Effect of Specific Immune Mouse Serum on the Growth of *Salmonella enteritidis* in Nonvaccinated Mice Challenged by Various Routes

F. M. COLLINS

Trudeau Institute, Inc., Saranac Lake, New York 12983

Received for publication 2 October 1968

*Salmonella enteritidis* was injected intravenously, intraperitoneally, or subcutaneously into specific pathogen-free mice. The number of organisms in the blood, liver, spleen, peritoneal cavity, and draining inguinal lymph node was determined by daily enumeration. Opsonization of the organism with hyperimmune serum increased the rate of phagocytosis, resulting in rapid blood clearance together with an alteration in the relative numbers of organisms accumulating in the liver and spleen. Serum treatment also brought about a substantial increase in the number of bacteria killed during the first 60 min of the infection. However, the survivors of this initial period of inactivation then multiplied rapidly in the liver and spleen, ultimately resulting in the death of the animal from a generalized infection. Attempts to passively protect mice with hyperimmune serum were uniformly negative. The effects of treatment of the virulent *S. enteritidis* with hyperimmune serum were consistent with the general thesis that cellular rather than humoral factors play the major role in the expression of an effective antibacterial immunity against salmonella infections.

Living salmonella vaccines produce more effective immunity in mice than do killed saline suspensions of the same organisms (6–10). The mechanism involved in the expression of such antibacterial immunity is essentially cellular in nature and does not depend upon the presence of specific humoral protective factors (12), at least for intravenously challenged mice (4, 6, 8). Nevertheless, a number of claims have been made that mouse "protective" antibodies are produced after the use of certain immunizing procedures (12, 13, 20, 21). In particular, Jenkin (11) showed that pig serum opsonins consisted of at least three antibody populations which varied in their specificities for virulent and avirulent strains of *Salmonella typhimurium*. In a subsequent paper (21), a macroglobulin isolated from immune peritoneal macrophages was shown to passively protect mice challenged intraperitoneally with *S. enteritidis*. Recently, Kenny and Herzberg (16) reported the production of both bacitracidal and haemagglutinating antibodies within 4 days of administration of either living or heat-killed salmonella vaccines. These workers claimed that killed vaccines could induce effective protection against both intraperitoneally and subcutaneously injected *S. typhimurium* or *S. enteritidis*. The use of these routes of challenge was recommended despite the fact that Blanden et al. (1) had criticized the use of the intraperitoneal route of challenge in mouse protection experiments because massive extracellular multiplication makes the interpretation of the results equivocal. Blanden et al. (1) recommended the use of the intravenous route of challenge as the best means of dissociating the cellular and humoral components of the resistance mechanism. The same type of criticism also holds for the use of the intraperitoneal route of challenge as a means of demonstrating serum protection against *S. enteritidis* infections (3). Immunity against salmonella infections is best assessed not in terms of the ultimate survival of an increased proportion of the vaccinated mice but by the presence of a bac- tericidal mechanism capable of progressively eliminating the challenge organism without the development of a clinical infection (4, 6, 8).

The predominating influence of cellular as opposed to humoral factors in the development of resistance to salmonella infections has been unequivocally established in the case of the intravenously challenged animal (1, 6, 8, 17). In view of the continuing dissent regarding the general significance of these findings (16), it was con-
sidered important to investigate the interplay of cellular and humoral factors in the development of immunity to a subcutaneous challenge in which the infection follows an invasive route via lymphatics and thus approaches more closely the conditions which obtain in naturally acquired infections. To make this study meaningful, it was necessary to make a simultaneous comparison of the host's response to challenge by three commonly used routes of infection. In this and an ensuing paper (5), a study is made of the effect of specific immune serum on the growth of *S. enteritidis* in normal or immunized mice after subcutaneous, intraperitoneal, and intravenous inoculation. The results of these studies indicate that, once phagocytosis is complete, humoral factors play only a minor role in the expression of effective anti-salmonella immunity, irrespective of the route of infection.

