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Variability of Protection in Inbred Mice Induced by a Ribosomal Vaccine Prepared from *Salmonella typhimurium*

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Ribosomal vaccines prepared from *Salmonella typhimurium* were effective immunogens in A/J inbred mice and C_3H/He_{Tex} inbred mice. However, ribosomal vaccines were not protective in $C_{57}BL/6J$ inbred mice. A/J mice were protected against lethal challenge by attenuated *S. typhimurium* live-cell, ribosomal, phenol, and heat-killed vaccines. C_3H/He_{Tex} mice were protected by live-cell, ribosomal, and phenol vaccines but not the heat-killed vaccine. Only the live-cell vaccine gave significant protection in the $C_{57}BL/6J$ inbred mice. A comparison of the kinetics of infection in sham-immunized mice and mice immunized with ribosomes showed that ribosome preparations elicited protection against *Salmonella* infection in mice inherently sensitive and resistant to *Salmonella*.

Subcellular fractions have recently been recognized as potential alternatives to living, whole-cell vaccines. Protection of mice against lethal challenge by subcellular fractions was first observed with *Mycobacterium tuberculosis* (47, 48). Further investigation led to the discovery that the mycobacterial ribosomal fraction was the immunogen responsible for protection against experimental tuberculosis (49-51).

Investigators have now examined ribosomal fractions from numerous bacteria and fungi for their protective ability. Ribosomal immunogens derived from *M. tuberculosis* (27), *Salmonella typhimurium* (3, 15, 16, 19, 20, 23, 29, 32, 33, 39-43, 45), *Staphylococcus aureus* (46), *Pseudomonas aeruginosa* (34), *Streptococcus pneumoniae* (37, 38), *S. pyogenes* (30), *Neisseria meningitidis* (36), *Vibrio cholera* (14, 18), *Brucella abortus* (8), *Listeria monocytogenes* (23), *Francisella tularensis* (1), and *Histoplasma capsulatum* (11) have all been shown to be protective against challenge with the homologous organism. Some investigators have shown that ribosomal preparations may be as effective immunogens as vaccines currently available (1, 11, 14, 16, 22, 29, 30, 32, 42, 47, 48, 50).

Differences in host resistance to facultative intracellular parasites have been shown for eukaryotic and prokaryotic organisms (4, 5, 12, 13, 23, 25, 26, 29, 35, 44). Examination of the genetic resistance to *S. typhimurium* infection in inbred mice has been reported in the literature (13, 23, 25, 26, 29). However, little is known of the mechanism by which strains of mice differ in their resistance to salmonella infection. Since ribosomes from *S. typhimurium* have been shown to elicit protective immunity against the homologous organism and *Salmonella* is widely used in genetic resistance studies, an investigation was undertaken to determine whether the ability to immunize against salmonella infection with ribosomal preparations correlates with the sensitivity of the inbred mouse strain to salmonella infection.

MATERIALS AND METHODS

Animals. Male C_3H/He mice were obtained from Texas Inbred, Houston, Tex. Male A/J and $C_{57}BL/6J$ mice were obtained from Jackson Laboratory, Bar Harbor, Me. Adult male mice weighing 18 to 24 g were used in all experiments. Mice were housed 10/cage and given mouse chow and water ad libitum.

Organisms. *S. typhimurium* strain SR-11 was supplied by L. J. Berry, University of Texas, Austin. *S. typhimurium* strain RIA was obtained from N. Bigley, Chicago Medical School, Chicago, Ill. Cultures were maintained on brain heart infusion agar (Difco). The mean lethal dose (LD_{50}) was determined by the method of Reed and Muench (28). The LD_{50} for SR-11 in the inbred mouse strains were as follows: C_3H/He_{Tex}, 1; A/J, 1; and $C_{57}BL/6J$, <10. The LD_{50} of the RIA strain was $>10^7$ for all three strains of inbred mice. Survival was measured over 30 days.

