INFECTION AND IMMUNITY, Oct. 1979, p. 131-136
0019-9567/79/10-0131/06$02.00/0

Vol. 26, No. 1

Gamma-Irradiated Scrub Typhus Immunogens: Broad-Spectrum Immunity with Combinations of Rickettsial Strains

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Received for publication 12 June 1979

Scrub typhus immunogens were prepared from *Rickettsia tsutsugamushi* strains Karp, Kato, Gilliam, Kostival, and Buie by exposing frozen infected yolk sac suspensions to 300 krad of gamma radiation. Mouse protection tests showed that each of the irradiated immunogens protected C3H/HeDub mice against high challenge levels of Karp and Gilliam, but that none of these single-strain immunogens were capable of protecting against all five of the challenge strains. Broad-spectrum protection was achieved by using combinations of three strains of irradiated rickettsiae in a vaccination regimen of three injections at 5-day intervals. A comparison of vaccination efficacy employing three such combinations (Karp-Gilliam-Kato, Karp-Kostival-Kato, and Buie-Kostival-Kato) indicated that both sequential administration of strains on successive vaccination days and multiple injections of trivalent mixtures produced protective responses superior to those obtained with single-strain immunogens. Trivalent mixtures of rickettsiae exhibited a striking synergistic effect on the immune response of C3H/HeDub mice and elicited a protective response against Kato challenge that could not be obtained with any single-strain immunogen. Mice vaccinated with the trivalent Karp-Gilliam-Kato immunogen resisted challenge with more than 10^3 50% mouse lethal doses of Karp and Gilliam for 12 months, and were resistant to similar levels of challenge with Kato and Buie for 6 months.

Epidemiological investigations have revealed the existence of numerous strains of *Rickettsia tsutsugamushi* that can be differentiated on the basis of serological tests and virulence characteristics in laboratory animals. In some regions endemic for scrub typhus, a single strain appears to be dominant (13), but other reports indicate that several different strains may coexist in rather small geographical areas (3, 7, 14). This antigenic diversity has been a major consideration in the development of a scrub typhus vaccine. Although single-strain formalinized rickettsiae have been shown to elicit significant levels of protection against homologous strain challenge in laboratory animals (12), gamma radiation-inactivated scrub typhus immunogens protected mice against challenge with both homologous and heterologous strains of *R. tsutsugamushi* (5). Subsequent studies from this laboratory (6) defined the optimum temporal regimen for vaccination and examined the development and duration of the protective immune response in BALB/cDub (BALB) mice. Animals vaccinated with gamma-irradiated Karp immunogen were resistant to a 10^4 50% mouse lethal dose (MLD_{50}) homologous challenge for 9 months. However, resistance to the heterologous Kato strain waned rapidly, indicating the necessity to prolong effective heterologous protection and to assess resistance against other strains of scrub typhus rickettsiae. Such a study was facilitated by the recent discovery that C3H/HeDub (C3H) mice are susceptible to lethal infection with strains of *R. tsutsugamushi* to which BALB mice are naturally resistant (11).

In this study, we determined the comparability of data obtained with BALB and C3H mice and then used the latter animal model to expand the number of rickettsial strains we could use for challenge and immunization. In addition to Karp and Kato, we have used Gilliam, the third of the classical prototype strains of *R. tsutsugamushi* (1), Buie, a strain shown to produce broadly cross-reactive antisera in rabbits (2), and Kostival, one of the more effective formalinized immunogens in cross-vaccination studies (12). The immunogens were used as single strains and three-strain combinations in investigations that concluded with a 1-year study in which the durability and scope of scrub typhus immunity in vaccinated mice were assessed and contrasted with the durability of heterologous

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strain immunity occurring after sublethal infection.

MATERIALS AND METHODS

Mice. Female BALB and C3H mice (Flow Laboratories, Dublin, Va.), 18 to 22 g, were used throughout this study.

