

Salmonella abony-*Salmonella typhimurium* Recombinant Nonvirulent for the Mouse

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A previous genetic investigation involving a mouse-nonvirulent *Salmonella abony* donor (high frequency of recombination) and a virulent *S. typhimurium* recipient indicated that two unlinked "low-virulence" loci determined nonvirulence. A nonvirulent recombinant was analyzed to determine the basis for its nonvirulence. The recombinant was smooth (like the parental strains) and prototrophic. The doubling time in mouse serum of the recombinant and the *S. abony* parent (both streptomycin-resistant) was longer than that of the wild-type streptomycin-sensitive ancestor of the *S. typhimurium* recipient. The virulent recipient also grew poorly in serum. However, the nonvirulence of the recombinant was probably not due to its inheritance of the streptomycin-resistance allele from the donor, because other recombinants were streptomycin-resistant but still virulent. Unlike the nonvirulent *S. abony* (but like the *S. typhimurium*), the recombinant was insusceptible to rapid intravenous clearance in normal mice. It therefore appears that neither of the "low-virulence" loci determine diminished virulence by enhancing phagocytosis. Clearance of the recombinant was enhanced by opsonization with immune serum. Counts of viable bacteria in the blood, liver, and spleen of normal mice after intravenous challenge showed that the recombinant, like the *S. abony* donor, failed to proliferate in the tissues, whereas the virulent *S. typhimurium* did so markedly. It is concluded that the nonproliferation of the recombinant was determined by one or both of the "low-virulence" loci from the nonvirulent *S. abony* donor.

There are two regions of the bacterial chromosome which specify nonvirulence for mice (i.e., inability to kill with a few bacteria) of a smooth strain of *Salmonella abony* (11). This was inferred from the segregation of nonvirulence in crosses between the nonvirulent strain of *S. abony* ($LD_{50} = 10^8$ bacteria intraperitoneally) as the chromosomal Hfr (high frequency of recombination) donor and a virulent strain of *S. typhimurium* ($LD_{50} = <50$ bacteria) as the recipient (F^-). One nonvirulence locus was mapped between the gene for inositol fermentation (*inl*) and the gene controlling one of the steps in the biosynthesis of methionine (*metA*), and the other was mapped between the gene controlling high-level resistance to streptomycin (*strA*) and one of the genes (*aroD*) specifying the biosynthesis of aromatic amino acids (11, 14, 17). Recombinants which inherited either of these two loci were of intermediate virulence, i.e., with LD_{50} values

between those of the *S. abony* and *S. typhimurium* strains. When a recombinant of intermediate virulence was backcrossed to the *S. abony* donor, an F_2 recombinant, which also inherited the second nonvirulence locus of the donor, became as nonvirulent as the *S. abony* parent.

This paper compares various characteristics of a completely nonvirulent *Salmonella* recombinant with those of its parental strains, in an attempt to understand the physiological basis of its nonvirulence.

MATERIALS AND METHODS

Culture media. Nutrient broth consisted of Lab-lemco Beef Extract (Oxoid), 0.5%; Bacto-Peptone (Difco), 1%; and NaCl, 0.5%. Nutrient agar was nutrient broth solidified with 2% agar (Difco).

Diluent. Phosphate buffer (0.1 M, pH 7.4) was used.

Bacterial strains. Table 1 shows the characteristics of the strains used. All the strains have the Kauffmann-White O antigens 1, 4, 5, and 12. *S. abony* SW1444 is an Hfr strain and probably identical to *S. abony* SW1391 (13). *S. typhimurium* C5 is the mouse-virulent prototrophic wild-type strain described previously (6). *S. typhimurium* M206 is mouse-nonvirulent and sensitive to the bactericidal action of serum (12). *S.*

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TABLE 1. Characteristics of strains

Strain	Genotype ^a									Mouse virulence (LD ₅₀ , bacteria/mouse)	Source or origin	Reference
	<i>his</i>	<i>metA</i>	<i>aroD</i>	<i>inl</i>	<i>rha</i>	<i>ara</i>	<i>strA</i>	<i>H1</i>	<i>H2</i>			
SW1444	+	-	-	+	+	+	r	b	e, n, x	5 × 10 ⁷	P. H. Mäkelä	11, 14
C5	+	+	+	-	-	+	s	i	1, 2	<10	D. Rowley	6
C5P	-	+	+	-	-	-	s	i	1, 2	<10	C5	11
M206	+	+	+	-	+	+	s	i	1, 2	5 × 10 ⁶	D. Rowley	6
C5R	+	+	+	+	+	-	r	b	1, 2	10 ⁷	SW1444 × C5P	11
Lister	+	+	+	+	+	+	s	b	1, 2	10 ⁶	R. Bhagwan Singh	—

