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Detection of *Ehrlichia chaffeensis* in Human Tissue by Using a Species-Specific Monoclonal Antibody

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A mouse monoclonal antibody (MAb 1A9) was produced and used in detection of *Ehrlichia chaffeensis* in human tissues including kidney, liver, and lung by using an indirect immunohistologic stain. MAb 1A9 was specific to *E. chaffeensis* and did not react with other bacteria, including *Ehrlichia canis*, which is the organism most closely related to *E. chaffeensis*. It reacted with an epitope present in two surface proteins of *E. chaffeensis* with molecular masses of 29 and 27 kDa. *E. chaffeensis* was easily detected in human tissue by immunohistology with MAb 1A9. This study demonstrates that our MAb can provide a specific and simple method for detection of *E. chaffeensis* in clinical specimens for establishing an etiologic diagnosis of human ehrlichiosis; it may also provide a tool for the investigation of immunopathologic characteristics in infected patients.

Ehrlichiae are members of the family *Rickettsiaceae* and are obligate intracellular bacteria that infect mammalian hematopoietic cells (24). Most species of *Ehrlichia* are known as animal pathogens. Previously, *Ehrlichia sennetsu* was the only known *Ehrlichia* sp. causing human infection (24). A new human disease, human ehrlichiosis, caused by an *Ehrlichia* species was identified in the United States (16, 19) and possibly in Europe (20). The disease is characterized as a systemic febrile illness with headache, myalgia, and gastrointestinal symptoms and is associated with leukopenia, thrombocytopenia, and mild to moderate hepatitis (14, 15). *Ehrlichia chaffeensis* was isolated from the blood of a patient with ehrlichiosis (7) and is proposed as the etiologic agent of human ehrlichiosis (1). The 16S rRNA gene sequence comparisons indicate that *E. chaffeensis* is most closely related to *Ehrlichia canis*, *Ehrlichia ewingii*, and *Cowdria ruminantium* but is more distantly related to the other *Ehrlichia* spp. (1, 2, 25). The vector of *E. chaffeensis* is not known. Ticks are strongly suspected to be the vectors and hosts of *E. chaffeensis* since most patients had histories of tick exposure prior to the onset of the symptoms (15, 16, 19, 21) and because of the recent report of polymerase chain reaction amplification of *E. chaffeensis* DNA in a *Dermacentor variabilis* tick (3). The diagnosis of human ehrlichiosis mainly depends on seroconversion to *E. canis* or *E. chaffeensis* in convalescence (8, 14). In this study, a species-specific monoclonal antibody (MAb) to *E. chaffeensis* was produced and used in detection of *E. chaffeensis* in human tissue including kidney, liver, and lung.

MATERIALS AND METHODS

Cultivation of bacteria. *E. chaffeensis* and *E. canis* were cultivated in DH82 cells (9). *Ehrlichia risticii* and *E. sennetsu* were cultivated in mouse macrophage (ATCC P388D1) cells (23). *Cowdria ruminantium* grown in bovine endothelial cells was a gift of Patrick J. Kelly (University of Zimbabwe, Harare, Zimbabwe). *Chlamydia* spp. and *Coxiella burnetii* were cultivated in L929 cells. *Afipia* spp. and *Rochalimaea* spp. were cultivated on tryptic soy agar supplemented with 5% defibrinated sheep blood (22). *Afipia* spp. were incubated at 30°C, and *Rochalimaea* spp. were cultivated in 95% CO_2 atmosphere at 37°C. Other bacteria were cultivated on blood agar at 37°C.

MAb production. Eight-week-old female BALB/c mice were immunized with *E. chaffeensis*-infected cells. *E. chaffeensis* was inactivated in 0.1% formaldehyde at 4°C overnight prior to immunization. Mice were immunized with *E. chaffeensis* intraperitoneally three times at 1-week intervals. In the third week after immunization, the mice were boosted by injection into the tail vein. After 72 h, collected splenocytes were fused with SP 2/0 Ag-14 cells with polyethylene glycol (molecular weight, 1,450; Sigma Chemical Co., St. Louis, Mo.) as previously described (17).