Rickettsiae. The Karp, Gilliam, Kato, Kostival, and Buie strains of *R. tsutsugamushi* were propagated, stored, and quantified using methods previously reported (4). Only those suspensions having titers $\geq 10^8$ MLD_{50} per g of yolk sac, as determined in susceptible mice, were used. The 50% endpoints were calculated from mortality data by the Spearman-Karber method (8).

Immunogens. Radiation-inactivated immunogens were prepared by exposing frozen 20% yolk sac suspensions to 300 krad of gamma radiation in a ^{60}Co gamma irradiator (Gamma Cell 220, Atomic Energy of Canada Ltd., Ottawa, Canada) as previously described (5). Immunogen mass was quantified in MLD_{50} units based on mouse titration of unirradiated organisms.

Vaccination and challenge. All injections were administered intraperitoneally in a standard volume of 0.2 ml. Immunogenicity of irradiated suspensions was determined by administering a 1,000-MLD_{50} homologous strain challenge dose to groups of mice that had been vaccinated 24 days previously with a single dose of immunogen diluted in 10-fold increments over an appropriate dose range. The 50% protective dose was expressed in MLD_{50} units by calculating the difference between the $\log_{10}$ of the 50% protective dilution and the $\log_{10}$ of the MLD_{50} of the unirradiated suspension (5). The level of immunity, or immunity index, induced by vaccination by the standard regimen of three injections at 5-day intervals was determined by challenge titration in vaccinated and control mice on day 31. The immunity index was calculated by subtracting the $\log_{10}$ of the MLD_{50} in vaccinates from that in controls (15). For comparison of vaccination methods or immunogen combinations when multiple-strain challenge was employed, composite immunity indices were obtained from mean MLD_{50} values calculated after summing mortality data for all challenge strains at each challenge dose.

RESULTS

Effect of gamma radiation on lethality and immunogenicity of rickettsiae. Since we used the Karp strain of *R. tsutsugamushi* and BALB mice for most of our previous scrub typhus immunogen studies (5, 6), it was necessary to determine if other rickettsial strains were inactivated by gamma radiation in a manner similar to Karp, and to test the comparability of results generated in C3H mice with those established in BALB mice. Consequently, suspensions of Karp, Kato, and Gilliam were exposed to gamma radiation doses in the range of 0 to 300 krad, and MLD_{50} titrations were performed simultaneously at each dose in both mouse strains. The individual titers of the Karp and Kato suspensions were almost identical in BALB and C3H mice, and regression analyses (Table 1) indicated that the radiation dose-lethality curves for the rickettsial strains were quite similar and unaffected by choice of mouse model. The generation of complete data for Gilliam was precluded by the natural resistance of BALB mice to lethal infection with this strain of *R. tsutsugamushi* (11). Corresponding results among mouse and rickettsial strain combinations also were observed in the 50% protective dose values obtained with 300-krad gamma-irradiated immunogens (Table 2). Protective doses for homologous strain challenge were quite similar for Karp immunogen titrated in either mouse strain, for Kato immunogen in BALB mice, and for Gilliam immunogen in C3H mice. However, a 50% protective dose for Kato in C3H mice could not be calculated. The highest concentration of Kato immunogen used failed to protect the animals from subsequent challenge. This apparent inability of C3H mice to develop protective immunity to Kato challenge was reproducible. However, other values were comparable for the two mouse strains, and so we decided that the benefit derived from broadened

TABLE 1. *Regression analysis of radiation dose-lethality curves of Karp, Kato, and Gilliam in BALB and C3H mice*

R. tsutsugamushi strain	Mouse strain	$\log_{10}$ MLD_{50} of unirradiated suspension/g of yolk sac	100% lethal gamma dose (krad)[a]	Slope ($\log_{10}$ MLD_{50}/g of yolk sac per krad)	Correlation coefficient
Karp	BALB	−8.7	180	5.0×10^2	0.98
	C3H	−8.8	170	5.2×10^2	1.00
Kato	BALB	−8.6	190	4.4×10^2	1.00
	C3H	−8.5	190	4.4×10^2	1.00
Gilliam	C3H	−9.4	180	5.2×10^2	1.00

[a] Point at which calculations indicate there will be ≤1 lethal rickettsia per g of irradiated yolk sac.

