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Monoclonal Antibody Binding to a Surface-Exposed Epitope on *Cowdria ruminantium* That Is Conserved among Eight Strains

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Monoclonal antibodies (MAb) binding to *Cowdria ruminantium* elementary bodies (EB) were identified by enzyme-linked immunosorbent assay, and surface binding of one MAb (446.15) to intact EB was determined by immunofluorescence, immunogold labeling, and transmission electron microscopy. MAb 446.15 bound an antigen of approximately 43 kDa in immunoblots of eight geographically distinct strains. The MAb did not react with *Ehrlichia canis* antigens or uninfected bovine endothelial cell lysate and may be useful in diagnostic assays and vaccine development.

Heartwater is an often fatal tick-borne disease of domestic and wild ruminants caused by *Cowdria ruminantium*, and it remains a major constraint to efficient livestock production in sub-Saharan Africa (22, 30). In some sub-Saharan African countries, *Amblyomma variegatum* is the most important vector (30), while in Zimbabwe, *Amblyomma hebraeum* is most important. Heartwater has been detected in the Caribbean islands and is a threat to the mainlands of North and South America, which have potential tick vectors (1, 2, 31). Detection of *C. ruminantium* depends on diagnostic tests; however, the current tests lack specificity, as evidenced by the detection of antibodies in sera from regions where heartwater does not occur (15). Some of the lack of specificity is caused by cross-reacting antigens in *Ehrlichia* species (7). In addition, the development of effective vaccines is constrained by a lack of knowledge of both the required target antigens and immunologic effector mechanisms (25–27, 29). The observation that protective immunity can be induced in some goats and sheep with culture-attenuated or inactivated organisms (12, 17, 19) indicates that induction of protective immune responses in ruminants by using subunits is possible. Toward this end, a protein that can induce a protective response against *C. ruminantium* challenge in some mice was identified using a DNA vaccine vector (21). This report describes a monoclonal antibody (MAb) that reacts with a surface-exposed epitope on *C. ruminantium* elementary bodies (EB) that is conserved among eight disease-causing strains of the organism. The use of MAb 446.15 for surface protein identification may be useful in the development of more specific diagnostic reagents for heartwater, and the protein could be evaluated as a component of a subunit vaccine.

(This study was done with the permission of the Director of the Kenya Agricultural Research Institute.)

Development of MAbs to *C. ruminantium* EB. The Crystal Spring strain of *C. ruminantium* was grown and maintained in culture as described previously (32). Bovine pulmonary artery endothelial cells (BPA 593) (18) were infected with EB and harvested when the cytopathic effect was approximately 80 to 90%. Harvested cells were sonicated for 1 min and centrifuged at 500 × *g* for 10 min to remove cellular debris. EB were pelleted from the supernatants by centrifugation at 13,000 × *g* and washed three times with phosphate-buffered saline (PBS) (0.01 M sodium phosphate, 0.14 M NaCl; pH 7.4). One hundred micrograms of EB was mixed with an equal amount of Freund's complete adjuvant and used to immunize mice intramuscularly. After three booster immunizations using Freund's incomplete adjuvant, mice with the highest immunofluorescence assay (IFA) antibody titer were given a final intravenous injection containing 50 μg of EB without adjuvant. Three days later, spleen cells were harvested and fused with cells of the P3X63.Ag8.653 mouse myeloma line (ATCC CRL 1580) at a ratio of 10:1 as previously described (24). An enzyme-linked immunosorbent assay (ELISA) using EB as the antigen immobilized on ELISA plates was used to identify hybridomas secreting antibody to these organisms. Briefly, 96-well plates were coated with approximately 5 μg of sonicated EB antigens per well overnight at 4°C. The plates were washed once with PBS–0.05% Tween 20 and blocked with PBS–0.05% Tween 20–1% nonfat dry milk. Hybridoma supernatants were applied to each well and bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse immunoglobulins diluted 1:800. Bound antibodies were visualized using 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid (ABTS) and phosphate citrate buffer with urea-hydrogen peroxide. The optical densities were read at 419 nm using a Multiscan ELISA reader. Selected hybridomas were cloned three times by the limiting dilution method using ascitic fluid produced in pristane-primed BALB/c mice. The MAbs were purified using 50% ammonium sulfate precipitation followed by fractionation on DEAE cellulose before use in other assays (20).