**MATERIALS AND METHODS**

**Organisms.** *S. enteritidis* 5694, *S. enteritidis* 5694 SM®, *S. enteritidis* Se795, *S. enteritidis* Se795 SM®, and *S. gallinarum* 9240 have been described in earlier papers (6, 8). The SM® strains were resistant to streptomycin (10 μg/ml). The L.D₅₀ values for the five strains, determined as described previously (3), are listed in Table 2.

**Animals.** Specific pathogen-free (COBS) mice (Charles River Farms, Inc.) were maintained under conditions described previously (6). Eight-week-old female mice were used throughout the study.

**Preparation of antiserum.** Mice were vaccinated with three weekly doses of 10⁴ ethyl alcohol-killed *S. enteritidis* and then challenged intravenously with 10 L.D₅₀ of living *S. enteritidis* 5694 7 days later. The survivors were injected intravenously 10 days later with approximately 10⁴ living *S. enteritidis* 5694 (L.D₅₀ = 350 organisms) once each week for 3 weeks. The mice were bled from the tail 7 days later, and the serum was pooled and stored at −20°C.

*Opsonization of S. enteritidis.* *S. enteritidis* 5694 was grown in digest broth (Difco) for 4 hr at 37°C. The culture was diluted 1:5 in saline and mixed with an equal volume of 1:5 immune mouse serum. The bacterial suspension was kept at 4°C for 30 min, subjected to sonic vibration (Bronwill Biosonic II) for 5 sec to break up the agglutinated bacteria, and then diluted suitably before challenge. Unopsonized bacteria were subjected to sonic vibration and then diluted similarly in saline before injection into mice by the intravenous, intraperitoneal, or subcutaneous routes. The number of viable bacteria in the challenge dose was checked by plating suitable serial dilutions on digest-agar (Difco) plates.

*Enumeration of the in vivo population.* The number of bacteria in the blood, liver, and spleen was determined by the methods described previously (17). The peritoneal cavity was washed out with 2 ml of cold saline, and the number of bacteria present was determined immediately by plating samples on digest-agar plates (1). The washout was then centrifuged at 600 × g for 10 min to deposit cell-associated bacteria. The number of bacteria remaining in the supernatant fluid indicated the size of the extracellular bacterial population. Viable counts were also made on the right, inguinal lymph node, which was carefully dissected out and homogenized in saline. Suitable dilutions were plated on digest-agar.

**Serology.** H and O agglutinin titers were measured by the methods described by Kauffmann (15). Hemagglutinin titers were determined against *S. enteritidis* lipopolysaccharide (Difco) by use of the method described in Kabat and Mayer (14). The bactericidal antibody titer was determined, as described previously, by the use of absorbed guinea pig serum as complement source (2).

**RESULTS**

Growth of unopsonized *S. enteritidis* in normal mice: intravenous challenge. Although the growth of unopsonized *S. enteritidis* in the liver and spleen after intravenous injection has been described (8), the presence of organisms in the peritoneal cavity and in the lymph nodes was not then reported. The growth curves shown in Fig. 1 reveal that the growth of the highly virulent strain of *S. enteritidis* 5694 in the livers and spleens of specific pathogen-free mice, as well as the per cent mortality and the mean time to death (Tables 1 and 2), were essentially similar to those reported for conventional mice (3, 7, 8). However, the early occurrence of extensive growth in the peritoneal cavity and the presence of organisms in the inguinal lymph node (and, presumably, in other lymph nodes throughout the body) indicate that, once established in the liver and spleen, infection spreads rapidly to the remainder of the reticuloendothelial system. Approximately half of the bacteria detected in the peritoneal cavity were in the extracellular phase, and there was no apparent increase in the percentage of phagocytized bacteria as the infection proceeded. The rate of intraperitoneal growth paralleled that observed in the liver and spleen (Fig. 1).

