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Propagation of Granulocytic *Ehrlichia* spp. from Human and Equine Sources in HL-60 Cells Induced To Differentiate into Functional Granulocytes

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***Ehrlichia* spp. from human and equine sources in the northeastern United States were detected by PCR, isolated, and propagated in the HL-60 promyelocytic leukemia cell line. Growth of *Ehrlichia* from both equine and human sources was enhanced by addition of retinoic acid, which causes granulocytic differentiation of the HL-60 cells. DNA sequencing of a portion of the 16S rDNA gene supported the hypothesis that the same pathogen was responsible for both equine and human granulocytic ehrlichiosis.**

One characteristic of the newly emergent pathogens of the genus *Ehrlichia* is their selection of cells of myelocytic origin as targets. *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis, infects macrophages (1). *Ehrlichia equi*, *E. phagocytophila*, and the agent that causes human granulocytic ehrlichiosis (HGE) infect granulocytes; these organisms are closely related or may be the identical species (3, 10, 11). It has been reported recently that the HGE pathogen can be propagated in the HL-60 promyelocytic leukemia cell line (5). HL-60 cells continuously proliferate in suspension culture and, hence, are a convenient tissue culture system in which to further characterize the propagating *Ehrlichia*. One additional property of the HL-60 cell line is its ability to terminally differentiate in response to certain inducers. HL-60 cells can be induced to differentiate down a monocytic pathway and, consequently, exhibit biochemical and functional characteristics of mature macrophages following exposure to inducers such as phorbol esters, vitamin D_3 derivatives, and anthracycline antineoplastic agents (12). Alternatively, this cell line possesses the ability to be induced by dimethyl sulfoxide, retinoic acid, and sodium butyrate to differentiate and, consequently, exhibit the biochemical and functional characteristics of granulocytes (2). In both cases, induction produces a terminal differentiation in which cell proliferation ceases in conjunction with the appearance of the differentiated phenotype.

In this report, we describe the isolation and propagation of *Ehrlichia* spp. obtained from the blood of infected humans and horses in Connecticut and southeastern New York. The pathogens were grown and passaged in HL-60 cell cultures which were either left to proliferate continuously or were terminally differentiated down either the monocytic or the granulocytic pathway. We obtained 11 such isolates, each of which grew as well or better in terminally induced granulocytic HL-60 cells than in uninduced cells or terminally induced monocytic cells. Furthermore, each isolate was found to be closely related to *E. equi* by 16S rDNA sequence analysis.

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MATERIALS AND METHODS

HL-60 cell culture. HL-60 cells were maintained in continuous culture by growth in RPMI 1640 liquid medium supplemented with 20% fetal bovine serum, pyruvate, nonessential amino acids, and glutamine (RP-U medium) in 12.5- or 25-cm^2 tissue culture flasks in a 5% CO_2 incubator at 37°C. Undifferentiated HL-60 cells were serially passaged twice weekly at dilutions of 1:10 and 1:40. At each passage, aliquots of the cells were induced to undergo granulocytic differentiation by the addition of 1 μM all-*trans*-retinoic acid (RA; Sigma Chemical Co., St. Louis, Mo.) and monocytic differentiation by the addition of 2 μM 25-OH vitamin D_3 (VD; Calbiochem, San Diego, Calif.). Induced cultures were grown for a minimum of 3 days to permit differentiation and were maintained for no more than 1 week.

Specimen collection. Suspected ehrlichiosis cases occurred in Connecticut and southwestern New York. For humans, the criteria for obtaining a sample were fever, headache, either thrombocytopenia or neutropenia, and the absence of prior treatment with doxycycline. For horses, the criteria were fever, the absence of respiratory involvement, and the absence of prior treatment with tetracycline derivatives. Blood samples were obtained by venipuncture from horses and humans with these symptoms and were collected in tubes containing K_3-EDTA.

