Rocky Mountain Spotted Fever Vaccine in an Animal Model

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Formalin-killed, purified *Rickettsia rickettsii* vaccine was evaluated in a guinea pig model of *R. rickettsii* infection. Vaccinated guinea pigs were partially protected by the vaccine when challenged with virulent, viable rickettsiae. Greater protection was observed when higher doses of vaccine were given and when frequent booster injections were administered. Stimulation of cell-mediated immunity to the vaccine antigens was variable and also appeared to be achieved more reproducibly with booster vaccinations. Serum antibody was elicited by high doses of vaccine and by booster vaccinations. The presence of serum antibody was useful in predicting immunity to challenge with *R. rickettsii*.

Rocky Mountain spotted fever (RMSF) is an acute febrile illness caused by *Rickettsia rickettsii*. The disease has a significant mortality rate and is a disease for which a vaccine is needed to protect individuals living in endemic areas. The vaccines which were available previously have not reliably prevented infection by *R. rickettsii* (2, 13). Previous vaccines consisted of killed *R. rickettsii* harvested either from infected ticks (13) or from embryonated hen eggs (2). Both vaccines contained nonrickettsial antigens of eggs or whole macerated ticks and offered incomplete protection against disease.

The purpose of this investigation was to evaluate a Formalin-inactivated *R. rickettsii* vaccine Sheila Smith strain (SS strain), chick embryo cell culture origin (freeze-dried lot no. 1), supplied by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, Md. (5) in a guinea pig model. Infection of guinea pigs with *R. rickettsii* gives a characteristic course of febrile illness and death (6). The vaccine was administered intradermally, subcutaneously or intramuscularly. Animals were challenged at various times after vaccination by injection with virulent *R. rickettsii*. Daily rectal temperatures were recorded, and blood was taken from survivors for antibody determinations and lymphocyte transformation studies.

MATERIALS AND METHODS

Determination of optimal route of vaccination. Forty-eight adult male Hartley guinea pigs (weight, >500 g) were bled three days before vaccination to obtain 1- to 2-ml prevaccination serum samples. All animals were ear-tagged for identification purposes. On day 0, 12 guinea pigs were vaccinated by intradermal, subcutaneous, or intramuscular route of injection. Six animals

were inoculated by each route and received 0.5 ml of a 1:3 dilution of vaccine. The other six animals received 0.5 ml of a 1:100 dilution. Vaccine was a Formalin-inactivated SS strain, chicken embryo cell culture origin, freeze-dried, lot no. 1, as supplied by USAMRIID. Twelve animals in the control group were injected intramuscularly with 0.5 ml of saline. On day 10 postvaccination, a sample of four animals was bled for lymphocyte transformation. This was repeated on days 21 and 30 postvaccination. Postvaccination antibody titers were assayed by indirect immunofluorescence with serum collected on day 28. On day 30, all animals were challenged intraperitoneally with 1.0 ml of blood-spleen suspension from *R. rickettsii*-infected guinea pigs. The infectious dose of the blood-spleen suspension was quantified and standardized in terms of tissue culture median infectious dose as determined by immunofluorescent demonstration of *R. rickettsii*. A challenge dose of 2×10^2 of tissue culture median infectious dose per ml was prepared by dilution in sucrose phosphate glutamate before injection. Daily rectal temperatures were recorded starting on day 5 after infectious challenge and continuing for a week. The duration of fever and the percentage of animals with fever were recorded. Blood samples for lymphocyte transformation were taken from survivors on days 12, 20, and 27 after inoculation. Convalescent antibody titers were determined by indirect immunofluorescence on serum taken on day 27 postchallenge.

Evaluation of long-term protection. Male Hartley strain guinea pigs (250) were vaccinated with Formalin-inactivated SS strain chicken embryo cell culture origin vaccine provided by USAMRIID. All animals in the long-term study were vaccinated subcutaneously. This study was initiated first because of the duration of the study, and animals were vaccinated subcutaneously on the basis that this was the route previously used for all three RMSF vaccines in humans. (See Table 4 for the schedule used.)

