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Development of a Murine Model of Chronic *Salmonella* Infection

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The invasive disease caused by *Salmonella typhimurium* in mice resembles the acute phase of human typhoid fever caused by *Salmonella typhi*, and experimental murine salmonellosis is a widely used experimental model for systemic salmonellosis. In this paper we demonstrate that murine *S. typhimurium* infection can also be used to model the development of the chronic carrier state that develops in humans after infection with *S. typhi*. We describe a virulent variant of *S. typhimurium* that has decreased expression of AgfA fibers under all environmental conditions studied and that causes a chronic carrier state in BALB/c mice after peroral inoculation. The chronic carrier state is associated with persistence of bacteria in the small intestine, spleen, and liver, and chronic infection continues despite the development of protective immunity to challenge with virulent *Salmonella*.

At least 2 to 5% of cases of typhoid fever in humans caused by *Salmonella typhi* are complicated by the development of a chronic carrier state (1, 3, 8). The chronic carriers are asymptomatic and do not demonstrate defects in humoral immune responses (8, 10, 17). Even though chronic carriers often have biliary tract disease, its presence is not an absolute requirement for development of the carrier state (3, 18). Although the carrier state is characterized by a persistence of organisms in the small intestine, variable numbers of bacteria are excreted in the feces (1, 3, 8, 12, 21).

Experimental murine salmonellosis is a widely used experimental model for acute systemic salmonellosis in humans because the invasive disease caused by *Salmonella typhimurium* in mice resembles the acute phase of human typhoid fever caused by *S. typhi* (17). In this paper we describe an *S. typhimurium* variant that also causes a chronic carrier state in mice after peroral inoculation, suggesting that murine salmonellosis may additionally be useful as a model for the human carrier state. The chronic infection of mice we describe is associated with persistence of bacteria in the small intestine, spleen, and liver and continues despite the development of an anti-*Salmonella* specific humoral response and despite the development of protective immunity to challenge with virulent *Salmonella.*

The *S. typhimurium* variant causing murine chronic infection has altered regulation of AgfA fiber expression. AgfA fibers are thin, aggregative organelles composed of a 17-kDa subunit protein that bind soluble fibronectin (5); they were first discovered in *Salmonella enteritidis* (6). Available nucleotide sequence data from *Escherichia coli* (11) and *S. enteritidis* (4) indicate that curli of *E. coli* and AgfA fibers of *Salmonella* are encoded by highly conserved gene clusters. In *E. coli*, the expression of curli organelles is strongly influenced by environmental conditions (2, 25). Although the role of AgfA fibers in salmonellosis is unknown, recent advances suggest that AgfA fibers of *S. typhimurium* mediate bacterial attachment to mouse intestinal epithelial cells (30).

Bacteria and manipulations. *S. typhimurium* SR-11 variants χ3181, χ4666, and χ4665 were originally isolated by Hank Lockman and Roy Curtiss III, Department of Biology, Washington University, St. Louis, Mo. Bacteria were grown in Luria agar or colonization factor antigen (CFA) agar (9) interchangeably, with ampicillin (100 μg/ml) or tetracycline (20 μg/ml) as required. To detect fiber-producing bacteria on the plates, CFA agar was supplemented with the dyes Congo red (15 μg/ml) and brilliant blue G (15 μg/ml). *S. typhimurium* SL2965 (29) was used as the source of the Tn*10* element encoding tetracycline resistance; integration of the Tn*10* element into χ4666 had no phenotypic effect, as expected (29). The sensitivity of *Salmonella* strains to lipopolysaccharide-specific bacteriophages was determined by the drop-on-lawn method on Luria agar plates (32). Transductions using the bacteriophage P22HT*int* 105 were performed essentially as described previously (28). *S. typhimurium* TT10381 (*hisD1284*::MudK *hisA9944*::MudI) (16) was used to propagate the transducing bacteriophage used for construction of the *agfA-lacZ* fusion; *agf-lacZ* was constructed by transducing the MudK insertion element containing *lacZ* into the *agf* operon, and the location of the insertion was determined by restriction mapping.

Biochemical assays. The ability of bacteria to bind ^{125}I-labeled soluble bovine fibronectin (Sigma) was measured as described by Olsen et al. (26) after bacterial growth for 48 h. Lipopolysaccharide structure was analyzed by P22 sensitivity (32) and confirmed by using proteinase K-treated whole-cell lysates electrophoresed and stained as described previously (13). β-Galactosidase activity was determined in triplicate as described by Miller (22) on bacterial cultures grown on CFA agar plates for 48 h.

