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Detection and Characterization of Mouse Monoclonal Antibodies to Epidemic Typhus Rickettsiae

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A solid-phase immunofluorometric assay was used to detect mouse monoclonal antibodies to epidemic typhus rickettsiae, *Rickettsia prowazekii* (the immunizing antigen), and to murine typhus rickettsiae, *Rickettsia typhi*, a related antigen. Of the 649 hybridoma cultures obtained, 628 contained antibodies either to *R. prowazekii* or to both *R. prowazekii* and *R. typhi*. A total of 72 cultures were cloned by limiting dilution and yielded 137 antibody-producing clones. Of these, 104 produced antibodies specific for *R. prowazekii*, 22 produced antibodies that reacted with *R. prowazekii* and *R. typhi*, and 11 produced antibodies that reacted with *R. prowazekii*, *R. typhi*, and *R. canada*. The immunoglobulin isotypes of the mouse monoclonal antibodies produced were identified by a related indirect immunofluorometric assay technique with fluorescein isothiocyanate-conjugated antisera specific for each isotype. Antibodies were also evaluated by indirect fluorescent antibody tests, and antibodies from selected clones were found to neutralize rickettsial toxic activity in mice.

Because all typhus group rickettsiae possess common antigens, it is difficult to distinguish the three species in this group (*Rickettsia prowazekii*, *Rickettsia typhi*, and *Rickettsia canada*) with conventional polyclonal antibodies. Species-specific antisera would greatly facilitate epidemiological studies of typhus infections in areas where epidemic typhus rickettsiae, *R. prowazekii*, and murine typhus rickettsiae, *R. typhi*, coexist, and strain-specific antisera would be exceptionally useful for comparing *R. prowazekii* isolates from suspected zoonotic reservoirs of epidemic typhus infection (2, 12) with human isolates. Obviously, monospecific antisera to these organisms could also provide useful tools for studying the mechanisms of infection and immunity in both diseases.

Because hybridoma technology is especially suited for producing antibodies with the required specificity, we recently began studies designed to produce and characterize monoclonal antibodies specific for *R. prowazekii*. During these studies, we found it necessary to develop reliable and sensitive techniques for screening culture fluids for rickettsial antibodies. An immunofluorometric assay (IFMA) was developed which was well suited for this purpose. Culture fluids were initially screened for typhus antibodies by the IFMA technique with both epidemic and murine rickettsial antigens. The immunoglobulin isotypes of the monoclonal antibodies were determined by a similar IFMA procedure. The monoclonal antibodies in concentrated culture fluids or ascitic fluids were further characterized by conventional indirect fluorescent antibody (IFA) techniques with a battery of rickettsial antigens, and homologous toxic activity neutralization titers were determined for 21 representative monoclones.

MATERIALS AND METHODS

Antigen preparation. (i) Immunogens. *R. prowazekii* (Breinl) and *R. typhi* (Wilmington) were cultivated in embryonated chicken eggs by established procedures (5). Yolk sac suspensions of the organisms were inactivated with gamma irradiation (total dose 3×10^6 rad from a cobalt 60 source). Rickettsiae were then purified from the yolk sacs by a sequential centrifugation process described previously (14). Briefly, 20% suspensions of infected yolk sacs were mixed with an equal volume of 50% (wt/wt) sucrose solution and centrifuged at 15,000 rpm for 1 h. The pellet was suspended in 0.01 M phosphate-buffered saline (PBS), pH 7.2, and centrifuged through a 30% (wt/wt) sucrose cushion. The pellet was again suspended in PBS, pH 7.2, and the rickettsiae were purified twice by isopycnic banding in a potassium tartrate-glycerol gradient. The resulting purified rickettsial suspension is referred to as the "whole cell" immunogen. In addition to the whole cell suspension, a second immunogen was prepared by extracting these purified organisms with diethyl ether (24), collecting the extracted organisms by high-speed centrifugation, washing the cells with PBS, suspending them in the same buffer, and rebanding the extracted organisms in a potassium tartrate-glycerol gradient as described above.

