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Antigenic Modification, Rosette-Forming Cells, and *Salmonella typhimurium* Resistance in Outbred and Inbred Mice

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To assess the separate contributions of host T cells and the physical state of the antigen in the development of effective *Salmonella* resistance, glutaraldehyde-treated and untreated protein- and ribonucleic acid-rich extracts (E-RNA extracts) of virulent *Salmonella typhimurium* SR-11 or attenuated *S. typhimurium* RIA were used to immunize *Salmonella*-resistant and *Salmonella*-susceptible strains of mice for the purpose of determining whether antigen-specific T-cell or B-cell responses were formed and, if so, which responses predominated. The resistance imparted to each mouse strain after vaccination with *S. typhimurium* RIA was used as the standard for comparison. The inbred mouse strains C57BL/6 and DBA/2 and their F_1 hybrid (strain BDF_1), outbred ICR Swiss mice, and endotoxin-resistant C3H/HeJ mice were examined for the capacity to develop resistance to lethal *Salmonella* infections, as well as the ability to generate antigen-reactive T cells. Only the BDF_1, C3H/HeJ, and ICR Swiss mice were able to develop resistance to challenge infections mediated by the virulent SR-11 strain of *S. typhimurium* after vaccination with the living, attenuated RIA strain of *S. typhimurium* or immunization with E-RNA extracts. We developed an assay to identify the antigen-reactive rosette-forming lymphocytes present in lymph nodes and spleens of immunized mice. Levels of 0.2% or higher of theta antigen-bearing, antigen-reactive rosette-forming cells were found in the lymph nodes or spleens or both of only the BDF_1, C3H/HeJ, and ICR Swiss mice (i.e., in the "*Salmonella* responder" strains). Mouse strains C57BL/6 and DBA/2, which failed to develop resistance to lethal infections after immunization with the *S. typhimurium* RIA vaccine or with the E-RNA extracts, lacked effective numbers of antitheta antigen-sensitive rosette-forming cells. Modification of the effective E-RNA extracts by polymerization with glutaraldehyde resulted in a marked diminution in their abilities to induce resistance to salmonellosis in the two responder mouse strains tested (BDF_1 and ICR Swiss), even though detectable levels of antibody were induced.

In *Salmonella*-infected mice immune opsonins (41) and cytophilic antibody (28, 42) cause early blood clearance and inactivation of the infecting microbial population, as described by Collins (9), whereas the cell-mediated immune response provides hosts with protection from replicating virulent bacteria within reticuloendothelial cells (26). Strains of mice differ greatly in their responses to infection by facultative intracellular bacteria and in the development of immunity (6, 7, 16, 21, 30-32, 35, 37, 40, 44). Hormaeche (21) has suggested that responsiveness to *Salmonella* is polygenic. In ICR Swiss outbred mice, extracts rich in ribonucleic acid and protein (E-RNA extracts) isolated from the virulent SR-11 and RIA vaccine strains of *Salmonella typhimurium* induce marked resistance to *Salmonella* challenge infection, as does the *S. typhimurium* RIA vaccine (1a, 45). These mice exhibit measurable effector T-cell responses (46), a macrophage-bound cytophilic macroglobulin, and little, if any, antibody reactive with the E-RNA extracts (28). Cross-linking of the proteins present in these extracts with glutaraldehyde in attempts to stimulate antibody production produced substances with diminished capacities to induce *Salmonella* resistance in ICR Swiss mice (Bigley et al., submitted for publication).

The goal of this study was to assess the separate contributions of the host and the physical state of the antigen to the development of pro-

tective *Salmonella* immunity. It was necessary first to establish a method for detecting lymphoid cells bearing specific antigen receptors (4); we developed a rosette technique for the detection and enumeration of antigen-reactive lymphoid cells in the lymph nodes and spleens of mice after vaccination or immunization. Using this method, we measured the numbers of antigen-reactive rosette-forming cells (RFCs) and the ability to withstand lethal *Salmonella* infections in ICR Swiss mice and in four inbred mouse strains after injection of either the RIA vaccine or glutaraldehyde-treated or untreated E-RNA extracts isolated from *S. typhimurium* SR-11 or *S. typhimurium* or both.

(Some of the results were taken from a Ph.D. thesis submitted by A.E. to the University of Health Sciences/Chicago Medical School and from an M.S. thesis submitted by D.P.K. to Wright State University.)

MATERIALS AND METHODS

Animals. Noninbred ICR Swiss mice and inbred C57BL/6, DBA/2, and BDF_1 (C57BL/6 × DBA/2) mice were obtained from A. R. Schmidt Co.-Sprague-Dawley, Madison, Wis. C3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Female mice (12 to 16 g) were used throughout this study.

Bacterial strains. *S. typhimurium* strains SR-11 and RIA were obtained originally from L. Joe Berry, Bryn Mawr College, Bryn Mawr, Pa. The method for determining the 50% lethal dose (LD_{50}) of *S. typhimurium* SR-11 has been described previously (1a, 15, 44).

Preparation of protein-rich fractions (E-RNA extracts) from lysates of strains SR-11 and RIA. The method used was that described by Smith and Bigley (44). Protein determinations were performed by the method of Lowry et al. (25). Bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) was used to establish standard curves. Deoxyribonucleic acid analyses were performed as described by Dische (12), using the sodium salt of deoxyribonucleic acid (Nutritional Biochemicals Corp., Cleveland, Ohio) as a standard. Ribonucleic acid determinations were performed by the orcinol test for ribonucleic acids (11), using bovine liver ribonucleic acid (Nutritional Biochemicals Corp.) as the standard. The E-RNA fractions used in this study contained ratios of ribonucleic acid to protein of 13:7 and 11:9 and less than 5% diphenylamine-positive material; disc gel electrophoresis of these samples showed the same protein bands that have been reported previously (44).

