INFECTION AND IMMUNITY, Nov. 1984, p. 289–294
0019-9567/84/110289-06$02.00/0

Vol. 46, No. 2

Production and Characterization of Monoclonal Antibodies to *Rickettsia rickettsii*

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Received 21 March 1984/Accepted 19 July 1984

Five mouse ascitic fluids (MAFs) containing monoclonal antibody to *Rickettsia rickettsii* were produced from three original fusions by murine hybridoma technology. The five MAFs were fractionated and purified; each contained monoclonal antibody of the immunoglobulin G2a subclass. Each monoclonal antibody-containing MAF was titrated by indirect immunofluorescence against three *R. rickettsii* isolates from humans and four other spotted fever group rickettsiae. Each MAF was also titrated in the complement fixation, latex agglutination, microagglutination, and indirect hemagglutination tests. Two of the MAFs were examined for their ability to prevent fever and rickettsemia in susceptible guinea pigs after a 1:100 dilution of each was mixed with viable *R. rickettsii*, and all five MAFs were titrated in the mouse toxicity phenomenon assay. All MAFs had high indirect immunofluorescence titers to the three strains of *R. rickettsii* (1:200,000 to 1:800,000), reduced indirect immunofluorescence titers to *R. montana*, and were nonreactive with *R. akari*, *R. sibirica*, and *R. conorii*. Each MAF was able to fix complement in the presence of spotted fever group antigen reagent and agglutinate a suspension of purified *R. rickettsii*, and each was negative in both the latex agglutination and the indirect hemagglutination tests. The two MAFs which were tested proved to be capable of preventing rickettsemia and death in guinea pigs, and each MAF was able to prevent death in mice at dilutions ranging from 1:40 to 1:80.

Rickettsia rickettsii, the etiological agent of Rocky Mountain spotted fever, continues to be a major cause of human morbidity and mortality in the continental United States (5). A detailed knowledge of the biochemical structure of *R. rickettsii*, its antigenic constituents, and whether there is strain variation at the structural subunit level, are questions which remain unresolved.

Murine hybridoma technology has provided a powerful tool for obtaining useful information on the immunochemical makeup of complex organisms. Monoclonal antibodies have been used to identify surface proteins on *Pseudomonas aeruginosa* (18), to differentiate strains of *Bacteroides intermedius* (12), to identify and classify microorganisms (6), and to purify a relevant antigen from *Babesia bovis* (25).

To our knowledge, monoclonal antibodies to spotted fever group (SFG) rickettsiae have not been described. To investigate the relationship among strains of *R. rickettsii* and between *R. rickettsii* and other SFG organisms, we have produced monoclonal antibodies against *R. rickettsii*. This paper describes how these antibodies were produced and how they were characterized by various serological and biological methods.

MATERIALS AND METHODS

Rickettsiae. The Sheila Smith (SS) strain of *R. rickettsii* was passed an undetermined number of times in guinea pigs and embryonated hen eggs and three times in L-929 mouse fibroblasts before its use as a live, whole immunogen. Clinical isolates were obtained in L-929 mouse and RK-13 rabbit kidney cells as previously described (14, 17). Rickettsiae were propagated in various cell lines for preparing stock pools and fluorescent antigen (Table 1).

Fluorescent antigen. A portion of each rickettsia-host suspension was centrifuged at 5,000 × *g* for 30 min. The pellet was suspended in 5 ml of phosphate-buffered saline (0.1 M Na_2HPO_4 [anhydrous], 0.1 M NaH_2PO_4, 0.15 M NaCl, [pH 7.2 to 7.4]). The suspension was applied to 2 wells of a 12-well Teflon-coated slide and examined under a standard light microscope. The suspension was diluted with phosphate-buffered saline until 1 drop contained well dispersed cells. The diluted suspension was then dispensed onto 12-well slides, allowed to air dry, acetone fixed for 10 min at room temperature, checked for the presence of SFG rickettsiae by a direct immunofluorescent system, and stored at −70°C.

