although only one of the groups of vaccinated mice was protected completely against a lethal challenge, each of the other four groups was protected to some degree (Table 6). The less complete protection conferred by the Δ*flg-25* live vaccine than by the auxotrophic live vaccines probably resulted from the much smaller (10^2-CFU versus 10^6-CFU) immunization dose used. The smaller dose was necessitated by the less complete attenuation caused by the Δ*flg-25* mutation. Furthermore,

TABLE 6. Effect of immunization of normal mice by i.p. injection of attenuated live strains on the outcome of subsequent burn and challenge with virulent strains[a]

Expt	Immunization			Challenge		No. dead/total (days to death)
	Strain	Relevant genotype	Dose (CFU)	Strain	Dose (CFU)	
1	None			SL3201	10^2	5/5 (2, 2, 3, 3, 3)
	St36	Δ*flgABCDE25*	10^2	SL3201	10^5	7/10 (2, 2, 3, 3, 4, 6, 9)
	None			SL1344	10^2	5/5 (2, 3, 3, 4, 4)
	St36	Δ*flgABCDE25*	10^2	SL1344	10^5	5/10 (3, 6, 7, 7, 8)
	None			SL1420	10^2	5/5 (2, 2, 3, 4, 4)
	St36	Δ*flgABCDE25*	10^2	SL1420	10^5	8/10 (2, 2, 2, 2, 2, 3, 3, 4)
	None			SR-11	10^2	5/5 (2, 2, 2, 2, 2)
	St36	Δ*flgABCDE25*	10^2	SR-11	10^5	6/10 (2, 2, 2, 2, 3, 9)
	None			LT2	10^2	5/5 (2, 2, 3, 5, 5)
	St36	Δ*flgABCDE25*	10^2	LT2	10^5	6/10 (3, 3, 4, 5, 5, 5)
2	None			SL5328	10^3	5/5 (2, 2, 2, 2, 2)
	SL5327	Δ*pur*	10^6	SL5328	10^6	1/5 (2)
	SL5329	*galE*	10^6	SL5328	10^6	4/5 (2, 3, 6, 10)
3	None			SL1420	10^2	5/5 (2, 2, 3, 4, 4)
	SL1479	*aroA*	10^6	SL1420	10^6	2/5 (1, 4)
	None			SL1344	10^2	5/5 (2, 2, 4, 5, 5)
	SL3261	Δ*aroA*	10^6	SL1344	10^6	0/5

[a] Groups of 10 (experiment 1) or 5 (experiments 2 and 3) normal mice were immunized i.p. as indicated. Three weeks later they were burned and challenged s.c. at the burn site as indicated. At the time of challenge in experiments 1 and 2, groups of five nonimmunized mice were also burned and challenged s.c. at the burn site with each of the challenge strains as indicated. The data for nonimmunized mice in experiment 3 are those shown in Table 4.

since vaccination was highly effective in several cases, we infer that the burn injury at the time of challenge did not abrogate the already developed protective capacity engendered by vaccination. Consequently, we think that the nonvirulence of a candidate live-vaccine strain in the BMM argues for its probable innocuousness even if administered to a subject with reduced immunological or cellular defenses. For this reason and because it requires only small numbers of relatively inexpensive outbred mice, the BMM may be of use in the development of live-vaccine strains.

In this study we have shown that the virulence of *S. typhimurium* in the BMM closely parallels that observed in models that use inbred *Salmonella*-susceptible mice. Strains bearing a variety of mutations (certain auxotrophies, *galE*, Δ*flg-25*) were shown to exhibit comparable attenuation in the two models. The BMM also seemed to be useful in assessing the vaccine potential of attenuated live strains. We used a single stock of outbred mice for our investigation of the extent of the loss of virulence in the BMM which resulted from auxotrophy mutations and other mutations in *S. typhimurium*. We think it likely that similar results would be obtained with outbred mice from other sources, but this hypothesis would need to be investigated to validate their use in the testing of candidate live-vaccine strains. An advantage of the BMM is the virulence in it of strains, e.g., LT2 and RIA, which are of low and/or variable virulence, as tested by parenteral administration, in normal (nonburned), genetically susceptible inbred, and outbred mice. The particular advantages of the use of strain LT2 have already been cited. These features, coupled with the use of relatively inexpensive mice, the short observation period, and the readily scored endpoint (lethality), suggest that the BMM can serve many purposes in studies of salmonellosis.

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