Early Antibody Response in Mice to Either Infection or Immunization with *Salmonella typhimurium*

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After immunization with either live or heat-killed *Salmonella typhimurium*, mice responded with an extremely rapid production of bactericidal antibody which was correlated with the appearance of immunity to a heavy challenge dose (100 LD$_{50}$) of the virulent bacteria. Inactivation of sera with mercaptoethanol along with Sephadex fractionation indicated that the observed bactericidal activity was associated with a macroglobulin which was completely mercaptoethanol-sensitive. The unexpected finding, that a heat-killed vaccine gave excellent protection from a challenge dose which killed all immunized control mice, seriously challenges the theory attributing immunity against typhoid infection entirely to a cellular host factor produced only in response to a live vaccine.

The early immunological responses to infection obviously play a role in the ability of the host to overcome the virulent pathogen. However, with a minimal antigenic stimulus, circulating antibodies formed during the early stages of primary gram-negative bacterial infections are not easily detected by classical antibody assays such as precipitation or agglutination (16, 21). Turner et al. (20) studied the immune response in mice to primary infection with an avirulent *Salmonella typhimurium*, and were able to detect antibodies 5 days after infection, using the opsonic test as an assay for circulating antibodies. The bactericidal reaction, which involves the action of specific antibody and complement on gram-negative bacteria (17), is an extremely sensitive method which can detect circulating antibodies in low concentration. Consequently, it is an excellent tool for assaying antibodies formed during the early course of a primary gram-negative bacterial infection. Using the bactericidal reaction, Michael (14) has shown that antibodies elicited by *Escherichia coli* endotoxin can be detected in mouse serum 2 days after antigenic stimulation, and Landy et al. (12) were able to measure antibody activity in rabbits within 60 hr after injection of *S. enteritidis* polysaccharide, obtained by aqueous ether extraction. Evidence on salmonella infections presented by Hobson (7), Mitsuhashi et al. (15), and Ushiba et al. (22) has indicated a lack of protection by heat-killed vaccines. The present paper describes the antibody response to a minimal infection as measured by bactericidal activity. A comparison is made with the antibody response to and the protective effect of a heat-killed vaccine. The time sequence of the appearance of protection against a subsequent severe infection after immunization with both procedures is described.

**Materials and Methods**

Bacterial strain. The organisms employed were derived from *S. typhimurium* strain 7, and were designated Suc LL and Suc EE (6). The LD$_{50}$ dose of Suc LL was 5.0 X 10$^4$ and the LD$_{50}$ of a Suc EE strain of reduced virulence was 10$^4$; both were determined by the intraperitoneal route.

Mouse strain. Specific pathogen-free, white, female Swiss mice (Charles River Mouse Farms, Wilmington, Mass.) were employed. Representative samples of the normal stock and experimental animals were checked repeatedly by culture on S S Agar (Difco) for the presence of *S. typhimurium* in liver and spleen homogenates and in cecal contents. The organism was never isolated from unoinoculated mice or from mice which had received injections of heat-killed cells. Also, bactericidal antibodies against *S. typhimurium* could not be detected in undiluted sera of unoinoculated mice.

Immunization and collection of sera. Mice were inoculated intraperitoneally with either live Suc EE (10$^9$) or heat-killed (100 C for 1 hr) Suc EE (10$^4$ or 10$^5$) organisms. In mice immunized with live organisms, the resultant mortality was approximately 20%, so that immunized mice were survivors of a minimal virulent infection. Some animals from each group were exsanguinated under anesthesia; their blood was collected from the axillary artery, pooled, and allowed to clot at room temperature. The serum was separated and stored at -25 C.

Protection tests. At various intervals after immunization, the immunity of some of the mice was challenged by intraperitoneal infection with a 100 LD$_{50}$
dose of Suc LL. A control group of unimmunized mice was inoculated at the same time. At the time of challenge, groups of six mice per time period were bled and the bactericidal activity of the serum was measured. Bacterial counts of the livers were determined, and cultures of cecal material were made on S S agar. Deaths were followed for 21 days after challenge, and protection was measured as per cent survival. Unimmunized mice which were inoculated at the same time as immunized mice, acting as controls for the challenge infection, invariably yielded 100% mortality.