Glutaraldehyde polymerization of E-RNA extract preparations. Cross-linking of the E-RNA extracts was achieved by modifying the procedures of Avrameas and Ternynck (1), as described elsewhere (1a). Glutaraldehyde-treated E-RNA extracts are referred to below as poly(E-RNA).

Spent medium fractions. Spent medium fractions were prepared by the method of Plant et al. (38). We found that these spent medium fractions contained the same 18 bands of protein-staining material (44) that were present in the E-RNA fractions as determined by polyacrylamide gel electrophoresis (J. M. Margolis and N. J. Bigley, unpublished data). The spent medium protein fraction used in this study contained less that 2% ribonucleic acid and deoxyribonucleic acid.

Immunization. ICR Swiss, C57BL/6, DBA/2, and BDF_1 mice were each inoculated intraperitoneally with 10^5 *S. typhimurium* RIA cells. The bacteria were grown in brain heart infusion broth and harvested as described elsewhere (1a, 44). Other mice of these same strains were divided into groups and immunized subcutaneously with either 200 μg (based on protein content) of E-RNA extract from strain RIA or strain SR-11 or 200 μg of glutaraldehyde-treated strain SR-11 E-RNA extract in Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). After 15 days, the inoculated mice were examined for levels of serum antibody, migration inhibition factor of spleen cells, rosette-forming activity of lymphoid cells, and protection against lethality by *S. typhimurium* SR-11.

Challenge of immunized mice with virulent *S. typhimurium* SR-11. To determine the efficacy of immunizations with E-RNA extracts, glutaraldehyde-treated E-RNA extracts, and RIA vaccine, mice were challenged 15 to 18 days after immunization with 100 LD_{50} of *S. typhimurium* SR-11 (based upon the LD_{50} for each strain of mice) prepared from an 8-h brain heart infusion broth culture. Each animal was injected intraperitoneally with the challenge dose of *Salmonella* in 0.2 ml of sterile saline. Infected mice were isolated from noninfected animals to prevent accidental infection and were checked for signs of salmonellosis for 35 days, after which the number of survivors in each group was determined.

Conjugation of antigen to erythrocytes. Antigen-coated mouse erythrocytes were used both for rosette formation and for titrations of immune serum by passive hemagglutination. These cells were prepared by the methods of Gold and Fundenberg (20) and Giuliano et al. (19). Blood was obtained from the retroorbital sinus of each mouse with a capillary tube and was collected in heparinized tubes (5 U of heparin per ml of blood; heparin sodium; Fisher Scientific Co., Chicago, Ill.). The freshly drawn blood was washed three times in sterile isotonic saline and then centrifuged for 1 min with a Clay-Adams Sero-Fuge at 250 × *g*. E-RNA extracts were conjugated to mouse erythrocytes by mixing 0.1-ml amounts of the extracts, each containing 1 mg of protein per ml, with 0.1-ml volumes of washed packed cells. Chromic chloride (Fisher Scientific Co.) in normal saline was added quickly (0.1 ml of a 0.1% solution), and the mixture was incubated for 4 min at room temperature. The cells were then washed three times in 20 to 40 volumes of sterile isotonic saline and finally suspended in sufficient saline to produce a 2% suspension. For the rosette assays to detect antigen-reactive splenic lymphocytes from C3H/HeJ mice, sheep erythrocytes were coated with the spent medium protein antigen as described below.

Titration of immune serum. The levels of serum antibody in immunized animals were determined by passive hemagglutination (5). Erythrocytes obtained

from unimmunized animals of each mouse strain were washed three times with saline and coated with the SR-11 E-RNA extract by chromic chloride treatment as described previously (19, 20). Portions (0.2 ml) of a 2% suspension of washed antigen-coated cells were added to 0.5-ml amounts of immune serum (from the same mouse strain as the test erythrocytes) serially diluted in normal saline. The mixtures were incubated at 37°C for 1 h, after which the tubes were centrifuged for 1 min with a Clay Adams Sero-Fuge and read for hemagglutination.

Isolation of spleen and lymph node cells. Spleens and inguinal, mesenteric, brachial, and axillary lymph nodes were removed aseptically and placed into cold barbital-buffered saline (pH 7.2). The spleens or lymph nodes from five mice were pooled and forced through a steel sieve (40 mesh).

The lymphoid cells used for rosette formation were separated by centrifugation on a Ficoll-Hypaque gradient; 5-ml amounts of the cell suspensions were carefully layered over 5-ml volumes of Ficoll-Hypaque solution (9% Ficoll-34% Hypaque, 24:10) and were centrifuged at 800 × *g* for 20 min. The cells at the interface were removed with a Pasteur pipette and washed three times with barbital-buffered saline, and the cell pellet was suspended in barbital-buffered saline. Viable cell numbers were determined by the trypan exclusion test (3).

Macrophage migration inhibition technique. We used the method of Smith and Bigley (44) for spleen cell migration inhibition assays. Migration indices were then determined by the method of George and Vaughan (18), as follows: migration index = [(area of migration with antigen)/(area of migration without antigen)] × 100.

Formation of antigen-reactive RFCs. The techniques which we used to form antigen-reactive RFCs were modifications of the procedures of Theis et al. (46) and Elliott et al. (17). Spleen or lymph node cells prepared as described above were adjusted to a concentration of 10^8 lymphoid cells per ml; 0.1-ml samples of the cell suspensions were mixed with 0.1-ml volumes of 2% antigen-coated mouse erythrocytes from the same mouse strain as the lymphoid cells and 0.8-ml volumes of saline in siliconized culture tubes (10 by 75 mm). The tubes were centrifuged for 2 min at 250 × *g* and then transferred to a 37°C water bath for a 30-min incubation. After incubation, T-cell rosettes were stabilized by adding 0.1 ml of a 1% solution of sodium azide. The pellet containing lymphoid cells and erythrocytes was carefully dispersed and mixed with equal parts of trypan blue before scanning for cell viability and cell numbers in a hemacytometer counting chamber. Approximately 5×10^3 lymphocytes were scanned; the percentage of RFCs was determined. A cell was considered to be an RFC if it was a lymphocyte that bound three or more erythrocytes.

