INFECTION AND IMMUNITY, Jan. 1983, p. 262–269
0019-9567/83/010262-08$02.00/0

Vol. 39, No. 1

Gamma-Irradiated Scrub Typhus Immunogens: Development of Cell-Mediated Immunity After Vaccination of Inbred Mice

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Received 9 August 1982/Accepted 14 October 1982

Mice immunized with three injections of gamma-irradiated Karp strain of *Rickettsia tsutsugamushi* were evaluated for the presence of cell-mediated immunity by using delayed-type hypersensitivity, antigen-induced lymphocyte proliferation, and antigen-induced lymphokine production. These animals also were evaluated for levels of circulating antibody after immunization as well as for the presence of rickettsemia after intraperitoneal challenge with viable Karp rickettsiae. After immunization with irradiated Karp rickettsiae, a demonstrable cell-mediated immunity was present as evidenced by delayed-type hypersensitivity responsiveness, lymphocyte proliferation, and production of migration inhibition factor and interferon by immune spleen lymphocytes. Also, a reduction in circulating rickettsiae was seen in mice immunized with irradiated rickettsiae after challenge with 1,000 50% mouse lethal doses of viable, homologous rickettsiae. All responses except antibody titer and reduction of rickettsemia were similar to the responses noted in mice immunized with viable organisms. Antibody levels were lower in mice immunized with irradiated rickettsiae than in mice immunized with viable rickettsiae. Furthermore, mice that were immunized with viable rickettsiae demonstrated markedly lower levels of rickettsemia after intraperitoneal challenge compared with either mice immunized with irradiated rickettsiae or nonimmunized mice.

Previous studies in this laboratory have demonstrated that mice can be protected effectively against lethal infection with *Rickettsia tsutsugamushi* by administration of gamma-irradiated, yolk-sac-grown homologous rickettsiae (3–5). It also has been shown that the most effective protection to homologous as well as heterologous challenge with strains of *R. tsutsugamushi* is acquired after administration of a vaccine consisting of the Karp, Kato, and Gilliam strains of rickettsiae (5). Importantly, the development of protective immunity was associated with the development of splenic lymphocytes able passively to transfer immunity to normal mice, and administration of vaccine under optimal conditions resulted in little or no antibody production (3), thus suggesting that resistance to challenge in these animals was mediated by a cell-mediated immunity (CMI).

Immunity also can be elicited in mice and can be established by administration of viable *R. tsutsugamushi* subcutaneously (s.c.) (8, 13). This protective immunity is apparently due to the development of a CMI, as demonstrated by passive transfer of resistance with thymus-derived lymphocytes (T-cells) (25), development of delayed-type hypersensitivity (DTH) (13), and in vitro lymphocyte proliferation (LP) in response to *R. tsutsugamushi* antigens (T. R. Jerrells and J. V. Osterman, manuscript in preparation).

The presence of CMI after infection with other rickettsiae as well as with *R. tsutsugamushi* has been demonstrated by LP assays (14, 15), and the production of lymphokines, including migration inhibition factor (MIF) and macrophage-activating factor, after the interaction of sensitized T-cells and antigen (9, 19). Furthermore, these activities have been demonstrated in experimental systems after the administration of nonviable rickettsiae (15).

As an understanding of the immunity developed by administration of inactivated rickettsiae is of critical importance in the development of an effective vaccine to scrub typhus, this study was designed to assess development of a specific CMI in BALB/c mice that received gamma-irradiated Karp immunogen. To detect antigen-responsive T-cells, DTH responses to irradiated, tissue-culture-grown Karp rickettsiae were examined as well as antigen-induced LP and

production of the lymphokines MIF and interferon (IFN) in lymphocyte culture supernatants.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from Flow Laboratories, Inc. (Dublin, Va.) and used at the age of 8 to 12 weeks.