For the measurement of *S. typhimurium*-specific immunoglobulin G (IgG), polystyrene microtiter plates (Dynatech Laboratories) were coated with an *S. typhimurium* sonicate (cleared by centrifugation and filter sterilized, but not otherwise fractionated) at a concentration of 7 μg/ml. *Salmonella*-specific IgG in the serum of infected mice was detected by

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TABLE 1. Phenotypic characteristics of *S. typhimurium* SR-11 strain χ3181 and its phase-locked variants

Strain[a]	Phase switch ability[b]	Colony morphology (37°C)	Result at 26°C			Result at 37°C		
			Expression of fibers on cell surface	Fibronectin binding (%)[c]	Transcription of *agfA* (U[d])	Expression of fibers on cell surface	Fibronectin binding (%)[c]	Transcription of *agfA* (U[d])
χ3181	Yes	Glistening and rugose[e]	Yes	30.6	444.9	No	1.0	9.3
χ4666	No	Rugose	Yes	26.6	686.4	Yes	47.4	1065.3
χ4665	No	Glistening	Yes	15.2	50.7	No	1.3	0.4

[a] Bacteria were grown for 48 h prior to assays.
[b] Ability to switch between glistening and rugose colonies.
[c] Percentage of total added fibronectin bound.
[d] β-Galactosidase units.
[e] Each colony is either glistening or rugose; the percentage of each type varies from experiment to experiment depending on phase switching.

using a goat anti-mouse IgG peroxidase conjugate (Sigma); ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] (Boehringer Mannheim) was used as the chromogenic substrate (31). A standard curve, generated with murine IgG (Sigma), was used to quantitate the relative concentration of IgG in the serum samples.

Mice. Six-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine) were maintained by the Division of Comparative Medicine at Washington University School of Medicine in accordance with all applicable federal guidelines. Prior to peroral inoculation, mice were deprived of food overnight and of water for 3 h; they were then fed 50 μl of 1% sodium bicarbonate followed by log-growth-phase bacteria suspended in 10 to 20 μl of phosphate-buffered saline.

Characterization of *S. typhimurium* SR-11 strains χ3181, χ4666, and χ4665. Wild-type *S. typhimurium* SR-11 strain χ3181 when grown at 37°C forms unremarkable, glistening colonies on Luria agar plates but occasionally undergoes a phase switch yielding rugose colonies; these rugose variants can back-switch at a lower frequency to the parental glistening colony morphology. The spontaneous rugose variant χ4666, isolated after repeated subculturing in Mueller-Hinton broth, has a rugose colony morphology at 37°C and is unable to back-switch to the parental glistening colony morphology. Variant χ4665, also isolated after repeated subculturing, produces glistening colonies at 37°C but is unable to switch to the rugose form. All three strains (see Table 1) are prototrophic, have a complete lipopolysaccharide structure, and carry the *Salmonella* virulence plasmid (data not shown).

Electron microscopic analysis of negatively stained samples shows that bacteria from rugose colonies of χ3181 and χ4666 produce abundant amounts of thin, aggregative fibers morphologically identical to the AgfA fimbriae of *S. enteritidis*; immunoblot analysis of whole-bacterial-cell lysates from rugose colonies reveals a 17-kDa protein reactive with antiserum against the CsgA protein of *E. coli*, while no such protein band is detectable in lysates from glistening colonies (data not shown). In addition, N-terminal sequence analysis of fibers purified by the method of Collinson et al. (6) yields a sequence identical to that of AgfA (30).

Because the ability to express AgfA fimbriae in *S. enteritidis* is associated with the ability of the bacteria to bind soluble fibronectin (5), we quantitated the ability of the *S. typhimurium* variants under various growth conditions to bind iodinated fibronectin (Table 1). Fibronectin binding by χ4665 and the parent strain, χ3181, showed a temperature dependence, although the level of binding by χ4665 was always lower than that of χ3181. Fibronectin binding by χ3181 and χ4665 could be completely inhibited by 0.1 M NaCl in the growth medium (data not shown). In contrast, fibronectin binding by χ4666 was constitutive regardless of temperature, and inhibition of binding by χ4666 could be achieved only by using 0.4 M NaCl (as expected, at this higher osmolarity, the morphology of strain χ4666 changed from rugose to glistening [data not shown]).