Treatment of lymphoid cells with antitheta serum. Antitheta serum (lot 231-20-1; Litton Bionetics, Kensington, Md.) was produced in AKR mice immunized with thymocytes from C3H mice. This serum was diluted 1:10 in incomplete RPMI 1640 medium and frozen at −80°C until needed. The T-cell specificity of this antitheta serum was verified by its cytotoxicity titer of 1:80 or more for C3H thymocytes, its cytotoxicity titer of 1:40 or more for C3H spleen cells, and its lack of cytotoxicity for C3H bone marrow cells at a dilution of 1:10; pretreatment of C3H spleen cells with this antitheta serum and complement inhibited the mitogenic response to phytohemagglutinin, whereas the mitogenic response to bacterial lipopolysaccharide was not affected. In our hands, a 1:10 dilution of this serum in the presence of a 1:6 dilution of agarose-adsorbed guinea pig complement diminished viability of lymph node and spleen cell suspensions from ICR Swiss, DBA/2, C57BL/6, and BDF_1 mice by approximately 50%.

In experiments with C3H/HeJ mice, rabbit anti-mouse thymocyte serum (lot 11940; Cappel Laboratories, Cochranville, Pa.) was used. The T-cell specificity of this serum was determined by using indirect immunofluorescence with goat anti-rabbit immunoglobulin G labeled with fluorescein isothiocyanate (Cappel). This method showed that in spleen cell suspensions (10^7 cells), the percentage of T cells was 57.2 ± 4.7%; after treatment with a 1:10 dilution of rabbit antithymocyte serum and a 1:6 dilution of agarose-adsorbed guinea pig complement, the percentage of T cells was 36.5 ± 5.2%.

Antitheta serum treatment was performed by a modification of the procedure of Wilson and Miller (47). Spleen and lymph node cells from immune animals (10^7 cells per ml) were incubated with equal volumes of a 1:10 dilution of either antitheta serum or antithymocyte serum at 37°C for 30 min. The cells were then centrifuged at 250 × *g* for 5 min, washed three times in RPMI 1640 medium, and resuspended in 1 ml of fresh medium. These cells were then exposed to 1 ml of a 1:6 dilution of agarose-adsorbed guinea pig complement. The mixture was incubated for an additional 30 min at 37°C and then washed two times with RPMI 1640 medium.

RESULTS

Detection of antigen-reactive RFCs. ICR Swiss mice were sacrificed 15 to 18 days after immunization, and the numbers of antigen-reactive lymphocytes in their spleens and lymph nodes were compared with the numbers found in animals sham-immunized with phosphate-buffered saline (pH 7.1) in incomplete Freund adjuvant. Sham-immunized mice did not contain detectable RFCs reactive with E-RNA extracts. The lack of antigen-specific background lymphocytes in mouse spleens has been reported for other antigens; Cunningham (11) showed that the number of antigen-specific spleen cells in immunized mice varied from zero for soluble or haptenic antigens to 33 to 500 antigen-specific cells per 10^8 spleen cells for heterologous erythrocytes. As Table 1 shows, greater numbers of RFCs were usually present for the homologous E-RNA extract than for a heterologous E-RNA extract. This table also shows the number of RIA-reactive RFCs present after injection of the same amount (200 μg) of SR-11 poly(E-RNA) extract. When these numbers were compared

TABLE 1. *Antigen-specific RFCs in immunized ICR Swiss and C57BL/6 mice*

Immunizing extract	E-RNA on erythrocytes	% Of antigen-reactive RFCs in:[a]			
		ICR Swiss mice		C57BL/6 mice	
		Spleens	Lymph nodes	Spleens	Lymph nodes
RIA E-RNA	RIA	0.14	0.28	0.06	0.13
	SR-11	0.10	0.26	0.07	0.06
SR-11 E-RNA	SR-11	0.10	0.37	ND[b]	0.07
	RIA	0.13	0.20	0.18	0.10
SR-11 poly(E-RNA)	RIA	0.21	0.20	ND	ND
RIA vaccine	RIA	0.31	0.21	0.11	ND
	SR-11	0.20	0.10	0.07	0.04

[a] Mean values of three separate experiments; each experiment was performed in triplicate.
[b] ND, Not done.

with the numbers of RFCs present in comparable cell suspensions from ICR Swiss mice immunized with the conventional antigenic extracts (either RIA or SR-11), we observed little difference in the spleen and lymph node preparations from the three groups of mice. Since maximum protection against lethal disease occurs after active infection or after immunization with vaccines which are capable of establishing sublethal infections (9), it was of interest to determine the number of antigen-reactive RFCs in mice after vaccination with 10^5 live cells of *S. typhimurium* RIA. ICR Swiss mice were immunized intraperitoneally, and more RFCs were present in spleens than in lymph nodes 15 to 18 days after immunization (Table 1).

Development of antigen-reactive RFCs in vaccinated ICR Swiss mice. ICR Swiss mice were immunized intraperitoneally with 10^5 *S. typhimurium* RIA cells, and the lymphoid organs from animals in groups of five were examined after varying times. Figure 1 shows the number of antigen-binding lymphoid cells in the spleens and lymph nodes of immunized mice over a 15-day period. Detectable levels of antigen-reactive RFCs appeared first in the spleens of immunized animals by day 4. At this time lymph nodes did not contain cells capable of binding erythrocytes coated with SR-11 E-RNA extract. This behavior was reversed 6 days after immunization, at which time lymph nodes contained slightly higher numbers of RFCs than did the spleens from the same animals. Throughout the remaining period of observation, the highest levels of RFCs were maintained in the splenic lymphoid population.