(ii) Antigens for the IFMA and IFA tests. The whole cell immunogens prepared from suspensions of *R. prowazekii* and *R. typhi* were used as the solid-phase reactants in the IFMA tests as follows. The concentration of purified antigens was adjusted in PBS, pH 7.2, to give an optical density of 0.5 at 660 nm in a 1-cm light path. These antigens for use in IFMA were then stabilized by the addition of 0.1% Formalin and stored frozen in small volumes. The formalinized cells were diluted for use at a ratio of 1/50 in 0.01 M PBS, pH 8.0, that was supplemented with 0.25% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA-NaN_3). The antigens for the IFA test were 10% (wt/vol) suspensions of infected yolk saks in PBS.

Monoclonal antibody production. Young adult female BALB/c mice (two groups of 10 each) were immunized with *R. prowazekii* antigens. The groups were initially immunized by simultaneous footpad and subcutaneous inoculation of either whole cell antigen or ether-treated antigen diluted to an optical density of 0.5 at 660 nm as described above. Total initial dose was 0.5 ml per mouse. After approximately 3 weeks, 3 days before hybridoma formation, both groups of mice were given an intravenous booster inoculation of 0.2 ml of whole cell antigen. Hybridomas were produced by fusion with a nonsecretor mouse myeloma cell line (SP2/0) by the method of Kearney et al. (11). Spleens from two mice primed and boosted with whole cell antigen were pooled, and three spleens from mice primed with the ether-treated antigen and boosted with whole cell antigen were pooled separately, resulting in two groups of cells for hybridoma production. Selected antibody-producing cultures, identified by IFMA (see below), were expanded in cell culture and stored frozen until time permitted cloning. Cells from 72 of these cultures, shown by the IFMA to be producing antibody to *R. prowazekii*, were expanded in cell culture from the frozen state and cloned by limiting dilution. The resulting monoclonal antibody-producing cultures were in turn expanded in cell culture, and the antibodies in these culture fluids were concentrated five- to tenfold by precipitation with 2 M $(NH_4)_2SO_4$. The concentrated antibodies were dialyzed against PBS containing 0.1% NaN_3 and were stored at $-20°C$ until they were assayed. Selected clones were subsequently inoculated intraperitoneally into pristane-primed BALB/c mice for specific antibody production in ascitic fluids. These ascitic fluids were also stored frozen until tested. Culture fluids and ascitic fluids were evaluated by IFA and toxic activity neutralization tests; culture fluids were additionally tested by the IFMA procedure (see below).

Fluorescein-conjugated antisera. Lyophilized goat anti-mouse immunoglobulin A (IgA) and IgM fluorescein isothiocyanate (FITC)-conjugated antisera were purchased from Research Plus Laboratories, Denville, N.J. These conjugates were rehydrated with distilled water, centrifuged at $10{,}000 \times g$ for 15 min, and stored in small volumes at $-20°C$. Mouse subclass-specific antisera (goat anti-mouse IgG1, IgG2a, IgG3, and rabbit anti-mouse IgG2b) were purchased from Litton Bionetics, Kensington, Md., and the IgG fractions of these unconjugated antisera were isolated by fractionation on DEAE-Sephadex. The IgG fraction of goat anti-mouse IgG (Fc specific) was purchased from U.S. Biochemicals, Cleveland, Ohio, and the IgG fraction of rabbit broadly specific anti-mouse immunoglobulins was purchased from Cappel Laboratories, Cochranville, Pa. The IgG fractions of all unconjugated antisera were labeled with FITC by the dialysis method. The molar fluorescein-to-protein ratios for all conjugates produced were between 3 and 4 as determined by the method of Brusman (3).

The immunoglobulin class and subclass specificities and potencies of the conjugated antisera were evaluated by the method of Phillips et al. (18), in which stable antigen standards consisting of purified mouse myeloma immunoglobulins covalently bound to polyaminostyrene beads were used to quantify the homologous and heterologous potencies of the conjugates. The titers of the conjugates were determined with 0.1 ml of 3.75×10^8 IgG1-, IgG2a-, IgG2b-, IgG3-, IgM-, and IgA-coated beads per ml, representing both kappa and lambda light chains. The working dilution for each conjugate was chosen as that dilution which gave a fluorescent intensity of one-half the plateau fluorescence brightness for homologous staining obtained when beads were saturated with both antigen and conjugated antibody. Background fluorescence was determined by using solid-phase beads coated with pooled normal IgG of the same animal species as the respective conjugates. The conjugates described by the manufacturer as subclass specific sometimes exhibited substantial reactivity to other immunoglobulin subclasses. These unwanted specificities were largely removed by a single absorption with mouse myeloma immunoglobulins (20 to 30 μg of the indicated IgG per ml of conjugate) without substantially influencing the homologous specificities.