Preparation of *Ehrlichia*-infected leukocytes. Fresh anticoagulated blood samples brought to the laboratory within 24 h of collection were used as a source for leukocytes (WBCs) in attempts to transfer *Ehrlichia* from infected peripheral blood cells to tissue culture cells. The blood was centrifuged at 2,500 rpm (1,000 × *g*) for 10 min and the buffy coat was harvested, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 2 ml of RP-U. WBC densities obtained from the buffy coat preparation were determined with a hemocytometer. The WBCs were then used to infect cultures of HL-60 cells and to prepare DNA for PCR-based detection of *Ehrlichia* DNA.

Propagation of infected cultures. The propagation of *Ehrlichia* in HL-60 cultures employed the strategy schematized in Fig. 1. Cocultures were set up by adding aliquots of 2×10^6 WBCs to three cultures, each of which contained 1 ml of HL-60 cells at 10^6 cells/ml and 5 ml of medium in 12.5-cm^2 tissue culture flasks. One culture contained uninduced HL-60 cells and 5 ml of RP-U; the second contained HL-60 cells induced for 2 to 4 days with RA and 5 ml of RP-U plus 1 μM RA (RP-RA); the third contained HL-60 cells induced for 2 to 4 days with VD and 5 ml of RP-U plus 2 μM VD (RP-VD). The tissue culture flasks were maintained at 37°C in a 5% CO_2 incubator.

Two days after the initial cocultures were established, each was subdivided by removing 2 ml of the cell suspension to a new flask with 4 ml of the appropriate fresh medium. Cultures which tested positive by PCR for the presence of *Ehrlichia* were maintained and passaged. The infected, uninduced cultures of HL-60 cells growing in RP-U were attended to every 4th or 5th day, according to a protocol (Fig. 1) that alternated between subdivision of the culture (0.5 ml of old culture plus 5 ml of fresh RP-U) and passage with uninfected, uninduced HL-60 cells (0.5 ml of old culture plus 0.5 ml of logarithmically growing HL-60 cells at a density of 10^5 cells per ml plus 5 ml of fresh RP-U). Infected cultures containing RA or VD as inducing agents were passaged weekly. For granulocytically induced cells growing in RP-RA, each passage combined 0.5 ml of the old culture with 1 ml of uninfected, RA-induced HL-60 cells at a density of 2×10^5 cells per ml and 5.0 ml of fresh RP-RA. For monocytically induced cells growing in RP-VD, each passage combined 0.5 ml of the old culture with 1 ml of uninfected, VD-induced HL-60 cells at a density of 2×10^5 cells per ml and 5.0 ml of fresh

