Analysis of T-Cell-Dependent and -Independent Antigens of *Rickettsia conorii* with Monoclonal Antibodies

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Four monoclonal antibodies from euthymic mice and two monoclonal antibodies from athymic mice were directed against antigens of *Rickettsia conorii*, as shown by both indirect immunofluorescence and an enzyme immunoassay. There was extensive cross-reactivity with other spotted fever group rickettsiae. Euthymic monoclonal antibodies 3-2 and 9-2 (immunoglobulin G2a [IgG2a]) and 27-10 (IgG1) distinctly outlined the acetone-fixed rickettsial surface, as determined by indirect immunofluorescence; only monoclonal antibody 3-2 reacted with the intact rickettsial surface, as determined by colloidal gold-protein A negative-stain electron microscopy. Athymic monoclonal antibodies 32-2 and 35-3 (IgM) and euthymic monoclonal antibody 31-15 (IgG3) all demonstrated an irregular, extrarickettsial morphology, as determined by immunofluorescence, and ultrastructural cell wall blebs that were readily shed from the rickettsial surface. Monoclonal antibody 3-2, the only antibody to confer protection in lethally challenged mice, reacted with a high-molecular-weight protein in Western immunoblots. Monoclonal antibodies 31-15, 32-2, and 35-3 reacted with a "ladder" of proteinase K-resistant, lipopolysaccharidelike antigens. None of the monoclonal antibodies stabilized the ultrastructural rickettsial slime layer, but both athymic and euthymic polyclonal antibodies to *R. conorii* did. This is, to the best of our knowledge, the first report of the production of monoclonal antibodies to *R. conorii* and their use for antigenic analysis.

Rickettsia conorii is the etiologic agent of boutonneuse fever, a fever-producing exanthem that occurs over much of Africa, southern Europe, and the Middle East. The incidence of clinical illness has risen sharply during the past decade, and severe and fatal cases have occurred (33). Moreover, there is serologic evidence for a very high prevalence of undiagnosed infections (14, 24).

R. conorii, a member of the spotted fever group (SFG) of rickettsiae, is immunologically and genetically closely related to *R. rickettsii*, the etiologic agent of Rocky Mountain spotted fever (4, 6, 32). As *R. conorii* confers cross-protection against subsequent *R. rickettsii* challenge of guinea pigs, the comparative antigenic composition of both rickettsiae is relevant to vaccine development and to the study of immunity to SFG rickettsioses (4, 40). It has been shown that athymic nude mice produce a nonprotective antibody response (21, 28). It was hypothesized that monoclonal antibodies to T-cell-independent antigens could be produced by cells from nude mice and would help define antigens that were unlikely candidates for a vaccine but might be helpful in the characterization of the putative slime layer. Thus, monoclonal antibodies to *R. conorii* were developed from spleen cells from both euthymic and athymic BALB/c mice in an effort to find protective and diagnostically useful antibodies.

MATERIALS AND METHODS

Rickettsiae. *R. conorii* (Malish 7 strain) for immunization of mice was obtained from the American Type Culture Collection, Rockville, Md., and cultivated in rabbit kidney cells (RK-13). The cell culture harvest contained 1.8×10^6

PFU/ml when assayed by a plaque assay in primary chicken embryo cell cultures (42). SFG rickettsiae as a source for microdot antigens for indirect immunofluorescent-antibody (IFA) titrations consisted of a yolk sac suspension of *R. conorii* Malish 7 strain, a South African strain of human origin; a yolk sac suspension of *R. conorii* Eth 2476 strain, an Ethiopian tick isolate obtained from the Rocky Mountain Laboratories, Hamilton, Mont.; a yolk sac suspension of *R. conorii* Indian tick typhus strain, an Indian tick isolate obtained from the American Type Culture Collection; a yolk sac suspension of *R. rickettsii* Sheila Smith strain, a Montana human isolate obtained from C. L. Wisseman, Jr., of the University of Maryland, Baltimore; *R. rickettsii* 84JG and 81WA, made in a yolk sac suspension and L cells, respectively, in our laboratory from isolates from fatal cases of Rocky Mountain spotted fever in North Carolina; a yolk sac suspension of *R. sibirica* strain 246, a USSR tick isolate obtained from the American Type Culture Collection; a primary chicken embryo cell suspension of *R. slovaca* B strain, a Czechoslovakian tick isolate obtained from the Rocky Mountain Laboratories; *R. akari*, obtained from the Centers for Disease Control, Atlanta, Ga.; and a primary chicken embryo cell suspension of TT-118, a Thailand tick isolate obtained from C. L. Wisseman, Jr.

Mice. Male BALB/c mice, 6 to 8 weeks old, and nude (*nu/nu*) mice on a BALB/c background were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Female C3H/HeJ mice, 8 to 12 weeks of age, were purchased from Jackson Laboratory, Bar Harbor, Maine.

Immunization of mice, fusion, cloning, and monoclonal antibody production. BALB/c mice were immunized by intraperitoneal inoculation of 3×10^4 PFU of viable *R. conorii* (Malish 7 strain) and boosted once or twice intraperitoneally with the same dose of rickettsiae before spleens were harvested for fusions. Nude mice were also inoculated with 3×10^4 PFU of *R. conorii* 5 days prior to collection of

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spleen cells. Spleen cells were fused to P3 × 63-Ag8.653 myeloma cells with polyethylene glycol and cultivated in microtiter wells, supernatant fluids were screened for antibodies to *R. conorii* by indirect immunofluorescence, and positive clones were subcloned by limiting dilutions and subsequently expanded to provide cells for the production of ascites fluids in pristane-primed BALB/c mice irradiated with 450 rads as described previously (23, 30). Ascites fluids containing monoclonal antibodies were fractionated by ammonium sulfate precipitation.