On day 396 after the first vaccination, four or five animals from each group were challenged intraperitoneally with 1.0 ml of a 1:50 dilution of a guinea pig

blood-spleen suspension containing 2×10^2 tissue culture median infectious doses of virulent *R. rickettsii*. Challenged guinea pigs were examined for fever starting on day 5 postinoculation and continuing through day 12. Whole blood samples were taken from survivors on day 14 postinoculation for lymphocyte transformation tests. Serum samples were collected 28 days after challenge and tested by indirect immunofluorescence for convalescent antibody titers. Ten of the survivors were then sacrificed, and the spleens were removed aseptically for lymphocyte transformation tests. The remaining animals were challenged 1.5 and 2 years after initial vaccination.

Determination of optimal dose of vaccine and requirements for booster vaccination. Adult male guinea pigs (64) were bled for prevaccination sera and divided into groups of 8 to 12 animals which were vaccinated with a vaccine dose of a 1:3, 1:20, or 1:100 dilution. One-half of the animals were given booster vaccinations of the same dose 30 days after the first vaccination and one-half received no booster vaccination. Ten control animals were given saline as a sham vaccine and were challenged by the same procedure as the vaccinated animals.

One-half of the guinea pigs were challenged by inoculation of *R. rickettsii* intraperitoneally; the other half were challenged by intradermal inoculation. After challenge with virulent *R. rickettsii* on day 30 after the last dose of vaccine, animals were observed for morbidity and mortality, and daily rectal temperatures were recorded on days 4 to 12 after rickettsial challenge. All survivors were bled on day 28 postchallenge to examine for antibody to *R. rickettsii* by indirect immunofluorescence assay.

Lymphocyte transformation. Blast transformation studies were performed with guinea pig lymphocytes obtained from the peripheral blood or spleen from vaccinated animals. The methods were previously described by Pavia et al. (11). Phytohemagglutinin was used for mitogenic stimulation, and Formalin-killed rickettsiae (FRR) were used as a specific stimulant. Stimulation indices of greater than 10 were considered significant for mitogen responses, and indices of 3 or greater were considered significant for responses to FRR (8).

Serological studies. Antibody to *R. rickettsii* was measured by indirect immunofluorescence with rickettsiae fixed to slides and a fluorescein-labeled rabbit anti-guinea pig serum (12). Detection of antibodies at a 1:16 titer was considered a significant antibody response. A Leitz-Ortholux UV microscope with incident beam illumination was used with barrier and exciter filters for fluorescein.

RESULTS

Effect of intramuscular injection of RMSF vaccine. The results from a group of experiments in which RMSF vaccine was given intramuscularly are given in Table 1. The animals were challenged 30 days after intramuscular administration of the vaccine with virulent rickettsiae. In the group of guinea pigs that received the higher dose of vaccine (1:3 dilution), all animals developed antibody titers of $\geq 1:16$ by 28 days, and all animals survived challenge with virulent organisms. Blood was taken from two animals of the group, and lymphocytes were cultured in vitro with mitogen and FRR. The animals responded to mitogen but gave poor responses to rickettsial antigens. Animals receiving a lower dose of vaccine (1:100) failed to develop detectable antibodies by 28 days after administration of the vaccine, except for one guinea pig which developed an antibody titer of 1:16. However, all animals survived, and there was no suppression of lymphocyte transformation by mitogen.

Effect of subcutaneous injection of RMSF vaccine. Studies on guinea pigs vaccinated by the subcutaneous route demonstrated that with the higher dose of vaccine (1:3) two of six animals developed fever upon challenge with virulent organisms but all survived (Table 2). Two of the vaccinated animals produced significant antibody titers by day 28 after vaccination, and five of six guinea pigs had detectable antibody responses by 28 days after challenge. Lymphocyte transformation studies indicated that representative animals of the group responded to mitogens, and one animal responded to FRR in vitro. Only one guinea pig vaccinated with a 1:100 dilution of vaccine had antibody detected at a titer of 1:16 28 days after vaccination, but five of six animals had significant antibody levels at 28 days after challenge. Three of six animals developed fever upon challenge, but all guinea pigs survived. Lymphocytes from guinea pigs of this group responded in vitro to mitogens but not to FRR under the conditions used in these experiments.