Immunogen. The Karp strain (egg passage 52) of *R. tsutsugamushi* was plaque purified (22) and propagated in embryonated hen eggs. Infected yolk sacs were prepared as 20% suspensions, and the organisms were inactivated by irradiation with 200 krads (by using ^{60}Co with a Gamma cell 220 [Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada]), as previously described (3). Before irradiation, the number of viable rickettsiae was determined by titration in mice, and the 50% mouse lethal dose (MLD_{50}) was calculated by the method of Spearman and Karber (7). Mice to be vaccinated were given three doses of 2×10^7 MLD_{50} of irradiated rickettsiae diluted in brain heart infusion broth intraperitoneally (i.p.) at 5-day intervals (4). Immunological studies were initiated 7 days after the last vaccine dose. Mice were immunized with viable Karp rickettsiae by the administration of 1,000 MLD_{50} s.c. as previously described (13).

Antigen preparation. Karp rickettsiae were grown in irradiated L-929 cells as previously described (2). Rickettsiae were liberated from the cells by homogenation, and, after centrifugation at $240 \times g$ for 10 min, the rickettsiae were concentrated by centrifugation at $5,000 \times g$ for 60 min to remove cell debris. The resulting pellet was suspended in phosphate-buffered saline, dispensed in samples, and stored at −70°C. Before use as an antigen, the sterility of each lot was determined with standard bacteriology culture media, and the preparations were exposed to 200 krads of gamma radiation as described above. Antigen preparations were quantitated for total protein by the technique of Lowry et al. (17).

DTH assay. The development of DTH was evaluated by the procedure of Lefford (13, 16). Briefly, mice to be tested were given 1 μCi of tritiated thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per 10 g of body weight 24 h before testing. Animals were injected intradermally with 25 μl of pyrogen-free saline in one ear and with 25 μl of irradiated antigen diluted to contain 100 μg of total protein per ml in pyrogen-free saline in the other ear. After another 24 h, the mice were sacrificed by CO_2 asphyxiation, and a circular tissue piece was removed from each ear and digested with Protosol (New England Nuclear Corp.). Incorporation of radiolabel into diluent- and antigen-injected ears was determined by scintillation counting (Prias; Packard Instrument Co., Inc., Downers Grove, Ill.), and data were expressed as ear ratios determined by dividing counts per minute (CPM) in the antigen-injected ear by CPM in the diluent-injected ear.

LP assay. Spleen lymphocytes were evaluated for proliferative responses to Karp antigen by using a microculture assay as previously described (12). Single-cell suspensions were prepared from at least three spleens per group in RPMI 1640 medium supplemented with 50 μg of gentamicin per ml, 1% fresh glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, all obtained from MA Bioproducts (Walkersville, Md.). The cells were washed twice and adjusted to 5×10^6 viable cells per ml in RPMI 1640 medium supplemented as described above and containing 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Individual wells of a 96-well microtiter plate (Falcon Plastics, Oxnard, Calif.) received 0.1-ml aliquots of cells, and quadruplicate wells received 0.1 ml of either medium or Karp antigen diluted to the desired concentration in RPMI 1640 medium without added serum, making the final serum concentration 5%. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 h. Tritiated thymidine (1 μCi) was added to each well for the final 6 h of culture. Cells were harvested onto filter strips by using a multiple harvesting system, and incorporated radioactivity was determined by scintillation counting as described above. Data were expressed as net CPM, derived by subtracting CPM of cultures receiving medium from CPM of cultures receiving antigen, and as stimulation indexes, derived by dividing CPM of antigen-stimulated cultures by CPM of cultures receiving medium.