Electrophoretic separation and Western immunoblotting of polypeptides. The Laemmli system for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used (22). Antigens of *R. conorii* (Malish 7 strain) and *R. rickettsii* (Sheila Smith strain) were prepared by cultivation of rickettsiae in yolk sacs of embryonated hen eggs (SPAFAS, Inc., Norwich, Conn.) and clone E6 Vero cells in 850-cm^2 roller bottles and 150-cm^2 flasks. Yolk sacs of 4-day-old chicken embryos were inoculated, and embryos died after 6 days of incubation at 35°C (36). Yolk sacs were harvested after further incubation for 24 h at 35°C, examined for rickettsiae, and homogenized in a Waring blender. Inoculated cell cultures were incubated at 35°C for 7 to 10 days prior to harvesting, with monitoring of the condition of the cell monolayers by phase-contrast microscopy and of rickettsial quantity by immunofluorescence. Scraped cell monolayers were pelleted at 18,000 × *g* for 20 min, suspended in sucrose-phosphate-glutamate buffer (0.218 M sucrose, 3.8 mM KH$_2$PO$_4$, 7.2 mM K$_2$HPO$_4$, 4.9 mM L-glutamic acid [pH 7.0]), sonicated in an E/MC Corp. model 450 apparatus for 90 s (8) to break the host cells, digested with 0.5 μg of DNase (Sigma Chemical Co., St. Louis, Mo.) per ml for 1 h at 37°C, pelleted, and purified in a 30%–36%–42% Renografin discontinuous gradient by the method of Hanson et al. (16). Rickettsiae were also prepared from homogenized yolk sacs by the method of Weiss et al. prior to similar Renografin purification (41). Antigens were dissolved in one of three ways: (i) dilution in final sample buffer (0.0625 M Tris base [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue, 5% 2-mercaptoethanol) and incubation at 37°C for 30 min, (ii) dilution in final sample buffer and heating at 100°C for 5 min, or (iii) treatment with proteinase K (type XI, Sigma) (0.7 μg/μl of sample) at 65°C for 1 h prior to dissolution in final sample buffer (1, 2, 18).

Several dilutions of each antigen were separated electrophoretically to determine the optimal antigen concentration. Electrophoresis was performed at 10 mA for the 1.5% acrylamide stacking gel and at 125 V per gel with a constant current for the 15% acrylamide separating gel. Electrophoretically separated polypeptides were transferred from gels to 0.2-μm-pore nitrocellulose paper (Sartorius) by electrophoresis at 195 mA for 1 h followed by 50 mA for 3 h on a Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, Calif.) as previously described (15, 37). Antigens were detected on the nitrocellulose paper by immunoperoxidase staining after blocking of protein-binding sites with 0.05% Tween 20 in phosphate-buffered saline (PBS) for 30 min (19). After the blotted nitrocellulose filter strips were incubated with a 1:50 or 1:100 dilution of monoclonal antibody for 1 h and washed three times for 10 min each time in Tween 20-PBS, they were incubated for 30 min with either biotinylated anti-mouse immunoglobulin M (IgM) (μ chain specific) or biotinylated anti-mouse IgG (light and heavy chain specific), washed for 30 min in Tween 20-PBS, incubated with avidin-biotin-peroxidase complexes (Vectastain ABC kit),

washed in PBS, and reacted with color reagent (4-chloro-1-naphthol in methanol) and hydrogen peroxide. Some gels were stained with Coomassie blue or silver stain (Bio-Rad) by previously described methods (25).

After the failure to demonstrate clearly the antigens with which monoclonal antibodies 3-2 and 9-2 reacted, an alternative method developed by Dasch et al. that used less harsh denaturing conditions was used (10). The modifications were the dissolution of a Renografin-purified light band that had been frozen in distilled water and thawed once in final sample buffer at 4°C, prolonged SDS-PAGE at 4°C for 13 h, transfer of polypeptides at 4°C for 1.5 h at 195 mA followed by 2 h at 100 mA, and blocking of nonspecific protein binding with 5% milk in 0.05 mM Tris buffer (pH 7.5) overnight at 4°C. Monoclonal antibodies 3-2 and 9-2 and mouse sera were diluted in 3% milk in Tris buffer as described above for reaction with blotted antigens.

IFA assay. Microdots of various SFG rickettsial strains were incubated with serial dilutions of monoclonal antibodies, and the antibody reaction was detected by immunofluorescence after staining with anti-mouse immunoglobulin-fluorescein isothiocyanate conjugate (32).

EIA. The enzyme immunoassay (EIA) was performed with two types of rickettsial antigen preparation: (i) corpuscular antigen from Renografin density gradient banding and (ii) dissolved antigen. Intact rickettsial corpuscular antigen consisted of the same light band of Renografin density gradient-purified rickettsiae that was used for immunoblotting, except that formaldehyde at a final concentration of 0.1% was added and the mixture was maintained at 4°C for 3 days to inactivate rickettsial infectivity. Hypotonically released antigen was prepared from heavily infected Vero cell cultures. Cells containing *R. conorii* were scraped from flasks and pelleted at 18,000 × *g* for 20 min. Hypotonic buffer (1 mM Tris hydrochloride, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetracetic acid [EGTA], 1 mM MgCl$_2$ [pH 7.6]) was added, and the suspension was sonicated in the same apparatus as that described above in an ice bath for 90 s. After recentrifugation at 18,000 × *g*, a final concentration of 0.1% formaldehyde was added to the supernatant, which was maintained at 4°C for 3 days and is the final product (hypotonically released antigen).