^a Abbreviations (17): *his*, *metA*, and *aroD* denote genes determining biosynthesis of histidine, methionine, and aromatic amino acids (tyrosine and phenylalanine), respectively; *inl*, *rha*, and *ara* denote genes specifying utilization of inositol, rhamnose, and arabinose, respectively; *strA* denotes gene controlling streptomycin resistance (1 mg/ml); *H1* and *H2* denote genes specifying synthesis of flagellar antigens of phase 1 and phase 2, respectively; + = ability to synthesize or utilize; - = inability to synthesize or utilize; r = resistance; s = sensitivity.

typhimurium C5P is a double mutant selected from C5 and previously described as C5 *his ara* (11). C5R is the completely nonvirulent recombinant obtained by recombination between SW1444 as donor and C5P as recipient (11). It was derived as follows. A recombinant selected for the donor *his*⁺ allele also inherited the donor *strA* resistance allele. Its virulence was intermediate between that of the donor and the recipient (LD₅₀ = 10⁸ bacteria), as previously reported (11). This recombinant was then crossed with the *S. abony* donor, selection being made for the *rha*⁺ allele of the donor. C5R, the *rha*⁺ recombinant from this backcross, also inherited the *inl*⁺ and *H1-b* loci from the donor as unselected markers. Its mouse virulence was similar to that of the donor (LD₅₀ = 10⁸ bacteria, i.e., nonvirulent), as previously reported (11). The recipient alleles still present in the recombinant were *ara*⁻ and *H2-1, 2*. This strain will be referred to as recombinant C5R. *S. paratyphi* B Lister was the strain used in the Institute for Medical Research, Kuala Lumpur, for the preparation of typhoid-paratyphoid vaccine.

Preparation of bacterial cultures. Nutrient broth cultures, incubated for 18 hr at 37 C without shaking, were used in all experiments.

Mice. Random-bred Swiss white mice (of both sexes) weighing 18 to 22 g, obtained from the Institute for Medical Research, Kuala Lumpur, were used.

Mouse virulence of bacterial strains. Broth cultures were diluted and 0.2-ml dilutions were injected intraperitoneally into groups of 10 mice. The mice were housed in groups of 10. Mortality was recorded daily for 28 days, and the LD₅₀ dose was calculated (15).

Clearance of viable bacteria from the mouse bloodstream. The technique was similar to that described previously (1, 9), except that recovery of viable bacteria was measured rather than recovery of ³²P label. Heparin was not used, because it has been reported that there is no significant difference in the rate of clearance of bacteria in normal and heparinized mice (1). A sample (0.2 ml) of a broth culture was injected into a tail vein (5 × 10⁷ to 10 × 10⁷ bacteria/mouse). At intervals, 0.05 ml of blood was obtained from the

retro-orbital venous plexus with a calibrated glass pipette, diluted, and plated in duplicate on nutrient agar. For each bacterial strain, the logarithm of the arithmetic mean of the counts from three to five mice for a particular time was plotted against time, and the best-fit straight line through the points was calculated; its slope, which measures the rate of clearance, is the "phagocytic index" (*K*) and may alternatively be calculated from the equation $K = (\log_{10}C_1 - \log_{10}C_2) / (T_2 - T_1)$, where *C*₁ and *C*₂ are the counts at times *T*₁ and *T*₂ in minutes (9). The calculations were made by using an IBM360 model 50 computer at Stanford University School of Medicine. The *K* values are expressed with two standard errors of the slope. In control experiments, it was determined that the percentage recovery of viable bacteria immediately after inoculation (assuming a mouse blood volume of 2 ml) was 75 to 95% of the inoculum.

Mouse serum. Normal mice were bled from the retro-orbital venous plexus and the blood was pooled. After clotting, the serum was collected by centrifugation and stored at -20 C. To obtain immune mouse serum, animals were inoculated intraperitoneally with 10⁶ viable bacteria of the immunizing strain, and were bled 2 weeks later.

Opsonization of bacteria. One milliliter of undiluted immune serum and an equal volume of broth culture were mixed and held at 4 C; after 20 min, 0.4 ml of this mixture was inoculated intravenously into mice and the rate of clearance was determined as described.

Immune mice. Mice were inoculated intraperitoneally with 10⁶ viable bacteria of the nonvirulent strain C5R. Twelve days later they were used in clearance tests.

Liver and spleen counts. Groups of mice were inoculated intravenously in the tail vein with 5 × 10⁶ to 10 × 10⁶ bacteria/mouse. At intervals, three mice for each bacterial strain were killed and their livers and spleens were removed aseptically. They were homogenized at 2,000 rev/min for 60 to 90 sec in a Stir-R tissue homogenizer (Tri-R Instruments, Jamaica, N.Y.). Samples of diluted homogenate were spread on agar in duplicate for viable count.

Bacterial growth rate in normal mouse serum. Broth cultures diluted in buffer were mixed with undiluted serum to obtain two concentrations of serum: 90% (9 parts serum, 1 part bacterial suspension) and 10% (1 part serum, 9 parts bacterial suspension). The amount of broth carried over during dilution was 0.1% into the 90% serum-mixture and 0.9% into the 10% mixture. The initial bacterial concentrations were adjusted to about 10^6 bacteria/ml. The mixtures were held in a water bath at 37 C, and at intervals samples were diluted and plated on agar in duplicate for viable count.

