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Antibody Response and Protection Induced by Immunization with Smooth and Rough Strains in Experimental Salmonellosis[1]

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The antibody response of mice to a smooth strain of *Salmonella typhimurium* was shown previously to be extremely rapid and potent. As measured by the complement-mediated bactericidal reaction, it was also found to be highly specific as well as reproducible. Experiments which studied the effects of antigen type (live or heat-killed), antigen dose, and the route of immunization indicated that the most rapid and highest antibody response was achieved with live, smooth organisms injected by the intraperitoneal route. Living vaccines of rough strains of either *S. typhimurium* or *S. enteritidis* induced antibodies directed against the corresponding smooth organisms. The response to the rough strains was apparently due to antibody production rather than to the simple release of preformed natural antibody. The duration of protection conferred by the rough strain vaccines was closely correlated with the endotoxic content of the immunizing strain. Smooth heat-killed vaccines and a rough live vaccine protected against homologous but not heterologous challenge. In contrast, immunization with a smooth live vaccine protected mice against both homologous and heterologous challenge infections. Protection was not due to a local effect in the peritoneal cavity, since mice were also protected against subcutaneous challenge. The secondary antibody response, induced in immunized animals by the virulent challenge infection, was demonstrated to be rapid and potent, and hence a factor to be considered in protection.

In a previous report (8), the antibody response of mice to immunization with either live or heat-killed vaccines of *Salmonella typhimurium* was described. Antibody was measured by the extremely sensitive complement-mediated bactericidal reaction. Immunization or infection induced an extremely rapid and potent antibody response which would have been overlooked if one tested only for O agglutinins. Protective immunity, measured by survival to 100 LD_{50} challenge doses of the virulent organism, paralleled antibody level in mice previously immunized with either live or heat-killed bacteria. The present report extends these findings in regard to the reproducibility of the antibody response and the specificity of the antibody produced.

Ushiba et al. (16) reported that protection was induced by a live rough vaccine in the absence of O agglutinins. This result implied that anti-O antibody is not important in protection against challenge infection. Since the agglutinin response was not detectable soon after immunization, whereas the bactericidal antibody response was detectable (8), we investigated the antibody response to rough vaccines by the more sensitive method. Results of this investigation showed that the presence of the antibody response was demonstrable and that it was specific for the smooth homologous strain.

Since challenge infection itself constitutes a secondary stimulus in immunized animals, it was of interest to measure the antibody response to infection in animals immunized with either live smooth or rough vaccine. The response was shown to be rapid and potent, and a factor to be considered in immunity.

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MATERIALS AND METHODS

Bacterial strains. Four strains of *S. typhimurium* were employed in this investigation. Strain Suc LL (6) had an LD_{50} of 5×10^4 when injected intraperitoneally (ip) or subcutaneously (sc) into specific pathogen-free mice. This strain was used as the challenge organism for the smooth *S. typhimurium*. A strain of Suc EE (6) of reduced virulence was employed as the immunizing strain for the smooth *S. typhimurium*. It has an LD_{50} of 10^6 when injected ip into mice. Strain TV119, originally obtained from B. A. D. Stocker and kindly donated to us by J. W. Shands, is a rough variant of *S. typhimurium*. This mutant contains ketodeoxyoctonate (KDO), heptose, and other basal sugars as core constituents of the cell wall but lacks the O-specific side chain polysaccharide. This organism is classified as an R_{II} rouB mutant (2). The LD_{50} of this strain was 10^8 when injected ip into mice. Strain SL1102 was also obtained from B. A. D. Stocker's collection. This rough mutant contains KDO only as a core constituent, i.e., it lacks the heptose, the basal sugars, and the O-specific side chain polysaccharide (5). It is classified as chemotype Re (10). This mutant had an LD_{50} greater than 10^9 in mice when injected ip.

Three strains of *S. enteritidis* were obtained from Kazuhisa Saito. Strain no. 11 is a smooth organism (16), having an LD_{50} of 5×10^4 when injected ip into mice. Strain no. 11RX is a rough variant of strain no. 11 and has an LD_{50} of 10^6 for mice when injected ip. This mutant contains KDO, heptose, glucose, and galactose, but lacks mannose, rhamnose, and tyvelose (13).

From the known sugar content of the cell walls of TV 119, SL1102, and 11RX, and from the data concerning mouse lethality for these mutants, they can be classified according to degree of "roughness" as: SL1102 > TV119 > 11RX. Strain RB1 is an attenuated R-type mutant of no. 11 (15) which exhibited an LD_{50} of 5×10^6 when injected ip.

Serum-sensitive strains of *Escherichia coli* B and *S. typhosa* 0901 were obtained from Louis Muschel.

Mouse strain. Salmonella-free white, female CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) were employed in our experiments (8). Both control and experimental animals were checked repeatedly for the presence of *S. typhimurium* and *S. enteritidis* by culture of liver and spleen homogenates and cecal contents on SS agar (Difco). The organisms could not be isolated from uninoculated mice or mice that were immunized with heat-killed bacteria. Bactericidal antibodies directed against the test organisms could not be detected in undiluted sera of uninoculated mice, except in one batch of animals in which the titer (2.7) versus *S. typhimurium* was just detectable.

Immunization and collection of sera. Mice were immunized by ip, sc, or intravenous (iv) inoculation with either the live or the heat-killed (100 C for 1 hr) bacteria. Sample animals from each group were bled at intervals after immunization. The mice were exsanguinated under anesthesia, and their blood was collected from the severed axillary artery by means of a Pasteur pipette. Blood from four to six mice was pooled and allowed to clot at room temperature; the serum was separated by centrifugation. The pooled sera were stored at −25 C. Mice immunized with live, smooth bacteria were survivors of a minimal virulent infection which resulted in 10 to 25% mortality in different experiments.

Protection tests. The immunity of mice was challenged at various intervals after immunization by either ip or sc inoculation of 100 LD_{50} doses of the designated organism. Deaths were monitored for 21 days after challenge and protection was measured as percentage of survival. Nonimmunized mice, acting as controls for the lethality of the challenge infection, invariably were killed by 100 LD_{50} doses.

Bactericidal assay. Bactericidal antibodies were assayed as previously described (8). Briefly, 0.025 ml of a serum dilution (ranging from undiluted to 10^{-5} dilution) was added to a mixture of 0.05 ml of precolostral calf serum which was used as the source of complement and 0.025 ml of a bacterial suspension (10^5 bacteria per ml) in a 6-mm well of a plastic plate. The plate was incubated in a 37 C water bath for 1 hr, and 0.025-ml samples were removed and diluted into 1.0 ml of sterile saline. A 0.1-ml amount of this dilution was plated onto either SS agar or Trypticase Soy Agar (TSA, BBL) plates. Colonies were counted after 18 hr of incubation at 37 C. The bactericidal titer is expressed as the reciprocal of the serum dilution needed to kill 50% of the original inoculum. Smooth organisms were always employed as test bacteria in the assay system.

