INFECTION AND IMMUNITY, Sept. 1971, p. 177-188
Copyright © 1971 American Society for Microbiology

Vol. 4, No. 3
Printed in U.S.A.

Uridinediphosphogalactose-4-Epimerase Deficiency in *Salmonella typhimurium* and Its Correction by Plasmid-Borne Galactose Genes of *Escherichia coli* K-12: Effects on Mouse Virulence, Phagocytosis, and Serum Sensitivity

V. KRISHNAPILLAI[1]

Department of Medical Microbiology, Stanford University School of Medicine, Palo Alto, California 94305

Received for publication 4 May 1971

The synthesis of smooth lipopolysaccharide (LPS) in relation to mouse virulence and resistance to serum bactericidal activity in vitro and to rapid intravenous clearance in vivo was studied in *Salmonella typhimurium* by using a virulent [median lethal dose (LD_{50}) = 10^2], smooth, and genetically marked strain, a uridinediphosphogalactose epimerase-deficient mutant of it which was, therefore, rough, and a derivative of the mutant made smooth again by acquisition of the galactose-positive genes of *Escherichia coli*. The mutant was of reduced virulence (LD_{50} = 10^6) but the smooth derivative regained the virulence character typical of the parent. The nonsmooth phenotype also made the mutant, but not the smooth relatives (parent and derivative), susceptible to serum bactericidal activity and also to rapid intravenous clearance by phagocytosis by the liver. The mutant was similarly treated by germfree mice (expected to be relatively free of opsonizing antibodies). The clearance of the mutant could be impaired by prior intravenous inoculation of homologous bacteria or their LPS but was reversible by preopsonization of the second inoculum with nonimmune mouse serum, suggesting that the initial inoculum preempted the opsonizing antibodies. Independent evidence of clearance specificity was also provided in mixed inoculum experiments on impaired mice by the rapid clearance of an antigenically unrelated heptose-deficient mutant while maintaining the decelerated clearance of the epimerase mutant. The latter, however, was converted to accelerated clearance by the intravenous inoculation during the impaired state of anti-epimerase mutant immune mouse serum.

In various *Salmonella* species, mutants lacking uridine diphosphate (UDP)-galactose-epimerase (and formerly termed M mutants) are found to be much less virulent than their smooth parent strains (28, 31), presumably because they are rough, synthesizing lipopolysaccharide (LPS) of the chemotype termed Rc (Fig. 1). The availability of an epimerase-deficient mutant, derived from a smooth, mouse-virulent *Salmonella typhimurium* strain, and of its smooth derivative obtained by infecting it with an *Escherichia coli* K-12 F′-*gal*$^+$ plasmid (16) made possible a direct test of the effect of a known defect in LPS structure on virulence for the mouse, susceptibility to phagocytosis (as measured by rate of intravenous clearance of bacteria), and susceptibility to the bactericidal activity of serum. The role of "normal antibody" in the very rapid clearance of the epimerase-deficient cells was studied by the use of genetically marked, transductional derivatives of the epimerase-deficient strain, by experiments on the effect of opsonization by normal or immune mouse serum, and by experiments on the "impairment" of clearance caused by prior intravenous injection of homologous viable bacteria or LPS.

MATERIALS AND METHODS

Bacterial strains. The smooth, mouse-virulent *S. typhimurium* strain TV253 (*col El-30 leu1051 cysG1175 his C1150 malB479*) was derived from strain M7471, which is of the "FIRN" subtype, i.e., nonfimbriate, rhamnose- and inositol-negative (19). The UDP-galac-

---

[1] Present address: Department of Genetics, Faculty of Science, Monash University, Clayton 3168, Victoria, Australia.

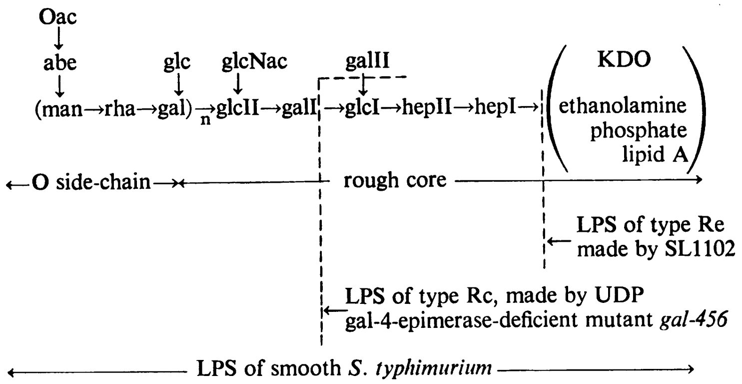

FIG. 1. *Lipopolysaccharide (LPS) structure of S. typhimurium. See (17, 24) for details. Abbreviations: abe, abequose; Oac, O-acetyl; man, mannose; rha, rhamnose; gal, galactose; glc, glucose; glcNac, N-acetyl glucosamine; KDO, 2-keto-3-deoxyoctonic acid; hep, heptose; n, number of repeating units of O side-chain.*

tose-epimerase-deficient mutant *gal-456*, isolated from TV253, and the corrected, smooth derivative of the mutant designated *gal-456* (F′-1-*gal*$^+$) have been described elsewhere (16). Strains *gal-456 cys*$^+$ and *gal-456 his*$^+$ were transductants obtained from *gal-456* as described elsewhere and were nonlysogenic for phage P22 and were confirmed to retain the identical phage pattern of *gal-456* (16). Strain TV253 *his*$^+$ was a *his*$^+$ transductant obtained from the smooth parent strain, TV253, and it was also nonlysogenic for phage P22 and confirmed to retain the identical phage pattern of TV253 (16). Strain SL1102 is a rough mutant, *rfaE543*, of a genetically marked *S. typhimurium* LT2 line (10); it synthesizes LPS lacking heptose and all sugars distal to it (Fig. 1). Strain SL1102 is resistant to high concentrations of streptomycin. *S. paratyphi* B strain Lister (15) and *S. enteritidis* strain Br′203C (30) are both smooth but serum-sensitive.

Media. These were as described (16). Nutrient agar was supplemented with streptomycin (1 mg/ml) where appropriate. Phosphate buffer (0.1 M, *p*H 7.4) was used as diluent.

LPS from UDP-galactose-4-epimerase-deficient mutant. Purified LPS was prepared by the method of R. G. Wilkinson by hot phenol extraction and Mg^{2+} precipitation (25) from an LT2 epimerase-deficient mutant, number G-30 (27). A solution of 20 mg/ml, made by heating lyophilized LPS in 0.85% NaCl at 100 C for 5 to 10 min, was diluted in buffer before use.

Mice. Female mice (18 to 22g) of the conventional (specific-pathogen-free) and germ-free lines of an inbred strain, CF no. 1, were purchased from Carworth Inc., New City, N.Y. Conventional mice were used in all experiments unless otherwise stated. The germ-free mice were used immediately upon arrival.

Virulence titrations. Mice were inoculated intraperitoneally with 0.2-ml volumes of serial decimal dilutions in buffer of overnight broth cultures (15). Mortality was recorded daily for 28 days, and the median lethal dose (LD_{50}) dose was calculated (26).