RESULTS

Smooth-rough character. The smooth or rough character of *Salmonella* strains can be determined by the pattern of resistance to phages which distinguish the qualitative nature of the bacterial lipopolysaccharide (18). The strains shown in Table 1 were tested with the phages used in B. A. D. Stocker's laboratory. The parental strains C5P and SW1444, and the recombinant C5R, were fully smooth (Table 2), as were C5 (wild-type ancestor of C5P) and *S. paratyphi* B Lister. The insensitivity of strain Lister to phages P22 and P22h was presumed to be due to its lysogeny. Strain M206 was sensitive to several rough-specific phages, which suggests that this strain was at least partly rough.

Growth rate in normal mouse serum. Certain streptomycin-resistant mutants of a mouse-virulent strain of *S. typhimurium* have a 3 to 7 min longer doubling time in nutrient broth, and are less virulent than the parent (7). One such mutant was found to be less capable of proliferation in the mouse than was the virulent parent (7). We therefore tested the recombinant and the parental *Salmonella* to determine whether they differed in growth rate, since the virulent parent C5P was streptomycin-sensitive, whereas the other parent, SW1444, and the recombinant,

C5R, were both nonvirulent and streptomycin-resistant. Growth in mouse serum would be a closer analogy to growth in the host than growth in nutrient broth, and so normal mouse serum was used at two concentrations (90 and 10%), with the 90% concentration approximating whole serum. For comparison with the auxotrophic derivative C5P, the streptomycin-susceptible wild-type C5 was also tested. Figure 1 shows the growth curves and the calculated doubling times. Growth in 10% mouse serum (in phosphate buffer) was slower and the final yield less than in 90% serum for all four *Salmonella* strains. The histidine-requiring C5P, though mouse-virulent, did not grow well in mouse serum (decrease in growth rate when viable count reached about 10^6), probably because of limitation of histidine in mouse serum (on addition of histidine, C5P grew normally). The doubling times in 90% serum were: C5P (streptomycin-sensitive, histidine-requiring, virulent), 33 min (in serum supplemented with histidine); C5 (streptomycin-sensitive, prototrophic, virulent), 27 min; C5R (streptomycin-resistant, prototrophic, nonvirulent), 34 min; SW1444 (streptomycin-resistant, methionine-, phenylalanine-, and tyrosine-requiring, nonvirulent), 43 min.

Rate of clearance from the blood in normal mice. Soon after intravenous inoculation of certain nonvirulent *Salmonella* into normal mice, the bacteria are rapidly removed from the circulation, whereas virulent *Salmonella* are only slowly cleared (3, 9). The rates of clearance of the recombinant and parental *Salmonella* strains were therefore tested in normal mice. It was expected that the nonvirulent recombinant C5R and the nonvirulent parent SW1444, but not the virulent parent C5P, would be rapidly phagocytized. Figure 2 shows the clearance of these

TABLE 2. Pattern of resistance to phages^a

Strain	Smooth-specific phages			Smooth- and rough-specific phage (Felix O)	Rough-specific phages					
	P22	P22h	9Na		6SR	Br2	Ffm	Br60	P221	C21
SW1444	+	+	+	+	-	-	-	-	-	-
C5	+	+	+	+	-	-	-	-	-	-
C5P	+	+	+	+	-	-	-	-	-	-
M206 ^b	-	-	+	+	-	-	+	+	+	-
C5R	+	+	+	+	-	-	-	-	-	-
Lister	-	-	+	+	-	-	-	-	-	-

^a Nutrient agar plates were surface-inoculated and spotted with 0.01 ml of phage lysate (10^8 plaque-forming units/ml). The plates were incubated at 37 C and examined at 5 and 18 hr (B. A. D. Stocker, personal communication; 18). Symbols: + = lysis; - = no lysis.

^b The M206 strain that was used in clearance tests (Fig. 2) was no longer available. The strain shown was a culture subsequently provided by D. Rowley.

Salmonella strains during the first 5 min after inoculation (determined by viable counts of blood samples), and the calculated "phagocytic indices" (K). As expected, the nonvirulent *S.*