IFMA of culture fluids for rickettsial antibodies. Each culture fluid was analyzed by mixing either 0.05 or 0.1 ml of sample with 0.1 ml of whole cell antigen suspensions in disposable, round-bottom, borosilicate test tubes. The mixture of whole cell antigens and culture fluids was incubated for 1 h in a 37°C water bath. The following wash procedure was used: Unbound mouse immunoglobulin was removed by suspending the cells in 2.0 ml of PBS-BSA-NaN_3 and then centrifuging the mixture at $1{,}950 \times g$ for 15 min at 20°C. The supernatant fluids were decanted. This wash procedure was then repeated. After the second wash, 0.05 ml of prediluted conjugate was added to the cells, and the suspension was mixed on a vortex apparatus and incubated again for 1 h at 37°C. The cells then were washed as described above, except that the buffer was 0.01 M PBS, pH 8.0, containing 0.15 mg of Tween 20 (PBS-Tween 20) per ml, and the reaction mixture was washed three times. The final pellet was suspended in 1.0 ml of PBS-Tween 20 to measure the fluorometric response. (It was noted that after three washes the fluorescence intensity for a given sample remained constant with additional washes, indicating that all the unbound conjugate had been removed and that there was no additional loss of bound antibody or organisms with further washing.) Background fluorescence was determined by testing fresh tissue culture media. Initially the positive control sample consisted of a dilution of guinea pig antiserum to *R. prowazekii* that demonstrated cross-reactivity with *R. typhi* and was recognized by the anti-mouse broadly specific immunoglobulin conjugate. Later, positive control samples were prepared by diluting with PBS–BSA–NaN_3-specific immune ascitic fluids from mice that had antibody-producing hybridoma

tumors. Small volumes of this diluted material were frozen, so a fresh set could be used for each assay.

A Gilson Spectra/Glow Filter Fluorometer (Gilson Medical Electronics, Middleton, Wis.) equipped with a quartz-halogen lamp and a 100-mV digital display unit was used for all fluorometric measurements. For excitation, two interference filters were used in tandem: each filter transmitted at least 50% of the incident blue-green light at 492 nm and less than 1% of the incident light below 484 nm or above 496 nm. A 520-nm barrier filter (Corning no. 3-69; Corning Glass Works, Corning, N.Y.) was used to isolate the fluorescent light. Fluorometer stability and fluorescence output were monitored with standard solutions of fluorescein diacetate in 0.1 M NaOH.

IFA of monoclonal antibodies. IFA was performed as described by Newhouse et al. (13). Antigens were as follows: five strains of *R. prowazekii* (Breinl, Madrid E, the erythromycin-resistant Madrid E strain), and two isolates from flying squirrels (F-16 and GvV-250), all of which have been described previously (25); *R. typhi* (Wilmington); *R. canada* (ATCC 2678); and *R. rickettsii* (Sheila Smith). Mouse monoclonal antibodies were detected with FITC-labeled rabbit broadly specific anti-mouse immunoglobulin.

Toxic activity neutralization. Rickettsial toxic activity neutralization tests were performed with a yolk sac suspension of *R. prowazekii* (Breinl) according to the general procedure described by Henderson and Topping (9). Serial twofold dilutions of the respective monoclonal antibodies were prepared in PBS, pH 7.2, beginning with a 1/16 dilution; these were mixed with an equal volume of an infected yolk sac suspension that had been prediluted (1/12) in Snyder I diluent (10) to a concentration containing four mouse mean lethal doses per 0.25 ml. The reaction mixtures were incubated at room temperature for 30 min. Groups of four young adult male Swiss mice (approximately 25 g each) were then inoculated intravenously with 0.25 ml of the antibody-rickettsial mixtures, and deaths were recorded the following day. Toxic activity neutralization titers were then calculated by the Reed-Muench formula (19) with the endpoint expressed as the reciprocal of the highest dilution of antibody which protected 50% of the animals against toxic death. Control mice were inoculated with infected yolk sac suspension that had been incubated with PBS while the neutralization incubations were being performed. All mice in such control groups routinely died after inoculation.