Influence of virulence and inoculum size on the growth of *S. enteritidis* in vivo. In order to sustain an argument that will be developed in a later section, it was necessary to examine the influence of virulence and inoculum size on the growth characteristics of *S. enteritidis* in vivo. The size of the intravenous challenge inoculum of a highly virulent strain of *S. enteritidis*, when tested over a wide range, was found to have little effect on the slope of the liver or spleen growth curves, but it did affect the mean time to death (Table 2). In this study, no attempt was made to follow the early growth of an intravenous inoculum of less than 10⁶ organisms because of the technical difficulties associated with the enumeration of small numbers of bacteria in vivo. However, the growth
rates observed after detectable numbers of organisms had emerged indicated that the initial growth rate of organisms in minimally infected mice does not differ from that observed with larger doses (Table 2).

As reported by Roantree (19), the virulence of various strains of salmonellae appears to depend directly on the in vivo growth rate of the organism rather than on any detectable differences in antigenic makeup. This is clearly seen in the liver and spleen growth curves observed for strains of S. enteritidis of decreasing virulence (Table 2; Fig. 2). The data in Table 2 show that a correlation exists between the slope of the growth curve in vivo and strain virulence as estimated by LD50, mean time to death, or per cent mortality. The level of protection afforded by a given immunizing regimen can be assessed in terms of a significant change in the slope of the growth curve and the progressive elimination of the challenge population. The use of a challenge organism of low intrinsic virulence (and, therefore, having a slower rate of growth in vivo) may appear to show protective value for a given immunizing preparation, which is quite apparent when a more virulent strain is used. The importance of these findings in relation to the mouse protection studies, which have been performed by different workers with strains of widely differing virulence, will be discussed later.

It should be noted at this stage, however, that the virulence measurements differ from those previously reported for the same bacterial strains (3). The reason has been shown to depend upon the use in the present study of specific pathogen-free COBS rather than conventional mice.

**Intraperitoneal challenge.** Injection of unopsonized bacteria by the intraperitoneal route resulted in the growth curves shown in Fig. 3. Only a fraction of the injected organisms could be recovered 1 hr after injection. Some of these organisms obviously had left the peritoneal cavity, as shown by their presence in blood, liver, and spleen. Others presumably were inactivated while still in the peritoneal cavity. However, the peritoneal cavity was never completely cleared of viable bacteria and, in fact, a considerable number of both extra- and intracellular bacteria persisted there throughout the experiment. Within 10 min of injection, the heart blood contained 10^8 bacteria per ml. By 4 hr, the size of the liver and spleen populations actually exceeded that still present at the site of inoculation (Fig. 3). From that point, the numbers of organisms in the liver and spleen increased steadily, and mice commenced dying by day 6 (Table 1). By this time, there was also an extensive inguinal lymph node population, indicating, once again, the presence of a fulminating, generalized infection. As the infection reached its terminal stages, the peritoneal infection increased rapidly until it equalled that present in the livers and spleens of the moribund animals.

**Subcutaneous challenge.** When the unopsonized inoculum was introduced subcutaneously over the inguinal region, the growth curves shown in Fig. 4 were obtained. The population present in the draining inguinal node increased only for the first 2 days. By the 2nd or 3rd day of the infection, increasing numbers of bacteria were detected in the blood, liver, and spleen. It is clear that infection from a subcutaneous site spreads slowly, but once
TABLE 1. Progressive mortality in mice challenged with opsonized and unopsonized S. enteritidis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc of organism</th>
<th>Route of challenge</th>
<th>No. of deaths on day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Unopsonized</td>
<td>$10^4$</td>
<td>IV</td>
<td>0</td>
</tr>
<tr>
<td>Opsonized</td>
<td>$10^4$</td>
<td>IV</td>
<td>0</td>
</tr>
<tr>
<td>Opsonized, +0.7 ml of immune serum</td>
<td>$10^4$</td>
<td>IV</td>
<td>0</td>
</tr>
</tbody>
</table>

* IV, intravenous; IP, intraperitoneal; SC, subcutaneous.