abony parent SW1444, though smooth, was rapidly cleared ($K = 0.137$), whereas the virulent parent C5P was not ($K = 0.015$). However, contrary to expectation, the nonvirulent recombinant C5R was not rapidly cleared ($K = 0.007$). Before the significance of this result could be assessed, two points had to be considered. First, it was possible that the mice used in these tests were somehow different from mice used by other investigators. Second, the rate of clearance determined by enumeration of viable bacteria may differ from those determined by using the ^{32}P label technique described by Jenkin (8). The rates of clearance of the *S. typhimurium* strains C5 and M206, which had previously been characterized by ^{32}P label-monitoring in other mice (8), were therefore examined with the mice used in these studies by enumeration of viable bacteria (Fig. 2). The nonvirulent strain M206 was rapidly cleared ($K = 0.163$) and the virulent strain C5 was only slowly cleared ($K = 0.010$). These K values closely resemble those of Jenkin (8). We conclude that clearance experiments as carried out by us give the same kind of results as ^{32}P label experiments. The result with C5R therefore indicates that some nonvirulent *Salmonella* strains are not rapidly cleared. It was thought of interest to see how another *Salmonella* strain, identical to the recombinant C5R in its Kauffmann-White antigens (1, 4, 5, 12: b ↔ 1, 2), and like it of low virulence ($\text{LD}_{50} = 10^8$ bacteria), would fare in the circulation of normal mice. The clearance of such a strain (*S. paratyphi* B strain Lister) is shown in Fig. 2; like C5R, it was very slowly cleared ($K = 0.010$).

Thus, the smooth, nonvirulent *Salmonella* (*S. abony* SW1444) was rapidly cleared even though it does not obviously differ in O-somatic antigens from a virulent *Salmonella* which is not cleared (*S. typhimurium* C5P) (8, 9). Some nonvirulent smooth strains (recombinant C5R and *S. paratyphi* B Lister) are not rapidly cleared, which implies that susceptibility to clearance is not the only cause of nonvirulence in smooth *Salmonella*.

Effect of antibody on rate of clearance. The slow clearance of some bacteria from the mouse circulation results from absence of antibody (1, 8). The slow elimination of C5P and C5R might therefore be due to the absence of antibody directed against these strains in the serum of normal mice. To test this, the clearance in normal mice of bacteria opsonized by prior exposure to immune mouse serum was examined (Table 3). For comparative purposes, the K values (from Fig. 2) of nonopsonized bacteria were also included. Untreated cells of strains C5P and C5R were scarcely cleared in 5 min

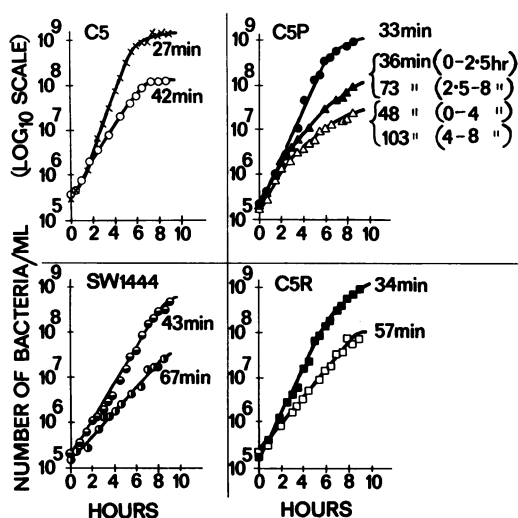


FIG. 1. Growth curves of *Salmonella* in mouse serum at 37 C. The time (minutes) refers to doubling time. Symbols: X, strain C5 in 90% serum; O, strain C5 in 10% serum; ●, strain C5P in 90% serum (supplemented with 20 μg of histidine per ml); ▲, strain C5P in 90% serum; △, strain C5P in 10% serum; ●, strain SW1444 in 90% serum; ▲, strain SW1444 in 10% serum; ■, strain C5R in 90% serum; □, strain C5R in 10% serum.

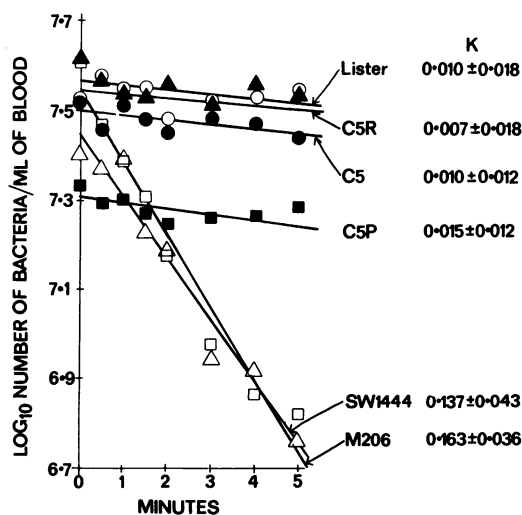


FIG. 2. Rate of intravenous clearance of *Salmonella*. Symbols: ▲, strain Lister; ○, strain C5R; ●, strain C5; ■, strain C5P; △, strain SW1444; □, strain M206.

TABLE 3. Effect of antibody on intravenous clearance of bacteria

Strain	Normal mice ^a (nonopsonized)	Immune mice (nonopsonized)	Normal mice	
			Opsonized with C5R immune serum	Opsonized with SW1444 immune serum
C5P	0.015 ± 0.012 ^b	0.140 ± 0.030	0.178 ± 0.024	0.244 ± 0.026
C5R	0.007 ± 0.018	— ^c	0.158 ± 0.046	0.189 ± 0.040
SW1444	0.137 ± 0.043	—	—	0.180 ± 0.046

^a The *K* values are from Fig. 2 for comparison.