RESULTS

A total of 649 fusion cultures were initially obtained from the two hybridizations. Of this total, 628 cultures were shown by IFMA tests to contain antibodies either to *R. prowazekii* or to both *R. prowazekii* and *R. typhi*; 195 of these cultures were from mice primed and boosted exclusively with whole cell antigen, whereas 433 were from mice primed with ether-extracted cells but boosted with whole cell antigen. Figure 1 shows the relative fluorescence intensities of each fusion culture fluid for *R. prowazekii* and *R. typhi*. Figure 1A illustrates the response of hybridoma cultures produced when whole cell antigen was the sole immunogen, and Fig. 1B shows the response of cultures derived from animals that were primed with ether-extracted antigen. Analysis of accumulated data from background fluorescence showed that a fluorescence of twice the mean background (<0.01), routinely used as our discrimination point to distinguish positive from negative reactions, represented at least six standard deviation units of the population of background fluorescence values.

A total of 72 cultures that preferentially demonstrated antibody to *R. prowazekii* by IFMA were expanded and cloned by limiting dilution to give 155 monoclones. A summary of the specificity of the antibody in the concentrated culture fluids from these clones for the two antigens is shown in Table 1. Eighteen clones lost their ability to produce antibody when they were expanded in cell culture. Of the 137 remaining clones, 104 cultures produced antibodies specific for *R. prowazekii*, 12 cultures produced antibodies that reacted with both *R. prowazekii* and *R. typhi* with equal fluorescence intensity, and 21 cultures produced antibodies that reacted with both antigens, but the reactivity to *R. prowazekii* was much greater than the reactivity to *R. typhi*. All of the 137 monoclonal antibodies in the culture fluids were found to be IgG and represented the four known subclasses of mouse IgG: IgG2b predominated (51 of 137 clones), although there were also ample clones of IgG1 (33 clones), IgG3 (29 clones), and IgG2a (17 clones). Seven of the monoclonal cultures apparently produced antibodies of more than one isotype; these clones are under further study.

Concentrated tissue culture fluids (142) were also tested for antibody specificity by IFA. There was complete agreement betweeen the two assays, with two exceptions where the supernatant fluids were negative for antibody activity by IFMA and were positive by IFA (data not shown).

IFA and homologous toxic activity neutralization titers of selected culture fluid concentrates or ascites fluids are shown in Table 2. All subclasses of mouse IgG were represented with no obvious relation between immunoglobulin subclass and serological or toxic activity neutralization specificity. Note, however, that most of the antibodies which neutralized toxic activity were species specific (all except clone 44-87-9). It may also be significant that antibodies from one clone (43-58D) did not neutralize *R. prowazekii* toxic activity, even though it gave a very high titer in IFA tests (≥8,192), which apparently was species specific.

Antibodies which were presumed to be group specific were also tested for their ability to

