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Detection of *Rickettsia rickettsii* Antibodies in Human Sera by Crossed Immunoelectrophoresis

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To identify *Rickettsia rickettsii* antigens of immunological importance, we examined sera from patients with serologically confirmed cases of Rocky Mountain spotted fever by crossed immunoelectrophoresis for antibodies to antigens extracted from the R strain of *R. rickettsii* with the detergent Triton X-100. Sixteen antigens were identified in the detergent extract by crossed immunoelectrophoresis with a hyperimmune rabbit serum raised against whole rickettsiae. When the rabbit antiserum was placed in the reference gel and patient sera were placed in the intermediate gel, antibodies to one or more antigens were detected in 61 of 71 North Carolina sera, all of 7 Oklahoma sera, and 9 of 10 Montana sera obtained from 1 day to 40 years after onset of Rocky Mountain spotted fever. Antibodies to antigens 1 and 16 were found as early as 1 day after onset of illness, and antibody to 16 was found in 20 of 29 sera obtained within the first 7 days of illness. Antibodies to antigens 2 and 3 generally did not appear until the third week of illness but were found in six of seven serum samples collected 4 to 40 years after onset of Rocky Mountain spotted fever. Antibodies to *R. rickettsii* antigens 1, 7, 8, and 16 were found in sera from patients with illnesses caused by other etiological agents. Four of the Oklahoma and Montana sera from Rocky Mountain spotted fever patients, but none of the North Carolina sera, had antibodies to antigen 12. Sera containing antibodies against antigens 3 and 14 prevented death of mice challenged with two 50% lethal doses of *R. rickettsii*.

Crossed immunoelectrophoresis (CIE) with an intermediate gel is a useful tool for the study of antibodies to antigens extracted from eubacteria, viruses, chlamydiae, fungi, and protozoa by procedures which do not denature protein antigens (8, 10, 12, 20, 22). To determine whether CIE might be of value in the definition of the humoral antibody response of Rocky Mountain spotted fever (RMSF) patients to particular *Rickettsia rickettsii* antigens, we examined sera from patients primarily from North Carolina and from several other states for comparison. We hoped to accomplish several objectives in our study of patient sera. First, we wanted to identify *R. rickettsii* antigens which were recognized by antibodies from patients. Second, we wanted to determine the kinetics of the antibody response. Third, we wished to know whether the presence of specific antibodies could be correlated with the severity of illness of the patient or the type of antibiotic administered. Finally, we hoped to determine whether any of the antibodies identified could be associated with the protective activity of the serum. Our efforts to achieve these goals are described in the following report.

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MATERIALS AND METHODS

Sera. As part of an epidemiological study of RMSF (26), 64 serum specimens were obtained from 38 patients with confirmed cases of RMSF within 18 months of the onset of illness during the years 1979 to 1981 in Rowan County and Cabarrus County, North Carolina. Patients with confirmed cases of RMSF were defined as those who had ≥4-fold rises in antibody titers by the indirect hemagglutination (IHA) test or by the microimmunofluorescence (micro-IF) test, or *R. rickettsii* immunoglobulin M antibodies detected by the micro-IF test (26). In addition, the patients had to have a clinical presentation compatible with the diagnosis of RMSF. Sera were obtained from seven individuals who had had clinically diagnosed RMSF 4 to 40 years earlier. Seventeen sera representing 13 RMSF patients from Oklahoma and Montana were also examined.

Several kinds of sera were studied for comparison. Sera were included from six patients from Rowan County or Cabarrus County who in 1981 developed RMSF-like symptoms but were seronegative for RMSF at two bleedings done first within 2 days of onset and then from 13 to 49 days later. Also included were six sera from six patients who had either Q fever, tularemia, or Colorado tick fever and resided in the western United States.

Animals. Locally obtained New Zealand white rabbits of either sex, weighing 2 to 3 kg each, and 4-week-old male Swiss mice of the Rocky Mountain Laboratories strain were used for our experiments.

Rickettsiae. *R. rickettsii* strain R, isolated from *Dermacentor andersoni* ticks collected in Bitter Root Valley, western Montana, was used for these studies (4).

Cultivation of rickettsiae. Rickettsiae were grown either in embryonated chicken eggs (H & N Inc., Redmond, Wash.) or in monolayers of L cells by methods previously described (1, 19).

Purification of rickettsiae. Rickettsiae used for inoculation of rabbits were grown in L cells, inactivated with Formalin, and purified by sucrose density gradient centrifugation in a zonal rotor (1). Rickettsiae used for extraction were grown in embryonated eggs and purified by Renografin density gradient centrifugation without prior Formalin treatment (25).

Preparation of detergent extract. Purified rickettsiae were suspended to a concentration of 15 to 30 mg (wet weight) per ml of 10 mM phosphate-buffered saline containing 1% Triton X-100 and sonicated at 0°C for 60 s (model 8845-2 Ultrasonic Cleaner; Cole-Parmer Instrument Co., Chicago, Ill.). The sonicated product was placed in a 56°C water bath for 10 min and then held at room temperature for 1 h and then overnight at 4°C. The supernatant fraction obtained after centrifugation for 30 min at 13,200 × *g* and 4°C was used as the antigen for CIE. Antiserum to normal yolk sacs did not precipitate any of the components of the extract in CIE tests.