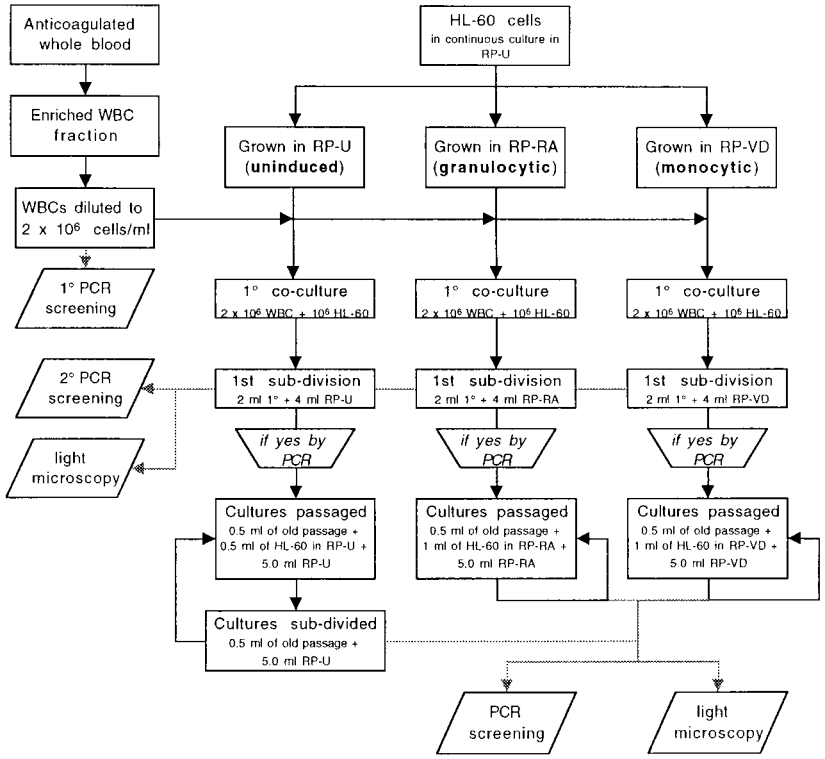

FIG. 1. Flow chart of culture protocol. The scheme for the procedures to establish and propagate *Ehrlichia* used initial coculturing of WBCs from humans and horses with symptoms of ehrlichiosis and uninduced, RA-induced, or VD-induced HL-60 cells.

RP-VD. The maintenance of *Ehrlichia* in these cultures was assayed by PCR or by cytological staining and light microscopy.

Microscopic detection of *Ehrlichia* spp. For light microscopy, 0.1-ml aliquots of cells in culture were applied to a slide by using a cytospin (Shandon Scientific, Pittsburgh, Pa.) preparation at 1,000 rpm. Slides were stained sequentially with Wright's and Giemsa stains. For electron microscopy, cells were fixed in suspension in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer and then immediately pelleted by centrifugation. The pellets were poststained with 1% aqueous osmium tetroxide, dehydrated with ethanol, and embedded in Epon resin. Sections, contrasted with uranyl acetate and lead citrate, were viewed and photographed on an EM410 transmission electron microscope (Phillips, Mahwah, N.J.) operating at 80 kV. Electron microscopy was performed by the Center for Cell Imaging at the Yale University School of Medicine.

PCR identification of *Ehrlichia* spp. The initial screening of each blood sample for the presence of *Ehrlichia* employed a PCR-based assay for the detection of *Ehrlichia* DNA. DNA was extracted by either of two methods. In the first, DNA was prepared from 200 μl of WBC suspension by standard proteinase digestion, sequential organic extraction with phenol and chloroform-isoamyl alcohol, and ethanol precipitation. Recovered DNA was resuspended in 200 μl of double-deionized H_2O. In the second method, DNA was prepared from 100 μl of whole blood by cell lysis and proteolysis followed by affinity chromatography according to the manufacturer's recommendations (QIAamp blood kit; Qiagen Inc., Chatsworth, Calif.). Affinity-purified DNA was eluted into 200 μl of double-deionized H_2O. Following DNA extraction by either method, 2.5-μl aliquots of 1:2, 1:20, and 1:200 dilutions of the recovered DNA were analyzed for the presence of ehrlichial 16S rDNA.

PCR-based detection of *Ehrlichia* DNA in the HL-60 cell cultures was carried out on plateau phase cultures at a density of 1.2×10^6 to 1.5×10^6 HL-60 cells/ml. Target DNA was prepared as previously described for 16S rDNA-based phylogenetic studies (8). Cells in 500 μl of culture were washed once with PBS; the cell pellet was resuspended in 100 μl of a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 20 μg of proteinase K per ml; and the lysate was incubated for 1 h at 50°C. The proteinase K was inactivated by incubation at 100°C for 10 min. PCR was run with 2.5 μl of 1:5 and 1:100 dilutions of the cell extract.

All amplification reactions were carried out with primers EHR521 and EHR747, which amplify a portion of the 16S rDNA gene (9). All reactions had final volumes of 50 μl, containing final concentrations of 40 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.04% (wt/vol) gelatin, 250 μM each deoxynucleoside triphosphate, 0.5 μM each primer, and 2 U of *Taq* DNA polymerase. Following an initial denaturation at 95°C for 2 min, PCR was run for 35 cycles, each consisting of 20 s at 92°C, 30 s at 57°C, and 45 s at 72°C. A final elongation at 72°C was carried out for 6 min. PCR products were identified by 2% agarose gel electrophoresis and ethidium bromide staining. Positive controls included DNA obtained from three *Ehrlichia* species: *E. equi*, *E. risticii* (both kind gifts of Cynthia Holland, Protatek Laboratories, Phoenix, Ariz.), and *E. chaffeensis* (a kind gift of Jacqueline Dawson, Centers for Disease Control and Prevention, Atlanta, Ga.). DNA from each of these species was successfully amplified with the EHR521-EHR747 primer pair (data not shown). Three or four negative controls, including samples containing no target DNA, horse WBC DNA, and DNA from induced and uninduced HL-60 cells, were included with each set of amplification reactions. The success of the standard laboratory practices to prevent end product contamination of amplification reactions was attested to by the absence of false positives among the negative controls.