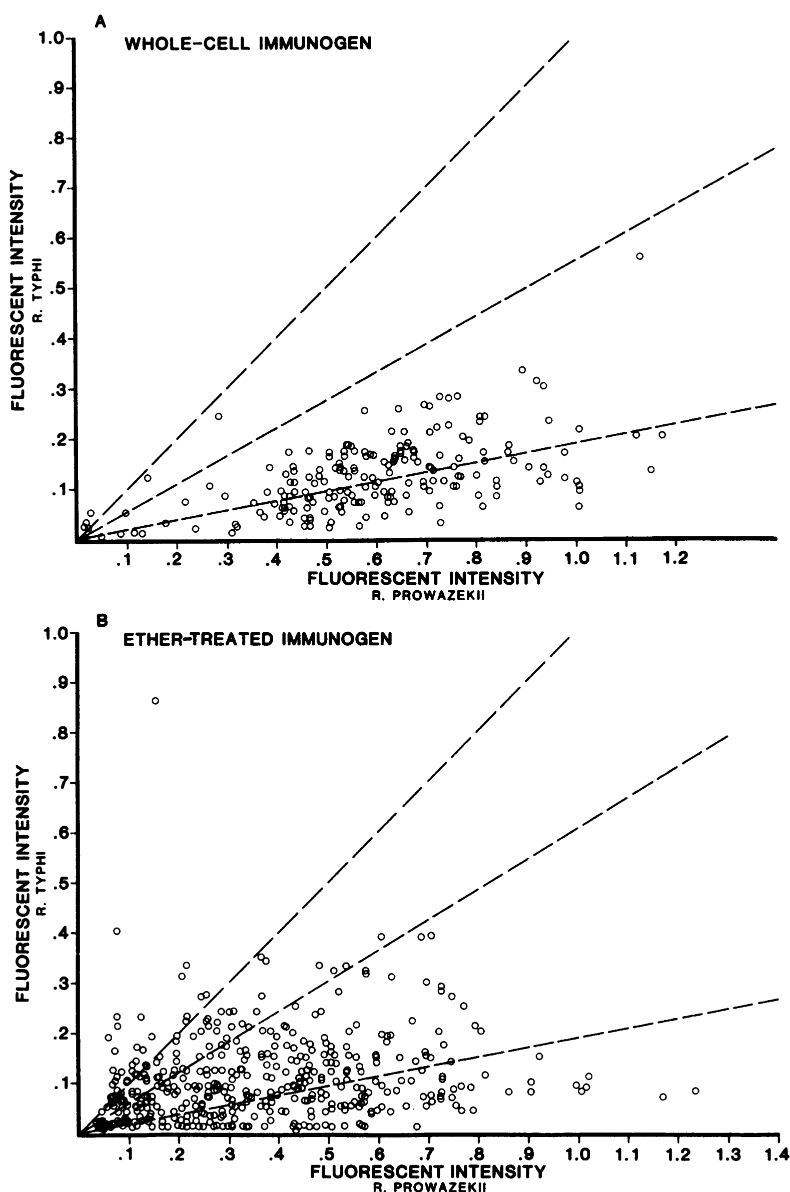

FIG. 1. Relative fluorescent intensity of antibodies to *R. prowazekii* and *R. typhi* by IFMA tests of hybridoma culture fluids. Broken lines were drawn in arbitrary locations to facilitate comparison of the overall patterns of reactivity.

neutralize toxic activity of murine typhus rickettsiae. Generally speaking, endpoints with these determinations were not as sharp as the epidemic typhus titrations. Culture fluid from clone 44-87-9 had a murine typhus neutralization titer of approximately 1:128 which was roughly equivalent to the epidemic typhus neutralization titer of 1:171. The remaining group-specific antibodies all had low neutralization titers to murine typhus rickettsiae (≤1:32) and epidemic typhus rickettsiae (<1:16). Because none of the antibodies provisionally classified as species specific reacted with murine typhus rickettsiae in IFA, IFMA and, in some instances, complement fixation (CF) tests (see below), we did not test them for their ability to neutralize the toxic activity of murine typhus rickettsiae.

We also screened selected culture fluids and ascitic fluids for rickettsial antibodies by the CF technique. Thirty specimens which were positive to typhus antigens both by the IFA and IFMA procedures were tested against *R. prowa-*

TABLE 1. Reactivity of monoclonal antibodies in concentrated culture fluids to typhus rickettsiae by IFMA

No. of clones	Antigen for the following species:		Interpretation of results
	R. prowazekii	*R. typhi*	
18	−	−	Negative
104	+	−	Species specific
12	+	+	Group specific
21	+[a]	+	Differential

[a] Reactivity to *R. prowazekii* was much greater than the reactivity to *R. typhi*.