Indirect immunofluorescence assay (IFA) was used to screen hybridoma clones and test the specificity of the MAb. In brief, the antigens were dotted onto the slides by using a pen. Each dot contained one species of bacteria, and each well of the slides contained four dots. The antigens were fixed in acetone for 10 min. The antigens were incubated with 20 μl of hybridoma culture supernatant (for screening hybridoma clones) or 20 μl of diluted mouse ascites (for testing the specificity of the MAb) for 30 min at 37°C. The slides were rinsed once with phosphate-buffered saline (PBS), immersed in PBS for 10 min, and then rinsed with distilled water. The slides were dried and incubated with 20 μl of fluorescein (dichlorotriazin-amino-fluorescein)-conjugated goat anti-mouse immunoglobulin G (IgG), IgA, and IgM (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.; diluted 1:100) at 37°C for 30 min. The slides were washed as described above and dried. The slides were mounted with coverslips and examined by using a UV light microscope with ×400 magnification. The strategy for screening hybridoma clones was designed to select the species-specific MAb to *E. chaffeensis*. Thus, the supernatant of each hybridoma clone was tested by using antigens of *E. chaffeensis*, *E. canis*, *E. risticii*, and *E. sennetsu* simultaneously, and the hybridoma clones secreting antibodies reacting with only *E. chaffeensis* were chosen.

The specificity of the MAb was tested by using IFA with

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the following bacteria: *E. canis*, *E. risticii*, *E. sennetsu*, *Cowdria ruminantium*, *Afipia felis* (ATCC 49714, ATCC 49715, and ATCC 49716), *Afipia broomeae* (ATCC 49717, ATCC 49718, and ATCC 49719), *Afipia clevelandensis* (ATCC 49720), *Afipia* sp. ATCC 49721, *Rochalimaea henselae*, *Rochalimaea quintana*, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae*, *Coxiella burnetii* (nine-mile strain) phase I and phase II, *Rickettsia rickettsii*, *Rickettsia typhi*, *Rickettsia prowazekii*, *Acinetobacter lwoffii*, *Citrobacter diversus*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Pseudomonas maltophilia*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. The MAb was also tested by an immunoalkaline phosphatase technique with paraffin-embedded *E. canis*- and *E. ewingii*-infected dog tissue; *E. sennetsu*-infected mouse tissue; and *Rickettsia rickettsii*-, *Rickettsia typhi*-, *Coxiella burnetii*-, and *Chlamydia trachomatis*-infected human tissue, as well as acetone-fixed *Ehrlichia phagocytophila*-infected bovine buffy coat blood (courtesy of G. Liz, University of Neuchatel, Neuchatel, Switzerland).

Isotyping of MAbs was determined by double immunodiffusion with a MAb typing kit (Sigma).

Purification of *E. chaffeensis*. *E. chaffeensis* was purified according to the method described previously (5). Briefly, *E. chaffeensis*-infected cells were mechanically disrupted, and cell debris was removed by centrifugation at 150 × *g* for 10 min. Ehrlichiae in the supernatant were harvested by centrifugation at 7,000 × *g* through a 25% sucrose gradient.

Western blot (immunoblot). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-Protein II vertical electrophoresis cell (Bio-Rad) with a 4% stacking gel and a 12.5% separating gel (18). The purified *E. chaffeensis* samples were solubilized in sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% sucrose, 0.002% bromophenol blue in 0.0625 M Tris, pH 6.8). The samples used were either at ambient temperature or boiled for 5 min. Proteinase K-digested *E. chaffeensis* was also used to characterize the protein nature of the epitope recognized by the MAb. Purified *E. chaffeensis* was digested with proteinase K in 0.1 M Tris (pH 7.8)–0.5% SDS at 56°C for 1.5 h, and then 1 volume of 2× sample buffer was added and boiled for 5 min. Purified *E. canis* was grown in duplicate cultures of the same type of cell line as *E. chaffeensis*, used as a negative control, and treated the same as the samples of *E. chaffeensis*. SDS-PAGE and Western blot were performed at room temperature. Twenty-five micrograms of protein of each sample prepared as described above was loaded into each lane of the gel, run at 30 mA, and transferred to nitrocellulose membranes at 60 mA for 1 h. The membranes were blocked in 5% nonfat milk in PBS overnight and then incubated with mouse ascites at a 1:200 dilution in 1% nonfat milk in PBS for 1 h. After being washed with Tris-buffered saline, the membranes were incubated with peroxidase-labelled goat anti-mouse IgG and IgM conjugate (Immunotech, Marseille, France; diluted 1:200). Bound antibodies were detected by using 4-chloro-1-naphthol (Sigma).