Preparation of cultures. Fernbach flasks containing 1.5 liters of brain heart infusion broth (Difco) were inoculated with 100 ml of an exponential broth culture of *S. typhimurium*. The flasks were incubated at 37°C for 8 h on a New Brunswick Scientific shaker. Cells were collected by centrifugation at 30,000 × *g* in a Sorvall RC-2B centrifuge equipped with an SZ-14 continuous-flow rotor and were

washed with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, containing 0.01 M $MgCl_2$ (TMB). Cells were stored in 50-g lots at −80°C.

Isolation of ribosomes. Washed cells in 100-g amounts were suspended in 400 ml of 0.01 M TMB. Deoxyribonuclease was added to the cell suspension at a concentration of 2 μg/ml. The bacterial cell suspension was disrupted in 75-ml stainless steel flasks containing 40 g of 0.017-mm glass beads by shaking for two 5-min periods in a Braun MSK cell homogenizer. Cells were cooled by circulating liquid CO_2 through the vibrating chamber of the homogenizer. The cell extract was filtered through a sintered-glass funnel to remove the glass beads. The resulting filtrate was centrifuged at 27,000 × *g* for 30 min to remove intact cells and cell debris. The supernatant fluid (SF-1) was centrifuged for 30 min at 27,000 × *g* to remove any remaining cell debris. This supernatant fluid (SF-2) was centrifuged at 125,000 × *g* for 1 h. The supernatant fluid (SF-3) from the ultracentrifugation was filtered through a sterile Nalge disposable filter containing a 0.45-μm grid membrane. Ribosomes were pelleted by centrifugation at 125,000 × *g* for 3 h. The ribosome pellet was homogenized in TMB buffer and checked for sterility by plating on blood agar.

Whole-cell vaccines. *S. typhimurium* strain RIA was grown in brain heart infusion broth for 8 h or until it reached exponential growth. Live whole cells were stored at a concentration of 1 × 10^9 bacteria/ml at −80°C. Phenol-killed RIA vaccines were prepared by adding 90% phenol to a 24-h culture of *S. typhimurium* strain RIA to obtain a final concentration of 0.5%. The phenol-treated culture was incubated for 24 h at 37°C and then checked for sterility by plating on blood agar. Heat-killed RIA vaccines were prepared by boiling the cells for 30 min, washing two times with sterile saline, and then resuspending in sterile saline. All vaccines were stored at a final concentration of 1 × 10^9 bacteria/ml at −80°C.

Immunizations and challenge. All mice were immunized intraperitoneally with 0.2 ml of the appropriate vaccine preparation. Each mouse received two injections of equal concentration of antigen 14 days apart unless otherwise stated. Ribosome doses were calculated in micrograms of protein. Mice were challenged with 100 LD_{50} 14 days after immunization.

Bacterial clearance by infected mice. At various intervals after intraperitoneal challenge with 100 LD_{50} of strain SR-11, ribosome- and sham-immunized mice were sacrificed. A 0.1-ml sample of blood was aspirated from the heart by cardiac puncture. Tenfold dilutions were made in sterile phosphate-buffered saline (PBS) (pH 7.2), and 0.1-ml portions were incorporated into Trypticase soy agar pour plates. The spleens were removed and washed three times in sterile PBS. The spleen tissue was weighed and then homogenized with 10 ml of cold, sterile PBS. One-milliliter portions of the appropriate 10-fold serial dilutions were incorporated into pour plates. All plates were incubated at 37°C for 24 h. Random colonies were verified as *S. typhimurium* by slide agglutination, using Difco *Salmonella* group B antiserum.

Biochemical assays. Protein was determined by the method of Lowry et al. (21), using bovine serum albumin fraction V as the standard. RNA was measured by the orcinol method (9), and deoxyribonucleic acid was measured by the diphenylamine procedure (2). *Escherichia coli* ribonucleic acid and pancreatic deoxyribonucleic acid served as standards.

Statistical evaluation. Significance levels for protection were determined by the Fisher exact probability test by the method of Siegel (31).