Detection of antigen-reactive RFCs in C57BL/6 and ICR Swiss mice. ICR Swiss and C57Bl/6 mice were sacrificed 15 to 18 days after immunization, and the numbers of antigen-reactive lymphocytes in their spleens and lymph nodes were compared with the numbers found in unimmunized control animals. Sham-immunized mice of both strains did not contain detectable RFCs reactive with E-RNA extracts (Table 1). ICR Swiss mice immunized with RIA E-RNA extract or vaccinated with 10^5 *S. typhimurium* RIA cells contained higher numbers of antigen-reactive RFCs in their spleens and lymph nodes than did C57BL/6 mice. After immunization with SR-11 E-RNA extract, C57BL/6 mice contained fewer antigen-reactive RFCs than did the comparable immunized ICR mice. Even after vaccination with the attenuated RIA strain of *S. typhimurium*, C57BL/6 mice were less capable than ICR Swiss mice of responding immunologically to *Salmonella*.

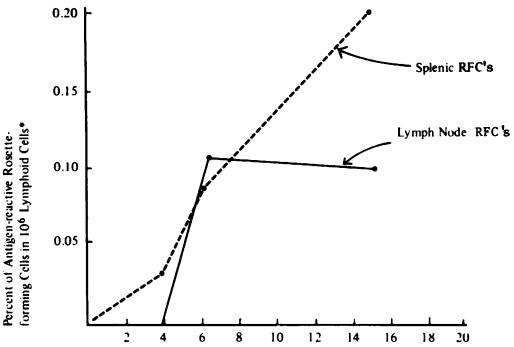

FIG. 1. *Development of antigen-reactive RFCs in the spleens and lymph nodes of ICR Swiss mice immunized with 10^5 S. typhimurium RIA cells. Each point on the graph represents the mean value of three separate tests; each test was performed in triplicate.*

Nature of the RFCs produced in response to E-RNA extract and poly(E-RNA). To determine the nature of the antigen-reactive RFCs found in the lymph nodes and spleens of immunized mice, cells were treated before rosette formation with antitheta serum and agarose-absorbed guinea pig complement. As Table 2 shows, after this treatment the numbers of antigen-reactive RFCs in mice immunized with E-RNA extracts and RIA vaccines were greatly

reduced, suggesting the T-lymphocyte nature of the majority of the RFCs counted in these samples. All RFCs remaining in the lymphoid cell population after such treatment had large numbers of erythrocytes clustered around the central mononuclear cells; the remaining rosettes were either the B-cell type of rosettes described by others (17) or rosette formers resulting from the presence of cytophilic antibody adsorbed to the cell surface. The latter possibility is likely since workers in our laboratory have shown previously that cytophilic macroglobulin is produced regularly in ICR Swiss mice immunized with E-RNA extracts and RIA vaccine (28). Table 2 also shows that antitheta serum treatment did not remove the majority of the RFC activity in lymph node suspensions from ICR Swiss mice immunized with the poly(E-RNA) antigen. This observation suggests that poly(E-RNA) extracts induce primarily a B-cell (antitheta serum-resistant) response in immunized mice.

Nature of the immune response and host protection. Table 3 shows the relationship between antibody formation and the type of antigen-reactive RFCs present in immunized animals when SR-11 E-RNA extract was used as the test antigen. Readily detectable levels of serum antibody (titer, 128) were associated with antitheta serum-resistant lymphoid cell populations in ICR Swiss mice. Animals immunized with E-RNA extracts and RIA vaccine contained an extremely small antitheta serum-resistant population (B cells) and a concomitant lack of detectable serum antibody.

Table 4 shows the survival of immunized ICR Swiss and C57BL/6 mice after intraperitoneal challenge with 100 LD_{50} of *S. typhimurium*. Only ICR Swiss mice containing T-cell RFCs (antitheta serum sensitive) after immunization were able to withstand infection by virulent *Salmonella*. poly(E-RNA), which stimulated production of detectable serum antibody and antitheta serum-resistant RFCs reactive with erythrocytes coated with SR-11 E-RNA extract in ICR Swiss mice, failed to provide adequate levels of protection against lethal infection. Only untreated E-RNA extracts and RIA vaccine were able to induce an immune response capable of protecting ICR Swiss mice. Upon vaccination, C57BL/6 mice were able to produce an antibody response to SR-11 poly(E-RNA), and they did not develop detectable levels of non-T-cell RFCs or resistance to lethal *Salmonella* infection.

TABLE 2. *Effect of antitheta serum on the formation of antigen-reactive RFCs in lymph node cell suspensions from immunized ICR Swiss mice*

Immunizing substance	E-RNA on erythrocytes	% Of RFCs[a]	
		Untreated	Antitheta serum treated
RIA E-RNA	RIA	0.24	0.04
	SR-11	0.20	0.06
SR-11 E-RNA	SR-11	0.17	NF[b]
SR-11 poly(E-RNA)	SR-11	0.20	0.15
RIA vaccine	SR-11	0.13	0.02

[a] Mean values of three separate experiments; each experiment was performed in triplicate.
[b] NF, None found.

TABLE 3. *Antibody production and antitheta serum-resistant RFCs in immunized ICR Swiss mice*

Immunogen	% Of RFCs[a]		Serum antibody titer
	Untreated	Antitheta serum treated	
SR-11 E-RNA	0.23	0.04	NF[b]
RIA E-RNA	0.28	NF	NF
RIA vaccine	0.25	0.02	NF
SR-11 poly(E-RNA)	0.20	0.15	128

[a] Mean values of three separate experiments; each experiment was performed in triplicate.
[b] NF, None found.

