JOURNAL OF CLINICAL MICROBIOLOGY, July 1995, p. 1704–1711 Vol. 33, No. 7
0095-1137/95/$04.00+0

Isolation and Characterization of a New Strain of *Ehrlichia chaffeensis* from a Patient with Nearly Fatal Monocytic Ehrlichiosis

J. STEPHEN DUMLER,[1]* SHENG-MIN CHEN,[2] KRISTIN ASANOVICH,[1]
ELLEN TRIGIANI,[1] VSEVOLD L. POPOV,[2] AND DAVID H. WALKER[2]

Department of Pathology, University of Maryland Medical Center, Baltimore, Maryland 21201,[1] and Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0609[2]

Received 13 February 1995/Returned for modification 15 March 1995/Accepted 31 March 1995

***Ehrlichia chaffeensis* is the causative agent of human monocytic ehrlichiosis, a disease that ranges in severity from asymptomatic infection to death. Only one isolate of *E. chaffeensis* has been made, the Arkansas strain, upon which all characterizations of the agent of human monocytic ehrlichiosis have been based. We report the isolation and characterization of a new strain of *E. chaffeensis*, the 91HE17 strain, which was cultivated from a patient with a nearly fatal illness. The new isolate grows best in culture with careful control of pH. The two isolates are nearly identical as determined by light and electron microscopy and have significant antigenic identity in fluorescent-antibody and immunoblot assays using polyclonal antisera and the *E. chaffeensis*-specific monoclonal antibody 1A9. Isolate 91HE17 had 99.9% nucleotide sequence identity with the Arkansas strain in the 16S rRNA gene. Parts of the *Escherichia coli* GroE operon homologs had identical restriction enzyme digestion patterns, and a 425-bp region of the GroEL gene had at least 99.8% sequence identity between the *E. chaffeensis* Arkansas and 91HE17 strains. Isolate 91HE17 lacked an epitope identified in *E. chaffeensis* Arkansas by the monoclonal antibody 6A1. This new *E. chaffeensis* isolate is very similar to the Arkansas strain and provides the opportunity to substantiate the existence of diversity among ehrlichiae which infect humans. Specific factors which differ among strains may then be compared to assess their potential contributions toward cellular pathogenicity and ultimately toward the development of disease in humans.**

Human ehrlichial infections are increasingly recognized in the United States and worldwide. Ehrlichiae that cause human disease include the mononuclear phagocyte pathogens *Ehrlichia sennetsu* and *E. chaffeensis* and a granulocytic ehrlichia closely related to *E. phagocytophila* and *E. equi* (1, 6, 25). Human monocytic ehrlichiosis in the United States appears to be caused by *E. chaffeensis* (2, 16). Many cases of monocytic ehrlichiosis are now identified, but only one isolate of *E. chaffeensis* has been reported (10). Thus, all investigations and serologic confirmation of infection by *E. chaffeensis* depend on the use of a single strain (10, 18). This isolate, the Arkansas strain, was obtained from a military recruit with mild signs and symptoms (10). Monocytic ehrlichiosis has a wide spectrum of clinical findings, ranging from asymptomatic infections to severe or fatal disease (12, 17, 18, 23). The pathogenetic mechanisms of human monocytic ehrlichiosis are not well understood and may relate to differences in virulence of ehrlichial strains, variable host responses to infection by *E. chaffeensis*, or both.

We isolated an *Ehrlichia* strain from a patient with nearly fatal disease and meningeal involvement. The new ehrlichia is closely related to the *E. chaffeensis* Arkansas strain as determined by morphologic, genetic, and antigenic analyses and thus represents only the second strain of *E. chaffeensis* isolated. Although most data support the new isolate as a variant strain of *E. chaffeensis*, the two strains have different metabolic requirements for cultivation, and an epitope present in the *E. chaffeensis* Arkansas strain is absent in the 91HE17 strain. Whether these differences in biologic behavior and antigenicity are associated with the greater severity of illness noted in the infected patient is unknown. The new *E. chaffeensis* strain offers an opportunity for comparative analysis to determine the degree of diversity between these two strains.

* Corresponding author.

MATERIALS AND METHODS

Patient and culture. The clinical case for the patient in this study has been previously reported (14). Briefly, a 72-year-old man developed respiratory distress, acute renal failure, and severe lethargy associated with thrombocytopenia, elevations in serum aspartate transaminase concentration, and cerebrospinal fluid (CSF) mononuclear cell pleocytosis. The diagnosis was confirmed by the immunocytologic demonstration of *E. chaffeensis* in morulae in CSF mononuclear cells, PCR amplification of *E. chaffeensis* 16S rRNA genes in CSF and blood, and serology. After doxycycline therapy, the patient defervesced rapidly, had improved sensorium, and recovered completely after 4.5 weeks of hospitalization.

A sample of clotted blood obtained 2 days prior to antibiotic therapy was stored at 4°C. One milliliter of CSF was also saved from before therapy, and both samples were delivered on ice to the University of Texas Medical Branch, Galveston. Upon receipt, 2 ml of clotted blood was removed from below the serum separator gel by sterile technique and homogenized in tissue culture medium (Eagle minimal essential medium [EMEM] supplemented with 10% fetal bovine serum [FBS], 2 mM L-glutamine, and 0.075% bicarbonate) with approximately 50 strokes in a Dounce homogenizer. The resulting homogenate and 1 ml of CSF diluted in 2 ml of tissue culture medium were separately overlaid onto 25-cm^2 tissue culture flasks which contained confluent layers of DH82 canine histiocyte cells (generously provided by Jacqueline Dawson, Centers for Disease Control and Prevention [CDC], Atlanta, Ga.). The samples were incubated with the DH82 cells at 37°C for 3 h with gentle rocking. After this incubation, the inocula were removed, and the cell layers were gently rinsed once with tissue culture medium to remove nonadherent cells and blood components. Our previous experience with whole homogenized blood showed that approximately half of the attached DH82 cells would become dislodged. Thus, the inocula were then replaced with 5 ml of tissue culture medium supplemented with approximately 10^7 uninfected DH82 cells to maintain confluency, and the culture flasks were sealed tightly in ambient air and returned to the incubator. The medium was changed twice each week, and the cells were examined for the presence of morulae weekly by staining with LeukoStat (Fisher Scientific, Houston, Tex.). If morula-like structures were identified, immunocytologic methods

using biotinylated human anti-*E. chaffeensis* were used (12–14). *E. chaffeensis* Arkansas strain (generously provided by Jacqueline Dawson) was propagated in DH82 cells as previously described (10).

Infected cells were passaged onto three flasks containing uninfected confluent DH82 cells. To determine optimal culture conditions, 10^6 DH82 cells containing 0.5% infected cells were incubated in 24-well plates with 1 ml of tissue culture medium supplemented with various concentrations of FBS or L-glutamine with 0.22% bicarbonate in a humidified atmosphere with 5% CO_2. As control, 25-cm^2 flasks newly seeded with the 0.5% isolate 91HE17-infected DH82 cells were incubated in EMEM supplemented with 10% FBS, 2 mM L-glutamine, and 0.075% bicarbonate and were maintained in the ambient air of the tightly sealed flask at 37°C.