Induction of a sterile inflammation in the peritoneal cavity of mice. A sterile inflammation was induced by ip injection of 4.0 ml of a 2.0% sterile solution of starch in 0.85% saline. At various intervals (0, 6, 10, and 14 days) after injection of the starch, the resistance of the mice was challenged by ip inoculation of 100 LD_{50} doses of *S. typhimurium* Suc LL. Deaths were monitored for 21 days after challenge.

Rate of antibody response. The rate constant for the formation of circulating antibody during the interval from injection to peak titer was computed by the formulation of Taliaferro et al. (14), $K = (\ln A_2 - \ln A_1)/(t_2 - t_1)$, where $A_1 + A_2$ are the bactericidal titers measured at days $t_1 + t_2$ after immunization. K_1 is the initial rate; $K_2 + K_3$ are rates computed for changes in slope after K_1, if such changes occurred.

RESULTS

Bactericidal antibody response. The bactericidal antibody response in mice to both live and heat-killed smooth *S. typhimurium* was described previously (8). The reproducibility of this response in independent experiments is presented in Fig. 1. Each curve represents the response of one experimental group of animals and each point on the curve represents the titer of sera pooled from four to six mice. Titers were measured against *S. typhimurium* Suc LL, the virulent strain. Portions of the curves that extend to base line represent titers of less than 1.0, i.e., no detectable bactericidal antibody in undiluted sera.

The intensity of the response, as measured by peak titer, and the rate of response, as measured

by initial slope, were always greater in animals immunized with live bacteria (Fig. 1A versus Fig. 1B). The peak titers were higher by 10- to 100-fold, and the average K values were also greater with the live vaccine (initial $K = 3.77$ for 10^5 live bacteria versus 0.69 for 10^8 heat-killed bacteria). The decline from peak titer was consistently evident in animals inoculated with live cells, but the level ultimately rose to the peak level by 35 days. The peak titer level was maintained throughout the observation period in animals immunized with 10^8 heat-killed cells.

The live vaccine (actually a minimal virulent infection) multiplied throughout the initial post-inoculation period. This was indicated by quantitative culture of the liver and by the fact that the population reached a peak at 6 days (1.6 bacteria per liver) and declined thereafter (by the 20th day the population was 3.0×10^5) and by day 27 and thereafter it was undetectable. Thus, an increase in antigenic mass occurred during the period of most rapid antibody production. This increase in mass was much more effective than a roughly equivalent mass (10^8) administered in the killed state in one dose. This is further borne out by the minimal antibody response (Fig. 1C) to the initial mass (10^5), when it was administered in the killed state.

The variation in bactericidal titers between individual animals within a group was tested. The average of five individual determinations was in close agreement with titers measured in sera pooled from four to six mice.

In a series of experiments in which the route of injection, the antigen type, and the antigen dose were varied, 10^5 live bacteria, administered by the ip route, induced both the highest level of antibody and the most rapid response (Fig. 2). The K values of Table 1 represent a measurement of the rate of the response occurring before the peak titer was reached. For example, the antibody response in mice to an ip injection of 10^5 live bacteria showed an initial rapid rate of rise ($K_1 = 2.50$) which was followed by a slower rate of rise ($K_2 = 0.16$) to the peak titer. This same type of response was reported by Taliaferro et al. (14) for immune hemolysin produced in rabbits to Forssman antigen, except that the hemolysin response was slower. As compared to the response to an ip injection of 10^5 live bacteria, an intravenous injection of the same number of organisms was almost as great (peak titer 1.1×10^3), but the initial rate of the response was slower and the length of time to reach peak titer was more than 1 week longer. This same length of time to reach peak titer was evident in the response to an iv injection of 10^8 heat-killed cells, but not in the response to iv injections of a small number (10^2) of live bacteria or a small number (10^5) of heat-killed cells. The initial response to 10^5 live bacteria inoculated by the ip route was almost twice as rapid as the response to 10^8 heat-killed cells and six times as rapid as the response to the small dose (10^5) of heat-killed cells.

Specificity of the antibody response. To demonstrate that the observed antibody response was

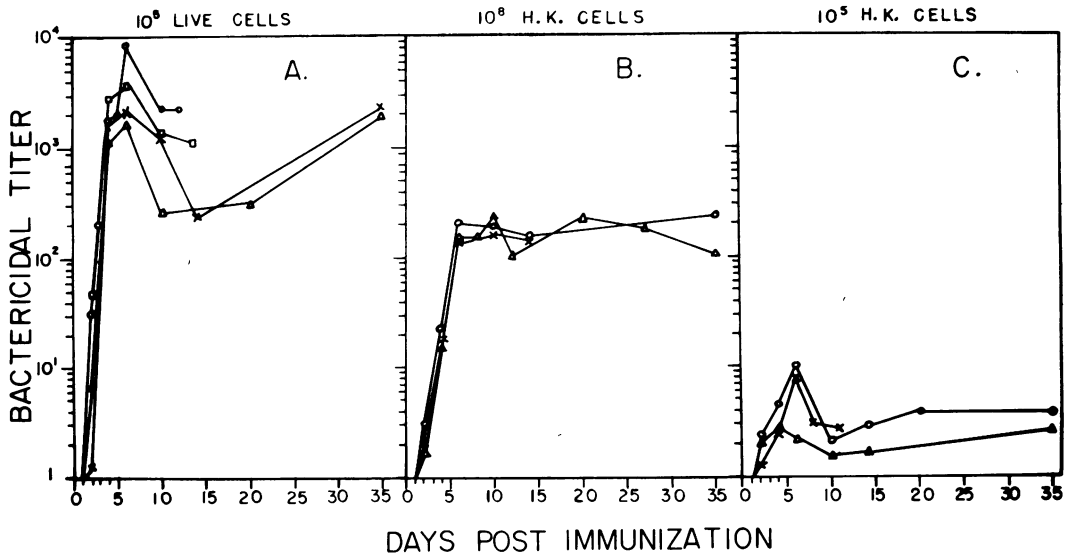

FIG. 1. *Bactericidal antibody response in mice at intervals after ip immunization with (A) 10^5 live, (B) 10^8 heat-killed, or (C) 10^5 heat-killed Salmonella typhimurium. Each curve represents an independent experiment.*