Sera. Pooled serum from nonimmunized (normal) conventional or from germ-free mice was collected as described (15). Anti-*S. typhimurium* and anti-*S. enteritidis* immune mouse sera (from mice vaccinated with heat-killed smooth bacteria) were obtained from E. P. Ornellas of this department. Anti-*gal-456* immune mouse serum was from mice inoculated intraperitoneally at weekly intervals with 1.8×10^5, 1.3×10^6, and 6.5×10^6 live bacteria of strain *gal-456* and bled two weeks after the final inoculation. Fetal calf serum was purchased from Hyland Laboratories, Los Angeles, Calif.

Adsorption of fetal calf serum. A 400-ml amount of *gal-456* culture [grown for 18 hr in Brain Heart Infusion broth (Difco) and having a viable count of 1.4×10^9/ml] was centrifuged, washed twice in saline, heated at 70 C for 2 hr, and washed three times, and the cell pellet was resuspended in 0.5 ml of saline. Twenty milliliters of serum was mixed with one-third of the cell suspension. After 6 hr at 4 C the, serum was freed of cells by centrifugation, and the adsorption was repeated twice more.

Titration of mouse serum by the bactericidal test. Mouse serum titrations were performed as described (30). Bacteria (grown in Difco Brain Heart Infusion broth) were sensitized by mixing about 2,500 cells in 0.1 ml of buffer with 0.4 ml of a dilution of mouse serum (presumed antibody source) and incubating for 30 min at 37 C and then for 2 hr at 4 C. To 0.1 ml of the suspension of sensitized bacteria, 0.4 ml of undiluted fetal calf serum (complement source) was then added. At 1 and 2 hr at 37 C, 0.1-ml samples were plated on nutrient agar (Difco). Three controls were always included: in the complement control, mouse serum was replaced by buffer; in the buffer control, both the mouse and fetal calf sera were replaced by buffer; in the mouse serum control, calf serum was replaced by buffer. zero-time counts were made by plating from the mouse serum control tube. The number of viable bacteria in the buffer controls at the end of 2 hr was always about the same as at zero time.

Measurement of clearance of intravenously inoculated viable bacteria from the blood stream. A 0.2-ml amount of a bacterial suspension was injected into the tail vein of a mouse and then, at intervals, 0.05 ml of blood was obtained from the retro-orbital venous plexus, diluted, and plated on appropriate agar media for the enumeration of recovery of viable bacteria

(15). As a precaution against intravascular clotting (1) heparin (100 units) was inoculated intravenously 5 to 10 min before inoculation of test bacteria. In experiments in which viable bacteria or LPS was inoculated, to effect immunological impairment, the heparin was inoculated together with the material used for impairment. When the inoculum was a mixture of 2 to 3 different strains, viable counts of the component strains were made by simultaneous platings on appropriate selective or indicator media. The clearance rate or "phagocytic index," *K*, was determined by calculating the slope of the regression line of viable count against time. The slope and its standard error (SE) and the intercept were calculated by the method of least squares on an IBM360 model 50 computer. In some experiments, the mice were killed (5 min after inoculation of bacteria), and viable counts were made from dilutions of homogenates of liver and of spleen (15).

Opsonization in vitro. One milliliter of undiluted serum and an equal volume of broth culture were mixed and held at 4 C for 20 min, and then 0.2 ml of the unwashed mixture of bacteria and serum was inoculated intravenously into mice (15). Preopsonization of *gal-456 cys*$^+$ with anti-*gal-456* immune mouse serum was done with 1:1,000 dilution of the serum (since under these conditions its agglutinating titer, observed microscopically, was 1:512), and, after 60 min, the bacteria were freed of unabsorbed antibodies by membrane filtration and rinsing with ice cold buffer and were then kept on ice until inoculated.

RESULTS

Mouse virulence. Table 1 shows that the smooth, parental *gal*$^+$ strain TV253 was highly virulent for the mouse by the intraperitoneal route. The LD_{50} calculated by the method of Reed and Muench (26) was 90 bacteria with a mean death time of 10 days. The smooth *his*$^+$ transductional derivative of TV253 also retained its virulence ($LD_{50} = < 11$ bacteria). A point that is worth making regarding the mouse virulence of highly mouse-virulent strains was the observation of "interference," a phenomenon somewhat analogous to that described in *Bordetella pertussis* infections (6–9). It is shown here by strains TV253 and *gal-456* (F′-1-*gal*$^+$) by the lower mortality encountered in groups of mice challenged with about 10^3 bacteria relative to mortality at lower doses. The lower mortality is usually accompanied by a longer mean time to death as shown by TV253 and TV253 *his*$^+$. Interference has also been observed with the unrelated strain C5 of *S. typhimurium* (14; *unpublished data*), and, although its characteristics are not well understood, there is preliminary evidence to suggest that it is immunological in nature (*unpublished data*). However, the epimerase-deficient mutant *gal-456* was much less virulent (Table 1), for in groups inoculated with doses corresponding to those used for strain TV253 there was either no or only a single death in any group. But, with higher doses, *gal-456* produced mortality, the LD_{50} being 1.1×10^6 bacteria, with a mean death time of about 6 days. The *cys*$^+$ and *his*$^+$ transductional derivatives of *gal-456* were also of low virulence, like *gal-456* itself. The spleens and livers from mice that died after challenge with *gal-456* were cultured on EMB galactose agar to determine whether deaths were due to proliferation of *gal*$^+$ and, therefore virulent, revertants. All colonies were galactose-negative even from mice that died after inoculation of only 10^4 to 10^5 bacteria. But the acquisition of the galactose-positive genes via the F′ plasmid of *E. coli* restored complete virulence to *gal-456*, as shown by the fact that the smooth, cured derivative *gal-456* (F′-1-*gal*$^+$) had an LD_{50} of 130 bacteria and a mean time to death of about 9 days, the virulence character typical of the original parent TV253.

Bactericidal activity of serum. Nonsmooth *Salmonella* strains are killed by normal mammalian sera (other than mouse serum which is deficient of a complement component) because such sera contain both complement and, probably, antibody against their somatic lipopolysaccharide (23). By contrast, smooth *Salmonella* strains are often unaffected, either because of absence of homologous O antibody in the sera or because their cells are little affected by complement even in the presence of homologous antibody. Strain *gal-456*, its smooth parent, and its cured derivative were therefore tested for sensitivity to the bactericidal effect of mouse serum, with fetal calf serum added as a source of complement reputedly free of antibody. It was found (Table 2) that cells of strain *gal-456* were killed (survival ca. 1%) by 2 hr of contact with fetal calf serum at 37 C, in the absence of any other source of antibody; by contrast, there was no killing of the *gal*$^+$ parent or the cured derivative strain, which indeed multiplied about fivefold during the 2-hr incubation period. This suggested that the fetal calf serum contained not only complement but also antibody against the type Rc (galactose-deficient) lipopolysaccharide of *gal-456* cells. Fetal calf serum absorbed three times with heat-killed *gal-456* cells did not kill *gal-456* cells and permitted their fourfold increase during 2 hr (Table 2). The absorbed fetal calf serum retained complement activity, as shown by its efficacy in another system, viz., the complement-dependent killing of a serum-sensitive smooth *S. enteritidis* strain Br′203C (30) by homologous immune mouse serum. Although *gal-456* cells multiplied in undiluted, absorbed fetal calf serum or undiluted serum from conventional mice, they were killed if both were present together (Table 2). Serum from conventional mice diluted 1 in 25 caused more than 50% mortality and even at 1 in 125 almost entirely pre-