Preparation of reference rabbit antiserum. Antisera to *R. rickettsii* were prepared in four rabbits inoculated intravenously with 50 μg of rickettsiae in 67 mM phosphate buffer (pH 7.4) containing 0.85% NaCl on days, 0, 2, 37, 39, 53, 67, 86, 88, 107, 164, 168, 170, 172, 203, and 205, subcutaneously with 50 μg in complete Freund adjuvant on days 8, 23, and 151, and subcutaneously with 50 μg in phosphate-buffered saline on days 35, 52, 65, 84, 105, 163, and 200. Globulins in pools made from sera obtained on days 60 and 72, 95 and 116, and 60, 95, 116, 179, and 212 were precipitated with 40% saturated ammonium sulfate, suspended in and dialyzed against barbital buffer (ionic strength, 0.05; pH 8.6) containing 0.02% sodium azide, and stored at 4°C. The final volume was about one-third that of the original serum. Qualitatively, all of the pools were essentially the same, but the relative concentrations of the various antibodies in the several pools differed slightly.

CIE. CIE was performed essentially by the method of Weeke (23). The detergent extract of *R. rickettsii* was electrophoresed in the first dimension on slides (5 by 5 cm) covered with 3.6 ml of gel composed of 1% agarose (Litex, Glostrup, Denmark)–0.5% Triton X-100–barbital buffer (pH 8.6; ionic strength, 0.05; 0.02% sodium azide) at 6 V/cm for 80 min in a Multiphor cell (LKB Instruments, Inc., Rockville, Md.) at 8°C. The electrode compartments contained barbital buffer without detergent. Electrophoresis was

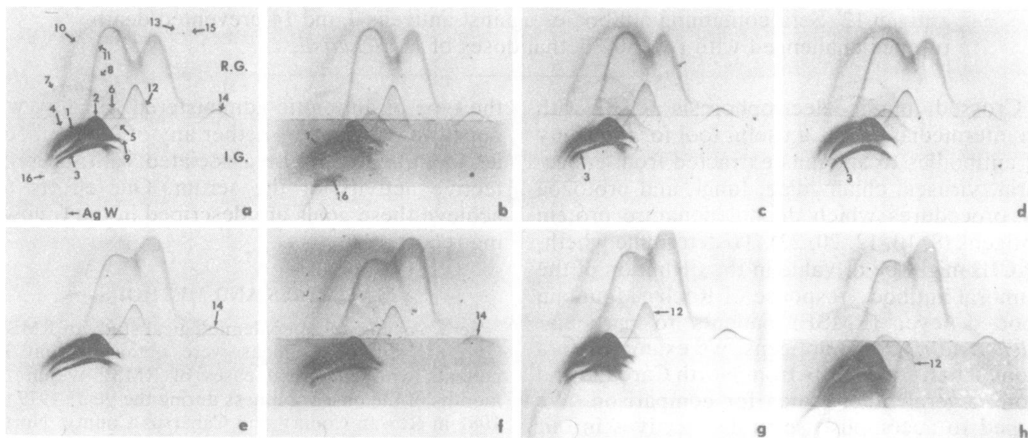

FIG. 1. Antibodies detected in sera of RMSF patients by CIE. The Triton X-100 extract of *R. rickettsii* containing 25 μg of protein was first electrophoresed for 80 min (towards the right) at 6 V/cm and then overnight (towards the top) at 2 V/cm against 29 μl of *R. rickettsii* rabbit antiserum per cm^2 in the reference gel of all slides. Slides with patient serum in intermediate gel (23 μl/cm^2), shown in b, d, f, and h, are to be compared with their respective control slides with control human serum in the intermediate gel (23 μl/cm^2), shown in a, c, e, and g, electrophoresed in the same run. (a) Control slide showing immunoprecipitates identified with reference antiserum. Ag W, Antigen well; R.G., reference gel; I.G., intermediate gel. (b) CIE pattern showing antibodies 1 and 16 in patient serum R80-218A; (d) CIE pattern showing antibody 3 in patient serum R80-203B; (f) CIE pattern showing antibody 14 in patient serum R81-224B; (h) CIE pattern showing, among other antibodies, antibody 12 in serum from Montana patient. These runs were made over a period of several months. Some changes were noted in the CIE patterns during this interval, particularly in the bimodal peaks of antigen 11.

conducted in the second dimension at 2 V/cm for about 16 h against either a pool of 25 patient sera negative for *R. rickettsii* antibodies, as a control, or serum from a patient with RMSF in the intermediate gel (23 μl/cm^2) and hyperimmune rabbit serum to *R. rickettsii* in the reference gel (29 μl/cm^2). After electrophoresis, the slides were washed in two changes of saline for a total of 24 h and two changes of distilled water for a total of 30 min, dried, and then stained for 10 min with 0.5% Coomassie brilliant blue R-250 in ethanol-acetic acid-water (9:2:9). Excess stain was removed with the solvent only.