Quantitation of rickettsiae. Rickettsiae were quantitated in cell culture as previously described (14).

Culture cells. A passage of Sp2/0-Ag14 (23) plasmacytoma cells was obtained from Howard M. Reisner, Department of Pathology, University of North Carolina–Chapel Hill School of Medicine, and cultured in antibiotic-free RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% heat-inactivated (at 56°C for 60 min) fetal bovine serum (Sterile Systems, Inc., Logan, Utah) and containing 8-azaguanine (Sigma Chemical Co., St. Louis, Mo.). Selective medium consisted of RPMI 1640 medium with 15% fetal bovine serum which contained hypoxanthine and aminopterin as described by Oi and Herzenberg (19).

Immunization of mice. Six- to eight-week-old BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were injected intravenously with a 2×10^6 50% tissue-culture-infective dose of *R. rickettsii* SS from an aliquoted pool in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). Three weeks later, mice received a 3.1×10^6 50% tissue-culture-infective dose of *R. rickettsii* SS from the original pool.

Fusion and cloning. Spleen cells from two mice were pooled and fused with SP2/0-Ag14 myeloma cells (1:1) by using polyethylene glycol 1500 (Sigma) as the fusing agent

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(19). After incubation for 18 h at 36°C in an atmosphere of 4% CO_2 and 65 to 75% relative humidity, 0.1 ml of selective medium was added to each well. Selective medium was changed at 72-h intervals thereafter. The supernatants of wells with viable growth were screened for immunoglobulin M (IgM) and IgG antibodies to *R. rickettsii* by the indirect fluorescent antibody (IFA) assay (8, 21). Cells from antibody-positive wells were expanded, cryopreserved, cloned, and recloned by limiting dilution (19) in low-serum medium with 4% fetal bovine serum (Hybridoma Sciences, Inc., Atlanta, Ga.).

Ascites production. BALB/c mice were injected intraperitoneally with 500 mg of Pristane (0.5 ml; Aldrich Chemical Co., Inc., Milwaukee, Wisc.) 4 to 6 weeks before intraperitoneal injection of 3 to 5 × 10^6 cloned cells in low-serum medium without fetal bovine serum. Mouse ascites fluid (MAF) was collected from days 10 through 30 after cell injection and defibrinated with 2 M glacial acetic acid (11), and the globulin fraction was precipitated with $(NH_4)_2SO_4$ to 50% saturation.

Isotype determination. Each MAF fraction was purified on a protein-A–agarose (E-Y Laboratories, San Mateo, Calif.) column (22). The column was equilibrated with 0.1 M Na_2HPO_4 adjusted to pH 8.2 with 0.1 M citrate (starting buffer). The sample was dialyzed into starting buffer, applied to the column, and 2- 3-ml fractions were collected. The column was eluted with starting buffer until the first peak appeared, and then a pH gradient was run with 30 ml each of starting buffer and citrate buffer in a gradient mixer. After the gradient was completed, the pH of each fraction was determined and then adjusted to 7.0 to 7.5 with 1 M Tris-hydrochloride, pH 8.5. Each fraction was tested for antibody to *R. rickettsii* in the IFA assay, and each was examined in an Ouchterlony gel against rabbit antiserum (Miles Laboratories, Inc., Elkhart, Ind.) specific for each of the respective mouse IgG subclasses.

Serological characterizations. Each MAF sample was titrated in the IFA assay and in the complement fixation (CF) (4), microagglutination (MA) (10), latex agglutination (LA) (13), and indirect hemagglutination (IHA) (20) tests.

