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Characterization of Murine Antibody Response to *Salmonella typhimurium* by a Class-Specific Solid-Phase Radioimmunoassay

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A heavy-chain class-specific, solid-phase radioimmunoassay was developed to characterize the murine antibody response to *Salmonella typhimurium*. The specificity of the assay was verified by quantitation of the extent of binding of anti-*S. typhimurium* antibodies to other bacterial genera and species and by cross-adsorption studies. The sensitivity of the procedure was also examined, and it was determined to be substantially more sensitive than either the passive hemagglutination or the whole-cell agglutination technique. The method was subsequently used to analyze the murine antibody response to *S. typhimurium*. Groups of mice were prebled and then immunized with live *S. typhimurium* via different routes. The animals were bled weekly for 12 weeks, and then sera were assayed for antibodies directed against whole bacteria or purified lipopolysaccharide. Anti-*Salmonella* antibodies of the immunoglobulin M class appeared in the serum approximately 2 to 3 weeks after immunization, and then immunoglobulin G anti-*Salmonella* antibodies appeared which constituted the major part of the long-term response. Immunoglobulin A was not a major component of the serum antibody response. The antibodies were primarily directed against the lipopolysaccharide determinants, but a small percentage of the response was directed against other cell surface components. Qualitatively and quantitatively similar anti-*Salmonella* antibody responses were observed in sera of outbred and inbred strains of mice.

Previous studies by Mackaness and his colleagues (reviewed in reference 6) suggested that the immune response to the facultative intracellular bacterium *Salmonella typhimurium* is primarily regulated by T cells and macrophages and that antibodies, and hence, B cells, play a less important role in resistance to the organism. The relegation of an antibody-dependent immune mechanism to a secondary status was based on two observations. First, passive transfer of immune mouse serum to outbred mice did not protect them from lethal challenge (5). Second, outbred mice infected 3 weeks previously with a sublethal dose of *S. typhimurium* were resistant to lethal challenge, although little (17) or no (2) anti-*Salmonella* O antibodies were demonstrable in their sera. However, recent studies from this laboratory (20) suggested that B cells may, in fact, be required for the expression of resistance to *S. typhimurium*. Mice with an X-linked B-cell functional defect (CBA/N mice and F_1 male progeny derived from CBA/N females) were found to be *Salmonella* susceptible (50% lethal dose, ≤20), whereas immunologically normal, histocompatible CBA/Ca mice and F_1 female progeny of CBA/N mice were resistant (50% lethal dose, 8×10^3). Since other studies had demonstrated that CBA/N mice and their immune-defective F_1 male progeny have poor or no antibody response to certain T-independent and T-dependent antigens (4, 18, 21, 27, 28), although their T-cell and macrophage functions are generally normal (24, 28), the results of this study suggested that the susceptibility of CBA/N mice to *Salmonella* might be a consequence of a defective anti-*Salmonella* antibody response. In support of this theory, recent data from this laboratory demonstrated that the *Salmonella* resistance of immune-defective F_1 male mice could be significantly increased by passive transfer of immune F_1 female serum (A. D. O'Brien, I. Scher, and E. S. Metcalf, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, E69, p. 62; manuscript in preparation). To further evaluate this hypothesis, sera from both immune-defective F_1 male mice and immunologically normal F_1 female littermates were analyzed for anti-*Salmonella* antibodies at various times after sublethal challenge by standard serological methods (8). However, no antibody was detected by either a whole-cell agglutination assay or passive hemagglutination in the sera of normal

or immune-defective infected mice even after 3 weeks postchallenge. In addition, the bactericidal assay with which Kenny and Herzberg (12, 13) demonstrated an early antibody response in mice challenged with 10^5 live salmonellae could not be used in the present studies because the virulent challenge strain, *S. typhimurium* strain TML (TML), is not killed by antibody and complement (unpublished data).

Since it was possible that the established serological methods might be too insensitive to detect small amounts of anti-*Salmonella* antibody, we sought to design a more sensitive assay for quantitation of these antibodies. This paper describes such a technique. Because of its sensitivity, the method permits the analysis of sera from normal and immune-defective mice challenged with a low dose ($<10^2$) of live organisms. Moreover, the assay is specific and can be used to distinguish the heavy-chain class of anti-*Salmonella* antibodies in the sera of infected outbred and inbred strains of mice.