Immunocytology and immunofluorescence. Immunocytologic confirmation of infection was performed by a modification of a previously described method (12, 14). Briefly, cells in the supernatants of 91HE17 strain-infected cultures were cytocentrifuged onto glass slides and fixed for 10 min in cold acetone. These slides were incubated with biotinylated human anti-*E. chaffeensis* globulin, biotinylated normal human globulin, biotinylated canine anti-*Ehrlichia canis* globulin, or biotinylated normal dog globulin, then reacted with fast red-naphthol phosphate substrate, and counterstained with Mayer's hematoxylin and examined by light microscopy. The *E. chaffeensis*-specific monoclonal antibody 1A9 was used similarly except that detection of bound antibody with biotinylated anti-mouse immunoglobulin M (IgM) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added before the streptavidin-alkaline phosphatase step (30). Monoclonal antibodies were also tested by an indirect fluorescent-antibody assay as described above except that bound antibody was detected with fluorescein-labeled anti-mouse IgG or IgM (Kirkegaard & Perry Laboratories) and then stained with Evans blue counterstain and examined by fluorescent microscopy.

Electron microscopy. Confluent layers of DH82 cells infected with the 91HE17 isolate were fixed in situ with a mixture of 1.25% formaldehyde, 2.5% glutaraldehyde, and 0.03% trinitrophenol in 0.05 M cacodylate buffer, pH 7.3 (19). After fixation, cells were scraped from the flask in 0.1 M cacodylate buffer, pelleted by centrifugation, and further processed as a pellet as previously described (9). Grids were examined in a Philips 201 electron microscope.

Density gradient purification of ehrlichiae. After approximately 95% of the DH82 cells contained ehrlichiae, all cells from 6 to 24 150-cm^2 flasks were removed by scraping. The infected cells were pooled and centrifuged at 13,000 × *g* for 20 min at 4°C to pellet all cells and any free ehrlichiae. The pellets were then resuspended in 10 ml of sucrose phosphate glutamine buffer (SPGn) (200 mM sucrose, 50 mM potassium phosphate, 1 mM glutamine buffer; pH 7.4), placed on ice, and lysed by sonication (Branson Sonifier; low setting for 1 to 2 min). Lysis was monitored by examining LeukoStat-stained smears for intact cells, and if identified, sonication for an additional 30 s to 1 min was performed. The cell debris was removed by centrifugation at 1,500 × *g* for 10 min at 4°C, the supernatant was mixed with DNase I and RNase A (GIBCO BRL, Bethesda, Md.), both at a final concentration of 50 μg/ml, and the mixture was incubated for 45 min at 37°C. The resulting suspension was overlaid onto a cushion of 30% diatrizoate meglumine and ultracentrifuged at 87,000 × *g* for 75 min at 4°C. The pellet was washed three times in SPGn, assessed for the presence of individual ehrlichiae and host cell contamination by LeukoStat staining, and assayed for protein concentration by the micro-bicinchoninic acid method (Pierce, Rockford, Ill.). Aliquots were adjusted to 2 mg of protein per ml and stored in 1-ml volumes at −70°C. An uninfected DH82 cell control was prepared similarly; however, since no pellet could be obtained after ultracentrifugation, that step was omitted, and DH82 cell sonicates were used.

PCR and sequence analyses. DNAs from *E. chaffeensis* Arkansas strain and isolate 91HE17 were extracted at different times in a laminar flow biohazard containment cabinet, and reagents, PCR master mixes, and other pre-PCR manipulations were prepared and performed in separate containment cabinets. The PCR amplification was carried out in a separate laboratory, and all agarose gel electrophoresis was performed in another separate post-PCR laboratory. Post-PCR samples were never allowed in the pre-PCR area, and dedicated instruments were available for both pre- and post-PCR handling of samples.

Preparation of DNA templates. Approximately 10^7 DH82 cells infected with the 91HE17 isolate were harvested from cell cultures when 90 to 95% contained morulae. The cells were centrifuged at 1,500 × *g* for 3 min in a microcentrifuge, and the pellet was then resuspended in lysing buffer (10 mM Tris-HCl–1 mM EDTA [pH 8.0] supplemented with 1% sodium dodecyl sulfate [SDS] and 100 μg of proteinase K per ml). After incubation at 37°C overnight, the sample was boiled for 5 min to inactivate the proteinase K and centrifuged at 16,000 × *g* for 5 min to pellet the insoluble material. The DNA in lysing buffer was purified by phenol-chloroform-isoamyl alcohol extraction followed by precipitation with 3 M sodium acetate and cold absolute ethanol, drying, and resuspension in sterile, deionized water (1).

***E. chaffeensis* 16S rRNA gene-specific primers.** A modified PCR using primers specific for *E. chaffeensis* was performed as previously described using primers HE1 and HE3 (2). Amplification was performed on an automated thermal cycler for 3 cycles of 94°C for 2 min, 48°C for 1 min, and 68°C for 4 min and then 37 cycles of 90°C for 2 min, 52°C for 1 min, and 68°C for 4 min. Amplified products were separated by electrophoresis in 1% agarose gels and stained with ethidium bromide. Each PCR amplification included an *E. chaffeensis* Arkansas strain cell culture control, an uninfected DH82 cell DNA control, and a negative control with all reagents except with PCR reagent water substituted for DNA template.

Universal eubacterial primers. In order to amplify the 16S rRNA gene for comparative sequence analysis, universal eubacterial primers were used as previously described (1, 29). The same DNA template prepared for amplification of isolate 91HE17 DNA using *E. chaffeensis* Arkansas strain primers and the same cycling parameters were used except that combinations of universal eubacterial primers were substituted. To avoid amplification of extraneous or contaminating eubacterial rRNA genes present in reagents, the master mix was modified to contain 25 μg of 8-methoxypsoralen per ml and was UV irradiated by placing the tube with the master mix on a transilluminator (Foto/UV-15; Fotodyne, Hartland, Wis.) for 6 min (22). Three pairs of these universal primers were used to amplify part or all of the 16S rRNA gene for subsequent sequencing. The pair EC9 and EC10 amplifies a 733-bp fragment on the 3′ end, EC11 and EC12 amplify a 767-bp fragment on the 5′ end, and EC9 and EC12 amplify a 1,474-bp fragment comprising nearly the entire length of the 16S rRNA gene (1). Each reaction included *E. chaffeensis* Arkansas strain genomic DNA as a positive control and PCR reagent water as a negative control. Freshly amplified PCR products were cloned into a plasmid vector for simple sequence analysis (TA cloning kit; Invitrogen, San Diego, Calif.). Transformed clones containing inserts of the correct sizes corresponding to the 3′ end, 5′ end, and nearly the entire gene were selected after rapid alkaline lysis (15) followed by *Eco*RI digestion.

Partial amplification of the GroE operon and restriction enzyme fragment analysis. Gene segments of the *E. chaffeensis* Arkansas strain homologs of the *Escherichia coli* GroEL and GroES protein genes were amplified by PCR (26). The primer pair p15-*Bam*3–p15-*Xba*2 amplifies a 286-bp fragment containing most of the 3′ end of the noncoding intergenic region between the GroES and GroEL genes and part of the 5′ end of the GroEL homolog in *E. chaffeensis* Arkansas. The primer pair p15-*Bam*–p15-*Xba* amplifies a 470-bp fragment of the *E. chaffeensis* GroEL gene homolog. The PCR was performed as described for amplification of the 16S rRNA gene. Each reaction included *E. chaffeensis* Arkansas strain genomic DNA as a positive control and an uninfected DH82 cell DNA control and PCR reagent water as negative controls.

