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Gamma-Irradiated Scrub Typhus Immunogens: Development and Duration of Immunity

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The development and duration of immunity to lethal scrub typhus infection was studied in BALB/c mice vaccinated with gamma-irradiated *Rickettsia tsutsugamushi*, strain Karp. One intraperitoneal injection containing approximately 10^8 50% mouse lethal doses (MLD_{50}) of irradiated organisms elicited an immune response protective against challenge with 10^5 MLD_{50} of viable Karp. The same mass of immunogen given in three injections at 5-day intervals increased homologous (Karp strain) protection 25-fold and heterologous (Kato strain) protection 60-fold. Further temporal expansion of the immunization regimen did not increase protection. Subcutaneous vaccination provided significant, but lower, levels of protection than were achieved by intraperitoneal immunization, but the levels of cell-transferable immunity elicited by the two routes were approximately the same. Immunologically specific protection after intraperitoneal vaccination developed rapidly enough to provide resistance against simultaneous challenge with 200 MLD_{50} of Karp. Homologous immunity was protective against a 10^6-MLD_{50} challenge 7 days after completion of the three-injection regimen, remained at that level for 3 months, dropped to 10^4 MLD_{50} by 9 months, and was effective against a 50-MLD_{50} Karp challenge at 12 months. Protection against heterologous challenge was first observed on day 17 and peaked on day 38, when the mice resisted a 10^5-MLD_{50} Kato challenge. Thereafter, heterologous protection waned rapidly and was not significant at 6 months.

In a recent report (8), we contrasted the protection achieved against experimental infection with scrub typhus rickettsiae in mice vaccinated with formalinized or gamma-irradiated immunogens. Irradiated organisms protected the majority of mice against challenge with 10,000 50% mouse lethal doses (MLD_{50}) of either the immunizing strain or a heterologous strain of *Rickettsia tsutsugamushi*. This level of heterologous protection has been observed in mice surviving experimental infection (2, 22), but was much greater than that achieved after vaccination with formalinized preparations (19). We also observed two additional properties shared by normal and gamma-irradiated scrub typhus rickettsiae, but not by formalin-killed organisms: the capacity to enter and persist for short periods of time within cultured L-929 cells, and the ability to stimulate cell-transferable immunity. Other investigators have shown that mice which recover from experimental scrub typhus infection harbor viable rickettsiae for extended periods of time while remaining immune to exogenous challenge (10, 15); work with other intracellular pathogens has indicated that latent infection may contribute to the maintenance of cell-mediated immunity (16); also, this laboratory has demonstrated that cell-mediated immunity is important in protection against lethal infection with heterologous strains of *R. tsutsugamushi* (21). Since irradiated organisms stimulated substantial levels of immunity and exhibited two properties potentially important for maintenance of immunity, we felt that further studies were indicated to determine the rate of development and the duration of immunity to homologous and heterologous challenge.

Our previous study used vaccination and challenge schedules similar to those employed by others with formalinized immunogens (25). Although this approach allowed a direct comparison of our results with theirs, our current knowledge of the different characteristics of irradiated rickettsiae suggested that studies should be initiated to explore the effects of different regimens on the ability of irradiated Karp strain immunogens to induce protection. In addition, we wished to measure the contribution of progressive increases in antigenic mass, because this has been shown to be a factor in stimulating humoral responses (6, 17) and recent work has indicated that a threshold mass must be reached to initiate

protective cell-mediated immunity in experimental scrub typhus infections (20). Use of the subcutaneous route for immunization with gamma-irradiated rickettsiae also appeared interesting because other investigators have shown that subcutaneous inoculation of viable *R. tsutsugamushi* produces a nonlethal infection in mice with development of heterologous immunity (2, 22), whereas subcutaneous vaccination with formalinized organisms has failed to elicit significant protection (1, 11, 18).

Therefore, using the inbred BALB/c mouse model, we have continued our investigations on vaccination with 300-Krad gamma-irradiated Karp immunogens, exploring the relationships between antigenic mass, route of inoculation, and regimen on the induction of immunity, and using that knowledge in a long-term study to examine the maintenance of protection in vaccinated animals.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 18 to 22 g, were obtained from Flow Laboratories, Dublin, Va.