DNA sequencing. PCR products were directly sequenced with the EHR521 and EHR747 primers, *Taq* FS DNA polymerase, and fluorescent dideoxy terminators (Perkin-Elmer, Foster City, Calif.) by a cycle sequencing method. The resultant DNA fragments were separated by electrophoresis and analyzed with an automated 373A Stretch DNA sequencer (Applied Biosystems, Perkin-Elmer). Sequencing was performed in the W. M. Keck facility at Yale University. Sequences obtained by using downstream and upstream primers were compared and resequenced if necessary to resolve all heterologies. Sequences were aligned with Megalign (Lasergene, DNAStar, Madison, Wis.) and compared to *Ehrlichia* sequences entered in GenBank for the HGE agent (U02521), *E. equi* (M73223), *E. phagocytophila* (U10873), and *E. chaffeensis* (M73222).

RESULTS

Between August and December 1995, 17 human and 29 horse samples likely to be infected with *Ehrlichia* were received. In the initial PCR screen using proteinase K digestion, organic extraction, and ethanol precipitation to purify DNA, 2 (11.8%) of the human samples and 19 (65.5%) of the horse samples tested positive. Concomitantly with the DNA extractions, WBCs from the buffy coat of all samples were prepared and introduced into cocultures containing either undifferentiated, RA-induced, or VD-induced HL60 cells. Cultures were subdivided once after 48 h in culture. After an additional 5 days in culture, cells were removed and DNA was extracted

