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Intracellular Growth of *Afipia felis*, a Putative Etiologic Agent of Cat Scratch Disease

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The organism *Afipia felis*, which is thought to be an etiologic agent of cat scratch disease, is a gram-negative rod that is clearly seen in infected tissue but is very difficult to isolate from clinical specimens; there has been only one report to date of the successful isolation and maintenance of the bacterium on artificial medium. We have found that *A. felis* will attach, invade via phagocytosis, and multiply intracellularly within the phagosomes of primary human monocytes and HeLa cells. Once in the cell, the bacterium appears to change morphologically, becoming longer and more pleomorphic, and loses its ability to grow on an artificial medium. Unique proteins have been identified in both the intra- and extracellular variants of *A. felis*. Convalescent-phase sera from patients with cat scratch disease react poorly with intracellular and extracellular bacteria, suggesting a poor humoral response. The tissue culture protocol presented has been used to isolate 14 new strains of *A. felis* and has for the first time permitted study of the pathogenesis of this unique organism.

Cat scratch disease (CSD) is most often described as a benign subacute regional lymphadenitis after cutaneous inoculation, typically a cat scratch. Some investigators have suggested that CSD is the most common cause of chronic lymphadenopathy in children and adolescents, and it is now recognized more often in adults (1, 25). Although CSD is normally self-limiting, with spontaneous resolution after a period of weeks or months, it can progress to a severe, systemic, or recurrent infection (13) and may be life threatening in immunocompromised persons (4). Although the disease is susceptible in vitro to certain aminoglycoside antibiotics (7), the efficacy of antibiotic therapy has not been convincingly demonstrated in controlled clinical trials.

The disease was first described in 1950 by Debré and his colleagues in France (10), and since that time a number of organisms, including mycobacteria (5), chlamydiae (12) and several viruses (21, 34), have been proposed as possible etiologic agents. In 1983, Wear et al. demonstrated by microscopy the presence of delicate pleomorphic, gram-negative bacilli in the lymph nodes of 39 patients with CSD (35). The presence of these bacilli, clearly seen with the Warthin-Starry silver impregnation stain, has since been confirmed by others (23, 26). In 1985 Gerber and associates cultured from the lymph node of a patient with CSD a highly pleomorphic, gram-positive bacterium which they believed to be the same organism as that shown with the Warthin-Starry stain (15). They subsequently identified this organism as a member of the genus *Rothia*. In 1988, English et al. (13) reported the isolation of what is believed to be a causative agent of CSD, a pleomorphic gram-negative bacillus from the lymph node of a patient with suspected CSD. More recently, Moss et al. (29) and Brenner et al. (7) described other characteristics of this organism, including its unique fatty acid and biochemical profiles and its name, *Afipia felis*. English et al. also reported that antibodies made to *A. felis* B91-007352 reacted with several specimens from patients suspected of having CSD (13). No other laboratory has reported the isolation of additional strains of this bacterium, although lymph nodes and other specimens from patients with CSD are observed microscopically to contain large numbers of bacilli. In addition, the results of Western immunoblots, whole-cell enzyme-linked immunosorbent assays, and direct and indirect fluorescent-antibody tests on tissue biopsy specimens with antibodies made to the agar-grown *A. felis* have been routinely negative (2).

Numerous studies have been performed with tissue culture invasion as perhaps a more precise measure of survival, growth, and pathogenesis in such bacterial intracellular parasites as *Mycobacterium*, *Shigella*, *Salmonella*, *Yersinia*, *Legionella*, *Listeria*, and *Chlamydia* species (11, 30). More recently, tissue culture studies with *Salmonella* (14), *Yersinia* (19), and *Chlamydia* (32) species have examined the mechanisms of adherence and invasion and detected the synthesis of proteins specific to these processes and to survival within the host cell. All of these systems made use of a uniform population of mammalian cells that could be infected under defined conditions and modified as necessary and for which the results could be quantitated accurately (17). Since intracellular bacteria have been observed microscopically within cells lining the capillary walls of lymph nodes and within macrophages in clinical tissue from patients with suspected CSD (13), we used similar tissue culture strategies with a variety of tissue culture lines of endothelial, epithelial, and lymphocyte origin. We found several tissue culture lines in which the organism survives and two that support its growth. We have cultured additional strains of *A. felis* in these tissue culture cell lines (3), and we have begun to examine differences between the intracellular and extracellular growth of this organism to identify putative virulence factors.