TABLE 2. *Immunogenicity of 300-krad gamma-irradiated suspensions of R. tsutsugamushi*

R. tsutsugamushi immunogen strain	Protective dose of immunogen ($\log_{10}$ PD_{50})[a]	
	BALB[b]	C3H[b]
Karp	5.4 ± 0.3[c]	5.5 ± 0.3
Kato	5.7 ± 0.4	≥8.2
Gilliam	ND[d]	6.3 ± 0.4

[a] $\log_{10}$ PD_{50} = the number of MLD_{50} units of immunogen required to protect 50% of vaccinated mice against a 10^3-MLD_{50} homologous strain challenge.
[b] Mouse strain.
[c] Value ± standard deviation.
[d] ND, Not determined.

challenge studies warranted further use of C3H mice.

Protection with single-strain immunogens. The use of C3H mice provided an opportunity to examine a matrix of homologous and heterologous protective capacities with immunogens prepared from *R. tsutsugamushi* Karp, Gilliam, Kato, Kostival, and Buie. Animals were vaccinated using the standard three-injection regimen and were subdivided on day 31 for homologous and heterologous challenges. The results of the titrations, conducted in vaccinates and controls, are expressed as immunity indices in Table 3. All of the immunogen preparations, including the Kato immunogen, were effective in protecting the animals against Karp and Gilliam challenge. Levels of cross-protection indicated considerable antigenic similarity between Gilliam and Kostival and, to a lesser extent, between Karp and Buie. Further, Kostival and Buie appeared to be the most omnipotent of the immunogen strains tested, providing protection against all challenge strains except Kato. None of the immunogen strains examined, including Kato, provided detectable protection against Kato challenge, although a similar experiment employing irradiated Kato to vaccinate BALB mice yielded immunity indices of 6.4 ± 0.2 after Kato challenge and 5.2 ± 0.3 after Karp challenge.

Protection with three-strain combinations of immunogens. Three combinations of strains and two methods of effecting the combination within the standard vaccination regimen were examined. The combinations tested were: Karp-Gilliam-Kato (KGKt), Karp-Kostival-Kato (KKosKt), and Buie-Kostival-Kato (BKosKt). Vaccination was accomplished either by sequential administration of single-strain immunogens on days 0, 5, and 10 or by injection of a trivalent mixture on each of these days. The total mass of immunogen injected was similar regardless of the method of combination of strains. As in previous experiments, vaccinated animals were subdivided and challenged on day 31. Immunity indices for challenge with each strain and composite immunity indices for each strain combination are shown in Table 4. Individual immunity indices indicate that use of three immunogen strains for vaccination, regardless of strains combined or method of combination employed, resulted in detectable levels of protection against Kato and moderate to excellent protection against challenge with the other four strains. For each combination, composite immunity indices achieved with injection of the trivalent immunogen mixture were higher than those obtained by sequential injection of the single-strain suspensions. These differences in composite values were primarily due to the protection achieved against Kato challenge, where individual immunity indices indicate that protection levels increased 100- to 1,000-fold with use of trivalent immunogens instead of sequential injection. Finally, composite immunity indices for the three trivalent immunogens indicate that the protective efficacies of KGKt and BKosKt were similar and significantly higher than that of KKosKt.