Production and assay of MIF. Spleen cells from normal mice or mice immunized with viable or irradiated rickettsiae were adjusted to 5×10^6/ml in RPMI 1640 medium supplemented as described above and containing 10% fetal bovine serum, and 5-ml aliquots were cultured in conical centrifuge tubes. Medium or antigen was added to the appropriate tubes, and the cultures were incubated for 72 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the cultures were centrifuged at $200 \times g$ for 10 min, and the supernatant was collected and filtered through a 0.45-μm filter and stored at −40°C until assayed. In some experiments cells were pulsed with antigen by incubation of cells and antigen for 2 h followed by centrifugation to pellet the cells, which were then suspended in antigen-free medium. Cultures were handled from this point as described above. These experiments demonstrated results identical to those of experiments in which antigen was left in the culture for the entire 72 h (data not shown). The presence of MIF in the culture supernatants was detected by using an agarose droplet macrophage migration inhibition as described by Padarathsingh et al. (23). Peritoneal exudate cells were obtained by peritoneal lavage, as previously described (12), of normal mice 72 h after injection with 1.0 ml of brain heart infusion broth. The peritoneal exudate cells were washed twice with RPMI 1640 medium and finally adjusted to 2×10^8 cells per ml in an agarose (Marine Colloids, Rockland, Maine)-RPMI 1640 mixture containing 10% fetal bovine serum. To individual wells of flat-bottom microtiter plates (Costar, Boston, Mass.) 2-μl droplets of cells in agarose were added and allowed to solidify for 5 min at room temperature. Quadruplicate cell-containing wells received medium or supernatants from lymphocyte cultures which were diluted in RPMI 1640 medium containing 10% fetal bovine serum. Plates were incubated for 48 h at 37°C in a humidified 5% CO_2 atmosphere, and the resulting migration patterns were traced with a projection device (Plaque Viewer; Bellco Glass, Inc., Vineland, N.J.). The extent of migration was determined by planimetry as described previously (1). Data were expressed as migration indexes, which were calculated

by dividing migration in the presence of antigen-stimulated supernatant by migration in the presence of nonstimulated supernatant. A positive response was defined as a migration index of ≤0.80.

Quantitation of IFN. Murine L-929 cells (American Type Culture Collection, Rockville, Md.) were adjusted to 5×10^5 viable cells per ml, using trypan blue exclusion as the criterion for viability. The medium was minimal essential medium supplemented with 1% glutamine, 50 μg of gentamicin per ml, and 5% heat-inactivated fetal bovine serum (Microbiological Associates, Walkersville, Md.). Cells and twofold dilutions of spleen cell supernatants were added to the wells in 0.1-ml volumes and incubated for 18 h at 37°C in a humidified 5% CO_2 incubator. A series of dilutions of the murine reference IFN (G-002-904-511; National Institutes of Health, Bethesda, Md.) was included in each test. After 24 h, the supernatant was removed and replaced with fresh medium containing vesicular stomatitis virus (Indiana strain) at a multiplicity causing complete cytopathic effects in unprotected cells within 48 h. At that time, cultures were observed for reduction of cytopathic effects. The highest dilution still protecting 50% of the cells provided the IFN titer. One antiviral unit in our assay corresponded to approximately 1.3 reference IFN units.

In preliminary experiments, immune mice or spleen cells were tested with a preparation of uninfected L-929 cells as previously described (13), and these studies demonstrated no significant reactivity in any of the assays used.

Detection and quantitation of rickettsemia. Animals that were immunized by the standard procedure described above were challenged i.p. with 1,000 MLD_{50} of viable Karp rickettsiae 14 days after the last injection. At the same time, nonimmunized mice also were challenged. At appropriate intervals after challenge, three to five animals from each group were bled from the retroorbital sinus into heparinized capillary tubes; the blood was pooled and diluted in cold brain heart infusion broth, and 0.2 ml was injected i.p. into five recipient mice per dilution. Rickettsial numbers were expressed as MLD_{50} per 0.2 ml of whole blood, calculated as described above.

Antibody quantitation. Levels of circulating antibody were quantitated in serum samples by using an indirect immunofluorescence assay as previously described (24).

RESULTS

The administration of a total rickettsial mass of 6×10^7 MLD_{50} of irradiated Karp rickettsiae given in three equal doses 5 days apart rendered BALB/c mice resistant to challenge with 1,000 MLD_{50} of viable Karp rickettsiae, even if challenged as early as 3 days after the last injection of immunogen (Table 1). Animals immunized with this regimen demonstrated protective immunity up to 60 days after the last immunogen injection, when the experiments were terminated (data not shown), which is essentially as previously described (4).