agfA has been shown to be cotranscribed with an upstream *agfA*-like gene named *agfB* (4); therefore, in order to help correlate fibronectin binding with transcription of *agfA* in *S. typhimurium* SR-11, we introduced a MudK-*lac* element into the cloned *S. typhimurium agfB-agfA* region and transduced the corresponding construct as a reporter into strains χ3181, χ4665, and χ4666. The β-galactosidase activity measured from χ4666 was constitutive (Table 1). In contrast, while both χ3181 and χ4665 showed temperature-dependent β-galactosidase activity, the activity from χ4665 was markedly reduced (Table 1). Even though a component of these measurements may reflect translation and the intrinsic stability of the AgfA-LacZ fusion protein itself, the strain-to-strain variation of β-galactosidase activity suggests that transcription of *agfA* is under altered regulation in strains χ3181, χ4665, and χ4666.

***S. typhimurium* χ4665 causes chronic infection in BALB/c mice.** Virulence assays with BALB/c mice demonstrated similar 50% lethal dose (LD_{50}) values between parent strain χ3181 and the variant χ4666 and only a 12-fold-higher LD_{50} for χ4665 after peroral inoculation (Table 2). Interestingly, all three variants remained equally virulent after intraperitoneal inoculation (Table 2).

As shown in Fig. 1A, mice surviving peroral infection with χ3181 or χ4666 completely cleared the infection by 5 weeks, a time course for resolution of infection that correlates well with published data demonstrating that the development of specific immunity to *S. typhimurium* in mice begins within 3 weeks after infection (19, 20, 23). In contrast, peroral infection with χ4665 resulted in prolonged colonization of the livers and spleens of surviving mice. This prolonged infection, which persisted for at

TABLE 2. Mouse virulence of χ3181 and its phase-locked variants

Strain[a]	LD_{50} after inoculation[b]		Ability to cause chronic infection
	Peroral	Intraperitoneal	
χ3181	8×10^3	<20	No
χ4666	2×10^3	<20	No
χ4665	1×10^5	<20	Yes

[a] After overnight culture in Luria broth in a tightly capped tube, the bacteria were subcultured into Luria broth and grown without aeration prior to inoculation into mice.
[b] LD_{50} determinations were performed with serial 1/2-log dilutions of bacteria, and at least five BALB/c mice per inoculation dose. LD_{50}s were then calculated by the method of Reed and Muench (27).