^b "Phagocytic index" (*K*).

^c Not done.

(*K* = 0.015 and 0.007, respectively; Fig. 2), but they were very rapidly cleared (*K* for C5P = 0.178 – 0.244, and for C5R = 0.158 – 0.189) if they had been exposed, prior to inoculation, to serum from mice immunized 2 weeks previously either with C5R or with SW1444. C5P was also rapidly cleared from the circulation of mice which had previously been actively immunized against C5R (*K* = 0.140). The rapid clearance of C5P and C5R after opsonization with immune serum prepared against C5R or SW1444 can be explained in terms of the identity of the Kauffmann-White O antigens of strains C5P, C5R, and SW1444. These results indicate that the non-clearance of C5P and C5R in normal mice is probably due to absence of sufficient opsonizing antibody in their sera.

Rate of intravenous clearance determined over 30 min. Strains C5P and C5R were not cleared appreciably from the mouse's circulation in the 5-min test (Fig. 2), and so clearance was examined over a longer period (Fig. 3). The rates of clearance of C5P and C5R differed only slightly from those obtained over the 5-min period (C5P, *K* = 0.015 for 5 min and 0.015 for 30 min; C5R, *K* = 0.007 for 5 min and 0.016 for 30 min). With both strains, however, only about 30% of the bacteria remained in the mouse's circulation at 30 min. In contrast, 99% of the bacteria of the nonvirulent parent SW1444 had been cleared by 30 min. (Two *K* values for SW1444 are recorded, since the rate decreased after 10 min.)

Blood counts of recombinant and parental *Salmonella* over a long period of time. As the clearance of the virulent parent C5P and the nonvirulent recombinant C5R did not differ over a 30-min period, viable counts were made over a considerably longer period (Fig. 4). The geometric mean of counts from five mice at each time period are shown. For all three *Salmonella* strains, the number of viable bacteria per milliliter of blood decreased in the first few hours after introduction. Even strains C5P and C5R,

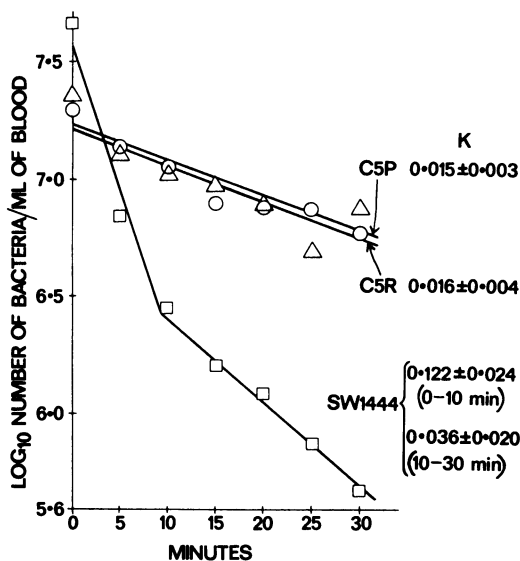


FIG. 3. Rate of intravenous clearance in 30 min. Symbols: Δ , strain C5P; \circ , strain C5R; \square , strain SW1444.

which were scarcely phagocytized initially (Fig. 2 and 3), slowly disappeared from the circulation, with less than 5% remaining 2 hr after challenge. The decrease in blood viable count terminated by 6 hr. From this time onwards, the behavior of the three *Salmonella* strains was quite different. The virulent parent C5P rapidly increased in numbers in the blood till it reached 10^7 bacteria/ml, 30 to 36 hr after challenge, by which time nearly all the remaining challenged mice died. In the case of the nonvirulent recombinant C5R, there was little change in the blood viable count till 48 to 72 hr. In mice challenged with the nonvirulent donor parent SW1444, the bacterial population in the blood did not increase appreciably. While these experiments were in progress, groups of mice (15 per strain) that were similarly challenged were set aside to determine cumulative mortality (Fig. 4). The period

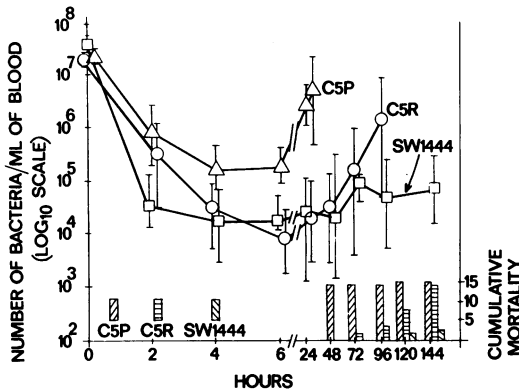


FIG. 4. Distribution of *Salmonella* in the blood after intravenous challenge with 5×10^7 to 10×10^7 bacteria/mouse. Five mice were analyzed at each time. Cumulative mouse mortality at different times after challenge with the same dose are also shown. Symbols: Δ , strain C5P; \circ , strain C5R; \square , strain SW1444. The vertical bars represent the range of counts about the geometric mean.

of maximum mortality coincided with the appearance of large numbers of bacteria in the blood.

