INFECTION AND IMMUNITY, Oct. 1989, p. 3243–3246
0019-9567/89/103243-04$02.00/0

Vol. 57, No. 10

Identification of an Immunodominant Antigenically Conserved 32-Kilodalton Protein from *Cowdria ruminantium*

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Western blotting (immunoblotting) of *Cowdria ruminantium* antigens with goat or mouse antiserum identified a periodate-resistant, proteinase K-sensitive immunodominant antigen of 32,000 daltons. This protein, designated Cr32, could be demonstrated in goat choroid plexus infected with one of two different *Cowdria* stocks. Antisera against nine different *Cowdria* stocks from Africa and the Caribbean region recognized Cr32, which indicates that this protein contains conserved antigenic determinants.

Cowdriosis, or heartwater, caused by the tick-borne rickettsial pathogen *Cowdria ruminantium*, is an important infectious disease affecting domestic and wild ruminants (17, 19). The disease is endemic in sub-Saharan Africa and has recently been discovered in the Caribbean region (16), thus posing a threat for livestock on the American mainland (18). *C. ruminantium* stocks differ in antigenic composition, virulence, pathogenicity for mice, serotype, and rickettsial infection level within brain capillaries (4, 10, 19). For the development of improved methods of vaccination against cowdriosis, protective immunogens, which are conserved among the many different *Cowdria* stocks, need to be identified. We have studied *C. ruminantium* antigens by Western blot (immunoblot) analysis and found an antigenically cross-reactive 32,000-dalton *Cowdria* protein.

Nine stocks of *C. ruminantium* were used: one each from Senegal (10), Sudan (Um Banein; 9), Kenya (Kiswani; 11), Nigeria (Ifé), and Guadeloupe (Gardel; 20) and four stocks from South Africa (Ball 3 [8], Kümm [6], Kwanyanga [12], and Welgevonden [4]). All isolates were stored in liquid nitrogen as infected blood stabilates as previously described (19). Polyvalent antisera were raised in goats and mice by intravenous inoculation of thawed blood stabilate followed by antibiotic treatment to protect the animals against an otherwise lethal infection. All animals were challenged after 4 weeks, and sera were collected another 2 weeks later, unless reported otherwise.

Cowdria antigens were obtained from infected choroid plexus and larger brain blood vessels of goats. Blood vessels infected with *Cowdria* species (Senegal and Welgevonden stocks) were dissected from the brains of goats that had succumbed to cowdriosis. The vessels were stored in sucrose-phosphate-glutamate (SPG) buffer (2) at −20°C until used. Before the assay, the material was washed in SPG and ultrasonically disrupted in SPG on ice with a Branson Sonifier in four cycles of 30 s each with intervals of 1 min. The sonically treated material was centrifuged for 10 min at 1,000 × *g*, and the supernatant was centrifuged for 30 min at 20,000 × *g*. The pellet was resuspended in 1.0 ml of SPG and stored at −20°C. Choroid plexus and larger brain blood vessels were also obtained from noninfected control goats. The material was ultrasonically disrupted, and a pellet from the 20,000 × *g* centrifugation was prepared as described above.

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Cowdria extracts were heated at 100°C for 5 min in sample buffer (0.6 M Tris hydrochloride buffer, pH 6.8, containing 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) and then applied onto 7.5 to 20% polyacrylamide gradient gels. Western blotting was performed by a modification of the method of Burnette (3). Electrophoretic transfer was accomplished at 20 V for 16 h followed by 1 h at 60 V. Thereafter, the blots were stained for 20 s in 0.2% Ponceau S in 3% trichloracetic acid, cut into strips, and destained. Blots were incubated in quenching buffer (0.25% gelatin in phosphate-buffered saline) for 1 h, washed in phosphate-buffered saline-Tween 20, and incubated with goat or mouse antiserum diluted 1:100. Blots were again washed, and binding of the antibody was localized with rabbit anti-goat immunoglobulins conjugated with horseradish peroxidase or with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) diluted 1:750 for 80 min. Binding of the conjugate was visualized by immersing the blots for 30 to 60 s into 150 ml of H_2O containing 300 mg of sodium nitroprusside (Sigma Chemical Co., St. Louis, Mo.), 120 mg of O-dianisidine (Sigma), and 100 μl of 30% hydrogen peroxide.

