Effect of Vaccination Schedule on Immune Response of *Macaca mulatta* to Cell Culture-Grown Rocky Mountain Spotted Fever Vaccine

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The effect of vaccination schedule on the immune response of *Macaca mulatta* to formalin-inactivated chicken embryo cell culture (CEC)-grown *Rickettsia rickettsii* vaccine was studied. Schedules consisted of inoculation on day 1 only, on days 1 and 15, on days 1 and 30, on days 1, 8, and 15, or on days 1, 15, and 45. Humoral antibody measured by microagglutination and indirect immunofluorescence and resistance to challenge with 10⁴ plaque-forming units of yolk sac-grown *R. rickettsii* were assessed. Seroconversion was noted in all monkeys after the first dose of vaccine. A second dose administered 8 or 15 days after the primary infection, or a third given 7 or 30 days after the second, produced no long-term effect on antibody titer. Only monkeys given two doses of vaccine at a 30-day interval showed an increase in antibody titer during the period before challenge. Vaccination with one, two, or three doses of CEC vaccine prevented development of rash and rickettsemia after challenge. The two-dose schedules appeared to induce the highest degree of resistance to challenge, as indicated by unaltered hematological parameters and body temperature in monkeys. The one- and three-dose schedules were somewhat less effective, in that some challenged monkeys within each group displayed febrile and leukocyte responses associated with Rocky Mountain spotted fever infection. Our data suggest that administration of two doses of CEC vaccine at 15- or 30-day intervals is the immunization schedule of choice.

A formalin-inactivated Rocky Mountain spotted fever (RMSF) vaccine prepared from *Rickettsia rickettsii* grown in chicken embryo cell culture (CEC) has been developed (3). This CEC vaccine is more immunogenic and contains less protein than the commercially available yolk sac vaccine (4). In monkeys inoculated with a single dose, the CEC vaccine was more efficacious than the commercially available vaccine for protection against rickettsemia and death (4). Administration of two or three injections of vaccine, a procedure commonly used for immunization with inactivated vaccines, would increase the antigenic mass administered and might provide better resistance to infection. A single dose of vaccine and multiple-dose schedules similar to those used for the immunization of humans with other inactivated vaccines were tested and compared for efficacy in rhesus monkeys.

MATERIALS AND METHODS

Rickettsiae. The method used for the preparation of yolk sac-grown *R. rickettsii* (Sheila Smith strain)

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was that described by other investigators (7). The suspension of rickettsiae used for challenge was titrated by the method of Weinberg et al. (8). Rickettsemia titers were measured in heparinized blood by the method of Wike and Burgdorfer (9). Heparinized blood samples were stored at -70°C until tested.

Vaccine. Preparation and efficacy testing of the CEC-grown inactivated RMSF vaccine (Sheila Smith strain) have been reported previously (3, 4).

Experimental design. (i) **Vaccination schedule.** Five vaccination schedules were used. The number of inoculations and time intervals between inoculations were both varied. Schedules consisted of inoculation on day 1 (group II), on days 1 and 15 (group III), on days 1 and 30 (group IV), on days 1, 8, and 15 (group V), or on days 1, 15, and 45 (group VI); group I was unvaccinated controls. Each administered dose contained 0.5 ml of CEC vaccine and was given subcutaneously. The vaccination schedule was designed in such a manner that the last dose was administered precisely 30 days before the day of challenge.

(ii) **Animals.** Twenty-four well-conditioned, healthy rhesus monkeys (*Macaca mulatta*) of random sex and weight (range, 3 to 5 kg) and with no history of exposure to *R. rickettsii* were allocated to six groups. One group of four nonvaccinated monkeys constituted the nonimmune control group,

whereas five groups of monkeys received one or more doses of the CEC vaccine. Before each vaccination and to challenge, monkeys were bled and the antibody titers of the serum were determined. Temperature determinations and sampling procedures were performed in the morning before feeding. Vaccinated and control monkeys were challenged subcutaneously with 10^4 plaque-forming units of chick yolk sac-grown rickettsiae (Sheila Smith strain) 30 days after the last vaccination. This dose was equivalent to approximately 10^4 50% rhesus monkey median infectious doses. Fever was defined as a temperature $\geq 40^\circ\text{C}$. Blood samples for rickettsemia titrations were obtained daily, and for hematological samples were obtained on alternate days.