TABLE 2. Variations in the growth rate and virulence of S. enteritidis and S. gallinarum in specific pathogen-free mice

<table>
<thead>
<tr>
<th>Organism</th>
<th>LD$_{50}$</th>
<th>Challenge dose</th>
<th>In vivo growth rate</th>
<th>MTD (days)</th>
<th>Per cent mortality at day 28</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. enteritidis 5694</td>
<td>$3.5 \times 10^3$</td>
<td>$1.1 \times 10^4$</td>
<td>0.97</td>
<td>4.3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>S. enteritidis 5694</td>
<td>$3.5 \times 10^3$</td>
<td>$3.8 \times 10^4$</td>
<td>0.90</td>
<td>4.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>S. enteritidis 5694</td>
<td>$3.5 \times 10^3$</td>
<td>$4.0 \times 10^4$</td>
<td>1.00</td>
<td>10.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>S. enteritidis 5694 SM$^R$</td>
<td>$5.0 \times 10^3$</td>
<td>$2.0 \times 10^4$</td>
<td>0.48</td>
<td>6.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>S. enteritidis Se795</td>
<td>$5.0 \times 10^4$</td>
<td>$2.0 \times 10^4$</td>
<td>0.60</td>
<td>5.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>S. enteritidis Se795 SM$^R$</td>
<td>$5.0 \times 10^4$</td>
<td>$3.5 \times 10^4$</td>
<td>0.26</td>
<td>&gt;28</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>S. gallinarum 9240</td>
<td>$3.0 \times 10^4$</td>
<td>$2.0 \times 10^4$</td>
<td>0.30c</td>
<td>&gt;28</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Slope = log increase of combined liver and spleen populations/time in days.

** Mean time to death of 10 mice.

^c Slope of growth curve reversed on day 3 to $-0.3$.

...disseminated it grows in the liver and spleen at a rate comparable to that seen in intraperitoneally infected animals. The delay in the emergence of a generalized infection is reflected in the longer time to death (Table 1). As a rule, the peritoneal cavity became infected only when the disease had progressed to its terminal stages.

Having established the growth pattern for S. enteritidis in specific pathogen-free mice, attention was turned to the influence of hyperimmune serum on the growth of S. enteritidis after introduction of the opsonized organisms by one of three possible routes of infection.

Preparation of immune mouse serum. Mice vaccinated first with ethyl alcohol-killed S. enteritidis and then with living S. enteritidis were totally resistant to an intravenous challenge by as many as $10^7$ living, virulent bacteria. Serum from such mice should, therefore, contain “protective” antibodies in high titer. In fact, this serum did contain both H and O agglutinating antibody in higher titer than is found in convalescent mice (Table 3). High titers of hemagglutinating and bactericidal antibodies were also detected in the hyperimmune serum. Therefore, it was considered suitable for studying the fate of preopsonized bacteria and examining the influence of passive serum transfer on the behavior of intravenously injected organisms.

Effect of opsonization of S. enteritidis on the course of an intravenous infection. When normal mice were challenged intravenously with approximately $10^4$ opsonized S. enteritidis (50 to 100 LD$_{50}$), the growth curves shown in Fig. 1 were obtained. The only obvious effect of the pretreatment of the bacteria with specific antibody was a more rapid blood clearance and a significant shift in the proportion of organisms present in the liver and spleen compared to that observed for corresponding unopsonized bacteria (Fig. 1). The im-
In an effort to determine whether the antibody still adhering to the opsonized bacteria at the time of injection was limiting in this system, mice challenged with the serum-treated *S. enteritidis* were injected intravenously with 0.5 ml of pooled immune serum 30 min later, followed by a further injection of 0.2 ml the following day. Once again, the only alteration to the in vivo growth pattern was the elimination of the early bacteremia and a shift in the relative proportions of organisms present in the liver and spleen (Fig. 5). The passively administered immune serum was not able to prevent the challenge population from reaching toxic levels in a high proportion of the infected mice (Table 1).

**Intraperitoneal challenge of mice with opsonized *S. enteritidis***. The growth curve in Fig. 3 shows that opsonization of *S. enteritidis* prior to intraperitoneal injection increased the rate of phagocytosis and the subsequent killing of the challenge inoculum so that less than 2% of the original inoculum was still viable at 4 hr. Despite the increased mortality, bacteria continued to be present in the peritoneal cavity throughout the experiment (Fig. 3). Bacteria were detected in the blood within 10 min of injection and, although the initial liver and spleen populations were smaller than those present in corresponding experiments using unopsonized bacteria (Fig. 3), they still reached levels sufficient to kill most of the mice by day 10 (Table 1). The bacterial numbers in the peritoneal cavity remained small for the first 5 days of the infection. This may explain the slower rate of increase in the liver and spleen due to the reduced numbers of bacteria being released from the peritoneal cavity.