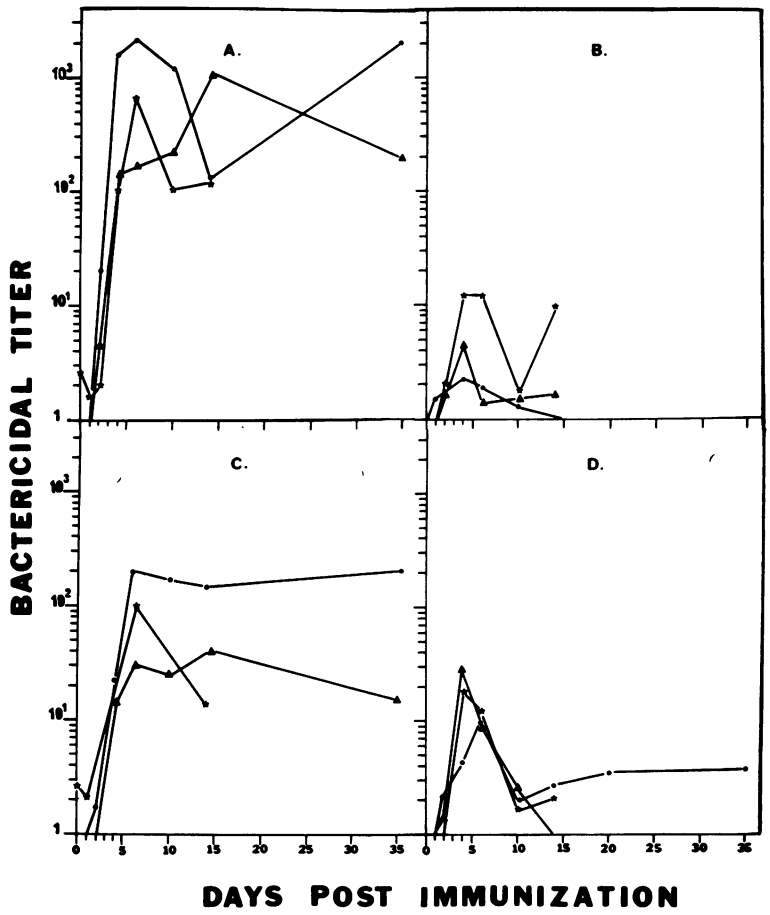

FIG. 2. *Bactericidal antibody response to various doses of live and heat-killed Salmonella typhimurium Suc EE administered by different routes. (A) 10^5 live bacteria, (B) 10^2 live bacteria, (C) 10^8 heat-killed cells, and (D) 10^5 heat-killed cells. Immunization: (●) intraperitoneal, (★) subcutaneous, (▲) intravenous.*

directed against the immunizing organism, experiments were designed to test the specificity of the response. Mice were immunized with either *S. typhimurium* or *E. coli*, and the sera from these mice were tested against homologous and heterologous organisms in the bactericidal assay system (Table 2). The bactericidal antibody was distinctly more active in the homologous systems than in the heterologous systems tested. This occurred when a small dose (10^5) of the live or a large dose (10^8) of the heat-killed smooth *S. typhimurium* were the immunizing organisms. The antibody response to a small dose (10^5) of heat-killed cells was minimal and the homologous and the heterologous titers were quite similar. However, the slight rise in the homologous titer at 6 days is probably significant, as it was reproducible on repeated testing.

Absence of a "local effect" in the peritoneal cavity. In a previous study (8), mice immunized intraperitoneally with 10^8 heat-killed *S. typhimurium* Suc EE were well protected against an ip challenge infection (100 LD_{50} doses of Suc LL). Evidence concerning salmonella infections has indicated a lack of protection by heat-killed vaccines (7, 12, 16). Ushiba et al. (16) observed a prolonged time to death with heat-killed vaccines in mice immunized and challenged by the ip route. This type of immunity conferred by the heat-killed vaccines appeared to be due to a local clearing of the challenge infection from the peritoneal cavity by peritoneal macrophages (1, 15). Recently, Blanden et al. (3) confirmed this observation. It seemed possible that the solid protection conferred by the heat-killed vaccine in the previous report (8) was the result of a local effect in the peritoneal cavity, i.e., a residuum of the inflammatory response produced by the vaccine inoculation. To test the effect of the presence of inflammatory cells in restricting a challenge in-

TABLE 1. *Bactericidal antibody response of mice to various doses of either live or heat-killed Salmonella typhimurium administered by the intraperitoneal, subcutaneous, or intravenous routes*

Route of injection	Antigen dose	Peak bactericidal titer	Days to peak titer	K values[a] measured from day 0 to peak titer		
				K_1	K_2	K_3
ip	10^5 live	2.2×10^3	6	2.50	0.16	—
sc		6.8×10^2	6	1.92	0.90	—
iv		1.1×10^3	14	1.73	0.08	0.60
ip	10^2 live	2.3	4	0.46	0.12	—
sc		1.2×10^1	4	0.62	—	—
iv		4.6	4	0.38	—	—
ip	10^8 heat-killed	2.0×10^2	6	1.20	—	—
sc		1.0×10^2	6	0.80	—	—
iv		4.0×10^1	14	1.30	0.40	0.03
ip	10^5 heat-killed	1.0×10^1	6	0.41	0.37	—
sc		2.8×10^1	4	1.44	—	—
iv		1.8×10^1	4	1.50	—	—

[a] K = rate constant determined by the equation $K = \ln A_2 - \ln A_1/t_2 - t_1$ (Taliaferro et al., 14) where A_1 and A_2 are the bactericidal titers measured at days t_1 and t_2 after immunization.

fection, a sterile inflammatory exudate was induced by injecting mice ip with 4.0 ml of a sterile 2.0% starch solution in 0.85% NaCl. At 0, 6, 10, and 14 days after the starch injections, mice were infected ip with 100 LD_{50} doses of the virulent strain *S. typhimurium* Suc LL. The results indicated that there was no significant protection of these mice against the virulent infection in terms of either survival or mean time to death, even though the number of peritoneal exudate cells in mice receiving the starch injections was 10-fold greater than the number found in normal mice.

As another test of the possibility of a local effect, mice were immunized ip with either 10^5 live or 10^8 heat-killed *S. typhimurium* Suc EE, and the immunity of these mice was challenged with 100 LD_{50} doses of Suc LL injected by the sc route. Mice immunized with either the live or the heat-killed vaccine were as well protected when challenged by the sc route as they were when challenged by the ip route (8).

