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Characterization of the Virulence and Antigenic Structure of *Salmonella typhimurium* Strains with Lipopolysaccharide Core Defects

M. B. LYMAN, J. P. STEWARD, AND R. J. ROANTREE*

Department of Medical Microbiology and Fleischmann Laboratories of Basic Medical Sciences, Stanford University School of Medicine, Stanford, California 94305*

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The virulence and antigenic characters of *Salmonella typhimurium* strains, identical except for known lipopolysaccharide core defects, were compared. Smooth strains multiplied extensively and killed most mice. Deep rough strains containing only heptose I or heptose I and II in the rough core were completely eliminated after 6 h, whereas more superficial rough strains containing additional core sugars could be detected in low numbers (10^4 colony-forming units/g of tissue) for at least 7 days postinjection. Normal human serum exhaustively absorbed with certain rough strains was tested for ability to kill other rough strains. Two strains with the most superficial defects (*rfaJ*, *rfaL*) each had a unique serological character; strains with deeper defects showed much cross-reactivity. Similarities between the susceptibility of strains to the bactericidal effect of specifically absorbed serum correlated, in some cases, with similarities in in vivo behavior.

Rough *Salmonella* whose lipopolysaccharide lacks the O-specific polysaccharide usually possess a mutation in one of two chromosomal regions. The *rfb* cluster controls synthesis of enzymes necessary for biosynthesis of the O-specific oligosaccharide repeat units; bacteria with an *rfb* mutation make core polysaccharide chains which are not "capped" by O chains. A rough phenotype may also result from a mutation in any one of the *rfa* genes, most of which are located in a cluster between *cysE* and *pyrE*. Such bacteria have defects in some reaction involved in the biosynthesis of the lipopolysaccharide core; e.g., *rfaG* mutants lack a glucosyl transferase required for attachment of the proximal glucose unit (Fig. 1). A mutation that prevents attachment of an inner core component (other than a branch unit), e.g., heptose I or heptose II, will prevent subsequent addition of all distal sugars (for review, see reference 8). A set of five strains, each genetically identical except for the depth of their rough lesions, was derived by Kuo and Stocker (4) by ES18 transduction of the *rfa* mutations into an LT2 *pyrE* recipient. The *rfa* mutation was co-transduced with the donor's *pyrE*$^+$ gene. The set includes strains with defects associated with the *rfaL*, *rfaJ*, *rfa*(R-res-2), *rfaG*, and *rfaF* genes (see Fig. 1). One rough strain, SL1102, used in these experiments is not one of the nearly isogenic set. It is an LT2 derivative and has a mutation in the *rfaE* locus, which is remote from the *cysE-pyrE* segment. The purpose of this work was to characterize these *rfa* mutants with respect to mouse virulence and antigenic character.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. For the core defects associated with each *rfa* mutation, see Fig. 1.

Serum. The serum used was collected as a single batch from a healthy human and stored at −14 C except during absorptions. Absorptions were completed within 1 month of collection, and testings were completed within 2 months. Absorbed sera retained full bactericidal activity against our standard *Escherichia coli* O:89 test strain, A14 (7), as well as against other *Salmonella* strains in the test series.

Serum absorption. The absorbing bacteria were prepared in the following way. Four 150-ml overnight stationary brain heart infusion cultures were centrifuged; the pellets were combined, suspended in 70 ml of saline, and incubated at 70 C in a water bath for 3 h. The heat-killed bacteria were washed once and resuspended in 3 ml of saline, to a final concentration of approximately 10^{11}/ml. This bacterial suspension was mixed with 30 ml of human serum kept at 4 C in an ice bath. After 2 h, the serum was centrifuged in the cold to pellet the bacteria. For most strains, 12 such absorptions were required to remove completely the serum bactericidal activity for the absorbing strain. Except during centrifugation, the serum was kept at 4 C to preserve complement.

Bactericidal tests. A 0.3-ml amount of buffer containing about 3×10^4 bacteria diluted from an overnight stationary brain heart infusion culture was added to 2.7 ml of freshly thawed unabsorbed or absorbed normal human serum. As control, 0.3 ml of buffer containing about 3×10^3 bacteria was added to 2.7 ml of saline. At 0 time, the tube containing serum was placed at 37 C, and starting counts were obtained by plating 0.1-ml samples from the control. At 1 and 2 h, 0.1-ml test samples were removed and plated for colony-forming units on blood agar base (Oxoid CM55) agar. In a micro-test, a calibrated platinum loop was used to deliver 0.001 ml of bacterial suspension (ca. 10^5 bacteria) to 0.1 ml of serum. This mixture was incubated at 37 C; 0.001-ml-loopful samples were removed at 1 and 2 h and incorporated, by gentle swirling, into melted agar for a pour plate.