As Fig. 2 shows, lymph nodes from mice initially immunized with unpolymerized SR-11 E-RNA extract showed a marked hyperproliferation of the cells in the thymus-dependent paracortical areas, whereas lymph nodes from mice immunized with SR-11 poly(E-RNA) contained numerous and distinct lymphoid follicles with active germinal centers; little, if any, stimulation of the thymus-dependent paracortical areas was observed in the latter tissues.

ICR Swiss mice immunized with either 100 μg of SR-11 E-RNA extract or 100 μg of glutaraldehyde-treated SR-11 E-RNA extract were challenged 18 days later with 100 LD_{50} of virulent *S. typhimurium* SR-11. The lymph nodes were removed from the survivors 37 days after challenge and fixed by David Schwartz, The University of Health Sciences, Chicago, Ill. Microscopic examination of the tissues was done by Raoul Fresco, Stritch School of Medicine, Maywood, Ill.

Cellular basis for inherited resistance against murine salmonellosis. Inbred C57BL/6, DBA/2, and BDF_1 mice are markedly susceptible to infection by *S. typhimurium*; LD_{50}s of less than five microorganisms were observed for all three strains during this investigation, which is in agreement with values reported by Robson and Vas (40). Hormaeche (21) observed intravenous LD_{50} (log 10) values for *S. typhimurium* C5 (virulent) of 1.62 cells in DBA/2 mice and 1.7 cells in C3H mice. LD_{50} values of

TABLE 4. *Inverse relationship between antibody formation and protection in ICR Swiss and C57BL/6 mice*

Mouse strain	Immunizing substance	% Of non-T-cell rosettes[a]	Antibody titer	% Survival at 35 days after 100 LD_{50} of *S. typhimurium* SR-11
ICR Swiss	SR-11 E-RNA	0.04	NF[b]	83 (50/60)[c]
	SR-11 poly(E-RNA)	0.20	128	20 (6/30)[d]
	RIA vaccine	0.02	NF	85 (51/60)
	None	NF	NF	0 (0/60)
C57BL/6	SR-11 poly(E-RNA)	ND[e]	64	0 (0/20)
	RIA vaccine	NF	2	0 (0/20)
	None	NF	NF	0 (0/20)

[a] Mean values of three separate experiments; each experiment was performed in triplicate.
[b] NF, None found.
[c] Numbers in parentheses indicate number surviving/number tested.
[d] $P < 0.01$ by chi-square test.
[e] ND, Not done.

10^2 cells (intraperitoneal) and 10^3 cells (intravenous) were observed for *S. typhimurium* SR-11 in the markedly more resistant ICR Swiss mice. Of the strains of mice examined in this study, only BDF_1 (C57BL/6 × DBA/2) and ICR Swiss animals developed resistance to lethal salmonellosis after immunization with the strain RIA vaccine. The data in Fig. 3 provide possible reasons for the differences in the responses of these four mouse strains to protein-rich *Salmonella* extracts after immunization. ICR Swiss mice exhibited higher numbers of RFCs after immunization than did the more susceptible C57BL/6 and DBA/2 strains.

Comparison of spleen cell migration inhibition, antigen-reactive lymph node cells, and host resistance. Table 5 shows the abilities of the F_1 hybrid (BDF_1) mouse strain and its parental strains to develop lymph node RFCs, spleen cell migration inhibition, and protective host resistance. ICR Swiss mice were also included in this comparison. These mice were examined for the capacity to form antigen-reactive RFCs in lymph nodes, the capacity to exhibit spleen cell migration inhibition responses, and the capacity to resist lethal *Salmonella* infections after injection of the RIA vaccine or injection of glutaraldehyde-modified E-RNA extracts (Table 5). Since the glutaraldehyde-treated extracts stimulated formation of antibody titers ranging from 64 to 128 in the inbred and ICR Swiss mice, the role of antibody formation in host resistance was evaluated in the mice receiving the SR-11 glutaraldehyde-treated poly(E-RNA). As Table 5 shows, only the BDF_1 inbred mice and the ICR Swiss control mice showed marked survival to challenge infections, lymph node RFCs at or above the 0.2% level, and reduced spleen cell migration. Animals (DBA_2, BDF_1, and ICR Swiss) forming only antibody to the poly(E-RNA) did not survive challenge infections, did not form lymph node RFCs up to the 0.2% level, and did not exhibit spleen cell migration inhibition after vaccination. Vaccination with living *S. typhimurium* RIA cells also did not improve the capacity of either the DBA/2 or the C57BL/6 parental strain to mount these responses.

RFCs and host protection in endotoxin-resistant C3H/HeJ and ICR Swiss mice. C3H/HeJ mice are as susceptible to lethal *Salmonella* infections as DBA/2, C57BL/6, and BDF_1 (DBA/2 × C57BL/6) mice, in that the LD_{50} of *S. typhimurium* SR-11 is less than 10 *Salmonella* cells. Of all the mice used in this study, only C3H/HeJ mice are endotoxin resistant (13, 39, 43). We wanted to determine whether endotoxin resistance influenced the ability of an animal to form antigen-reactive RFCs and to survive a challenge infection of 100 LD_{50} of virulent *S. typhimurium* SR-11 2 weeks after vaccination with 10^5 cells of the RIA vaccine. As Table 6 shows, C3H/HeJ mice were able to develop antigen-reactive rosette-forming T cells when the spent medium extract was used as the test antigen and were able to withstand a challenge infection. Again, no background rosettes were observed when nonimmune C3H/HeJ splenic lymphocytes were added to sheep erythrocytes coated (chromic chloride treatment) with the spent medium protein antigen.