TABLE 1. *Dose/mortality estimations for mice inoculated intraperitoneally with bacteria*

Strain	Inoculum	Mortality[a]	Mean time to death[b] (days)	LD_{50}[c] (no. of bacteria)
TV253	1.9×10^4	8/8	8	9.0×10^1
	1.9×10^3	3/8	15	
	1.9×10^2	6/8	10	
	1.9×10^1	4/8	10	
TV253*his*$^+$	1.1×10^4	5/5	6	$<1.1 \times 10^1$
	1.1×10^3	5/5	12	
	1.1×10^2	5/5	8	
	1.1×10^1	5/5	8	
gal-456[d]	9.1×10^6	9/10	5	1.1×10^6
	9.1×10^5	4/10	6	
	9.1×10^4	1/10	12	
	9.1×10^3	1/10	9	
gal-456 cys$^+$	7.2×10^6	4/5	9	1.2×10^6
	7.2×10^5	1/5	9	
	7.2×10^4	2/5	17	
gal-456 his$^+$	1.1×10^7	3/5	5	5.5×10^6
	1.1×10^6	0/5	0	
	1.1×10^5	1/5	7	
gal-456(*F′-1-gal*$^+$)	10^4	8/10	14	1.3×10^2
	10^3	4/10	10	
	10^2	6/10	9	
	10^1	5/10	11	

[a] Number of mice dead/number of mice challenged.

[b] Mean of times to death of all mice which died before termination of experiment, i.e., 28 days.

[c] Estimated by the method of Reed and Muench (26).

[d] No mortality was observed in additional groups of five mice inoculated with smaller doses, i.e., 9.1×10^2, 9.1×10^1, and 9.1×10^0 bacteria.

TABLE 2. *Survival of cells of strain gal-456 and its smooth parent and F′ derivative*[a]

Strain	Viable count after 2 hr of incubation in[b]								
	Absorbed fetal calf serum[c]	Conventional mouse serum[c]	Absorbed fetal calf serum[c] plus conventional mouse serum at concn				Absorbed fetal calf serum[c] plus germ-free mouse serum at concn		
			1:1	1:5	1:25	1:125	1:1	1:10	1:100
TV253	470	1,100	1,030						
gal-456	403	930	14	9	40	156	5	15	152
gal-456(F′-1-*gal*$^+$)	326	1,020	870						

[a] Incubated 2 hr at 37 C with absorbed fetal calf serum, serum from nonimmunized conventional or germ-free mice, or both.

[b] Values represent per cent of zero-time count.

[c] Used undiluted. (But in control tubes the omitted serum component was replaced by buffer.)

vented multiplication. By contrast, the galactose-positive parent and F′ derivative strains multiplied in the presence of even undiluted conventional mouse serum plus absorbed fetal calf serum (Table 2). The serum from germ-free mice, which would presumably have had less antigenic stimulation by bacterial LPS, likewise killed *gal-456* cells when supplemented with absorbed fetal calf serum, a 1:10 dilution causing 85% mortality and even a 1:100 dilution restricting multiplication. It was considered that the survival and multiplication of cells of the galactose-positive parent and F′ derivative strains in the presence of undiluted conventional mouse serum plus absorbed fetal calf serum might result not from the absence of anti-O antibody in these sera but from an inherent resistance of the smooth cells to complement killing, even in the presence of mouse homologous (anti-O) antibody. Therefore, cells of these strains were tested for susceptibility to killing by fetal calf

TABLE 3. *Survival of cells of strains TV253, gal-456(F′-1-gal$^+$), and a serum-sensitive smooth strain incubated at 37 C with fetal calf serum and dilutions of immune mouse serum*

Strain	Time of incubation (hr)	Viable count, after incubation in[a]			
		Fetal calf serum	Fetal calf serum[b] plus immune mouse serum[c] at concn		
			1:100	1:1,000	1:5,000
TV253	1	301	45	20	30
TV253	2	801	111	53	67
gal-456(F′-1-*gal*$^+$)	1	312	50	50	30
gal-456(F′-1-*gal*$^+$)	2	683	110	121	85
Lister[d]	1	342	4	1	5
Lister[d]	2	727	<1	<1	<1

[a] Values represent per cent of zero-time count.

[b] Fetal calf serum was unabsorbed and used undiluted.

[c] Immune mouse serum was a pool from mice immunized with heat-killed smooth *S. typhimurium*.

[d] *S. paratyphi* B strain Lister was a serum-sensitive but smooth strain (15) with the same O-antigen constitution as *S. typhimurium* strain TV253.

serum plus various dilutions of homologous mouse immune serum, i.e., anti-4,5,12 serum, from mice vaccinated with heat-killed smooth *S. typhimurium* cells (Table 3). In samples taken after 1 hr both strains appeared somewhat sensitive, in that all dilutions (1:100, 1:1,000, and 1:5,000) of immune serum caused some killing (survivals 20 to 50%). After 2 hr of incubation, however, the viable counts had increased somewhat in all serum dilutions, but even the 1:5,000 dilution of immune serum prevented the ca. sevenfold multiplication which occurred in the control tubes containing fetal calf serum but no anti-O antibody. A known serum-sensitive smooth strain of the same O-antigen constitution, i.e., *S. paratyphi* B strain Lister, included as a control, was killed (survival < 1%) by exposure to even the 1:5,000 dilution of mouse immune serum plus complement (Table 3). Thus, the smooth parent and derivative strains of *gal-456* are somewhat sensitive to the bactericidal and bacteriostatic action of complement plus mouse anti-O antibody, and the failure of serum from conventional mice, supplemented with fetal calf serum, to kill or inhibit their multiplication (Table 2) was interpreted to be due to the absence of anti-4, 5, 12 bactericidal antibody from the serum of such mice. However, this interpretation may not be correct if the prozone phenomenon (20) was operative since dilutions of mouse serum were not tested against smooth strains (Table 2).

Rates of clearance of bacteria inoculated intravenously. As strain *gal-456* synthesizes LPS of chemotype Rc (Fig. 1), owing to its deficiency of UDP-galactose-epimerase (16), it was expected that it, but not its smooth parent TV253, would be rapidly cleared from the bloodstream after intravenous inoculation. Therefore, the clearance rates of a *his*$^+$ transductional derivative of TV253 (=TV253 *his*$^+$) and of a *cys*$^+$ transductional derivative of *gal-456* (=*gal-456 cys*$^+$) were measured in two separate groups of conventional mice (Fig. 2). As expected, the smooth, virulent transductional derivative of the parent strain TV253 was not detectably cleared ($K = 0.003 \pm 0.007$), whereas the nonvirulent epimerase-deficient *gal-456 cys*$^+$ was very rapidly cleared ($K = 0.316 \pm 0.031$).