After electrophoresis through 0.9% agarose gels, PCR products were visualized with ethidium bromide. Aliquots of the PCR products from amplification by the p15-*Bam*3–p15-*Xba*2 primer pair were digested to completion with the restriction enzymes *Bam*HI, *Sac*I, and *Xmn*I. Likewise, aliquots of the p15-*Bam*–p15-*Xba* primer pair PCR products were digested to completion with the restriction enzyme *Ssp*I. The restriction enzyme digests were then analyzed by agarose gel electrophoresis and were compared with undigested PCR products. PCR-amplified *E. chaffeensis* Arkansas strain GroE gene segments served as a control. For sequence analysis, PCR products (470 bp) of the GroEL gene homolog were cloned into plasmids as described above.

Sequence analyses. Sequence analysis was performed by two different methods. Fluorescent automated dideoxynucleotide sequence analysis (Applied Biosystems Inc., Foster City, Calif.) and *Taq* polymerase cycle sequencing were performed at the Biopolymer Laboratory, University of Maryland School of Medicine. To confirm the results of automated sequencing, a separate sequence was generated by a combination of manual methods (performed at the CDC), including double-stranded sequencing with T7 DNA polymerase (Sequenase; U.S. Biochemicals, Cleveland, Ohio) and a *Taq* polymerase cycle sequencing protocol (GIBCO BRL). Primers were ^{35}S or ^{32}P end labeled and were created on the basis of the known sequence of *E. chaffeensis* Arkansas strain or the emerging sequence of isolate 91HE17. Where discrepancies between duplicate sequencing reactions occurred, or when the emerging isolate 91HE17 sequence was discrepant with the established sequence for *E. chaffeensis* Arkansas, a third sequencing reaction was performed by the automated method to obtain consensus.

SDS-PAGE and immunoblots. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (20). The density gradient-purified *E. chaffeensis* Arkansas and 91HE17 strains and uninfected DH82 cell sonicate were suspended in final sample buffer (8% 2-mercaptoethanol, 40% glycerol, and 0.4% bromphenol blue in 60 mM Tris-HCl–1 mM EDTA–2% SDS buffer, pH 6.8) at a protein concentration of 2 mg/ml. Initially, 20 μl was loaded in wells of 5% stacking–12.5% separating polyacrylamide gels. Subsequently, the quantity of protein per lane surface area was calculated for use in preparative gel electrophoresis. Each gel included prestained molecular size standards (GIBCO BRL). Gels were electrophoresed at 15 mA or 100 V until the dye front eluted from the gel. Electrotransfer of proteins was performed according to the method of Towbin et al. (27) with modifications (4, 7). Briefly, proteins in SDS-PAGE gels were transferred at a constant 24 V in phosphate buffer for 2 h at 4°C. The nitrocellulose was dried and cut into 4-mm-wide strips for use in immunoblots. The strips were stored at −20°C until used.

Antibodies. To assess antigenic cross-reactivity between isolate 91HE17 and *E. chaffeensis* Arkansas strain, human, rabbit, and mouse polyclonal antisera and monoclonal antibodies derived after immunization of mice with *E. chaffeensis* Arkansas strain were used. One serum specimen with an indirect fluorescent-antibody assay titer of 1,280 shown to contain antibodies reactive with *E. chaffeensis* Arkansas strain by immunoblot (7) was collected from a patient convalescing from human monocytic ehrlichiosis. The second convalescent-phase human antiserum was obtained from the patient infected with isolate 91HE17 and had a titer of 320 (14). Mouse polyclonal antisera were prepared by intra-

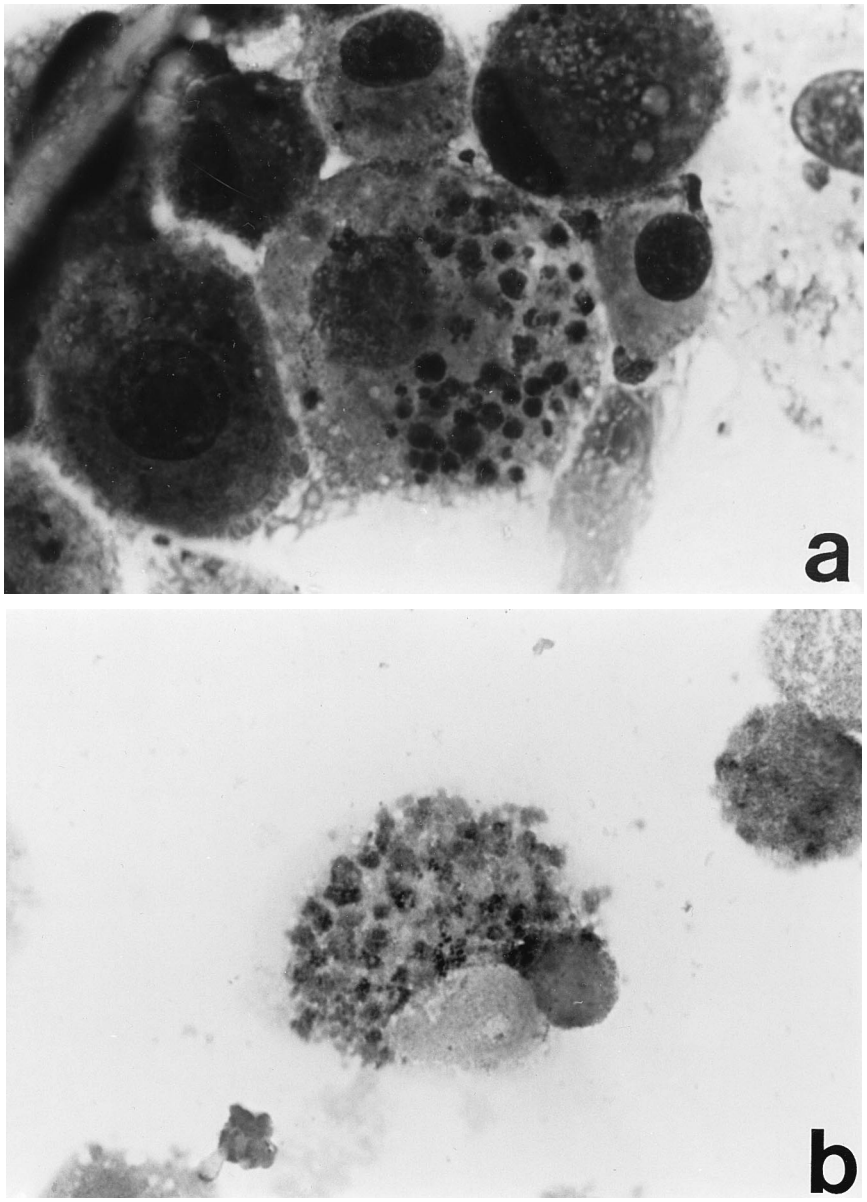

FIG. 1. *E. chaffeensis* 91HE17 cultivated in DH82 canine histiocyte cells. Cells have multiple small, intracytoplasmic morulae which usually contain many ehrlichiae. (a) LeukoStat-stained preparation; magnification, ×1,200. (b) Immunoalkaline phosphatase-stained preparation with the *E. chaffeensis*-specific monoclonal antibody 1A9 and hematoxylin counterstain; magnification, ×1,200.

peritoneal and subcutaneous primary and booster immunizations with *E. chaffeensis* Arkansas strain or isolate 91HE17 suspended in Ribi adjuvant (Ribi ImmunoChem, Hamilton, Mont.). Rabbit polyclonal antisera were prepared as previously described (7). Control antisera included sera from normal human subjects, preimmunization sera from rabbits, sera from normal unimmunized mice, and sera from mice immunized with a lysate of DH82 cells suspended in Ribi adjuvant. Monoclonal antibodies reactive with *E. chaffeensis* Arkansas strain were produced by standard methods and are described elsewhere (8). Monoclonal antibody 1A9 (30) was kindly provided courtesy of X. Yu and D. Raoult, Marseille, France.