**Subcutaneous challenge of mice with opsonized *S. enteritidis***. The only apparent influence of the hyperimmune serum treatment on the in vivo behavior of subcutaneously injected *S. enteritidis* was an initially faster rate of accumulation of bacteria in the inguinal node (cf. the size of the 10-min counts in the opsonized and unopsonized animals in Fig. 4). By day 4, bacteria were detected in the liver and spleen, and thereafter the organisms continued to grow in all the organs tested until the conclusion of the experiment. Whereas *S. enteritidis* was less virulent for the specific pathogen-free mice when injected subcutaneously than by either of the other two routes of inoculation, serum treatment was still not able to prevent extensive growth of this organism in vivo.

**DISCUSSION**

In view of the importance of effective control of salmonella infections in man and animals, as well as the continuing controversy over the role of
antibody in the mechanism of host immunity, it is surprising that so little data exists in regard to the influence of immunization on the growth patterns of the organism in vivo (19). The present comparison of the growth of *S. enteritidis* introduced by different routes revealed that bacteria which survive initial inactivation ultimately reach the spleen and liver regardless of the route of inoculation. There they multiply until they reach toxic proportions. However much the time course may differ, the end result is the same. This course of events is not restricted to organisms of high virulence; Mitsuhashi and his colleagues (18) reported some time ago that even attenuated salmonellae rapidly spread throughout the reticuloendothelial system to produce an infection which may persist for weeks. The present study amply confirms this picture. Thus, the criticism made by Kenny and Herzberg (16), that the intravenous route for vaccination or for challenge was objectionable because organisms introduced in this way are lodged in the liver and spleen without prior involvement of the lymphatic system, seems irrelevant.

Animals challenged intraperitoneally were found to harbor large numbers of bacteria in liver and spleen within 10 min of injection. This is in agreement with Ushiba et al. (22), who earlier noted a rapid transfer of bacteria from the peritoneal cavity to the spleen. Thus, the assumption by some workers (11, 20) that the decrease in numbers of viable bacteria in intraperitoneally challenged mice represents inactivation of the organisms due to treatment with "protective" antibody may be questioned in the absence of quantitative data on the size of the populations in liver and spleen. Irrespective of the real importance of immune serum under such circumstances, the end result is invariably the emergence of surviving bacteria which enter a phase of rapid

**Fig. 3.** Growth of unopsonized *S. enteritidis* 5694 (left) and opsonized *S. enteritidis* 5694 (right) after intraperitoneal injection into normal mice. See the legend to Fig. 1 for the key to the various curves.
multiplication in liver, spleen, and peritoneal cavity whether or not they have been treated with antibody.

In the present study, it was noted that growth of extracellular S. enteritidis in the peritoneal cavity paralleled that of the intracellular organisms, suggesting that multiplication of the pathogen within the phagocytic cells was not affected greatly by its intracellular environment. The initial "leakage" of S. enteritidis to the liver and spleen observed in the first 10 min, together with the continuing presence of a bacteremia throughout the experiment, suggests that free bacteria were leaving the peritoneal cavity throughout the infection. It is possible that these migratory bacteria were intracellular at the time of their transfer to the liver, spleen, and elsewhere. However, the marked reduction in the size of the extracellular peritoneal population when mice were challenged with opsonized bacteria, taken in conjunction with the smaller liver and spleen populations found in these mice (Fig. 3), suggests that it is the extracellular bacteria in the peritoneal cavity which can easily pass through the lacunae of the diaphragm to enter the lymphatics and so reach the liver and spleen via the peripheral blood. Thus, the only real contribution of opsonic anti-

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**TABLE 3.** Agglutinating, hemagglutinating, and bactericidal antibody titers in vaccinated mouse sera

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>H agglutinating antibody</th>
<th>O agglutinating antibody</th>
<th>Hemagglutinating antibody</th>
<th>Bactericidal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl alcohol-killed and living S. enteritidis</td>
<td>160&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80</td>
<td>10,240</td>
<td>10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Convalescent mouse serum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td>10</td>
<td>160</td>
<td>10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inverse of titers.
<sup>b</sup> Survivors from an LD<sub>50</sub> determination.