MATERIALS AND METHODS

Animals. Female C3H/HeNCrlBr (C3H/HeN) and outbred CD-1 mice were purchased from Charles River Laboratories, Wilmington, Mass. (CBA/N × DBA/2)F_1 female mice were the kind gift of Irwin Scher. SWXL-4 and B6D2 male mice were the generous gift of Ben Taylor. C3H/HeJ and C57BL/6 female mice were purchased from Jackson Laboratories, Bar Harbor, Maine. All mice were 8 to 16 weeks of age.

Antigens. TML was originally isolated from a patient with salmonellosis (9). Acetone-killed and dried (AKD) bacteria of this strain were prepared by the method of Landy (16). *Salmonella enteritidis* strain 795 was a gift of Lou Baron, Walter Reed Army Institute of Research, Washington, D.C. *Salmonella typhi* strain QS was a gift of Samuel Formal, Walter Reed Army Institute of Research. *Escherichia coli* strain HS (nontypable) was previously isolated from the stool of a healthy adult (7). *Streptococcus pneumoniae* type 3 was a gift of John Robbins, Bureau of Biologics, Bethesda, Md. Gram-negative bacterial cultures were grown overnight in Penassay broth (Difco Laboratories, Detroit, Mich.) at 37°C with shaking. *S. pneumoniae* was grown on blood agar plates, and the organisms were suspended in saline. Bacteria for coating plates in the radioimmunoassay (RIA) were harvested from cultures by centrifugation and washed twice in 0.15 M phosphate buffer, pH 7.2.

Lipopolysaccharide (LPS) from TML was prepared by the procedure of Romeo et al. (22). To verify that this phenol-water extract of TML LPS was free of lipid A-associated protein, a mitogenesis assay (23) was performed with C3H/HeJ spleen cells. The B cells from these mice proliferate poorly in the presence of protein-free preparations of LPS (32), but blast formation is observed when C3H/HeJ spleen cells are incubated with LPS preparations which contain lipid A-associated protein (31). The stimulation indices for duplicate cultures of C3H/HeJ spleen cells exposed to 0.1, 1, and 10 μg of TML LPS were 1.2, 1.5, and 2.0, respectively. In contrast, the stimulation index for C3H/HeJ spleen cells exposed to 10 μg of a butanol extract of *E. coli* strain K235 LPS, which contains lipid A-associated protein (19), was 10. Moreover, spleen cells from C57BL/6 mice responded well to the TML LPS preparation. The stimulation indices for duplicate cultures of C57BL/6 spleen cells exposed to 0.1, 1, and 10 μg of this LPS preparation were 3.8, 14.8, and 35.7, respectively.

A phenol-water extract of LPS from *E. coli* strain K235 was a gift of Stefanie Vogel, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

Immunizations and collection of test antisera. Mice were prebled and then immunized intraperitoneally (i.p.), subcutaneously (s.c.), or intravenously (i.v.) with 50 organisms from an overnight culture. Plate counts on tryptic soy agar (Difco Laboratories) were performed to confirm the number of viable bacteria in the challenge inoculum. Mice were individually bled from the retro-orbital plexus at various times. Since TML-infected mice may have bacteremia, the sera were filtered twice and stored frozen at −70°C. For some experiments, sera from individual mice were pooled before freezing. Control, normal sera from the prebled mice (NMS) were also stored frozen before assay. Rabbit anti-TML antiserum was obtained from animals 8 weeks after biweekly i.v. injections of 5 × 10^6 TML AKD organisms. A second series of rabbits were infected i.v. with five doses of 10^5 live TML organisms each week for 6 weeks and subsequently exsanguinated.

Whole-cell bacterial agglutination and passive hemagglutination assays. The procedure of Eisenstein and Angerman was employed to determine passive hemagglutination titers of mouse anti-TML serum, NMS, rabbit anti-TML serum, and normal rabbit serum (8). The same serum samples were tested for whole-cell bacterial agglutinins by a modification of the procedure of Eisenstein and Angerman (8). Serial twofold dilutions of test serum in 0.5 ml of saline were added to tubes (12 by 75 mm). To each tube was added 0.5 ml of a suspension of TML containing 5 × 10^8 organisms per ml. The tubes were incubated at 50°C overnight and read.