To determine whether the absolute number of bacteria inoculated intravenously affected subsequent events in the blood, mice were inoculated with 5×10^5 to 10×10^5 bacteria/mouse (i.e., with 5×10^4 to 10×10^4 LD₅₀ of C5P, 0.05 to 0.1 LD₅₀ of C5R, or with 0.01 to 0.02 LD₅₀ of SW1444) instead of with 5×10^7 to 10×10^7 bacteria/mouse (as in Fig. 4). The results (Fig. 5) showed that all three *Salmonella* strains were removed from the circulation by 24 hr (count reduced to <1% of initial level), but by 48 hr there was a divergence in the behavior of the strains. In mice inoculated with the virulent strain C5P, the numbers of viable bacteria in the blood increased rapidly, by 72 hr exceeding 10^6 bacteria/ml, and all the mice died within 96 hr of inoculation. In mice inoculated with the nonvirulent C5R, small numbers of bacteria were recoverable till 72 hr, after which time blood samples were sterile (<20 bacteria/ml) till the end of the experiment (day 13). Most mice challenged with the nonvirulent SW1444 gave sterile blood cultures after 24 hr.

Recovery of viable bacteria of the recombinant and parental *Salmonella* in the liver and spleen. The phagocytic cells of the reticuloendothelial system (RES) in the liver and the spleen are principally responsible for the removal of bacteria from the blood (1, 2). It was therefore of interest to study the counts of viable bacteria in the liver and spleen of intravenously inoculated mice (Fig. 6 and 7).

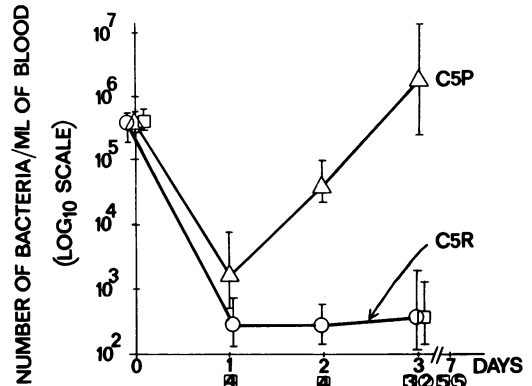


FIG. 5. Distribution of *Salmonella* in the blood after intravenous challenge with 5×10^5 to 10×10^5 bacteria/mouse. Five mice were analyzed at each time. Symbols: Δ , strain C5P; \circ , strain C5R; \square , strain SW1444. The vertical bars represent the range of counts about the geometric mean. Values below the axis line indicate that the blood sample was sterile (no viable bacteria in 0.05-ml blood sample plated), and the numbers within the symbols indicate the numbers of such mice.

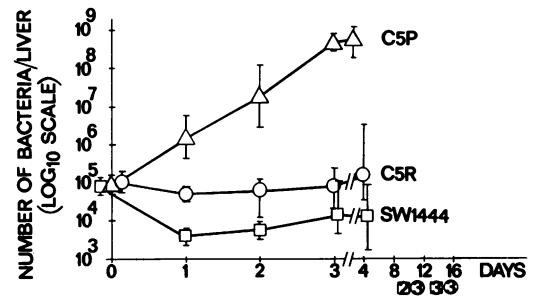


FIG. 6. Distribution of *Salmonella* in the liver after intravenous challenge with 5×10^5 to 10×10^5 bacteria/mouse. Three mice were analyzed at each time. Symbols: Δ , strain C5P; \circ , strain C5R; \square , strain SW1444. The vertical bars represent the range of counts about the geometric mean. The values below the axis line indicate that the livers when cultured yielded <500 bacteria/liver, and the numbers within the symbols indicate the numbers of such mice.

The mice were inoculated with 5×10^5 to 10×10^5 bacteria/mouse. For each bacterial strain, three mice were killed per time. The zero-time values refer to counts on the organs of mice killed immediately after challenge. The number of viable bacteria of the virulent strain C5P recovered from these organs increased, and by 72 hr the bacterial population reached 10^8 to 10^9 per organ. All of the remaining mice challenged with the virulent strain died during the next 24 hr. The nonvirulent recombinant C5R and the

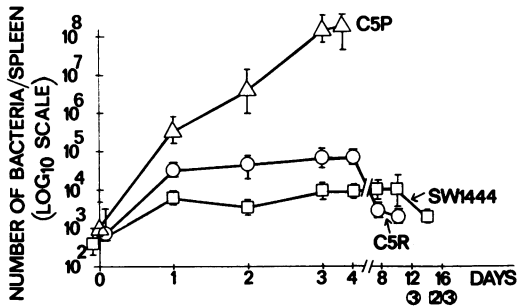


FIG. 7. Distribution of *Salmonella* in the spleen after intravenous challenge with 5×10^6 to 10×10^6 bacteria/mouse. Three mice were analyzed at each time. Symbols: Δ , strain C5P; \circ , strain C5R; \square , strain SW1444. The vertical bars represent the range of counts about the geometric mean. The values below the axis line indicate that the spleens when cultured yielded < 100 bacteria/spleen, and the numbers within the symbols indicate the numbers of such mice.