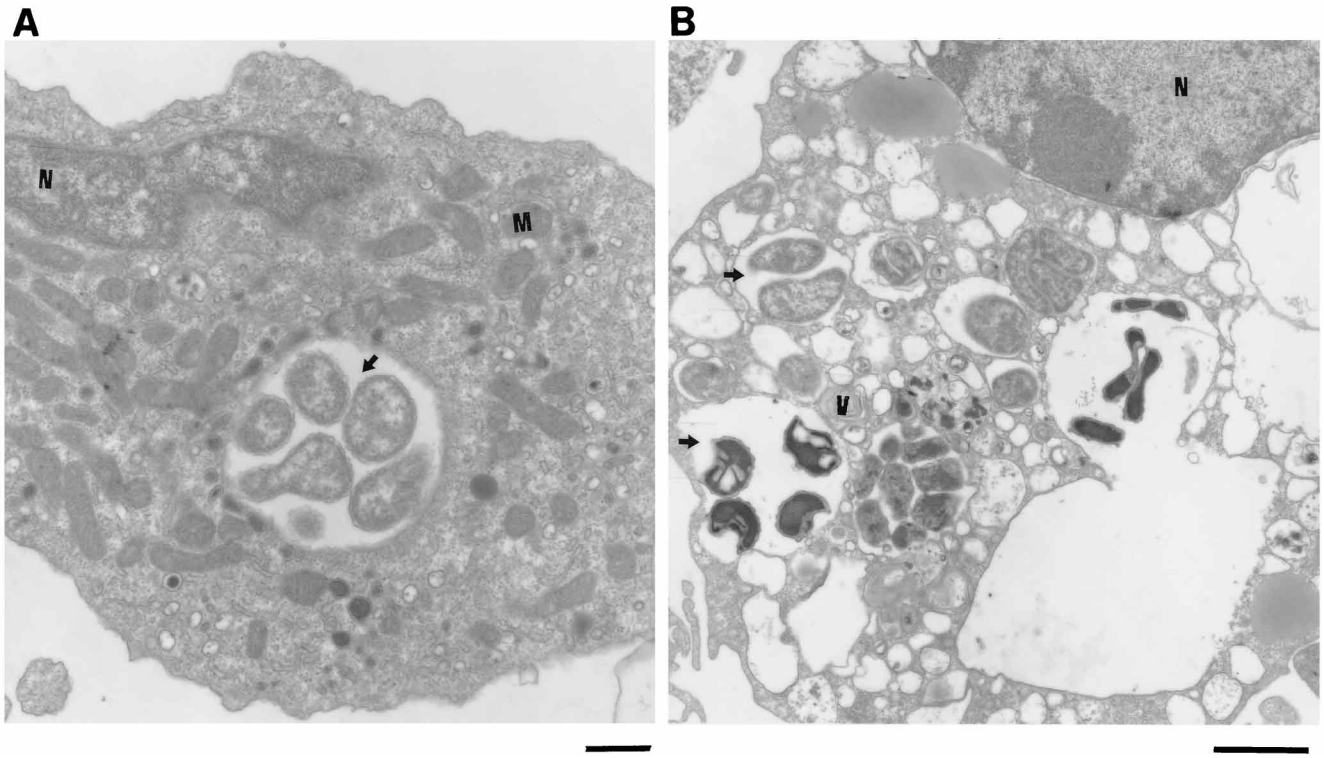

FIG. 2. Micrographs of *Ehrlichia* in HL-60 cells. (A) An *Ehrlichia* morula in uninduced HL-60 cells. (B) Morulae in HL-60 cells induced with 1 μM RA. The morulae (arrows), nuclei (N), mitochondria (M), and multilamellar vesicles (V) are indicated. Bar, 1 μm.

and again analyzed by PCR. In addition to the positive samples from the initial PCR, *Ehrlichia* DNA was recovered from cultures from one human and three horse samples which had tested negative. To retest these four negative DNA samples, the initial DNA extracts from the buffy coat were reextracted with phenol and chloroform-isoamyl alcohol and amplified by PCR; each subsequently tested positive. Therefore, the sensitivity of the initial screening method was less than 100%.

To improve the sensitivity of the initial screen, we switched to a method employing direct lysis of whole blood and affinity chromatography to recover DNA. This technique was applied to the next 12 samples obtained after December 1995. Four positive samples were detected by affinity chromatography. Each sample which tested positive in the initial screen remained positive when cocultures were tested. Of the eight DNA extracts which were negative following affinity chromatography, all cocultures established from the WBCs remained negative for *Ehrlichia* following the initial period of HL-60 cell coculture propagation. To ascertain if the affinity chromatography also yielded false-negative DNA preparations, the eight negative column eluates were subjected to an additional organic extraction step similar to that used for reextraction of the previous false negatives. DNA from all eight extracts was amplified and tested negative. Although the second method appeared superior as an initial test (sensitivity, 100%), the number of samples tested was too small to demonstrate a statistically significant difference ($P = 0.060$) in the sensitivity of the two DNA extraction techniques employed for initial screening.

We began HL-60 cocultures on 16 of the 25 PCR-positive samples. Sustained growth of *Ehrlichia* in culture, defined as detection by PCR and by light microscopy in subsequent HL-60 passages, was observed in 11 cases (3 human and 8 horse). Infected HL-60 cell cultures, in which ehrlichial DNA remained detectable while morulae were observed in approximately 5% of the cells, were maintained for periods up to 6 months. The five unsuccessfully infected cultures lost all cytological and molecular traces of *Ehrlichia* infection after the second passage, i.e., following 2 weeks in culture. Sustained infection was observed in electron micrographs, in which the *Ehrlichia* appeared mostly within membrane-bound vacuoles, displayed the characteristic wavy outer wall, and were found in both reticular and pyknotic forms (Fig. 2). Differences in HL-60 cell phenotypes also were apparent in these micrographs. The uninduced cells appeared with more condensed nuclei, multiple mitochondria with fully articulated cristae, and a smattering of vesicles (Fig. 2A). In contrast, RA-induced HL-60 cells had more extended nuclei with visible perinuclear gaps, fewer and more disrupted mitochondria, and many more, larger, and frequently multilamellar vesicles, all features characteristic of terminally differentiated cells (Fig. 2B).