RIA. RIA is a modification of the method originally designed by Segal and Klinman (29) for the detection of murine antitumor antibodies. Polyvinyl U-bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) served as a solid phase for this assay. Fifty microliters of washed TML organisms (2 × 10^9 viable organisms per ml) was added to each well and allowed to air dry overnight at room temperature. Fixation of the bacteria to the wells was accomplished by the addition of 0.2 ml of 0.15% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0) followed by a 5-min incubation period at room temperature. The glutaraldehyde was removed by suction, and the fixation reaction was terminated by the addition of 0.2 ml of 0.15 M glycine in 0.015 M phosphate buffer (pH 7.0) containing 1% (vol/vol) agamma horse serum (GIBCO Laboratories, Grand Island, N.Y.). The glycine solution was removed by shaking, and the wells were filled with 10% horse serum in distilled water. After a minimum

of 0.5 h of incubation at room temperature, the horse serum was removed, and the plates were washed twice with 1.5% horse serum in phosphate-buffered saline (PBS-HS). Duplicate samples containing 40 μl of serial twofold dilutions of mouse serum from *Salmonella*-immunized mice were added to each well. Duplicate 40-μl samples of a 1:10 dilution of NMS and duplicate 40-μl samples of phosphate buffer alone were also added to wells. All serum dilutions were prepared in 10% horse serum in PBS-HS (PBS-HS-HS). The plates were incubated for 2 h at room temperature. Subsequently, the serum samples were removed by shaking, and the plates were washed twice with PBS-HS. Specific anti-*Salmonella* antibody was detected by the addition of 25 μl of affinity-purified class-specific ^{125}I-labeled rabbit anti-mouse immunoglobulin M (IgM) (μ), IgG (γ), or IgA (α) diluted in PBS-HS-HS (10). The dilution of the ^{125}I-labeled immunoglobulins was calculated to give 100,000 counts per well. After 1 h of incubation at room temperature, the unbound radiolabeled conjugates were removed by suction, and the plates were washed eight times under tap water. The plates were allowed to dry, and then the wells were individually cut out and counted in a Searle gamma counter, model 1285.

Other bacterial strains and antisera were also tested with this assay. These bacteria were fixed to plates as described for TML, and the antisera were diluted as described for the anti-TML serum. When anti-LPS activity was to be evaluated, 25 μg of LPS in 50 μl of phosphate buffer was added to each well. After air drying overnight, the LPS was fixed to the plate by the glutaraldehyde procedure outlined above, or the plates were immediately washed two times under tap water. The remainder of the assay was carried out as described for the bacterium-coated wells. The two methods for the attachment of LPS to the plate appeared to give similar results.

Iodination of myeloma proteins. Affinity-purified heavy-chain class-specific rabbit anti-mouse IgM, IgG, and IgA sera were the kind gifts of Fred Finkelman, Uniformed Services University of the Health Sciences. The heavy-chain class-specific rabbit anti-mouse IgG has been shown to contain IgG1, IgG2, and IgG3 subclasses (F. Finkelman, personal communication). These antisera were iodinated by a modification of the chloramine-T procedure with 1 mCi of carrier-free ^{125}I (Amersham Corp., Arlington Heights, Ill.) (11, 14).

Adsorption studies. Confluent lawns of TML and *S. enteritidis* were prepared on tryptic soy agar plates. Ten milliliters of sterile saline was added to each plate, and the cells were then harvested and collected by centrifugation at 10,000 × *g* for 15 min. Three milliliters of the antiserum to be adsorbed was added directly to the cell pellet obtained from two plates. Anti-TML serum and anti-*S. enteritidis* serum were each adsorbed with TML and *S. enteriditis*. After the sera and organisms were mixed, the mixtures were gently agitated at room temperature for 45 min. Subsequently, the mixtures were centrifuged, and the sera were removed. The adsorption of each serum sample was repeated with a fresh bacterial pellet of the appropriate species, and the resultant adsorbed serum was filtered twice and checked for sterility by plating 0.1 ml of adsorbed serum on tryptic soy agar plates. The serum samples were stored at −70°C.