Rickettsiae. The Karp strain (49th to 56th egg passages) and Kato strain (161st to 198th egg passages) of *R. tsutsugamushi* were propagated, stored, and quantified by methods previously reported (3). Only those suspensions having a titer $\geq 1 \times 10^8$ MLD_{50}/g of yolk sac were used.

Immunogens. Radiation-inactivated immunogens were prepared by exposing frozen 20% yolk sac suspensions of the Karp strain to 300-Krad gamma radiation in a ^{60}Co gamma irradiator (Gamma Cell 220, Atomic Energy of Canada Limited, Ottawa, Canada) as previously described (8). Immunogen mass is reported in MLD_{50} based on lethal titer in BALB/c mice before irradiation.

Vaccination and challenge. Unless otherwise stated, all injections were administered intraperitoneally (i.p.) in a standard volume of 0.2 ml, and challenge doses were injected 21 days after completion of the vaccination regimen. On the day of challenge, standard suspensions of either Karp (homologous strain) or Kato (heterologous strain) were titrated in vaccinated and in normal control animals, using dilutions in 10-fold increments calculated to encompass dose ranges, as appropriate, between 10^{-1} and 10^7 MLD_{50}. Deaths occurring 6 to 28 days postchallenge were quantified. The $\log_{10}$ MLD_{50} and standard deviation were calculated by the Spearman-Kärber method (9), and immunity indexes (25) were obtained by subtracting the $\log_{10}$ MLD_{50} observed by titration in the control mice from that seen in the test group.

Spleen cell and serum transfer. When required, one mouse equivalent of serum or washed, unfractionated spleen cell suspension was transferred from vaccinated donors to normal recipients by i.p. injection (21). After 8 h, the Karp and Kato challenge seeds were titrated in donor and control mice, and the recipients were challenged with those dilutions containing 10^1 to 10^4 MLD_{50} of rickettsiae.

Transfer of disrupted spleen cells. Homogenized spleen cell suspensions prepared from vaccinated mice as previously described (20) were transferred to normal mice by i.p. injection. Recipients were observed for deaths occurring 6 to 28 days after transfer, and survivors were challenged with 10^3 MLD_{50} of Karp and observed for an additional 28 days.

RESULTS

Effect of rickettsial mass and multiple injections on development of protection. Two groups of mice were vaccinated with the same mass of immunogen, 2.5×10^8 MLD_{50} of irradiated rickettsiae. One group was given the entire dose in a single injection of 0.6 ml, and the other group received the dose in a series of three injections of 0.2 ml administered at 5-day intervals. The results of homologous and heterologous challenge are shown in Table 1. While both immunization procedures elicited high levels of immunity to homologous challenge, immunity indexes indicated that protection was heightened 25-fold by using the multiple-injection regimen. The difference in protection was even greater when heterologous challenge was used. Although individual mice that received the immunogen in a single injection were capable of resisting 10^5 MLD_{50} of Kato, the majority suc-

TABLE 1. *Effect of injection regimen on the capacity of a mass of gamma-irradiated rickettsiae to induce a protective immune response in mice*

Challenge strain	No. of immunogen injections[a]	No. of survivors/no. of vaccinated mice challenged							$\log_{10}$ MLD_{50} in vaccinated mice[b]	$\log_{10}$ MLD_{50} in control mice[b]	Immunity index[c]
		10^{4d}	10^7	10^6	10^5	10^4	10^3	10^2			
Karp	1	0/5	0/5	1/5	5/5	5/5	5/5	5/5	−4.0 (±0.2)	−9.6 (±0.2)	5.6 (±0.3)
	3	0/5	3/5	5/5	5/5	ND[e]	ND	ND	−2.6 (±0.2)	−9.6 (±0.2)	7.0 (±0.3)
Kato	1	ND	0/5	0/5	1/5	1/5	1/5	2/5	−5.8 (±0.4)	−8.0 (±0.2)	2.2 (±0.5)
	3	ND	0/5	0/5	0/5	4/5	5/5	5/5	−4.0 (±0.2)	−8.0 (±0.2)	4.0 (±0.3)

[a] Total mass delivered, regardless of regimen, was 2.5×10^8 MLD_{50} of irradiated Karp strain *R. tsutsugamushi*.

[b] Values (± standard deviation) are based on dilution factors used to achieve the approximate challenge doses noted.

[c] Immunity index = $\log_{10}$ MLD_{50} in vaccinated mice − $\log_{10}$ MLD_{50} in control mice.

[d] Approximate challenge dose (MLD_{50}) administered on day 31.