Specificity of the protective response. The antibody induced by both live and heat-killed vaccines of *S. typhimurium* showed a high degree of specificity. Consequently, it was of interest to determine whether the specificity would be reflected in immunity, as measured by protection against a virulent challenge infection. Mice im-

TABLE 2. *Bactericidal activity of mouse anti-Salmonella typhimurium or anti-Escherichia coli serum versus homologous and heterologous test organisms*

Immunizing organism	Bactericidal titer[a]						Bactericidal test organism
	2 days[b]	4 days	6 days	10 days	12 days	14 days	
E. coli (10^8 heat-killed cells)	1.2	3.7	2.0	1.2	ND[c]	1.6	*S. typhimurium*
S. typhimurium (10^8 heat-killed cells)	3.0	22	200	190	ND	150	*S. typhimurium*
S. typhimurium (10^8 heat-killed cells)	1.3	16	54	18	21	ND	*E. coli*
	12	44	290	160	180	ND	*S. typhimurium*
S. typhimurium (10^8 heat-killed cells)	4.7	18	47	ND	26	ND	*S. typhosa*
	1.6	15	150	230	100	ND	*S. typhimurium*
S. typhimurium (10^5 heat-killed cells)	2.1	3.4	1.3	ND	1.8	ND	*S. typhosa*
	1.0	2.4	7.6	ND	2.7	ND	*S. typhimurium*
S. typhimurium (10^5 live cells)	ND	2.3	1.3	3.7	ND	1.0	*S. typhosa*
	ND	1,600	5,200	330	ND	240	*S. typhimurium*

[a] Titer expressed as the reciprocal of the serum dilution needed to kill 50% of the original inoculum. In all cases, titers measured at 0 days postimmunization (unstimulated mice) were <1.0.

[b] Days postimmunization.

[c] ND = not determined.

munized with 10^8 heat-killed *S. typhimurium* Suc EE, were well protected against a heavy challenge infection with the homologous organism, whereas similarly immunized animals were not well protected against a comparable heterologous challenge infection with *S. enteritidis* no. 11 (Fig. 3). The heterologous test showed little antibody activity and minimal protection. The reciprocal experiment, in which *S. enteritidis* immunization and *S. typhimurium* challenge were used, gave similar results (Fig. 4). Therefore, the specificity which was demonstrated with antibody induced by heat-killed vaccines, was reflected in the specificity as determined by challenge infection. However, mice immunized with 10^5 live, smooth *S. typhimurium* were as well protected when their immunity was challenged by the homologous strain of *S. enteritidis* as they were when challenged by the heterologous strain of this organism, even though the antibody titers for the homologous and the heterologous systems were quite different (Fig. 5).

Effect of challenge infection on antibody production. An experiment was designed to test whether the challenge infection itself was acting as a stimulus for a secondary antibody response in mice previously immunized. Mice previously immunized with 10^5 live *S. typhimurium* Suc EE were given a heavy challenge infection of 100 LD_{50} doses of Suc LL by the ip route, at 2, 4, 6, 10, and 35 days after primary infection (Fig. 6). Antibody levels were subsequently followed for 10 days after challenge. With the exception of mice challenged at days 2 and 10 following primary infection, the challenge infection induced significantly higher antibody titers, approximately

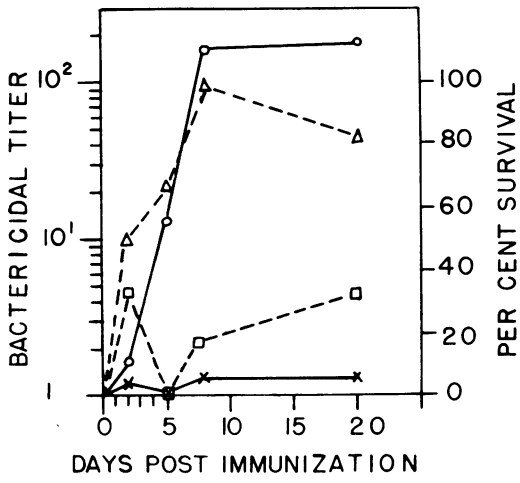

FIG. 3. *Specificity of the antibody response to immunization. Bactericidal titer (left axis) and the protective response (% survival, right axis) of mice immunized ip with 10^8 heat-killed Salmonella typhimurium. Bactericidal titer (○) determined with S. typhimurium as the test organism; bactericidal titer determined with S. enteritidis no. 11 as the test organism (×); % survival of mice challenged ip with 100 LD_{50} doses of S. typhimurium (△); survival of mice challenged ip with 100 LD_{50} doses of S. enteritidis no. 11 (□).*

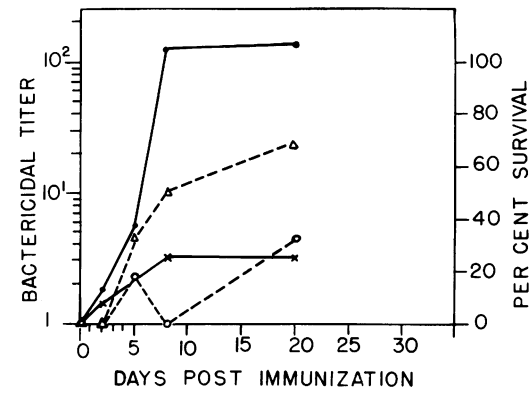

FIG. 4. *Specificity of the antibody response to immunization. Bactericidal titer (left axis) and the protective response (% survival, right axis) of mice immunized ip with 10^8 heat-killed S. enteritidis no. 11. Bactericidal titer determined with S. enteritidis as the test organism (●); bactericidal titer determined with S. typhimurium as the test organism (×); % survival of mice challenged ip with 100 LD_{50} doses of S. enteritidis no. 11 (△); % survival of mice challenged ip with 100 LD_{50} doses of S. typhimurium (○).*

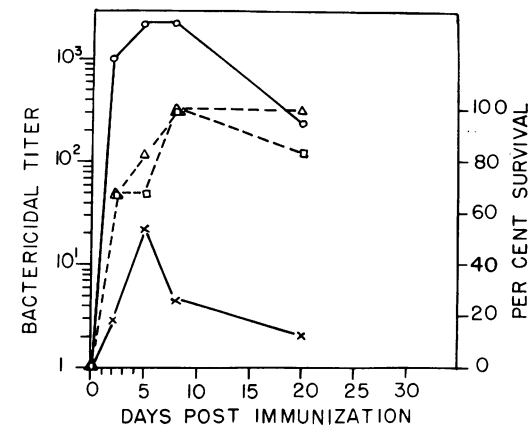

FIG. 5. *Specificity of the antibody response to immunization. Bactericidal titer (left axis) and the protective response (% survival, right axis) of mice immunized ip with 10^5 live Salmonella typhimurium. Bactericidal titer determined with S. typhimurium as the test organism (○); bactericidal titer determined with S. enteritidis no. 11 as the test organism (×); % survival of mice challenged ip with 100 LD_{50} doses of S. typhimurium (△); % survival of mice challenged ip with 100 LD_{50} doses of S. enteritidis no. 11 (□).*