Effect of intradermal injection of RMSF vaccine. All guinea pigs receiving a 1:3 dilution of

TABLE 1. Effect of intramuscular injection of RMSF vaccine into guinea pigs^a

Group no.	Vaccine dose	Microimmunofluorescence antibody titer range (GMT) at:		Outcome of challenge as no. of animals febrile/no. of animals inoculated	Lymphocyte transformation (stimulation index) for:	
		Day 28 postvaccination	Day 28 postinfection		PHA	FRR
I	1:3	1:16–1:256 (1:102)	1:128–1:512 (1:256)	0/6	39, 51	1.2, 3.1
II	1:100	Neg–1:16 (1:9)	1:16–1:2,048 (1:81)	1/5	15, 60	2.3, 2.5

^a Abbreviations: PHA, phytohemagglutinin stimulation; FRR, Formalin-killed *R. rickettsii* antigenic stimulation; GMT, geometric mean titer of $<1:16$ assigned an arbitrary value of 1:8, Neg, $<1:16$ antibody titer.

TABLE 2. Effect of subcutaneous injection of RMSF vaccine into guinea pigs^a

Group no.	Vaccine dose	Microimmunofluorescence antibody titer range (GMT) at:		Outcome of challenge as no. of animals febrile/ no. of animals inoculated	Lymphocyte transformation (stimulation index) for:	
		Day 28 postvaccination	Day 28 postinfection		PHA	FRR
I	1:3	Neg-1:512 (1:37)	Neg->1:2,048 (1:372)	2/6	35, 98	2.1, 3.4
II	1:100	Neg-1:16 (1:9)	Neg-1:2,048 (1:311)	3/6	33, 66	2.3, 2.6

^a Abbreviations: GMT, geometric mean titer of <1:16 assigned an arbitrary value of 1:8; PHA, phytohemagglutinin stimulation; FRR, Formalin-killed *R. rickettsii* antigenic stimulation; Neg, <1:16 antibody titer.

RMSF vaccine intradermally survived challenge with *R. rickettsii* at 30 days after vaccination (Table 3). Five of six guinea pigs had significant postvaccination antibody titers before challenge. One animal failed to make detectable antibody even after challenge. In this experiment, the animal without detectable antibody (<1:16) failed to develop fever and survived. Two guinea pigs receiving the 1:100 dilution of vaccine also failed to show detectable antibody before challenge; however, one of those animals did produce antibody after challenge. Three of five animals developed fever, and one animal died 11 days after challenge without detectable antibody. It also should be noted that one guinea pig which failed to make detectable antibody survived without developing fever. Results of in vitro lymphocyte transformation indicated that responses to mitogens were within the usual range, i.e., >10, but no response to rickettsial antigen was detected.

Controls for vaccination experiments. Twelve guinea pigs served as controls for the vaccine studies. All controls were given sterile pyrogen-free saline on day 1, were bled 28 days later, and were challenged with virulent organisms on day 30. None of these guinea pigs had detectable antibody before challenge. However, five animals had significant levels 28 days after challenge. Seven of the 12 animals developed fever, and two animals died from the infection. It is interesting that one animal never developed a fever yet did synthesize antibodies (titer of 1:2,048) after challenge. In addition, two ani-

mals developed neither fever nor detectable antibodies and survived the infection. None of the animals studied responded to rickettsial antigens in vitro, but they did respond to mitogens.

Effect of long-term vaccination with RMSF vaccine. Data on 10 groups of guinea pigs with different vaccination schedules over 366 days are given in Table 4. All animals were injected subcutaneously with a low dose of vaccine (1:100), a high dose of vaccine (1:3), or saline. All animals were challenged at 13 months, 1.5 years, and 2 years after the initial injection. Animals in groups VII and VIII (Table 4) that received three doses of either a 1:3 or 1:100 dilution of vaccine all survived challenge at 13 months, and only one animal developed a fever.

In contrast, animals receiving two doses of vaccine (Table 4, groups V and VI) differed in protection from disease according to vaccine dose. Three of four animals receiving a 1:3 dose of vaccine survived challenge at 13 months and made antibody. Lymphocytes from these surviving animals responded strongly to rickettsial antigens and mitogens in vitro. Animals which received two vaccinations with a 1:100 dose (on days 1 and 366) all developed fever upon challenge and four of five animals died. There was a poor in vitro lymphoblastic response to FRR in the sole survivor in group VI (Table 4). The results of challenge after 1.5 and 2 years were generally similar to those at 13 months. Among the nonvaccinated controls, 16 of 20 animals developed fever, and 14 of the 16 febrile animals died.