(iii) **Data.** Seven consecutive daily prechallenge rectal temperatures from each monkey were used to establish group mean base-line values. Each daily group mean temperature was subtracted from the group mean base-line value to determine degrees of change from base line. A mean degree change from base line was calculated for each group for each day before and after challenge. Four blood samples from each monkey were drawn during the week before challenge to establish group mean base-line leukocyte, neutrophil, and lymphocyte counts. Each of the daily mean hematological values was divided by the respective group mean base-line value to obtain a normalized percentage of base line, which was calculated for days -1, +4, +6, +8, +10, and +13 for each group. Groups were compared by one-way analysis of variance and least significant difference. Differences were considered significant when $P < 0.05$.

Serology. (i) **MA.** Microagglutination (MA) assays for rickettsial antibody were performed as described by Fiset et al. (1). The rickettsial test antigen was prepared from a suspension of *R. rickettsii* (Sheila Smith strain) grown in duck embryo cell cultures which was purified by differential centrifugation and ether extraction to remove cellular debris. Serum samples were stored at -70°C until tested, at which time they were thawed and twofold dilutions were made.

(ii) **Indirect immunofluorescence (IIF).** WI-38 cells grown on cover slips in Leighton tubes were

infected with *R. rickettsii* (Sheila Smith strain) at a multiplicity of five rickettsiae per cell. At 72 h post-infection, cells were fixed and stored in acetone at -70°C . Twofold dilutions of serum samples were made with phosphate-buffered saline, pH 7.3, and applied to infected cells for 25 min in a moist staining chamber. After washing with phosphate-buffered saline, fluorescein-conjugated goat anti-monkey globulin (prepared in our laboratory) was applied for 30 min. Slides were washed three times in phosphate-buffered saline and mounted with phosphate-buffered glycerol, pH 7.3. Stained cells were observed with a Zeiss microscope fitted with BG-38 and BG-12 exciter filters and $10\times$ ocular and $40\times$ Planapochromat objectives. The fluorescent antibody titration end point was considered to be the highest dilution of test serum that gave definite fluorescence of rickettsiae in the cells. The lowest dilution of the immune sera showed no positive staining with uninfected WI-38 cells. The conjugate itself at the working dilution showed no positive staining with infected WI-38 cells.

Geometric mean titers were determined to evaluate both MA and IIF group responses; end points below the initial dilution of 1:2 were considered to represent a titer of 1.

RESULTS

Antibody response. Prevacination MA titers for all monkeys were 1:4 or less, and prevaccination IIF titers were 1:32 or less. Such titers are commonly found in our monkey colony and do not appear to affect the disease. Seroconversion, as indicated by a positive MA titer, was observed for all vaccinated monkeys (Table 1). Postvaccination MA titers for each animal were at least two dilutions higher than the prevaccination MA titer. The geometric mean MA titer for each vaccinated group before challenge was significantly greater ($P < 0.05$) than the corresponding prevaccination value (Table 1). Likewise, geometric mean values for MA and IIF titers of prechallenge sera from all vaccinated groups were significantly greater (P

TABLE 1. Vaccination schedule and prechallenge antibody responses of groups of four monkeys each

Group	No. of vaccinations	Total dose (ml)	Day of vaccination			Geometric mean MA titer ± 1 SE ^a	Geometric mean IIF titer ± 1 SE
			Initial	2nd	3rd		
I	0	0				2 $\pm$ 0.3 ^b	5 $\pm$ 4
II	1	0.5	1			13 $\pm$ 2.6 ^c	600 $\pm$ 352
III	2	1.0	1	15		13 $\pm$ 1.3 ^d	430 $\pm$ 84
IV	2	1.0	1	30		38 $\pm$ 10.4 ^c	1,200 $\pm$ 240
V	3	1.5	1	8	15	16 $\pm$ 5.1 ^e	360 $\pm$ 40
VI	3	1.5	1	15	45	8 $\pm$ 0 ^e	430 $\pm$ 117

^a SE, Standard error.