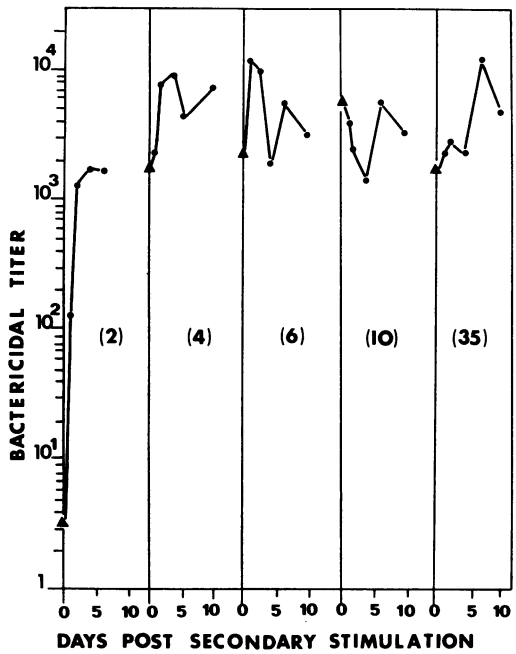

FIG. 6. *Bactericidal antibody response of mice to a secondary stimulus in the form of a challenge infection. Numbers in parentheses represent days after primary stimulation when mice were given a challenge infection. Response to the primary infection (10⁵ live Salmonella typhimurium ip (▲); response to the challenge infection (●) (100 LD₅₀ doses of S. typhimurium ip).*

a 5- to 10-fold increase over the primary response. (The secondary response on day 2 was no greater than it would have been to a primary stimulus.)

Immune response of mice immunized with rough mutants of Salmonella. Ushiba and co-workers (16) showed that a rough, R-type variant (no. 11RX) for *S. enteritidis* could confer solid immunity to mice against a challenge infection of the virulent, smooth S-type organism (no. 11). Subsequently, Ushiba et al. (15) were able to isolate cell-bound antibody from peritoneal macrophages taken from mice immunized with the S-type vaccine but not from mice immunized with an R-type vaccine. They also observed a marked "clearance phenomenon" in the peritoneal cavity of mice immunized with the S-type vaccine but not in mice immunized with the R-type vaccine. The implication was that immunization with rough *S. enteritidis* was protective in the absence of specific antibody directed against smooth bacteria. Nevertheless, the possibility existed that antibody directed against bacterial surface components was present in the sera of mice immunized with the R-type vaccine, in concentrations low enough to escape detection by the methods employed. In the present investigation, during the periods of greatest protection againt a heavy challenge infection of the S-type bacteria, strain no. 11 (*see* Fig. 7), mice immunized with 10^5 live *S. enteritidis* 11RX (an R-type vaccine) produced bactericidal antibody directed against the smooth virulent strain no. 11. A degree of protection (50 to 67% survival) was observed at a time when negligible amounts of bactericidal antibody were detected at 10 to 35 days after immunization. This does not necessarily exclude antibody from

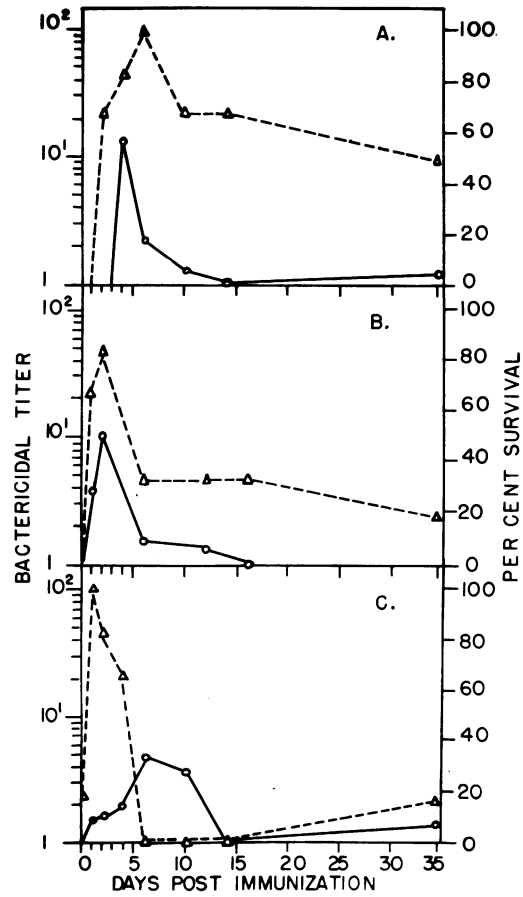

FIG. 7. *Bactericidal antibody (○, left axis) and protective (△, % survival, right axis) responses of mice at various intervals after immunization (ip) with rough strains of Salmonella. (A) Immunization: 10⁵ live S. enteritidis no. 11RX. Challenge: 100 LD₅₀ doses of S. enteritidis no. 11. (B) Immunization: 10⁷ live S. typhimurium TV119. Challenge: 100 LD₅₀ doses of S. typhimurium Suc LL. (C) Immunization: 10⁸ live S. typhimurium SL1102. Challenge: 100 LD₅₀ doses of S. typhimurium Suc LL. Challenge infections were administered by ip route. Bactericidal activity was tested against the corresponding challenge organism in each case.*

participating in the protective response, because, even though only low levels resulted from the primary stimulation by the R-type vaccine, the challenge inoculum (a smooth virulent organism) can induce a rapid and potent antibody response. This was demonstrated (Fig. 8) in the following way. Mice were immunized as before with 10^5 live no. 11RX. On days 2, 10, 14, 25, and 35 after immunization, these mice were given a heavy challenge infection of the virulent organism, strain no. 11. Antibody titers were followed for 4 to 6 days after challenge. In every case, except for day 25, mice responded to challenge infection by a 100-fold increase in bactericidal antibody within 4 days after challenge. The *K* values (Table 3) for the rapid rise to peak titer at days 2 to 4 after immunization or challenge indicated that the response to the challenge infection was indeed rapid. Yet the response was not quite as rapid as the response to primary infection with 10^5 live smooth *S. typhimurium*, administered by the ip route (*see* Table 1), but more rapid than the primary response to the immunization ($K = 1.0$ versus other *K* values in Table 2).