DISCUSSION

The contribution of a host to *Salmonella* resistance is complex. The net growth rate of *Salmonella* cells in the livers and spleens of inbred mice during the first 4 days of infection is high in some susceptible host strains, yet it is low in other susceptible strains and in all resistant mouse strains tested by Hormaeche (21). The trait determining low net growth rate is dominant and functions before detection of cell-mediated immunity (21). The earliest detection

of a cell-mediated immune response in mice occurs 4 days after injection of *Salmonella* either intravenously (1a, 9) or subcutaneously (1a, 45). *Salmonella*-specific delayed hypersensitivity responses can develop in some susceptible mouse strains, as well as in resistant mouse strains (21). *Salmonella* resistance appears to occur in mouse strains in which low net growth rates of *Salmonella* occur along with positive footpad reactivity (a measure of cellular immunity). This study was focused on the abilities of murine hosts to generate antigen-reactive T-cell responses in lymphoid tissues; these responses were assessed by measuring the number of theta antigen-bearing antigen-reactive RFCs present in the lymph nodes or spleens or both of five mouse strains at 2 to 2.5 weeks after vaccination with *S. typhimurium* RIA or immunization with

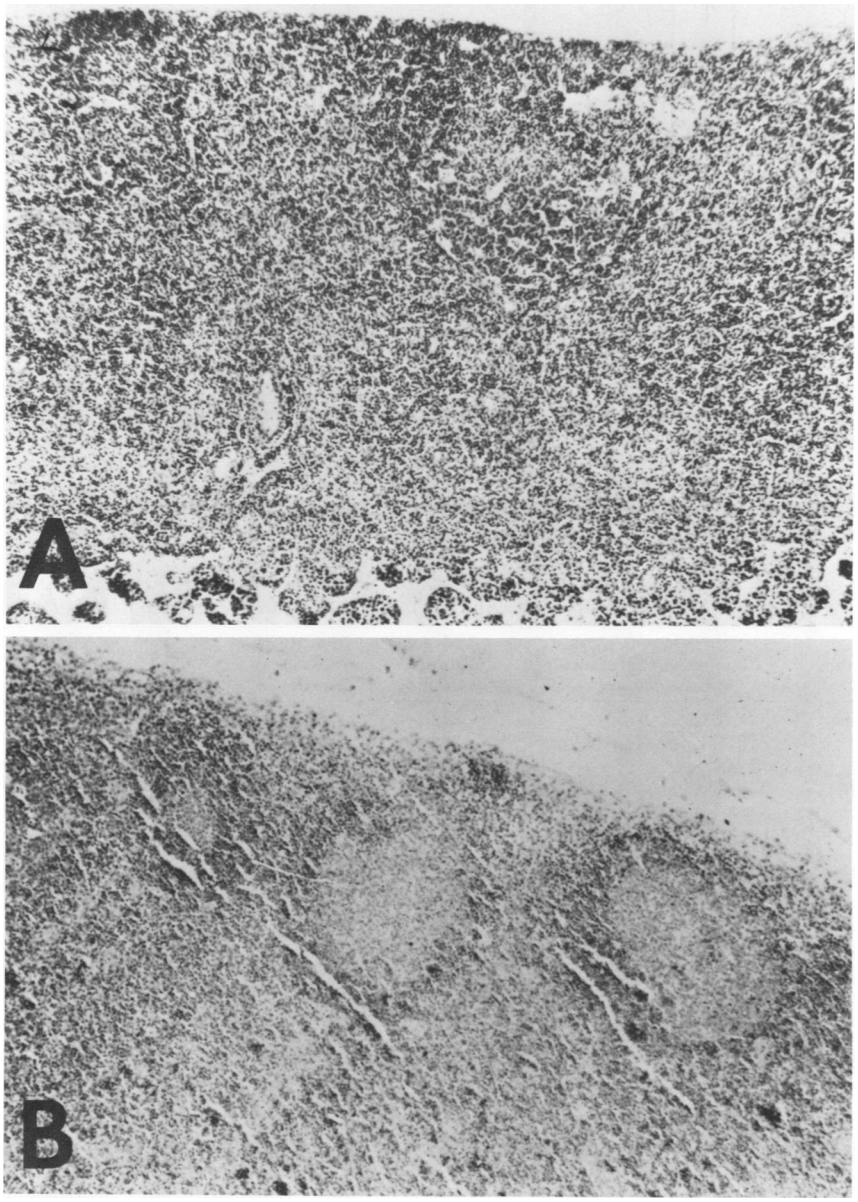

FIG. 2. *Lymph node sections of immunized and challenged ICR Swiss mice. (A) Mice initially immunized with untreated SR-11 E-RNA extract. (B) Mice initially immunized with glutaraldehyde-treated SR-11 E-RNA extract.*