Immunoblot staining was performed with modifications of a standard method (7). Blotted strips were incubated sequentially in blocking buffer (phosphate-buffered saline [PBS] with 0.05% Tween 20, 0.5% nonfat dry milk, and 1% normal goat serum [Sigma Chemical Co., St. Louis, Mo.]), diluted sera (1:50 to 1:100), and biotinylated secondary antibodies (all diluted 1:200 in blocking buffer), with each step separated from the next by extensive washes in PBS with 0.05% Tween 20. Biotinylated secondary antibodies included goat anti-human immunoglobulin (IgG plus IgA plus IgM), horse anti-mouse IgG, goat anti-mouse IgM, and goat anti-rabbit IgG (all from Kirkegaard & Perry Laboratories). The strips were then reacted with streptavidin-alkaline phosphatase (diluted 1:1,000; Dako, Carpinteria, Calif.), and bound antibody complex was detected with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP/NBT). The migration of *E. chaffeensis* Arkansas strain and isolate 91HE17 antigens was determined by a standard curve generated with molecular size standards.

Adaptation of 91HE17 strain to HEL cells. The 91HE17 strain was adapted for growth in the continuous human HEL fibroblast cell line as described previously (3). Briefly, infected DH82 cells from one 25-cm^2 flask were cocultivated with a confluent monolayer of HEL cells in a 25-cm^2 flask containing EMEM with 5% FBS, 2 mM L-glutamine, and 0.22% bicarbonate at 37°C in a 5% CO_2 atmosphere. The medium in the flask was changed once per week. Infection was allowed to continue until the monolayer began to slough, when an aliquot of cells was removed for examination by LeukoStat stain (Fisher). If more than 95% of the cells were infected, the monolayer was passaged onto four new flasks with confluent, uninfected HEL cells.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the 16S rRNA gene of the 91HE17 strain of *E. chaffeensis* is U23503.

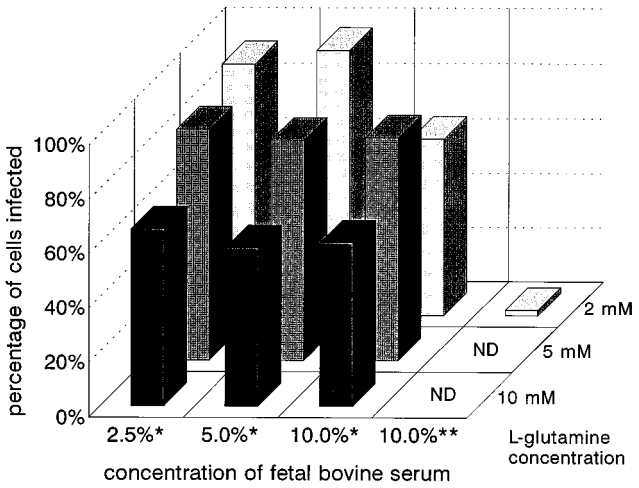

FIG. 2. Effects of pH control, concentration of L-glutamine, and concentration of FBS on growth of the 91HE17 isolate. 91HE17-infected DH82 cells were initially propagated in medium supplemented with 2 mM L-glutamine, 10% FBS, and 0.075% bicarbonate and ambient air, and cultures rapidly become acidic. Not more than 2% of the cells became infected after the initial cultivation in this medium. When 0.5% of the cells were infected, aliquots were incubated under standard conditions (**) or in medium supplemented with 0.22% bicarbonate and cultivated in 5% CO_2 (*). With the latter medium, cultures were supplemented with L-glutamine at the indicated concentrations in various combinations with FBS at the indicated final concentrations. The acidity of the medium was well controlled when supplemented with 0.22% bicarbonate and incubated in 5% CO_2 (*). The medium was replaced on days 3 and 5. Ehrlichial growth was determined by calculating the percentage of infected cells present in LeukoStat-stained cytocentrifuged cells after 7 days of cultivation. ND, not done.

RESULTS

Culture. By LeukoStat staining, morulae consistent with *Ehrlichia* species were first detected in approximately 0.5% of cells incubated in EMEM supplemented with 0.075% bicarbonate and held in ambient air on day 36 after inoculation of cultures. By day 46, approximately 80% of the cells in the culture contained morulae (Fig. 1a), and subcultures were prepared. Thereafter, the ehrlichiae grew poorly, rarely achieving infected-cell proportions of >20%, and routinely, only 0.5 to 2% of cells contained morulae. Empirically, growth of the new isolate was observed to occur more slowly in acidic conditions after prolonged incubation of DH82 cells. Combinations of various concentrations of L-glutamine, FBS, and bicarbonate were tested for their ability to influence ehrlichial proliferation. The most dramatic increase in ehrlichial number was detected in cultures containing medium with 0.22% bicarbonate and maintained in 5% CO_2 (Fig. 2) that had a more effective control of pH, since the indicator dye rarely changed color. 91HE17 strain ehrlichiae also grew to larger quantities with lower concentrations of FBS, while higher concentrations of L-glutamine seemed to be inhibitory. Thus, isolate 91HE17 was thereafter cultivated in DH82 cells incubated in EMEM supplemented with 5% FBS, 2 mM L-glutamine, and 0.22% bicarbonate and held in 5% CO_2.

Immunocytology. Immunocytology with the biotinylated human anti-*E. chaffeensis* globulin and biotinylated dog anti-*E. canis* globulin indicated the presence of an *Ehrlichia* strain closely related to both of these species. Ehrlichiae were also stained by monoclonal antibodies 1A9 (30) (Fig. 1b) and 6A1 (8). The 1A9 monoclonal antibody does not react with *E. canis* (30), indicating a very close antigenic relationship between *E. chaffeensis* Arkansas strain and isolate 91HE17. The absence of immunofluorescence of isolate 91HE17 with monoclonal antibody 6A1 indicated that there were at least some antigenic differences between the two isolates.

Ultrastructure of the 91HE17 isolate. Electron microscopic examination 5 days after infection of DH82 cells showed many vacuoles (morulae) ranging from 0.7 to 3.5 μm in diameter that contained from one to many ehrlichiae. As for the Arkansas strain of *E. chaffeensis* (9, 24), these ehrlichiae were present in two forms, reticulate and dense-core cells (Fig. 3A and B). Both types were spherical or ovoid, ranging from 0.6 to 1.3 μm in maximum diameter for reticulate cells and 0.5 to 1.0 μm in maximum diameter for the dense-core cells, and were surrounded by a trilaminar cytoplasmic membrane and a loose, wavy cell wall. Both reticulate and dense-core cells were seen in the process of binary fission (Fig. 3A and B). Vacuoles with single reticulate cells only were occasionally present; however, larger morulae often contained a uniform population of reticulate or dense-core cells (Fig. 3A). Some giant ehrlichiae were present (Fig. 3C) and were fragmented by membrane invaginations or wrapped by cell wall or cell envelope protrusions (not shown). Occasional spheroplast-like cells were also seen (Fig. 3C). Many morulae contained small vesicles (50 to 120 nm in diameter) and tubules (20 nm in diameter and up to several micrometers in length) that originated from the cell walls of large reticulate cells, as described previously for *E. chaffeensis* Arkansas strain (9, 24).

