Absorption of *Rickettsia rickettsii* Antibodies by *Rickettsia rickettsii* Antigens in Four Diagnostic Tests

KARIM E. HECHEMY,^{1*} ROBERT L. ANACKER,² NANCY L. CARLO,¹ JOHN A. FOX,¹ AND
HASSAN A. GAAFAF¹

*Centers for Laboratories and Research, New York State Department of Health, Albany, New York 12201,¹
and National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana
59840²*

Received 20 August 1982/Accepted 10 November 1982

Immunoabsorption profiles were determined for *Rickettsia rickettsii* antigens used in four confirmatory tests for detection of antibodies to Rocky Mountain spotted fever. A human serum reactive in the four tests was absorbed with each test antigen and then reexamined by all four tests. The results indicated that the whole organism and complement-fixation antigen absorbed the whole array of antibodies to *R. rickettsii*, whereas erythrocyte-sensitizing substance coated on latex or erythrocytes did not. With erythrocyte-sensitizing substance coated on latex there was a decrease in titer when tested by the latex, indirect hemagglutination, and complement fixation tests but none when tested by the microimmunofluorescence test. With erythrocyte-sensitizing substance coated on sheep erythrocytes there was a decrease in titer when tested by indirect hemagglutination or complement fixation but none when tested by latex or microimmunofluorescence. These findings confirm that erythrocyte-sensitizing substance contains more than a single antigenic substance and plays a major role in the complement fixation test and a minor role in the microimmunofluorescence test.

In the last few years a number of specific serological tests have been developed for detection of antibodies to Rocky Mountain spotted fever. Five tests considered confirmatory by the Centers for Disease Control (3) are complement fixation (CF), microimmunofluorescence (micro-IF), latex (latex-*Rickettsia rickettsii*), indirect hemagglutination (IHA), and microagglutination. These tests use either whole antigens or antigenic fractions from *R. rickettsii*. The CF test uses group-specific solubilized antigen (13). Both the micro-IF and microagglutination tests use the whole *R. rickettsii* organism. The latex-*R. rickettsii* and IHA tests use erythrocyte-sensitizing substance (ESS) obtained by boiling the CF antigen or the whole organism with sodium hydroxide (1, 4) without further purification.

Differences in sensitivity among these tests have been reported (7, 11). Through immunoabsorption studies we have examined the possible role of four of the antigen preparations in causing such differences among the micro-IF, CF, latex-*R. rickettsii*, and IHA tests. To assess the reactivity of these tests, a serum reactive in all four was used. This report presents the immunoabsorption profiles of the various *R. rickettsii* antigens with this serum and examines especially the role of ESS.

MATERIALS AND METHODS

Antigen preparation. The R strain of *R. rickettsii* was cultivated in L cells, purified by zonal centrifugation, and lyophilized as described by Anacker et al. (1). ESS was prepared by suspending the rickettsiae in saline (1 mg/ml) and adjusting the solution to 0.2 N with sodium hydroxide, boiling it for 30 min, and then dialyzing against isotonic phosphate-buffered saline (4).

CF antigen (13) was provided by Theodore Tzianabos of the Centers for Disease Control, and the micro-IF antigen (12) was provided by Robert Philip of the Rocky Mountain Laboratory.

The latex-*R. rickettsii* reagent was prepared by a forced-adsorption technique (K. E. Hechemy and R. L. Anacker, J. Immunoassay, in press), which consisted of precipitating ESS on latex by using 95% ethanol containing 0.5% sodium acetate at 25°C for 30 min, leaving the mixture overnight at 5°C, centrifuging at 5,000 × g, and discarding the ethanolic supernatant. The ESS-coated latex particles were suspended in twice the original volume of glycine-buffered saline (GBS), pH 8.1, and more ESS from the same batch used to coat the latex particles was added (approximately 30% of the volume used to precipitate on latex). Finally, GBS, pH 8.1, containing 0.1% fatty acid-free bovine albumin (Sigma Chemical Co., St. Louis, Mo.) was added. The use of forced adsorption rather than passive adsorption ensured optimum reactivity and batch-to-batch reproducibility (Hechemy and Anacker, in press).