RESULTS

Dose response. To determine whether ribosomes could induce protection in the three inbred mouse strains, mice were immunized intraperitoneally with varying concentrations of ribosomes. One group of 10 mice received a single injection, whereas another group of 10 mice received a booster injection of equal concentration of ribosomes 14 days after the first immunization. The results for the single immunization are presented in Table 1. $C_{57}BL/6J$ mice were not protected by a single immunization with ribosomes at doses ranging from 25 to 400 μg of total protein. C_3H/He_{Tex} mice showed 50 to 80% protection, depending on the dose of ribosomes injected. A/J mice showed survival rates of 80 to 100%, depending on the dose used. Table 2 shows the protective ability of ribosomes in the inbred mouse strains that had received a booster immunization 14 days after the initial injection. None of the doses of ribosomes employed elicited any significant protection in the $C_{57}BL/6J$ mice. The use of a booster immunization in the C_3H/He_{Tex} mice increased the survival rates when compared with mice receiving a single injection of ribosomes. Maximum protection in the C_3H/He_{Tex} mice was observed when 400 μg of total protein was used. A/J mice receiving a booster immunization showed the same survival rates as the A/J mice receiving only a single injection. Therefore, to

TABLE 1. *Protective ability of ribosomes in selected inbred mouse strains*

Immunizing dose (μg of protein)	Survival[a] (%)		
	$C_{57}BL/6J$	C_3H/He_{Tex}	A/J
400	20	80[b]	100[b]
200	0	80[b]	100[b]
100	0	50[b]	100[b]
50	0	66.67[b]	80[b]
25	0	ND[c]	90[b]
0	0	0	0

[a] Mice were challenged with 100 LD_{50} of SR-11 14 days after immunization.

[b] $P \leq 0.025$.

[c] ND, Not determined.

obtain optimal protection by ribosomes in both the C_3H/He_{Tex} and A/J mice, a dose of 200 μg of ribosomes followed 2 weeks later with a booster of equal concentration was used as the immunization schedule.

Comparative immunogenicity of *S. typhimurium* vaccines. To compare the immunogenicity of the ribosomal preparations with that of other *Salmonella* vaccines, mice were immunized with the appropriate antigen and given a booster immunization 2 weeks later. The results of these experiments are presented in Table 3. Only the live, attenuated RIA vaccine was able to provide significant protection in the $C_{57}BL/6J$ mice. The ribosomal, phenol, and heat-killed vaccines gave no significant levels of protection in the $C_{57}BL/6J$ mice. In contrast, significant levels of protection were observed in C_3H/He_{Tex} mice immunized with either the live, attenuated RIA vaccine or ribosomes. In the A/J mice, live RIA cells and ribosomes provided high levels of protection. The slightly different levels between mice immunized with the live RIA and ribosomes were not significantly different in either the A/J or C_3H/He_{Tex} mice. Phenol-killed vaccine provided equal levels of protection in the A/J and C_3H/He_{Tex} mice. However, the heat-killed vaccine gave significant levels of protection only in the A/J mice. Levels of protection achieved by the phenol and heat-killed vaccines were significantly lower than those observed in mice immunized with the live RIA or ribosomes.

TABLE 2. *Effect of multiple immunization on the protective ability of ribosomes in selected inbred mouse strains*

Immunizing dose (μg of protein)	Survival[a] (%)		
	$C_{57}BL/6J$	C_3H/He_{Tex}	A/J
200 (2 times)	0	100[b]	100[b]
100 (2 times)	0	100[b]	90[b]
50 (2 times)	0	87.5[b]	90[b]
25 (2 times)	0	83.33[b]	100[b]
0 (2 times)	0	0	0

[a] Mice were challenged with 100 LD_{50} of SR-11 14 days after the final immunization.
[b] $P \leq 0.005$.