The method used for the EIA is a modification of that described previously (9). The suitable dilution of antigen in Tris buffer (pH 8.0) was determined by block titration to be a 1:50 to 1:100 dilution of the antigen preparations. Diluted antigen (75 μl) was added to each well of a 96-well microtiter plate, incubated overnight at room temperature, and washed once with Tris buffer-Tween 20 (0.15 M NaCl, 0.02 M Tris base, 0.02% Tween 20 [pH 8.0]). Excess binding sites were blocked with 150 μl of 3% bovine serum albumin in Tris buffer per well for 2 h. After removal of the blocking solution, serial dilutions of monoclonal antibodies from 1:90 to 1:590,490 were added (75 μl/well) and incubated at room temperature for 2 h followed by four washes. A 1:400 dilution of peroxidase-labeled second antibody against mouse immunoglobulins (Cooper Biomedical, Inc., West Chester, Pa.) was added (75 μl per well) and incubated for 2 h followed by four washes. The peroxidase substrate, [2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate)] and hydrogen peroxide were added and allowed to develop for 15 min. The results were read in a model MR 600 microplate reader (Dynatech Laboratories, Inc., Alexandria, Va.) at 490 nm.

Identification of immunoglobulin classes and subclasses. The mouse immunoglobulin classes and subclasses were determined by an EIA. The wells of a microtiter plate were

TABLE 1. Reciprocal IFA titers of monoclonal antibodies to SFG rickettsiae

Antigen	Titer of monoclonal antibody:					
	3-2	9-2	27-10	31-15	32-2	35-3
Pathogenic rickettsiae						
R. rickettsii						
Sheila Smith	81,920	81,920	−[a]	2,560	5,120	163,840
84JG	81,920	20,480	40	1,280	10,240	81,920
81WA	81,920	81,920	20	1,280	2,560	81,920
R. conorii						
Malish 7	327,680	20,480	20	5,120	2,560	81,920
Ethiopian (Eth 2476)	655,360	40,960	−	2,560	2,560	81,920
Indian	163,840	20,480	20	2,560	5,120	163,840
R. sibirica 246	40,960	10,240	−	80	2,560	20,450
R. akari	−	5,120	10	320	640	163,840
Nonpathogenic rickettsiae						
TT-118	81,920	5,120	40	−	−	−
R. slovaca B	81,920	5,120	40	320	1,280	20,480

[a] −, Nonreactive at a titer of 1:10.

coated with a first layer of a 1:50 dilution of corpuscular *R. conorii* antigen in Tris buffer (pH 8). The second layer was a 1:50 dilution of the monoclonal antibody to be typed in Tris buffer (pH 8) with 1% bovine serum albumin. The third layer comprised the various rabbit anti-mouse subclass antisera (Bio-Rad) followed by a 1:3,000 dilution of peroxidase-conjugated anti-rabbit immunoglobulin serum in Tris buffer (pH 8) with 1% bovine serum albumin and was reacted with the peroxidase substrate and hydrogen peroxide as in the EIA described above.

Evaluation of animal protection by monoclonal antibodies. Monoclonal antibodies prepared by ammonium sulfate precipitation of BALB/c ascites fluid (1:10 final dilution in PBS) and 2, 5, or 10 50% lethal infectious doses (final concentration) of *R. conorii* were mixed, allowed to react at room temperature for 1 h, and inoculated intraperitoneally into groups of four C3H/HeJ mice (3, 13, 23). Mice were observed for morbidity and mortality for 28 days.

Electron microscopy. Ultrastructural examination for the stabilization of the slime layer of *R. conorii* by antibody was performed by the method of Silverman et al. (35) with slight modifications. *R. conorii* (Malish 7 stain) cultivated in clone E6 Vero cells for 5 days was scraped into medium containing 1% fetal bovine serum and 10% monoclonal antibody or serum, including Swiss Webster and nude (*nu/nu*) mouse anti-*R. conorii* hyperimmune serum, normal mouse serum, and monoclonal antibodies 9-2, 3-2, 31-15, 32-2, and 35-3. The Vero cells were then broken open in a Ten Broeck blender and sonicated in the previously described apparatus for 90 s. After incubation at room temperature for 1 h, the suspensions were fixed in 2.5% glutaraldehyde for 1.5 h, pelleted, washed twice in Sorenson buffer (0.1 M PBS [pH 7.3]), postfixed in 2% OsO_4 for 1 h, dehydrated in a series of concentrations of ethanol, infiltrated with propylene oxide-Epon-Araldite (Mollenhauer no. 2) overnight, and embedded in Epon-Araldite at room temperature for 2 h and then for 3 days at 60°C (26). Ultrathin sections were cut on an ultramicrotome, and grids were stained with lead citrate-uranyl acetate (39) and examined in a Zeiss 10A electron microscope.

The location of the antigenic epitopes of *R. conorii* reac-

tive with our monoclonal antibodies was investigated ultrastructurally. Renografin-purified *R. conorii* was diluted 1:10 in PBS, and 7 μl was placed on a Formvar-coated nickel grid and allowed to adsorb for 1 min (1, 5). The excess fluid was then removed. Monoclonal antibodies and control polyclonal antibodies were added to each grid as follows: monoclonal antibodies 3-2, 9-2, 31-15, 32-2, and 35-3 diluted 1:50 in PBS and monoclonal antibody 27-10 and preinoculation and anti-*R. conorii* mouse sera diluted 1:10 in PBS. After incubation in a moist chamber for 30 min, the fluid was removed, and the grid was rinsed in PBS. Colloidal gold (15 nm)-labeled protein A (Life Sciences Products, Piscataway, N.J.) diluted 1:16 was added to the grids and incubated at room temperature for 30 min. The excess fluid was then removed, and the grids were rinsed. The grids were prepared for negative-stain electron microscopy by the addition of 7 μl of 1% phosphotungstic acid in 0.1 M phosphate buffer (pH 7.5) for 20 s and the subsequent removal of excess fluid. After being air dried, the grids were examined in a Zeiss 10A transmission electron microscope.