Clearance of the epimerase-deficient mutant and its cured derivative, inoculated together into mice. It was next of interest to determine whether correcting the epimerase defect of *gal-456* by the F′-1-*gal*$^+$ plasmid from *E. coli* would prevent its rapid removal from the bloodstream. Three mice were injected intravenously with a mixture of *gal-456* and *gal-456* (F′-1-*gal*$^+$) so that each mouse received about 5 × 10^4 bacteria of each

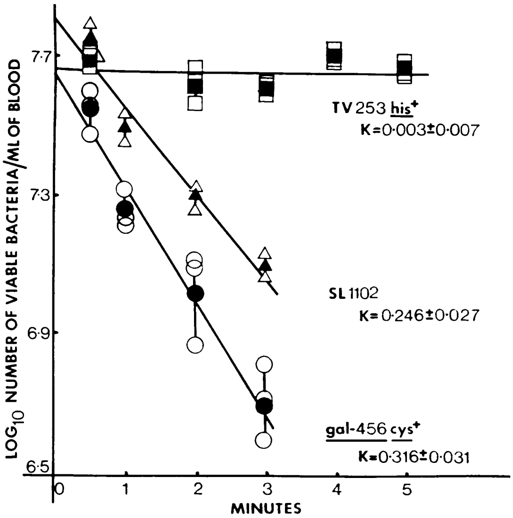

FIG. 2. *Rates of intravenous clearance of bacteria. Three separate groups of untreated mice were inoculated with about 10^8 viable bacteria per mouse. The slopes are regression lines (±1 standard error) calculated as described in the text. The open symbols indicate counts obtained from individual mice, and the closed symbols represent the geometric means. Symbols:* △ *and* ▲*, strain SL1102;* ■ *and* □*, strain TV253 his*$^+$*;* ○ *and* ●*, strain gal-456 cys*$^+$*.*

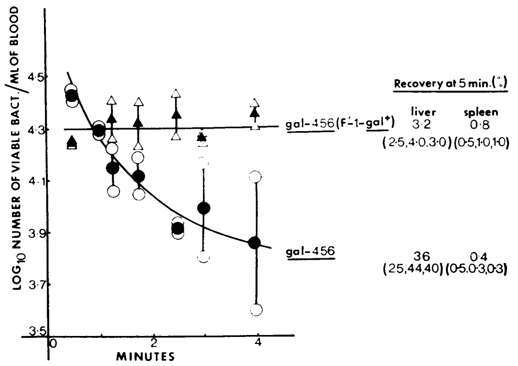

FIG. 3. *Intravenous clearance in untreated conventional mice of a mixture consisting of an equal number of cells of the epimerase-deficient mutant gal-456 and its cured derivative gal-456 (F'-1-gal+). Mice were inoculated with about 10^5 viable bacteria per mouse of the mixture. The slopes are regression lines fitted by eye. The open symbols indicate counts obtained from individual mice, and the closed symbols represent the geometric means. The per cent of the inoculum of bacteria recovered from the spleens and livers 5 min after inoculation are also shown (by the individual values and their arithmetic means). Symbols:* ○ *and* ●, *strain gal-456;* △ *and* ▲, *strain gal-456 (F'-1-gal+).*

strain. After a number of blood samples had been obtained, they were killed at 5 min and spleen and liver counts were made (Fig. 3). As expected the cured F' derivative was not rapidly removed, whereas *gal-456* was. The rapid disappearance of viable *gal-456* cells from the circulation evidently resulted in large part from their retention within the liver, for an average of 36% (range 25 to 44%) of the viable count of the inoculum was found in this organ (Fig. 3). By contrast, only 3.2% (range 2.5 to 4%) of the inoculum of *gal-456* (F'-1-*gal*+) was recovered from the liver. The number of bacteria recovered from the spleen was small for both strains, i.e., 0.4% of inoculum for *gal-456* and 0.8% for *gal-456* (F'-1-*gal*+).

Clearance of gal-456 and gal-456 (F'-1-gal+) inoculated together into germ-free mice. As opsonizing antibodies accelerate the clearance of bacteria from the bloodstream (1, 12), it seemed that rapid clearance of *gal-456* cells from the bloodstream of conventional mice might result from the presence in their sera of antibodies active on the type Rc (epimerase-deficient) LPS of *gal-456* cells. It was surmised that the sera of germ-free mice might lack such antibodies because of lack of antigenic stimulation by bacteria. Mixed inoculum experiments were therefore made in germ-free mice (Fig. 4). In all four mice, *gal-456* was cleared about as rapidly as in conventional mice, whereas the cured derivative was not detectably removed. The recovery of *gal-456* cells from the livers of the germ-free mice (48, 43, 37, and 40 of inoculum, with a mean of 42%) was about the same as in the case of conventional mice, as also were the recoveries of *gal-456* from the spleen and of the cells of the cured derivative from the liver and spleen. Thus it seems that the germ-free mice either possess adequate amounts of opsonizing antibodies active on *gal-456* or rapid clearance of "rough" bacteria of this sort is not dependent on possession of opsonizing antibodies.

Impairment of clearance of epimerase-deficient cells by prior inoculation of homologous viable bacteria and its partial reversal by preopsonization. If the very rapid clearance of *gal-456 cys*+ (Fig. 2) was due to the presence of opsonizing antibodies in the serum of conventional mice, then prior intravenous inoculation of an excess of the homologous viable bacteria should reduce the rate of clearance of a subsequent intravenous inoculum (12, 13), and preopsonization (in vitro) of the second inoculum with conventional mouse serum should restore clearance in such impaired mice (13). To distinguish the first and second viable inocula, transductional derivatives of *gal-456* were used. The first inoculum consisted of 5 × 10^{10} live *gal-456 his*+ cells. (This large dose was used because preliminary experiments showed

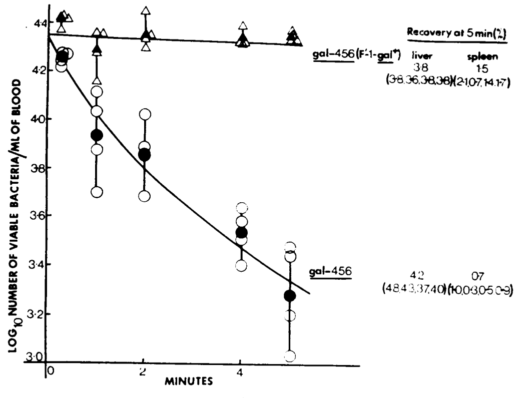

FIG. 4. *Intravenous clearance in untreated germ-free mice of a mixture consisting of an equal number of cells of the epimerase-deficient mutant gal-456 and its cured derivative gal-456 (F'-1-gal+). Mice were inoculated with about 10^5 viable bacteria per mouse of the mixture. The slopes are regression lines fitted by eye. The open symbols indicate counts obtained from individual mice, and the closed symbols represent the geometric means. The per cent of the inoculum of bacteria recovered from the spleens and livers 5 min after inoculation are also shown (by the individual values and their arithmetic means). Symbols:* ○ *and* ●, *strain gal-456;* △ *and* ▲, *strain gal-456 (F'-1-gal+).*

that inocula of 5 × 10^9 to 10^{10} cells did not cause substantial impairment of clearance of the second inoculum. The first inoculum would by itself have killed the mice in a few hours, but it seemed unlikely that its toxic effects would influence the outcome of the clearance experiments since these were completed within 8 min after the first inoculum was given.) Five minutes after the first inoculum, three mice were injected intravenously with nonopsonized *gal-456 cys*$^+$ and three with *gal-456 cys*$^+$ preopsonized by incubation in normal conventional mouse serum. In the latter case, the mice were inoculated with the bacterial-serum mixture. The results (Fig. 5) showed that, as expected, the prior inoculation of 5 × 10^{10} *gal-456 his*$^+$ cells interfered with the clearance of the nonopsonized *gal-456 cys*$^+$ (K = 0.042 ± 0.021, as compared to clearance in nonimpaired mice, K = 0.316 ± 0.031; Fig. 2). Preopsonization of *gal-456 cys*$^+$ cells in normal mouse serum accelerated clearance in impaired mice, K for the opsonized cells being 0.118 ± 0.009 and for nonopsonized cells 0.042 ± 0.021 (Fig. 5).