TABLE 3. *Comparative protection induced by various Salmonella vaccines*

Vaccine	Survival[a] (%)		
	$C_{57}BL/6J$	C_3H/He_{Tex}	A/J
SR-11 ribosomes[b]	10[c]	90	80
Live RIA[d]	60[c]	100[c]	100
Phenol-killed RIA[d]	0	60[c]	60
Heat-killed RIA[d]	0	0	60
Controls[e]	0	0	0

[a] Mice were challenged with 100 LD_{50} of SR-11 14 days after the final immunization.
[b] Mice were injected with 200 μg of protein two times.
[c] $P < 0.05$.
[d] Mice were immunized with 2×10^6 bacteria two times.
[e] Controls received 0.2 ml of TMB buffer two times.

Bacterial clearance in infected mice. The clearance of the bacterial challenge in ribosome- and sham-immunized mice was compared. At various intervals after challenge with 100 LD_{50}, mice were examined for the number of viable *Salmonella* in the spleen and blood. Figure 1A illustrates the bacterial growth in spleens from sham-immunized mice. Bacterial growth in the spleens of control C_3H/He_{Tex} and $C_{57}BL/6J$ mice was identical. No restriction of bacterial multiplication could be detected during week 1 of the postchallenge period. By day 8 postchallenge bacteria levels had reached 10^7 to 10^8 cells/spleen. Spleens from A/J control mice displayed some restriction of bacterial growth. The A/J mice survived for significantly longer periods of time, even though large numbers of salmonella could be recovered from their spleens.

Figure 1B illustrates the bacterial growth in spleens from mice immunized with ribosomes. In all three strains of inbred mice, no bacterial multiplication was evident for the first 3 to 5 days. Some bacterial multiplication occurred, but it was ultimately cleared by day 7 to 8. In the $C_{57}BL/6J$ mice immunized with ribosomes, logarithmic bacterial multiplication was detectable after day 8. Approximately 10^8 bacteria/spleen were recovered at death. In contrast to the $C_{57}BL/6J$ mice, C_3H/He_{Tex} mice immunized with ribosomes showed significantly lower numbers of bacteria than did the controls. In the C_3H/He_{Tex} mice, clearance began at approximately day 10, but low numbers of organisms could be recovered throughout the remainder of the postchallenge period. A/J mice immunized with ribosomes also showed a restriction of bacterial multiplication in their spleens. Both the A/J and the C_3H/He_{Tex} immunized mice had low numbers of *Salmonella* in their spleens after day 10. The bacterial population in the spleens of A/J mice were always 10- to 100-fold lower than the numbers observed in spleens from C_3H mice. Figure 2 illustrates the bacterial clearance from blood of mice immunized with ribosomes or those sham immunized. Figure 2A shows bacterial clearance in sham-immunized controls. Bacterial growth in blood from C_3H/He_{Tex} and $C_{57}BL/6J$ sham-immunized mice was similar to the previously