Biological characterization. Randomly bred male Hartley strain guinea pigs 8 to 12 weeks old and ICR/Swiss Webster white mice were obtained from the Animal Resources Branch, Centers for Disease Control, Atlanta, Ga. Two monoclonal antibody-containing MAF samples were tested for their ability to protect guinea pigs from fever and rickettsemia (1). Portions of the eighth L-929 passage of *R. rickettsii* SS were mixed with immune MAF, control MAF, or buffer, and incubated at room temperature for 1 h; 1.0 ml per animal was injected intraperitoneally into groups of six guinea pigs for each mixture (see below). Each MAF was diluted 1:100 in buffer, and this 1:100 dilution was used to react with the rickettsiae. The tissue culture infectivity of each mixture was determined after the 1-h incubation. Rectal temperatures were recorded daily, and animals were bled by cardiac puncture on day 1 after injection and every other day thereafter through day 18. Serum was separated by centrifugation (800 × *g*), and portions were stored in liquid nitrogen vapor until titration in L-929 cells for rickettsiae and by the IFA assay for antibody.

Dilutions of each MAF fractionate in BHI broth, control MAF, and BHI were mixed 1:1 with a pool of *R. rickettsii* SS. the mixtures were incubated for 1 h at room temperature and then injected intravenously in 0.3-ml volumes into mice (3). Mice were observed for the occurrence of toxic death for 18 h after injection (3).

TABLE 1. Cell lines used for rickettsial propagation

Agent	Host cell line[a]
R. rickettsii SS	L-929
R. rickettsii LNC	L-929 RK-13
R. rickettsii DDH	L-929
R. akari Kaplan	CE
R. sibirica ATCC-VR-151	CE E-6
R. conorii Str 7	L-929 E-6
R. montana ATCC-VR-611	L-929 E-6

[a] L-929, mouse fibroblasts; RK-13, rabbit kidney; CE, primary chicken embryo; E-6, African green monkey kidney (clone E-6).

TABLE 2. Reciprocal IFA titers of monoclones to SFG rickettsiae

Antigen	Titer of MAF fractionate:				
	E11-1G8	E11-2F1	E12-2H11	E11-1G2	E11-1F12
R. rickettsii SS	600,000	400,000	200,000	400,000	800,000
R. rickettsii LNC	200,000	200,000	100,000	200,000	100,000
R. rickettsii DDH	600,000	400,000	100,000	200,000	600,000
R. montana ATCC VR611	320	320	40	640	640
R. akari Kaplan	<8	<8	<8	<8	<8
R. sibirica ATCC VR151	<8	<8	<8	<8	<8
R. conorii Str 7	<8	<8	<8	<8	<8

RESULTS

After 5 weeks in selective medium, 17 of the 576 original cultures contained IgG antibodies to *R. rickettsii* only. Nine of the expanded hybrids continued to produce antibody. Three of these were cloned, and six cultures from the first cloning were recloned. Six of the subclones were used for injection into mice after expansion and cryopreservation. Five of the six groups of mice produced ascites, with the pooled MAF samples of each group having an IFA titer to *R. rickettsii* SS of >1:16,384.

The 50% ammonium sulfate fractionate of each MAF pool was purified on a protein-A agarose column under a pH gradient. Fractions which fell under the second peak were the only fractions which contained antibody to *R. rickettsii*. In the Ouchterlony gels, the fractions under the second peak formed a line of identity with the anti-IgG2a antiserum only. The unpurified MAF fractionate formed precipitin lines in the gels with anti-IgG1 and IgG3.

IFA titers of the MAF fractionates to *R. rickettsii* SS ranged from 1:200,000 to 1:800,000 (Table 2). The titers against two other strains of *R. rickettsii* (LNC and DDH) (14, 17) were within a threefold range of the homologous strain (SS) titer, with one exception. The titer of MAF E11-1F12 versus the *R. rickettsii* LNC human isolate was consistently

TABLE 3. Reciprocal titers of MAF globulin fractions to *R. rickettsii* in CF, MA, LA, and IHA tests[a]

Test	Titer of MAF fractionate				
	E11-1G8	E11-2F1	E12-3H11	E11-1G2	E11-1F12
CF	256	256	64	256	128
MA	64	64	16	64	64
LA	<8	<8	<8	<8	<8
IHA	<16	<16	<16	<16	<16

[a] Controls titers: CF (guinea pig), 128 (immune) <8 (normal); MA (guinea pig), 32 (immune); <4 (normal); LA (human), 64 (immune), <8 (normal); IHA (human), 128 (immune), <16 (normal).

one-eighth that of its titer with SS. Table 2 also presents titration data on each MAF fractionate against other SFG rickettsiae in the IFA assay. No MAF sample was reactive with *Rickettsia akari*, *R. sibirica*, or *R. conorii*. Each MAF sample did react with *R. montana*, although at significantly lower titers (1:40 to 1:640) than those obtained with *R. rickettsii*.