RESULTS

Standardization of RIA. In preliminary studies, optimal conditions for coating of the microtiter wells with antigen were determined. Overnight TML cultures were washed in various solutions (PBS, saline, or phosphate buffer, pH 7.2) before addition to the wells. Phosphate buffer, pH 7.2, was chosen because it did not contribute significantly to the background counts of the assay. The optimal concentration of TML organisms to be added to the wells was also determined by a checkerboard titration, with different numbers of organisms being tested against dilutions of NMS and immune mouse serum. Fifty microliters of 2×10^9 TML organisms per ml was established as the optimal concentration of antigen, since this concentration gave the highest titers with the immune serum. In a similar set of checkerboard titrations, the optimal dilutions of iodinated rabbit anti-mouse class-specific immunoglobulin reagents were determined. Based on these studies, the ^{125}I-labeled conjugates were henceforth diluted to give 100,000 counts in the 25 μl added to the wells. Furthermore, because the half-life of ^{125}I is 60 days, the radioiodinated materials were recounted and appropriately diluted before each assay. Preliminary experiments also revealed that the maximum binding of mouse serum to antigen-coated wells occurred by 2 h of incubation. In addition, the initial studies showed that a 1-h exposure of radiolabeled rabbit anti-mouse immunoglobulins to antigen-bound murine serum allowed consistent uptake of the radioiodinated conjugate without appreciable elevation in background radioactivity (phosphate buffer-coated wells).

To detect any variation among assays, a reference immune serum was included in each test. This positive control was prepared as follows. Fifty outbred CD-1 mice were prebled and then immunized i.p. with 50 TML organisms. After 28 days, the mice were exsanguinated, and the pooled serum was analyzed for anti-TML antibody. This serum pool served as a positive control for all subsequent experiments. Samples of the normal serum obtained before this immunization and samples of the immune serum were stored frozen at −70°C. Freshly thawed samples of NMS and immune serum were used for each experiment. An analysis of the counts per minute bound by the reference CD-1 anti-TML serum and NMS, as determined on eight different occasions, is shown in Fig. 1. The background contributed by the iodinated anti-immunoglobulin reagents was minimal; control wells which

contained phosphate buffer or the serum diluent PBS-HS-HS instead of immune serum had very few counts. However, NMS did exhibit some *Salmonella*-binding activity which was primarily of the IgM class. Nevertheless, in comparison with the immune serum, the level of antibody activity in the NMS was low. Based on these binding studies, the titers of antibody in immune serum were taken as the highest dilution which bound ≥1.5 times the counts per minute of a 1:10 dilution of NMS. By this criterion, the IgM and IgG titers of the CD-1 anti-TML reference serum in each of the eight experiments were 1:80 and 1:1,280, respectively.

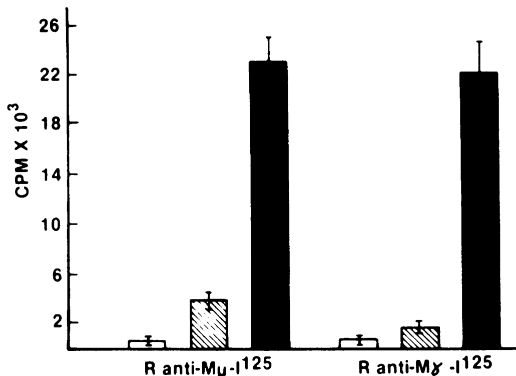

FIG. 1. *Comparison of the extent of binding of normal and immune sera to S. typhimurium-coated wells. Samples of a 1:10 dilution of normal or immune serum or diluent were incubated on S. typhimurium-coated wells for 2 h. After washing, iodinated rabbit anti-mouse IgM or IgG was added to the wells. After 1 h, the plates were washed and counted in a gamma counter. Bars represent the standard error of the mean. Symbols: □, diluent, phosphate buffer, or PBS-HS-HS; ▧, 1:10 dilution of NMS; ■, 1:10 dilution of immune serum.*

Specificity of the assay. To determine the specificity of anti-TML RIA, two types of studies were carried out. In the first set of experiments, the activity of the reference CD-1 anti-TML serum was tested on microtiter plates to which bacteria other than TML had been fixed. Groups A through E in Table 1 illustrate the results of this type of specificity experiment. The three other gram-negative organisms tested, two heterologous *Salmonella* species (*S. enteritidis* and *S. typhi*) and *E. coli*, did bind CD-1 anti-TML serum to some extent. The cross-reactivity was most evident in the IgM titer of the CD-1 anti-TML serum on *S. typhi*, but the homologous binding (binding to TML) was still four times greater than the binding of the heterologous antigen. In all other instances, the homologous binding was at least eight times greater than the heterologous binding for IgM and at least 32 times more effective for IgG titers. The CD-1 anti-TML serum did not bind to the gram-positive bacterium *Streptococcus pneumoniae* (Table 1, group E).