In further studies, we examined mice immunized with irradiated Karp rickettsiae for the presence of a CMI. In initial experiments we used DTH reactivity to examine immunized mice for the presence of antigen-responsive lymphocytes. Mice receiving irradiated immunogen demonstrated DTH reactivity at 7 days after the last immunogen dose; this reactivity was greatest at 14 to 21 days and remained throughout the study (Table 2). It is important to note that the reactivity of immunized animals was comparable to that of animals immunized by s.c. administration of viable rickettsiae. Nonimmunized, noninfected animals, when tested with Karp antigen, demonstrated no reactivity, as expected. Immunized mice tested with a homogenate of L-929 cells demonstrated no reactivity (ear ratio of 1.08 ± 0.03).

Additional studies were performed with in vitro assays to monitor antigen-responsive lym-

TABLE 1. Protection of BALB/c mice against homologous i.p. challenge after administration of irradiated immunogen[a]

Day of challenge[b]	% Survival[c]
3	100
7	100
14	100
21	100
Nonimmunized	0

[a] Mice were immunized via three i.p. injections of irradiated Karp rickettsiae (2×10^7 MLD_{50} per injection) at 5-day intervals.

[b] Mice were challenged i.p. at the indicated time after the last administration of immunogen with 1,000 MLD_{50} of Karp rickettsiae.

[c] Survival at 28 days after challenge; five animals were challenged at each time point.

TABLE 2. DTH responses of mice immunized with gamma-irradiated Karp strain of *R. tsutsugamushi*

Day after last immunogen injection[a]	Ear ratio[b]
Nonimmunized	0.89 ± 0.07
7	1.27 ± 0.13
14	1.78 ± 0.18
21	1.44 ± 0.10
28	1.24 ± 0.17
Immunized s.c.[c]	1.64 ± 0.10

[a] Mice were immunized via three i.p. injections of irradiated Karp rickettsiae (2×10^7 MLD_{50} per injection) at 5-day intervals.

[b] Mean ± one standard error of CPM in antigen-injected ear/CPM in diluent-injected ear (10 mice per group). Results for all immunized groups are significantly different from those of nonimmunized controls at $P \leq 0.05$ by Student's t test.

[c] Mice were injected s.c. with 1,000 MLD_{50} of viable rickettsiae from the same batch used for the vaccine and were tested 28 days after immunization.

phocytes. Initially, the ability of spleen lymphocytes to proliferate in response to Karp antigen was investigated. The development of LP paralleled the development of DTH reactivity (Table 3) in that significant proliferation (stimulation index of ≥2.0) was evident as early as 7 days after the last administration of vaccine, and mice remained reactive through at least 43 days (data not shown) after the vaccine regimen. In contrast to DTH reactivity, a marked peak of proliferation was not evident during this study, and LP reactivity was fairly uniform throughout the study, with only slight changes noted. It would appear that LP reactivity developed somewhat later than DTH reactivity, with the greatest reactivity evident at 21 days after the last dose of vaccine and remaining high thereafter. Also, immune spleen cells stimulated with homogenized, uninfected L-929 cells showed no significant response during 72 h (data not shown).

TABLE 3. Development of LP activity after administration of irradiated Karp strain of *R. tsutsugamushi*

Day after last immunogen injection[a]	Net CPM ± SE[b]	SI[c]
Nonimmunized	4,695 ± 506	1.3
7	9,718 ± 947	2.2
14	11,327 ± 493	2.0
21	11,810 ± 397	4.0
28	13,111 ± 270	2.8
Immunized s.c.[d]	36,867 ± 304	9.0

[a] Mice were immunized via three i.p. injections of irradiated Karp rickettsiae (2×10^7 MLD_{50} per injection) at 5-day intervals.

[b] Results are for quadruplicate cultures. Values represent maximal stimulation. Results for all immunized groups are significantly different from CPM of nonstimulated cultures at $P \leq 0.05$ by Student's t test.

[c] SI, Stimulation index. Values of ≥2.0 were considered to be a positive response.

[d] Mice were injected s.c. with 1,000 MLD_{50} of viable rickettsiae from the same batch used for the vaccine and were tested 28 days after immunization.