None of the rickettsiae could be differentiated on a qualitative basis in the IFA assay. The staining pattern was a completely circumferential rim fluorescence for each positive rickettsia. Quantitatively, *R. rickettsii* could be easily differentiated from any of the other SFG rickettsiae.

The data from titrations of each monoclone in the CF, MA, LA, and IHA tests are shown in Table 3. Each monoclone was able to fix complement in the presence of SFG antigen in the CF test. Titers ranged from 1:64 to 1:256. The MA results indicate that each monoclone will agglutinate a purified suspension of *R. rickettsii* at relatively low titers (1:16 to 1:64). None of the monoclones was reactive in either the LA or the IHA tests. Both of these tests rely on reactivity with the so-called erythrocyte-sensitizing substances.

The two monoclones which were tested proved to be capable of preventing rickettsemia and death, and they also prevented fever in five of six guinea pigs which received mixtures of viable *R. rickettsii* and each of the respective monoclones (Figs. 1 and 2).

After an incubation period of 1 h at room temperature, each of the mixtures contained tissue-culture-infective rickettsiae. The mixtures of Snyder I buffer, control (Lassa) MAF, MAF E11-1G8, and MAF E11-2F1 with rickettsiae contained $10^{5.4}$, $10^{4.5}$, $10^{3.0}$, and $10^{2.5}$ 50% tissue-culture-infective dose per ml of tissue-culture-infective rickettsiae, respectively.

All of the animals which received buffer or control MAF-rickettsiae mixtures became febrile (>39.7°C) (Fig. 1). Fever in the buffer-rickettsiae group began on day 5 and persisted through day 10, with deaths occurring on days 5, 6, 9, 10, and 12. A febrile reaction began on day 4 and lasted through day 9 in the control MAF-rickettsiae group, with deaths on days 9, 10, and 12. All animals in these two groups were anorectic on day 6 and had bilateral hemorrhagic necrosis of the scrotum on day 8. By day 13 after challenge, only one animal remained alive in each of these groups.

One animal in each of the E11-1G8 and E11-2F1 groups became febrile (Fig. 1). The animal in the E11-1G8-rickettsiae group had a fever of 40.0°C on day 9, 40.2°C on day 10, and was afebrile thereafter. The E11-2F1-rickettsiae group febrile reactor reached 39.7°C on day 8 and defervesced by day 9. Controls injected with each respective MAF or buffer were afebrile throughout the experiment.

Levels of rickettsemia in each group are presented in Fig. 2. Rickettsiae were isolated from the blood of every animal in both the buffer and control MAF-rickettsiae groups. Low levels of rickettsemia ($10^{0.7}$ to $10^{1.2}$) were present on day 5 after infection. Rickettsemia peaked on day 7 in the buffer-rickettsiae group and on day 9 in the control MAF-rickettsiae group. Rickettsemia dropped to undetectable levels in the survivor of each group by day 14. No animal in either group which received immune MAF-rickettsiae had detectable rickettsemia through day 13.

The mean reciprocal IFA titers of each group of animals are shown in Fig. 3. Antibody was detectable as early as day 7 and peaked on day 24. All animals that received rickettsiae in any of the mixtures responded immunologically.

The mouse toxicity phenomenon (3) neutralization results are presented in Table 4. Each MAF was able to prevent death in all mice at dilutions which varied from 1:40 (E11-1H11) to 1:80 (E11-1F12). Clear-cut endpoints were obtained with two MAFs, whereas the other three partially protected against the MTP at dilutions greater than their optima. In the control groups, all BHI-rickettsiae recipients, and 10 of 14 control MAF-rickettsiae recipients died.