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MATERIALS AND METHODS

Bacterial strains. B91-007352 (kindly provided by the Armed Forces Institute of Pathology, Bethesda, Md.) was isolated from the lymph node of a patient with CSD (13). B91-007352 was maintained on buffered charcoal-yeast extract (BCYE)-agar plates (Carr-Scarborough Microbiologicals, Decatur, Ga.) at 32°C in 4% CO_2 and stored in Luria-Bertani broth plus 20% glycerol at −70°C.

Tissue culture lines. Published photomicrographs of biopsy specimens from suspected CSD infections have shown bacteria surviving and perhaps multiplying within the cells lining capillary walls of lymph nodes and within macrophages (13, 35). In choosing tissue culture cell lines that would most likely support the survival and intracellular growth of our isolates, we focused on these sources and on epithelial cells, since the point of infection is usually a skin lesion. We selected for analysis a primary cell culture of human monocytes and cell lines of human macrophage (U937), human foreskin (FS9), mouse connective tissue (L929), human epithelial carcinoma (A431), human colon carcinoma (Caco-2), human vascular endothelial (HMEC-1), and human cervical carcinoma (HeLa) origins.

The cell lines used were maintained and supplied by the Biological Products Branch, National Center for Infectious Diseases. Cells were grown in minimal essential medium (MEM; Flow Laboratories, McLean, Va., or GIBCO Laboratories, Grand Island, N.Y.) plus 10% fetal bovine serum (FBS; Hazleton Research Products, Inc., Lenexa, Kans.) or Iscove's modified Dulbecco's medium (GIBCO) supplemented with 7.5% sodium bicarbonate and 10% FBS at 37°C in 5% CO_2. Human monocytes, supplied by Thomas Rowe, Retrovirus Diseases Branch, National Center for Infectious Diseases, were obtained from human peripheral blood by a double adherence method with a Ficoll gradient (6) followed by removal and pelleting of the monocyte band (200 × *g* for 10 min). Cells were resuspended in macrophage medium (Iscove's medium supplemented with 2 mM L-glutamine, 1% antibiotics [penicillin and streptomycin], 10% heat-inactivated human AB+ serum, and 10% giant cell tumor supernatant). Cells were counted by the trypan blue dye exclusion method; 10^8 viable cells were added to T150 flasks (Costar, Cambridge, Mass.) and allowed to adhere for 1 h at 37°C. Monolayers were then washed in phosphate-buffered saline (PBS), removed with trypsin-EDTA, pelleted, resuspended, and counted; the cell density was then adjusted to 10^6 cells per ml. Twenty-four-well plates (Costar) were inoculated with 1 ml of cell suspension per well and incubated for 1 to 24 h at 37°C; then the monolayers were washed three times with Iscove's medium, and the macrophage medium was replaced. Monocytes were infected 7 to 10 days after the initial isolation.

Antibiotic sensitivity. All antibiotics were added to BCYE plates at concentrations of 600 μg/ml (streptomycin) or 100 μg/ml (amikacin, neomycin, kanamycin, tobramycin, spectinomycin, and vancomycin). Plates were spread with approximately 5×10^7 bacteria and incubated at 32°C in 4% CO_2 for 5 days.

Tissue culture infection. The CSD tissue culture assay was based on that developed for *Shigella flexneri* by Clerc and Sansonetti (9). Monolayers of 10^5 cells per ml in 3 ml of MEM with 10% FBS were grown in six-well plates (Costar). In carrying out these studies, we found that the number of times the host cells had been passaged was important. HeLa cells passaged >40 times appeared to lose their ability to become infected and to support the growth of this organism. HeLa cells from passages 28 through 38 appeared to be optimal.