Concentrations of the various antibodies in the sera of patients were estimated basically by the procedure described previously (10). First, the position of each immunoprecipitate under standard conditions was determined by CIE of the detergent extract on four slides with rabbit antiserum in the reference gel and the negative control serum in the intermediate gel. Then, the changes in positions of the precipitates were noted when various amounts of the reference rabbit antiserum were substituted for the control serum in the intermediate gel. Finally, the positions of the various precipitates were determined after CIE with 150 μl of patient sera in the intermediate gel. The levels of the patients' antibodies which retarded migration of their particular antigens a distance greater than 2 standard deviations from the average position of the precipitates in the control slides were expressed as microliters of reference rabbit antiserum required to retard migration of the *R. rickettsii* antigens during CIE to the same degree.

Serological tests. The IHA test was performed with fresh sheep erythrocytes coated with an erythrocyte-sensitizing substance (3). Sera were absorbed with sheep erythrocytes before testing.

All sera were also tested for *R. rickettsii* antibodies by the micro-IF test. Details of the test and the preparation of the antigen were described previously (16).

Protection of mice against *R. rickettsii*. The ability of patient sera to prevent early death of mice challenged with viable *R. rickettsii* was determined by the method of Bell and Stoenner (5). Although the mechanism of injury has not been defined, this phenomenon is referred to in the literature as toxic death. Patient serum or the negative human control serum (both held at 56°C for 30 min), diluted in Snyder I solution (11), were incubated for 1 h at room temperature with two 50% lethal doses of *R. rickettsii* per 0.5 ml of final mixture. Twenty-eight-day-old RML male mice were inoculated intravenously with 0.5 ml of the serum-rickettsiae mixture, and deaths were recorded at 24 h.

RESULTS

Detection of antibodies. Antibodies to 11 different *R. rickettsii* antigens were detected in sera from patients with RMSF by CIE with intermediate gel. Of the 71 North Carolina sera, 61

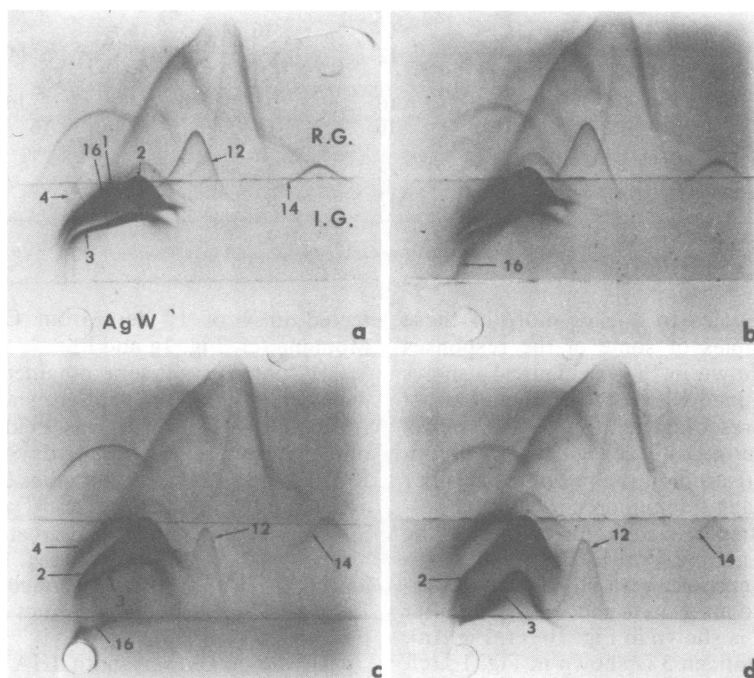

FIG. 2. Antibodies detected in serial bleedings from Montana patient with RMSF. Conditions for electrophoresis were same as those described in the legend to Fig. 1. Antibodies observed in patient serum are noted by arrows. (a) Control slide with pooled negative human sera in the intermediate gel (I.G.). Ag W, Antigen well; R.G., reference gel. (b) Patient serum obtained 12 days after onset of RMSF in the intermediate gel; (c) patient serum obtained 18 days after onset in the intermediate gel; (d) patient serum obtained 26 days after onset in the intermediate gel.