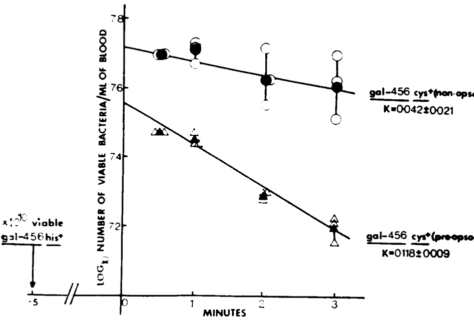

FIG. 5. *Rates of intravenous clearance in conventional mice impaired 5 min previously with viable cells of gal-456 his*$^+$. *Two groups of such impaired mice were inoculated either with nonopsonized gal-456 cys*$^+$ *(1.1* × 10^8 *per mouse) or with preopsonized gal-456 cys*$^+$ *(6.2* × 10^7 *per mouse). The preopsonization was with undiluted normal conventional mouse serum (see text), and mice were injected with the serum-bacterial mixture. The slopes are regression lines (±1 standard error) calculated as described in the text. The open symbols indicate counts obtained from individual mice, and the closed symbols represent the geometric means. Symbols:* ○ *and* ●, *strain gal-456 cys*$^+$ *(nonopsonized);* △ *and* ▲, *strain gal-456 cys*$^+$ *(preopsonized).*

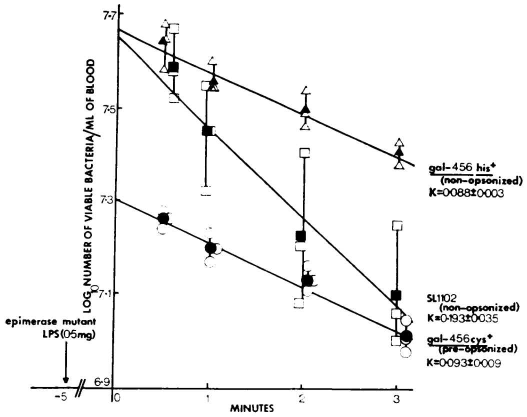

FIG. 6. *Rates of intravenous clearance in conventional mice impaired 5 min previously with 0.5 mg per mouse of type Rc LPS (from an epimerase-deficient mutant). Such impaired mice were each intravenously inoculated with a mixture consisting of preopsonized gal-456 cys*$^+$, *nonopsonized gal-456 his*$^+$, *and nonopsonized SL1102 (3.5* × 10^7, *9.7* × 10^7, *and 9.0* × 10^7 *cells, respectively, per mouse). The preopsonization was with 1:1,000 dilution of immune mouse serum (see text), and before inoculation the bacteria were freed of unabsorbed antibodies by membrane filtration and were rinsed with ice-cold buffer. The slopes are regression lines (± 1 standard error) calculated as described in the text. The open symbols indicate counts obtained from individual mice, and the closed symbols represent the geometric means. Symbols:* ○ *and* ●, *strain gal-456 cys*$^+$ *(preopsonized);* △ *and* ▲, *strain gal-456 his*$^+$ *(nonopsonized);* □ *and* ■, *strain SL1102 (nonopsonized).*

Impairment of clearance of epimerase-deficient bacteria by inoculation of homologous LPS and effect of preopsonization with immune serum. In the experiment recorded in Fig. 5, preopsonization of *gal-456* cells in normal mouse serum, although it accelerated their clearance in impaired mice (K = 0.118 ± 0.009 for preopsonized cells; K = 0.042 ± 0.021 for nonopsonized cells), did not restore the very rapid clearance (K = 0.316 ± 0.031; Fig. 2) seen in nonimpaired mice. The incompleteness of the reversal of impairment by opsonization might have resulted from rapid dissociation of antibody from the serum-treated *gal-456 cys*$^+$ cells when the mixture of bacteria and serum was diluted by intravenous injection; the large excess of *gal-456 his*$^+$ cells, injected 5 min earlier, would take up nearly all such dissociated antibody. In a further experiment (Fig. 6), specific impairment was achieved by injecting 0.5 mg of type Rc LPS from an epimerase-deficient mutant of *S. typhimurium* LT2 (*see above*), instead of by injection of genetically marked live bacteria. Immune mouse serum from mice vaccinated with live *gal-456* cells (*see above*) was used for opsonization, instead of normal mouse serum, with the idea that the induced anti-Rc antibodies from such mice would be less liable to dissociation on dilution. The immune serum was used at a dilution of 1 in 1,000 (to avoid O agglutination during opsonization); the period allowed for opsonization was extended from 20 to 60 min, and the bacterial cells were membrane-filtered and rinsed in the cold at the end of opsonization to avoid inoculation of unadsorbed antibody. Five minutes after the LPS injection, each of three mice was inoculated intravenously with a mixture of three components, in approximately equal parts: nonopsonized *gal-456 his*$^+$ cells, *gal-456 cys*$^+$ cells preopsonized in immune serum, and cells of SL 1102, a rough mutant of a different LPS type, Re, i.e., heptose-deficient (Fig 1). A preliminary experiment (Fig. 2) showed that SL1102 cells were rapidly cleared (K = 0.246 ± 0.027) after intravenous inoculation into normal mice. The nonopsonized *gal-456 his*$^+$ cells were much less rapidly cleared (K = 0.088 ± 0.003) than in normal mice (K = 0.316 ± 0.031); thus, prior injection of 0.5 mg of LPS of type Rc is sufficient to cause specific impairment. The SL1102 (heptose-deficient) cells were cleared at a rate (K = 0.193 ± 0.035) not significantly different from the rate observed in nonimpaired mice (K = 0.246 ± 0.027; Fig. 2). Additionally, the rapid clearance of SL1102 was correlated with a high recovery of these cells in the livers of impaired mice (40, 28 and 32% of inoculum, with a mean of 33%). These facts show that the injection of LPS of type Rc caused a specific, not a general, impairment of ability to clear rough bacteria from the blood stream. Contrary to expectation, preopsonization of *gal-456* cells in (diluted) immune serum had no detectable effect on their rate of clearance, the rate for preopsonized *gal-456 cys*$^+$ cells (K = 0.093 ± 0.009) not differing significantly from the rate for the nonopsonized *gal-456 his*$^+$ cells (K = 0.088 ± 0.003).