Virulence determinations. Female CF1 mice (Carworth Farms, New City, N.Y.) were injected intraperitoneally with approximately 10^5 organisms of each strain suspended in buffered saline. At 6 h and at 3, 7, and 21 days after injection, five mice were killed, and the liver and spleen of each were removed and homogenized in buffer to make a 10-ml total volume. The numbers of colony-forming units were determined by plating 0.1 to 0.5 ml of tissue homogenate on eosin methylene blue agar. The least number of bacteria detectable per organ pool was 10 (when 0.5 ml was plated) or 50 (when 0.1 ml was plated). For intraperitoneal mortality determinations, mice were injected with 10^5 bacteria and observed for 21 days.

TABLE 1. *Bacterial strains*[a]

Strain	Genotype	Source of *rfa* allele	Reference
SL1515	*pyrE125 rfa*$^+$ smooth recipient		6
SL3770	*pyrE*$^+$ *rfa*$^+$ smooth transductant		4
SL3749	*pyrE*$^+$ *rfaL446*	TV225	4, 9
SL3750	*pyrE*$^+$ *rfaJ417*[b]	TV161	4, 9
SL3748	*pyrE*$^+$ *rfa*(R-res-2)*432*[c]	TV148	3, 4, 9
SL3769	*pyrE*$^+$ *rfaG471*	SL1032	4, 9
SL3789	*pyrE*$^+$ *rfaF511*	SL1181	4, 9
SL1102[d]	*rfaE543*		4, 9

[a] All strains are derivatives of *S. typhimurium* LT2. Strains indicated *pyrE*$^+$ were made by transduction of *pyrE*$^+$ and indicated mutant *rfa* locus (or of *pyrE*$^+$ only, in the case of the control smooth transductant SL3770) from the donor strain, using phage ES18. SL1515 was the transductional recipient used for the other strains in this table.

[b] Gene *rfaJ417*, formerly indicated *rfa*(R-res-1)*417*, determines phage resistance pattern R-res-1, now known to indicate failure to add glucose II (3).

[c] The lipopolysaccharide of strain TV148, carrying gene *rfa*(R-res-2)*432*, has been shown by methylation analysis (3) to lack galactose I.

[d] SL1102 is a rough mutant, *rfaE543*, deficient in formation of the heptose I unit, of a smooth parent strain, SL1027, having several nutritional and other mutations (4): the mutated *rfa* locus is remote from the *cysE-pyrE* segment.

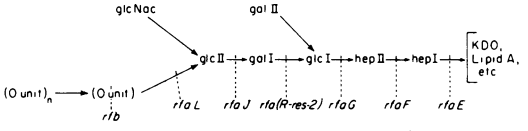

FIG. 1. *Main features of Salmonella lipopolysaccharide structure and symbols assigned to genes concerned with formation of individual units of core.*

RESULTS AND DISCUSSION

Bactericidal tests using absorbed normal human serum. The antigenic character of the *rfa* mutants was tested by determining the bactericidal activity of absorbed normal human serum against each. Samples of the serum were absorbed with each strain until the bactericidal activity against the absorbing strain was completely removed; these absorbed serum samples were then tested for killing activity against the other rough mutants. The results are shown in Table 2. Unabsorbed serum killed all rough strains (survival <1%), as expected, and 94% of smooth strain SL3770 (*pyrE*$^+$). Superficial rough strains SL3749 (*rfaL*) and SL3750 (*rfaJ*) absorbed activity quite specifically, indicating that each is antigenically distinct from the other and from all the other rough strains tested. The absorption patterns of SL3748 (*rfa* R-res-2) and SL3769 (*rfaG*), on the other hand, were very similar in that each removed killing activity against the other and against SL3789 (*rfaF*). Thus, although strain SL3748 (*rfa* R-res-2) supposedly contains a terminal glucose I

TABLE 2. *Characterization of antigenic structure by determining bactericidal activity of absorbed normal human serum*[a]

Absorbing strains	% Survival of test strains					
	SL3749 *rfaL*	SL3750 *rfaJ*	SL3748 R-res-2	SL3769 *rfaG*	SL3789 *rfaF*	SL1102 *rfaE*
SL3749 *rfaL*	100	0	5-10	0	0	0
SL3750 *rfaJ*	0	100	0	0	0	0
SL3748 *R-res-2*	0	10	100	100	100	0
SL3769 *rfaG*	0	0	100	100	100	0
SL3789 *rfaF*	0	0	0	0	100	10-20

[a] Number given is percentage of 10^6 bacteria/ml surviving for 2 h at 37 C. Zero, therefore, means no growth in the sample tested (survival ca. <0.005%).