Before infection, the medium was removed and the monolayers were washed one time in PBS. Iscove's medium without FBS (3 ml) was then added to each well. Bacteria grown on BCYE were suspended in Iscove's medium to an optical density at 600 nm of 0.45; 30 μl was added to each well, giving an approximate multiplicity of infection of 10. Some infected monolayers were centrifuged (Beckman model TJ-6) at 1,200 rpm for 10 min to potentially enhance attachment (9). However, this centrifugation did not seem to have any effect on attachment or uptake and was therefore not included in the routine protocol. Cultures were incubated at 37°C in 5% CO_2 for 18 h. The medium was then removed, and the monolayers were washed once in PBS. Then 3 ml of Iscove's medium containing streptomycin (200 μg/ml), tobramycin (100 μg/ml), and 10% FBS was added to each well. Gentamicin is used in most systems to kill extracellular bacteria, since aminoglycosides are theoretically unable to enter or are inactive within eucaryotic cells (19). However, B91-007352 was resistant to gentamicin and was also found to be resistant to kanamycin, neomycin, vancomycin, and amikacin and partially resistant to spectinomycin at a concentration of 100 μg/ml but sensitive to streptomycin (200 μg/ml) and tobramycin (100 μg/ml). Streptomycin and tobramycin in combination were used in all subsequent experiments. Cultures were incubated as described above and examined at 24-h intervals by removing the medium from a single well, washing once with PBS, scraping the cells from the plate surface with a cell scraper (Costar), and applying 10 μl of the cell suspension to a microscope slide. Slides were then dried at 37°C, stained by the Gimenez technique (20), and examined under a light microscope. After 72 h of incubation, the Iscove's medium with antibiotics was removed and replaced with fresh medium without antibiotics. Infected cells were then allowed to incubate for an additional 72 h. These cells could be stored as long as 6 weeks at 4°C, and the bacteria remained viable.

Tissue cultures in suspension (U937) were likewise infected with 10 μl of bacterial suspension per ml of tissue culture cells. After 18 h of incubation, the cell suspension was centrifuged at 225 × *g* for 5 min, washed in PBS, and resuspended in MEM plus antibiotics. At each time point, 1 ml of suspension was centrifuged briefly (maximum speed, Eppendorf microcentrifuge) and 10 μl of the cell pellet was applied to a slide for staining.

Electron microscopy. Infected cells in suspension or scraped from tissue culture wells were prepared for electron microscopy by pelleting at 1,000 × *g* for 5 min, washing the cell pellets with PBS, and resuspending the cells in 2% glutaraldehyde fixative. The cells were held at 4°C for 1 h before they were pelleted again, washed, and resuspended in ice-cold PBS. The cells were postfixed in 2% osmium tetroxide, dehydrated in graded solutions of ethanol, passed through propylene oxide, and embedded in Epon resin. Sections 1 μm thick were stained with 0.1% (wt/vol) sodium borate for examination by light microscopy. Sections 40 to 60 nm thick were stained with uranyl acetate and lead citrate for examination in a transmission electron microscope (EMU-4; RCA, Camden, N.J.).

Measure of intracellular growth. HeLa cell monolayers in T25 flasks were washed with PBS, overlaid with Iscove's medium without serum, and infected with B91-007352 at a multiplicity of infection of 10. Controls included the inoculum in the absence of the HeLa cell monolayer and an intact monolayer with cytochalasin D (5 μg/ml) added 30 min

before infection and maintained throughout the experiment. After 18 h, the medium was removed from each flask; the monolayers were washed once with PBS, and fresh medium with 10% FBS, streptomycin, and tobramycin was added. At 48 h after the addition of antibiotic, the monolayers were scraped from the flasks and centrifuged (10 min, 8,000 × *g*). Pellets were washed twice in PBS to remove antibiotics, resuspended in 1 ml of PBS, and sonicated for 15 to 20 s at a setting of 2.5 with the microtip of a cell disrupter (Heat Systems-Ultrasonics, Inc.); 100 μl was plated on BCYE. The remainder of the sample was added to a new HeLa cell monolayer, and the process was repeated as described above. The control without HeLa cells was scraped, centrifuged, and resuspended, and the entire amount was plated.

Computer-enhanced image analysis. Gimenez-stained slides were examined, and intracellular bacteria were directly counted by using a barrier filter (cutoff of 450 to 490 nm) and a ×40 Plan Neofluar objective on a Zeiss Aziovert inverted microscope equipped with epifluorescence. Emitted fluorescence was transmitted to a video camera and IM 3000 digital image analysis system (Analytical Imaging Concepts, Irvine, Calif.) containing 256 gray levels for pixel discrimination.