Acceleration of clearance of epimerase-deficient bacteria in impaired mice by intravenous inoculation of immune mouse serum. Because of the failure of preopsonization in dilute immune serum to reverse impairment (Fig. 6), the effect of intravenous inoculation of undiluted immune serum during clearance was next tested; this procedure has been shown to accelerate the rate of clearance of cells that were initially poorly phagocytized (3). Each of three mice was inoculated intravenously with 0.6 mg of LPS of type Rc and 5 min later with a mixture of *gal-456* and SL1102 cells. Blood samples were taken at intervals. At 4 min, 0.1 ml of undiluted anti-*gal-456* immune mouse serum was injected intravenously and further blood samples were taken up to 8 min. It was found in all three mice (Fig. 7) that the homologous LPS impaired the clearance of the epimerase-deficient *gal-456* cells (K = 0.068 ± 0.021 for times 0.25 to 3 min) but not that of the SL1102 cells (K = 0.179 ± 0.029); thus before serum infusion the heptoseless cells were cleared more rapidly than the epimerase-deficient cells.

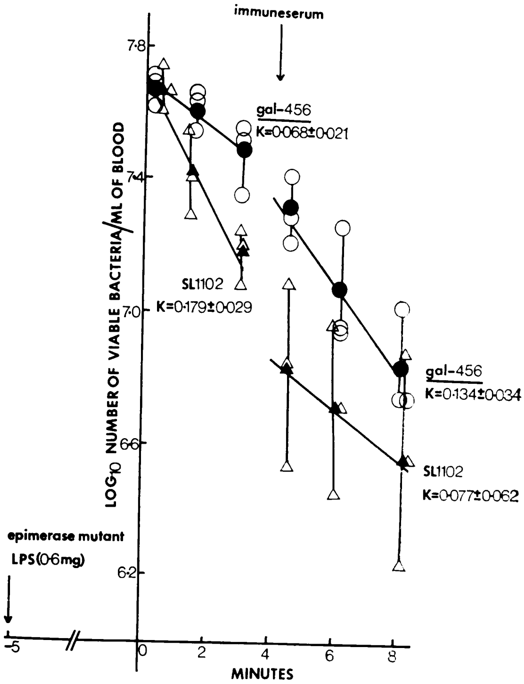

FIG. 7. *Rates of intravenous clearance in conventional mice impaired 5 min previously with 0.6 mg per mouse of type Rc LPS (from an epimerase-deficient mutant). Such impaired mice were each intravenously inoculated with a mixture consisting of gal-456 and SL1102 (1.4 × 10^8 and 1.8 × 10^8 cells, respectively, per mouse). At 4 min, undiluted anti-gal-456 immune mouse serum was injected intravenously. The slopes are regression lines (± 1 standard error) calculated as described in the text. The open symbols indicate counts obtained from individual mice, and the closed symbols represent the geometric means. Symbols:* ○ *and* ●, *strain gal-456;* △ *and* ▲, *strain SL1102.*

Comparison of the rates of clearance before and after the introduction of immune serum showed that, as expected, *gal-456* cells were more rapidly phagocytized after serum administration (K = 0.068 ± 0.021 for samples taken up to 3 min; K = 0.134 ± 0.034 for samples taken between 4 and 8 min). By contrast, clearance of the heptose-negative strain, SL1102, was slower after injection of immune serum (K = 0.179 ± 0.029 for times 0.25 to 3 min; K = 0.077 ± 0.062 for times 4 to 8 min). This difference presumably does not result from any effect of the injected serum on the rate of clearance of heptose-negative cells but is instead a consequence of the decrease in rate of clearance of any type of nonsmooth cells observed in experiments prolonged beyond 4 min or so, as indicated by the flattening-out of the plots of log viable count against time in such experiments (Fig. 3 and 4).

DISCUSSION

The strains used for mouse experiments, i.e., the mouse-virulent parent strain TV253, its UDP-galactose-epimerase-deficient mutant *gal-456*, and the "cured" derivative of the latter, were isogenic except for their *gal* genes and for the *his C* or *cys G* genes, in the case of the transductional derivatives of TV253 and *gal-456*. But, as these derivatives had the same virulence (Table 1) and phage patterns (16) as their *his*⁻ *cys*⁻ parents, these marker characters may be disregarded in considering the behavior of the strains after inoculation into mice. As was expected, the almost complete lack of epimerase in *gal-456*, with consequent inability to form smooth LPS, resulted in a very great reduction in virulence ($LD_{50} \geqq 10^6$, compared with $LD_{50} \leqq 100$ for the parent strain). But, on conversion to smoothness, the mutant regained the virulence character typical of the parent strain. The decreased virulence associated with inability to synthesize smooth LPS corresponds to that reported for epimerase-deficient mutants of various *Salmonella* species by Saito (28) and by Ushiba and Kitasato (31), of *E. coli* 0111:B4 by Medearis et al. (18), and for mutants of *S. typhimurium* strains LT2 and 395MS with various other defects in LPS synthesis (5, 22). The epimerase-deficient mutant was not totally nonvirulent since some mice died from inocula of ca. 10^4 to 10^5 cells, and evidently died as a result of multiplication of the mutant cells themselves rather than from growth of a galactose-positive (and therefore virulent) revertant since only galactose-negative bacteria were recovered from post-mortem cultures. It is not known whether the residual virulence of *gal-456* was related to the leakiness of its enzyme defect (16). This point could be determined by testing the virulence of mutants with deletions of the galactose genes derived from a mouse-virulent strain.

The nonsmooth phenotype resulting from the epimerase defect of *gal-456* made it susceptible to rapid removal from the circulation (phagocytic index $K = 0.316 \pm 0.031$; Fig. 2), whereas the smooth parent strain ($K = 0.003 \pm 0.007$; Fig. 2) and the cured derivative (Fig. 3) were not removed at a measurable rate. The different rates of clearance of *gal-456* and its galactose-positive relatives were observed both in comparisons of clearance rates in groups of mice each inoculated with a single strain and in comparisons of rates of clearance of galactose-positive and galactose-negative bacteria in mice inoculated with a mixture of the two strains (Fig. 3). Other investigations, including those of Nakano and Saito (21) and Hofman et al. (11) have shown that nonsmooth *Salmonella* injected intravenously into normal mice or newborn colostrum-deprived piglets are very rapidly removed from the circulation (and are correspondingly susceptible to rapid uptake by phagocytosis if injected intraperitoneally), whereas most smooth *Salmonella* strains are cleared only slowly (11, 15, 21; but see reference 15 for an instance of an apparently smooth *Salmonella* strain which was rapidly cleared from the circulation of nonimmunized mice). Medearis and his colleagues (18) observed similar susceptibility to phagocytosis of cells of an epimerase-deficient mutant of *E. coli* 0111:B4 inoculated intraperitoneally into normal mice, and Dlabac (4; *personal communication*) found that cells of an epimerase-deficient mutant of *S. typhimurium* LT2 were efficiently removed during perfusion of the livers of normal rats or newborn piglets. In both of these instances, bacteria grown in the presence of galactose were less rapidly removed, as a consequence of the restoration of smooth phenotype by utilization of exogenous galactose for LPS synthesis. In these experiments, counts of viable bacteria in the livers and spleens of mice killed 5 min after inoculation showed that a high proportion (25 to 44%) of the inoculated *gal-456* cells were recoverable from the liver but only 0.3 to 0.5% from the spleens. If one assumes that the liver of a 20-g mouse weighs about 1.35 g and its spleen about 0.15 g (2), it appears that on an equal-weight basis liver retains about nine times as many *gal-456* viable bacteria as spleen. Similar observations have been made in respect of clearance of a rough strain of *S. typhi* (3), but the liver was only twice as efficient as spleen in uptake of heat-killed *S. enteritidis* (2) and was no more efficient in the case of live cells of a smooth strain of *S. typhi* (3).