Immunoelectron microscopy. Immunoelectron microscopy was performed as previously described (4). One microliter of purified *E. chaffeensis* was placed on 300-mesh copper grids coated with Formvar (Sigma) and allowed to dry. Grids were blocked with 1% bovine serum albumin in PBS for 15 min and then sequentially incubated with MAb (diluted 1:1,000 in 1% bovine serum albumin in PBS); biotin-labelled goat anti-mouse IgG, IgM, and IgA (Sigma; diluted 1:1,000); and streptavidin colloidal gold (Sigma; diluted 1:100), each for 30 min at room temperature. The grids were washed three times with 1% bovine serum albumin in PBS after each step and examined with a JEOL 1200 electron microscope for the presence and location of colloidal gold. Mouse ascites produced by SP 2/0 Ag-14 cells were used as negative controls.

Immunohistologic detection of *E. chaffeensis* in human tissue. Human kidney, liver, and lung autopsy tissues were obtained from two patients with human ehrlichiosis, and case reports are presented elsewhere (9a, 13). In brief, the first patient died after corticosteroid therapy for presumed thrombotic thrombocytopenic purpura and an intracerebral hemorrhage, and the second patient died after a prolonged febrile illness terminating in multiple secondary bacterial, viral, and fungal infections. Immunoalkaline phosphatase labelling of *E. chaffeensis* in human tissue was performed as previously described (6). The experimental procedures were performed at room temperature except where the temperature is noted. Four-micrometer tissue sections were cut on silane-coated slides (Sigma) and heated to 45°C for 30 min prior to being deparaffinized in xylene as described elsewhere (12). The slides were then rehydrated in three baths of decreasing concentrations of methanol (100, 95, and 80%) for 2 min each time and then rinsed in PBS (pH 7.4) for 5 min. The slides were then blocked for 10 min in a humidity chamber in 5% normal goat serum (Sigma)–0.5% nonfat dried milk in PBS (PBSM). Excess blocking solution was drained, and the slides were incubated in a humidity chamber with the mouse anti-*E. chaffeensis* MAb diluted 1:1,000 in PBSM-normal goat serum buffer for 1 h. The slides were rinsed twice in PBS and washed for 5 min prior to incubation in biotinylated goat anti-mouse IgM (Vector Laboratories) diluted 1:100 in PBSM-normal goat serum buffer for 30 min. After two additional rinses and a 5-min wash in PBS, the slides were incubated in a 1/100 dilution of alkaline phosphatase-conjugated streptavidin (Dako) in PBSM for 30 min. The slides were rinsed as described above and incubated for 10 min in fast red-phosphate chromogen solution (Sigma), washed in running water, counterstained with Mayer's hematoxylin for 10 min, blued in Scott's solution for 10 s, washed in water, covered with Crystal Mount (BioMeda, Foster City, Calif.), air dried, and mounted with glass coverslips. Negative controls were performed by using mouse anti-*Afipia felis* MAb (26).

RESULTS

MAb characterization. The hybridoma clone designated 1A9 produced a MAb which reacted by IFA with *E. chaffeensis* but not with *E. canis*, *E. risticii*, or *E. sennetsu* and was selected and cloned by limiting dilution. The isotype of MAb 1A9 was determined as IgM by double immunodiffusion. The specificity of MAb 1A9 was further tested by using mouse ascites. IFA demonstrated that MAb 1A9 reacted with none of the test bacteria at a titer of 1:8, although homologous antigen reaction could reach a titer of 1:32,768.

Western blot. MAb 1A9 reacted with both native and heat-denatured antigens of *E. chaffeensis* but did not react with proteinase K-digested *E. chaffeensis* antigen (Fig. 1). This strongly suggested that MAb 1A9 reacted with a protein antigenic epitope rather than with lipopolysaccharide. The patterns of MAb 1A9 reacted with heat-denatured and native antigens of *E. chaffeensis* were different (Fig. 1). MAb 1A9 reacted with two bands of the heat-denatured antigen of