^b All prevaccination titers = 1:4 or less.

^c $P < 0.001$ compared to prevaccination titer.

^d $P < 0.05$ compared to prevaccination titer.

^e $P < 0.01$ compared to prevaccination titer.

< 0.025 and < 0.01, respectively) than for the nonimmune control group.

Comparisons between vaccination schedules suggested that only those monkeys in group IV (vaccinated on days 1 and 30) had increased MA titers after the second dose of vaccine (Fig. 1). Sequential doses administered at shorter intervals (8 or 15 days) failed to enhance MA response; 30 days after completion of immunization, MA titers were essentially the same regardless of immunization schedule. To represent temporal course of MA response to a single vaccine dose, MA data from individuals in groups III, V and VI, and II and IV were plotted; geometric mean values are shown in Fig. 2. Maximum titers were detected at 15 days post-immunization, with somewhat lower values in monkeys 30 days after immunization. It should be noted, however, that the magnitude of the prechallenge antibody titer of individual monkeys tended to be unrelated to their protection against development of disease (Fig. 3).

Clinical response. Clinical responses after challenge are summarized in Table 2. All non-immune control monkeys (group I) became febrile within 5 days, three of four developed detectable rickettsemia and rash, and one of these three died on day 8 after inoculation. The monkey that died had a prechallenge MA titer of 1:2 and no detectable IIF titer. Six monkeys in the vaccinated groups (II, V, and VI) also became febrile, but none exhibited a rash or had detectable rickettsemia. Although mean time for onset of illness was not appreciably different, mean duration of fever appeared shorter for the six vaccinated responders than for the nonimmune control monkeys. The mean base-line temperature for all monkeys before

FIG. 1. Temporal course of geometric mean MA titers ($\pm$ standard error of the mean) for each vaccinated group of monkeys.

FIG. 2. MA response at 8 days (group V), 15 days (groups III and VI), or 30 days (groups II and IV) after a single dose of vaccine. Dashed line represents corresponding geometric mean prevaccination MA titers. The horizontal line at each time is the geometric mean for the combined groups.

FIG. 3. Prechallenge MA and IIF titers for individual monkeys ($\circ$, nonfebrile; $\bullet$, febrile). Geometric mean titer is shown for each group as a horizontal line.

challenge was $39.4 \pm 0.3^\circ\text{C}$. Mean temperature for the nonimmune control group after challenge was elevated on days 4 through 9, with the maximum value, 1.3°C above base-line temperature, on day 6 (Fig. 4). Mean temperatures for groups II and VI increased on days 5 through 7, whereas those for groups III, IV, and V showed little change.

The prechallenge mean value for peripheral neutrophils was $3,800 \pm 170$, and for lymphocytes, $6,900 \pm 220$. Nonimmune monkeys developed neutrophilia on days 4 through 10 post-challenge (Fig. 5A). The day 10 neutrophil count for nonimmune monkeys was significantly greater than the base-line value or for groups III, IV, V, and VI ($P < 0.05$), indicating that the neutrophil response was more prolonged in nonimmune monkeys. Elevated neutrophil counts for groups II and VI reflect responses of two and three febrile monkeys, respectively, in these groups. Group V, with one febrile monkey, and groups II and IV, with no febrile responders, had relatively stable mean neutrophil counts. Lymphopenia on days 6 through 10 accompanied neutrophilia in the nonvaccinated group, and the value for day 8 was significantly ($P < 0.05$) different from values for groups II, III, IV, and V. Likewise, the

day 8 lymphocyte count for group VI (three febrile monkeys) was significantly ($P < 0.05$) lower than values for groups II, III, IV, and V. In contrast, the day 8 lymphocyte counts for groups on the two-dose schedule (III and IV) were significantly ($P < 0.05$) higher than the base-line value.