Reactivity of MAbs with intact EB. The initial determination of whether the MAbs bound to the surface of intact *C. ruminantium* EB was done using indirect immunofluorescence. Briefly, 100 μl of free EB (or EB-infected endothelial cells fixed in acetone) was washed in PBS and then reacted with 100 μl of MAb 446.15.22 (20 μg/ml). The mixtures were incubated for 30 min at room temperature, washed three times with PBS,

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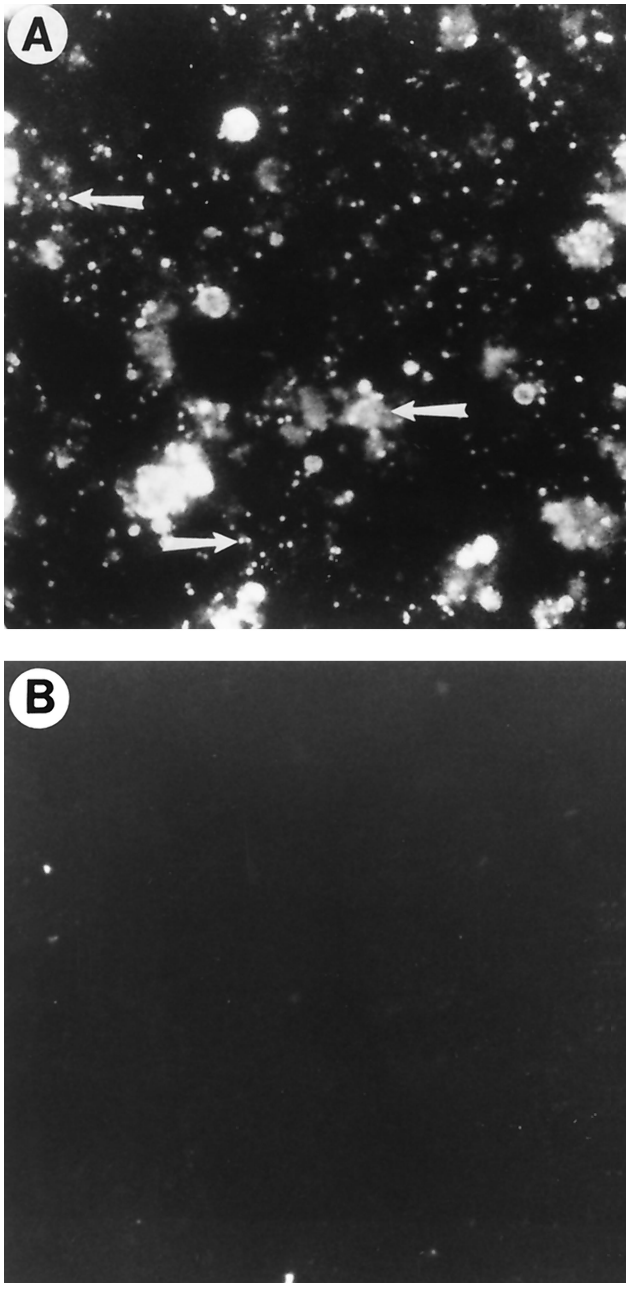

FIG. 1. Binding of monoclonal antibody 446.15 to epitopes on *C. ruminantium* acetone-fixed free EB in IFAs. (A) Arrows show MAb-stained free and clumped EB. Also shown are colonies of stained EB in endothelial cells. (B) No reactivity was observed from isotype control MAb WM25. Magnification, ×600.

and reacted with 100 μl of fluorescein-conjugated rabbit antibody to mouse immunoglobulins (Organon Teknika, Durham, N.C.) diluted 1:100 in PBS. The mixture was incubated for 30 min at room temperature, washed three times with PBS, and examined by fluorescence microscopy. The specificity of the immunofluorescence was controlled by reacting EB with an isotype control (immunoglobulin M [IgM]), MAb WM25. Seven IgM MAbs (320.1.8, 442.3.21, 443.3.2, 446.15.22, 447.3.26, 447.3.15, and 447.3.24) binding to *C. ruminantium* EB were identified by IFA. All the MAbs caused clumping of the EB, indicating that they were reacting with epitopes on the EB surface (Fig. 1A). The isotype control MAb WM25 did not bind to EB (Fig. 1B). Evan's blue (1%) was used in IFAs as the quenching agent. MAb 446.15.22 (designated 446.15) was selected for more detailed evaluation.