TABLE 3. Effect of intradermal injection of RMSF vaccine into guinea pigs^a

Group no.	Vaccine dose	Microimmunofluorescence antibody titer range (GMT) at:		Outcome of challenge as no. of animals febrile/ no. of animals inoculated	Lymphocyte transformation (stimulation index) for:	
		Day 28 postvaccination	Day 28 postinfection		PHA	FRR
I	1:3	Neg-1:2,048 (1:100)	Neg-1:1,024 (1:252)	0/6	26, 48	2.3, 2.8
II	1:100	Neg-1:16 (1:9)	Neg-1:1,024 (1:81)	3/5 ^b	49, 85	1.3, 2.2

^a Abbreviations: GMT, geometric mean titer of <1:16 assigned an arbitrary value of 1:8; PHA, phytohemagglutinin stimulation; FRR, Formalin-killed *R. rickettsii* antigenic stimulation; Neg, <1:16 antibody titer.

^b Outcome of challenge was death for one animal.

TABLE 4. Effect of long-term vaccination of guinea pigs with RMSF vaccine

Group no.	Vaccination schedule (days)	Vaccine dose	Response at 13 mo as no. of animals febrile/no. of animals inoculated ^a	Response at 1.5 yr as no. of animals febrile/no. of animals inoculated ^a	Response at 2 yr as no. of animals febrile/no. of animals inoculated ^a
I	1	1:3	2/4 (1)	0/5	3/3 ^b
II	1	1:100	4/5 (4)	4/4 (4)	3/5 (3)
III	1, 30	1:3	1/5 (1)	0/5	1/4
IV	1, 30	1:100	4/5 (2)	5/5 (4)	4/5 (3)
V	1, 366	1:3	1/4 (1)	0/5	1/4 (1)
VI	1, 366	1:100	5/5 (4)	3/3 (1)	4/4 (4)
VII	1, 30, 366	1:3	0/4 (0)	1/4	0/2
VIII	1, 30, 366	1:100	1/4 (0)	3/5 (2)	0/4
IX and X	None	Saline	7/8 (5)	5/6 (5)	4/6 (4)

^a Number of animals with fatal courses are given in parentheses.^b All three animals were febrile for only 1 day.

Determination of optimal dose and requirement for booster vaccination. The results of various doses and booster regimens of vaccine are presented in Table 5. All doses of vaccine were given subcutaneously. The highest dose of vaccine was most effective, and the lowest dose was least effective. The effects of booster vaccination were apparent at vaccine dilutions of 1:20 and 1:100, at which dilutions fewer booster-vaccinated animals developed a fever after challenge.

Determination of immunity parameters which are predictors of protection. Animals that were vaccinated once with vaccine dilutions of 1:3, 1:20, or 1:100 and were challenged 30 days later showed some correlation between postvaccination antibody response and protection from disease due to *R. rickettsii* (Table 6). Twelve of 33 (36%) animals vaccinated once and with antibody titers of $\leq 1:16$ became febrile. Only 1 of 14 (7%) animals vaccinated once and with antibody titers of $\geq 1:64$ became febrile, and 10 of 11 unvaccinated animals, which were, of course, seronegative, became febrile on challenge.

Animals that were vaccinated twice 30 days apart with dilutions of vaccine of 1:3, 1:20, or 1:100 and that were challenged 30 days after the booster vaccination showed a correlation of antibody titer with response to challenge with *R. rickettsii*. One of three animals (33%) with an antibody titer of $\leq 1:16$ developed a fever after challenge. Two of nine animals (22%) with an antibody titer of 1:128 developed a fever after challenge. None of 20 animals (0%) with an antibody titer of $\geq 1:256$ became febrile.