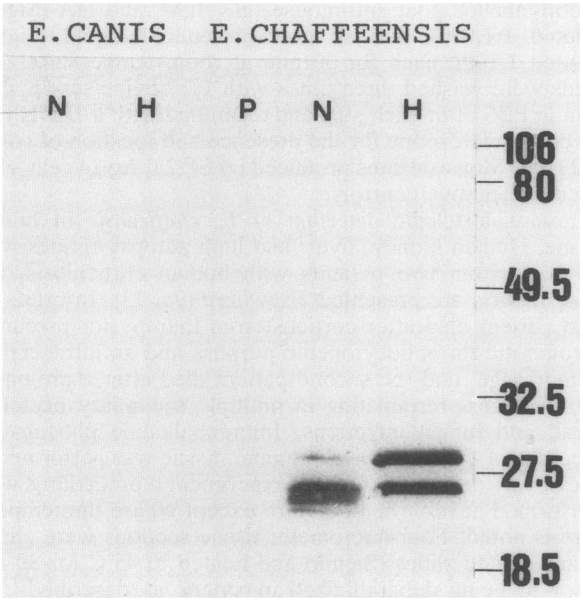

FIG. 1. Western blot of *E. chaffeensis* and *E. canis* antigens reacted with MAb 1A9. N, native antigen; H, heated antigen; P, proteinase K-digested antigen.

E. chaffeensis with molecular masses of 29 and 27 kDa. However, it reacted with four bands of native antigen of *E. chaffeensis*; two of them corresponded to the 29- and 27-kDa heat-denatured proteins, and two other protein bands with molecular masses of 26 and 25 kDa were unique to native samples. MAb 1A9 did not react with *E. canis* antigen and confirmed the results of IFA.

Immunoelectron microscopy. The *E. chaffeensis* cells incubated with MAb 1A9 were significantly labelled with colloidal gold (Fig. 2). However, very few gold beads associated with *E. chaffeensis* incubated with mouse ascites produced by nonfused SP 2/0 Ag-14 cells (data not shown). This electron microscopic appearance suggested that the proteins of *E. chaffeensis* recognized by MAb 1A9 were surface associated.

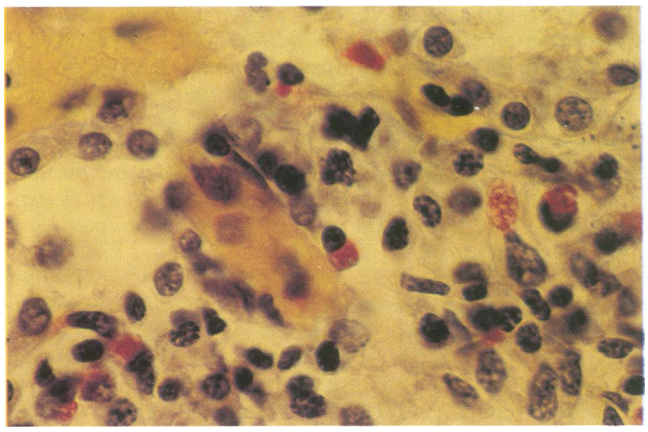

FIG. 3. Immunohistologic stain of human kidney with mouse anti-*E. chaffeensis* MAb. *E. chaffeensis* morulae appear as red aggregates of small bacteria within the cytoplasm of macrophages and mononuclear cells between kidney tubules (magnification, ca. ×2,940).

Evaluation of MAb in detection of *E. chaffeensis* in human tissue by immunohistologic stain. *E. chaffeensis* was easily detected within mononuclear cells of the human kidney, liver, and lung by using mouse anti-*E. chaffeensis* MAb (Fig. 3). *E. canis*, *E. ewingii*, *E. sennetsu*, *E. phagocytophila*, *Rickettsia rickettsii*, *Rickettsia typhi*, *Coxiella burnetii*, and *Chlamydia trachomatis* were not detected in infected dog, mouse, or human tissues or in bovine neutrophils by mouse anti-*E. chaffeensis* MAb, whereas the *E. canis* was clearly demonstrated by using a polyclonal antiserum obtained from a patient convalescing from human ehrlichiosis (10, 11).