Mercaptoethanol reduction of sera. Sera were reduced and alkylated according to the procedure of Chan and Deutsch (4). Samples were dialyzed for 48 hr against phosphate-buffered saline (PBS), pH 7.0, containing 0.01 M 2-mercaptoethanol (ME) (Eastman Organic Chemicals, Kingsport, Tenn.). These sera were subsequently dialyzed for 48 hr against 0.02 M iodoacetamide (Mann Research Laboratories, New York, N.Y.) in PBS to prevent reassociation of the reduced globulin. Finally, the sera were dialyzed for 48 hr against 0.85% NaCl. Control sera were treated in the same manner, except that ME was omitted from the dialysis buffer.

Sephadex fractionation of sera. Serum samples (1.0 to 1.5 ml), pooled from 6 to 10 mice, were placed on a column (2.0 by 100 cm) containing G-200 Sephadex (Pharmacia Fine Chemicals, Inc., New Market, N.J.) equilibrated with 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0 (5). The proteins were eluted from the gel at a flow rate of 5 ml/hr. Fractions were collected in 2.5-ml volumes, and protein was determined by absorbance at 280 mλ in a Beckman DU spectrophotometer.

Agglutination of sera. As previously described (19), twofold dilutions of antiserum (0.5 ml) were mixed with an equal volume of heat-killed bacteria (5 × 10⁸ per milliliter) in 12-mm culture tubes (13 by 100 mm). The tubes were incubated at 37 C for 1 hr and in a refrigerator (4 C) overnight. Agglutination was measured by the settling pattern, and the titer was reported as the reciprocal of the highest dilution showing activity.

Bactericidal assay. Bactericidal antibodies were assayed by a modification of the plate count method (11) with the use of a Microtiter kit containing Perspex plastic plates with 6-mm wells, calibrated (0.025 ml) dropping pipettes, calibrated (0.025 ml) loops, and plastic adhesive tape (Cooke Engineering Co., Alexandria, Va.). From a calibrated pipette, 0.025 ml of a serum dilution (dilutions ranging from undiluted to 10⁻³) was added to 0.05 ml of precooled calf serum, as the source of complement, and 0.025 ml of a bacterial suspension (10⁶ Suc LL cells per milliliter) in a well of the plastic plate. The plate was covered with the plastic adhesive tape and incubated in a water bath (37 C) for 1 hr, after which time 0.025-ml samples were removed by means of a calibrated loop and diluted in 1.0 ml of saline. A 0.1-ml amount of this suspension was plated directly onto an S S agar plate, and colonies were counted after 18 hr of incubation at 37 C. Duplicate assays were performed on all samples tested, and reported values represent an average of the two tests. Bactericidal activity is expressed as the reciprocal of that serum dilution needed to kill 50% of the original inoculum. The control, containing complement but no antibody, was assumed to represent 100% survival. The variation in bactericidal titers between animals within a group was not tested. The response, as determined with pooled sera obtained at similar times postimmunization, was strikingly similar in replicated experiments.

**RESULTS**

Bactericidal antibody response and protection of mice from death. Bactericidal antibodies could be detected in the sera of mice within 2 days after immunization with either live or heat-killed bacteria (Fig. 1). In each case, the bactericidal titer

![Fig. 1. Bactericidal activity of sera (O, left axis) and resistance to challenge (Δ, right axis) infection (10⁶ dose) of mice at various intervals after immunization with (A) 10⁶ live, (B) 10⁶ heat-killed, and (C) 10⁶ heat-killed Salmonella typhimurium.](http://jb.asm.org/)

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rose rapidly until it reached a peak value at 6 days after immunization. Thereafter, the titer dropped at 10 to 14 days and rose again to the maximal value at 35 days, except in the case of mice immunized with 10⁴ heat-killed cells. Titters measured at 76 days after inoculation were similar to those measured at 35 days. The number of organisms cultured from the livers of mice infected with 10⁴ live bacteria reached a peak at 6 days (2.0 × 10⁸ cells). After this time, the number gradually decreased until no organisms could be isolated from liver homogenates 27 days after infection.