Another parameter of interest was the production of lymphokines by antigen-responsive lymphocytes obtained from animals receiving irradiated immunogen. Culture supernatants from spleen cells stimulated with irradiated rickettsiae were assayed for the lymphokines MIF and immune IFN. Lymphocytes from vaccinated mice produced MIF activity in culture supernatants when challenged with Karp antigen at all doses tested if obtained 14 days after the last immunogen dose (Table 4), and these results correlated with DTH and LP reactivity. At 7 days after the last injection, MIF was produced at the higher antigen doses. It also is evident from these data that mice immunized with viable rickettsiae also demonstrated MIF production in vitro and that this reactivity could be elicited in culture with relatively small antigen doses. Lymphocyte supernatants also were examined for the presence of immune IFN by using reduction of cytopathic effects in L-929 cell monolayers due to vesicular stomatitis virus infection (Table 4). As with MIF activity, IFN activity was evident only in relatively high titers in lymphocyte cultures prepared from spleen cells taken from mice 14 days after the completion of the vaccine regimen. Spleen cells taken 7 days after the last injection produced only minimal amounts of IFN, and the greatest production of IFN (1,280 U/ml) was obtained by culturing lymphocytes from animals 14 days after completion of the vaccination schedule. Furthermore, as it is possible to measure IFN production in vivo (20), additional experiments were undertaken to determine whether IFN production in the whole animal correlated with activity noted in lymphocyte culture supernatants. Serum samples were obtained from immunized mice (21 days after the last injection) 4 h after intravenous challenge with 500 μg of Karp antigen. Immunized mice produced IFN titers of 1,280 U/ml compared with IFN levels of ≤40 U/ml in nonimmunized mice challenged with the same antigen concentration. In preliminary experiments, peak levels of IFN were produced 4 h after antigen challenge (B. A. Palmer, unpublished observation).

To determine whether mice immunized with irradiated Karp rickettsiae were able to limit the levels of circulating rickettsiae after challenge, blood rickettsial titers were determined after challenge of immunized and nonimmunized mice. A representative experiment is presented in Fig. 1. Circulating rickettsiae were detectable in immunized mice after challenge, but at 7 and 10 days after infection, these levels were significantly lower ($P \leq 0.05$ by Student's t test) than levels detected in nonimmunized mice. Nonimmunized mice uniformly died 10 to 11 days after challenge, but at this time the levels of circulating rickettsiae in immunized animals peaked and declined to low or undetectable levels by 14 days after infection. This experiment was repeated on three separate occasions with similar results. As it was possible that an increase in the dose of irradiated immunogen would provide a more efficient mechanism for limiting a rickettsemia, animals were immunized with an irradiated immunogen preparation containing 8×10^8 MLD_{50} per injection given in three doses 5 days apart. These animals were challenged 14 days after the last injection, and the levels of circulating rickettsiae at 7 and 10 days after infection were determined and compared with levels detected in nonimmunized mice and mice immunized by s.c. infection with viable Karp rickettsiae. A

TABLE 4. Production of lymphokines by spleen lymphocytes obtained from mice immunized with gamma-irradiated Karp strain of *R. tsutsugamushi*

Day after last immunogen injection[a]	Antigen dose (μg/ml)	MI[b]	IFN concn (U/ml)
Nonimmunized	100	0.97	40
	10	0.97	<40
	1	1.04	<40
7	100	0.65	160
	10	0.75	80
	1	0.91	<40
14	100	0.44	>1,280
	10	0.37	1,280
	1	0.63	640
21	100	0.52	320
	10	0.60	80
	1	0.64	80
28	100	0.49	640
	10	0.50	640
	1	0.69	160
Immunized s.c.[c]	100	0.59	640
	10	0.71	160
	1	ND[d]	80

[a] Mice were immunized via three i.p. injections of irradiated Karp rickettsiae (2×10^7 MLD_{50} per injection) at 5-day intervals.

[b] MI, Migration index in presence of lymphocyte supernatant diluted 1:10. Values of ≤0.80 and mean areas of migration significantly different from those in the presence of control supernatants are considered to be a positive response.

[c] Mice were injected s.c. with 1,000 MLD_{50} of viable rickettsiae from the same batch used for the vaccine and were tested 28 days after immunization.

[d] ND, Not determined.