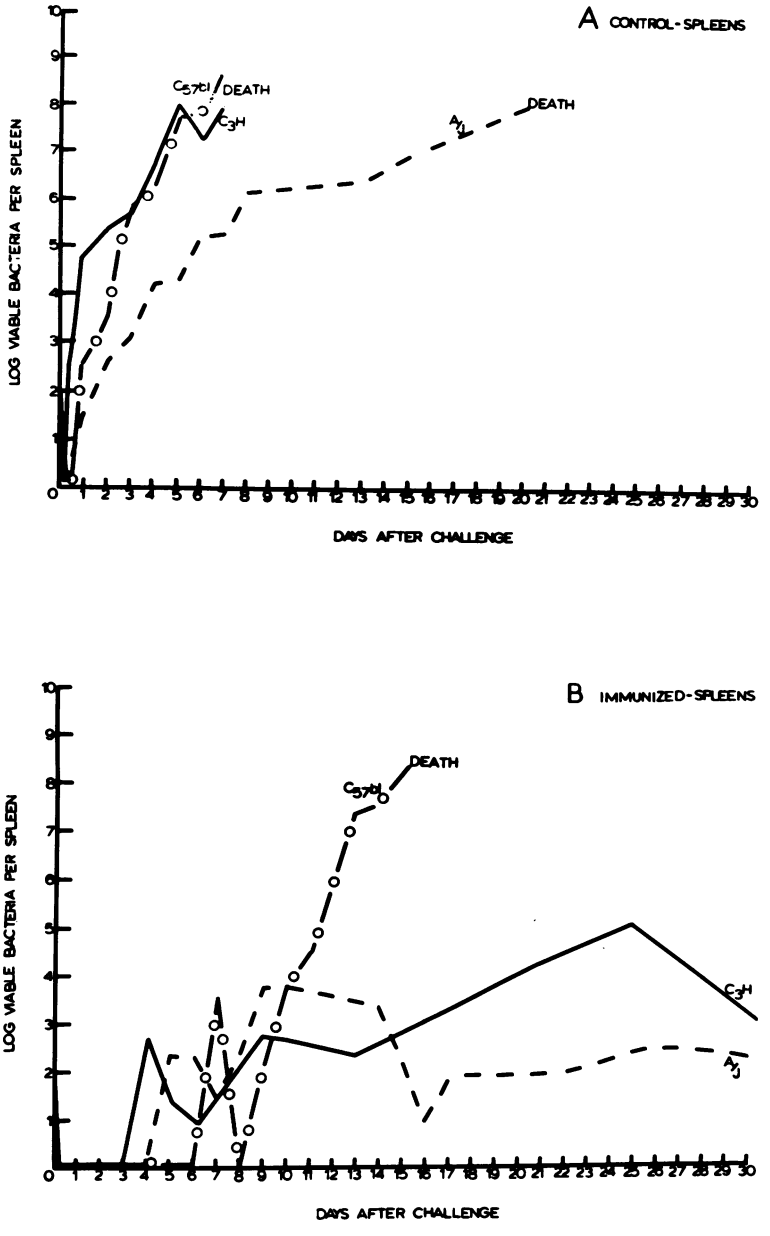

FIG. 1. *Log_{10} viable count (colony-forming units) of S. typhimurium SR-11 in spleens from control mice (A) and spleens from mice immunized with ribosomes (B). Each point represents the average of two mice. Challenge dose, 100 LD_{50} of S. typhimurium SR-11 intraperitoneally. Symbols: ——, C_3H/He_{Tex}; - - -, A/J; O, $C_{57}BL/6J$.*

observed results with spleens. Growth of *Salmonella* in the blood of control A/J mice was similar to that observed in spleens from control A/J mice (see Fig. 1A). Figure 2B illustrates bacterial miltiplication in the blood of mice immunized with ribosomes. $C_{57}BL/6J$ mice showed an initial clearance of organisms. This clearance was short-lived, and bacteria began to multiply until numbers of *Salmonella* reached lethal levels between days 13 and 15. The C_3H/He_{Tex} immunized mice also displayed an initial clearance. Even though detectable numbers of bacteria could be recovered after day 10, the bacteremia remained limited. In the A/J immunized mice, no detectable bacteremia was observed after day 9.