RESULTS

From the fusion of spleen cells from infected euthymic BALB/c mice, 27 hybridomas secreting antibodies detected by the IFA assay on microdots of Vero cells infected with *R. conorii* were established. From the fusion of spleen cells from infected athymic nude (*nu/nu*) BALB/c mice, three clones producing antibodies to *R. conorii* were obtained. We arbitrarily selected four subclones (3-2, 9-2, 27-10, and 31-15) from the euthymic mice and two subclones (32-2 and 35-3) from the athymic nude mice to investigate in detail.

To assure that the antibodies which were selected were really monoclonal, we used three methods. First, according to Poisson analysis, the subclones were chosen from the 96-well plate that contained more than 37% negative wells. The second method is based on the results of isoelectric focusing, which demonstrated patterns for the monoclonal antibodies that indicated molecular homogeneity, as compared with polyclonal antibody controls (34). Third, determinations of the immunoglobulin classes and subclasses of

TABLE 2. EIA titers of monoclonal antibodies to *R. conorii*

Antigen	Titer of monoclonal antibody:					
	3-2	9-2	27-10	31-15	32-2	35-3
Dissolved	$-^a$	1:90	–	–	1:90	1:810
Corpuscular	1:90	1:270	1:270	1:270	1:590,490	1:590,490

^{a} –, Nonreactive at a titer of 1:90.

the monoclonal antibodies were as follows: monoclonal antibodies 3-2 and 9-2, IgG2a; monoclonal antibody 27-10, IgG1; monoclonal antibody 31-15, IgG3; and monoclonal antibodies 32-2 and 35-3, IgM.

Reactions of monoclonal antibodies with nondenatured rickettsial antigens were examined by IFA titration and EIA titration. The IFA titers of monoclonal antibodies to different strains of four pathogenic and two nonpathogenic SFG rickettsial species are shown in Table 1. All of these monoclonal antibodies recognized the epitopes present on the four pathogenic SFG rickettsiae tested, except for monoclonal antibody 3-2, which did not react with *R. akari*, and monoclonal antibody 27-10, which reacted with only some of the strains of *R. rickettsii*, *R. conorii*, and *R. akari* at very low titers. The morphology demonstrated by IFA titration with monoclonal antibodies 3-2, 9-2, and 27-10 was that of a distinct outline of the rickettsial surface. In contrast, monoclonal antibodies 31-15, 32-2, and 35-3 yielded a markedly different, irregular extrarickettsial pattern of staining that was consistently distinguished in blind evaluations.

The results of EIA titration are shown in Table 2. It appears that the nude mouse monoclonal antibodies and monoclonal antibody 9-2 recognized the epitopes of *R. conorii* that can be detached easily from the rickettsiae.

The results of Western immunoblotting of the rickettsial antigens with which the monoclonal antibodies reacted are

demonstrated in Fig. 1 to 4. Monoclonal antibody 3-2 did not react with any denatured antigen. The two visible bands were not specific, for they were also present with control, nonrickettsial monoclonal antibodies and normal mouse serum. Monoclonal antibody 9-2 appeared to recognize a faint band of 84 kilodaltons (kDa) when rickettsial antigens were dissolved at 37°C for 30 min. Monoclonal antibody 27-10 reacted specifically with four weak bands (115, 100, 84, and 74 kDa) when antigens were dissolved at 37°C for 30 min and only one band (115 kDa) when antigens were dissolved at 100°C for 5 min. Monoclonal antibody 31-15 reacted specifically with eight major bands (53, 49, 47, 45, 36, 33, 29, and 26 kDa) when antigens were treated at 37°C and only five bands (47, 45, 43, 31, and 29 kDa) when antigens were treated at 100°C. Monoclonal antibody 35-3 reacted specifically with 12 bands when antigens were dissolved at 37°C and 9 bands when antigens were dissolved at 100°C. Monoclonal antibody 32-2 recognized specific bands with estimated molecular weights the same as those recognized by monoclonal antibody 35-3 (Fig. 1 and 3). Dissolution, electrophoresis, and immunoblotting of the rickettsial antigens at 4°C successfully revealed two high-molecular-weight, heat-sensitive protein antigens which reacted with monoclonal antibodies 3-2 and 9-2 (Fig. 2). The estimated molecular masses of these antigens were 112 (monoclonal antibody 3-2) and 128 (monoclonal antibody 9-2) kDa. Normal mouse serum did not react with either of these two polypeptides, although the cold method resulted in moderate nonspecific reactivity with other antigens. Because the anti-mouse IgG-biotin conjugate reacts with both light and heavy chains, the IgM monoclonal antibodies were demonstrated in those immunoblots. However, the heavy-chain-specific biotin con-
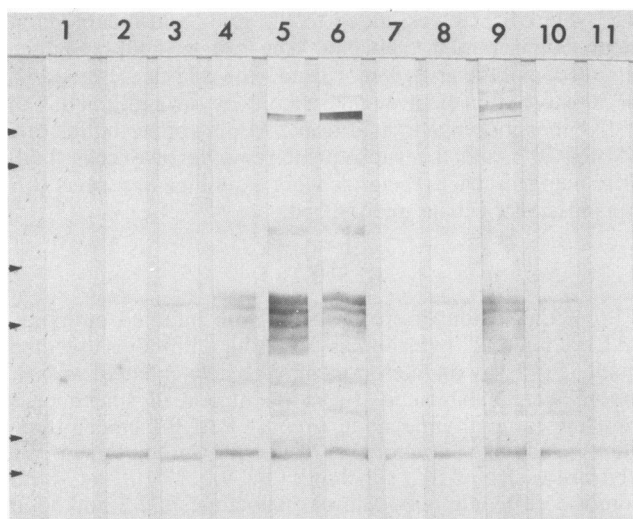