In the second set of specificity experiments, CD-1 anti-TML serum and CD-1 anti-*S. enteritidis* serum were adsorbed with homologous and heterologous *Salmonella* species. Adsorption of the reference CD-1 anti-TML serum with TML effectively eliminated the antibodies of the IgM class and reduced the IgG titer in one experiment by 16-fold and in a second study by 64-fold (Table 1, group F). However, adsorption of the CD-1 anti-TML serum with *S. enteritidis* (Table 1, group G) did not reduce either the IgM or IgG titers of the reference serum. In the reciprocal studies, groups H through K, in which CD-1 anti-*S. enteritidis* serum was adsorbed with *S. enteritidis* or TML, similar results were obtained.

These two sets of specificity experiments

TABLE 1. *Specificity of RIA*

Group	Source of antiserum	Bacterial strain used for adsorption	Bacterial strain coating plate	Reciprocal titer	
				IgM (μ)	IgG (γ)
A	CD-1 anti-TML		TML	80	1,280
B	CD-1 anti-TML		*S. enteritidis*	<10	40
C	CD-1 anti-TML		*S. typhi*	20	20
D	CD-1 anti-TML		*E. coli*	10	20
E	CD-1 anti-TML		*S. pneumoniae*	<10	<10
F	CD-1 anti-TML	TML	TML	<10 (<10)[a]	80 (20)[a]
G	CD-1 anti-TML	*S. enteritidis*	TML	80	1,280
H	CD-1 anti-*S. enteritidis*		*S. enteritidis*	80	160
I	CD-1 anti-*S. enteritidis*	*S. enteritidis*	*S. enteritidis*	<10	10
J	CD-1 anti-*S. enteritidis*	TML	*S. enteritidis*	20	40
K	CD-1 anti-*S. enteritidis*		TML	<10	20
L	CD-1 anti-*S. pneumoniae*		*S. pneumoniae*	80	80
M	CD-1 anti-*S. pneumoniae*		TML	<10	<10

[a] The results of two separate experiments are shown.

strongly suggested that the antiserum raised in CD-1 mice after immunization with TML was indeed anti-*Salmonella* antibody. However, it remained a concern that any immune serum might bind to TML-coated plates and that the titers observed might be merely a reflection of nonspecific binding by high levels of immunoglobulin. Therefore, murine antiserum was raised against an unrelated bacterial component, pneumococcal capsular polysaccharide. This was accomplished by i.p. immunization of CD-1 mice with a 1:100 saline dilution of Pneumovax (Merck Sharpe & Dohme, West Point, Pa.), a vaccine which contains 14 pneumococcal polysaccharide capsular types, including type 3. Mice were bled 10 days later, and their pooled serum was assayed for anti-*S. pneumoniae* antibody on *S. pneumoniae* type 3-coated wells. This particular immunization protocol was chosen because these vaccinated mice are protected from lethal type 3 pneumococcal infection (unpublished data). It is evident (Table 1, group L) that this assay can detect anti-*S. pneumoniae* antibodies. More important, however, is the finding that this immune serum did not bind to TML (Table 1, group M). These observations support the conclusions discussed above that this RIA detects specific anti-*Salmonella* antibodies rather than nonspecific components of immune serum.

Detection of anti-*Salmonella* antibodies in inbred strains of mice. Many of the previous studies of the immune response to *Salmonella* have used outbred mice. Since studies of the genetic regulation of immune responsiveness require the use of inbred strains of mice, it was of interest to determine whether the salmonella RIA could detect anti-*Salmonella* antibodies in such strains. Therefore, (CBA/N × DBA/2)F_1 females and C3H/HeN females were immunized i.p. with 50 TML organisms. After 28 days, the mice were exsanguinated, and their sera were assayed for anti-TML antibodies. In one case, AKD TML organisms were used to immunize C3H/HeN mice. The results of this study are presented in Table 2. The titers for CD-1 anti-TML are also shown for comparison. Both (CBA/N × DBA/2)F_1 and C3H/HeN mice immunized with TML organisms produced IgM and IgG anti-*Salmonella* antibodies. Moreover, C3H/HeN mice immunized with AKD TML organisms elicited significant anti-*Salmonella* antibody titers of both the IgM and the IgG heavy-chain classes. Thus, it appears that both of the inbred strains of mice tested can elicit a significant anti-*Salmonella* antibody response which is at least as good as, and in some cases better than, the anti-*Salmonella* antibody response observed in outbred mice. Two of the sera, the reference CD-1 anti-TML serum and the (CBA/N × DBA/2)F_1 female serum pool, were also assayed for the presence of anti-*Salmonella* antibodies of the IgA heavy-chain class. As shown in Table 2, the serum anti-TML response was primarily IgM and IgG. Thus, specific IgA antibody did not constitute a major portion of the response 28 days after i.p. inoculation of TML organisms.