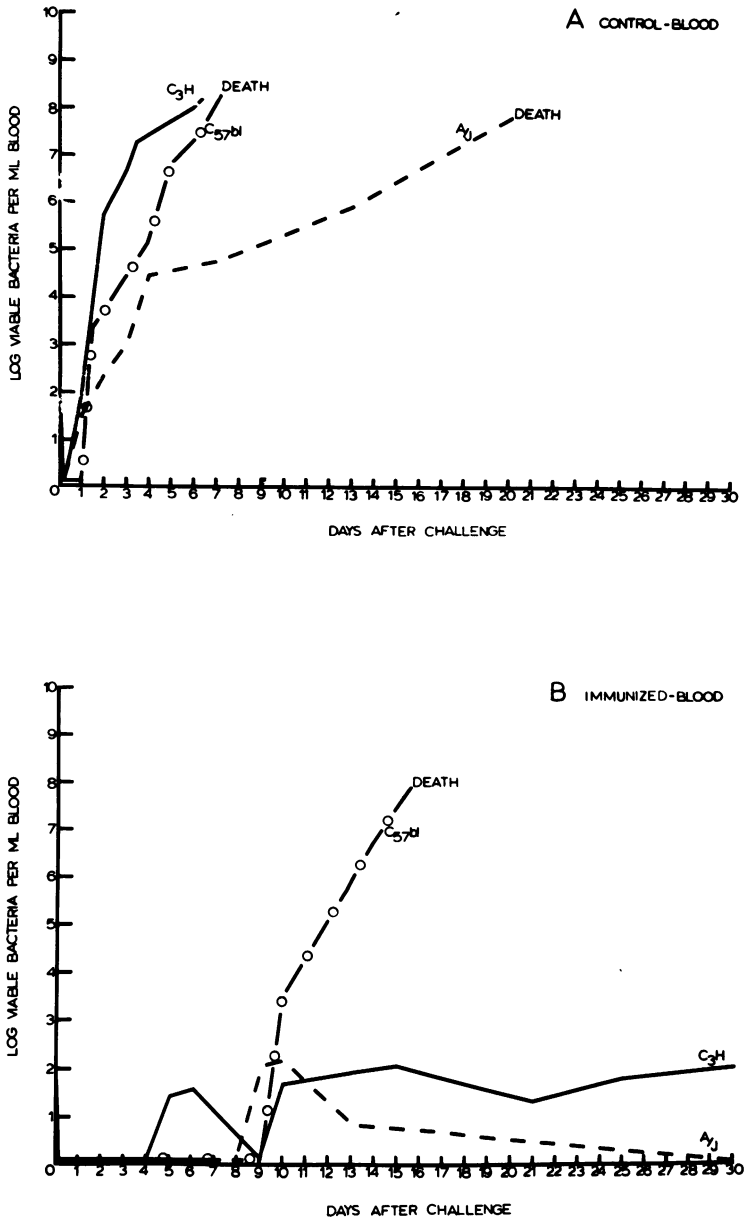

FIG. 2. *Log_{10} viable count (colony-forming units) of S. typhimurium SR-11 in blood from control mice (A) and blood from mice immunized with ribosomes (B). Each point represents the average of two mice. Challenge dose, 100 LD_{50} of S. typhimurium SR-11 intraperitoneally. Symbols: ——, C_3H/He_{Tex}; - - -, A/J; ○, $C_{57}BL/6J$.*

DISCUSSION

Since the first research with *Salmonella* ribosomes began, investigators have used an outbred mouse for the experimental animal of choice. Early work by Webster (44) and Gowen (12) showed that there exists a genetic basis for resistance to salmonella infection. Groschel et al. (13) also reported that the host response to salmonella infection is dependent on such factors as route of infection and the state of the reticuloendothelial system. Recent studies by Medina et al. (23) have shown that protection induced by salmonella ribosomes may in part

depend on the strain of mouse used. Whether a mouse strain can be judged as being sensitive or resistant to salmonella infection should not depend on the LD_{50}. Even though the LD_{50} for A/J mice is <10 organisms when given by the intraperitoneal route, Robson and Vas (29) and this paper have shown that the kinetics of infection in A/J mice qualifies these mice as resistant to salmonella infection. To distinguish between mice resistant and sensitive to salmonella infection, an examination of the kinetics of infections must be undertaken. The present study has shown that the A/J mice are resistant to *Salmonella* and the C_3H/He_{Tex} and $C_{57}BL/6J$ mice are sensitive to *Salmonella*. A consideration of the mouse strain used may be of some importance when investigating the mechanism by which salmonella ribosomes induce immunity.