FIG. 1. Protein immunoblotting of antigens of *R. conorii* dissolved at 100°C for 5 min, separated by SDS-PAGE, reacted with antibodies (lane 1, 3-2; lane 2, 9-2; lane 3, 27-10; lane 4, 31-15; lane 5, 32-2; lane 6, 35-3; lane 7, 17-12 [to a Vero cell cytoskeletal antigen]; lane 8, 10-2 to *Acholeplasma laidlawii*; lane 9, euthymic mouse anti-*R. conorii* hyperimmune serum; lane 10, euthymic mouse anti-*R. rickettsii* hyperimmune serum; lane 11, normal euthymic mouse serum), and developed with anti-mouse IgG–avidin-biotin-peroxidase complexes. Molecular mass markers (92, 66, 45, 31, 21, and 14 kDa) are marked by arrowheads.
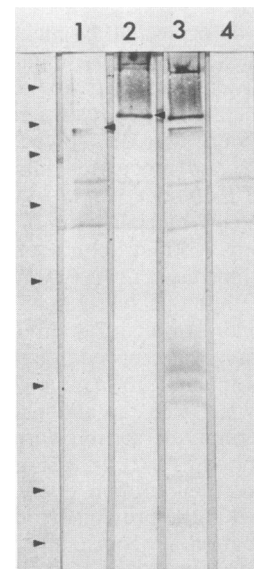

FIG. 2. Protein immunoblotting of antigens of *R. conorii* dissolved at 4°C, separated by SDS-PAGE at 4°C, reacted with antibodies (lane 1, 3-2; lane 2, 9-2; lane 3, euthymic mouse anti-*R. conorii* hyperimmune serum; lane 4, normal euthymic mouse serum), and developed with anti-mouse IgG–avidin-biotin-peroxidase complexes. Monoclonal antibody 3-2 reacted with a 112-kDa polypeptide (arrowhead); monoclonal antibody 9-2 reacted with a 128-kDa polypeptide (arrowhead); polyclonal immune serum reacted with both polypeptides; and normal mouse serum reacted with neither polypeptide. Molecular mass markers (200, 116, 92, 66, 45, 31, 21, and 14 kDa) are marked by arrowheads at left.

jugate revealed rickettsial antigen bands only for the IgM monoclonal antibodies.

After treatment of the rickettsial antigens with proteinase K, monoclonal antibodies 32-2 and 35-3 were still able to react with five bands between 36 and 48 kDa (Fig. 5).

The profile of rickettsial components in SDS-PAGE revealed 31 and 33 bands stained with Coomassie blue after dissolution at 37 and 100°C, respectively. Silver-stained gels revealed a different profile of rickettsial components than did Coomassie blue-stained gels. Silver-stained gels showed low-molecular-weight bands more distinctly than did Coomassie blue-stained gels.

The results of mouse protection tests demonstrated that monoclonal antibody 3-2 conferred complete protection against a lethal challenge with *R. conorii* (Table 3).

Electron microscopy of pellets of *R. conorii* released in the presence of monoclonal and polyclonal antibodies revealed the presence of an extracellular layer of material compatible with a slime layer only when polyclonal anti-*R. conorii* immune sera from both euthymic and athymic mice were used (Fig. 6). Anti-*R. conorii* monoclonal antibodies and normal mouse serum failed to stabilize the extracellular layer around *R. conorii*.

Colloidal gold-protein A examination of *R. conorii* reactive with the monoclonal antibodies demonstrated that monoclonal antibody 3-2 recognized an epitope on the surface of *R. conorii*, monoclonal antibodies 31-15, 32-2, and 35-3 reacted with surface antigens which were shed from the rickettsial surface, as has been described for the group-reactive complement fixation antigen (38), and monoclonal antibodies 9-2 and 27-10 did not react demonstrably with *R. conorii* (Fig. 7). Monoclonal antibody 17-12, which is reactive with a cytoskeletal component of Vero cells, did not
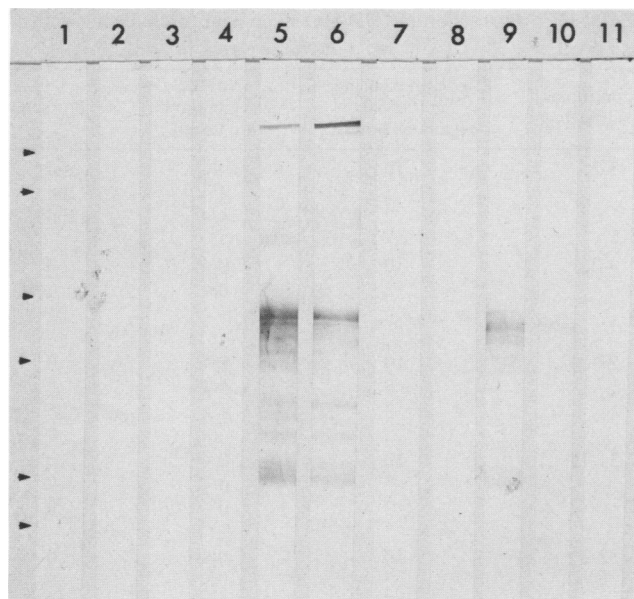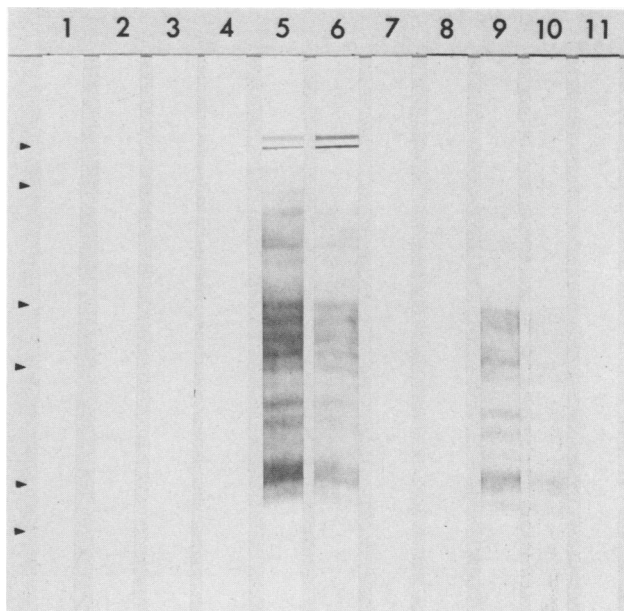