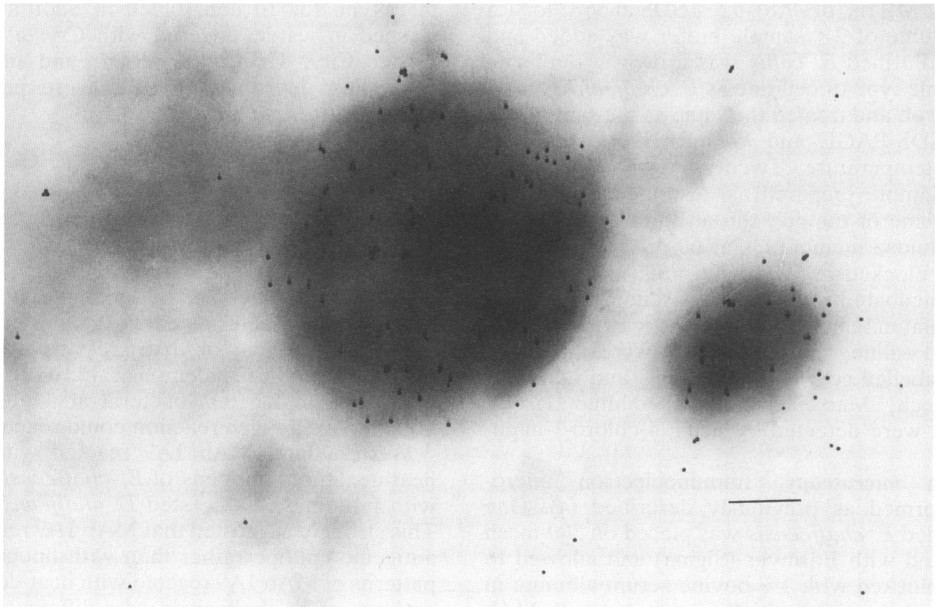

FIG. 2. Electron micrographs of immunogold-coated *E. chaffeensis* reacting with MAb 1A9. Bar, 0.2 μm.

DISCUSSION

E. chaffeensis is the agent of the newly described human ehrlichiosis in the United States (16, 19) and probably in Europe (20). This bacterium exhibits wide cross-reactions with *E. canis* (7) and *Cowdria ruminantium* (17a), which are very closely phylogenetically related as determined by 16S rRNA sequencing (3, 25). In fact, human ehrlichiosis was considered first as an *E. canis* infection (19). Isolation of *E. chaffeensis* is extremely difficult (7), and the diagnosis of human ehrlichiosis depends on seroconversion to *E. chaffeensis* or *E. canis* in convalescence (8, 14). A recent study demonstrated that *E. chaffeensis* was easily detected in human tissues by an immunohistologic stain with human convalescent serum (11). Moreover, the specificity of our MAb reaction in fixed tissues from human kidney, liver, and lung and from other mammals, including an *E. ewingii*-infected dog and *E. phagocytophila*-infected cattle, has been verified (13). Immunohistologic demonstration of *E. chaffeensis* in human tissue offers a direct means for establishing the etiologic diagnosis. We believed that MAb should be more specific and sensitive than polyclonal antibodies in detection of *E. chaffeensis* in human tissue. Our MAb is specific to *E. chaffeensis* because it reacted with *E. chaffeensis* and did not cross-react with *E. canis*, *E. risticii*, *E. sennetsu*, and *Cowdria ruminantium* among other rickettsiae and intracellular pathogens of human importance by IFA. It also did not react with *E. ewingii* in infected dog tissues or with *E. phagocytophila* in infected bovine buffy coat blood by this immunohistologic stain. MAb 1A9 recognized an epitope which is present on several proteins, including two which are heat labile. These proteins could be candidates for a specific serodiagnostic test for *E. chaffeensis* infection. Moreover, these proteins are apparently surface exposed, are specific for *E. chaffeensis*, and differentiate it from *E. canis* and *E. ewingii*, which are pathogenic only for dogs, and from *Cowdria ruminantium*, which is pathogenic for cattle (24).

The recent introduction of a polymerase chain reaction using oligonucleotide primers specific for *E. chaffeensis* on fresh peripheral blood or frozen clotted blood has allowed a method for rapid diagnosis of human ehrlichiosis. Unfortunately, this method, in spite of its capability for extreme sensitivity, is available only in specialized research facilities and may suffer from frequent false-positive reactions because of extraneous DNA contamination. Immunohistology is a well-established method for the etiologic diagnosis of infectious diseases in routinely obtained tissue. Moreover, this uniform and easy methodology for rapid confirmation of human ehrlichiosis is achievable in standard laboratories. The sensitivity of this MAb method for immunohistology is still undetermined. The immunohistologic method for demonstration of *E. chaffeensis* in paraffin-embedded, formalin-fixed human tissue is useful for establishing a specific and rapid diagnosis in some cases of human ehrlichiosis.

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