Renografin gradient. Intracellular bacteria were purified from HeLa cells by using a modification of the Renografin density gradient described by Howard et al. for purifying *Chlamydia trachomatis* (18). Infected HeLa cells suspended in 0.01 M PBS (pH 7.4) were sonicated with three 10-s bursts (with the cell disrupter microtip and a setting of 2.5) and then centrifuged at 500 × *g* for 10 min. The supernatant was centrifuged at 10,000 × *g* for 30 min, and the pellet was resuspended in PBS and layered on 30% Renografin. The Renografin gradient was centrifuged for 1 h at 31,000 × *g*. The pellet was resuspended in PBS, and a second centrifugation was performed. The purified bacteria were then mixed with solubilization buffer, boiled, and applied to a polyacrylamide gel for protein analysis.

SDS-polyacrylamide gel electrophoresis. Whole-cell protein preparations of *A. felis* were applied to 12.5% polyacrylamide separating gels (with 5% polyacrylamide stacking gels) for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (24). The solubilization buffer consisted of 2% SDS (BDH Chemicals Ltd., Poole, United Kingdom), 4% 2-mercaptoethanol, and 10% glycerol in Tris buffer (pH 6.8). Samples were electrophoresed at 30 mA of constant current. Gels were then fixed overnight in 25% isopropanol–7% acetic acid and silver stained, Coomassie blue stained as described by Laemmli (24), or transferred to nitrocellulose for Western blot (immunoblot) analysis.

Western blots. The procedures used for Western blotting were based on those of Towbin et al. (33). Protein bands were transferred to nitrocellulose by using a Transblot semidry transfer cell as recommended by the manufacturer (Bio-Rad, Richmond, Calif.). After incubation with acute- and convalescent-phase sera from suspected and culture-confirmed CSD cases and controls, bands were detected with a 1:3,000 dilution of horseradish peroxidase-labeled goat anti-human immunoglobulin G (Bio-Rad).

RESULTS

Cell culture and the invasion assay. Human monocytes were successful in supporting the growth of *A. felis* (Fig. 1). Observed microscopically, the organisms elongated, became pleomorphic, appeared to multiply within the cytoplasm of the host cells, and, in some cases, stained gram positive, possibly because of the production of polyphosphate and lipid storage compounds (data not shown). The intracellular bacteria appeared similar to certain variants observed occasionally within an agar-grown population and sometimes within a single colony.

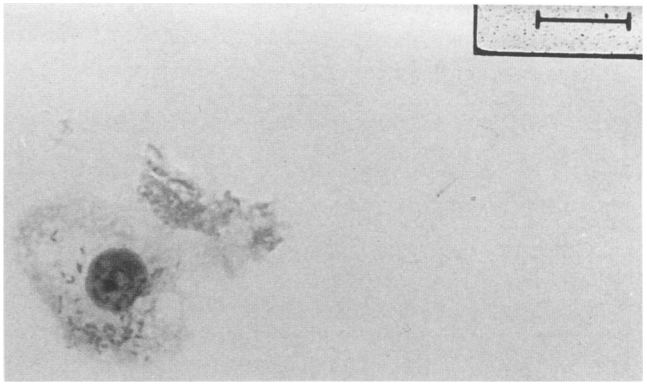

FIG. 1. Gimenez-stained human macrophage infected with *A. felis* 66 h after infection and 48 h after the monolayer was washed and medium with antibiotics was added. Bar, 10 μm.

The HeLa human cervical carcinoma cell line, which is more easily maintained than human monocytes, was also successful in supporting the growth of *A. felis* and produced the same morphological changes in the organism. Immediately after infection, only a few bacteria were observed within or associated with the host cells. Eighteen hours after the initial infection, large numbers of bacteria were seen in the extracellular spaces (Fig. 2A). At this point, antibiotics (streptomycin and tobramycin) were added. Twenty-four hours later, the extracellular space was clear; bacteria were seen only attached to or within the host cells (Fig. 2B). Host cell viability, which was determined by subculturing, was not affected by whether antibiotics were maintained throughout the remainder of the experiment or were washed off within 24 h of their addition. After each successive 24-h assay point, an increase in the number of bacteria within the infected HeLa cells was visible (Fig. 2C and D). Bacteria in the extracellular spaces may be progeny of a small number of organisms surviving the antibiotic treatment. After 72 h of infection, bacteria could be seen associated with nearly 100% of the cells of the HeLa monolayer.