Cell-mediated immunity as measured by lymphocyte transformation on exposure to FRR was not highly correlated with protection on challenge. One animal with a postvaccination stimulation index of less than 2 remained afebrile upon challenge. Two of four guinea pigs with postvaccination stimulation indices in the range of 2 to 3 became febrile after challenge. One of

the two guinea pigs with a significant postvaccination stimulation index, more than 3, nevertheless developed a fever after challenge.

DISCUSSION

It is apparent from these studies that vaccination of guinea pigs with the higher dose (1:3) of vaccine is protective against challenge with viable *R. rickettsii* under certain conditions. First, a single dose is partially protective if given within 30 days before challenge with virulent organisms. The frequency of vaccination is somewhat less important for long-term protection since it appears that animals receiving three doses of vaccine over 366 days were not more resistant to challenge with virulent *R. rickettsii* than were animals that received high-dose vaccine at 1 and 30 days and at 1 and 366 days. Lower doses (1:100) of vaccine did not seem to provide protection against challenge, except in experiments when vaccine was administered three times over a year. In this situation, most of the animals remained afebrile upon challenge and none of the animals died. Determination of

TABLE 5. Determination of optimal dose and requirement for booster

Group no.	Vaccine dose ^a	Booster ^b	Outcome of challenge ^c
XI	1:3	No	0/8
XII	1:3	Yes	0/11
XIII	1:20	No	3/12
XIV	1:20	Yes	1/10
XV	1:100	No	7/12 (1)
XVI	1:100	Yes	2/11
XVII	Saline	No	9/10 (7)

^a Vaccine was given by subcutaneous route.^b Booster of same dose given on day 30 after first inoculation.^c Outcome of challenge given as the number of febrile animals per the total number of animals inoculated. Number of deaths is given in parentheses.

TABLE 6. Correlation of prechallenge antibody titer and response to challenge with *R. rickettsii*

Postvaccination titer ^a	Outcome of challenge after ^b :	
	One vaccination	Two vaccinations
≤1:16	12/33	1/3
1:64–1:256	1/12	2/13
≥1:512	0/2	0/16

^a Reciprocal titer of indirect immunofluorescent antibody to *R. rickettsii*.

^b Outcome of challenge given as the number of febrile animals per the total number of animals challenged.

the relationship between the dose of vaccine which affords protection to guinea pigs and the vaccine dose which affords similar protection to humans remains problematical. If the mass of protective vaccine were related to the mass of the recipient, then a 70-kg man would require much more vaccine than a 500-g guinea pig. In initial human studies of the vaccine, 0.5-ml doses of the 1:3 dilution were administered (1). This dose contained 0.37 mg of protein. Since this was the dose which we demonstrated as protecting guinea pigs, it is conceivable that a larger dose might be required to confer protection to humans. Moreover, the requirement for booster vaccinations to maintain long-term protection from infection is a severe drawback for the achievement of immune protection for a relatively large population at risk.

In contrast to studies by Kenyon et al. (3), a small portion of the animals used in this study did develop lymphocyte transformation responses to rickettsial antigens after vaccination. It should be pointed out, however, that Kenyon et al. (3) were using whole blood lymphocyte cultures instead of enriched lymphocyte populations, and their cultures were established in guinea pig serum which may contain nonspecific inhibitors of lymphocyte transformation (9). The vaccinated animals also developed specific antibody to *R. rickettsii*. The higher dose of vaccine was effective in eliciting a higher titer of antibody, and the level of antibody was a reliable predictor of protection. Several negative observations were made, and they warrant further discussion. There was no evidence of suppression of lymphocyte transformation to mitogens by vaccination nor was suppression of lymphocyte transformation to mitogens observed after challenge of these animals. This is in contrast to the report of Oster et al. (10) who reported a transient depression of lymphocyte responses within a few days after infection with *R. rickettsii*. It is possible that we did not observe the decreased lymphocyte responses because we were studying lymphocyte transformation 28

days after challenge, and the depressed responses noted by Oster et al. (10) were seen in the first 10 days after infection. No immunopathological sequelae of vaccination or postvaccination challenge were observed in these experiments, and no systemic toxicity of the vaccine was noted.