***E. chaffeensis* PCR and sequencing.** PCR of isolate 91HE17 DNA using the *E. chaffeensis*-specific primers HE1 and HE3 generated the expected 389-bp fragment (2), which was not produced in PCRs containing uninfected DH82 cell DNA or PCR reagent water only (Fig. 4). When the eubacterial primer pairs EC9-EC10, EC11-EC12, and EC9-EC12 were used for PCR of DNA from isolate 91HE17-infected cells, they generated a 733-, 767-, or 1,474-bp product, respectively, identical in size to those generated with control *E. chaffeensis* Arkansas strain DNA (not shown). Control PCRs with no template (water only) and DH82 DNA did not produce any bands even after 80 rounds of amplification. Complete sequence analysis was performed twice, and a third nearly complete sequence was also generated to ensure accurate results and to avoid potential problems with *Taq* polymerase errors. Complete concurrence in the generated 1,435-bp sequence was obtained for each analysis. The entire sequence of isolate 91HE17 was aligned for maximal homology with the published sequence for *E. chaffeensis* Arkansas strain by use of the sequence alignment program of the PC Gene software package (IntelliGenetics Inc., Geneva, Switzerland). The aligned sequence had 99.9% similarity to that of *E. chaffeensis* Arkansas strain and differed in two nucleotide positions, a single substitution at position 744 (T in Arkansas strain versus G in 91HE17) and an insertion in isolate 91HE17 not present in *E. chaffeensis* Arkansas strain (a G at position 883).

PCR, restriction enzyme analysis, and partial sequence analysis of the isolate 91HE17 GroEL gene and GroES-GroEL intergenic region. As expected, PCR amplification of the GroEL genes and GroES-GroEL intergenic regions from isolate 91HE17 and *E. chaffeensis* Arkansas strain produced 470- and 286-bp fragments, respectively. Restriction enzyme analysis of these PCR products generated restriction enzyme-cleaved fragments of identical molecular sizes from isolate 91HE17 and *E. chaffeensis* Arkansas strain (Fig. 5) consistent with the computer-predicted molecular sizes. A search of GenBank GroE nucleotide sequences as of December 1994 revealed none capable of being amplified with these primers that would result in the predicted restriction enzyme cleavage pattern derived for *E. chaffeensis*.

The 470-bp PCR product (425 bp excluding the incorpo-

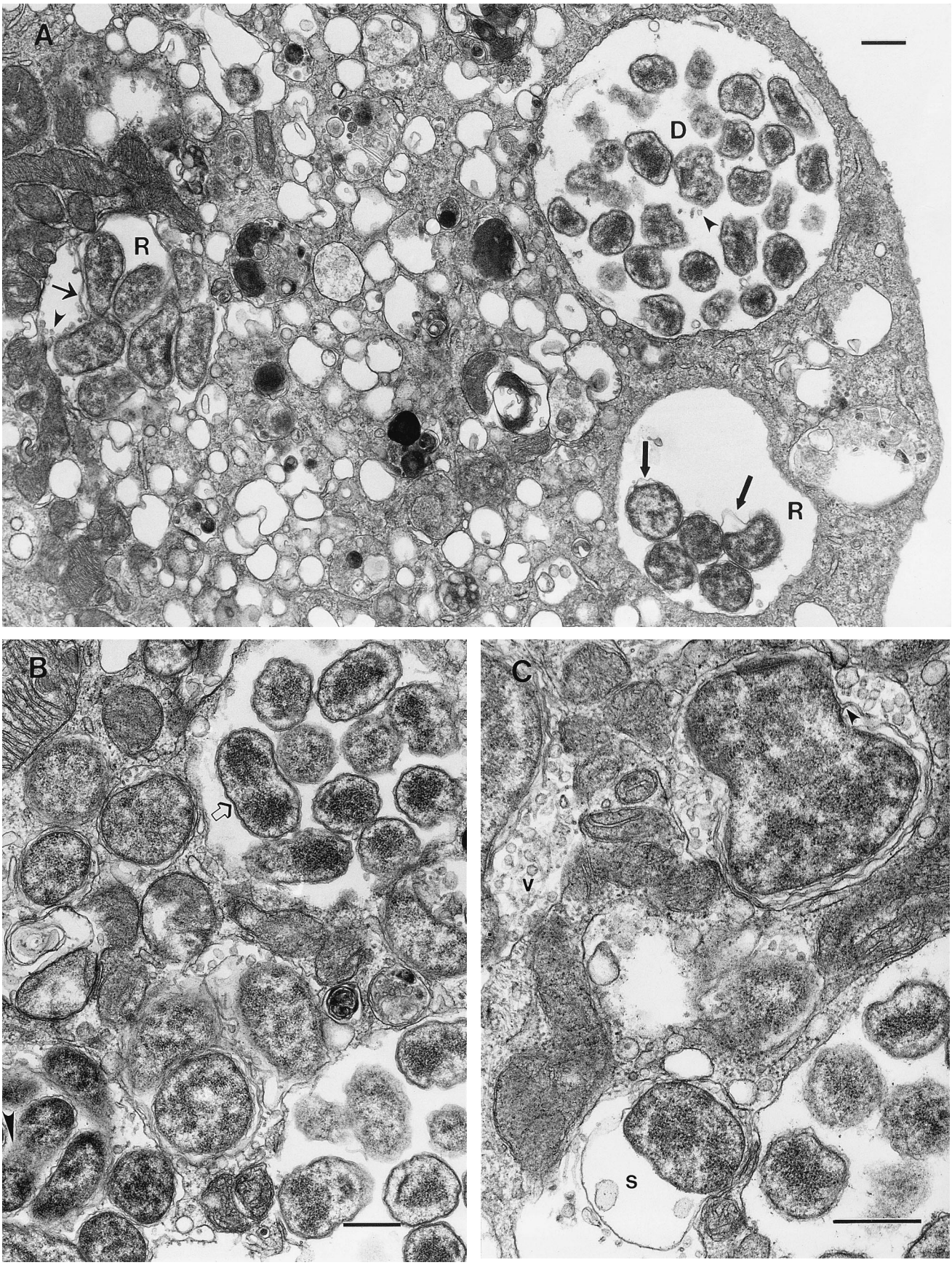
A
D
R
R
B
C
V
S

FIG. 3. Ultrastructure of *E. chaffeensis* 91HE17 in DH82 cells 5 days after infection (bars = 0.5 μm). (A) Three large morulae that contain either reticulate (R) or dense-core (D) cells are present in the cytoplasm of an infected DH82 cell. Some reticulate cells have expansions of the periplasmic space (long arrows). Morulae usually contain tubules (short arrow) and vesicles (arrowheads). (B) Cytoplasm of heavily infected DH82 cell with large and small morulae. Both reticulate and dense-core ehrlichial cells have wavy cell walls. Binary fission is present in both types of ehrlichial cells (arrow). (C) Abnormal giant reticulate cell in a morula. The cell wall of the giant reticulate cell is forming a long tubular protrusion into the vacuolar space (arrowhead). The vacuolar cavity is filled with tubules and vesicles (v). A spheroplast-like reticulate cell (s) with a wide expansion of the periplasmic space is also present in a separate vacuole.

rated oligonucleotide primers) obtained from amplification of part of the GroEL gene homolog of the 91HE17 isolate of *E. chaffeensis* had at least 99.8% identity with the same region in the Arkansas strain. Excluding a single nucleotide position that was ambiguous in all sequencing reactions, the partial GroEL gene sequences of the 91HE17 isolate and the Arkansas strain of *E. chaffeensis* were identical.