It is well established that opsonization of smooth *E. coli* by immune serum results in their rapid uptake by the liver (1), and susceptibility of *gal-456* to killing by normal mouse serum (plus an antibody-free source of complement) reported here indicated that the sera of the normal nonimmunized mice which were used here contained antibody with affinity for LPS of chemotype Rc, characteristic of *Salmonella* lacking UDP-galactose-epimerase. It was therefore suspected that the rapid clearance of *gal-456* cells from the circulation of normal mice resulted from their in vivo opsonization by such antibody. It seemed that germ-free mice, having received less antigenic stimulation from perhaps serologically related *E. coli*, might lack such anti-Rc antibodies. However, mixed-inoculation experiments in germ-free mice, of the same line as the conventional mice, showed the same rapid removal of *gal-456* cells, with recovery of a large fraction of the inoculum from the liver. This indicates either that phagocytosis of *gal-456* cells by the liver is not dependent on their opsonization or that germ-free mice have adequate concentrations of opsonizing antibody.

If the rapid removal of *gal-456* cells was due to in vivo opsonization by Rc-specific antibody, then prior injection of a large enough dose of bacteria or LPS of antigenic type Rc by preempting the antibody should prevent rapid clearance. As predicted, the intravenous inoculation of either 5×10^{10} live *gal-456* his^+ cells or of 0.5 to 0.6 mg of LPS of type Rc reduced the rate of clearance of *gal-456* cells (or its transductional derivative) injected 5 min later (K = 0.316 ± 0.031 in nonimmunized mice, but K = 0.042 ± 0.021, 0.088 ± 0.003, or 0.068 ± 0.021 in three groups of mice preinjected with bacteria or LPS of type Rc; Fig. 2, 5–7). The number of live bacteria or weight of LPS injected to preempt antibodies might themselves have killed the mice in a few hours, but, as the clearance experiments were completed within 8 to 13 min of the first injection, it does not seem likely that the toxicity of this material would have affected the rate of clearance of the test bacteria. Furthermore, it was found that the prior injection of 0.5 mg of Rc LPS did not retard clearance of epimerase-deficient cells by a nonspecific mechanism, such as saturation of the cells of the reticuloendothelial system, since it did not cause any significant reduction in rate of clearance of cells of the antigenically different, heptose-deficient strain, SL1102, included as one component of the test inoculum (K = 0.246 ± 0.027 in nonimpaired mice, K = 0.193 ± 0.035 in LPS-treated mice; Fig. 2 and 6) although prior injection of 0.6 mg of Rc LPS slightly reduced the clearance of SL 1102 (K = 0.246 ± 0.027 in nonimpaired mice; K = 0.179 ± 0.029 in LPS-treated mice; Fig. 2 and 7).

In the group of three mice pretreated with live bacteria of type Rc, the clearance rate of the test inoculum of *gal-456* cys^+ cells was 0.042 ± 0.021. In a second group given the same pretreatment, the test inoculum of *gal-456* cys^+ had been opsonized by incubation for 20 min in undiluted serum of conventional mice. This treatment resulted in clearance at a significantly more rapid rate (K = 0.118 ± 0.009; Fig. 5) but did not restore the rate observed in nonimpaired mice (K = 0.316 ± 0.031; Fig. 2). It was suspected that the incomplete restoration of normal rapid clearance might reflect low affinity of the postulated opsonizing antibody in the serum of nonimmunized mice, resulting in dissociation when the serum-bacteria mixture was diluted by inoculation into the mice. It was supposed that opsonizing antibodies in the serum of specifically immunized mice would have high affinity and, therefore, not dissociate on inoculation. However, in an experiment (Fig. 6) with mice pretreated with 0.5 mg of LPS, an inoculum of *gal-456* (genetically tagged) cells opsonized in immune mouse serum was cleared no more rapidly than the nonopsonized *gal-456* component of the mixed inoculum (K = 0.093 ± 0.009 for the cys^+ opsonized cells and 0.088 ± 0.003 for the nonopsonized his^+ component). It is not known why opsonization in immune serum failed to cause more rapid clearance; the immune serum was used at a dilution of 1:1,000 (to avoid O agglutination) and perhaps the concentration of antibodies or of adjuvant factors, e.g., complement components, in the diluted serum was insufficient for effective opsonization.

In mice which 5 min earlier had received 0.6 mg of type Rc LPS, the *gal-456* component of a mixed inoculum was, as expected, cleared more slowly than the heptose-negative SL1102 component (Fig. 7); injection of 0.1 ml of undiluted immune (anti-*gal-456*) mouse serum 4 min after the test inoculum resulted in acceleration of clearance of *gal-456* (K value for first 3 min = 0.068 ± 0.021; for last 4 min = 0.134 ± 0.034), whereas the clearance of the heptose-negative component became less rapid (K before injection of immune serum = 0.179 ± 0.029; after immune serum = 0.077 ± 0.062). The rates of clearance generally decline after the first 90% of the bacteria have been removed so that plots of log viable count against time if taken beyond this point are concave upwards (see Fig. 7, and references 1–3, 22), and it is therefore believed that the acceleration of clearance of *gal-456* after injection of immune serum was indeed caused by the opsonizing effect of the antibody in the serum.

All of the observations are compatible with the hypothesis that the rapid clearance of *gal-456* cells, largely by their uptake by the liver, results from the opsonizing effect of antibodies present in the serum of both conventional and germ-free mice. Furthermore, sera from both conventional and germ-free mice when supplemented with absorbed fetal calf serum proved bactericidal to *gal-456* cells. (Table 2). The killing of *gal-456* by normal mouse serum in the presence of specifically absorbed fetal calf serum (but by neither serum alone) was attributed to the presence of bactericidal antibodies against Rc LPS in the serum of both conventional and of germ-free mice, together with the sensitivity of *gal-456* to the bactericidal action of homologous anti-LPS antibody plus complement, a property typical of nonsmooth mutants. Similarly, the killing of *gal-456* by unabsorbed fetal calf serum alone was assumed to be due to the presence in such serum of antibody against Rc LPS. On the other hand, the lack of clearance of smooth cells (parent strain TV253 or cured derivative) in conventional and in germ-free mice (Fig. 2–4) and their survival in complement-supplemented conventional serum (Table 2) presumably reflect the absence of opsonizing and bactericidal antibodies against smooth *S. typhimurium* in such mice. Other authors have observed rapid clearance of rough *Salmonella* or *E. coli* or their susceptibility to in vitro phagocytosis or to serum killing in the absence or apparent absence of antibody (4, 18, 21, 29). The reasons for this difference from the results presented here are not known. The anti-Rc antibodies, opsonizing or bactericidal, in the serum of the CF no. 1 mice, conventional or germ-free, may have resulted from antigenic stimulation by serologically related material, such as live *E. coli* in the case of conventional mice or bacterial polysacchairde in the diet of the germ-free mice, or they may result from secretion of gamma globulins witn affinity for Rc LPS by a small proportion of antibody-synthesizing cells even in the absence of stimulation by any related antigen, a situation perhaps to be expected on the clonal selection hypothesis.