DISCUSSION

We have shown that monoclonal antibodies to a complex organism such as *R. rickettsii* can be produced by murine hybrid cell technology. A total of six monoclones were obtained after fusion and two clonings. Each monoclone was of the IgG class and IgG2a subclass. The 50% $(NH_4)_2SO_4$ fractionates of MAF of five of the monoclones had very high IFA titers to the homologous (SS) strain of *R. rickettsii* (Table 2). IFA titers of each MAF fractionate to two other strains of *R. rickettsii* (LNC and DDH) were within a two- to threefold range of the homologous titer with one exception. MAF E11-1F12 had a titer to the LNC strain which was one-eighth that of the homologous strain titer and one-sixth that obtained with the DDH strain. Whether the LNC strain possesses relatively less of a particular epitope which MAF E11-1F12 recognizes might be determined by comparing the

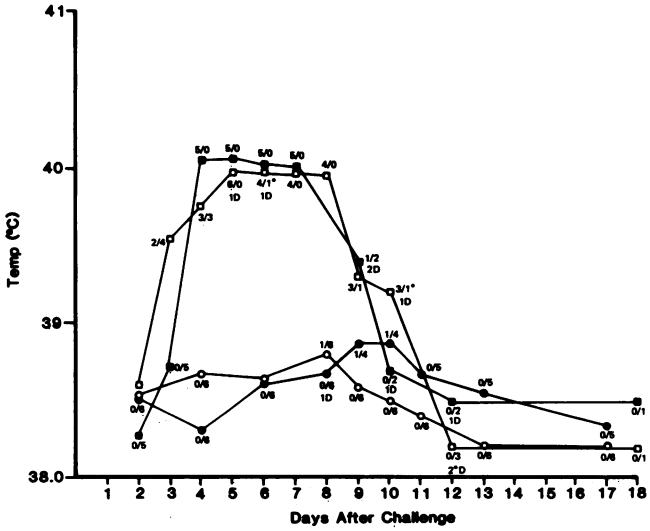

FIG. 1. Mean temperatures of guinea pigs inoculated with mixtures of *R. rickettsii* and monoclone E11-1G8 (●), monoclone E11-2F1 (○), Lassa MAF 52.93.4 (■), and buffer (□). No. D, Number dead; no./no., febrile/afebrile; *, hypothermic (<37.0°C).