[e] ND, Not determined.

cumbed at all dilutions tested. On the other hand, 80% of the mice that received the same mass of irradiated Karp delivered in a series of injections resisted 10^4 MLD_{50} of Kato and were protected completely against less severe challenge. The overall result, demonstrated by the immunity indexes, was a 60-fold increase in the level of protection against heterologous challenge achieved by using the multiple-injection regimen instead of a single immunogen injection. However, expanding the interval between the three injections (Fig. 1) did not result in further increases in protection against either homologous or heterologous challenge.

Immune response after subcutaneous vaccination. Using the standard regimen of three injections at 5-day intervals, mice were vaccinated by subcutaneous injection of irradiated rickettsiae. The results of challenge of vaccinates and of normal recipients of spleen cells from vaccinated donors are shown in Table 2. Significant levels of protection against homologous challenge were observed in both groups, but the protection achieved by cell transfer was approximately 100-fold lower than that seen in the donors. Cell transfer did not protect against heterologous challenge at the lowest dose used, and the heterologous protection observed in the vaccinates was inconsistent. Although the calculated immunity index suggested effective resistance against approximately 10^3 MLD_{50} of Kato, it is important to notice that substantial mortality occurred with challenge doses of 100 MLD_{50}. Serum transfer recipients succumbed to homologous and heterologous subcutaneous challenge at all doses, with no extension in survival times as compared with those observed in control mice.

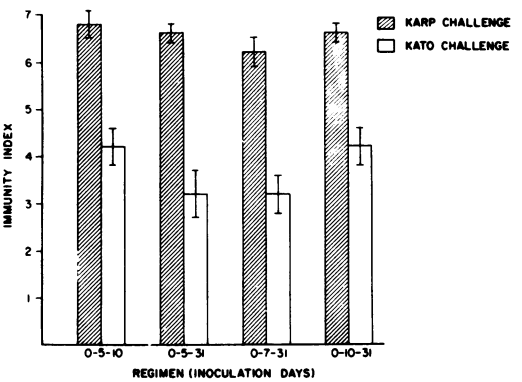

FIG. 1. *Effect of different multiple-injection regimens on the capacity of gamma-irradiated rickettsiae to induce a protective immune response in mice. Total mass of irradiated Karp immunogen in each regimen was 1.9 × 10^8 MLD_{50}, and the animals were challenged 21 days after completion of each vaccination regimen. Vertical brackets delimit ±1 standard deviation.*

Development and duration of immunity. The development and persistence of immunity induced by i.p. inoculation of irradiated Karp immunogens with the standard regimen is shown in Fig. 2. Resistance to homologous challenge developed quite rapidly, being effective against a 10^4-MLD_{50} challenge 5 days after the first injection of immunogen and reaching peak level 7 days after completion of the vaccination regimen. Animals remained resistant to $>10^6$ MLD_{50} for 3 months, and then immunity slowly declined. Resistance to challenge with 10^4 MLD_{50} of Karp was apparent at 9 months, and mice survived challenge with 50 MLD_{50} at the end of a year, when the experiment was terminated.

Immunity to heterologous challenge developed more slowly and waned much more rapidly than homologous immunity. Significant resistance to Kato was not achieved until day 17, when animals were protected against 10^4 MLD_{50} of rickettsiae. This level of protection was constant until day 38, when peak resistance was

TABLE 2. *Immunity and cell-transferable protection in mice vaccinated subcutaneously with gamma-irradiated Karp immunogens*

Challenge strain	Mouse status[a]	No. of survivors/no. of mice challenged							Log_{10} MLD_{50} in test mice[b]	Log_{10} MLD_{50} in control mice[b]	Immunity index[c]
		10^{7}[d]	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}			
Karp	Donor	0/5	2/5	4/5	5/5	5/5	4/4	ND[e]	−4.0 (±0.3)	−9.0 (±0.3)	5.0 (±0.4)
	Recipient	ND	ND	ND	1/5	1/5	4/5	5/5	−6.0 (±0.3)	−9.0 (±0.3)	3.0 (±0.5)
Kato	Donor	0/5	0/5	1/5	1/5	5/5	1/5	ND	−5.2 (±0.3)	−8.0 (±0.2)	2.8 (±0.4)
	Recipient	ND	ND	ND	0/5	0/5	0/5	0/5	ND		ND

[a] Donor is mouse vaccinated with a total mass of 3 × 10^8 MLD_{50} of irradiated Karp administered in three injections at 5-day intervals; recipient is normal mouse receiving i.p. injection of one mouse equivalent of spleen cells from donor mouse pool. Challenges and spleen cell transfers were performed on day 31.