Mice were also immunized with rough variants of *S. typhimurium* (*see* Fig. 7 B and C). At various intervals after ip immunization with either 10^7 live strain TV119 or 10^8 live strain SL1102, mice were given a 100 LD_{50}, ip challenge infection of the smooth, virulent *S. typhimurium* Suc LL. These rough organisms were able to confer a high degree of protection (67 to 100% survival) within 1 day after immunization, a day earlier than in the case of immunization with the smooth *S. typhimurium*. However, this protection was transient. The duration of the protection seemed to be correlated with the endotoxic content of the immunizing strain. Mice immunized with TV119 showed a very rapid bactericidal antibody response that reached a peak titer at 2 days after immunization

TABLE 3. *Bactericidal antibody response to a primary intraperitoneal infection with Salmonella enteritidis 11RX and to a secondary intraperitoneal challenge infection with S. enteritidis no. 11*

Stimulation	Day[a]	Bactericidal titer[b]	*K* values[c]
10^5 11RX.....	0	1.5×10^1	1.07
100 LD_{50} no. 11....	2	1.1×10^2	1.70
100 LD_{50} no. 11....	10	1.7×10^2	1.59
100 LD_{50} no. 11....	14	1.8×10^2	2.28
100 LD_{50} no. 11....	25	1.3×10^1	1.32
100 LD_{50} no. 11....	35	1.8×10^2	2.61

[a] Numbers represent the time of challenge at various days postimmunization.

[b] Bactericidal titer reached at 4 days postimmunization or postchallenge.

[c] *K* values measured during the most rapid rise in the response (2 to 4 days postimmunization or postchallenge).

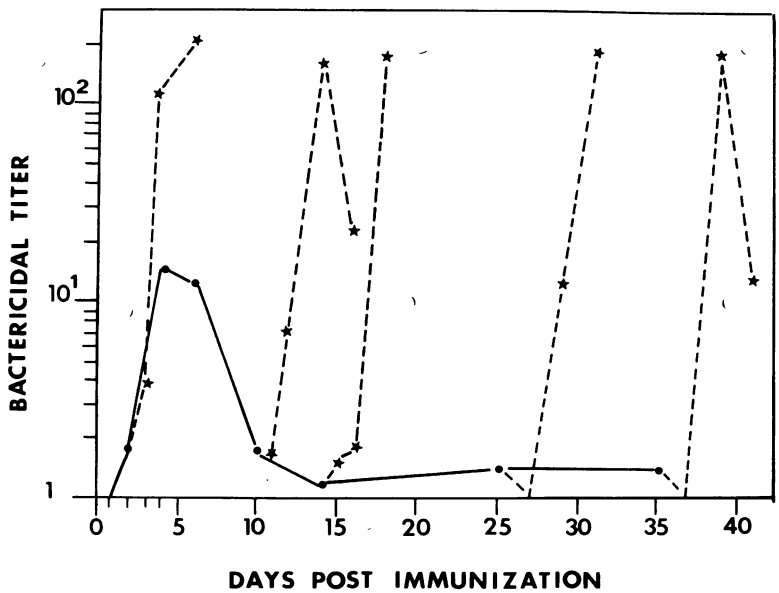

FIG. 8. *Bactericidal antibody response of mice immunized ip with 10^5 live Salmonella enteritidis no. 11RX and challenged ip with 100 LD_{50} doses of S. enteritidis no. 11 at 2, 10, 14, 25, and 35 days after immunization. Response to immunization (○); response to challenge (★).*

and dropped rapidly thereafter. This suggested that the rough mutant induced the release of preformed, "natural" antibody (11) to smooth bacteria and probably was unable to induce the production of antibodies directed against smooth organisms, owing to a lack of specific O-polysaccharide groups. To further test the ability of the rough strain to induce antibody formation, mice which were immunized previously with 10^7 TV119 were restimulated with the same dose of this rough organism 4 and 10 days after primary stimulation. The antibody response was followed for 6 days after secondary stimulation (*see* Fig. 9). The response to the secondary stimulus at 4 days, but not at 10 days, was apparently anamnestic because the titer reached a higher peak than in response to the primary stimulus. However, these were not typical anamnestic responses in that they did not appear to be more rapid than the primary response, and a longer latent period, or time before antibody production could be observed, was evident. However, the latent period may have been largely the result of antigen binding by the secondary inoculation. This was especially evident when the secondary inoculation was given 10 days after primary immunization. The observed secondary response would favor the idea that the rough organism was capable of inducing the formation of antibodies directed against smooth bacteria.

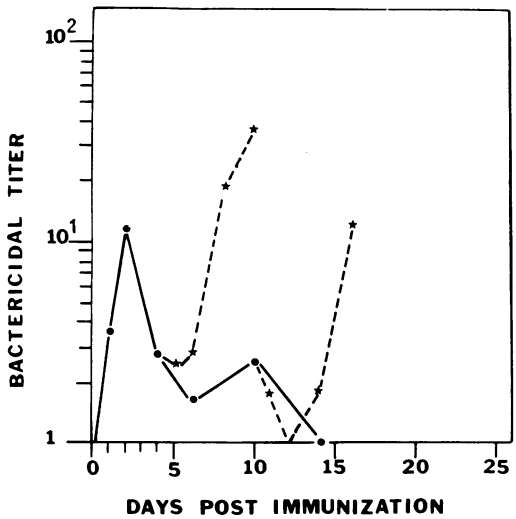

FIG. 9. *Bactericidal antibody responses to primary and secondary ip injections of 10^7 live Salmonella typhimurium TV119. Response to primary stimulation (●); response to secondary stimulation (★).*

Specificity of the antibody response to the rough organisms. Since the rough strains elicited antibodies directed against smooth bacteria, it was of interest to determine whether this response was specific for the corresponding smooth, virulent strains. Antibody responses to live vaccines of rough bacteria were more potent in homologous systems tested (Table 4) at peak titers (day 4 in *S. typhimurium* (7, 12, 16). It is conceivable that mice immunized with no. 11RX, day 2 in mice immunized with TV119, and days 6 and 10 in mice immunized with the extremely rough strain, SL1102). Apparently, some specificity was evident in these cases.

Immunization of mice with another rough variant of *S. enteritidis* no. 11, designated RB1, produced an antibody response similar to the response elicited by no. 11RX.