For protein digestion of the antigen (7), several blots were treated with proteinase K (10 mM Tris hydrochloride buffer, pH 7.5, containing 5 mM EDTA, 150 mM NACl, 0.5% sodium dodecyl sulfate, and 200 μg of proteinase K per ml) for 4 h at 37°C before the blots were quenched in 0.25% gelatin–phosphate-buffered saline. For periodate oxidation (7), blots were incubated with 0.05 M $NaIO_4$ in 0.01 M sodium acetate (pH 6.4) for 24 h at 4°C and then washed three times in 0.25% M sucrose–0.01 M Tris hydrochloride (pH 7.2). The blots were then treated with 0.25% gelatin–phosphate-buffered saline and processed as described above.

A predominant 32-kilodalton protein gave a strong signal in extracts that were infected with *Cowdria* (Senegal) stock probed with goat antisera raised against nine different *Cowdria* stocks (Fig. 1, lanes 4 to 12). This protein, designated Cr32 by abbreviations for genus, species, and molecular mass in kilodaltons, was not detected in noninfected extract probed with preimmune serum or *Cowdria* goat antiserum (Fig. 1, lanes 1 and 2) or in *Cowdria* species-infected extract probed with preimmune goat serum (Fig. 1, lane 3). Mouse antisera against two mouse-pathogenic *Cowdria* stocks (Kümm and Kwanyanga) also recognized Cr32 (Fig. 2, lanes 2 to 6), unlike preimmune mouse serum (Fig. 2, lane 1).

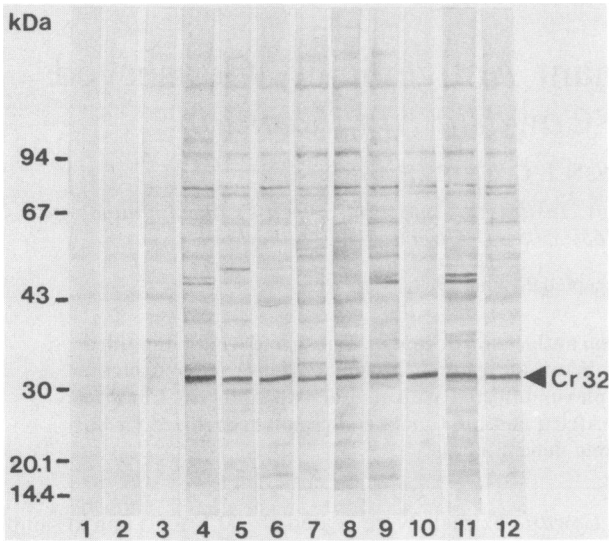

FIG. 1. Western blot analysis of *C. ruminantium* (Senegal) antigen from goat choroid plexus extract probed with nine different *Cowdria* goat antisera. Lane 1, Preimmune serum on noninfected plexus extract; lane 2, Senegal antiserum on noninfected plexus extract; lanes 3 to 11, *Cowdria* species-infected plexus extract with preimmune serum with Senegal, Welgevonden, Ball 3, Kümm, Kwanyanga, Um Banein, Gardel, and Kiswani antisera, respectively. Molecular mass markers in kilodaltons are indicated on the left.

Antibodies were detected for at least 3 months after infection with the Kümm stock and for more than 5 months with the Kwanyanga stock (Fig. 2). Finally, Cr32 in plexus extract infected with *Cowdria* species of another origin (Welgevonden stock) was also detected by all nine goat antisera, of which only six are shown in Fig. 3.