TABLE 1. Antibodies detected by CIE in sera from North Carolina patients with RMSF

Specimen no.[a]	No. of mos/ no. of days post-onset	IHA titer	Micro-IF titer[b]			CIE antibodies
			IgG	IgM	Ig	
C79-108A	0/5	<16	<16	<16	<16	—[c]
C79-108C	1/13	≥2,048	256	512	256	3, 4, 7, 8
C79-108D	2/27	ND[d]	256	64	256	3, 4
C79-118A	0/3	<16	<16	<16	<16	1, 4, 16
C79-118B	0/19	≥2,048	32	256	256	7
C79-118C	3/3	≥2,048	64	64	256	1, 4, 7, 16
C79-118D	16/27	256	32	tr	64	4, 7, 16
C80-138A	0/8	<16	<16	<16	<16	1, 7, 8, 16
C80-158A	0/2	<16	<16	<16	<16	1, 7, 8, 11, 16
C80-158B	0/18	1,024	512	128	128	1, 2, 3, 7, 11, 14, 16
C80-189A	0/5	<16	<16	<16	<16	16
C80-190A	0/1	<16	<16	<16	<16	1, 16
C80-301A	0/6	≥2,048	128	16	128	1, 16
C80-301B	0/20	≥2,048	1,024	256	512	1, 3, 7, 16
C80-301C	2/1	128	512	64	1,024	1, 3, 7, 16
R80-104A	0/3	16	<16	<16	<16	1, 16
R80-104B	0/18	2,048	128	512	2,048	1, 2, 3, 7, 16
R80-163A	0/5	<16	<16	<16	<16	—
R80-163B	0/19	≥2,048	1,024	512	2,048	7
R80-203A	0/3	<16	<16	<16	<16	—
R80-203B	5/27	256	512	tr	512	3, 11
R80-218A	0/1	32	<16	<16	<16	1, 16
R80-218B	0/25	512	16	64	128	16
R80-226A	0/6	16	64	<16	128	—
R80-226B	4/3	128	512	tr	1,024	3

contained antibodies to one or more of these antigens. Examples of some of the responses observed are shown in Fig. 1. Figure 1a shows the pattern obtained when the detergent extract was electrophoresed against the pooled control sera in the intermediate gel and the hyperimmune rabbit antiserum in the reference gel. For convenience, each immunoprecipitate was designated by a number which referred to both the antigen and the antibody present in the immunoprecipitate. An antiserum which had demonstrable antibodies to antigens 1 and 16 the day after onset of RMSF is shown in Fig. 1b (cf. Fig. 1a). A response to antigen 3 is shown in Fig. 1d (cf. Fig. 1c); this serum was collected 6 months after the symptoms first appeared. A serum in which only antibody 14 was observed is shown in Fig. 1f (cf. Fig. 1e). Antibody 12 was not found in any serum from North Carolina, but significant amounts of this and other antibodies were detected in 4 of 17 sera from Oklahoma and Montana (cf. Fig. 1g and h).

CIE patterns of sera obtained sequentially from one Montana patient are shown in Fig. 2. Antibody 16 (Fig. 2b) was evident in serum obtained from this patient 12 days after onset of RMSF. After 18 days, antibodies 2, 3, 4, 12, and 14 were also found (Fig. 2c). Twenty-six days after onset, this patient had greater amounts of antibodies 2, 3, 12, and 14 (Fig. 2d). Levels of antibodies 4 and 16 had declined by this time. Later serum specimens were not obtained from this patient.

CIE antibodies and both IHA and micro-IF titers for 44 sera obtained from North Carolina patients with RMSF within the first 18 months of illness are shown in Table 1. These sera were considered together because the same batch of antigen and the same pool of reference serum were used in CIE tests with these sera. Antibod-

TABLE 1—*Continued*

Specimen no.[a]	No. of mos/ no. of days post-onset	IHA titer	Micro-IF titer[b]			CIE antibodies
			IgG	IgM	Ig	
R80-231A	0/5	16	<16	<16	<16	16
C81-102A	0/2	<16	<16	<16	<16	16
C81-102B	0/17	1,024	64	128	512	11, 16
C81-103A	0/2	<16	<16	<16	<16	11, 16
C81-103B	0/15	512	16	<16	64	—
C81-105A	0/2	<16	<16	<16	<16	1, 4, 16
C81-105B	1/3	256	64	<16	64	2, 4
C81-109A	0/1	16	<16	<16	<16	11, 16
C81-109B	0/19	1,024	512	64	1,024	1, 3, 7, 8, 11
C81-120A	0/2	32	<16	<16	<16	1, 7, 11, 16
C81-120B	0/17	128	<16	<16	<16	1, 4, 7, 8, 11, 16
R81-200A	0/2	<16	<16	<16	<16	11, 16
R81-200B	0/15	512	<16	32	32	11
R81-201A	0/6	128	<16	<16	<16	10, 11, 16
R81-201B	0/24	≥2,048	32	1,024	1,024	1, 3
R81-224A	0/15	16	<16	<16	<16	16
R81-224B	0/20	≥2,048	256	256	1,024	14
R81-245A	0/1	16	<16	<16	<16	1, 11, 16
R81-245B	0/13	1,024	512	64	512	3, 11

[a] The first letter of the specimen number refers to the county of origin (C, Cabarrus County; R, Rowan County), the next two numbers indicate the year of illness, the next three numbers denote the patient code number, and the last letter refers to the sequence of blood samples (A, first sample; B, second sample, etc.).

[b] Because of the use of specific fluorescein-labeled goat anti-human globulins, microimmunofluorescence titers were specific for immunoglobulin G (IgG) antibodies, immunoglobulin M (IgM) antibodies, or broadly specific for all human globulins (Ig).