Sheep erythrocytes were coated directly with ESS

(RBC-ESS) (1) or were tanned (5) and then coated (RBCT-ESS). RBCT-ESS were further incubated with normal human serum for 1 h in a 37°C water bath. Both erythrocyte preparations were adjusted to 0.5% in normal human serum-phosphate-buffered saline for the IHA test and to 5.0% in GBS-0.1% fatty acid-free bovine albumin, pH 8.1, for immunoabsorptions.

Controls. The appropriate control for each antigen in the absorption experiments is listed in Table 1.

Serum. A serum from a Rocky Mountain spotted fever patient reactive by the four tests was used for the immunoabsorptions. The modal titers by micro-IF ($n = 20$) were 4,096 with goat anti-human immunoglobulin A (IgA) plus IgG plus IgM, heavy and light chains, coupled with fluorescein isothiocyanate (micro-IF-immunoglobulin); 4,096 with anti-human IgG (micro-IF-IgG); and 512 with anti-human IgM (micro-IF-IgM). The serum was not tested by microagglutination.

Serological tests. The micro-IF (12), CF (14), latex-*R. rickettsii* (6, 8), and IHA (1, 2) tests were performed as described.

One-step serum absorptions. The optimal conditions for each absorption experiment were determined and are described below. Controls for each test were included (Table 1). In each absorption a 1:64 dilution of the reactive serum was used, and the supernatant (absorbed serum) after each centrifugation was saved for assay by the four tests.

(i) **Whole *R. rickettsii*.** The serum dilution and antigen suspension (1 mg/ml) were mixed in equal volumes, incubated at 37°C for 5.5 h and then overnight at 5°C, and centrifuged at 5°C and $4,000 \times g$ for 30 min.

(ii) **CF antigen.** The same procedure was used as for the whole organism. The antigen titer was ≥ 16 .

(iii) **Latex-*R. rickettsii*.** The latex-*R. rickettsii* reagent (3 ml) was centrifuged at 5°C and $4,000 \times g$ for 30 min. The sediment was mixed with 0.6 ml of the serum dilution, incubated overnight at 37°C, and then centrifuged at 5°C and $4,000 \times g$ for 30 min.

(iv) **RBC-ESS and RBCT-ESS.** A portion (0.5 ml) of the serum dilution was mixed with 0.5 ml of the 5.0% RBC-ESS or RBCT-ESS suspension, incubated in a 37°C water bath overnight, and centrifuged at $2,500 \times g$ for 15 min.

Two-step serum absorption. To further decrease the antibody titers of the sera absorbed in the one-step procedure, two-step absorption by latex-*R. rickettsii* and RBC-ESS was performed. Portions of the absorbed sera from each step of both absorption experiments were tested by the four tests.

(i) **Latex-*R. rickettsii*.** In the first absorption step, 4.5 ml of latex-*R. rickettsii* reagent was centrifuged at 5°C and $4,000 \times g$ for 30 min. The supernatant was discarded. The pellet was suspended in 0.9 ml of the 1:64 serum dilution, incubated overnight at 37°C, and centrifuged at 5°C and $4,000 \times g$ for 30 min. The pellet was discarded. In the second absorption step, 3.0 ml of fresh latex-*R. rickettsii* reagent was centrifuged as above, and the pellet was suspended in 0.6 ml of the supernatant (absorbed serum) from step 1. This suspension was incubated and centrifuged as described in the first step.

(ii) **RBC-ESS.** In the first absorption step, 0.05 ml of sensitized erythrocytes was suspended in 2.0 ml of the serum, incubated overnight in a 37°C water bath, and

TABLE 1. Profile of one-step immunoabsorptions of rickettsial antibodies by rickettsial antigens for a representative trial