TABLE 2. *Production of anti-Salmonella antibodies by various strains of mice immunized with live or AKD TML organisms*

Strain of female mouse	Antigen used for immunization	Titer of pooled serum 28 days post-immunization		
		IgM (μ)	IgG (γ)	IgA (α)
(CBA/N × DBA/2)F_1	Live	1:320	1:640	<1:10
C3H/HeN	Live	1:160	1:1,280	NT[a]
C3H/HeN	AKD	1:80	1:160	NT
CD-1	Live	1:80	1:1,280	1:20 (1:10)[b]

[a] NT, Not tested.
[b] The results of two separate experiments are shown.

Characterization of the anti-*Salmonella* antibody response. The generation and detection of anti-TML antibodies after immunization by different routes were examined. Groups of 10 (CBA/N × DBA/2)F_1 females were immunized i.p., i.v., or s.c. with 50 TML organisms. Each group was bled on days 7, 14, 21, and 28 and at 6, 8, and 12 weeks after immunization. The serum was pooled from each group and assayed for anti-TML antibody activity. The geometric mean of antibody titers derived from individual mice was equivalent to the titers calculated from the pooled serum. Figure 2 shows the kinetics of the anti-TML response after inoculation of TML via different routes. It was evident that specific anti-TML antibody could be generated and detected regardless of the parenteral immunization protocol. In this experiment, IgM antibody was initially detected 2 to 3 weeks after immunization in the groups of mice which received TML organisms i.p. or s.c., whereas the IgG response did not appear until 3 to 5 weeks after immunization. By 6 weeks, the IgM titers reached a plateau, but the IgG response continued to rise. The response of the mice which received TML organisms i.v. followed the same general pattern, but the rise in both IgM and IgG titers occurred earlier than for i.p. or s.c. inoculations. This finding was expected since the organisms were injected directly into the bloodstream and presumably reached the spleen more rapidly than they did by other routes of inoculation.

The anti-TML antibodies were detected in this RIA with whole, fixed TML as a solid-phase immunoadsorbent. Although all of the antibody specificities which are raised in mice after immunization with whole salmonellae are not

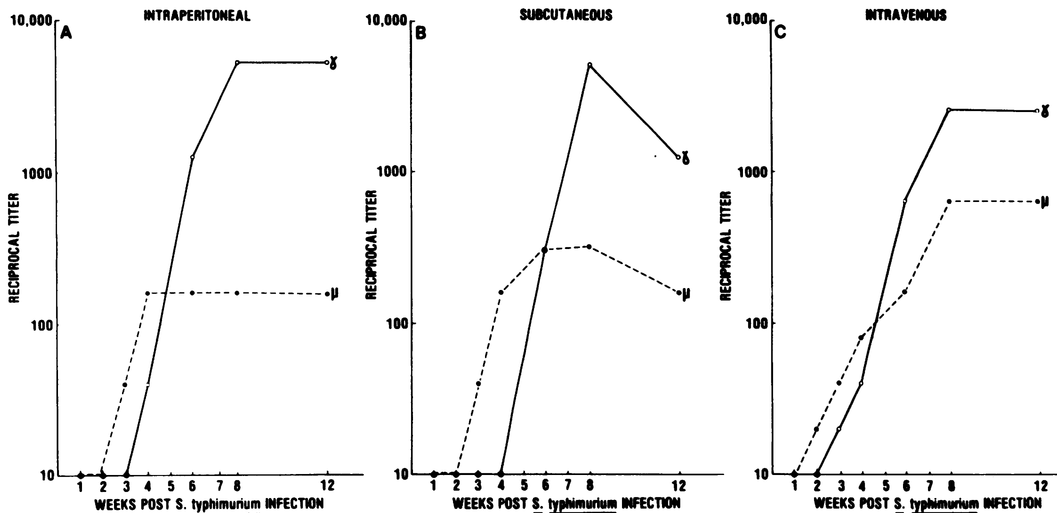

FIG. 2. *Duration of the IgM and IgG anti-TML antibody response. Groups of 10 mice were prebled and then immunized i.p., s.c., or i.v. with 50 TML organisms from an overnight culture. At various times, the mice were bled and their sera were analyzed for anti-TML antibody by RIA.* (- - -) *IgM titers;* (——) *IgG titers.*