Ehrlichial rDNA could be detected in all cultures during the first passage. However, the strength of the signal faded rapidly in all VD-induced cultures and disappeared altogether by the third passage (Fig. 3). In contrast, growth was sustained in uninduced and RA-induced HL-60 cell cultures. Two distinct patterns of sustained propagation of *Ehrlichia* were observed by PCR-based detection of 16S rDNA (Fig. 4). Of the 11 cultures with sustained growth in HL-60 cells, 3 (27.3%) displayed approximately equal growth of *Ehrlichia* in uninduced and RA-induced cells (Fig. 4B). Two of the three were cultures of *Ehrlichia* derived from horses and one was a culture of *Ehrlichia* derived from a human. In the remaining eight cultures, the propagation of *Ehrlichia* was better sustained in the RA-induced HL-60 cell cultures than in the uninduced cultures (Fig. 4A and C). Most of the *Ehrlichia* isolates (7 of 11,

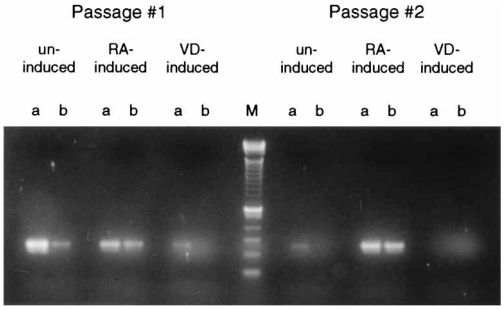

FIG. 3. PCR detection of *Ehrlichia* in early passages of HL-60 cells. Shown are results from the first and second passages of cocultures of WBCs from the isolate WA1 and uninduced, RA-induced, or VD-induced HL-60 cells. For each DNA extract, a 1:5 dilution (lanes a) and a 1:100 dilution (lanes b) were amplified. Lane M, 100-bp ladder used as molecular weight markers.

or 63.6%) were maintained for 6 months in culture with RA-induced HL-60 cells. However, four isolates (one human and three horse) were lost from both uninduced and RA-induced cultures during the course of culture. PCR-based detection of the human isolate was lost after four passages in both the uninduced and the RA-induced cultures. The loss of the PCR-based detection of *Ehrlichia* from all three equine isolates occurred first in uninduced cultures, in each case after 2 passages; in RA-induced cultures, PCR-detectable *Ehrlichia* rDNA disappeared following 5, 6, and 12 passages.

The amplified PCR products from all 3 human samples and from 15 of the 19 horse samples, including all 8 isolates that were successfully cultured, were sequenced. These 18 sequences were compared to each other and to those previously published. All 3 human sequences and 13 of 15 horse sequences were identical to those previously determined for *E. equi* (M73223), *E. phagocytophila* (U10873), and the HGE agent (U02521), as well as to the reference *E. equi* sample, which has been verified as *E. equi* by both cytological and immunological means. Two horse sequences (13.3%) differed from the consensus sequence by the substitution of an adenine for a guanine residue at a position which corresponds to 609 in U02521, 603 in M73223, and 590 in U10873.

DISCUSSION

Ehrlichia infections of humans and horses in Connecticut and New York were analyzed, and attempts were made to adapt the pathogen to suspension cell culture. The presence of *Ehrlichia* in blood obtained in suspected cases was detected by PCR for 3 of 17 human samples and 19 of 29 horse samples. Based on the DNA sequencing of a portion of the 16S rDNA PCR products, we conclude that these *Ehrlichia* isolates are either closely related to or identical with *E. equi*. The genetic similarity among these isolates should not be surprising, since all previous reports have demonstrated the similarity of the pathogens causing human and equine granulocytic ehrlichiosis (3, 5–7). Among the 18 PCR products we sequenced, there was close agreement with the consensus sequence, with only a single base pair difference, the presence of an adenine in place of the consensus guanine, in two cases. Both divergent isolates were derived from horses which, interestingly, were stabled in adjacent towns. However, *Ehrlichia* infections were detected in two other horses stabled in one of the towns, and these were infected with *E. equi* in which the 16S rDNA matched the consensus sequence. No *E. chaffeensis* was detected among the human subjects; the absence may only be a consequence of the small number of positive human samples, since *E. chaffeensis* infections have been reported in Connecticut based on results obtained by serological methods (4).