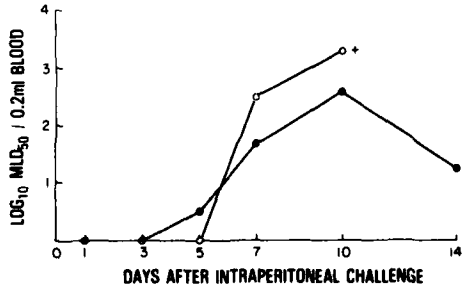

FIG. 1. Quantitation of rickettsemia after i.p. challenge of nonimmunized mice (○) or mice that received three injections of irradiated Karp immunogen (●) with 1,000 MLD_{50} of the Karp strain of *R. tsutsugamushi*. Each point represents the log MLD_{50} obtained from pooled blood samples obtained from five mice at each time point. +, Nonimmunized mice challenged with Karp rickettsiae died 10 to 11 days after challenge.

rickettsemia was still detectable in mice immunized with the maximum dose of irradiated Karp rickettsiae available (Table 5); however, no rickettsiae were detectable in the blood of mice immunized with viable Karp rickettsiae.

Serum samples obtained at various times after completion of the immunization scheme were examined for the presence of antibody with an indirect immunofluorescence assay. These data (Table 6) suggest that vaccination with a total irradiated rickettsial mass of 6×10^7 MLD_{50} does not lead to a marked antibody response at any of the time points examined. The maximal antibody response (antibody titer of 80) was detected 28 days after vaccination. Immunization with viable Karp rickettsiae, however, led to a markedly greater antibody response at 21 and 28 days after immunization than that noted in mice receiving irradiated immunogen.

DISCUSSION

Previous studies from this laboratory have demonstrated that administration of irradiated *R. tsutsugamushi* leads to the development of immunity to challenge with viable organisms in the relative absence of antibody production (3). These studies, along with the studies that demonstrated that inactivated *Coxiella burnetii* produce a measurable CMI when given to experimental animals (15), suggested the possibility that gamma-irradiated *R. tsutsugamushi* organisms induce a protective CMI when given to mice.

In the present study we confirmed that administration of irradiated *R. tsutsugamushi* protects BALB/c mice from lethal infection with the homologous immunizing strain of rickettsiae. We also examined mice immunized with irradiated rickettsiae for the presence of a CMI, using well-established techniques.

When animals were examined as early as 7 days after the last administration of immunogen, antigen-responsive T-lymphocytes were demonstrable, as evidenced by DTH reactivity, antigen-induced proliferation, and production of the lymphokines MIF and IFN. These responses reached optimal levels 14 days after the last administration of immunogen and remained relatively constant throughout the duration of the experiments.

It is important to note that the DTH and lymphokine responses seen in mice immunized with irradiated rickettsiae were similar to, although somewhat lower than, the responses of animals immunized by the administration of viable organisms. Antigen-induced LP responses were lower in mice immunized with irradiated organisms than in mice examined 28 days after administration of viable rickettsiae (Table 4).

TABLE 5. Levels of circulating rickettsiae after i.p. challenge of mice immunized with irradiated or viable organisms

Immunogen	MLD_{50} per 0.2 ml of blood on the following day after i.p. challenge:[a]	
	7	10
None	8.0×10^2	1.3×10^3
Irradiated[b]	8.0×10^1	2.0×10^2
Viable[c]	<10	<10

[a] Mice were challenged i.p. with 1,000 MLD_{50} of Karp rickettsiae 14 days after completion of immunizing injections.

[b] Karp rickettsiae at 8×10^8 MLD_{50} per 0.2 ml were irradiated with 300 krads and administered as described in the text.

[c] Karp rickettsiae (1,000 MLD_{50}) given s.c.

although positive results (stimulation index of ≥2.0) were obtained in these animals.