Comparison of immunoblot profiles of isolate 91HE17 and *E. chaffeensis* Arkansas strain. When *E. chaffeensis* Arkansas strain and isolate 91HE17 were examined by immunoblot analysis using polyclonal antibodies, the dominant immunoreactive bands shared by *E. chaffeensis* Arkansas strain and isolate 91HE17 included antigens at 88, 85, 68, 60, 55, and 38 kDa and a group of low-molecular-mass antigens at 30, 29, 28, and occasionally 25 and 22 kDa (Fig. 6). Although some variation in intensity of antigens was noted with different antibodies, no significant differences in molecular size and no novel antigens were noted among immunoblots reacted with polyclonal human, rabbit, or mouse antisera, regardless of the immunizing agent.

Adaptation of 91HE17 strain to HEL fibroblasts. After 7 days of cocultivation with DH82 cells heavily infected with 91HE17, residual cells with typical DH82 morphology could not be detected, but infected, adherent HEL fibroblast-like cells were present. The monolayers of HEL cells remained intact for 14 to 21 days, after which a cytopathic effect with foci of rounded, degenerated cells was seen. After 2 to 3 weeks, fibroblast-like cells containing up to 25 morulae per cell, usually in more than 95% of cells, were seen (Fig. 7). Numerous necrotic and lysed cells were evident, and free ehrlichiae were easily seen. The infection could be easily passaged by inoculating a portion of the infected cells onto uninfected monolayers of HEL cells and waiting 10 to 21 days until the majority of cultured cells were infected.

DISCUSSION

Since 1986, when human monocytic ehrlichiosis in the United States was documented (21), over 370 cases have been identified, mostly on the basis of serologic reactions with either *E. canis* or *E. chaffeensis* antigens (18, 28). The clinical spectrum of disease ranges from mild or asymptomatic infections to severe morbidity or fatal outcome (12, 17, 18). The pathogenic mechanisms of infection in humans are unknown, and the relative contributions of the bacteria and the host to disease severity are unclear. To date, all study of the agent of human monocytic ehrlichiosis has been based upon a single isolate from a patient with only a mild illness (10). The characterization of a second, variant strain of *E. chaffeensis* cultivated from the blood of a patient with a nearly fatal infection provides an opportunity to determine if isolates possess molecular differences which might explain the difference in severity of infection. Moreover, if present, such differences might provide valuable clues for studying ehrlichial pathogenesis.

Since no good animal model of monocytic ehrlichiosis caused by *E. chaffeensis* is available, in vitro antigenic and

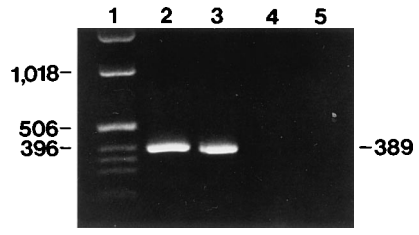

FIG. 4. Agarose gel electrophoresis of a PCR amplified segment of the *E. chaffeensis* 16S rRNA gene from both the Arkansas strain and isolate 91HE17 using the *E. chaffeensis*-specific PCR primers HE1 and HE3. Lane 1, 1-kb molecular size DNA standard; lane 2, *E. chaffeensis* 91HE17 strain; lane 3, *E. chaffeensis* Arkansas strain; lane 4, uninfected DH82 cells; lane 5, no template DNA (water only). Molecular sizes are indicated in base pairs. Ethidium bromide stain was used.

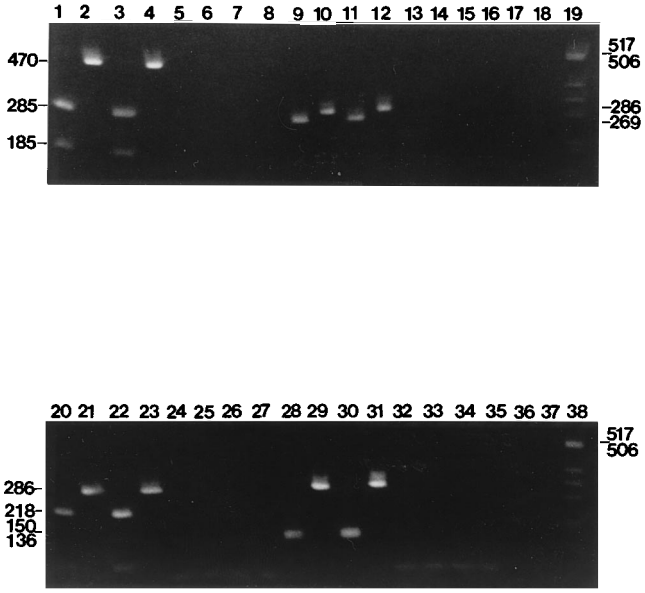

FIG. 5. Agarose gel electrophoresis of PCR-amplified parts of the *E. chaffeensis* GroEL and GroES (*E. coli*) homolog genes with and without restriction enzyme digestion. Primer pair p15-*Bam*–p15-*Xba* was used to amplify a 470-bp fragment of the *E. chaffeensis* GroEL gene homolog (lanes 1 to 8), and p15-*Bam*3–p15-*Xba*2 was used to amplify a 286-bp fragment of the *E. chaffeensis* GroES-GroEL intergenic region including part of the 5′ end of the GroEL gene homolog (lanes 9 to 16 and 20 to 35). Template DNA was prepared from density gradient-purified *E. chaffeensis* 91HE17 (lanes 1 and 2, 9 and 10, 20 and 21, and 28 and 29) and *E. chaffeensis* Arkansas (lanes 3 and 4, 11 and 12, 22 and 23, and 30 and 31) and from whole, uninfected DH82 cells (lanes 5 and 6, 13 and 14, 24 and 25, and 32 and 33), or no DNA template was used (water only in lanes 7 and 8, 15 and 16, 26 and 27, and 34 and 35). Lanes are arranged in pairs to demonstrate restriction enzyme digestion compared with an undigested, PCR-amplified control. Restriction enzymes used include *Ssp*I (lanes 1, 3, 5, 7, and 17), *Bam*HI (lanes 9, 11, 13, 15, and 18), *Sac*I (lanes 20, 22, 24, 26, and 36), and *Xmn*I (lanes 28, 30, 32, 34, and 37). Lanes 17, 18, 36, and 37 contain restriction enzyme controls in which no template DNA was added. Lanes 19 and 38 contain molecular size standards. Note the PCR products with identical molecular sizes after amplification of *E. chaffeensis* Arkansas and 91HE17 and identical-size fragments after restriction enzyme digestion. Fragment sizes are indicated in base pairs.