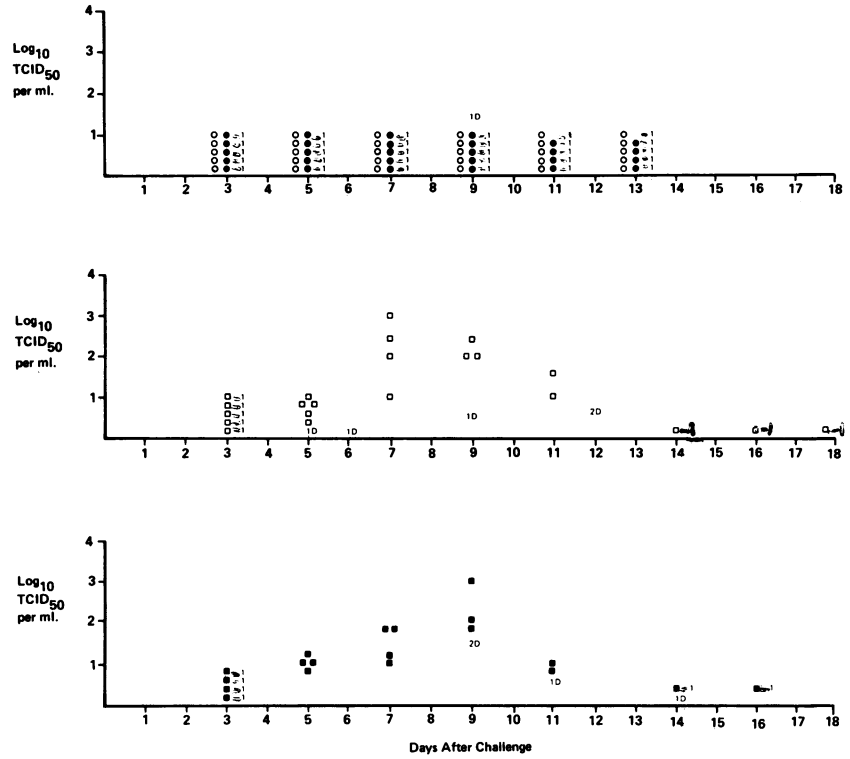

FIG. 2. Rickettsemia in guinea pigs inoculated with mixtures of *R. rickettsii* and monoclone E11-1G8 (●), monoclone E11-2F1 (○), Lassa MAF 52.93.4 (■), and buffer (□). −1, <1; no. D, number dead.

protein profile of the LNC isolate with those of the other strains of *R. rickettsii*. On a qualitative basis, all three strains of *R. rickettsii* were identical. Quantitatively, four of the five monoclones could not differentiate any of the strains of *R. rickettsii*. In a study which used polyclonal mouse antisera to compare human isolates of *R. rickettsii* from patients in North Carolina and Montana, Davis et al. (7) found no differences among any of the 12 strains which were examined. This limited serological evidence does not support the concept that strain variation might account for the spectrum of disease severity which is seen in Rocky Mountain spotted fever.

None of the monoclones was cross-reactive with three other species of SFG rickettsiae which cause human disease. *R. akari*, *R. sibirica*, and *R. conorii* did not react with any of the monoclones in the IFA assay, although each was reactive with a group-specific polyclonal antiserum. Thus, any of these monoclones can be used to distinguish *R. rickettsii* from any of the above rickettsiae.

All monoclones did react with *R. montana*, although at significantly lower titers than with any strain of *R. rickettsii*. These data indicate that *R. rickettsii* and *R. montana* share an epitope that is recognized by these monoclonal antibodies and that *R. akari*, *R. sibirica*, and *R. conorii* might not share

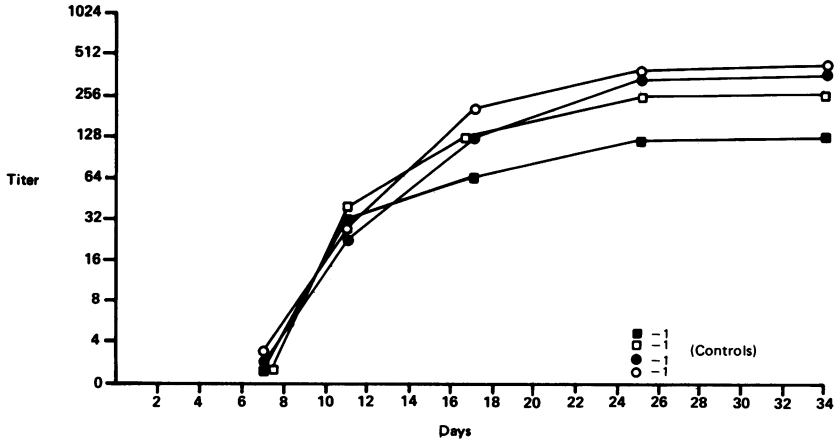

FIG. 3. Mean reciprocal IFA titers of guinea pig sera from animals inoculated with mixtures of *R. rickettsii* and monoclone E11-1G8 (●), monoclone E11-2F1 (○), Lassa MAF 52.93.4 (■), and buffer (□). −1: <4.