nonvirulent parent SW1444 failed to increase in number in the liver. A similar situation prevailed in the spleen except for a small increase in the first 24 hr after inoculation. The recombinant C5R appeared to fare a little better than SW1444 between days 1 and 4 in the spleen. Both nonvirulent strains were present in low numbers in the liver (< 500 bacteria/liver) by the 10th day, and in the spleen by the 16th day (< 100 bacteria/spleen). The significant point is that, when equivalent numbers of bacteria were introduced intravenously, the virulent strain C5P had multiplied within 72 hr to population levels 1,000-fold greater than the nonvirulent strains.

DISCUSSION

The lack of mouse virulence of certain strains of, for instance, *S. typhimurium*, results from known causes, such as a requirement for growth factors (e.g., adenine) which are absent from mouse tissue (6), or from roughness which is known to determine extreme susceptibility to phagocytosis (4). In other cases, the cause of lack of virulence is not apparent. An earlier investigation (11) concerned the genetic determination of the nonvirulence ($LD_{50} = 10^8$ bacteria by intraperitoneal inoculation) of a strain of *S. abony* which is smooth and which has an O-antigenic formula identical with that of a mouse virulent strain of *S. typhimurium* ($LD_{50} = < 50$ bacteria). In crosses of an Hfr derivative of this *S. abony* strain with the mouse virulent F⁻ *S. typhimurium*, the degree of virulence of recombinants suggested that the *S. abony* strain carried "low-virulence" genes in two regions of the chromosome. The present report describes

experiments designed to allow examination of the physiological basis of nonvirulence of the *S. abony* strain, by comparing the behavior in various tests of the two parental strains and a recombinant which was as nonvirulent as the *S. abony* donor.

The results of phage sensitivity tests showed that the nonvirulent *S. abony* parent SW1444 and the nonvirulent recombinant C5R were fully smooth, like the virulent *S. typhimurium* parent C5P (Table 2). All three strains were also resistant to the bactericidal action of normal human serum (*unpublished data*); results of this test have been interpreted as indicating a possible correlation between mouse virulence and insusceptibility to human serum (16). In contrast to the three strains mentioned above, the nonvirulent *S. typhimurium* strain M206 is sensitive to certain rough-specific phages (Table 2), and both this strain and *S. paratyphi* B Lister are very sensitive to killing by human serum (*unpublished data*); these results suggest possible causes of their nonvirulence.

Hobson (7) found that mutants of *S. typhimurium* which are highly resistant to streptomycin grew more slowly in broth than did the streptomycin-susceptible ancestor, and the mutants were of diminished virulence. Growth curves determined in 90% normal mouse serum (Fig. 1) showed that the two streptomycin-resistant strains tested (SW1444 and C5R), both nonvirulent, had longer doubling times than did the streptomycin-sensitive virulent strain C5 (the wild-type ancestor of C5P). These results are in agreement with those of Hobson (7). The growth rate of the virulent strain C5P was low, even in serum supplemented with histidine (the growth requirement of C5P), but it is possible that with a larger supplement of histidine it would have doubled faster. In any case, the nonvirulence of the recombinant C5R was unlikely to be due to inheritance of the streptomycin-resistance allele from the *S. abony* SW1444 donor, since some recombinants which inherited the same allele from SW1444 in crosses with another virulent subline of *S. typhimurium* C5 were still virulent (11).

When bacteria are injected intravenously into normal mice, cells of strains which are nonvirulent (to the mouse) are in general rapidly removed from the circulation by phagocytosis, whereas cells of mouse-virulent strains are much less rapidly cleared. This has been shown with *S. typhimurium* (3, 8), *S. gallinarum* (9), and with *Klebsiella pneumoniae* (9). Presumably, susceptibility to rapid clearance by phagocytosis is either the cause of the diminished virulence of the

strains concerned, or it results in some way from the character, e.g., roughness, which causes their low virulence. But not all strains of low virulence for the mouse are rapidly cleared, because there are strains (Vi-negative) of *S. typhi* (5), *S. adelaide* (5, 10), *Escherichia coli* O111B4 (1), and *S. paratyphi* B (this paper) which are smooth and yet poorly phagocytized. These exceptions have been noted previously (3, 5).