The isolation and propagation of *Ehrlichia* from the blood of 11 human and equine subjects and the sequencing of *Ehrlichia* from 18 specimens provide the largest set of *E. equi*-like organisms yet analyzed. The sequencing data lend strong support to the growing recognition that human and equine granulocytic ehrlichioses are caused by the same pathogen. However, the sequencing of a gene more divergent than the 16S rDNA gene may be necessary in order to conclude finally that *E. equi*, *E. phagocytophila*, and the agent of HGE are one and the same.

The establishment and propagation of so many isolates was facilitated by the existence of a tissue culture system based on growth of the pathogen in HL-60 cells (5). However, growth was enhanced and prolonged by the use of 1 μM RA, which induced granulocytic differentiation of HL-60. The RA-differentiated cell cultures proved as good as or better than the uninduced cell cultures as a tissue culture system for the propagation of *E. equi*, since infection was frequently maintained longer and at a higher relative level in the induced cell cultures. The similar patterns of growth in HL-60 cells of *Ehrlichia*, regardless of source, support the sequencing data in ascribing infections of different mammals to a common pathogen.

In our hands, some PCR-positive samples failed to grow in either the uninduced or the RA-induced HL-60 cultures. Furthermore, several of the cultures displayed diminished or disappearing levels of *Ehrlichia* during passage in culture. Among the possible reasons for these failures are isolate-specific differences in the *Ehrlichia*, low levels of pathogen in unsuccessful samples, and delays between venipuncture and initiation of the cocultures. One limitation of this study is that with the cur-

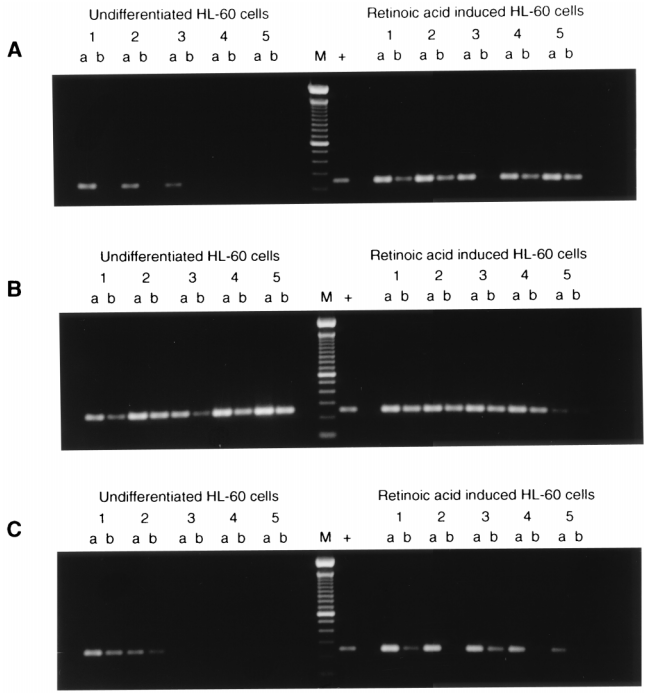

FIG. 4. PCR detection of *Ehrlichia* during 5 months of passage in uninduced or RA-induced HL-60 cells. At approximately monthly intervals, DNA was extracted from cell cultures and *Ehrlichia* DNA was amplified by PCR. For each DNA extract, a 1:5 dilution (lanes a) and a 1:100 dilution (lanes b) were amplified. The patterns for three different *Ehrlichia* isolates (WA2 [A], H05 [B], and H08 [C]) are shown. Lanes M, 100-bp ladder used as molecular weight markers; lanes +, positive controls.

rently available data, we cannot distinguish among these explanations. While these uncertainties point to the need to define experimentally the parameters necessary for long-term *Ehrlichia* propagation in cultured cells and the cellular factors associated with pathogenesis, the finding that propagation was enhanced in the RA-induced HL-60 cells provides a first step toward such a definition.

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