TABLE 4. Neutralization of toxic death in mice by monoclonal antibodies to *R. rickettsii*

MAF	Dilution[a]	No. injected	No. surviving	Titer
E11-1G8	1:100	3	0	
	1:80	3	2	
	1:50	3	3	1:50
	1:40	3	3	
	1:20	3	3	
E11-2F1	1:100	3	0	
	1:80	3	0	
	1:50	3	3	1:50
	1:40	3	3	
	1:20	3	3	
E11-1G2	1:100	3	0	
	1:80	3	1	
	1:50	3	1	1:40
	1:40	3	3	
	1:20	3	3	
E11-1H11	1:100	3	0	
	1:80	3	0	
	1:50	3	1	1:40
	1:40	3	3	
	1:20	3	3	
E11-1F12	1:100	3	0	
	1:80	3	3	
	1:50	3	3	1:80
	1:40	3	3	
	1:20	3	3	
BHI		15	0	
52.93'4	1:20	14	4	
BHI alone		10	10	
MAFs alone	1:20	12	12	

[a] Final dilution after mixing 1:1 with rickettsiae.

this epitope. In a serological analysis of polyclonal rabbit antibody against a purified surface component of *R. rickettsii*, Anacker et al. (1) demonstrated cross-reactivity between *R. montana*, *R. conorii*, *R. sibirica*, and the *R. ricketsii*-component antibody, with *R. akari* being nonreactive. The polyclonal antiserum of Anacker et al. and the monoclones in the present study probably recognize different epitopes, neither of which is present on *R. akari*. In a cross-protection study in guinea pigs, Feng and Waner (9) reported that the nonpathogenic *R. montana* protected animals from death when challenged with *R. rickettsii*.

Further serological characterization of each monoclone indicates that each will fix complement in the presence of the group CF antigen reagent, a complex mixture containing at least four major proteins (24), and moreover, that each will agglutinate a purified suspension of *R. rickettsii*. Considered together, the MA, IHA, and LA results provide indirect evidence that the monoclones are directed to some surface component other than the slime layer since both the LA and IHA tests use the carbohydrate erythrocyte-sensitizing substance as their antigen (20) and the erythrocyte-sensitizing substance has never been shown on purified organisms.

The biological characterization data indicate that the two monoclones which were tested can prevent rickettsemia and death in guinea pigs and can also reduce the severity and length of fever in animals which do become febrile. Although tissue-culture-infective rickettsiae remained in the monoclone-rickettsiae mixtures, the organisms were not able to proliferate to detectable levels (Fig. 2). This is probably due to opsonization, as shown in vitro with guinea pig leukocytes and antibody-coated and uncoated rickettsiae (15). However, the organisms in the mixture were able to persist at or proliferate to levels which were immunogenic (Fig. 3). At higher concentrations of antibody (<1:100), it might be possible to eliminate all tissue-culture-infective rickettsiae.

Each monoclone proved to be capable of preventing the mouse toxicity phenomenon. These data, along with the guinea pig data, suggest that the antigen(s) on viable *R. rickettsii* which are recognized may be involved in some step(s) in the pathogenic mechanism. This would probably involve either an attachment or penetration mechanism on the part of the organism. The fact that the number of tissue-culture-infective rickettsiae in the monoclone-rickettsiae mixtures was significantly less than that of the controls suggests that inactivation did take place. Although agglutination may have accounted for the reduction, the dilution of each monoclone (1:100) was greater than its maximum LA titer. This contrasts with the data of Kenyon and McManus (16), who showed that virtually all complexes of *R. rickettsii* and rhesus anti-*R. rickettsii* antibody were infectious for chicken embryo cultures unless the complexes were treated with an antirhesus immunoglobulin.

A proper dilution of each monoclone will clearly differentiate *R. rickettsii* from any of the other SFG organisms which were examined in the IFA assay. When tested against other SFG rickettsiae, they may prove to be useful in differentiating *R. rickettsii* from any of the other members of the group.

ACKNOWLEDGMENTS

We thank James D. Folds, Director of Laboratories, North Carolina Memorial Hospital, and J. N. MacCormack of the Division of Health Services, North Carolina Department of Human Resources, for performing the LA and IHA tests, respectively. We are also grateful to Ted Tzianabos and Martha Redus of the Viral and Rickettsial Zoonoses Branch, Division of Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga., for supplying the reagents for the agglutination and CF tests.

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