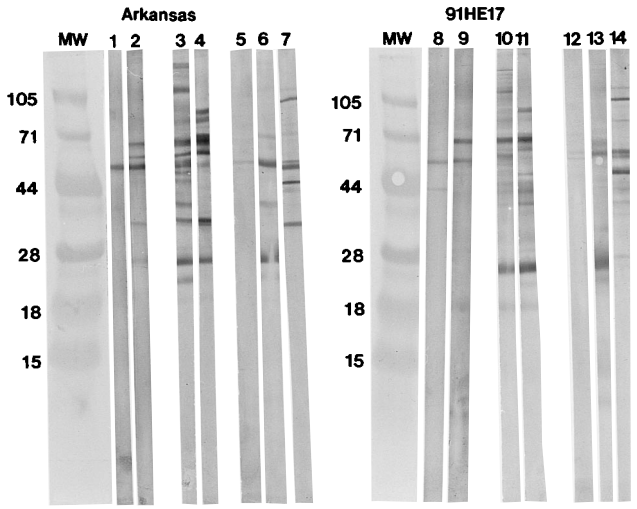

FIG. 6. Immunoblot analysis of heated, density gradient-purified *E. chaffeensis* Arkansas and 91HE17 using homologous, heterologous, and control antisera. Bound antibodies were detected with biotinylated secondary antibodies, streptavidin-alkaline phosphatase, and BCIP/NBT substrate. Lanes 1 to 7, heat-denatured Arkansas antigen; lanes 8 to 14, heat-denatured 91HE17 antigen. Antibodies used included preimmune rabbit serum (lanes 1 and 8), rabbit anti-*E. chaffeensis* Arkansas (lanes 2 and 9), mouse anti-*E. chaffeensis* Arkansas (lanes 3 and 10), mouse anti-*E. chaffeensis* 91HE17 (lanes 4 and 11), normal human serum (lanes 5 and 12), human anti-*E. chaffeensis* (lanes 6 and 13), and human anti-*E. chaffeensis* 91HE17 (lanes 7 and 14). Incubation of blotted Arkansas and 91HE17 strain antigens with unimmunized-mouse serum or mouse anti-DH82 cell antibody revealed no reactions (not shown). Molecular sizes (in kilodaltons) are shown on the left of each set of immunoblots.

molecular comparisons were performed. As determined by immunocytologic methods using polyclonal antibodies known to react with *E. chaffeensis*, the new 91HE17 isolate appeared similar to *E. chaffeensis* Arkansas strain (12, 14). The initial report of the case showed that the infecting ehrlichiae contained 16S rRNA gene nucleotide sequences specific for *E. chaffeensis* (14). Thus, it was suspected that the 91HE17 isolate was *E. chaffeensis*. The cultivated agent 91HE17 had the ultrastructural morphology of species in the *Ehrlichia* genus, including the presence of both reticulate and dense-core cells previously described for *E. chaffeensis* Arkansas strain (9, 24). Although a loose, wavy cell wall is also seen in the Arkansas strain, the 91HE17 isolate had a pronounced increase in cell wall material, as evident in the large amounts of vesicles and tubules within the vacuole. The significance of this finding is not certain, but it is clear that the morula cavity must contain abundant ehrlichial proteins.

The 91HE17 isolate had the same 16S rRNA gene nucleotide sequences as were used to prime PCR amplification of *E. chaffeensis* Arkansas strain and uncultivated *E. chaffeensis* from the blood of infected patients (2, 16). The complete nucleotide sequence of the 91HE17 isolate 16S rRNA gene establishes its phylogenetic position as most similar to *E. chaffeensis*, a finding further supported by sequence identity and identical restriction enzyme patterns of part of the GroE gene homologs present in both *E. chaffeensis* Arkansas strain and isolate 91HE17, similar immunoblot profiles of purified ehrlichial antigens reacted with homologous and heterologous antisera, and reactivity of isolate 91HE17 with the *E. chaffeensis*-specific monoclonal antibody, 1A9. Undoubtedly, isolate 91HE17 is a variant strain of *E. chaffeensis*.

The presence of two distinct nucleotide changes in the 16S rRNA gene shows that isolate 91HE17 possesses at least minimal differences from *E. chaffeensis* Arkansas strain. Phylogenetic typing by use of 16S rRNA and GroEL-GroES chaperonin (heat shock protein) gene sequencing is increasingly used as a method for identification and classification of bacteria of clinical significance. However, it is apparent that many organisms have distinct metabolic pathways or virulence mechanisms or cause disparate clinical conditions despite a high degree of 16S rRNA gene nucleotide similarity. It is unlikely that these rRNA gene changes of isolate 91HE17 are linked to the severities of infection seen in the two patients from whom the two *E. chaffeensis* isolates were obtained. However, these nucleotide changes may reflect other differences in the bacterial

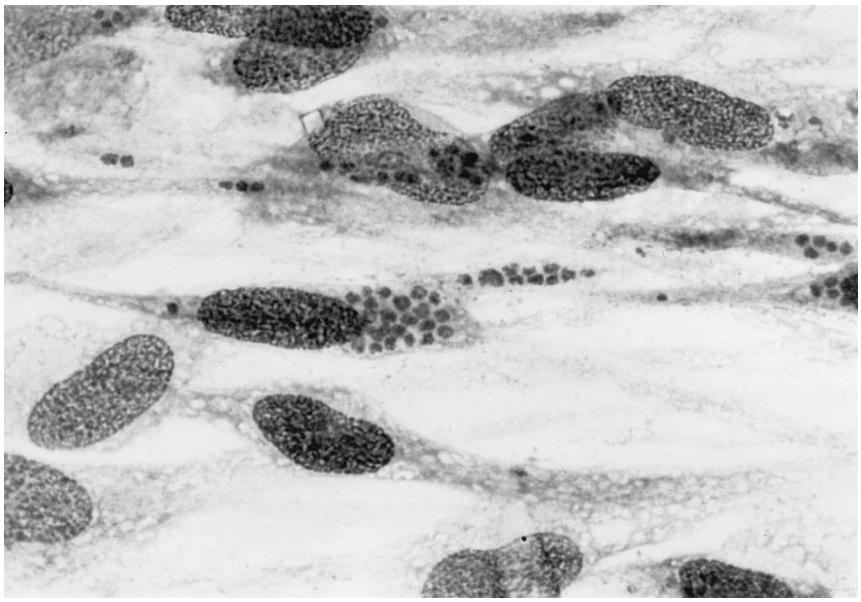

FIG. 7. *E. chaffeensis* 91HE17 infection of HEL fibroblast cells. Note the presence of numerous small morulae in the fibroblast-like HEL cells. The morulae are multiple and small, averaging approximately 1 to 4 μm in diameter (Wright stain; magnification, ×1,200).

genome which encode variant proteins associated with differences in virulence.

The differences between *E. chaffeensis* Arkansas strain and isolate 91HE17 are most likely the result of random polymorphisms and not related to mechanisms of virulence. Description of additional isolates would provide the data required to focus upon potential target molecules or genetic linkages with virulence for the study of ehrlichial pathogenesis. Unfortunately, no definite function has been described for any of the *E. chaffeensis* proteins identified. Monoclonal antibody 6A1 reacts with a 30-kDa protein of *E. chaffeensis* Arkansas strain but not the 91HE17 isolate in immunoblots, and differences in the molecular sizes of other *E. chaffeensis* proteins reactive with monoclonal antibodies are also present (8). Antigens in the molecular size range of 22 to 30 kDa, such as the one reactive with monoclonal antibody 6A1, have been suggested as species-specific proteins (30) and are therefore candidate molecules involved in the expression of disease in humans. However, the target of monoclonal antibody 6A1 is a minor, nonimmunodominant antigen, and its influence on virulence or pathogenesis of infection is not known.