Although the magnitude of the responses noted in mice receiving irradiated rickettsiae was similar to that noted in animals receiving viable organisms, the kinetics of the responses were different. In a previous report (13) DTH reactivity resulting from immunization with viable *R. tsutsugamushi* was found to peak at 14 days after immunization and to decline thereafter, which is in contrast to proliferative responses, which peaked somewhat later and remained for relatively long periods after immunization (T. R. Jerrells and J. V. Osterman, submitted for publication). It is possible that the chronic infection resulting from viable immunization results in the production of immunoregulatory mechanisms, which affect DTH and in vitro proliferation differently and, more importantly, are not induced by the administration of inactivated rickettsiae. As relatively large doses of irradiated immunogen do not produce this phenomenon, the presence of a chronic infection is apparently more important in the observed modulation of responses than simply the initial antigen mass administered.

TABLE 6. Development of circulating antibody after immunization of BALB/c mice with irradiated or viable Karp rickettsiae

Day after immunization[a]	Antibody titer[b] in mice immunized with:	
	Viable rickettsiae (1,000 MLD_{50} given s.c.)	Irradiated rickettsiae (2×10^7 MLD_{50} per injection)
7	40	40
14	80	80
21	160	80
28	1,280	80

[a] Day after last injection of irradiated Karp rickettsiae or after administration of viable Karp rickettsiae.

[b] Reciprocal of highest dilution of serum showing reactivity with homologous rickettsiae in the indirect immunofluorescence assay.

Spleen cells from mice immunized with viable *R. tsutsugamushi* produce the lymphokine macrophage-activating factor after exposure to specific rickettsial antigen (19). In the present study we elected to examine lymphocyte culture supernatants for the lymphokines MIF and IFN because of their traditional association with the presence of a CMI. Also, the direct applicability to human and primate systems or application to humans and primates of a modified technique (1, 18, 21), and the availability of microassay methods, which is a requirement for human and primate studies, make these assays attractive.

Gamma or immune IFN is produced by T-lymphocytes after mitogen or antigen stimulation and is felt to be a true lymphokine (6, 20, 26). The IFN produced by spleen cells obtained from mice immunized by viable and irradiated *R. tsutsugamushi* meets the criteria for immune IFN in that it is pH 2 labile, not neutralized by anti-α/β IFN sera, and produced in optimal quantities 48 to 72 h after antigen exposure (B. A. Palmer, F. Hetrick, and T. R. Jerrells, manuscript in preparation). Detection of antigen-responsive lymphocytes based on immune IFN production offers several advantages over that based on other lymphokines, including ease of assay, ability to quantitate in terms of international units, and availability of reference standards, and should prove to be useful in monitoring T-lymphocytes sensitized to *R. tsutsugamushi* antigens. With the exception of DTH reactivity, which is thought to be a sensitive and specific measure of T-lymphocyte antigen recognition, the parameters of CMI used in this study can, under certain conditions, involve B-lymphocytes (27). Although we feel it is unlikely that B-lymphocytes are contributing to the responses noted in mice which received irradiated immunogen, based on the relatively low antibody production, studies are currently in progress to show definitively that the responses are due to T-lymphocyte activation.

Although the survival of mice receiving irradiated immunogen was associated with a decreased number of circulating rickettsiae, it was clear that rickettsial replication did occur in these mice. To rule out a suboptimal immunizing dose, mice were given three injections of Karp rickettsiae at the maximum concentration available in our laboratory. In this experiment (Table 5) a demonstrable rickettsemia was still noted. Conversely, we found that low doses of immunogen (1.6×10^6 MLD_{50} per injection) provided protection against rechallenge, although no demonstrable antibody or cellular response was

elicited (data not shown). The levels of circulating rickettsiae in these animals were again lower, but not significantly, than those seen in nonimmunized mice (data not shown), and circulating rickettsiae were cleared after 10 days (Fig. 1). Also, mice immunized with viable rickettsiae did not develop a detectable rickettsemia after infection with homologous rickettsiae (Table 5). These data suggest that the administration of irradiated rickettsiae may prime the immune system and that a secondary response occurs quickly enough to clear the rickettsiae before a lethal infection occurs. It is also possible that immunization with viable organisms produces a more efficient cellular immunity than that with irradiated organisms, and this possibility was not ruled out in this study. However, one major difference noted in this study and previous studies (3) was the relatively low antibody levels in mice immunized with irradiated rickettsiae compared with the antibody response of mice immunized with viable rickettsiae. Other studies have shown that passive transfer of immune serum to naive mice protects against homologous *R. tsutsugamushi* challenge (25), and Humphres and Hinrichs (10) recently have shown that passive administration of immune serum to naive mice enhances the clearance of *C. burnetii*, apparently through an augmentation of the development of CMI. It is possible that the lack of circulating rickettsiae after challenge of mice immunized with viable rickettsiae is due to the presence of circulating antibody in these animals. The mechanism of this clearance simply may be due to a more rapid removal of rickettsiae antigen-antibody complexes by the reticuloendothelial system or to an antibody-mediated adjuvant effect, as described by Humphres and Hinrichs (10). The potential implications of these possibilities in the production of a scrub typhus vaccine are of importance, and further studies are in progress to attempt to answer these fundamental questions.