By the 2nd day after immunization with 10⁴ live organisms, considerable protection (67% survival) against a 100 LD₉₀ challenge dose was obtained. This protection reached a maximal value at 10 days (100% survival). Inoculation of mice with 10⁴ heat-killed cells also gave measurable protection (50% survival) within 4 days and maximal protection (100% survival) by day 10. Immunization with 10⁴ heat-killed cells gave only slight protection (30% survival) which was not long-lasting, indicating a dose dependency for protection with the heat-killed organisms.

Agglutinating antibodies could not be detected until 14 days after immunization with 10⁴ heat-killed organisms and 35 days after immunization with the live cells; sera from mice inoculated with 10⁴ heat-killed bacteria yielded no demonstrable agglutinating activity.

**Mercaptoethanol treatment of sera.** In an effort to determine the characteristics of the bactericidal antibodies formed during the course of infection of mice with *S. typhimurium*, serum samples taken at various intervals after inoculation of mice with 10⁴ live bacteria were subjected to reduction with ME and subsequent alkylation with iodoacetamide (IA). (See Table 1.) Bactericidal activity of the serum was completely ME-sensitive for 6 days after infection. After day 6, ME-resistant antibody appeared (9.0% of the total activity), but by day 40 the antibody activity was still predominantly distributed in the ME-sensitive fraction (94%), although some activity (6.0%) was associated with the ME-resistant antibody. Since the γ-M (ME-sensitive) antibody is a more active immunoglobulin in the bactericidal reaction than is the γ-G (ME-resistant) antibody (19), these data cannot be interpreted as representing quantities of the two immunoglobulins; they can only be interpreted as representing activities.

**Sephadex fractionation of sera.** It was apparent from the sensitivity to ME treatment that γ-M antibody was probably the component active in the early phase of infection (2 to 6 days). These data were by no means conclusive, since a ME-sensitive, γ-G mouse immunoglobulin has been reported (1). Therefore, portions of the same sera used in the ME experiment, but not treated with ME, were subjected to molecular-sieving chromatography on G-200 Sephadex. Selected fractions obtained from the column were subsequently tested for bactericidal activity (see Fig. 2). To simplify discussion of the data, the three peaks obtained upon Sephadex fractionation of sera will be referred to as the γ-M (first peak), the γ-G, (middle peak), and the albumin (last peak) fractions. Activity of sera collected from mice 6 days after infection with the live organism was located almost exclusively in the γ-M frac-

$\text{TABLE 1. Mercaptoethanol inactivation of mouse antiserum collected at various intervals after infection with Salmonella typhimurium}$

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Total activity$^a$</th>
<th>ME-resistant activity$^a$</th>
<th>Per cent of total activity after ME reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.8 × 10⁴</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1.1 × 10⁴</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1.6 × 10⁴</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2.7 × 10⁴</td>
<td>2.2 × 10⁴</td>
<td>0.8</td>
</tr>
<tr>
<td>20</td>
<td>3.0 × 10⁴</td>
<td>2.8 × 10⁴</td>
<td>9.0</td>
</tr>
<tr>
<td>40</td>
<td>2.0 × 10⁴</td>
<td>1.2 × 10⁴</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Units per milliliter determined by dividing the serum dilution needed to kill 50% of the original inoculum into 1.0.

**FIG. 2. Analysis of selected fractions of immune mouse anti-Salmonella typhimurium serum obtained by exclusion chromatography on G-200 Sephadex.** Sera collected (A) 6 days and (B) 40 days after infection with 10⁸ live bacteria; sera collected (C) 6 days and (B) 40 days after immunization with 10⁴ heat-killed cells. (Curves represent optical density values and bars represent bactericidal titer.)
tion, i.e., that fraction eluting first from the column (Fig. 2A). Although a small amount of activity was detected in the \( \gamma-G \) fraction, it could be attributed to the incomplete separation of the two components, as evidenced by overlapping peaks. On the other hand, activity was found in both the \( \gamma-M \) and the \( \gamma-G \) fractions from sera collected 40 days after infection (Fig. 2B), although the activity in the \( \gamma-M \) fraction was still higher than that observed in the \( \gamma-G \) fraction.

Sephadex fractionation of sera collected from mice after injection of \( 10^6 \) heat-killed cells gave similar results (see Fig. 2C and D).