Specificity of the protective response in mice immunized with the rough Salmonella enteritidis. Rough strains were able to elicit antibody responses directed against smooth bacteria. These responses were apparently specific for the smooth homologous strain. Therefore, the question arose as to whether this specificity was reflected in the

TABLE 4. *Specificity of the bactericidal reaction involving mouse anti-rough Salmonella serum versus smooth Salmonella as test organism*

Immunizing organism	Bactericidal titer[a]							Bactericidal test organism
	1 day[b]	2 days	4 days	6 days	10 days	14 days	35 days	
S. enteritidis no.	<1.0	<1.0	13	2.5	<1.0	1.7	<1.0	*S. enteritidis* no. 11
11RX (10^5 live cells)	1.4	<1.0	3.8	1.7	<1.0	<1.0	<1.0	*S. typhimurium* Suc LL
S. typhimurium TV 119	2.4	13	ND[c]	1.4	1.3	1.0	<1.0	*S. typhimurium* Suc LL
(10^7 live cells)	1.6	1.5	ND	1.0	1.0	<1.0	<1.0	*S. enteritidis* no. 11
S. typhimurium SL	1.5	1.5	1.9	5.0	4.1	<1.0	<1.0	*S. typhimurium* Suc LL
1102 (10^8 live cells)	<1	<1	3.7	1.1	<1.0	<1.0	<1.0	*S. enteritidis* no. 11

[a] Bactericidal titer expressed as the reciprocal of the serum dilution needed to kill 50% of the original inoculum.

[b] Days postimmunization.

[c] ND = not determined.

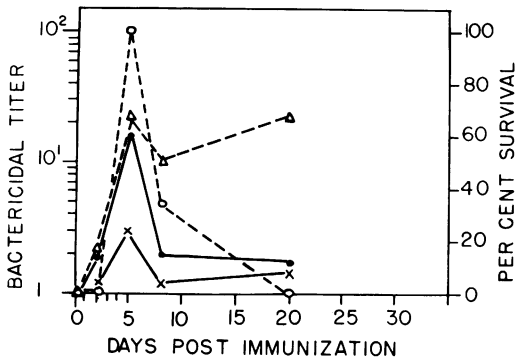

FIG. 10. *Specificity of the antibody (bactericidal titer, left axis) and the protective (% survival, right axis) responses of mice immunized ip with 10^5 live Salmonella enteritidis no. 11RX. Bactericidal titer determined with S. enteritidis no. 11 as the test organism (●); bactericidal titer determined with S. typhimurium Suc LL as the test organism (×); % survival of mice challenged ip with 100 LD_{50} doses of S. enteritidis no. 11 (△); % survival of mice challenged ip with 100 LD_{50} doses of S. typhimurium* (○).

protection of mice against virulent challenge infection with the smooth organism. Mice immunized ip with 10^5 live no. 11RX organisms were given a heavy infection of either the homologous smooth organism, *S. enteritidis* no. 11, or a heterologous smooth strain, *S. typhimurium* Suc LL, at various intervals after immunization. Sera were collected from the immunized mice at the time of challenge. Results are presented in Fig. 10. At 5 days after immunization, mice were well protected against both the homologous and the heterologous challenge infection. However, the protective response to the heterologous challenge was transient, and mice were still protected (70% survival) against the homologous challenge infection 20 days after immunization.

DISCUSSION

The extremely rapid bactericidal antibody response to immunization with a smooth strain of *S. typhimurium* was highly specific and reproducible in independent experiments. The most rapid initial response was achieved by an ip infection with live smooth cells. This response was twice as rapid, and the peak titer was 10- to 100-fold higher than the response to 10^8 heat-killed cells administered iv, even though the killed vaccine initially represented 1,000-fold more antigenic mass. As compared to an ip injection of 10^5 live bacteria, an iv injection of the same number of organisms induced a response that reached almost the same level, but the initial rate of antibody production was slower and the length of time to peak titer was more than 1 week longer. The sc route was almost as effective as the ip route. This suggests that the initial contact with lymphoid tissues enhanced antibody formation, since, by the iv route, initial contact is greatly diluted by the blood volume, and uptake by reticuloendothelial cells of clearance organs (spleen and liver) prevents antigen contact with antibody-forming cells.

Protection against virulent challenge infection paralleled antibody level in most cases and was demonstrable very early after vaccine administration. Early protection after immunization, in the absence of detectable antibodies, has been reported by others. (9; G. L. McCallum, H. C. Batson, M. S. Brown, and M. J. Carlson, Bacteriol. Proc., p. 110, 1951; G. L. McCallum, G. Edsall, and M. J. Carlson, Bacteriol. Proc., p. 112, 1952). It is likely that their method of detecting antibody by agglutination was too insensitive to demonstrate its presence during this period (8). However, in one case involving immunization against *Bacterium tularense*, antibody was detectable at 72 hr by passive transfer of protection, even though agglutinins were not demonstrable.

The very solid immunity, conferred by either the live or the heat-killed vaccines (8), was apparently not the result of any local effect produced by immunizing and challenging mice by the ip route, as was suggested by several investigations (1, 3, 15, 16). A sterile intraperitoneal inflammation did not offer mice protection from a subsequent virulent challenge infection with the smooth *S. typhimurium*. Furthermore, mice immunized with either the live or the heat-killed vaccines of *S. typhimurium* were as well protected against a subcutaneous challenge infection as they were against an ip challenge infection. Blanden et al. (3) showed that this local clearance phenomen could be overcome by inoculating larger doses of the challenge organism. Therefore, the absence of the clearance phenomenon in the present investigation may be due to the large challenge dose employed. Certainly, the virulence of the bacterial strain for a particular strain of mouse represents an important determinant of the outcome of immunization experiments. The mouse strain used in the present investigation was less susceptible to infection with the smooth strain of *S. typhimurium* than were other mouse strains previously employed (6). Many investigations have been carried out with mouse strains highly susceptible to infection with the smooth strain of *S. typhimurium* (7, 12, 16). It is conceivable that the immune apparatus of these strains is naturally unresponsive to antigenic stimulation (17). Susceptible mouse strains would tend to be selected

against in nature, and the "natural" response to salmonella infections may not be measured in such artifactual experimental animals.

Although valid objections have been made to the indiscriminate use of the ip route of injection in experimental salmonellosis (3, 15), caution should also be exercised in evaluating results obtained with iv challenge. Organisms entering through the oral, the ip, and the sc routes must pass the lymphatic barrier before entering the bloodstream and subsequently becoming disseminated. Experimental infections established through the iv route have limited significance, since the organisms do not have to pass through the local lymphatic barrier.

The protective response observed in the present investigation was specific when heat-killed vaccines of either the smooth *S. typhimurium* or the smooth *S. enteritidis* were the immunizing organisms. The protective response to a live vaccine of the rough *S. enteritidis* was nonspecific for the first 6 days after immunization but showed a high degree of specificity at 10 and 20 days after immunization. The protective response to immunization with the live smooth *S. typhimurium* was apparently nonspecific, even though the homologous and the heterologous antibody titers were quite different. This lack of specificity could be explained by destruction of a heat-labile common antigen in preparing the heat-killed vaccines. However, Ornellas et al. (Federation Proc., **26**:420, 1967) demonstrated a greater restriction of homologous infection than heterologous infection in mice immunized with either live or heat-killed vaccines. But their assessment was in terms of in vivo bacterial population, whereas our assessment was in terms of death, admittedly a less sensitive procedure. Collins et al. (4), using the same means of assessment, showed that living *S. montevideo* protected against antigenically unrelated *S. enteritidis*. However, their data indicate that a 100-fold larger dose of heterologous bacteria was required to achieve the same protection that is afforded by homologous organisms.