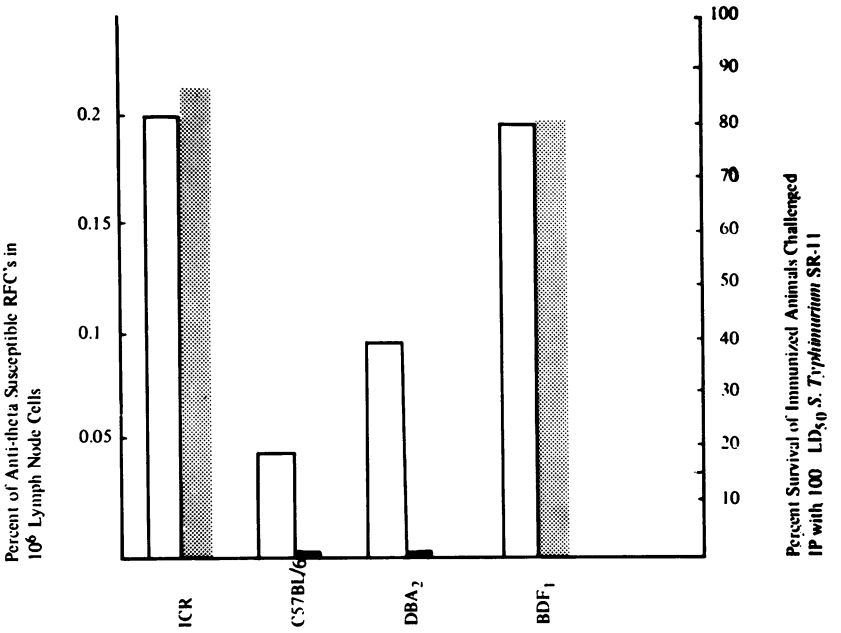

FIG. 3. *Comparison of the immune responses of inbred mouse strains C57BL/6, DBA/2, and BDF_1 (C57BL/6 × DBA/2) with the responses of outbred ICR Swiss mice. The percentage of theta-positive RFCs was determined by calculating the difference between the total number of RFCs observed and the number found after incubation with antitheta serum and complement. Protection studies were carried out by immunizing each group of animals with 10^5 RIA cells and then challenging with 100 LD_{50} of S. typhimurium SR-11. Solid and shaded bars, percent survival; open bars, percent theta-positive RFCs. Values are means of three separate experiments; each experiment was performed in triplicate. IP, Intraperitoneally.*

TABLE 5. *Comparison of the abilities of outbred and inbred mouse strains to exhibit antigen-induced spleen cell migration inhibition, antigen-reactive lymph node cells, and resistance to lethal salmonellosis*

Mouse strain	Immunizing substance	% Mean migration (spleen)[a]	% of RFCs (lymph node)[a]	Serum antibody titer	% Survival at 35 days after 100 LD_{50}
ICR Swiss	RIA vaccine	34.5	0.30	NF[b]	85 (17/20)[c]
	SR-11 poly(E-RNA)	70.0	0.14	128	0 (0/20)
	PBS[d]	100.0	NF	NF	0 (0/20)
C57BL/6	RIA vaccine	ND[e]	0.04	2	0 (0/20)
	PBS	100.0	NF	NF	0 (0/20
DBA/2	RIA vaccine	89.5	0.09	NF	0 (0/20)
	SR-11 poly(E-RNA)	90.2	NF	32	0 (0/20)
BDF_1	RIA vaccine	56.8	0.19	NF	80 (16/20)
	poly(E-RNA)	70.1	ND	128	15 (3/20)

[a] Mean values of three separate experiments; each experiment was performed in triplicate. The test antigen was SR-11 E-RNA extract.
[b] NF, None found.
[c] Numbers in parentheses indicate number surviving/number tested.
[d] PBS, Phosphate-buffered saline.
[e] ND, Not done.

E-RNA extracts prepared from *S. typhimurium* SR-11 and RIA. Coincident with the assessment of the ability of the various mouse strains to form detectable T-cell RFCs after vaccination or immunization, an evaluation was made of the type of cellular response stimulated by glutaraldehyde-modified E-RNA extracts.

The spleens and lymph nodes of the various strains of mice contained no detectable background RFCs for the E-RNA extract or spent medium protein-coated erythrocytes. Cunningham reported that the number of antigen-specific spleen cells in unimmunized mice varies from zero for some soluble and hapten antigens

to 33 to 500 antigen-specific cells per 10^8 spleen cells for heterologous erythrocytes (11).

In this study, the appearance of antigen-reactive RFCs occurred in the spleens of ICR Swiss mice as early as 4 days after injection of the RIA vaccine; three times as many RFCs were present on day 15. These findings correlate with our previous observation that immunized ICR Swiss mice also show positive footpad swelling responses 4 days post-immunization, which are characterized by mononuclear cell infiltration 48 h after footpad injection of antigen (45); spleen cells from the same mice showed a significant ($P < 0.01$) reduction in migration in the presence of the E-RNA extract (45). Similarly, Collins (9) and Hormaeche (21) reported that *Salmonella*-injected responder mice develop manifestations of delayed hypersensitivity as early as 4 days after vaccination.

The C57BL/6 and DBA/2 mice were susceptible to lethal *Salmonella* infections even after injection of the immunogenic E-RNA extracts or the RIA vaccine; 15 days after injection, these mice exhibited low levels (less than 0.1%) of antigen-reactive T-cell rosettes in their lymph nodes, suggesting that they were incapable of making an effective cell-mediated response. Boehme (3) reported a lack of thymic development and a lack of cellular activity in the subcapsular zones of lymphoid tissues in highly susceptible mouse strains. In strains of mice resistant to *Salmonella* infections, active cellular proliferation in these same tissues was observed (3); in such mouse strains, maximum resistance against lethal *Salmonella* infections is achieved by vaccination with *Salmonella* cells that are able to replicate within the lymphoid tissues of the host (2, 8, 9, 26, 27).