zekii, *R. typhi*, *R. canada*, *R. rickettsii*, and *Coxiella burnetii* antigens in the standard micro-CF procedure. Only 9 of 30 specimens were positive to typhus rickettsiae (titer, ≥1:8) by the CF test. Most of the remaining specimens were considered negative (no reaction at the starting dilution of 1:8), although a few ascitic fluids were anticomplementary (data not shown). It should be noted, however, that the specificity of the positive CF reactions was identical to that observed with the other procedures, i.e., fluids which were species specific by IFA and IFMA were also species specific with the CF technique. Similarly, specimens which were considered group specific were invariably group specific by all three tests. None of the 30 specimens reacted with the spotted fever group (*R. rickettsii*) or the Q fever group (*C. burnetii*) antigens. The relative insensitivity of the CF test in our hands, plus the known inability of mouse IgG1 to fix complement by the classical pathway, militated against additional CF testing.

DISCUSSION

Monoclonal antibodies are invaluable tools for the study of infectious agents. Not only do they have tremendous potential practical value as diagnostic reagents, but they also can be exceptionally useful probes for analyzing the topographical relationship of various antigens or for studying the mechanisms of infection and immunity in a given disease. Obviously, one of the principal requirements for the production of monoclonal antibodies is a sensitive and specific serological technique for detecting the low levels of immunoglobulin which usually are present in the initial culture fluids after hybridoma production. As there are few, if any, published data comparing the relative sensitivities of various techniques for detecting monoclonal antibodies with the rickettsiae, one of the principal objectives of our monoclonal antibody studies was to determine if IFMA is a suitable procedure for screening candidate clones. IFMAs have become increasingly important as a means of precisely measuring ligand binding (4, 7, 22, 23) and have been used by many investigators to screen antibodies to various antigens. The main reason for this is that such tests are sensitive enough to detect the low concentrations of nonprecipitating antibody that may be secreted by hybridoma cells in culture. We have developed and reported the use of several IFMAs to measure serum proteins (20, 21) to quantitate the specificity and potency of FITC-conjugated antibody reagents (18), to detect hybridoma antibody to influenza virus (17), to detect influenza virus (16), and to measure the immunoglobulin-class response to viral (8) and bacterial (1) infections. The results of this study indicate that the IFMA is also an efficient procedure for detecting and quantifying rickettsial antibodies in hybridoma culture fluids. The ability of the IFMA to precisely measure the relative reactivity of monoclonal antibodies with different antigens allows selection of clones of desired specificity and potency. Hundreds of samples can be tested in 1 day. We appreciated this advantage when we obtained 649 cultures in our initial hybridization attempt, and each culture needed to be tested against both the immunizing antigen (*R. prowazekii*) and a heterologous (*R. typhi*) antigen. Presumably, the large number of cultures that we obtained reflects the intrinsic immunogenicity of the rickettsiae. It may be significant that mice primed with ether-treated immunogen and boosted with whole cell immunogen elicited more antibodies that reacted with equal or greater fluorescent intensity to *R. typhi* than did mice primed and boosted with the whole cell immunogen. Because these results emanated from a single experiment, however, generalization is perhaps unwarranted. It also seems noteworthy that both immunization schedules elicited the production of all four isotypes of mouse IgG. This is noteworthy because published reports suggest that mouse IgG3 is produced in response to carbohydrate antigens (15).

We provisionally designated certain antibodies as group specific or species specific on the basis of their reactivity in the IFA and IFMA tests. Limited CF testing supported these tentative designations. Of special interest, however, were the specificity patterns of the neutralization tests. When neutralization tests are performed with conventional polyclonal antisera, homologous neutralization titers are much higher than the corresponding heterologous titers, thereby prompting the generalization that neutralizing antibodies are species specific. The results of our neutralization tests with monoclonal antibodies suggest that this generalization may not be universally true. Although most of the monoclonal antibodies that neutralized the toxic activity of epidemic typhus rickettsiae

TABLE 2. IFA and homologous toxic activity neutralization titers of representative monoclonal antibodies