Absorbent		Titer of absorbed serum determined by:					
Antigen	Control	Micro-IF tests			CF	Latex- <i>R. rickettsii</i>	IHA
		Micro-IF-immunoglobulin	Micro-IF-IgG	Micro-IF-IgM			
Whole <i>R. rickettsii</i>	GBS-0.1% fatty acid-free bovine albumin	512	≤ 64	≤ 64	≤ 32	≤ 64	≤ 64
		4,096	4,096	512	128	1,024	4,096
CF antigen	Egg yolk sac ^a	512	≤ 64	≤ 64	≤ 32	≤ 64	≤ 64
		8,192	2,048	512	AC ^b	256	1,024
Latex- <i>R. rickettsii</i>	Latex-normal rabbit serum ^c	2,048	512	256	≤ 32	128	≤ 32
		4,096	1,024	512	128	512	1,024
RBC-ESS	Erythrocytes ^e	4,096	1,024 ^d	256	≤ 64	512	≤ 64
		4,096	2,048	512	256	1,024	4,096
RBCT-ESS	Tanned erythrocytes ^f	4,096	1,024	256	AC	512	≤ 64
		4,096	2,048	256	AC	1,024	32,768

^a Normal egg yolk sac.

^b AC, Anticomplementary.

^c Latex-normal rabbit serum was prepared by mixing latex, rabbit serum, GBS, and GBS containing 0.1% fatty acid-free bovine albumin in the ratio (by volume) 72:1:61:10.

^d Results were variable.

^e Unsensitized 0.5% and 5.0% erythrocytes.

^f Unsensitized 0.5% and 5.0% tanned erythrocytes coated with human serum.

centrifuged at $2,500 \times g$ for 15 min. In the second step, 0.025 ml of sensitized erythrocytes was suspended in 1.0 ml of the RBC-ESS supernatant from step 1. This suspension was incubated in a 37°C water bath overnight and centrifuged at $2,500 \times g$ for 15 min.

RESULTS

In this study, absorption of antibodies by antigen was considered significant when there was a ≥ 4 -fold decrease in titer relative to the control.

The titers of sera absorbed with the whole *R. rickettsii* organism and with the CF antigen decreased ≥ 4 -fold in all four tests (Table 1).

When the serum was absorbed with the latex-*R. rickettsii* reagent, the titers by CF, latex-*R. rickettsii*, and IHA decreased ≥ 4 -fold. However, in six trials with the panel of three micro-IF tests, the decrease was only ≤ 2 -fold in four trials by micro-IF-immunoglobulin, in five trials by micro-IF-IgG, and in all six trials by micro-IF-IgM. The decrease in titer by micro-IF-immunoglobulin and micro-IF-IgG did not always occur within the same trial.

The titers of the RBC-ESS- and RBCT-ESS-absorbed sera decreased ≥ 4 -fold when tested by CF and IHA. With these sera, the titers by the latex-*R. rickettsii* test showed consistent two-fold decreases (5 of 5 trials). The titers by micro-IF-immunoglobulin and micro-IF-IgM also indicated a ≤ 2 -fold decrease (5 of 5 trials). The micro-IF-IgG results, however, were inconsistent. In three of five trials there was a ≥ 4 -fold titer decrease, which was not substantiated by a simultaneous decrease in micro-IF-immunoglobulin titer.

The two-step latex-*R. rickettsii* and RBC-ESS

serum absorptions were performed because of the lack of apparent significant absorption of (i) "micro-IF antibodies" when the reactive serum was absorbed with latex-*R. rickettsii* reagent, RBC-ESS, or RBCT-ESS and (ii) "latex antibodies" when the reactive serum was absorbed with RBC-ESS or RBCT-ESS (Table 1).

After two-step absorption with latex-*R. rickettsii* reagent, there was a ≤ 2 -fold decrease in serum titer when the absorbed sera from the second step were tested by micro-IF-IgG and micro-IF-IgM (Table 2). During the first step, the CF and IHA "antibodies" were removed, and the latex-*R. rickettsii* antibody titers decreased significantly; the second absorption step was required for maximal removal of the latex-*R. rickettsii* antibodies.

After two-step absorption with RBC-ESS, there were no decreases in the micro-IF-immunoglobulin and micro-IF-IgM titers relative to the control. The micro-IF-IgG results were equivocal: the decrease in titer was significant only at the first step. Titer reductions to ≤ 32 occurred for CF at the first step and for IHA at the second step. No titer decreases were seen at either step by the latex-*R. rickettsii* test.

DISCUSSION

Absorption with the whole *R. rickettsii* organism or CF antigen resulted in a decrease in titer by all four tests. Absorption of the micro-IF antibodies by the CF antigen indicates that the latter contains most, if not all, components of the antigenic array in the whole organism.