Duration of immunity after KGKt vaccination. Large groups of C3H and BALB mice were vaccinated with trivalent KGKt immunogen and challenged at intervals of 3 months over a period of 1 year (Table 5). With the exception of an unusually low value for Kato protection at 3 months, which may have been related to previously identified problems with Kato-C3H interactions, the levels of protection maintained by C3H mice over the first 6 months were relatively constant. The animals resisted challenge with over 10^5 MLD_{50} of Karp, Kostival, and Gilliam, 10^4 MLD_{50} of Buie, and 10^3 MLD_{50} of Kato. By 9 months, a 100-fold reduction in protection levels was observed for Karp, Buie, and

TABLE 3. *Protection of C3H mice vaccinated with monovalent gamma-irradiated scrub typhus immunogens*[a]

Immunogen strain	Total immunogen mass injected[b]	Immunity index[c]				
		Karp[d]	Buie	Kato	Kostival	Gilliam
Karp	4×10^8	6.1 ± 0.6[e]	1.8 ± 0.5	≤1.2	≤0.6	3.2 ± 0.4
Buie	1×10^8	5.0 ± 0.5	3.0 ± 0.4	≤1.0	1.4 ± 0.4	3.4 ± 0.4
Kato	2×10^8	4.7 ± 0.5	≤0.8	≤1.0	≤0.7	3.8 ± 0.5
Kostival	2×10^8	5.0 ± 0.5	1.6 ± 0.4	≤1.2	6.4 ± 0.4	6.0 ± 0.4
Gilliam	2×10^8	3.0 ± 0.4	≤0.6	≤1.6	5.7 ± 0.4	5.8 ± 0.2

[a] Mice were vaccinated with three intraperitoneal injections of 300 krad of gamma-irradiated immunogen given at 5-day intervals and were challenged on day 31.

[b] Expressed as MLD_{50} units based on titration before irradiation.

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

[d] Challenge strain.

[e] Value ± standard deviation.

Kato, although levels of resistance to Kostival and Gilliam were undiminished. No further decline in resistance was observed for Gilliam, Karp, and Buie at 12 months, but immunity to Kato challenge could not be detected at that time. Conversely, BALB mice remained immune to high levels of Kato challenge throughout the study, although they evidenced a gradual decline from immunity to a 10^6-MLD_{50} challenge at 3 months to protection against 10^3 MLD_{50} of Kato at 12 months.

Duration of immunity after sublethal infection. The durability of infection-induced immunity to heterologous challenge was studied in BALB mice that had sustained a sublethal infection with 100 50% mouse infectious doses of Gilliam. Although a few random deaths were observed after each challenge, the mortality data (Table 6) indicated that the vast majority of mice were protected for a year against Karp strain challenge doses exceeding 10^7 MLD_{50}. Meaningful immunity indices could not be calculated because most mice survived the largest challenge doses we used.

DISCUSSION

This study indicated that the conditions previously established for inactivation of *R. tsutsugamushi* strain Karp by gamma radiation (5) generally are applicable to other strains of scrub typhus rickettsiae. Use of these immunogens as single strains or multistrain combinations with broad-spectrum challenge delineated unexpected relationships among the rickettsial strains and revealed synergistic effects operative during multistrain immunization.

All single-strain immunogens elicited high levels of protection against Karp and Gilliam. This was interesting, because previous serological studies with these two strains, in which complement fixation tests (1, 14) and rickettsial-neutralization tests (2, 10) had been used, indicated that they were antigenically distinct, although a minor complement-fixing antigen common to

TABLE 4. *Protection of C3H mice vaccinated with three-strain immunogen combinations*[a]