[b] Values (± standard deviation) are based on dilution factors used to achieve the approximate challenge doses noted.

[c] Immunity index = $\log_{10}$ MLD_{50} in test mice − $\log_{10}$ MLD_{50} in control mice.

[d] Approximate challenge dose (MLD_{50}) administered to all animals 8 h after cell transfer.

[e] ND, Not done.

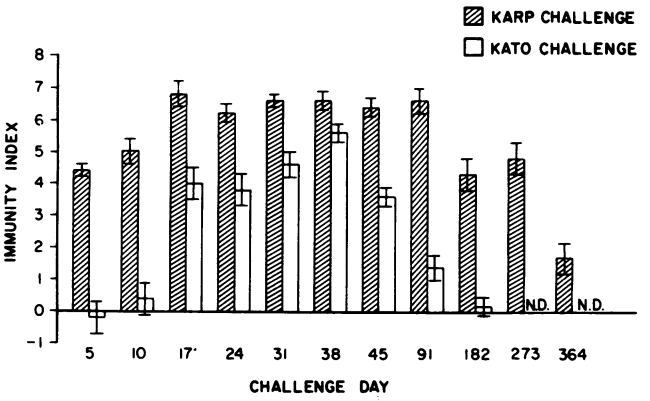

FIG. 2. *Development and duration of homologous and heterologous immunity achieved by vaccination with gamma-irradiated Karp. Vaccination regimen consisted of three i.p. injections of 10^8 MLD_{50} of immunogen given at 5-day intervals. Vertical brackets delimit ±1 standard deviation.*

observed and mice survived $>10^5$ MLD_{50} of Kato. Immunity then declined, evidencing a 100-fold decrease by day 45. Protection was very low on day 91 and insignificant 6 months after immunization.

At weekly intervals for the first 5 weeks after completion of the vaccination regimen, serum pools and spleen cell suspensions prepared from vaccinated animals were transferred to normal mice, which were challenged 8 h later with 10^1 to 10^4 MLD_{50} of either Karp or Kato. No protection was observed in serum recipients challenged with either strain, and cell transfer recipients died routinely from Kato challenge at all dosage levels. However, spleen cell transfer provided significant protection against Karp challenge, and immunity indexes indicate that the mean level of resistance transferred remained relatively constant, although some mice usually died at each challenge dose tested (Table 3).

Initial phase of the immune response. Since homologous protection was substantial 5 days after the first immunogen injection, it was of interest to determine the earliest time at which the gamma-irradiated rickettsiae induced a protective response. In the initial experiment, standard suspensions of Karp and Kato were each diluted in irradiated Karp immunogen and titrated in mice. The results (Table 4) indicated that the response induced by a single injection of immunogen was capable of masking the presence of 200 MLD_{50} of virulent Karp, but, as expected from the 5-day and 10-day heterologous challenge results (Fig. 2), did not alter the lethality of the Kato suspension. The transfer of homogenized spleen cells from survivors of 2 MLD_{50} or greater Karp challenge to normal recipients resulted in death, indicating that the donors had sustained and survived infections that would have been lethal for unprotected animals.

Although the strain specificity of the protection suggested that it was due to a specific immune response, a second experiment was performed to determine whether non-immunological factors contributed by either gamma-irradiated rickettsiae or yolk sac debris influenced the course of immediate infection with the homologous strain of *R. tsutsugamushi*. Irradiated yolk sac from normal embryonated eggs had no sparing effect on animals if injected at the same time as the challenge dose, but an irradiated suspension of Karp grown in the yolk sac of embryonated eggs protected 70% of the mice against challenge with 500 MLD_{50} of viable Karp (Table 5). Administration of immunogen 4 h before or 4 h after challenge resulted in 60% survival of animals. Again, homogenized spleen cells from the survivors were lethal for normal recipients, indicating that the donors had sustained active infections.