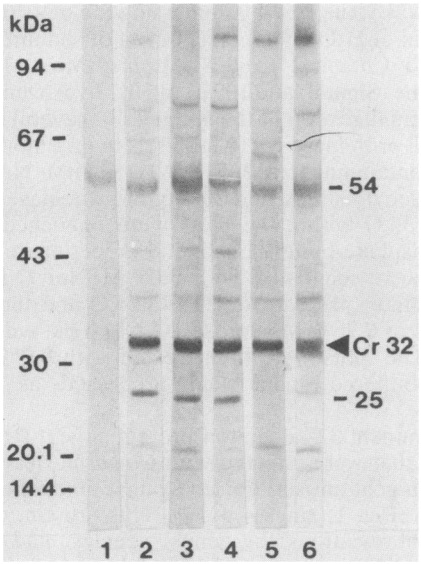

FIG. 2. Western blot analysis of *C. ruminantium* (Senegal) in goat choroid plexus extract probed with mouse antisera. Lanes contained preimmune serum (lane 1); Kümm antisera collected 45, 61, and 94 days postinfection (lanes 2, 3, and 4, respectively); and Kwanyanga antisera collected 44 days postinfection (lane 5) and 163 days postinfection (lane 6). Molecular mass markers in kilodaltons are indicated on the left.

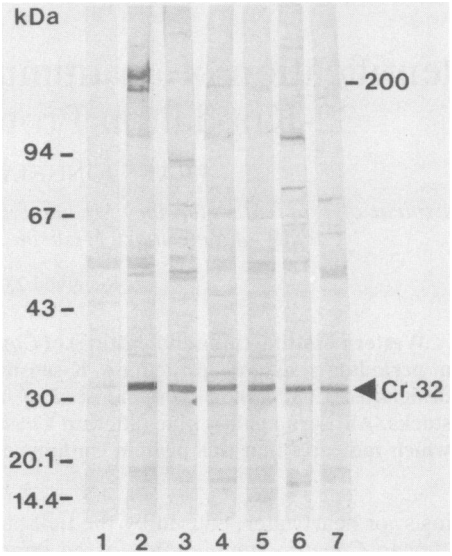

FIG. 3. Western blot analysis of *C. ruminantium* (Welgevonden) in choroid plexus extract analyzed with six different goat antisera. Lanes contained preimmune serum (lane 1) and Welgevonden (lane 2), Senegal (lane 3), Kwanyanga (lane 4), Kümm (lane 5), Um Banein (lane 6), and Ball 3 (lane 7) antisera. Molecular weight markers in kilodaltons are indicated on the left.

Proteinase K digestion of nitrocellulose-bound Cr32 completely abolished antibody binding, unlike oxidation of Cr32 with periodate. This shows that Cr32 epitopes are proteins in nature rather than surface lipopolysaccharides.

The lack of sufficient amounts of *Cowdria* antigen has long been prohibitive for antigenic analysis using Western blotting. Also, in this study, only the Senegal and Welgevonden stocks, with exceptionally high numbers of brain capillaries containing rickettsial organisms (4, 10) (Fig. 4), yielded sufficient antigens. The breakthrough in cultivating *Cowdria* species in endothelial cells (1) will undoubtedly become an excellent alternative. Our preliminary results, with *Cowdria* species-infected bovine umbilical endothelial-cell cultures, show that Cr32 is also the dominant protein in rickettsiae cultivated in vitro.

Serodiagnosis of *Cowdria* infection based on the apparently highly conserved genus-specific Cr32 protein looks promising. Current serological tests for the diagnosis of *Cowdria* infections in ruminants are unsatisfactory because of cross-reactive antigenic determinants with *Ehrlichia* sp. (5) and the occurrence of serotype-specific antibodies (F. Jongejan, L. A. Wassink, M. J. C. Thielemans, N. M. Perie, G. Uilenberg, Vet. Microbiol., in press). It remains to be shown whether Cr32 is genus specific and will not cross-react with antibodies to, for instance, *Ehrlichia* sp. Our preliminary results, however, indicate that antibodies to *Ehrlichia phagocytophila* do not recognize any epitopes on the Cr32 protein.

