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DIRECT CULTIVATION OF THE CAUSATIVE AGENT OF HUMAN GRANULOCYtic EHRlichiosis

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Abstract Background. Human granulocytic ehrlichiosis is a potentially fatal tick-borne infection that has recently been described. This acute febrile illness is characterized by myalgias, headache, thrombocytopenia, and elevated serum aminotransferase levels. The disease is difficult to diagnose because the symptoms are non-specific, intraleukocytic inclusions (morulae) may not be seen, and the serologic results are often initially negative. Little is known about the causative agent because it has never been cultivated.

Methods. We studied three patients with symptoms and laboratory findings suggestive of human granulocytic ehrlichiosis, including unexplained fever after probable exposure to ticks, granulocytopenia, and thrombocytopenia. Peripheral blood was examined for ehrlichia microscopically and with use of the polymerase chain reaction (PCR). Blood was inoculated into cultures of HL60 cells (a line of human promyelocytic leukemia cells), and the cultures were monitored for infection by Giemsa staining and PCR.

Results. Blood from the three patients, only one of whom had inclusions suggestive of ehrlichia in neutro-

phils, was positive for human granulocytic ehrlichiosis on PCR. Blood from all three patients was inoculated into HL60 cell cultures and caused infection, with intracellular organisms visualized as early as 5 days after inoculation and cell lysis occurring within 12 to 14 days. The identity of the cultured organisms was confirmed by immunofluorescence microscopy, PCR analysis, and DNA sequencing. DNA from the infected cells was sequenced in regions of the 16S ribosomal gene reported to differ between the agent of human granulocytic ehrlichiosis and closely related species, including *Ehrlichia equi* and *E. phagocytophila*, which cause infection in animals. The sequences from all three human isolates were identical and differed from the strain of *E. equi* studied in having guanine rather than adenine at nucleotide 84.

Conclusions. We describe the cultivation of the agent of human granulocytic ehrlichiosis in cell culture. The ability to isolate this organism should lead to a better understanding of the biology, treatment, and epidemiology of this emerging infection. (N Engl J Med 1996;334:209-15.)

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EHRlichia are intracellular organisms that may infect a variety of mammalian hosts.¹ The rapid emergence of a new human ehrlichial infection, human granulocytic ehrlichiosis, was recently reported. This infection was first recognized in north central Minnesota and Wisconsin^{2,3} and has now been reported in New York⁴ and Massachusetts.⁵ Preliminary studies⁴ suggest that human granulocytic ehrlichiosis is transmitted by *Ixodes scapularis* ticks, also a vector of Lyme disease.

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The University of Minnesota, University of Maryland, and University of California have applied for a patent on some of the cultivation techniques described for the agents causing granulocytic ehrlichiosis, with Drs. Dumler, Munderloh, Kurtti, Madigan, and Goodman listed as coinventors.

Human granulocytic ehrlichiosis is an acute, sometimes fatal, febrile syndrome. Unlike Lyme disease, it is usually accompanied by leukopenia, thrombocytopenia, and elevated serum aminotransferase levels. Little is known about the agent that causes human granulocytic ehrlichiosis, largely because it has not been isolated in culture.

CASE REPORTS

Patient 1

Patient 1 was a 61-year-old man from northwest Wisconsin with a 36-hour history of fever, myalgias, headache, and nausea. After a recent fishing trip he had removed ticks from himself. Examination showed him to be acutely ill and febrile (temperature, up to 38.5°C). The white-cell count was 5200 per cubic millimeter, the hemoglobin level was 152 g per liter, and the platelet count was 82,000 per cubic millimeter. No inclusions suggestive of ehrlichia were seen on blood smears. Treatment with 100 mg of doxycycline twice daily orally was initiated, with dramatic improvement within 24 hours. Treatment lasted two weeks, after which the patient was well.