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FIG. 4. Growth of unopsonized S. enteritidis 5694 (left) and opsonized S. enteritidis 5694 (right) after subcutaneous injection into normal mice over the inguinal region. See the legend to Fig. 1 for key to the various curves.
with organisms of decreasing virulence will reach toxic levels at progressively later time periods. Thus, moderate challenge doses of organisms of medium-to-low virulence will reach toxic levels at progressively later time periods. Intraperitoneally or subcutaneously introduced challenge populations of medium-to-low virulence may increase slowly enough to allow the host time in which to develop the cellular changes necessary to control the infection, at least in a proportion of the mice (1, 6, 8). It is clear from the present studies that serum treatment of such inocula will serve to accentuate this natural tendency. Since the primary effect of the immune serum will be to keep the numbers of extracellular organisms at a minimum, the effect we see in serum-treated animals is an overall slowing of the infection, presumably because intracellular organisms are not so readily disseminated throughout the reticuloendothelial system. The liver and spleen counts indicate, however, that the organisms there continue to multiply, but their numbers are not augmented by the constant arrival of organisms multiplying extracellularly in the peritoneal cavity and probably elsewhere. In consequence, the total numbers of organisms present in the animals increase at a slower rate, resulting in the survival of more of the challenged mice.

The foregoing considerations can explain the frequently reported finding (12, 13, 16, 21) that immune serum treatment of mice infected intraperitoneally or subcutaneously with opsonized S. enteritidis increases the survival rate compared to that observed in untreated animals challenged with nonopsonized bacteria. However, the interpretation of such data as indicative of a primary and major role for antibody in anti-salmonella immunity does not seem to be justified in view of present and earlier findings which show that the bacterial populations reach virtually the same levels in serum-treated and untreated animals whether or not the animal survives (8, 17). In the present study, mice failed to demonstrate an effective antibacterial immunity against an opsonized intravenous challenge even when subsequently injected intravenously with as much as 0.7 ml of hyperimmune serum. The donors of the hyperimmune serum were shown to be resistant to nearly a million \( LD_{50} \) of S. enteritidis \( 5694 \) immediately prior to collection of the antisera!

Despite the high agglutinating and bactericidal antibody titers of the hyperimmune serum, the failure to demonstrate passive serum protection could have been due to an inability to maintain effective levels of antibody over a sufficient period of time to protect the animals. Such a possibility was thought to be unlikely, but it was explored further by following the fate of opsonized S.

**Fig. 5. Growth of opsonized S. enteritidis 5694 after intravenous injection into mice that received a total of 0.7 ml of hyperimmune mouse serum intravenously.** See the legend to Fig. 1 for the key to the curves. The vertical arrow heads indicate the times of the serum injections.

body in this situation seems to be an initial reduction in the size of the population and the slower rate at which the infection spreads beyond the peritoneal cavity. At no time was immune serum able to suppress this migration, so that the degree of "protection" observed was, at best, marginal and resulted in little more than an increased time before the death of the host (Table 1).

The fact that a correlation exists between the virulence of an organism and its rate of growth in the liver and spleen (Table 2) means that the bacterial population present in mice challenged
enteritidis in mice previously immunized with living or killed salmonella vaccines. The results of these studies, which agree with the general thesis that cellular rather than humoral factors play the major role in the expression of effective anti-salmonella immunity, are reported in the ensuing paper (5).

ACKNOWLEDGMENTS

This investigation was supported by U.S. Public Health Service grant AI-07809 from the National Institute of Allergy and Infectious Diseases.

I thank Oliver Duprey and William Woodruff for their excellent technical assistance.

LITERATURE CITED