FIG. 4. Protein immunoblotting of antigens of *R. conorii* dissolved at 37°C for 30 min, separated by SDS-PAGE, reacted with antibodies (lane 1, 3-2; lane 2, 9-2; lane 3, 27-10; lane 4, 31-15; lane 5, 32-2; lane 6, 35-3; lane 7, 17-12 [to a Vero cell cytoskeletal antigen]; lane 8, 10-2 to *A. laidlawii*; lane 9, nude mouse anti-*R. conorii* serum; lane 10, nude mouse anti-*R. rickettsii* serum; lane 11, normal nude mouse serum), and developed with anti-mouse IgM–avidin-biotin-peroxidase complexes. Molecular mass markers (92, 66, 45, 31, 21, and 14 kDa) are marked by arrowheads.

react with the rickettsiae but did reveal a slight contamination of the rickettsial band with Vero cell components. The anti-*R. conorii* antibody containing polyclonal sera reacted with rickettsiae as expected, and normal sera did not cause rickettsial binding to colloidal gold-protein A.

DISCUSSION

This investigation produced an interesting array of monoclonal antibodies to *R. conorii*. Despite their immunodeficient state and susceptibility to fatal infection with *R. conorii* (Malish 7 strain) (28), athymic (*nu/nu*) mice provided spleen cells that were successfully fused with myeloma cells to produce antirickettsial antibodies. Both clones from athymic mice selected for further study appeared to be directed against rickettsial lipopolysaccharide (LPS)-like antigens, apparently the immunodominant T-cell-independent immunogen of *R. conorii*. No monoclonal antibodies to the rickettsial slime layer were obtained from this investigation. The monoclonal antibodies from nude mice were of the IgM class, as expected, and cross-reacted with antigens of *R. rickettsii*, *R. sibirica*, and *R. akari*, although not with the Thailand tick isolate antigen.

In contrast, three of the four monoclonal antibodies from euthymic mice selected for study were of the IgG2a and IgG1 subclasses, reacted with high-molecular-weight antigens in protein immunoblots, and demonstrated a distinct outline of the rickettsial surface by indirect immunofluorescence. The fourth euthymic monoclonal antibody (31-15) was of the IgG3 subclass (which often is stimulated by polysaccharide antigens [27]), reacted with the "ladder" of low-molecular-weight LPS-like antigens in protein immunoblots (1, 2, 18), and demonstrated morphology by colloidal gold-protein A

FIG. 3. Protein immunoblotting of antigens of *R. conorii* dissolved at 100°C for 5 min, separated by SDS-PAGE, reacted with antibodies (lane 1, 3-2; lane 2, 9-2; lane 3, 27-10; lane 4, 31-15; lane 5, 32-2; lane 6, 35-3; lane 7, 17-12 [to a Vero cell cytoskeletal antigen]; lane 8, 10-2 to *A. laidlawii*; lane 9, nude mouse anti-*R. conorii* serum; lane 10, nude mouse anti-*R. rickettsii* serum; lane 11, normal nude mouse serum), and developed with anti-mouse IgM–avidin-biotin-peroxidase complexes. Molecular mass markers (92, 66, 45, 31, 21, and 14 kDa) are marked by arrowheads.
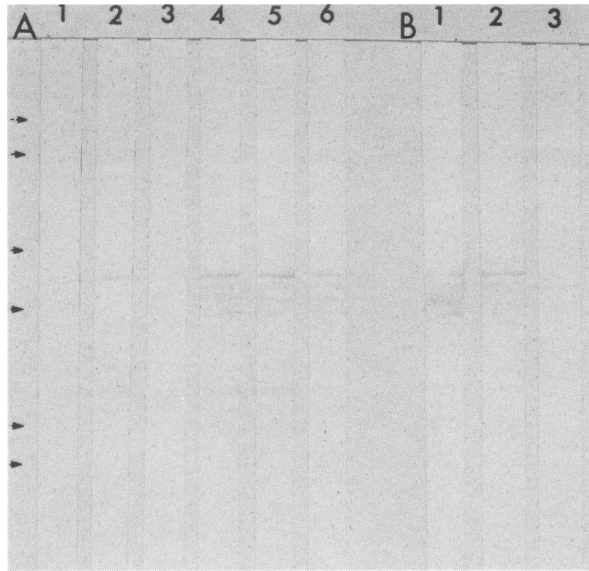
FIG. 5. Protein immunoblotting of antigens of *R. conorii* treated with proteinase K, dissolved at 100°C for 5 min, separated by SDS-PAGE, reacted with antibodies (panel A: lane 1, 9-2; lane 2, 27-10; lane 3, 31-15; lane 4, 32-2; lane 5, 35-3; lane 6, euthymic mouse anti-*R. conorii* serum), and developed with anti-mouse IgG–avidin-biotin-peroxidase complexes. In panel B, monoclonal antibodies 32-2 (lane 1) and 35-3 (lane 2) and nude mouse anti-*R. conorii* serum (lane 3) were developed with anti-mouse IgM–avidin-biotin-peroxidase complexes. Molecular mass markers (92, 66, 45, 31, 21, and 14 kDa) are marked by arrowheads.

negative-stain electron microscopy compatible with that of the cell wall-derived, group-reactive complement fixation antigen (38).