Method of combining immunogen strains	Immunogen strain combination	Total immunogen mass injected[b]	Individual challenge immunity index[c]					Composite immunity index[c]
			Karp[d]	Buie	Kato	Kostival	Gilliam	
Injection of one monovalent immunogen each day[e]	KGKt	6×10^8	5.6 ± 0.5[f]	3.6 ± 0.5	1.6 ± 0.5	2.8 ± 0.5	4.6 ± 0.4	3.6 ± 0.2
	KKosKt	8×10^8	5.6 ± 0.3	3.9 ± 0.6	1.0 ± 0.5	5.4 ± 0.2	6.0 ± 0.3	4.5 ± 0.2
	BKosKt	4×10^8	6.6 ± 0.4	4.8 ± 0.4	2.0 ± 0.4	5.4 ± 0.4	5.6 ± 0.3	4.9 ± 0.2
Injection of trivalent immunogen each day[e]	KGKt	5×10^8	6.8 ± 0.4	4.5 ± 0.5	4.6 ± 0.4	6.2 ± 0.2	6.2 ± 0.3	5.6 ± 0.2
	KKosKt	8×10^8	5.6 ± 0.4	3.4 ± 0.6	3.8 ± 0.6	5.4 ± 0.2	6.0 ± 0.3	4.9 ± 0.2
	BKosKt	4×10^8	6.4 ± 0.4	5.2 ± 0.4	4.2 ± 0.4	6.4 ± 0.4	6.4 ± 0.3	5.7 ± 0.2

[a] Mice were vaccinated by the standard regimen and challenged on day 31.
[b] Expressed in MLD_{50} units based on titration before irradiation.
[c] Immunity index = $\log_{10}$ MLD_{50} in vaccinated mice − $\log_{10}$ MLD_{50} in control mice.
[d] Challenge strain.
[e] Sequential injection was administered in order of presentation (for KGKt, Karp on day 0, Gilliam on day 5, and Kato on day 10). Trivalent immunogens were prepared by mixing thawed monovalent immunogens in equal volumes just before injection.
[f] Value ± standard deviation.

TABLE 5. *Duration of protection in mice vaccinated with KGKt trivalent gamma-irradiated scrub typhus immunogen*[a]

Mouse strain	Challenge strain	Immunity index[b]			
		3[c]	6	9	12
C3H	Karp	5.6 ± 0.4[d]	5.8 ± 0.5	3.8 ± 0.5	4.0 ± 0.7
	Buie	4.2 ± 0.6	4.2 ± 0.6	1.6 ± 0.5	1.4 ± 0.6
	Kostival	5.2 ± 0.3	5.6 ± 0.4	6.3 ± 0.4	ND[e]
	Gilliam	5.6 ± 0.5	5.6 ± 0.3	5.6 ± 0.5	5.1 ± 0.6
	Kato	1.8 ± 0.5	3.6 ± 0.5	1.7 ± 0.6	0.4 ± 0.3
BALB	Kato	6.0 ± 0.4	4.8 ± 0.3	5.8 ± 0.5	3.0 ± 0.5

[a] Mice were vaccinated by the standard regimen with 5.8×10^8 MLD_{50} units of KGKt immunogen per mouse.
[b] Immunity index = $\log_{10}$ MLD_{50} in vaccinated mice − $\log_{10}$ MLD_{50} in control mice.
[c] Months after immunization.
[d] Value ± standard deviation.
[e] ND, Not done.

TABLE 6. *Duration of immunity to Karp challenge in BALB mice convalescent from a sublethal Gilliam infection*[a]

Challenge month	No. of survivors/no. of immunized mice challenged					
	10^{7}[b]	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}
1	5/5	5/5	5/5	4/5		
6	5/5	5/5	5/5	5/5	4/5	5/5
9	5/5	4/5	4/5	5/5	5/5	5/5
12	4/5	5/5	4/5	4/5	5/5	5/5

[a] Mice were infected by intraperitoneal injection of 100 50% mouse infectious doses of Gilliam.
[b] MLD_{50} Karp strain challenge.

both strains had been detected (14).

The Buie and Kostival immunogens appeared to be broadly reactive, eliciting moderate to high levels of protection against Karp, Gilliam, and homologous strain challenge while showing low, but detectable levels of protection against each other. These observations agree with previous reports of broad reactivity for rabbit anti-Buie serum in cross-neutralization tests (2) and for formalinized Kostival immunogen in cross-vaccination studies (12).