As with *E. chaffeensis* Arkansas (4, 9, 11), the 91HE17 isolate may be cultivated in nonprofessional phagocytic cells in vitro. This situation will allow for careful quantitation and purification by plaquing and will provide an alternative model for evaluation of the biologic consequences of infection with these two variant strains. Preliminary investigations reveal significant and reproducible differences in the kinetics of development and morphologic appearance of plaques which develop in mouse embryo cells and L929 fibroblast cells infected with either *E. chaffeensis* Arkansas or *E. chaffeensis* 91HE17 (9). It is likely that continued endeavors to clone and characterize the molecular and protein constituents of both strains will provide the opportunity to address the question of whether variation in ehrlichial proteins among strains may be responsible for the increased pathogenicity, severe infections, and occasional mortality associated with *E. chaffeensis* infections in humans.

ACKNOWLEDGMENTS

We acknowledge the contributions of Burt Anderson, John Sumner, and Kim Sims of the CDC for their help with sequencing. Thanks to Julie Wen and Violet Han of UTMB for expert assistance with the electron micrographs, to Hui-Min Feng of UTMB for technical guidance, and to Abdu Azad and Marty Schriefer for helpful discussions.

This work was supported in part by an SRIS grant from the University of Maryland School of Medicine and by grant R01AI31431 from the National Institutes of Health.

REFERENCES

1. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson.** 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29:**2838–2842.
2. **Anderson, B. E., J. W. Sumner, J. E. Dawson, T. Tzianabos, C. R. Greene, J. G. Olson, D. B. Fishbein, M. Olsen-Rasmussen, B. P. Holloway, E. H. George, and A. F. Azad.** 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. **30:**775–780.
3. **Brouqui, P., M. L. Birg, and D. Raoult.** 1994. Cytopathic effect, plaque formation, and lysis of *Ehrlichia chaffeensis* grown on continuous cell lines. Infect. Immun. **62:**405–411.
4. **Brouqui, P., J. S. Dumler, D. Raoult, and D. H. Walker.** 1992. Antigenic characterization of ehrlichiae: protein immunoblotting of *Ehrlichia canis*, *Ehrlichia sennetsu*, and *Ehrlichia risticii*. J. Clin. Microbiol. **30:**1062–1066.
5. **Brouqui, P., and D. Raoult.** 1991. Effects of antibiotics on the phagolysosome fusion in *Ehrlichia sennetsu* infected P388 D1 cells, p. 751–757. *In* J. Kazár and D. Raoult (ed.), Rickettsiae and rickettsial diseases. Proceedings of the IVth International Symposium. Publishing House of the Slovak Academy of Sciences, Bratislava, Slovakia.
6. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
7. **Chen, S.-M., J. S. Dumler, H. M. Feng, and D. H. Walker.** 1994. Identification of the antigenic constituents of *Ehrlichia chaffeensis*. Am. J. Trop. Med. Hyg. **50:**52–58.
8. **Chen, S.-M., V. L. Popov, H.-M. Feng, and D. H. Walker.** Submitted for publication.
9. **Chen, S.-M., V. L. Popov, H.-M. Feng, J. Wen, and D. H. Walker.** 1995. Cultivation of *Ehrlichia chaffeensis* in mouse embryo, Vero, BGM, and L929 cells and study of *Ehrlichia*-induced cytopathic effect and plaque formation. Infect. Immun. **63:**647–655.
10. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley.** 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29:**2741–2745.
11. **Dawson, J. E., F. J. Candal, V. G. George, and E. W. Ades.** 1993. Human endothelial cells as an alternative to DH82 cells for isolation of *Ehrlichia chaffeensis*, *E. canis*, and *Rickettsia rickettsii*. Pathobiology **61:**293–296.
12. **Dumler, J. S., J. E. Dawson, and D. H. Walker.** 1993. Human ehrlichiosis: hematopathology and immunohistologic detection of *Ehrlichia chaffeensis*. Hum. Pathol. **24:**391–396.
13. **Dumler, J. S., W. L. Sutker, and D. H. Walker.** 1993. Persistent infection with *Ehrlichia chaffeensis*. Clin. Infect. Dis. **17:**903–905.
14. **Dunn, B. E., T. P. Monson, J. S. Dumler, C. C. Morris, A. B. Westbrook, J. L. Duncan, J. E. Dawson, K. G. Sims, and B. E. Anderson.** 1992. Identification of *Ehrlichia chaffeensis* morulae in cerebrospinal fluid mononuclear cells. J. Clin. Microbiol. **30:**2207–2210.
15. **Engebrecht, J., R. Brent, and M. A. Kaderbhai.** 1991. Minipreps of plasmid DNA, p. 1.6.1–1.6.10. *In* F. M. Ausubel, R. E. Brent, D. D. Moore, et al. (ed.), Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
16. **Everett, E. D., K. A. Evans, R. B. Henry, and G. McDonald.** 1994. Human ehrlichiosis in adults after tick exposure. Diagnosis using polymerase chain reaction. Ann. Intern. Med. **120:**730–735.
17. **Fichtenbaum, C. J., L. R. Peterson, and G. J. Weil.** 1993. Ehrlichiosis presenting as a life-threatening illness with features of the toxic shock syndrome. Am. J. Med. **95:**351–357.
18. **Fishbein, D. B., J. E. Dawson, and L. E. Robinson.** 1994. Human ehrlichiosis in the United States, 1985 to 1990. Ann. Intern. Med. **120:**736–743.
19. **Ito, S., and Y. Rikihisa.** 1981. Techniques for electron microscopy of rickettsiae, p. 213–227. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, Inc., New York.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade.** 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N. Engl. J. Med. **316:**853–856.
22. **Meier, A., D. H. Persing, M. Finken, and E. C. Böttger.** 1993. Elimination of contaminating DNA within polymerase chain reaction reagents: implications for a general approach to detection of uncultured pathogens. J. Clin. Microbiol. **31:**646–652.
23. **Petersen, L. R., L. A. Sawyer, D. B. Fishbein, P. W. Kelley, R. J. Thomas, L. A. Magnarelli, M. Redus, and J. E. Dawson.** 1989. An outbreak of ehrlichiosis in members of an army reserve unit exposed to ticks. J. Infect. Dis. **159:**562–568.
24. **Popov, S., S.-M. Chen, H.-M. Feng, and D. H. Walker.** 1994. Ultrastructure of *Ehrlichia chaffeensis* interaction with different cell lines, abstr. D-32. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
25. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. **4:**286–308.
26. **Sumner, J. W., K. G. Sims, D. C. Jones, and B. E. Anderson.** 1993. *Ehrlichia chaffeensis* expresses an immunoreactive protein homologous to the *Escherichia coli* GroEL protein. Infect. Immun. **61:**3536–3539.
27. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
28. **Walker, D. H., and J. S. Dumler.** 1994. Emerging and reemerging rickettsial diseases. N. Engl. J. Med. **331:**1651–1652.
29. **Wilson, K. H., R. B. Blitchington, and R. C. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942–1946.
30. **Yu, X., P. Brouqui, J. S. Dumler, and D. Raoult.** 1993. Detection of *Ehrlichia chaffeensis* in human tissue by using a species-specific monoclonal antibody. J. Clin. Microbiol. **31:**3284–3288.