Of the C57BL/6, DBA/2, and F_1 hybrid (BDF_1) mouse strains, only BDF_1 was capable of mounting an effective cell-mediated immune response against virulent *S. typhimurium* SR-11. The natural resistance of different inbred mouse strains to intravenous challenge with *S. typhimurium* appears to be polygenic (21). The fact that the F_1 hybrid (BDF_1) between C57BL/6 and DBA/2 is a *Salmonella* responder whereas the parent strains are not may be explained by the observations of Dorf and Benacerraf (14) and Katz et al. (22, 23) concerning gene complementation. The B10.A and B10 mouse strains are phenotypic nonresponders to the synthetic terpolymer GLϕ; the F_1 hybrid of these two strains (the recombinant 5R strain) is a phenotypic responder, having received the α gene from the B10.A parent and the β gene from the B10 parent (14, 22, 23). Responsiveness to GLϕ is an Ir gene-restricted response of the *H-2* genome (14). Similar to ICR Swiss mice, immunized or vaccinated BDF_1 mice demonstrated increased resistance to lethal salmonellosis, the presence of higher levels (0.2% or higher) of theta-positive antigen-reactive RFCs in lymphoid tissues, the inhibition of migration of spleen cells in the presence of antigen, and no detectable antibody to the test antigen (E-RNA extracts).

TABLE 6. *Host protection and percentages of antigen-reactive spleen RFCs in RIA-vaccinated and control ICR Swiss and C3H/HeJ mice*

Mouse strain	Treatment	% Of RFCs[a]		% Survival at 30 days after 100 LD_{50} of *S. typhimurium* SR-11
		Not treated	Antitheta serum and complement treated	
ICR Swiss	Immunized	0.27	0.07	100 (20/20)[b]
	Control	NF[c]	NF	0 (0/20)
C3H/HeJ	Immunized	0.22	0.06	70 (14/20)
	Control	NF	NF	0 (0/20)

[a] Mean values of three separate experiments; each experiment was performed in triplicate.

[b] Numbers in parentheses indicate number surviving/number tested.

[c] NF, None found.

The endotoxin-resistant C3H/HeJ mice also contained more than 0.2% antitheta serum-sensitive rosette-forming spleen cells after vaccination with the RIA vaccine strain, and 70% of these mice were able to withstand a challenge of 100 LD_{50} of virulent *Salmonella*. This latter observation suggests that the endotoxin resistance of the C3H/HeJ mice does not adversely affect the capacity of these mice to develop T-cell immunity and effective resistance against virulent *S. typhimurium*. Misfeldt and Johnson used four inbred strains of mice having different sensitivities to *Salmonella* infection and endotoxin to determine that endotoxin is not entirely responsible for the efficacy of *S. typhimurium* ribosomal vaccines (31). These same investigators also showed that the *Salmonella* immunity which develops in C3H/HeJ mice injected with ribosomal vaccines may depend upon the presence of bacterial cell wall proteins in the ribosomes (32). The levels of antitheta serum-susceptible antigen-reactive RFCs observed in this study (approximately 0.2%) correlated well with the capacities of the immunized responder mouse strains (ICR Swiss, DBA/2, and C3H/HeJ) to resist lethal *Salmonella* infections. In animals immunized with either the SR-11 E-RNA extract or the RIA vaccine, the majority of the antigen-reactive RFCs were T cells since they were sensitive to the cytotoxic effects of antitheta serum and complement. The impor-

tance of the T-cell response against facultative intracellular bacteria is well established (9, 24, 29, 34, 48). Moser et al. (33) have also observed that T-cell-mediated host responses directed against antigens of *S. typhimurium* other than lipopolysaccharides correlated well with the ability of murine hosts to withstand lethal *Salmonella* challenges.

The levels of antigen-reactive RFCs characterized as T cells in this study were reduced in animals immunized with glutaraldehyde-treated extracts; these same animals consistently made humoral antibody responses to E-RNA extracts and exhibited a concomitant reduction in the ability to survive a lethal challenge infection with *S. typhimurium* SR-11. Modulation of an immune response by antigen modification (10, 25) emphasizes the importance of antigen conformation in determining the type of response elicited. A histological examination of the lymph nodes from these animals 37 days after challenge revealed marked stimulation of follicles with active germinal centers typical of B-cell responses, in contrast to the hyperproliferative paracortical stimulation observed in mice initially immunized with untreated E-RNA extracts and subsequently challenged.

Although the antigen-reactive T cells reported here have not been shown to be involved directly in delayed hypersensitivity or in protective immunity, mice in whose spleens or lymph nodes or both such cells were found in concentrations of 0.2% did demonstrate positive spleen cell migration inhibition reactions and significant protective immunity. In this study, we found that the F_1 hybrid (BDF_1) between the intermediately susceptible DBA/2 strain (21, 40) and the highly susceptible C57BL/6 strain (30, 40) is able to produce both an effective delayed hypersensitivity response (as measured by spleen cell migration inhibition responses) and protective immunity after vaccination. These responses were similar in intensity to those which developed in the resistant ICR Swiss mice; immune lymph nodes of both the BDF_1 and ICR Swiss mice contained RFCs reactive with the E-RNA extracts at or above 0.2%. All mouse strains tested (C57BL/6, DBA/2, DBF_1, and ICR Swiss) showed readily detectable antibody responses after immunization with glutaraldehyde-modified E-RNA extracts; the antibody responses alone were not adequate to protect the mice against challenge infections. Antibody was able to prolong survival during the first 2.5 weeks after challenge but by itself was not able to prevent overgrowth of *Salmonella* in the mice (7). Prevention of overgrowth occurs in mice in which *Salmonella*-specific cell-mediated immunity exists (9). As this study shows, the *Salmonella*-specific theta antigen-bearing lymphocytes that are present in higher numbers in the responder host strains tested (ICR Swiss, BDF_1, and C3H/HeJ) than in the two nonresponder strains tested were directed against protein-containing *Salmonella* extracts (the E-RNA extracts, which contained an average of 40% protein, or the spent medium protein of Plant et al. [38], which is primarily protein). The nonresponder C57BL/6 and DBA/2 mice were able to generate only low levels of this type of lymphocyte response; if the antigen-reactive RFCs are responsible, even in part, for *Salmonella* resistance, this may explain the inability of these two *Salmonella* nonresponder strains to immunize effectively.

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