[c] —, Negative.

[d] ND, Not done.

ies were observed by CIE in all but five of these sera. Four of the negative sera were obtained within 6 days of onset, and the fifth was collected 15 days post-onset. Antibodies were not found in three of these five samples by the IHA or the micro-IF test. However, antibodies, particularly 1 and 16, were found in several sera shown to be negative by the IHA and micro-IF tests. Several of the paired sera indicated a sequential response of antibody types; antibodies 1 and 16 were found frequently during the first week of illness, and antibodies 2, 3, and 7 were often found in addition in later serum samples (see Table 1).

All of the above sera were obtained from patients with RMSF. Sera from patients with illnesses caused by other or unknown etiological agents were also tested by CIE (Table 2). Of the six sera from the western United States, three reacted with antigens in the extract. One of three sera from patients with Colorado tick fever gave evidence of an antibody which reacted with antigen 1, and both sera from Q fever patients reacted with antigen 8. All six sera from North Carolina patients without RMSF reacted with the detergent extract of *R. rickettsii*. Migration of antigens 1, 7, 8, and 16 was retarded by two or more of the sera.

Appearance and persistence of *R. rickettsii* antibodies. A summary of the frequency of *R. rickettsii* antibodies in these 44 sera is shown in Table 3. Although the number of samples within each time period was generally low, several trends in antibody response pattern were noted. Antibodies 1, 11, and 16 were found in a third or more of the patients during the first week of illness, and antibodies 1 and 16 were found in a high percentage of patients throughout the examination period. Antibodies 2, 3, and 14 were not detected until late in the second week or in

TABLE 2. Antibodies or antibody-like substances to *R. rickettsii* antigens detected by CIE in sera from patients with illnesses other than RMSF

Specimen no.[a]	Illness	Source	No. of mos/ no. of days post-onset	Antibodies detected
79-564	Colorado tick fever	South Dakota	0/21	—[b]
80-194	Colorado tick fever	Montana	0/1	1
80-199	Colorado tick fever	Montana	0/1	—
80-221	Tularemia	Wyoming	36/0	—
81-319	Q fever	Colorado	?[c]	8
81-329	Q fever	Wyoming	0/13	8
C81-134A	Undiagnosed	North Carolina	0/1	1, 7
C81-136A	Undiagnosed	North Carolina	0/2	7, 8
C81-139A	Undiagnosed	North Carolina	0/1	1
R81-229A	Undiagnosed	North Carolina	0/1	1
R81-233A	Pneumonitis	North Carolina	0/1	1, 16
R81-240A	Undiagnosed	North Carolina	0/1	1, 8, 16

[a] See Table 1, footnote *a*, for explanation of designations.
[b] —, Negative.
[c] ?, Unknown.

the third week. The response to antigen 7 was intermediate to the above two kinds of response; antibody 7 was present in a few individuals during the first 2 weeks, but most sera were not positive for this antibody until the third week. The other antibodies were present at such low frequencies that it was difficult to discern any particular pattern of response.

Most of the sera examined were obtained from RMSF patients during either the acute or the early convalescent stage of the disease. However, sera were collected from seven individuals who had had RMSF 4 to 40 years earlier. Six of these sera had antibodies to antigens 2 and 3; the seventh serum, taken 17 years after illness, had no antibodies detectable by CIE.

CIE titers of *R. rickettsii* antibodies. The titers of particular *R. rickettsii* antibodies in the 44 North Carolina sera are shown in Table 4. Compared with the reference rabbit antiserum, titers of antibodies 1, 7, and 16 were considerably higher than those of the other antibodies. Also, antibodies 1 and 16 were present more frequently than were the other antibodies, and antibodies 7 and 11 were the next-most-commonly occurring antibodies. As will be shown later, protective activity of the sera for mice was not correlated with the antibodies found most frequently.

TABLE 3. Frequency of occurrence of antibodies detected by CIE in sera from North Carolina patients with RMSF

Antibody	% Positive at following time post-onset (no. of sera tested)				
	0–7 days (20)	8–21 days (14)	22 days, 2 mo (4)	6–18 mo (6)	Total (44)
1	45	42.9	25	33.3	40.9
2	0	14.3	25	0	6.8
3	0	35.7	50	66.7	25
4	10	7.1	50	50	18.2
7	10	57.1	25	50	31.8
8	5	21.4	25	0	11.4
10	5	0	0	0	2.3
11	35	42.9	0	33.3	31.8
14	0	14.3	0	0	4.5
16	80	50	25	33.3	61.4

Clinical significance of *R. rickettsii* antibodies. An attempt was made to correlate the antibody responses of the RMSF patients with antibiotic therapy and severity of illness. An examination of the responses of patients given either tetracycline or chloramphenicol did not indicate obvious differences in response patterns, nor did the kinds of antibodies in the sera of patients who had been hospitalized differ from those of individuals who had not been hospitalized.