These studies also indicate that the guinea pig model of infection is an acceptable one. Our results on guinea pig mortality are similar to those of Kenyon et al. (4) in that approximately one-half of the infected, nonvaccinated control animals died during infection. Most of these animals developed fever within 5 days after inoculation. This apparently low mortality rate may be a result of the utilization of outbred guinea pigs in which individual, nonspecific resistance may have been a factor.

There is a definite need for a vaccine against RMSF, particularly in certain geographical regions and in patients with occupational or genetic risk factors. The mortality rate for RMSF in North Carolina in 1980 (7) was equal to the mortality rate for poliomyelitis in the United States in 1956 (14) (0.3 deaths per 100,000 population). The development of an effective vaccine for this obligate, intracellular bacterium requires more basic knowledge about the antigens of *R. rickettsii* and the components of the immune system which must be stimulated to confer immune protection against this disease. Acquisition of these data should be pursued.

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LITERATURE CITED

1. Ascher, M. S., C. Oster, P. Harber, R. Kenyon, and C. Pedersen, Jr. 1978. Initial clinical evaluation of a new Rocky Mountain spotted fever vaccine of tissue culture origin. *J. Infect. Dis.* 138:217–221.
2. Cox, H. R. 1939. Rocky Mountain spotted fever. Protective value for guinea pigs of vaccine prepared from rickettsiae cultivated in embryonic chick tissues. *Public Health Rep.* 54:1070–1077.
3. Kenyon, R. H., M. S. Ascher, R. A. Kishimoto, and C. E. Pedersen, Jr. 1977. In vitro guinea pig leukocyte reactions to *Rickettsia rickettsii*. *Infect. Immun.* 18:840–846.
4. Kenyon, R. H., R. A. Kishimoto, and W. C. Hall. 1979. Exposure of guinea pigs to *Rickettsia rickettsii* by aerosol, nasal, conjunctival, gastric, and subcutaneous routes and protection afforded by an experimental vaccine. *Infect. Immun.* 25:580–582.
5. Kenyon, R. H., and C. E. Pedersen, Jr. 1975. Preparation of Rocky Mountain spotted fever vaccine suitable for human immunization. *J. Clin. Microbiol.* 1:500–503.
6. Moe, J. B., D. F. Mosher, R. H. Kenyon, J. D. White, J. L. Stookey, L. R. Bagley, and D. P. Fine. 1976. Functional and morphologic changes during experimental Rocky Mountain spotted fever in guinea pigs. *Lab. Invest.* 35:235–245.
7. North Carolina Department of Human Resources. 1981. Epidemiologic notes and communicable disease morbidity

- report, Report no. 81-2, Epidemiology Section, Division of Health Services. North Carolina Department of Human Resources.
8. Oppenheim, J. J., S. Dougherty, S. P. Chau, and J. Baker. 1975. Use of lymphocyte transformation to assess clinical disorders, p. 87-109. In G. Vyas, D. Stites, and G. Brecher (ed.), Laboratory diagnosis of immunologic disorders. Grune and Stratton Publishers, New York.
 9. Oppenheim, J. J., and R. Rosenstreich. 1976. Signals regulating *in vitro* activation of lymphocytes. *Prog. Allergy* 20:65-159.
 10. Oster, C. N., R. H. Kenyon, and C. E. Pedersen, Jr. 1978. Suppression of cellular immune responses in guinea pigs infected with spotted fever group rickettsiae. *Infect. Immun.* 22:411-417.
 11. Pavia, C. S., J. D. Folds, and J. B. Baseman. 1977. Selective *in vitro* responses of thymus-derived lymphocytes from *Treponema pallidum*-infected rabbits. *Infect. Immun.* 18:603-611.
 12. Phillip, R. N., E. A. Casper, R. A. Ormsbee, M. G. Peacock, and W. Burgdorfer. 1976. Microimmunofluorescence test for the serological study of Rocky Mountain spotted fever and typhus. *J. Clin. Microbiol.* 3:51-61.
 13. Spencer, R. R., and R. R. Parker. 1925. Rocky Mountain spotted fever: vaccination of monkeys and man. *Public Health Rep.* 40:2159-2167.
 14. U.S. Department of Health, Education, and Welfare. 1956. Vital statistics of the United States, vol. II, p. 18. Public Health Service, National Office of Vital Statistics. U.S. Government Printing Office, Washington, D.C.