Cr32 appears to bear antigenic determinants which are shared by all nine *Cowdria* isolates tested thus far, which were collected from geographically widely separated areas on the African continent and from the Caribbean region. Five of these nine stocks were antigenically distinct on the basis of protective cross-immunity trials in goats, whereas the remaining four were fully cross-protective with reference

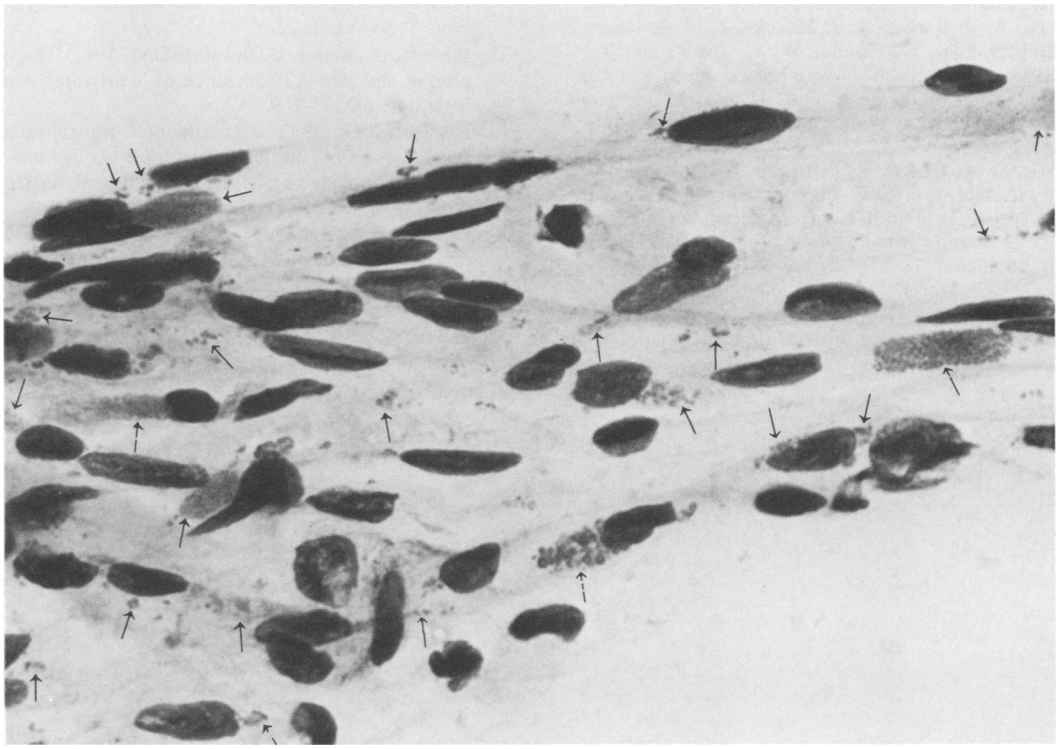

FIG. 4. Brain squash smear made from the cerebral cortex of a goat that had died from cowdriosis. The number of rickettsial colonies within the capillary endothelial cells is exceptionally high, which is typical for this Senegalese *Cowdria* isolate. Twenty-five colonies are indicated (arrows). Giemsa stain. Magnification, ×960.

stock Ball 3 (10, 19, 20). For instance, goats immune to *Cowdria* (Senegal) infection were susceptible to challenge with Welgevonden (F. Jongejan and M. J. C. Thielemans, unpublished data). Cr32 seems less important as a protective immunogen, since both Senegal and Welgevonden stocks contain the Cr32 protein. However, in recent studies on another tick-borne rickettsial pathogen, *Anaplasma marginale*, two surface proteins of 36 kilodaltons (15) and 105 kilodaltons (13) which are highly conserved among antigenically distinct *Anaplasma* isolates from Israel, Kenya, and the United States (14) have been identified. Nevertheless, immunization with the 36-kilodalton surface protein induced protection against both homologous and heterologous *Anaplasma marginale* challenge in cattle (15). This indicates the potential cross-protective ability of conserved surface proteins, which are common to antigenically different isolates.

We are presently raising monoclonal antibodies against *Cowdria* species-specific antigenic determinants. Monoclonal immunoaffinity chromatography of Cr32 would enable us to examine the potential immunoprophylactic and diagnostic values of this dominant protein.

This work was supported by a grant from the European Community (Directorate General XII) under contract TSD-A-249.

We thank Ben van der Zeijst, Evert Hensen, and Gerrit Uilenberg for encouragement and critical comments on the manuscript and J. Nieuwenhuijs for technical assistance.

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