Microscopic and electron microscopic data indicated that human macrophage primary cell cultures, HeLa cells, and HMEC-1 cells all supported the intracellular growth of B91-007352. In the cell lines U937, FS9, L929, and A431, *A. felis* invaded and survived for a limited period of time but did not replicate and was eventually eliminated by the host cells. Caco-2 cells, epitheliumlike cells from a human colon carcinoma, are often used as a model to study intracellular growth. However, we found no evidence of invasion or intracellular growth by *A. felis* in these cells.

Electron micrographs of several infected cell cultures clearly showed bacterial attachment and phagocytosis (Fig. 3A and B). The bacteria attached and invaded by what appeared to be typical phagocytosis. When cytochalasin D, one of a family of drugs known to inhibit host cell microfilament formation and hence phagocytosis, was added to HeLa cells, no microscopic evidence of bacterial invasion or multiplication was observed.

Once inside the cell, the bacteria appeared to replicate

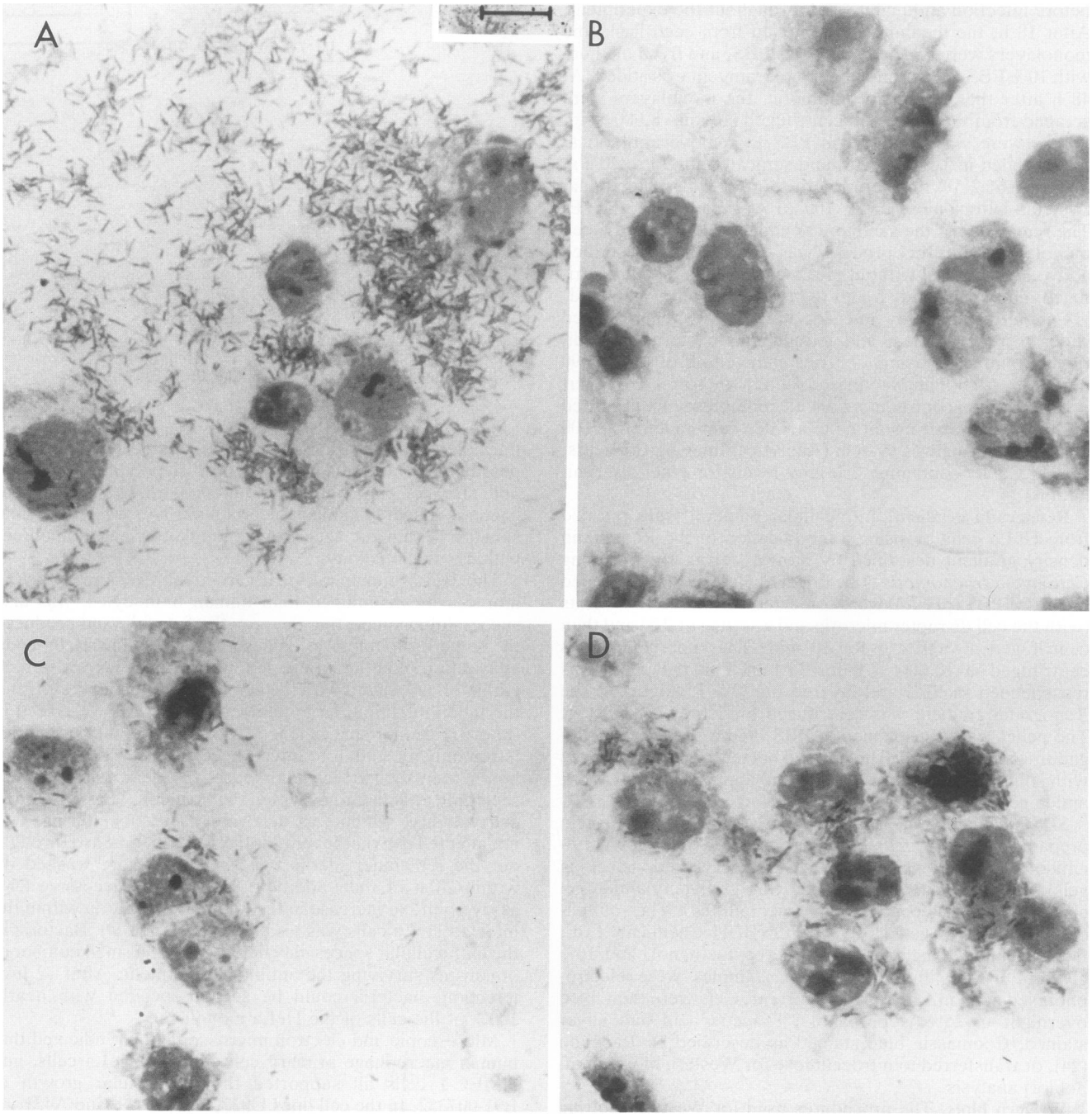

FIG. 2. Gimenez-stained HeLa cell monolayers infected with *A. felis* (multiplicity of infection, 10) 18 h after infection without antibiotics (A) and 42 h after infection and 24 (B), 48 (C), and 72 (D) h after the monolayer was washed and medium with antibiotics was added. Bar, 10 μm.

within the phagocytic vacuoles (Fig. 3C). These phagosomes then fused to form larger vacuoles, sometimes encompassing one-half of the cell volume. Eventually, the host cell membrane was degraded and the bacteria were released into the extracellular environment (Fig. 3D). Although such lysis was often seen, it may be attributed to the fact that the host cells are old at this point in the infection and their cell walls may be very weak.

By using computer-enhanced image analysis, bacteria within the host cells were enumerated. These numbers were used to calculate an intracellular growth curve, which rose steeply for the first 48 h after infection and more gradually in the next 48 h (Fig. 4A). Although large numbers of bacteria were clearly visible with the microscope, colony counts from the same samples sonicated and plated on BCYE agar declined dramatically over the same time period; within 48 h, less than 1% of the initial inoculum formed colonies on agar medium (Fig. 4B).

Western blot analysis. Western immunoblots with acute- and convalescent-phase sera from three culture-confirmed