This study demonstrated several important characteristics of *R. conorii*, an SFG rickettsia that had previously not been the subject of a study with monoclonal antibodies, protein immunoblotting, and electron microscopy. The existence of a rickettsial slime layer was reported in 1978 and has been the subject of considerable speculation but no new observations since then (35). Our demonstration of an amorphous extrarickettsial layer stabilized by antibodies from both athymic and euthymic mice by the method of Silverman et al. (35) extends the observed presence of this structure to another rickettsial species, *R. conorii*. The fact that monoclonal antibodies to T-cell-independent antigens, even of the IgM class, failed to stabilize the slime layer suggests that it is a polysaccharide substance distinct from the LPS-like antigens. A more direct investigation of this structure, e.g., by screening for more monoclonal antibodies until one reactive with the slime layer of *R. conorii* is obtained, is needed.

The production and investigation of three monoclonal antibodies to the LPS-like antigens of *R. conorii* allowed us to demonstrate that antibodies to these antigens do not protect C3H/HeJ mice against fatal infection, despite their presence on the rickettsial surface. Characteristics suggestive of LPS were the ladder distribution of antigens in SDS-PAGE, cell wall bleb ultrastructural morphology, heat-modifiable electrophoretic mobility, more distinct demonstration of low-molecular-weight bands by silver staining than by Coomassie blue staining, and persistent reactivity of the Western blots with monoclonal antibodies 31-15, 32-2, and 35-3 after proteinase K digestion (1). The failure of these monoclonal antibodies to protect C3H/HeJ mice from lethal

infection correlates with the observation of Anacker et al. that monoclonal antibodies to the LPS-like antigens of *R. rickettsii* do not protect mice in a very different noninfectious model, the so-called mouse toxin phenomenon (1, 2). In earlier studies, Anacker et al. described a polyclonal rabbit serum to one protein of *R. rickettsii* that protected both guinea pigs from infection and mice from intravenous toxic death (3). Lange and Walker conferred similar dual protection against guinea pig infection and mouse toxicity with a monoclonal antibody to *R. rickettsii* (23) which reacted with a 133-kDa rickettsial polypeptide (C. Spruill, R. Regnery, and J. Lange, personal communication).

Of the three monoclonal antibodies directed against high-molecular-weight proteins (3-2, 9-2, and 27-10), only monoclonal antibody 3-2 conferred complete protection of susceptible C3H/HeJ mice against challenges with 10, 5, and 2 50% lethal infectious doses of *R. conorii*. This antibody was also the only one of the three non-LPS-like antibodies to react demonstrably at the ultrastructural level with a surface antigen of *R. conorii*. The epitope of the protein antigen reactive with monoclonal antibody 3-2 was quite labile, as it was denatured to a nonreactive state by dissolution in sodium dodecyl sulfate–2-mercaptoethanol, even at 37°C, followed by electrophoresis and immunoblotting at room temperature. Less harsh conditions demonstrated that the protective monoclonal antibody reacted with an immunoblotted protein of 112 kDa. This antigen may be related to the 120-kDa protective typhus group antigens described by Dasch and co-workers (7, 11, 12). In contrast, nonprotective monoclonal antibody 9-2 reacted with a 128-kDa protein, and nonprotective monoclonal antibody 27-10 reacted best with a 115-kDa protein dissolved at 100°C. Weak reactions of monoclonal antibody 27-10 with proteins of 115, 100, 84, and 74 kDa dissolved at 37°C suggest the possibili-

TABLE 3. Protection of C3H/HeJ mice against challenge with *R. conorii*

Monoclonal antibody	*R. conorii* challenge (50% lethal infectious dose)	No. of survivors/total no. tested	% Protection
3-2	10	4/4	100
	5	4/4	100
	2	4/4	100
9-2	10	0/4	0
	5	0/4	0
	2	1/4	25
27-10	10	0/4	0
	5	0/4	0
	2	1/4	25
31-15	10	0/4	0
	5	0/4	0
	2	0/4	0
32-2	10	0/4	0
	5	0/4	0
	2	1/4	25
35-3	10	0/3	0
	5	0/4	0
	2	0/4	0
17-12	10	0/3	0
	5	0/3	0
	2	0/3	0

ties of repeated epitopes or degradation of the 115-kDa protein to smaller molecules, although the use of phenymethylsulfonyl fluoride, ε-aminocaproic acid, and other protease inhibitors did not alter the rickettsial profiles. Reaction of monoclonal antibody 9-2 with a particular epitope on the 128-kDa protein did not prevent the entry of *R. conorii* into cells and the establishment of a lethal infection in susceptible mice. The marked disparities in the relative titers of monoclonal antibodies 3-2 and 27-10 against whole *R. conorii* in the IFA and corpuscular antigen EIA are difficult to explain. These monoclonal antibodies are directed against different epitopes, as shown by the mouse protection data; however, the only apparent differences in the IFA and corpuscular
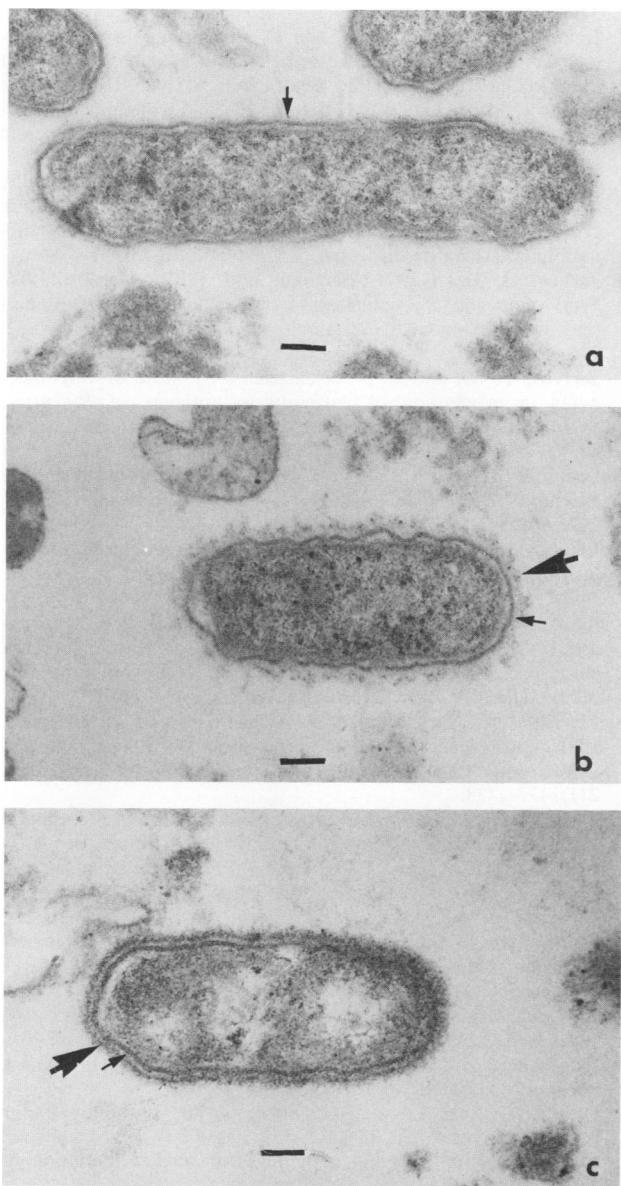