TABLE 3. *Survival of CF1 mice infected intraperitoneally with smooth and rough S. typhimurium strains*

Determination	Strain							
	SL1515	SL3770	SL3749	SL3750	SL3748	SL3769	SL3789	SL1102
Core defect	Smooth, *pyrE*$^-$	Smooth, *pyrE*$^+$	*rfaL*	*rfaJ*	*rfa* (R-res-2)	*rfaG*	*rfaF*	*rfaE*
Survival[a]	6/10	1/10	5/5	5/5	5/5	5/5	ND	ND

[a] Number alive at day 21/number injected with approximately 10^5 bacteria intraperitoneally. ND, Not determined.

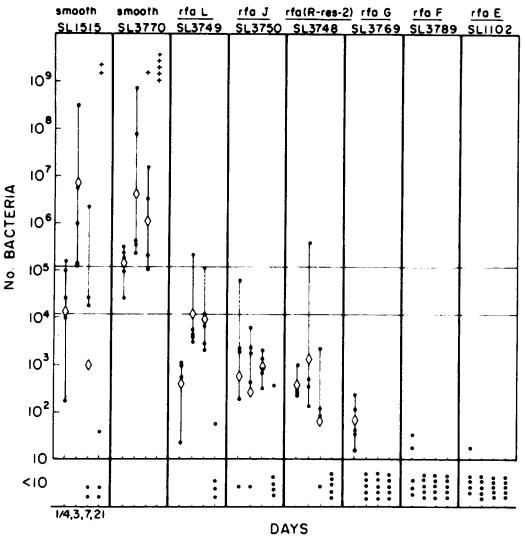

FIG. 2. *Bacterial counts on liver plus spleen pools of intraperitoneally infected mice. The injected strains are indicated at the top of each vertical column. The horizontal bar and arrows indicate the inoculum range. Mice were killed at 1/4, 3, 7, and 21 days after injection; each dot (●) represents the bacterial count obtained from the pooled liver and spleen of one mouse. The diamond (◆) is the geometric mean of the counts from (in most cases) five mice. The geometric means were calculated using 10^0 (i.e., 1.0) as the number of bacteria per organ pool for pools that gave sterile cultures (i.e., <10 colony-forming units/pool). Each cross (+) represents a mouse that had died by the time indicated.*

(with a galactose II branch), the heptose I and heptose II must be exposed sufficiently to absorb activity against the deeper mutants, which contain heptose as their terminal sugar (SL3789, *rfaF*, and SL3769, *rfaG*). Strain SL3789 *(rfaF)* absorbed activity against itself and, to a small degree, against the heptoseless strain SL1102 *(rfaE)*. This may indicate that deeper structures, such as ketodeoxyoctonate (KDO), are exposed in the one-heptose mutant, SL3789. That SL3789 *(rfaF)* absorbed killing activity against SL1102 *(rfaE)* and must, therefore, contain exposed deep-core lipopolysaccharide structures, correlates with the in vivo experiments, in which SL3789 *(rfaF)* and SL1102 *(rfaE)* behaved similarly and were unable to establish infection and were undetectable in most mice just 6 h after injection.

Virulence determinations. The survival of mice infected intraperitoneally with the smooth and *rfa* mutant *Salmonella typhimurium* strains is given in Table 3. A dose of 10^5 *rfa* mutant bacteria did not cause death or detectable illness in any of the mice. The smooth strains SL1515 *(pyrE$^-$)* and SL3770 *(pyrE$^+$)* killed 4 of 10 and 9 of 10 mice, respectively. The lesser virulence of SL1515 (also indicated by the counts in Fig. 2) could be a result of its pyrimidine requirement.

The bacterial counts obtained from the livers and spleens of mice at 6 h and at 3, 7, and 21 days after intraperitoneal injection with approximately 10^5 organisms are shown in Fig. 2. As expected, the smooth strains multiplied in vivo and were found in numbers between 10^5 and 10^8 at 6 h and 3 and 7 days. By day 21, most mice had died (see Table 3). SL3749 *(rfaL)*, SL3750 *(rfaJ)*, and SL3748 (*rfa* R-res-2) were recovered in numbers ranging from 10^2 to 10^5 per organ pool at 6 h and 3 and 7 days. Only approximately 10^2 colony-forming units of SL3769 *(rfaG)* were recovered from the organ pools of each of five mice at 6 h after injection and none at day 3 or thereafter. The two deepest rough strains, SL3789 *(rfaF)* and SL1102 *(rfaE)*, were recovered from only two of five and from one of five mice, respectively, at 6 h after injection, and none were recovered thereafter.

These results agree with those of Nakano and Saito (5), who found that *S. typhimurium* strains carrying deep lesions multiplied, in general, to a lesser extent than those with more superficial defects. Similar results were obtained by Edebo and Normann (1) and by Germanier (2). The strains used in the present study have the advantage, however, of being a comparable set with greater genetic homogeneity than those investigated previously. It looks as if there is a progressive loss of virulence with the loss of sugars from the lipopolysaccharide as one compares member strains of this set.

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