Absorption of sera with ESS, when coated on latex or erythrocytes, reduced the CF titer but

TABLE 2. Profile of two-step immunoabsorption of rickettsial antibodies by rickettsial antigens for a representative trial

Absorbent		Absorption step	Titer of absorbed serum determined by:					
Antigen	Control		Micro-IF tests			CF	Latex- <i>R. rickettsii</i>	IHA
			Micro-IF-immunoglobulin	Micro-IF-IgG	Micro-IF-IgM			
Latex- <i>R. rickettsii</i>	Latex-normal rabbit serum ^a	1	4,096	2,048	1,024	≤32	128	≤32
			4,096	2,048	1,024	128	512	4,096
		2	4,096	2,048	512	≤32	≤32	≤32
			4,096	4,096	1,024	128	512	4,096
RBC-ESS	Erythrocytes	1	8,192	2,048	2,048	≤32	512	2,048
			16,384	8,192	2,048	128	1,024	16,384
		2	8,192	2,048	1,024	≤32	512	≤32
			8,192	4,096	1,024	128	1,024	8,192

^a See Table 1, footnote c.

not the micro-IF titer, even after two-step absorption was performed. This indicates an antigenic relationship between ESS and soluble CF antigen, which corroborates the findings by Osterman and Eisemann (10). In addition, this shows that ESS plays a major role in the CF test and a minor role in the micro-IF test. The lack of decrease in the micro-IF titer could be a result of the micro amounts of ESS antigen coated on latex or erythrocytes, or the micro-IF antibodies could be directed primarily against other antigens, or both.

Differences in the test results when the serum was absorbed with ESS, a specialized antigen, depending upon whether it was coated on latex or erythrocytes, confirm that ESS contains more than a single antigenic fraction. The complexities of ESS have been shown by various authors. Anacker et al. (2) suggested the presence of at least two substances in ESS. Osterman and Eisemann (10) found that sedimented ESS extracted from radiolabeled *Rickettsia prowazekii* had more than one fraction. Murphy and others (9) showed that ESS from *Rickettsia mooseri* absorbed on erythrocytes elicited the production of antibody populations reactive with at least three separate antigens, indicating that the crude ESS preparation consisted of at least three antigens.

One explanation for the discrepancies of the RBC-ESS and latex-*R. rickettsii* immunoabsorption profiles may be the different methods used to absorb the ESS on erythrocytes and latex. In the former, the fraction(s) of the various components of ESS attaching to erythrocytes is selectively absorbed by the erythrocytes, whereas with latex the entire ESS is presumably precipitated onto the latex particle. This may explain in part the reactivity of the RBC-ESS-absorbed serum in the latex test. On the other hand, the serum was negative by IHA after absorption with latex-*R. rickettsii*.

Possibly one or more determinants on ESS adsorb to specific receptors on erythrocytes and therefore are unable to react with their specific antibodies. In contrast, when ESS is forcibly precipitated on latex, the determinants are randomly masked, and therefore some are always available to react with their specific antibodies.

Because Osterman and Eisemann (10) found ESS to be a carbohydrate-protein complex, we tanned sheep erythrocytes before ESS sensitization in an attempt to attach the protein to the erythrocytes. We had hoped to increase the affinity of the various components of ESS for erythrocytes, thus producing a reagent which would absorb all antibodies to ESS, similar to latex-*R. rickettsii* absorption. Serological test results indicated that we had successfully attached the ESS to tanned erythrocytes, and the

serum titers were higher than those with the RBC-ESS (32,768 versus 4,096). These higher titers indicated that both the protein and carbohydrate may be serologically active. When the serum was absorbed with RBCT-ESS, negative results by the latex-*R. rickettsii* test would indicate a role for the ESS protein component in serological reactions. However, the results obtained were similar to those for the serum absorbed with RBC-ESS. Osterman and Eisemann (10) have shown that the role of carbohydrate is complex. Periodate oxidation of ESS affected its ability to bind erythrocytes and to react with serum antibody, whereas enzymatic proteolysis did not. We also attempted to demonstrate the presence of various fractions in ESS by two consecutive absorptions, first with erythrocytes and then with latex-*R. rickettsii* reagent (data not shown). We found that the presence of hemoglobin in the supernatant after ESS absorption of the erythrocytes interfered with the latex-*R. rickettsii* reaction; the negative controls were positive. Therefore the investigation could not be completed. Further study is being conducted to determine whether any ESS remains after multiple absorptions with erythrocytes.