DISCUSSION

Previous studies on vaccination of mice with gamma-irradiated scrub typhus immunogens indicated that protection against homologous (Karp) challenge was heightened by increasing immunogen concentration and number of immunogen injections (8). The heightened protection could have been due either to temporal factors or to the accumulation of a larger amount of immunogen in the host. This study specifically addressed the influence of each of those two factors. The similarity of protection observed with the different temporal regimens was unexpected, since Plotz et al. (18), using formalinized scrub typhus immunogens, had observed a 10-fold increase in homologous protection with

TABLE 3. *Development of cell-transferable immunity to homologous strain challenge in mice vaccinated i.p. with gamma-irradiated Karp*[a]

Day of cell transfer	No. of survivors/no. of recipient mice challenged				Log_{10} MLD_{50} in recipient mice[b]	Log_{10} MLD_{50} in control mice[b]	Immunity index[c]
	10^{4}[d]	10^{3}	10^{2}	10^{1}			
17	0/5	1/5	4/5	3/5	−6.6 (±0.4)	−9.0 (±0.3)	2.4 (±0.5)
24	0/5	0/5	3/5	3/5	−7.0 (±0.3)	−8.8 (±0.2)	1.8 (±0.4)
31	0/5	2/5	1/5	4/5	−6.8 (±0.4)	−8.8 (±0.2)	2.0 (±0.4)
38	3/5	2/5	3/5	5/5	−5.6 (±0.4)	−8.6 (±0.2)	3.0 (±0.5)
45	1/3	0/4	3/5	0/5	−7.3 (±0.4)	−8.6 (±0.3)	1.3 (±0.5)

[a] Vaccination regimen consisted of three i.p. injections of 10^{8} MLD_{50} of immunogen given at 5-day intervals.
[b] Values (± standard deviation) are based on dilution factors used to achieve the approximate challenge doses noted.
[c] Immunity index = log_{10} MLD_{50} in recipient mice − log_{10} MLD_{50} in control mice.
[d] Approximate challenge dose (MLD_{50}) administered 8 h after cell transfer.

TABLE 4. *Survival of mice receiving simultaneous administration of immunogen and rickettsial challenge*

Challenge strain	No. of survivors/no. of vaccinated mice challenged[a]					Log_{10} MLD_{50} in vaccinated mice[b]	Log_{10} MLD_{50} in control mice[b]	Immunity index[c]
	2,000[d]	200	20	2	0.2			
Karp	ND[e]	10/10	10/10	9/9	10/10	≥−6.2	−9.0 (±0.3)	≥2.8
Kato	0/10	1/10	0/10	3/10	9/10	−8.1 (±0.2)	−8.2 (±0.2)	0.1 (±0.3)

[a] Mice were vaccinated with 10^{8} MLD_{50} of irradiated Karp in a single injection i.p.
[b] Values (± standard deviation) are based on dilution factors used to achieve the challenge doses noted.
[c] Immunity index = log_{10} MLD_{50} in vaccinated mice − log_{10} MLD_{50} in control mice.
[d] Number of MLD_{50} in challenge dose as determined by titration in control mice.
[e] ND, Not determined.

TABLE 5. *Effect of sequence of administration of immunogen and homologous challenge on mouse survival*

Sequence and time of injection			No. of survivors/no. challenged
0[a]	4	8	
	Irradiated normal yolk sac[b] + challenge[c]		0/10
	Immunogen[d] + challenge		7/10
Immunogen	Challenge		6/10
	Challenge	Immunogen	6/10

[a] Time of injections in hours.
[b] 20% suspension of normal yolk sac irradiated with 300-Krad gamma radiation.
[c] Challenge dose was 500 MLD_{50} of viable Karp, determined by separate titration in control mice.
[d] Immunogen consisted of 10^{8} MLD_{50} of irradiated Karp.

a similar temporal expansion. However, the levels of protection induced by their preparations were 100- to 1,000-fold lower than those observed after vaccination with gamma-irradiated immunogens. It is possible that the high protective level achieved with the initial three-injection regimen obscured differences in resistance due to temporal factors that were more apparent with the weakly immunogenic formalin-killed organisms.

At immunogen doses greater than 10^{7} MLD_{50}, heterologous protection levels appeared to be more responsive to the regimen used than to the total mass applied. When administered in a single injection, a dose of 10^{8} organisms yielded approximately the same level of protection against Kato challenge as reported previously for one injection of 10^{7} irradiated rickettsiae (8). On the other hand, protection increased 60-fold when this same rickettsial mass was delivered in a series of three injections. Thus, increasing the duration of exposure to antigen broadened the scope of immunity. Similar increases in cross-reactivity have been reported in other systems after prolonged exposure effected by multiple inoculations (14), active infection (16), or use of adjuvants (7). Again, we did not realize any further increase in protection when regimens employing expanded time intervals were used.