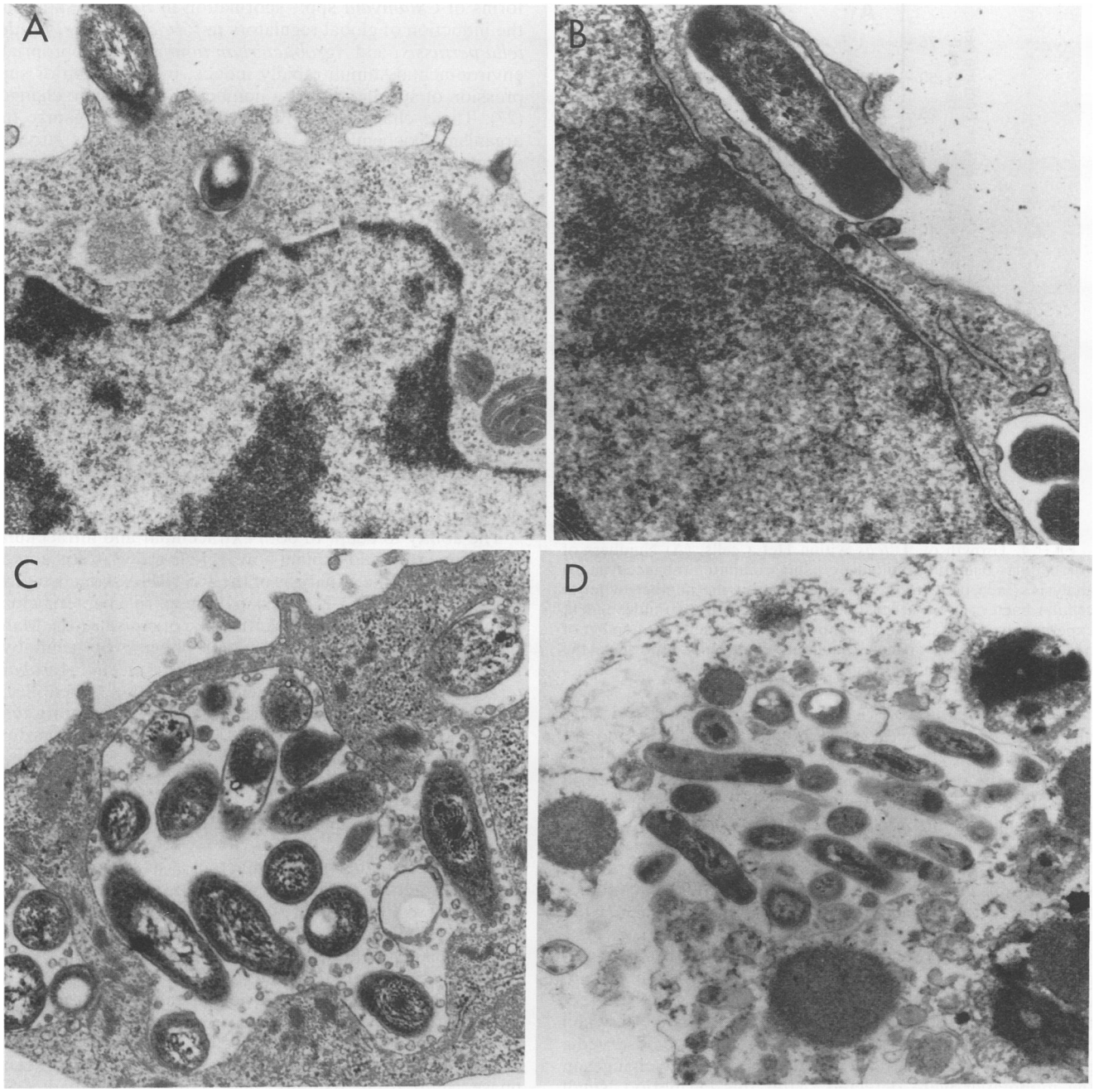

FIG. 3. Electron micrographs of HeLa cells infected with *A. felis*. (A) Attachment of bacterium to the host cell; (B) phagocytosis of bacterium by the host cell; (C) bacteria residing in a phagocytic vacuole; (D) degradation of the host cell membrane and release of the bacteria. Magnification, ×25,000.

and eight clinically defined patients with CSD showed no consistent banding patterns with the agar-grown or intracellularly grown bacteria.

DISCUSSION

Our data indicate that *A. felis* can survive and replicate intracellularly in a variety of fresh and immortalized tissue culture cells. Observed in clinical specimens, the bacteria appear to reside in macrophages or vascular endothelial cells. Electron micrographs and cytochalasin D inhibition studies indicate that the bacteria attach and invade by what appears to be a typical phagocytic mechanism. Once in the cell, bacteria apparently replicate within the phagocytic vacuoles, which then fuse to form larger vacuoles. It is unknown at this time whether lysosomal fusion is inhibited, as in *Legionella pneumophila* and *Chlamydia psittaci*, or whether the bacteria survive the infusion of lysosomal enzymes, as in *Yersinia pestis* and *Coxiella