Demonstration of MAb 446.15 binding to the surface of EB by immunogold electron microscopy. To verify that MAb 446.15 was binding to the EB surface, intact EB were reacted with MAb followed by antibodies to mouse IgM that were conjugated to gold particles. These EB were then embedded, and ultrathin sections were examined by transmission electron microscopy. Intact EB prepared as described above were incubated for 30 min in 1:10 and 1:100 dilutions of MAb 446.15, isotype control MAb WM25, or PBS. The suspension was washed three times with PBS containing 1% bovine serum albumin before incubation in gold conjugated to goat anti-mouse IgM antibodies (Biocell, Cardiff, United Kingdom) for 30 min. EB were then washed twice in PBS and fixed at room temperature by adding equal volumes of 4% glutaraldehyde and 0.4% picric acid in 0.2 M sodium cacodylate buffer (pH

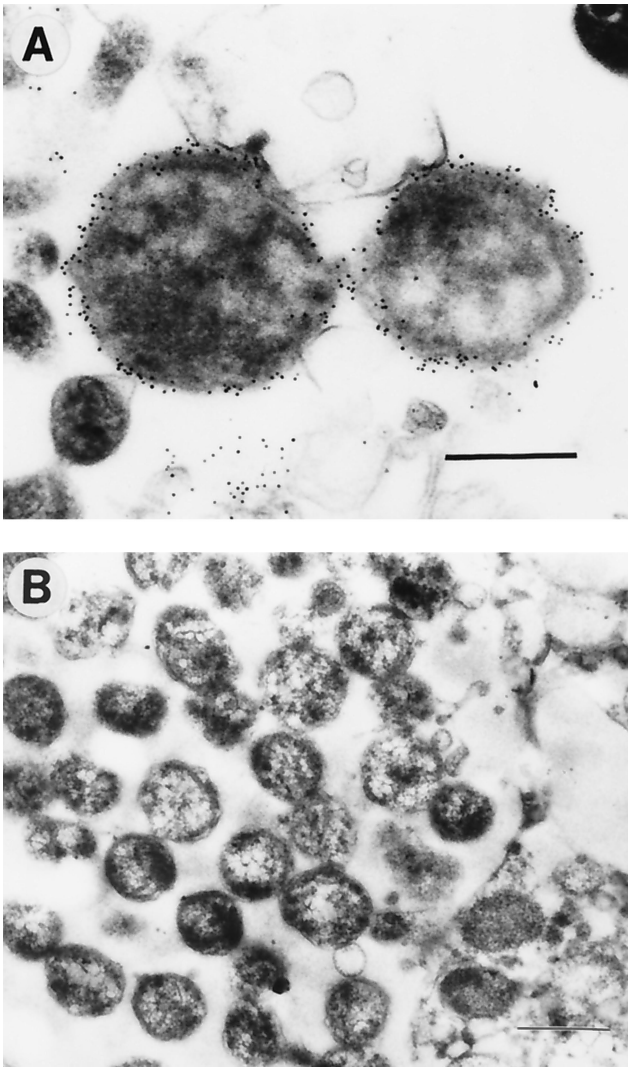

FIG. 2. Electron micrograph of MAb 446.15 binding to *C. ruminantium* EB. (A) Antibody specificity was visualized by the binding of gold-labeled goat anti-mouse immunoglobulin serum to the surface membranes of free EB. Bar = 0.25 μm. (B) No antibody binding was detected using isotype control MAb WM25. Bar = 0.5 μm.