The level of immunity observed in animals vaccinated subcutaneously was significant, but lower than that seen after intraperitoneal vaccination. On the other hand, the degree of resistance conferred by spleen cell transfer was quite similar for the two routes, suggesting that the overall systemic responses might be of similar magnitude and that the differences in resistance observed after challenge of the vaccinates were due to more localized phenomena. Unfortunately, mice have a high level of innate resist-

ance to lethal subcutaneous infection, rendering subcutaneous challenge studies impossible.

The rate of development of immunity to homologous challenge after i.p. vaccination was unexpectedly rapid. The results indicated that the immunogen generated a rapid, specific immune response capable of protecting mice against simultaneous homologous challenge. Specificity of resistance was demonstrated by the unaltered lethality of a simultaneous Kato challenge. However, a number of years ago, Edsall (5) noted reports of investigators who had observed protection against challenge with other infectious organisms or toxins administered concurrently with or shortly after vaccination. In some instances, early immune responses were implicated, although in others the protection appeared to be due to interference between vaccine and challenge doses for receptor sites, emphasizing the importance of incorporating proper controls to reveal contributions by nonimmunological factors. For this reason, we tested the effects of irradiated normal yolk sac and the sequence of administration of challenge and vaccination doses. Irradiated normal yolk sac did not modify the lethality of the Karp challenge, and it was not likely that resistance was due to an interference phenomenon, since administration of challenge either before or after injection of immunogen resulted in protection similar to that achieved when the two injections were administered simultaneously. Further, homogenized spleen cell transfers indicated that the animals had survived infection with fully virulent rickettsiae which remained harbored in the spleens of the immune survivors.

The level and duration of protection achieved with gamma-irradiated immunogens was superior to that achieved with formalinized rickettsiae, but less striking than that observed after experimental infection. The peak level of protection against homologous challenge approached 10^7 MLD_{50}, approximately 1,000-fold higher than reported for mice vaccinated with formalinized suspensions (1, 11, 18, 19, 25), and persisted for 3 months. On the other hand, we found resistance to be quite low at the end of a year, while Fox (10) reported that mice surviving experimental infection were resistant to homologous challenge with 10^4 MLD_{50} of rickettsiae at 610 days, when his experiments were terminated.

The peak level of heterologous protection exceeded 10^4 MLD_{50}, but the response developed slowly and waned rapidly, with no significant protection being observed beyond 3 months. Unfortunately, long-term studies on resistance to heterologous challenge after experimental infection of animals are not available. Kuwata (15) showed that rickettsiae persisted in mice infected with a relatively avirulent strain of *R. tsutsugamushi* for 180 days, but he examined the animals for resistance to heterologous challenge for no longer than 80 days, at which time they were immune. Long-term studies in human volunteers indicated that individuals convalescent from infection were resistant to homologous challenge for approximately 1 year, whereas susceptibility to heterologous challenge appeared within months (24).

In our initial report (8), we suggested that the differences in immunogenicity of gamma-irradiated and formalinized suspensions were related to their abilities to stimulate cell-mediated immunity. The results of the subcutaneous vaccination experiment reported in this study also support this hypothesis. However, it must be noted that the level of cell-transferable protection which we have observed is by no means as great, or as consistently effective, as that seen after active scrub typhus infection, where spleen cell transfer recipients routinely survive a 10^3-MLD_{50} heterologous strain challenge (21). As in our initial study, serum transfer did not protect against either homologous or heterologous challenge. However, our data do not exclude the possibility of supporting contributions by humoral factors.

Although gamma-irradiated immunogens prepared from the Karp strain of *R. tsutsugamushi* were more immunogenic than those inactivated by chemical treatment, the inability to induce persistent immunity to heterologous challenge remains a serious deficiency. We are now exploring the use of other scrub typhus immunogens and multiple-strain vaccination regimens to increase the level of resistance and duration of immunity to heterologous challenge. Further, the recent finding (13) that C3H mice are susceptible to the Gilliam and Kostival strains of *R. tsutsugamushi* will allow us to examine a broader spectrum of heterologous challenge strains in future studies.

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