In this study only the A/J mice were protected by all vaccines used. The C_3H/He_{Tex} mice were not protected by the heat-killed RIA vaccine. The live, attenuated RIA vaccine was the only vaccine able to induce significant protection in the $C_{57}BL/6J$ mice. The live RIA vaccine gave significant levels of protection in the C_3H/He_{Tex} and A/J mice. Although the level of protection achieved by the live, attenuated vaccine in the $C_{57}BL/6J$ was significant, it was lower than that observed in the A/J and C_3H/He_{Tex} mice. Vaccination with live, attenuated bacteria elicits cellular immunity which protects against lethal challenge (6, 7, 17). This phenomenon appears to hold true for the three inbred mouse strains used in this study. The lower level of protection observed in the $C_{57}BL/6J$ mice immunized with live cells may represent an inability to mount a cellular immune response to *Salmonella* infection equal to that observed in the A/J or C_3H/He_{Tex} mice (24, 25).

Ribosomal preparations appear to elicit an immunological response that protects the A/J and C_3H/He_{Tex} mice against lethal challenge. In contrast, $C_{57}BL/6J$ mice immunized with ribosomes were not protected against lethal challenge. These findings may indicate that the level of immunological response achieved by ribosomes in $C_{57}BL/6J$ mice is not as great as that achieved by the live, attenuated cells. However, to further examine this question it would be necessary to compare the levels of immunological response achieved by ribosomes or live cells at varying immunizing doses in mice challenged with graded doses of virulent bacteria.

A/J and C_3H mice immunized with phenol-killed vaccine showed similar levels of protection. However, the protection was significantly lower than that observed with either the live, attenuated cells or the ribosomal vaccine. The phenol-killed vaccine provided no protection in the $C_{57}BL/6J$ mice. The protection associated with the phenol-killed vaccine in our study differs from that observed by Robson and Vas (29), who showed that A/J mice were protected by a phenol-killed vaccine and the C_3H/He mice were not. These variations in results may be explained by the fact that immunization schedules were different and the immunized mice were challenged with 2×10^4 *S. typhimurium* Keller strain.

The heat-killed vaccine protected only the A/J mice. No protection was observed in the C_3H/He_{Tex} and the $C_{57}BL/6J$ mice. In contrast, Marecki et al. (22) have stated that the heat-killed vaccine prepared from the attenuated RIA strain was as effective an immunogen as the live attenuated vaccine at a challenge dose of 100 LD_{50}. However, Marecki et al. used the Swiss Webster outbred mice, and Robson and Vas (29) have reported that Swiss white mice behave similarly to A/J mice in vaccine-elicited resistance to salmonella infection. Protection induced by heat-killed vaccines may involve some other immunological mechanism than that associated with live or ribosomal vaccines (6, 7).

Investigators have shown that ribosomal vaccines are able to provide significant levels of protection. But neither the immunogenic moiety nor the mechanism by which they stimulate resistance is clearly understood. Many investigators have suggested that, in contrast to phenol-killed or heat-killed vaccines, ribosomes may be able to stimulate cellular immunity (33, 41).

In this study, an enhanced clearance of a lethal challenge has been shown in mice immunized with ribosomes. This clearance was observed in the A/J and C_3H/He_{Tex} mice. $C_{57}BL/6J$ mice that were not protected by ribosomes could not retard bacterial multiplication sufficiently to prevent death. When growth rates of bacteria in the spleens of control mice (Fig. 1) were examined, the C_3H/He_{Tex} and $C_{57}BL/6J$ mice were identical in their response to bacterial challenge. These findings indicate that the C_3H/He_{Tex} and $C_{57}BL/6J$ mice should be considered sensitive to salmonella infection. In contrast, Plant and Glynn (25) have shown that the C_3H/He mice are resistant to salmonella infection. The conflicting results may be explained by the differences of the route of injection and the challenge organism used in their studies.

An investigation of the defect in $C_{57}BL/6J$ mice that prevents them from being protected

by ribosomes may lead to an understanding of the mechanism by which salmonella ribosomal vaccines elicit protective immunity.

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