Another comparison, however, did reveal a positive correlation. It was noted during this investigation that most of the sera with high micro-IF titers of rickettsial immunoglobulin G antibodies also had antibody 3. Of the 55 sera (collected from 1979 to 1981) which had antibodies demonstrable by CIE, 16 had antibody 3 and a geometric mean micro-IF titer of 861. The geometric mean micro-IF titer of the remaining 39 sera lacking antibody 3 was only 7.3. Analysis of these results by the *t* test showed that this difference was significant at the 0.001 level, strongly suggesting that antigen 3 was a good immunogen in some individuals.

Protection by *R. rickettsii* antibodies. Some of the sera were incubated with *R. rickettsii* to

TABLE 4. Titers of antibodies in sera from North Carolina patients with RMSF

Antibody	No. of sera with titer (μl of antiserum) of[a]:							
	>400	400	200	100	50	25	12.5	0
1			10	8				26
2							3	41
3						2	9	33
4						2	6	36
7		2	6	6				30
8						5		39
10							1	43
11				1		12	1	30
14							2	42
16	5	6	8	4	4			17

[a] Results are expressed as microliters of reference rabbit antiserum causing an equivalent change in position of the immunoprecipitate in the CIE slide.

determine whether the serum could prevent death of Rocky Mountain Laboratories mice (Table 5). Sera containing only antibodies 1 and 16 or antibodies 1 and 16 in combination with antibodies 4 and 7 did not protect mice against challenge. However, all sera with antibody 3 either alone or in combination with other antibodies prevented death of the mice, at least at low serum dilutions. Included in the latter group was a serum which was obtained from an individual 39 years after recovery from RMSF. Results with antibody 7 were equivocal. Serum C79-118C, which had antibody 7 as well as antibodies 1, 4, and 16, did not protect mice, but serum R80-163B, in which only antibody 7 was detected, had a 50% protective dose of 80. Results from tests of sera C79-118A and C81-105B indicated that antibodies 2 and 4 had little, if any, protective activity. IHA titers were not directly correlated with protection, since several sera with high IHA titers (C79-118C, C80-301A) failed to protect mice. The role of other antibodies in protection could not be determined.

DISCUSSION

Our results indicate that antibodies to particular antigens in Triton X-100 extracts of *R. rick-*

TABLE 5. Protection of mice against lethal effects of *R. rickettsii* toxin by serum from North Carolina patients with RMSF

Serum[a]	Time post-onset	Antibodies detected by CIE	Titer		PD50[c]
			IHA	Micro-IF[b]	
C79-108D	87 days	3, 4	2,048	256	67
C79-118A	3 days	1, 4, 16	<16	<16	<40
C79-118C	93 days	1, 4, 7, 16	≥2,048	256	<40
C80-157B	24 days	3	128	16,384	≥538
C80-301A	6 days	1, 16	≥2,048	128	<40
R80-104A	3 days	1, 16	16	<16	<20
R80-104B	18 days	1, 2, 3, 7, 16	2,048	2,048	>320
R80-163A	5 days	—[d]	<16	<16	<40
R80-163B	19 days	7	≥2,048	2,048	80
R80-226B	123 days	3	128	1,024	202
C81-105B	33 days	2, 4	256	64	<40
R81-224A	15 days	16	16	<16	<40
R81-224B	20 days	14	≥2,048	1,024	508
RA79-204	39 yr	2, 3	64	128	95
RA79-213	4 yr	2, 3	16	64	50
Control			<16	<16	<20

[a] See Table 1, footnote *a*, for explanation of designations.
[b] Titers of immunoglobulin G and M antibodies.
[c] Reciprocal of dilution which protected 50% of the mice from death after challenge with two 50% lethal doses of *R. rickettsii*.
[d] —, Negative.

ettsii can be demonstrated in the sera of RMSF patients by CIE with an intermediate gel. Antibodies to 1 or more of 10 different *R. rickettsii* antigens were observed in most of the sera from North Carolina patients, and another antibody was found in patients from Oklahoma and Montana. Some of the antibodies (1 and 16) characteristically are present early in the course of disease (as early as 1 day after onset), whereas others (2, 3, and 14) cannot be detected until about the third week of illness. Two of the latter antibodies (2 and 3) can also be found in sera obtained many years after recovery from RMSF.