**Discussion**

Immunization of mice with a small dose of live or a large dose of heat-killed bacteria resulted both in a rapid production of bactericidal antibody, which reached a high titer within 6 days after infection, and in considerable protection from a 100 LD\(_{50}\) challenge dose of virulent bacteria. The height of the antibody response to infection was greater than the response to the killed vaccine. Although lasting protection was not achieved with a lower dose of the heat-killed organism (10\(^6\) bacteria), the response to this exceedingly small antigenic stimulus in the rapid production of a distinctly detectable bactericidal titer, along with the resultant partial protective effect, is indeed worth attention. The bacterial mass would be equal to approximately 0.1 \( \mu \)g of cells, as determined from volume and density values for *E. coli* (13). The observed antibody response appeared to be a primary response to the test organism, since, by the extremely sensitive bactericidal assay, antibodies directed against *S. typhimurium* could not be detected in the sera of uninoculated mice. Moreover, the organism could not be isolated (upon repeated attempts) from liver and spleen homogenates or from cecal contents of uninoculated mice. That the very early response was the result of antibody release rather than antibody production cannot be ruled out (14); however, the time sequence of the appearance of antibody favors production rather than release (26). Specificity tests now in progress indicate that the bactericidal response is specific for both heat-killed and live vaccines.

It is difficult to explain the success of the heat-killed vaccine in the light of reported failures of such vaccines to afford a protective immunity (7, 15, 22). Inability to confer protection with killed vaccines is one of the arguments used to support the concept of cellular immunity (8). Ushiba et al. (23) observed an “antilethal effect,” as evidenced in a prolonged time to death in mice immunized with heat-killed vaccines of *S. enteritidis*. This protective effect of the heat-killed vaccines was later attributed to the challenging of immunized mice by the intraperitoneal route (2). However, Hobson (7) reported that heat-killed vaccines of *S. typhimurium* afforded no significant protection to mice challenged by the intraperitoneal route, and concluded that resistance to a challenge infection conferred by live vaccines did not depend on any “local effect” in the peritoneal cavity, as these mice were also resistant to challenge by the intravenous route. The different results reported could be attributed to the particular strains of organism employed; e.g., in one case (3, 10), there appeared to be a heat-labile antigen involved in the virulence of the organism, which, when preserved in the process of preparing a killed vaccine, protected mice against a challenge infection. Another possibility would be the difference in the innate resistance or immune response of the particular strains of mice employed (24). In any case, the surprising result of the solid protective effect with the heat-killed vaccine certainly challenges the concept that only live vaccines can confer a solid immunity against typhoid infections.

Measuring the antibody response in mice by the opsonic assay, Turner et al. (20) detected an antibody response 5 days after infection, the titer of which reached a peak at 14 days. This activity appeared to be due to a macroglobulin, as determined by ME-inactivation. At this peak of the macroglobulin response, phagocytic cells taken from the mice were most effective in killing intra-cellular bacteria in vitro when compared with cells taken at other times. The authors postulated that this macroglobulin antibody was cytolytic and aided in the phagocytosis of the bacteria.

The bactericidal antibody observed in the present study was detected slightly earlier (2 days after infection), but its peak titer was reached at least 1 week earlier than the opsonic activity reported by Turner et al. (20). Since the bactericidal and the opsonic assays have similar sensitivities (19), this cannot be an explanation for the observed discrepancies in measurements made with the two systems. One explanation that can be offered is that the mice employed by Turner and co-workers might have been less responsive to antigenic stimulation than the mice used in the present investigation.

The peak of bactericidal activity was also demonstrated to be caused by a macroglobulin by virtue of ME inactivation and Sephadex fractionation of sera, and appeared to be correlated with the observed protection of the mice against a heavy challenge infection. The activity associated with the later response (14 to 35 days after immu-
nization) involved the presence of \( \gamma^-\)G as well as macroglobulin activity. Therefore, one cannot exclude the possibility of some protective role played by the \( \gamma^-\)G antibody. However, macroglobulin has usually been reported to be the predominantly active component in the immune response to administration of salmonella antigens in humans (21) and in rabbits (12, 25), although the route of injection may play an important role (18). The present report demonstrates that this is true in infection as well.

One interesting observation made from the present investigation was that mouse macroglobulin was completely sensitive to mercaptoethanol inactivation. Work presented by Turner et al. (20) supports this conclusion, but apparently this is not the case for rabbit macroglobulin, which seems to be only partially sensitive to inactivation by ME (19).

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