Patient 2

Patient 2 was a 66-year-old woman from north central Minnesota with a two-day history of fever, confusion, ataxia, and vomiting. She

did not recall having any tick bites, but two weeks earlier had walked through woods in northwestern Wisconsin. She had a temperature of 38.9°C and ataxia. A computed tomographic scan of the head and lumbar puncture revealed no abnormalities. Laboratory analyses revealed the following: hemoglobin level, 130 g per liter; white-cell count, 2600 per cubic millimeter, with 33 percent neutrophils, 58 percent bands, 8 percent lymphocytes, and 1 percent monocytes; platelet count, 33,000 per cubic millimeter; alanine aminotransferase level, 86 U per liter (normal, 0 to 33); lactate dehydrogenase level, 386 U per liter (normal, 91 to 232); and alkaline phosphatase level, 154 U per liter (normal, 34 to 114). One percent of the neutrophils contained inclusions suggestive of ehrlichia. Intravenous doxycycline was administered, and within 24 hours the patient was afebrile. She was given oral doxycycline at a dose of 100 mg twice daily for two weeks. The ataxia resolved, and she was well at this writing.

Patient 3

Patient 3 was a 64-year-old man from north central Minnesota with a history of lymphoid interstitial pneumonitis who had last received immunosuppressive treatment three years previously. Fever, rigors, and nausea developed, for which a regimen of amoxicillin and clavulanate was prescribed. The patient had no response and was admitted to another hospital. He had a white-cell count of 5700 per cubic millimeter, a platelet count of 95,000 per cubic millimeter, an aspartate aminotransferase level of 59 U per liter, and an alanine aminotransferase level of 62 U per liter. Chest x-ray films showed no change in the degree of interstitial fibrosis, and cultures of blood and sputum were negative. The patient was treated with erythromycin and ceftriaxone for 10 days, with improvement. Nine days later the fever recurred. The patient was readmitted, the antibiotics were re-instituted without improvement, and he was transferred to the University of Minnesota Hospital. He reported having removed multiple ticks from his dogs and having had laryngeal edema after tetracycline therapy. His temperature was 39.2°C, and he was somnolent. The white-cell count was 9200 per cubic millimeter, the hemoglobin level was 110 g per liter, and the platelet count was 179,000 per cubic millimeter. No babesia or ehrlichia were noted on blood smears. The alanine aminotransferase level was 88 U per liter, the bilirubin level was 1.4 mg per deciliter (normal, 0.1 to 1.2), the alkaline phosphatase level was 206 U per liter, and the lipase level was 942 U per liter (normal, 23 to 300). The cerebrospinal fluid was normal, and routine cultures were negative. Chloramphenicol was administered orally at a dose of 500 mg four times daily. Within 24 hours the patient became afebrile. By the fourth hospital day, the platelet count had increased to 306,000 per cubic millimeter, but the patient had dyspnea. An echocardiogram revealed a large pericardial effusion and tamponade. Pericardiocentesis yielded 650 ml of fluid, with 35,000 red cells per cubic millimeter and 790 white cells per cubic millimeter (53 percent neutrophils). Cultures and cytologic analysis were negative. Tests for serum antinuclear antibodies were positive at a titer of more than 1:320, and the level of antinative DNA was 184 IU per milliliter (normal, 0 to 99). There was no other clinical evidence suggestive of systemic lupus erythematosus. The patient was treated with chloramphenicol for 10 days and remained well four months later.

METHODS

Cultivation of Patients' Blood Samples and *Ehrlichia equi* in HL60 and Tick Cells

The HL60 leukemia cell line⁶ (American Type Culture Collection CCL240) was cultivated in RPMI 1640 medium supplemented with 10 percent heat-inactivated fetal-calf serum and 2 mM glutamine and maintained at 37°C in 5 percent carbon dioxide. Blood samples (100 μ l) were obtained from the patients, treated with EDTA as an anticoagulant, and immediately inoculated into 3 ml of HL60 cells adjusted to a density of 500,000 per milliliter. Cells were kept in 25-cm² culture flasks at a density of 200,000 to 600,000 per milliliter by feeding the cells with medium twice a week.

The tick-embryo cell line IDE8 was isolated from *I. scapularis*⁷; IDE8 cells, which support the growth of *E. equi* (unpublished data),

were maintained at 34°C and infected in the same manner as HL60 cells. Control and inoculated HL60 and IDE8 cultures were maintained in parallel.