Clone no.	Immunoglobulin isotype	Source[b]	IFA titer to[a]:								Neutralization titer for *R. prowazekii* Breinl	Presumed specificity[d]
			R. prowazekii strains:					*R. canada*	*R. typhi*	*R. rickettsiae*		
			Breinl	Madrid E	E[Rc]	F16	GvV-250					
43-15-14	IgG1	CF	128	256	256	64	256	<16	128	<16	<16	GR
43-82-14		CF	128	256	≥512	64	≥512	<16	64	<16	<16	GR
43-149-5		AF	2,048	≥512	≥512	≥512	≥512	<16	<16	<16	192	S
43-152-2		CF	64	256	256	64	256	<16	<16	<16	NT[e]	S
44-237-7		CF	≥512	≥512	256	256	256	<16	<16	<16	<16	S
43-58-D	IgG2a	AF	≥8,192	≥8,192	≥8,192	≥8,192	≥8,192	<16	<16	<16	<16	S
43-88-10		CF	32	64	64	32	128	<16	<16	<16	256	S
43-133-4		CF	256	≥512	≥512	−128	≥512	<16	<16	<16	≥128	S
43-133-24		CF	128	256	256	128	256	64	128	<16	<16	GR
44-87-9		CF	≥512	≥512	≥512	≥512	≥512	<16	256	<16	171	GR
43-82-3	IgG2b	CF	128	512	256	128	128	<16	<16	<16	128	S
43-82-23		CF	64	256	128	64	64	<16	64	<16	<16	GR
43-149-2		CF	≥512	≥512	≥512	256	≥512	<16	<16	<16	512	S
44-82-C		AF	1,024	1,024	1,024	1,024	1,024	<16	<16	<16	106	S
44-103-2		CF	≥512	≥512	≥512	≥512	≥512	<16	<16	<16	<32	S
44-252-17		CF	≥512	≥512	≥512	≥512	≥512	<16	<16	<16	96	S
44-279-A		AF	8,192	8,192	8,192	8,192	16,384	<16	<16	<16	4,096	S
43-88-6	IgG3	CF	32	128	128	32	128	<16	<16	<16	96	S
43-133-6		AF	128	≥512	≥512	128	≥512	128	128	<16	<16	GR
43-139-5		CF	64	128	64	64	64	<16	64	<16	<16	GR
44-25-4		CF	256	≥512	≥512	256	≥512	<16	<16	<16	<16	S
44-237-1		CF	256	≥512	≥512	≥512	≥512	<16	<16	<16	192	S

[a] See text for description of species and strains.
[b] CF, Culture fluid; AF, ascitic fluid.
[c] E^R, Erythromycin-resistant Madrid E strain.
[d] GR, Group specific; S, species specific.
[e] NT, Not tested.

appeared to be species specific, one antibody preparation (44-87-9) was clearly group specific. Perhaps more importantly, antibodies produced by clone 43-58D reacted at a higher titer in IFA and IFMA tests specifically with *R. prowazekii* but failed to neutralize the toxic activity of epidemic typhus rickettsiae. There could be several possible explanations for these findings; possibly there is more than one neutralization site, or possibly the topographical relationships of the various antigenic epitopes to functional areas of the rickettsiae affect neutralization. Immunoglobulins of all the IgG isotypes neutralized toxic activity, and the high IFMA and IFA potency of the nonneutralizing antibodies suggests that these are as avid as the neutralizing immunoglobulins. We are currently pursuing these interesting observations.

That we were able to produce potent antisera that apparently were specific for *R. prowazekii* is of potential practical significance. These antibodies could facilitate the identification of rickettsial isolates in areas where both epidemic typhus and murine typhus occur. The third species in the typhus group (*R. canada*) is presumed not to be of medical importance. Obviously, additional testing needs to be carried out with many different strains of these species to confirm these results. It is noteworthy that some of the monoclonal antibodies that were considered group specific, i.e., reacted with both *R. prowazekii* and *R. typhi*, also reacted with *R. canada*, whereas others did not. The evolutionary, taxonomic, and clinical significance of these observations is not apparent. We were disappointed that none of these monoclonal antibodies could distinguish among the various strains of *R. prowazekii*, but this was not particularly surprising in view of the known homogeneity of the surface proteins of reference strains (6). Regardless, the interesting array of monoclonal antibodies that were produced should be of value in the study of rickettsial diseases.

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