One important aspect of this investigation was the demonstration that the challenge infection itself induced a rapid and potent antibody response which would certainly augment any effect of antibody present at the time of challenge. The rapid rise of antibody occurred during the critical postchallenge period when most deaths occur in the control, nonimmune, animals. However, the size of the challenge inoculum, 10^7 bacteria, represents a sizable antigenic mass. It would be interesting to study the effect of a range of challenge doses on the secondary antibody response.

Findings of interest in the present investigation were those concerning the immune responses to rough bacteria. The work of Ushiba et al. (15) indicated that immunization with rough *S. enteritidis* was protective in the absence of antibody. However, mice immunized with live rough strains of either *S. typhimurium* or *S. enteritidis* produced low but detectable bactericidal antibody titers (directed against the corresponding smooth strains) which reached a peak 2 to 6 days after immunization. Because the titers fell rapidly after this peak of activity was attained, the possibility existed that these antigens (endotoxins) induced the release of preformed natural antibody rather than synthesis of antibody. Measurement of the homologous and the heterologous antibody responses to these rough antigens revealed that in each case the peak of activity was higher for the homologous systems. This would contradict the idea that antibody was released, since it would be expected that preformed antibodies directed against *S. typhimurium* and *S. enteritidis* would be released in similar amounts. Also, an "anamnestic" response, as evidenced in an increase in titer with secondary stimulation, occurred in mice given a secondary stimulus of the live rough *S. typhimurium* strain TV119. It was not a typical anamnestic response as defined by Taliaferro (14), since it was not more rapid than the response to primary stimulation. Nevertheless, the secondary response resulted in an increased level of antibody, which demonstrated that the rough strain was capable of inducing antibody formation directed against the smooth organisms. These experiments did not, however, distinguish between the possibility that the rough organisms contained undetectable smooth-cell determinants that induced the production of small amounts of anti-O antibody, or the possibility that the rough strain induced the production of anti-R antibodies and the smooth test strain contained a low number of rough determinants on the cell-wall surface.

The rough strains were capable of protecting mice against a challenge infection with the corresponding smooth virulent strain, and the duration of this protection was correlated with the endotoxic content of the immunizing strain. Experiments involving immunization with strains of varying degrees of roughness demonstrated that specific anti-O antibody cannot be eliminated from consideration in protection. This was also demonstrated in the finding that mice previously immunized with the rough *S. enteritidis* no. 11RX rapidly produced bactericidal antibody in response to a challenge infection of *S. enteritidis* no. 11. This response represented a 100-fold increase over titers formed against the immunizing strain.

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LITERATURE CITED

1. AKIYAMA, T., K. MAEDA, AND D. USHIBA. 1962. Studies on immunity of experimental typhoid. Challenge of mice passively immunized with antiserum through various routes (mechanism of immunization with killed vaccines). Japan. J. Bacteriol. **17**:829–834.
2. BECKMAN, I., T. V. SUBBAIAH, AND B. A. D. STOCKER. 1964. Rough mutants of Salmonella typhimurium. II. Serological and chemical investigations. Nature **201**:1299–1301.
3. BLANDEN, R. V., G. B. MACKANESS, AND F. M. COLLINS. 1966. Mechanisms of acquired resistance in mouse typhoid. J. Exptl. Med. **124**: 585–600.
4. COLLINS, F. M., G. B. MACKANESS, AND R. V. BLANDEN. 1966. Infection-immunity in experimental salmonellosis. J. Exptl. Med. **124**:601–619.
5. GEMSKI, P. JR., AND B. A. D. STOCKER. 1967. Transduction by bacteriophage P22 in nonsmooth mutants of *Salmonella typhimurium*. J. Bacteriol. **93**:1588–1597.
6. HERZBERG, M., M. J. JAWAD, AND D. PRATT. 1965. Correlation of succinate metabolism and virulence in *Salmonella typhimurium*. J. Bacteriol. **89**:185–192.
7. HOBSON, D. 1957. Resistance to reinfection in experimental mouse typhoid. J. Hyg.**55**:334–343.
8. KENNY, K., AND M. HERZBERG. 1967. Early antibody response in mice to either infection or immunization with *Salmonella typhimurium*. J. Bacteriol. **93**:773–778.
9. LARSON, C. L., J. F. BELL, AND C. R. OWEN. 1954. The development of resistance in mice immunized with soluble antigen derived from *Bacterium tularense*. J. Immunol. **73**:221–225.
10. LÜDERITZ, O., A. M. STAUB, AND O. WESTPHAL. 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. **30**:192–255.
11. MICHAEL, J. G. 1966. The release of specific bactericidal antibodies by endotoxin. J. Exptl. Med. **123**:205–212.
12. MITSUHASHI, S., M. KAWAKAMI, Y. YAMAGUCHI, AND M. NAGAI. 1958. Studies on the experimental typhoid. I. A comparative study of living and killed vaccines against the infection of mice with Salmonella enteritidis. Japan. J. Exptl. Med. **28**:249–258.
13. NAKANO, M. 1962. Mutants of Salmonella with unusually low toxicity for mice. Nature **196**:1118–1119.
14. TALIAFERRO, W. H., L. G. TALIAFERRO, AND B. N. JAROSLOW. 1964. Immune mechanisms. The hemolysin response, p. 5–8. *In* W. H. Taliaferro, L. G. Taliaferro, and B. N. Jaroslow [ed.], Radiation and immune mechanisms. Academic Press, Inc., New York.
15. USHIBA, D., T. NAKAE, T. AKIYAMA, AND Y. KISHIMOTO. 1966. Characterization of "clearance" factor and "cell-bound" antibody in experimental typhoid. J. Bacteriol. **91**:1705–1712.
16. USHIBA, D., K. SAITO, T. AKIYAMA, M. NAKANO, T. SUGIYAMA, AND S. SHIRONO. 1959. Studies on experimental typhoid: bacterial multiplication and host cell response after infection with *Salmonella enteritidis* in mice immunized with live and killed vaccines. Japan. J. Microbiol. **3**:231–242.
17. WEBSTER, L. T. 1933. Inherited and acquired factors in resistance to infection. II. A comparison of mice inherently resistant or susceptible to Bacillus enteritidis infection with respect to fertility, weight, and susceptibility to various routes and types of infection. J. Exptl. Med. **57**:819–843.