TABLE 2. Response to challenge with *R. rickettsii*

Group	No. of vaccinations	Febrile response			No. with rash and rickettsemia
		No. ill/total	Mean day, onset	Mean days, duration	
I	0	4/4	4.0	7.5	3 ^a
II	1	2/4	4.5	6.5	0
III	2	0/4	0	0	0
IV	2	0/4	0	0	0
V	3	1/4	6.0	2.0	0
VI	3	3/4	4.7	5.3	0

^a One monkey died.

FIG. 5. Mean normalized neutrophil (A) and lymphocyte (B) counts for six groups of monkeys. Symbols: Responses significantly different from corresponding base line ($P < 0.05$), $\star$; responses of vaccinated groups significantly different ($P < 0.05$) from the nonvaccinated group, large $\bullet$. The group VI, day 8 lymphocyte value is significantly different ($P < 0.05$) from values for vaccine groups II through V.

FIG. 4. Mean temperature response for groups I (○), II (●), V (◆), and VI (■). Groups III and IV had no febrile monkeys. The shaded band is the prechallenge group mean ± 1 standard error of the mean.

DISCUSSION

Although MA and IIF antibody titrations appear to be the most reliable of available methods for evaluating the humoral response to *R. rickettsii* (2), protection against challenge with the infectious organism is the most stringent criterion for vaccine efficacy. In the present studies, both humoral response and resistance of rhesus monkeys to challenge were used to determine the optimal schedule for administration of RMSF vaccine.

The course of *R. rickettsii* infection and the evaluation of RMSF vaccines in this primate model have been reported previously (4, 6). Clinical responses of nonvaccinated monkeys and monkeys receiving a single dose were similar to those seen in an earlier study (4). In the present study, all nonvaccinated control monkeys became clinically ill, exhibiting characteristic febrile responses, neutrophilia, and lymphopenia (6). Vaccination with one, two, or three doses of CEC vaccine prevented development of rash and rickettsemia after challenge. The two-dose schedules, however, appeared to induce the highest degree of resistance against infection, as was indicated by unaltered hematological parameters and body temperature in monkeys after challenge. The one- and three-dose schedules were somewhat less effective, in that some challenged monkeys within each group displayed some febrile and leukocyte responses associated with RMSF infections.

Seroconversion was noted in all monkeys after the first injection with the killed vaccine (Fig. 1). MA titers were maximum on day 15 and somewhat lower on day 30. A second dose of vaccine administered 8 or 15 days after the primary dose (groups III, V, and VI) afforded no long-term advantage in humoral antibody titer; likewise, a third injection given 7 or 30 days after the second (groups V and VI) produced no additional long-term effect on antibody titer. Indeed, during the 30 days preceding challenge of groups III, V, or VI, MA antibody titers decreased. Only group IV, given two doses of vaccine at a 30-day interval, showed an increase in antibody titer during the period before challenge. Perhaps revaccination at a shorter than 30-day interval does not allow subsequent time for processing of the initial antigen exposure to this particular vaccine preparation.

Data from individual monkeys suggest that an increase in MA or IIF titer is indicative of response to vaccine and probably of protection against fatal infection. These titers, however,

may not necessarily reflect the ability of the vaccinated individual to avoid clinical illness after exposure to an infective dose of *R. rickettsii*. Nonetheless, an important role for humoral antibody in protection against RMSF was demonstrated by Ricketts, who protected normal guinea pigs by passive transfer of homologous convalescent serum (5). Thus, it would appear that development of improved monitoring procedures is essential for evaluation of responsiveness to rickettsial vaccines. Exploration of the contribution of cellular mechanisms to development of resistance would also appear to be warranted, because establishment of a rickettsial infection is dependent upon intracellular multiplication of the infecting organism.

Despite limitations of the current methods for evaluating host response to vaccination, our data suggest that administration of two doses of RMSF vaccine at a 15- or, preferably, 30-day interval is the immunization schedule of choice. This was the only immunization regimen that entirely prevented the development of clinical illness in rhesus monkeys exposed to experimental infection with *R. rickettsii*.

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