As predicted by our preliminary lethality and immunogenicity studies, none of the immunogens, including the irradiated Kato suspension, protected the C3H mice against Kato challenge. However, the same Kato suspension elicited high levels of protection against Karp and Gilliam in C3H mice and against Kato in BALB mice, indicating that the limitation of the C3H model was restricted to use of Kato challenge. We can offer no explanation for the phenomenon at this time, although it does illustrate the importance of ascertaining the limitations of an animal model.

Previous studies with mixed, formalinized immunogens (12) indicated that a reduction in protection against each challenge strain occurred and was proportional to the dilution effect achieved by mixing the immunogens. Nevertheless, the demonstrable superiority of gamma-irradiated immunogens (5) and the pronounced cross-protection observed against Karp and Gilliam with the single-strain immunogens (Table 3) encouraged us to attempt the use of multistrain combinations in this study. We chose to use three immunogen strains because the three-injection regimen allowed combination of Karp, Gilliam, and Kato by sequential injection in single-strain form on successive vaccination days, as well as injection of a premixed suspension. We also examined two other combinations that employed the Buie and Kostival strains, since results of the single-strain study suggested they were slightly more immunogenic than Karp or Gilliam. The principal result of using any of the multistrain combinations was a vast improvement in the breadth and level of protection over that afforded mice vaccinated with any of the single-strain immunogens. For the first time, vaccinated C3H mice resisted significant levels of Kato challenge. This was particularly striking after immunization with the trivalent mixtures and could not be attributed to increased immunogen mass as less Kato was present in the KGKt mixture than had been employed in the single-strain vaccination studies. Protection against Buie challenge also increased when immunogen combinations were used, with the immunity index rising from 3.0 after single-strain Buie immunization to 4.5 with the KGKt trivalent mixture. The immunity indices achieved for Karp, Kostival, and Gilliam challenges after vaccination with trivalent mixtures were generally similar to those attained with single-strain immunogens against homologous strain challenge.

To evaluate fully the efficacy of the trivalent immunogens in mice, it was necessary to examine the duration of protective immunity. In a previous study (6) we reported that BALB mice vaccinated with Karp immunogen retained immunity to challenge with 10^{4} MLD_{50} of Karp for 9 months but became susceptible to challenge with low levels of Kato after only 3 months. In contrast, mice convalescent from sublethal *R. tsutsugamushi* infection have been shown to resist intraperitoneal inoculation of 10^{4} MLD_{50} of homologous strain organisms for at least 610 days (9), and in this study the majority of BALB mice convalescent from sublethal Gilliam infection resisted heterologous strain challenge with 10^{6} MLD_{50} of Karp for 1 year. In comparison, the trivalent KGKt gamma-irradiated immunogen elicited reduced, but significant levels of protection. C3H mice resisted challenge with 10^{3} MLD_{50} of Karp and 10^{4} MLD_{50} of Gilliam for 12 months, and it is likely that a similar level of protection was maintained against Kostival challenge. Immunity indices for Kato and Buie challenges remained at acceptable levels in C3H mice for only 6 months, although the vaccinated BALB mice continued to resist challenge with 10^{3} MLD_{50} of Kato for 12 months.

These vagaries in mouse response, most evident in the C3H-Kato interaction, emphasize the effect of the animal model on testing of scrub typhus immunogens. In this study, use of either BALB or C3H mice alone would have presented a biased view of the effectiveness of irradiated rickettsiae. Thus, sole use of BALB mice would have limited the number of challenge strains employed and prevented us from observing the

synergistic effect produced by vaccination with polyvalent immunogens. Conversely, use of only C3H mice would have led to the conclusion that radiation was totally unsuitable for the preparation of Kato immunogens. Interpretation of data from both strains of mice revealed that the KGKt trivalent immunogen induced protective immunity to broad-spectrum challenge for 6 months. Studies are currently in progress to prepare trivalent scrub typhus immunogen from rickettsiae grown in appropriate cell culture for testing in subhuman primates.

ACKNOWLEDGMENTS

We thank John Hallam for excellent technical assistance and Lottie Applewhite for editorial evaluation and suggestions.

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