It is not certain that the antibodies demonstrated by CIE within the first few days of illness, such as 1 and 16, are actually produced in response to the *R. rickettsii* infection or whether the precipitates observed by CIE are due to cross-reacting antibodies or even nonspecific reactions. Since antibodies to antigens 1, 7, 8, and 16 were observed in sera from patients with illnesses definitely or presumably caused by other infectious agents (Table 2), one cannot be sure that these antibodies, even in serologically confirmed RMSF patients, always developed specifically in response to *R. rickettsii* infection. Several observations support the suggestion that, in some cases, antibodies to antigens 1, 7, 8, and 16 may indeed be cross-reacting antibodies. First, the Weil-Felix test (24), used for many years for the serological diagnosis of RMSF, depends upon the agglutination of *Proteus* sp. organisms by cross-reacting antibody from RMSF patients. Conversely, there may be circulating in some individuals *Proteus* sp. antibodies (or other antibodies) which react with *R. rickettsii* antigens. Second, there is abundant evidence that there are many avirulent spotted fever group rickettsiae in nature (2, 7, 13, 14, 17). Since these other rickettsiae share one or more antigens with *R. rickettsii* (15), it is conceivable that some of the serum samples reacted with the soluble *R. rickettsii* antigens in the CIE test because the serum donors had previously been exposed to avirulent spotted fever group rickettsiae to an extent sufficient to stimulate antibody formation. Finally, Barbour et al. (submitted for publication; personal communication) demonstrated in their studies of Lyme disease that some human sera reacted with certain *R. rickettsii* antigens in immunoblotting tests. These sera presumably came from individuals who had not had RMSF. The presence of cross-reacting antibodies in some serum specimens may explain the findings (Table 1) that antibodies 1 and 16, the same antibodies found in non-RMSF cases, were detected by CIE, even though IHA and micro-IF tests were negative. It is possible that cross-reacting antibodies, in conjunction with specific antibodies present in the reference serum, could retard antigen migration in the CIE test, whereas these same cross-reacting antibodies in the absence of specific antibodies do not have sufficient affinity for the rickettsial antigens to produce positive results in the IHA and the micro-IF test.

Of some interest is the finding that antibody 12 was absent from sera of RMSF patients from Rowan County and Cabarrus County, North Carolina, but was easily demonstrated by CIE in sera of patients from Oklahoma and Montana. This finding suggests to us that *R. rickettsii* strains that are indigenous to North Carolina differ in antigenic composition from some *R. rickettsii* strains in Montana and Oklahoma in that the North Carolina strains lack antigen 12. Earlier serological comparisons of *R. rickettsii* strains from North Carolina and Montana failed to reveal antigenic differences (9, 15), but it is probable that the micro-IF test, used for these comparisons, is not as well suited as the CIE test to detect minor or subsurface differences.

Although most of the sera tested had from one to seven detectable *R. rickettsii* antibodies, we cannot be certain that the responses observed represent the full range and magnitude of responses of which humans are capable. First, patient antibodies in very low concentration relative to that of the reference rabbit antibodies would not be detected, because the position of the specific antigen-antibody precipitate would not be changed sufficiently to be considered significant. Second, all of the patients from whom sera had been collected in 1979 to 1981 had been treated with either tetracycline or chloramphenicol. Thus, it is possible that humans not treated with antibiotics would produce qualitatively and quantitatively greater antibody responses. However, it is probable that the antibodies that were demonstrated in human sera were antibodies to the most immunogenic components of *R. rickettsii*.

Our results may provide some information of value for the development of a subunit vaccine for RMSF. Our tests indicate the possible importance of antibody 3 and, in one instance, antibody 14 for the protection of mice. Perhaps other antibodies have protective activity. Certainly one cannot automatically expect results in mice to be duplicated in humans, but it may be suggested as a point of departure that an experimental vaccine might include some or all of the antigens associated with protection in mice. Previously it has been shown that the capacity of a vaccine to stimulate formation of antitoxin in guinea pigs was correlated with the ability of the vaccine to protect guinea pigs from RMSF after challenge with *R. rickettsii* (6). At present it is unclear just how important humoral immunity is

in host defense against spotted fever, but a variety of evidence indicates that antibodies may play a significant role in combating infection. Spencer and Parker (18) reported that sera from vaccinated rabbits, guinea pigs, or one human, when incubated with viable rickettsiae before intraperitoneal inoculation of normal guinea pigs, either prevented overt disease or ameliorated symptoms. Topping (21) protected experimentally infected guinea pigs and monkeys with fractionated rabbit antiserum after symptoms of RMSF had already appeared. In a trial conducted from 1940 to 1942, he showed that there were significantly fewer fatalities in a human population when first treated with this same antiserum up to 3 days after the appearance of rash than would be expected in an untreated group. Finally, Bell and Pickens (4) demonstrated that an otherwise lethal dose of *R. rickettsii* incubated with immune human or guinea pig sera was unable to kill mice. Our results, along with those from several other laboratories, suggest that the goal of developing a vaccine to stimulate the production of protective antibodies is a reasonable one.