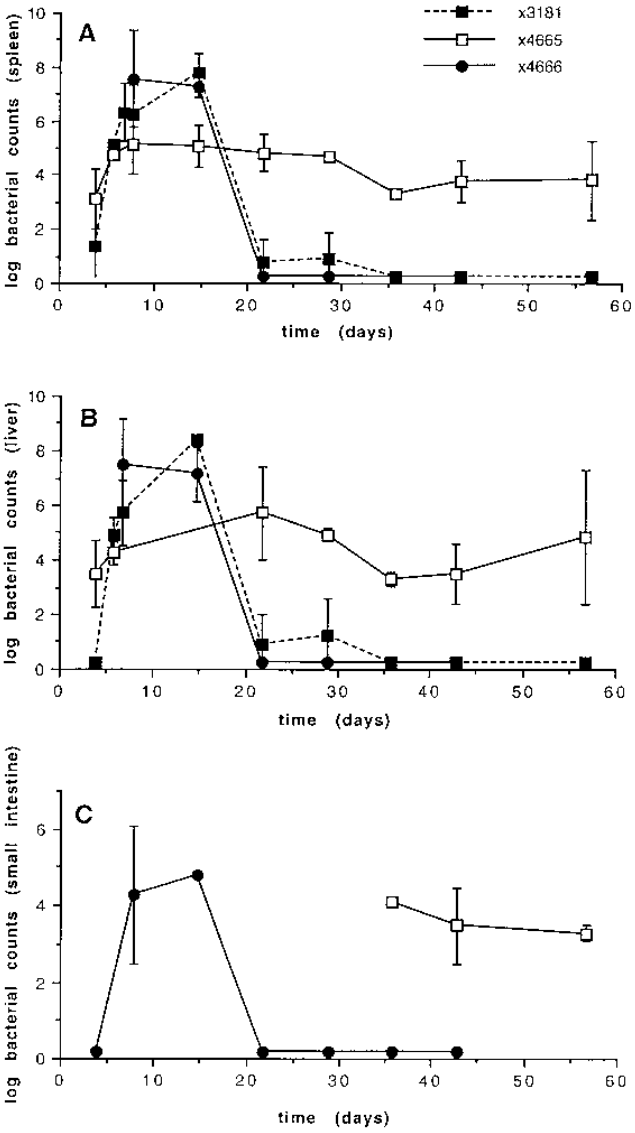

FIG. 1. Numbers of viable bacteria in the spleens (A), livers (B), and small bowel contents (C) of BALB/c mice at different time points after oral infection with the indicated *S. typhimurium* strains. Values are means for two to five mice pooled from four separate experiments. For these experiments, mice were inoculated perorally with the LD_{50} of the indicated strain (Table 1); the actual dosage was confirmed by plating serial dilutions of the inoculum. At the indicated time points, randomly chosen surviving mice were euthanized and viable counts were obtained. Bowel contents were collected from three separate portions of small intestine, each 2 cm long, by lavage with phosphate-buffered saline. Error bars represent 1 standard deviation.

least 12 weeks after inoculation (the duration of the experiment), was characterized by a plateau level of approximately 10^3 to 10^4 bacteria per organ (Fig. 1A and B), accompanied by marked splenomegaly (spleen weights up to 15 times normal). Chronic infection was never detected in mice orally infected with either χ4666 or χ3181.

Because the prolonged excretion of *S. typhi* by chronic human carriers is thought to be due to a continuous enterohepatic circulation (15, 21), we tested whether the chronic colonization of liver and spleen caused by χ4665 was associated with a prolonged presence of *S. typhimurium* in the small intestine. While neither χ3181 nor χ4666 could be recovered from the small bowel later than 2 weeks after oral inoculation (Fig. 1C and data not shown), χ4665 could be recovered for at least 12 weeks after infection (Fig. 1C). Similarly, χ4665 was occasionally recovered from the gallbladders of infected mice, while χ3181 and χ4666 never were (data not shown). Analysis of individual colonies of *S. typhimurium* recovered from the spleens and livers of mice chronically infected with χ4665 demonstrated no change in phenotype compared with the inoculating strain (data not shown).

TABLE 3. Mice chronically infected with χ4665 developed specific immunity against virulent *Salmonella*

Mouse group	No. of surviving mice/no. challenged with dose of *S. typhimurium* χ4666				
	20	200	10^3	10^4	10^5
Naive	0/3	0/3	0/3	ND[a]	ND
Chronically infected with χ4665	ND	ND	14/18	3/4	4/5

[a] ND, not done.

Chronically infected mice are immune to challenge with virulent *S. typhimurium*. We tested whether chronic disease caused by χ4665 was associated with a protective anti-*Salmonella* specific immune response. For this experiment, we inoculated mice with χ4665 perorally and then 4 weeks later withdrew a subgroup of these mice for inspection. All mice of this subgroup revealed typical characteristics of chronic infection, namely, 10^3 to 10^4 bacteria per liver and spleen with marked splenomegaly. The remaining mice received an intraperitoneal challenge with strain χ4666 at doses ranging from 10^3 to 10^5 bacteria. At serial time points, we then measured bacterial counts in the spleens and livers of these challenged mice. As Table 3 shows, mice chronically infected with χ4665 were immune to challenge doses roughly 10^2 to 10^4 times higher than the LD_{50} in control, naive mice. Furthermore, by 2 weeks after challenge, none of the mice harbored challenge strain χ4666 in their spleens, livers, or intestines, although the course of chronic infection by χ4665 was not affected (Table 4).

The anti-*Salmonella* specific antibody response was also compared in mice infected with either parent strain χ3181 or strain χ4665. As shown in Fig. 2, mice chronically infected with χ4665 in fact developed a markedly elevated antibody response compared with mice infected with χ3181.

Thus, the chronic murine *S. typhimurium* infection we ob-

TABLE 4. Response of mice chronically infected with χ4665 to challenge with χ4666

Group[a]	Tissue	Log count of χ4665 at wk after initial inoculation with χ4665[b]	
		6	8
Unchallenged	Spleen	3.50 ± 0.78	3.54 ± 1.44
Challenged	Spleen	3.08 ± 0.11	2.60 ± 0.65
Unchallenged	Liver	3.24 ± 1.11	4.58 ± 2.43
Challenged	Liver	5.08 ± 2.23	2.66 ± 0.41
Unchallenged	Small intestine	3.3 ± 1.0	3.10 ± 0.22
Challenged	Small intestine	5.21 ± 0.28	4.78 ± 0.18

[a] All mice were chronically infected with χ4665; challenged groups received χ4666 (harboring a silent Tn*10* element encoding tetracycline resistance) 4 weeks after inoculation with χ4665. Values for unchallenged groups are from the results shown in Fig. 1.