known, the major part of the response is presumed to be directed against LPS. To ascertain whether this is indeed the case, the titers of the reference CD-1 anti-TML antisera were determined in parallel on TML LPS- and TML-coated plates. The results (Table 3) indicate that the reference CD-1 anti-TML serum, which was raised by immunization with intact TML, contained anti-LPS antibody. Furthermore, the anti-TML response appeared to be directed against *Salmonella*-specific LPS components, since purified *E. coli* LPS bound the CD-1 anti-TML serum poorly.

Comparison of the sensitivity of RIA with other serological methods. Table 4 shows the results of an analysis of the reference CD-1 anti-TML serum, used throughout this study, by hemagglutination assay, whole-cell agglutination assay, and RIA. In addition, the anti-TML responses from two other strains of mice injected with 50 live TML organisms were compared by the three assay systems. Moreover, the kinetics of the anti-TML antibody response was observed by the three assays after immunization of C3H/HeN mice with 5×10^6 AKD TML organisms. The results of this study demonstrate that the RIA is at least 20 times more sensitive than either the hemagglutination or the whole-cell agglutination assay for the detection of murine serum anti-*Salmonella* antibodies.

TABLE 3. *Detection of anti-LPS antibody produced by immunization with TML organisms*

Expt no.	Antigen on plate	Titer	
		IgM (μ)	IgG (γ)
1	TML	1:80	1:1,280
	TML LPS	1:80	1:640
2	TML	1:80	1:1,280
	TML LPS	1:20	1:1,280
	E. coli LPS	1:10	1:10

DISCUSSION

The assay described herein is both sensitive and specific, and a large number of serum samples can be analyzed rapidly. In addition, the heavy-chain class of anti-*Salmonella* antibodies can be identified by this RIA, which allows a more complete characterization of the immune response to mouse typhoid than is possible when sera are assayed by the primarily IgM-specific (3) passive hemagglutination technique. Moreover, the RIA, unlike the bactericidal assay (12), does not depend on the sensitivity of the *Salmonella* test strain to killing by antibody plus complement. Finally, this RIA can be modified to determine antibody titers to other intact or subcellular components of bacteria.

An assay designed to detect antibodies to a complex natural antigen presents problems which are not necessarily applicable to chemically defined soluble antigens. The outer membranes of gram-negative bacteria contain a variety of complex antigenic determinants, at least some of which are shared by members of the same genus. For example, *S. typhimurium* belongs to Kauffman-White serological group B, whereas *S. typhi* belongs to group D. Although the "immunodominant" antigenic determinants

TABLE 4. *Comparison of RIA with other serological methods for detection of anti-Salmonella antibodies*

Animal	Immunization protocol	Agglutination titer	Passive hemagglutination titer	RIA	
				IgM (μ)	IgG (γ)
Mice					
CD-1	NMS (prebled)	<1:5	<1:2 (<1:2)[a]		
	28 Days post 50 TML organisms i.p.	1:5	1:32 (1:32)	1:80	1:1,280
B6D2	NMS (prebled)	<1:5	<1:2 (<1:2)		
	28 Days post 10^3 TML organisms s.c.	1:20	1:16 (1:64)	1:320	<1:5,120
SWXL-4	NMS (prebled)	<1:5	<1:2 (<1:2)		
	28 Days post 10^3 TML organisms s.c.	<1:5	1:16 (1:32)	1:80	<1:5,120
C3H/HeN	NMS (prebled)	<1:5	<1:2 (<1:2)		
	7 Days post 5×10^6 AKD TML organisms i.p.	<1:5	<1:2 (<1:2)	1:640	<1:10
	14 Days post 5×10^6 AKD TML organisms i.p.	<1:5	<1:2 (<1:2)	1:320	<1:10
	21 Days post 5×10^6 AKD TML organisms i.p.	<1:5	<1:2 (<1:2)	1:160	1:180
Rabbits	Normal rabbit serum (prebled)	<1:5	1:2 (1:4)		
	6 Weeks post 5×10^6 AKD TML organisms biweekly i.v.	1:320	1:16,384 (1:16,384)	NT[b]	NT
	6 Weeks post five doses 10^5 live TML organisms i.v.	1:2,560	1:1,024 (1:512)	NT	NT

[a] Overnight readings within parentheses.