burnetii* (30). While in the cell, *A. felis* elongates, becomes extremely pleomorphic, and synthesizes additional storage material and intracellular proteins. The replicating bacteria eventu-

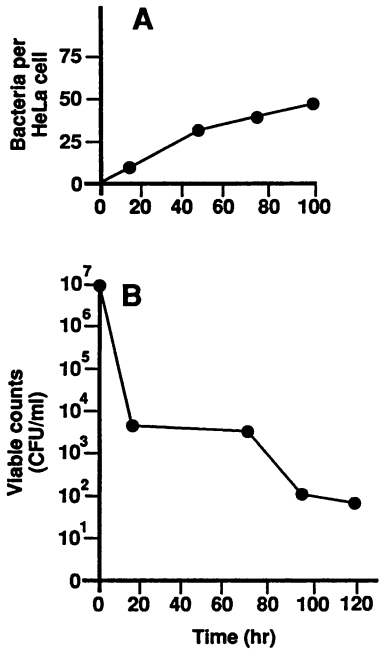

FIG. 4. Growth of *A. felis* within HeLa cells. (A) Numbers of intracellular bacteria counted by using computer-enhanced image analysis. Each point represents the mean of the number of intracellular bacteria from at least 30 infected cells in two independent experiments. (B) Viable counts showing a decrease in the number of bacteria growing on artificial medium. Each point represents the mean of four independent trials.

ally destroy the integrity of the host cell membrane and are released to the extracellular environment. Using a modification (3) of the previously described protocol, we cultured 30 clinical specimens (mainly lymph node biopsies and aspirates from patients with typical CSD) and were able to isolate *A. felis* from 14 specimens. These organisms appear to be quite similar morphologically to the organism described by Wear et al. (35) and English et al. (13). Three of these strains were analyzed in detail by Brenner et al. (7); the remainder will be discussed in detail elsewhere (3).