The MRK strain of *E. equi* was obtained from the blood of an infected horse⁸ and passed in vivo in horses several times. Buffy-coat blood from infected horses was used both to infect HL60 cells directly and to infect IDE8 tick cells, which were then used to infect HL60 cells secondarily. When 30 percent of the tick cells were infected, as determined by Giemsa staining, 0.25 ml was added to 9 ml of HL60 cells, and the cultures were maintained as described above.

Giemsa Staining and Immunofluorescence Microscopy

Slides of the cultured cells were dried, fixed in methanol for 10 minutes, and stained with Giemsa stain for 30 minutes at a pH of 6.8. An indirect immunofluorescence assay was performed after the slides had been fixed for 10 minutes in a 1:1 solution of methanol and acetone. The primary antibody applied was either control human serum (negative for *E. equi* antibody) or serum from a patient who had recovered from human granulocytic ehrlichiosis (*E. equi* titer, 1:40). The slides were counterstained with Evans blue and mounted with phosphate-buffered saline supplemented with 3 percent bovine-serum albumin, 10 percent glycerol, and 10 percent triethylenediamine.

Serologic Studies

Serum samples were assayed by an indirect immunofluorescence assay for the presence of antibodies against *E. equi* as described previously.⁸ Titers of more than 1:20 were considered positive because higher titers were not noted in uninfected horses or control patients and have been protective against experimental infection in horses.

PCR Analysis of Blood Samples and Cultured Cells

Blood samples and cultured cells were processed in a building where ehrlichia and their nucleic acids have never been present. To prevent contamination of samples for polymerase chain reaction (PCR), aerosol barrier pipette tips were used. A 100- μ l sample of blood was subjected to nucleic acid extraction with guanidium isothiocyanate chaotropic lysis (IsoQuik, ORCA Research, Bothell, Wash.). The template DNA for each assay, one third of the nucleic acids derived, was resuspended in 10 μ l of water. The other components of the PCR were as follows: 50 mmol of potassium chloride per liter; 10 mmol of TRIS buffer per liter at a pH of 8.3; 2 mmol of magnesium chloride per liter; 200 μ mol each of 2'-deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate per liter; 25 pmol of each primer; and 2.5 U of Amplitaq DNA polymerase per 100 μ l (Chiron, Cetus, Emeryville, Calif.). The primers PER 1 (5'TTTATCGCT-ATTAGATGAGCCTATG3') and PER 2 (5'TCTACTACAGGA-ATTCCGCTAT3') correspond to bases 187 to 211 and 616 to 638, respectively, of the reported sequence for the agent of human granulocytic ehrlichiosis (GenBank accession number U02521) and amplify a fragment of 451 base pairs (bp) from ehrlichia species other than *E. sensu. The primers GER 3 (5'TAGATCCTTAACGGAAGG-GCG3')* and GER 4 (5'AAGTGCCCGCTTAACCCGCTGGC3') correspond to bases 950 to 973 and 1077 to 1101, respectively, and amplify a 151-bp fragment from species of the *E. phagocytophila* group (e.g., *E. equi* and the agent causing human granulocytic ehrlichiosis) but not from monocytic ehrlichia, including the closely related *E. canis*. For PER 1 and PER 2, amplification was performed in a thermal cycler (model 9600, Perkin-Elmer, Norwalk, Conn.) with 5 minutes of denaturation at 95°C, followed by 40 cycles consisting of 11 seconds of denaturation at 94°C, 10 seconds of annealing at 45°C, and 15 seconds of extension at 72°C for all cycles but the 40th, in which extension lasted 7 minutes. For GER 3 and GER 4, amplification was performed in a Coy thermal cycler (Coy Laboratory Products, Ann Arbor, Mich.) with 5 minutes of denaturation at 95°C, followed by 40 cycles consisting of 1 minute of denaturation at 94°C, 1 minute of annealing at 50°C, and 1 minute of extension at 72°C for all cycles but the 40th, in which extension lasted 7 minutes. Multiple (≥ 4) negative controls were processed in parallel and included in every experiment.