In conclusion, our findings suggest that (i) ESS plays a major role in the CF test; (ii) ESS contains more than a single antigenic fraction, with various degrees of affinities for erythrocytes; and (iii) the micro-IF test measures antibodies to at least some antigens separate and distinct from ESS (R. L. Anacker, R. E. Mann, L. A. Thomas, and R. N. Philip, manuscript in preparation).

ACKNOWLEDGMENTS

We thank Walter Decher of the New York State Department of Health for performing the complement fixation test and Edith E. Michaelson for help in preparing the manuscript.

This work was supported in part by the National Institute of Allergy and Infectious Diseases-Biomedical Research Support grant 2S07-RR05649-15.

LITERATURE CITED

1. Anacker, R. L., R. K. Gerloff, L. A. Thomas, R. E. Mann, and W. D. Bickel. 1975. Immunological properties of *Rickettsia rickettsii* purified by zonal centrifugation. *Infect. Immun.* 11:1203-1209.
2. 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.
3. Centers for Disease Control. 1981. Rocky Mountain spotted fever—United States. 1980. *Morbidity and Mortality Weekly Report* 30:318.
4. Chang, S., J. C. Snyder, and E. S. Murray. 1953. A serologically active erythrocyte sensitizing substance from typhus rickettsiae II. Serological properties. *J. Immunol.* 70:215-221.
5. Conrath, T. B. 1972. Handbook of microtiter procedures, p. 259-260. Dynatech Corporation, Cambridge, Mass.
6. Hechemy, K. E., R. L. Anacker, R. N. Philip, K. T. Kleeman, J. N. MacCormack, S. J. Sasowski, and E. E. Michaelson. 1980. Detection of Rocky Mountain spotted

- fever antibodies by a latex-agglutination test. J. Clin. Microbiol. 12:144-150.
7. Hechemy, K. E., S. J. Sasowski, E. E. Michaelson, M. Zdeb, R. L. Anacker, R. N. Philip, K. T. Kleeman, C. Crump, J. Kudlac, J. M. Joseph, J. Patel, H. Dowda, Jr., G. Killgore, D. Young, J. H. Barrick, J. R. Hindman, L. B. Elliott, and R. H. Altieri. 1981. Rocky Mountain spotted fever: first-year evaluation of a latex agglutination test for antibodies to *Rickettsia rickettsii*, p. 159-169. In W. Burgdorfer and R. L. Anacker (ed.), *Rickettsiae and rickettsial diseases*. Academic Press Inc., New York.
 8. Hechemy, K., R. W. Stevens, and H. A. Gaafar. 1974. Detection of *Escherichia coli* antigens by a latex agglutination test. Appl. Microbiol. 28:306-311.
 9. Murphy, J. R., P. Fliset, L. B. Snyder, and C. L. Wiseman, Jr. 1979. Antibody to *Rickettsia mooseri* erythrocyte-sensitizing substance. Infect. Immun. 24:962-964.
 10. Osterman, J. V., and C. S. Elsemann. 1978. Rickettsial indirect hemagglutination test: isolation of erythrocyte-sensitizing substance. J. Clin. Microbiol. 8:189-196.
 11. Philip, R. N., E. A. Casper, J. N. MacCormack, D. J. Sexton, L. A. Thomas, R. L. Anacker, W. Burgdorfer, and S. Vick. 1977. A comparison of serologic methods for diagnosis of Rocky Mountain spotted fever. Am. J. Epidemiol. 105:56-67.
 12. 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.
 13. Tzianabos, T., E. L. Palmer, J. F. Obijeski, and M. L. Martin. 1974. Origin and structure of the group-specific complement-fixing antigen of *Rickettsia rickettsii*. Appl. Microbiol. 28:481-488.
 14. U.S. Department of Health, Education, and Welfare Public Health Service. 1974. Standardized diagnostic complement fixation method and adaptation to micro test. Center for Disease Control, Atlanta, Georgia.