[b] NT, Not tested by class-specific reagents. However, when the immune rabbit serum was tested with ^{125}I-labeled guinea pig anti-rabbit IgG, the titer was similar to the hemagglutination titer, i.e., 1:50,000.

for these two groups are different (4 and 9, respectively), they still share antigenic determinant number 12 (30). Moreover, it is possible that the 2-keto-3-deoxyoctulosonic acid and lipid A components of the LPS from these two groups of bacteria are structurally similar. Thus, anti-*S. typhimurium* antibodies raised against the live, intact bacterium may have antibodies which cross-react with other, closely related organisms. Moreover, gram-negative bacteria from different genera may also have identical or cross-reactive antigenic determinants. This conjecture is borne out in the results of the cross-binding and cross-adsorption studies. However, it is important to note that the binding of the antiserum to the homologous antigen was generally 8 to 32 times higher than the binding to the heterologous organisms and that the greatest reductions in antibody titers occurred when antisera were adsorbed with homologous rather than heterologous *Salmonella* species. Furthermore, by the criterion generally used for specificity, i.e., binding to a non-cross-reactive antigen (e.g., *S. pneumoniae*), the *Salmonella* RIA described in this report is highly specific.

Adult mice exposed to the environment have a gut flora which includes many different kinds of gram-negative organisms (25, 26). Thus, for the reasons which were described earlier in this report, there may be low levels of *Salmonella*-reactive antibodies in the prebled serum (i.e., NMS) of mice. As a consequence, the number of counts bound by NMS to *Salmonella* (TML)-coated wells are slightly higher than the number bound to phosphate buffer-coated wells. Since NMS represents the basis on which the titers of the immune sera were calculated (1.5 times the NMS titer of anti-TML serum), using the higher value of NMS results in an underestimation of the titer of the anti-TML serum being assayed. Thus, the titers presented in the results probably represent a conservative estimate of immune anti-*Salmonella* antibody titers.

The LPS molecule is considered to be a major antigenic determinant(s) on *Salmonella* organisms and, indeed, on gram-negative organisms in general. The results presented clearly support this tenet; the majority of the anti-*Salmonella* antibodies which bound to whole bacteria also bound to LPS. Since immunization with LPS

elicits an IgM polyclonal antibody response (1, 34, 35), at least part of the early IgM anti-*Salmonella* antibody response may be polyclonal in nature. Indeed, the minor cross-reactivity of anti-TML serum with heterologous organisms was almost entirely IgM. However, the major part of the anti-*Salmonella* antibody response appeared to be specific IgG anti-LPS. This finding is contrary to the current belief that LPS is a T-independent antigen which elicits primarily, if not exclusively, an IgM response (34). The discrepancy between this theory and the results of this study may reflect differences in the way LPS is presented to the murine host. It may be that LPS is a T-dependent antigen when administered to the host on the surface of a bacterium, whereas when purified LPS is administered alone the response may be T independent. An examination of the anti-LPS component of the murine anti-*Salmonella* antibody response in nude mice may aid in the elucidation of this hypothesis. These studies are currently in progress.

Presumably, the RIA could be altered to an enzyme-linked immunosorbent assay by substituting enzyme-conjugated rabbit anti-murine immunoglobulins for radioiodinated conjugates. Indeed, an enzyme-linked immunosorbent assay has been successfully employed to detect murine antibodies directed against purified components of salmonellae and against chemically synthesized O antigens (3, 15, 33). The RIA described herein and the enzyme-linked immunosorbent assay described by other investigators differ in the following aspects: the RIA is immunoglobulin class specific and requires less serum, and whole bacteria rather than subcellular components are used as the immunoadsorbent in the RIA. Nevertheless, the enzyme-linked immunosorbent assay technique described by other investigators is extremely sensitive and could easily be modified to determine the heavy-chain class specificity of the antibodies.

The RIA described herein will permit an extensive evaluation of the role of different heavy-chain class of antibodies in both natural and acquired resistance of mice to *S. typhimurium*. Finally, this technique should be applicable to the analysis of the humoral response to a wide variety of other bacteria.

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