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LITERATURE CITED

1. **Anacker, R. L., R. K. Gerloff, L. A. Thomas, R. E. Mann, W. R. Brown, and W. D. Bickel.** 1974. Purification of *Rickettsia rickettsii* by density gradient zonal centrifugation. Can. J. Microbiol. **20:**1523–1527.
2. **Anacker, R. L., T. F. McCaul, W. Burgdorfer, and R. K. Gerloff.** 1980. Properties of selected rickettsiae of the spotted fever group. Infect. Immun. **27:**468–474.
3. **Anacker, R. L., R. N. Philip, L. A. Thomas, and E. A. Casper.** 1979. Indirect hemagglutination test for detection of antibody to *Rickettsia rickettsii* in sera from humans and common laboratory animals. J. Clin. Microbiol. **10:**677–684.
4. **Bell, E. J., and E. G. Pickens.** 1953. A toxic substance associated with the rickettsias of the spotted fever group. J. Immunol. **70:**461–472.
5. **Bell, E. J., and H. G. Stoenner.** 1960. Immunologic relationships among the spotted fever group of rickettsias determined by toxin neutralization tests in mice with convalescent animal serums. J. Immunol. **84:**171–182.
6. **Bell, E. J., and H. G. Stoenner.** 1961. Spotted fever vaccine; potency assay by direct challenge of vaccinated mice with toxin of *Rickettsia rickettsii*. J. Immunol. **87:**737–746.
7. **Burgdorfer, W., D. J. Sexton, R. K. Gerloff, R. L. Anacker, R. N. Philip, and L. A. Thomas.** 1975. *Rhipicephalus sanguineus*: vector of a new spotted fever group rickettsia in the United States. Infect. Immun. **12:**205–210.
8. **Caldwell, H. D., C. C. Kuo, and G. E. Kenny.** 1975. Antigenic analysis of chlamydiae by two-dimensional immunoelectrophoresis. I. Antigenic heterogeneity between *C. trachomatis* and *C. psittaci*. J. Immunol. **115:**963–968.
9. **Davis, J. P., C. M. Wilfert, D. J. Sexton, W. Burgdorfer, E. A. Casper, and R. N. Philip.** 1981. Serologic comparison of *R. rickettsii* isolated from patients in North Carolina to *R. rickettsii* isolated from patients in Montana, p. 139–147. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, Inc., New York.
10. **Høiby, N., and N. H. Axelsen.** 1973. Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta Pathol. Microbiol. Scand. Sect. B **81:**298–308.
11. **Jackson, E. B., and J. E. Smadel.** 1951. Immunization against scrub typhus. II. Preparation of lyophilized living vaccine. Am. J. Hyg. **53:**326–331.
12. **Jepsen, S., and N. H. Axelsen.** 1980. Antigens and antibodies in *Plasmodium falciparum* malaria studied by immunoelectrophoretic methods. Acta Pathol. Microbiol. Scand. Sect. C **88:**263–270.
13. **Magnarelli, L. A., J. F. Anderson, R. N. Philip, W. Burgdorfer, and E. A. Casper.** 1981. Endemicity of spotted fever group rickettsiae in Connecticut. Am. J. Trop. Med. Hyg. **30:**715–721.
14. **Philip, R. N., and E. A. Casper.** 1981. Serotypes of spotted fever group rickettsiae isolated from *Dermacentor andersoni* (Stiles) ticks in western Montana. Am. J. Trop. Med. Hyg. **30:**230–238.
15. **Philip, R. N., E. A. Casper, W. Burgdorfer, R. K. Gerloff, L. E. Hughes, and E. J. Bell.** 1978. Serologic typing of rickettsiae of the spotted fever group by microimmunofluorescence. J. Immunol. **121:**1961–1968.
16. **Philip, 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.
17. **Philip, R. N., R. S. Lane, and E. A. Casper.** 1981. Serotypes of tick-borne spotted fever group rickettsiae from western California. Am. J. Trop. Med. Hyg. **30:**722–727.
18. **Spencer, R. R., and R. R. Parker.** 1925. Rocky Mountain spotted fever: vaccination of monkeys and man. Public Health Rep. **40:**2159–2167.
19. **Stoenner, H. G., D. B. Lackman, and E. J. Bell.** 1962. Factors affecting the growth of rickettsias of the spotted fever group in fertile hens' eggs. J. Infect. Dis. **110:**121–128.
20. **Svejgaard, E., and A. H. Christiansen.** 1979. Precipitating antibodies in dermatophytosis demonstrated by crossed immunoelectrophoresis. Acta Pathol. Microbiol. Scand. Sect. C **87:**23–27.
21. **Topping, N. H.** 1943. Rocky Mountain spotted fever. Further experience in the therapeutic use of immune rabbit serum. Public Health Rep. **58:**757–775.
22. **Vestergaard, B. F.** 1979. Quantitative immunoelectrophoretic analysis of human antibodies against herpes simplex virus antigens. Infect. Immun. **23:**553–558.
23. **Weeke, B.** 1973. Equipment, procedures, basic methods, p. 15–35. *In* N. H. Axelsen, J. Kroll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis. Methods and applications. Universitetsforlaget, Oslo, Norway.
24. **Weil, E., and A. Felix.** 1916. Zur serologischen Diagnose des Fleckfiebers. Wien. Klin. Wochenschr. **29:**33–35.
25. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by Renografin density gradient centrifugation. Appl. Microbiol. **30:**326–331.
26. **Wilfert, C. M., E. Austin, V. Dickinson, K. Kleeman, J. L. Hicks, J. N. MacCormack, R. L. Anacker, E. A. Casper, and R. N. Philip.** 1981. The incidence of Rocky Mountain spotted fever as described by prospective epidemiologic surveillance and the assessment of persistence of antibodies to *R. rickettsii* by indirect hemagglutination and microimmunofluorescence tests, p. 179–189. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, Inc., New York.