Our viability curve demonstrates that, when the agar-grown inoculum was added to permissive tissue culture cells, the viable count dropped precipitously but the direct count over the same period increased. Alternatively, when clinical specimens were added to HeLa cells, we observed a lag period of at least 4 days, during which time no changes in the monolayer were detected. After this lag period, small refractile microlesions appeared within cells of the infected monolayer, and soon thereafter colonies appeared on the agar plates (3). These observations may indicate that, after growth on solid media or intracellular growth (either in tissue culture or in clinical specimens), the bacterium has difficulty in adapting to growth in the alternate environment. This may also explain why *A. felis* has only once been reported to be successfully cultured from clinical specimens on laboratory media. In a biopsy specimen, the predominant bacterial population is intracellular. The subpopulation that is able to survive and grow on agar plates is extremely and perhaps undetectably low. Enhancement of viable intracellular bacteria in tissue culture may be required to increase this subpopulation to a detectable level.

Many examples of bacteria adapting to alternate environments exist. In the elementary body and reticulate body forms of *Chlamydia* spp., sporulation in *Bacillus* spp., and the induction of global regulators in *Vibrio cholerae*, *Bordetella pertussis*, and *Agrobacterium tumifaciens*, appropriate environmental stimuli rapidly induce, by activation or suppression of specific genes, a number of phenotypic changes (27). These changes completely or partially reorganize the organism biochemically and morphologically for survival and growth in the new environment. There are gram-positive pleomorphic variants of *A. felis* within an agar-grown population and even within a colony; these variants resemble the intracellular form of the bacteria by electron microscopy and gram stain. Silver stains of whole-cell protein profiles indicated marked differences between the proteins composing the agar-grown form of the organism and those composing its intracellular form. Whether these proteins are virulence factors, maintenance proteins, or shock proteins remains to be determined. Additionally, bacteria passaged once on agar plates from tissue culture require 7 days rather than 3 to grow to a detectable colony size (0.25 mm) and are still considerably smaller than colonies passaged multiple times on agar (1 mm). The first-passage organisms possess the unique fatty acid profile characteristic of *A. felis* (28), but the relative amounts of individual acids vary upon subsequent passage on agar medium (data not shown).

The ability to adapt to both extracellular and intracellular environments could explain how *A. felis* grows in soil as well as in human tissue. Analysis of the 16S rRNA sequence of *A. felis* places it in the α-2 subgroup of the purple bacteria along with a number of soil and rickettsialike organisms (31). Many organisms in this group are either obligate or facultative intracellular pathogens, e.g., *Bartonella*, *Brucella*, *Agrobacterium*, and *Rochalimaea* species. In addition, when HeLa cells infected with *A. felis* were stored at 4°C for more than 6 weeks, the bacteria remained viable, which is consistent with the fact that many soil organisms are facultative psychrophiles. This information supports the hypothesis that the cat acquires the organism from the soil, carries it orally and/or on its paws, and infects the human with a scratch or a bite.

Once the organism has infected a human through an animal scratch, it could be transported, possibly by macrophages, to regional lymph nodes. At this point the organism may spread to and invade adjoining cells (including vascular endothelial cells); much of this is consistent with the pattern of events observed in tissue culture. Cell-mediated immunity may play the major role in clearing the infection, as it does in cases of infection with *Listeria*, *Legionella*, and *Mycobacterium* species and other intracellular bacterial pathogens (22). Western immunoblots with acute- and convalescent-phase sera from patients with culture-confirmed CSD showed little reaction with agar-grown or intracellular bacteria. Convalescent-phase sera from patients infected with *Listeria monocytogenes* likewise showed poor responses to the bacterium in Western blots, enzyme-linked immunosorbent assays, and indirect fluorescent-antibody tests (16). The positive reaction to the commonly used skin test antigen in over 90% of patients with CSD (1, 8) strongly suggests a cellular immune response.

In this report, we demonstrate that *A. felis* multiplies intracellularly and that the intracellular and extracellular forms of the organism differ morphologically and perhaps functionally. The discovery of this intracellular lifestyle led to the development of a tissue culture isolation protocol and the subsequent isolation of 14 new strains of *A. felis* (3). Identification of the genetic mechanisms that allow only a subpopulation of intracellular *A. felis* to grow on artificial

media could greatly increase our understanding of the distinctions between facultative and obligate intracellular parasitic bacteria.

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