[b] Results are means ± standard deviations. No χ4666 organisms were recovered from any challenged animals from any site.

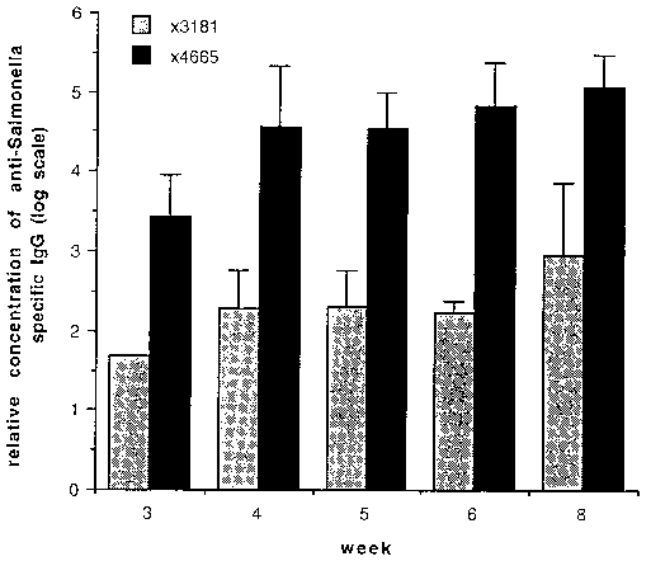

FIG. 2. Concentration of *S. typhimurium*-specific IgG in serum of mice infected with χ3181 or χ4665.

serve parallels closely the chronic human *S. typhi* carrier state. First, the chronic murine *S. typhimurium* carrier state caused by χ4665 develops after oral inoculation by a virulent strain, as does the human chronic *S. typhi* carrier state. Second, both the murine and human carrier states are characterized by a persistence of the organisms in the small intestine, with only variable recovery from the large intestine (references 1, 12, and 21 and data not shown). Third, the chronic carrier state is not due to a defect in the specific humoral immune response (8, 10, 17, 24, and this report); in fact, elevated titers of antibody against specific surface *Salmonella* antigens have been proposed as useful markers in identifying chronic human carriers (8, 10, 17). While the chronic human *S. typhi* carrier state has been shown to correlate with biliary tract disease, not all chronic human carriers have biliary tract disease (3, 18) and neither do mice chronically infected by χ4665 (data not shown).

Two other *S. typhimurium* strains that cause persistent infection in mice have been described. These include the temperature-sensitive mutant C5TS (14) and a *purE* derivative of strain HWSH (24). Although such mutants may shed light on details governing chronic infection, both strains are markedly attenuated (23, 24) and their relevance as physiologic models for salmonellosis is uncertain. In contrast, the virulent *S. typhimurium* SR-11 variant χ4665 described here is prototrophic and causes chronic infection after peroral inoculation, providing a model for study of the pathobiology of salmonellosis that resembles human disease.

The increased LD_{50} of χ4665 upon peroral challenge, without a corresponding change in LD_{50} upon intraperitoneal challenge, suggests that χ4665 diverges from the wild type during the intestinal or initiative phases of infection. If AgfA is a crucial adhesin in salmonellae (most *Salmonella* strains carry the *agfA* gene [reference 7 and our unpublished observations] even though they do not produce the fibers under standard laboratory conditions), changes in adhesive properties could explain the different behavior of the strain χ4665. Altered expression of the AgfA fiber could result in changes in the interaction of the bacterium with the epithelium of the small intestine, predisposing to persistence of the bacterium within the intestinal lumen and thereby providing a reservoir for establishment of an ongoing enterohepatobiliary circulation. Altered expression of the AgfA fiber could also change the interaction of the bacterium with components of the reticuloendothelial system. However, it is important to note that we cannot exclude the possibility that AgfA fibers act in concert with other gene products to produce chronic infection; the spontaneous variants we employ in our model system may have altered expression of a number of other proteins as a direct cause of, or as a direct consequence of, altered regulation of AgfA production.

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The first two authors contributed equally to this work.

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