FIG. 6. Transmission electron micrographs of pelleted *R. conorii*-infected Vero cells disrupted in the presence of normal mouse serum (a), nude mouse anti-*R. conorii* serum (b), and euthymic mouse anti-*R. conorii* serum (c). A modest slime layer (large arrow) is present in panel b; a dense slime layer (large arrow) is present in panel c. Small arrows indicate the outer envelope of the rickettsial cell wall. Uranyl acetate-lead citrate stain. Magnification, ×58,000. Bars, 100 nm.
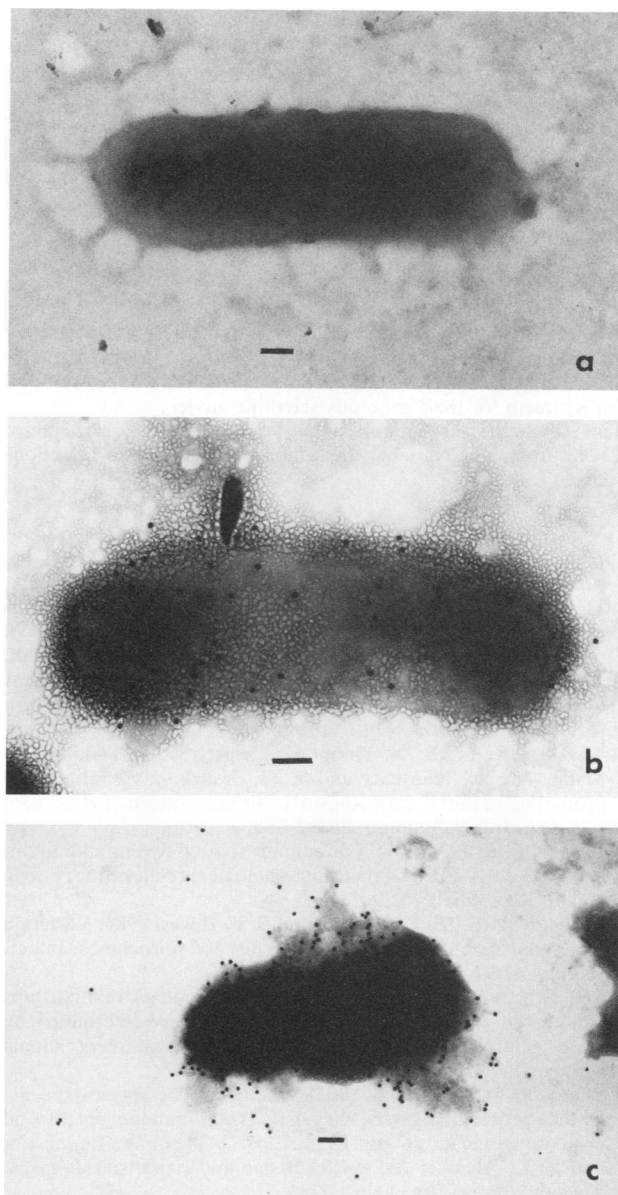

FIG. 7. Negative-stain electron micrographs of protein A-colloidal gold (15 nm)–treated *R. conorii* reactive with monoclonal antibody to a Vero cell cytoskeletal antigen (a) (×45,000), monoclonal antibody 3-2 directed against a surface protein of *R. conorii* (b) (×52,000), and monoclonal antibody 32-2 directed against blebs of cell wall in the process of being shed from *R. conorii* (×33,000). Bars, 100 nm.

antigen EIA that might account for the disparities are the fixatives, absolute acetone for 10 min and 0.1% formaldehyde for 72 h, respectively.

It is interesting that the protective monoclonal antibody cross-reacted with *R. rickettsii*, *R. sibirica*, and TT-118 but not with *R. akari*. This observation fits the previous observations that *R. conorii* and *R. rickettsii* are cross-protective in guinea pigs and mice (4, 13, 40) but that *R. akari* does not confer complete cross-protection against *R. rickettsii* in guinea pigs (17). The differences in reported protein profiles cannot be the explanation, as these proteins are very similar, particularly above 59 kDa (29, 31).

This investigation represents an important step in the

humoral analysis of the antigenic composition of *R. conorii* which complements the T-cell hybridoma approach (20). It is clear that the production of monoclonal antibodies to a larger array of epitopes of *R. conorii* is necessary to map the antigens of importance in immunity to boutonneuse fever. This approach will be fruitful not only in the study of the organism itself but also in producing reagents that are useful as diagnostic tools and for the study of antigens expressed by recombinant clones.

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