In the present study, clearance of intravenously inoculated viable bacteria was followed by making counts of viable bacteria in blood samples taken at short intervals after injection. When strains whose clearance in normal mice had been investigated by Jenkin (8) were tested in this way, the rates of clearance ("phagocytic indexes") determined from viable counts were essentially similar to those determined from clearance of radioactivity of intravenously inoculated viable bacteria labeled with ^{32}P (8; Fig. 2). We infer that the clearance (in terms of viable count) which we observed results, mainly or entirely, from removal by phagocytosis by fixed cells of the RES (liver and spleen mainly), rather than from killing in the bloodstream (e.g., phagocytosis by circulating leukocytes), and we have therefore calculated "phagocytic indexes." Earlier work cited above suggests that all *Salmonella* strains susceptible to rapid clearance are of low virulence, but that some (smooth) nonvirulent strains are not rapidly cleared. In the present work, the mouse-virulent (smooth) *S. typhimurium* parent strain C5P was, as expected, cleared very slowly in normal mice ($K = 0.015$; Fig. 2). The relatively nonvirulent *S. abony* parent SW1444, though smooth by the criteria used, was rapidly cleared ($K = 0.137$; Fig. 2) from such mice. Testing the virulence of recombinants from crosses of these two strains indicated that the low virulence of the *S. abony* parent ($\text{LD}_{50} = 10^6$ bacteria) was determined by the joint effect of two loci (chromosomal regions), one between *inl* and *metA* and the other between *strA* and *aroD* (11, 14, 17). The presence of one only of these "low-virulence" loci in a recombinant results in intermediate virulence ($\text{LD}_{50} = 10^5$ bacteria), as shown previously (11). The nonvirulent *S. abony* Hfr strain SW1444 is susceptible to rapid clearance, and it might therefore have been expected that one of the two "low-virulence" loci determined the susceptibility to rapid clearance of strain SW1444 and thus its diminished virulence. However, the smooth recombinant C5R, obtained from a backcross in such a way that it had both the *S. abony* "low-virulence" loci in an *S. typhimurium* genetic background, was as nonvirulent as the *S. abony* parent ($\text{LD}_{50} = 10^6$ bacteria; 11), but was no more

rapidly cleared ($K = 0.007$; Fig. 2) than the virulent *S. typhimurium* parent C5P. It therefore appears that both of the "low-virulence" loci determine diminished virulence by mechanisms other than susceptibility to rapid clearance. The physiological basis and genetic determination of the susceptibility of SW1444 to phagocytosis remain unknown. It is therefore not yet feasible to test whether this character would by itself affect virulence (e.g., in a recombinant lacking the two mapped "low-virulence" loci). The slow clearance of the recombinant C5R presumably reflects inefficient phagocytosis (owing to limitation of antibody), since cells of C5R become susceptible to rapid clearance upon opsonization with immune serum (Table 3).

Biozzi and his colleagues (3) reported that a nonvirulent *E. coli*-*S. typhimurium* recombinant was rapidly cleared from the blood, like its nonvirulent *E. coli* donor parent and unlike its virulent *S. typhimurium* recipient parent. Although the recombinant possessed the characteristic somatic and flagellar antigens of the virulent parent, minor differences in the surface antigens of such a recombinant and the recombinant C5R (described here) may account for the difference in behavior of the two types of recombinants in clearance tests.

The nonvirulent recombinant C5R and the virulent C5P were both poorly phagocytized initially in normal mice (Fig. 2 and 3), and we therefore made a study of later events in the hope of discovering which of these events were related to the final outcome of infection. When intravenous challenge doses were 5×10^7 to 10×10^7 viable bacteria, both C5P and C5R were slowly cleared from the mouse circulation but re-emerged in the bloodstream after a variable time (Fig. 4). The re-emergence was characteristic (i.e., occurred earlier with the virulent C5P than with the nonvirulent C5R), even though both of the strains finally killed their hosts. However, if the challenge dose was 5×10^5 to 10×10^5 bacteria, a different situation developed (Fig. 5). The numbers of virulent C5P in the blood increased and the challenged mice died, but both of these events occurred later than in the experiments with the larger dose. There was no resurgence of the nonvirulent C5R, no bacteria were detectable in the blood 5 to 7 days after challenge (Fig. 5), and all of the mice survived. This characteristic of a smaller dose level has previously been observed in the *Salmonella*-mouse infection system (7).

Prior to death of mice challenged intraperitoneally with small numbers of virulent *S. typhimurium*, the bacterial population in the organs (associated with bacteremia) amounts to

10^8 to 10^9 bacteria (7). This was confirmed here in the case of mice dying from challenge with the virulent parent strain C5P (Fig. 5-7). It may therefore be assumed that death of animals infected with the virulent *S. typhimurium* was associated with marked bacterial proliferation. On the other hand, the nonvirulent recombinant C5R and the nonvirulent parent SW1444 failed to proliferate markedly (Fig. 5-7). Similar observations have been made with a relatively non-virulent, streptomycin-resistant mutant of *S. typhimurium* (7).

One may conclude that the recombinant C5R lacks the ability to proliferate in the tissues of the mouse, presumably because it possesses one or both of the two "low-virulence" loci of its non-virulent *S. abony* parent SW1444. This inability to proliferate is not related to nutritional requirement, because C5R is prototrophic, is not associated with loss of the smooth character (as determined in phage sensitivity tests), and does not result from susceptibility to rapid intravenous clearance.

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