Taken together, we feel that the data generated in this study suggest that administration of irradiated *R. tsutsugamushi* to mice effectively produces a CMI, which in part may be responsible for clearance of viable rickettsiae after challenge. Furthermore, this study demonstrates that several assays are reliable indicators of immunity induced by irradiated *R. tsutsugamushi* immunogens in mice. It is important to note that the assays used in this study have been used to detect immunity to rickettsiae (28) or other antigens (1, 11, 18, 21; T. R. Jerrells, R. H. Neubauer, and H. Rabin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, E162, p. 69) in humans and primates and should be applicable to primate and human trials of *R. tsutsugamushi* vaccines.

ACKNOWLEDGMENTS

We thank Miriam Pedersen for expert technical assistance and Jan Jerrells for input in the preparation of this manuscript.

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A127886	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) "Gamma-Irradiated Scrub Typhus Immunogens: Development of Cell-Mediated Immunity After Vaccination of Inbred Mice		5. TYPE OF REPORT & PERIOD COVERED 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Thomas R. Jerrells, Bennie A. Palmer and Joseph Osterman		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research Washington, D.C. 20012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 612770/871AC/153/WWGD
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Ft Detrick, Fredrick, MD 21701		12. REPORT DATE 13. NUMBER OF PAGES 8
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

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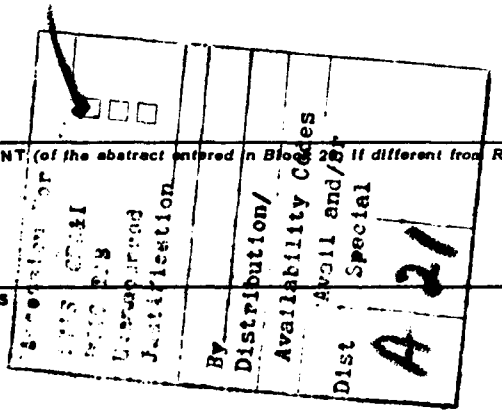

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18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Scrub typhus, Immunogens; cell mediated immunity; vaccine studies

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Mice immunized with three injections of gamma-irradiated Karp strain of Rickettsia tsutsugamushi were evaluated for the presence of cell-mediated immunity by using delayed-type hypersensitivity, antigen-induced lymphocyte proliferation, and antigen-induced lymphokine production. These animals also were evaluated for levels of circulating antibody after immunizations as well as for the presence of rickettsemia after intraperitoneal challenge with viable Karp rickettsiae. After immunization with irradiated Karp rickettsiae, a demonstrable cell-mediated immunity was present as evidenced by delayed-type hyper-

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sensitivity responsiveness, lymphocyte proliferation, and production of migration inhibition factor and interferon by immune spleen lymphocytes. Also, a reduction in circulating rickettsiae was seen in mice immunized with irradiated rickettsiae after challenge with 1,000 50% mouse lethal doses of viable homologous rickettsiae. All responses except antibody titer and reduction of rickettsemia were similar to the responses noted in mice immunized with viable organisms. Antibody levels were lower in mice immunized with irradiated rickettsiae than in mice immunized with viable rickettsiae. Furthermore, mice that were immunized with viable rickettsiae demonstrated markedly lower levels of rickettsemia after intraperitoneal challenge compared with either mice immunized with irradiated rickettsiae or nonimmunized mice.