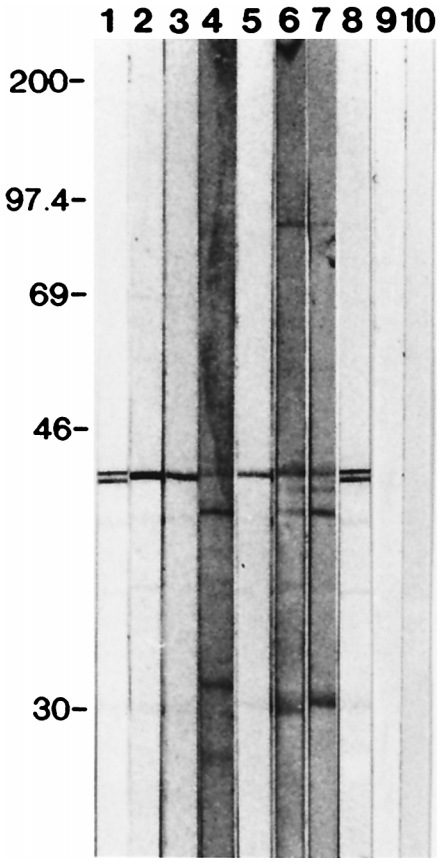

FIG. 3. Immunoblot of lysates from free *C. ruminantium* EB probed with MAb 446.15. *Cowdria* strains were Crystal Spring (lane 1), Welgevonden (lane 2), Ball (lane 3), Umbanein (lane 4), Nigeria (lane 5), Highway (lane 6), Zwimba (lane 7), and Plum Tree (lane 8). MAb 446.15 did not react with *E. canis* antigens (lane 9) or uninfected bovine endothelial cell lysates (lane 10). Values on the left are molecular masses (in kilodaltons).

7.3) for 3 min before washing three more times and postfixing in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Following another three washes, EB were block stained with aqueous 2% uranyl acetate for 4 h and processed with graded acetone into an Epon-araldite resin mix. Ultrathin sections (50 to 70 nm thick) were cut, mounted on copper grids, counterstained with Reynold's lead citrate, and viewed in a Zeiss (Oberkochen, Germany) EMICA. The presence of gold particles on the outer surface of EB further demonstrated that MAb 446.15 bound a surface-exposed epitope on these organisms (Fig. 2A). Similar results were obtained with MAbs 442.3.21 and 443.3.2 (data not shown). There were no significant amounts of gold particles in the control preparations incubated first with isotype control MAb WM25 or PBS followed by the gold-labeled second antibody (Fig. 2B). Immunogold staining for localizing antibody binding to surface antigens has been used previously with *Chlamydia trachomatis*, *C. ruminantium*, and *Babesia bigemina* (10, 13, 24).

Identification of the antigen bound by MAb 446.15 and conservation of the recognized epitope in *C. ruminantium* strains. To identify the antigen recognized by MAb 446.15 and to determine if the epitope is conserved on molecules of similar size, immunoblotting was done. Antigens from culture-adapted *C. ruminantium* from the Crystal Spring (3), Welgevonden (4), Ball 3 (5), Umbanein (6), Nigeria (14), Highway (3), Zwimba (14), and Plum Tree (16) strains were disrupted in lysis buffer containing 1% Nonidet P-40. The proteins were separated by electrophoresis on 7.5 to 17.5% polyacrylamide gels with sodium dodecyl sulfate. High-molecular-weight prestained protein standards (Bethesda Research Laboratories, Bethesda, Md.) were also electrophoresed. After electrophoretic transfer of proteins from the gel to a 0.45-μm-pore-size nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) (28), strips were cut and incubated overnight at room temperature with MAb 446.15 or the isotype control MAb WM25. Bound MAbs were visualized using peroxidase-conjugated goat anti-mouse immunoglobulins diluted 1:1,000 (Cappel, Durham, N.C.). MAb 446.15 bound to proteins from all eight strains tested (Fig. 3, lanes 1 to 8), and in each case a protein of approximately 43 kDa was recognized. However, other proteins were also recognized by MAb 446.15, including a 42-kDa protein in the Crystal Spring and Plum Tree strains (Fig. 3, lanes 1 and 8) and proteins of approximately 30 and 32 to 39 kDa in the Umbanein, Highway, and Zwimba strains (Fig. 3, lanes 4, 6, and 7). There was no reactivity when the isotype control MAb was reacted with proteins from the eight strains. In control reactions, MAb 446.15 did not react with *Ehrlichia canis* antigens loaded at 40 μg/well or with uninfected bovine endothelial cell lysates (Fig. 3, lanes 9 and 10).

One other *C. ruminantium* surface antigen that is widely conserved is major antigenic protein 1 (MAP1), which was originally described as a 32-kDa protein, although it varies in molecular mass, depending on the origin of the *C. ruminantium* isolate (7, 9, 11, 23). A unique region of this protein (MAP1b) is being evaluated in a diagnostic assay for heartwater.

Conclusions. Since MAb 446.15 identifies an epitope that was conserved on a 43-kDa protein in all eight strains tested, this MAb may be useful in a competitive ELISA to detect antibody in infected ruminants, or the epitope may be useful in a direct ELISA to detect antibody. Finally, the identified antigen is exposed on the surface of intact *C. ruminantium* and could serve as a target for an immune response that would prevent infection of host cells or promote EB destruction.

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