ACKNOWLEDGMENTS

I thank E. and Z. Schack and W. Nye for their help in the computer analysis of clearance data, E. P. Ornellas for provision of immune sera, and B. A. D. Stocker and D. G. MacPhee for valuable discussion.

This work was supported by Public Health Service research grant AI07168 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Benacerraf, B., M. M. Sebestyen, and S. Schlossman. 1959. A quantitative study of the kinetics of blood clearance of ^{32}P-labelled *Escherichia coli* and staphylococci by the reticulo-endothelial system. J. Exp. Med. 110:27–48.
2. Biozzi, G., J. G. Howard, B. N. Halpern, C. Stiffel, and D. Mouton. 1960. The kinetics of blood clearance of isotopically labelled *Salmonella enteritidis* by the reticulo-endothelial system in mice. Immunology 3:74–89.
3. Biozzi, G., and C. Stiffel. 1962. Role of normal and immune opsonins in the phagocytosis of bacteria and erythrocytes by the reticulo-endothelial cells, p. 249–266. *In* P. Grabar and P. Miescher (ed.), Mechanisms of cell and tissue damage produced by immune reactions. Benno Schwabe and Co., Basel.
4. Dlabac, V. 1969. On the significance of the lipolysaccharide structure of *Salmonella* mutants in different immune reactions. Colloq. Int. Centre Nat. Rech. Sci. 174:297–305.
5. Edebo, L., and B. Normann. 1970. Virulence and immunogenicity of mutant strains of *Salmonella typhimurium*. Acta Pathol. Microbiol. Scand. Section B 78:75–84.
6. Evans, D. G., and F. T. Perkins. 1954. The ability of pertussis vaccine to produce in mice specific immunity of a type not associated with antibody production. Brit. J. Exp. Pathol. 35:322–330.
7. Evans, D. G., and F. T. Perkins. 1954. Interference immunity produced by pertussis vaccine to pertussis infection in mice. Brit. J. Exp. Pathol. 35:603–608.
8. Evans, D. G., and F. T. Perkins. 1955. The production of both interference and antibody immunity by pertussis vaccine to pertussis infection in mice. Brit. J. Exp. Pathol. 36:391–401.
9. Fisher, S. 1955. Multiplication of *H. pertussis* in the mouse lung following intranasal infection. Aust. J. Exp. Biol. Med. Sci. 33:609–628.
10. Gemski, P., and B. A. D. Stocker. 1967. Transduction by bacteriophage P22 in non-smooth mutants of *Salmonella typhimurium*. J. Bacteriol. 93:1588–1597.
11. Hofman, J., V. Dlabac, and I. Trebichavsky. 1970. Significance of the surface structure of microorganisms in phagocytosis. Folia Microbiol. (Prague) 15:228.
12. Jenkin, C. R. 1962. An antigenic basis for virulence in strains of *Salmonella typhimurium*. J. Exp. Med. 115:731–743.
13. Karthigasu, K., and C. R. Jenkin. 1963. The functional development of the reticulo-endothelial system of the chick embryo. Immunclogy 6:255–263.
14. Krishnapillai, V., and L. S. Baron. 1964. Alterations in the mouse virulence of *Salmonella typhimurium* by genetic recombination. J. Bacteriol. 87:598–605.
15. Krishnapillai, V., and K. Karthigasu. 1969. *Salmonella abony-Salmonella typhimurium* recombinant nonvirulent for the mouse. J. Bacteriol. 97:1343–1351.
16. Krishnapillai, V., D. G. MacPhee, and B. A. D. Stocker. 1971. Properties of a *Salmonella typhimurium* mutant with an incomplete deficiency of uridinediphosphogalactose-4-epimerase. J. Bacteriol. 107:155–161.
17. Lindberg, A. A., T. Holme, C. G. Hellerqvist, and S. Svensson. 1970. Bacteriophage receptor development and synthesis of O-specific side chains after addition of D-galactose to the uridine diphosphate-galactose-4-epimerase-less mutant *Salmonella typhimurium* LT2-M1. J. Bacteriol. 102:540–547.
18. Medearis, D. N., B. M. Camitta, and E. C. Heath. 1968. Cell wall composition and virulence in *Escherichia coli*. J. Exp. Med. 128:399–414.
19. Morganroth, A., and J. P. Duguid. 1968. Demonstration of different mutational sites controlling rhamnose fermentation in FIRN and non-FIRN rha$^-$ strains of *Salmonella typhimurium*: an essay in bacterial archaelogy. Genet. Res. 11:151–169.
20. Muschel, L. H., 1965. Immune bactericidal and bacteriolytic reactions, p. 155–174. *In* G. E. W. Wolstenholme and J. Knight (ed.), Ciba foundation symposium on complement. J & A. Churchill Ltd., London.
21. Nakano, M., and K. Saito. 1968. The chemical compositions in the cell wall of *Salmonella typhimurium* affecting the clearance-rate in mouse. Jap. J. Microbiol. 12:471–478.
22. Nakano, M., and K. Saito. 1969. Chemical components in the cell wall of *Salmonella typhimurium* affecting its virulence and immunogenicity in mice. Nature (London) 222:1085–1086.
23. Nelson, B. W., and R. J. Roantree. 1967. Analyses of lipopolysaccharides extracted from penicillin-resistant, serum-sensitive *Salmonella* mutants. J. Gen. Microbiol. 48:179–188.
24. Nikaido, H. 1969. Structure of cell wall lipopolysaccharide from *Salmonella typhimurium*. 1. Linkage between O side chains and R core. J. Biol. Chem. 244:2835–2845.
25. Osborn, M. J., S. M. Rosen, L. Rothfield, and B. L. Horecker. 1962. Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of *Salmonella*. Proc. Nat. Acad. Sci. U.S.A. 48:1831–1838.
26. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent end points. Amer. J. Hyg. 27:493–497.
27. Rothfield, L., M. J. Osborn, and B. L. Horecker. 1964. Biosynthesis of bacterial lipopolysaccharide. II. Incorporation

of glucose and galactose catalyzed by particulate and soluble enzymes in *Salmonella*. J. Biol. Chem. 239:2788–2795.

28. Saito, K. 1951. On variation of mutabile type (murase) originated from rough phase of *Salmonella*; mode of variation and characteristics of each variant. Jap. Med. J. 4:347–359.

29. Sterzl, J., L. Mandel, I. Miler, and I. Riha. 1965. Development of immune reactions in the absence or presence of an antigenic stimulus, p. 351–370. *In* J. Sterzl (ed.), Molecular and cellular basis of antibody formation. Academic Press Inc., New York.

30. Steward, J. P., L. R. Collis, and R. J. Roantree. 1966. Effects of immunization of guinea pigs lacking bactericidal antibody against *Salmonella enteritidis*. J. Immunol. 97:224–230.

31. Ushiba, D., and B. Kitasato. 1952. Studies on mutabile type variation (murase) with special reference to influence of galactose on the variants. Keio J. Med. 1:269–281.