For analysis, a 15- μ l sample was electrophoresed in agarose gels. As a probe for the 450-bp product of the PER 1 and 2 primers, an internal *Hin*II fragment was obtained by digestion and gel purification of PCR-amplified *E. equi* DNA. Digoxigenin labeling of probes, Southern hybridization, and the chemiluminescence assay (Genius, Boehringer-Mannheim, Indianapolis) were performed as described previously.⁹

DNA Sequencing of Isolates of the Agent Causing Human Granulocytic Ehrlichiosis from Infected HL60 Cells

DNA was amplified with PCR as described for the PER 1 and 2 primers. However, the primer pairs used were PER 3 (5'-ATGCATTACTCACCCCTCTG3') and PER 4 (5'-TCCTGGCTCAGAACGAACGC3'), which span bases 1 to 20 and 92 to 111, respectively, of the 16S sequence of the agent causing human granulocytic ehrlichiosis, and PER 5 (5'-AAGCACTCCGCCTGGGGACT3') and PER 6 (5'-CCATGTCAAGGAGTGGTAAGG3'), which span bases 818 to 837 and 925 to 943, respectively. To minimize the amplification of cellular sequences, *Taq* polymerase was not added to the reaction mixtures until the temperature reached 95°C (so-called hot-start PCR). DNA was purified with Centricon-30 columns (Amicon, Beverly, Mass.), sequenced with primers PER 3, 4, 5, and 6, and dye-labeled with dideoxynucleotides (PRISM, Applied Biosystems, Foster City, Calif.). The sequence of both DNA strands was obtained with a DNA sequencer (model 373, Applied Biosystems).

RESULTS

Cultivation of the Agent of Human Granulocytic Ehrlichiosis

Complete cytopathic effects, with lysis of the HL60 cells, were noted 12 days after blood from Patient 1 was inoculated into HL60 cells, and PCR analysis of the cultured cells was strongly positive for human granulocytic ehrlichia. Organisms and degenerating cells were visualized, but the organisms could not be recovered by subcultivation. Control cultures and cultures of blood obtained one day after the initiation of doxycycline therapy showed no evidence of infection on Giemsa staining or PCR. Twelve days after the inoculation of blood from Patient 2 into HL60 cells, organisms were noted on Giemsa staining in almost 100 percent of the HL60 cells. A variety of forms were noted, ranging from what were presumed to be individual organisms to small, dense bodies to rounded masses of organisms similar to those seen in granulocytes (morulae) in some patients (Fig. 1A). Immunofluorescence microscopy (Fig. 1B) demonstrated specific intense staining of ehrlichial antigens in both morulae and smaller forms. Such staining was not observed in uninfected cells or cells incubated with control serum. In cultures inoculated with blood from Patient 3, rare morulae were first noted in both HL60 and IDE8 cells five days after infection. By day 14, more than 90 percent of HL60 cells contained morulae and other forms similar to those noted in Patient 2 (Fig. 1C), but less than 1 percent of IDE8 cells were infected. As of this writing, the isolates from Patients 2 and 3 have been continuously subcultivated in HL60 cells for 4 to 5 months by the addition of fresh cells every 7 to 10 days. All blood samples obtained from the three patients after the initiation of doxycycline therapy and from three other patients who were initially suspected of having ehrlichiosis but who were found not to have it on the

basis of PCR and blood-smear examination were culture-negative.

Cultivation of *E. equi* in HL60 Cells

Rare morulae were first noted 21 days after HL60 cells were inoculated with *E. equi* derived from IDE8. By day 49, 27 percent of cells contained morulae. As was true for the agent causing human granulocytic ehrlichiosis, these morulae had complex internal structures, and infected cells often contained several morulae (Fig. 1D); however, cell lysis was rarely observed. Immunofluorescence microscopy revealed intense and specific staining of morulae not seen with control cells or cells incubated with control serum. The rate of replication was slow in tick-cell-derived *E. equi*. In contrast, eight days after direct inoculation of HL60 cells with blood from a horse infected with the same *E. equi* strain, morulae were noted in 90 percent of the cells.

PCR Analysis of Blood Samples and Cell Cultures

Blood samples from Patients 1, 2, and 3 were analyzed by PCR with primer pair PER 1 and 2. The pretreatment blood samples from Patients 1 and 2 were strongly positive for ehrlichial DNA (Fig. 2, lanes 2 and 13), yielding bands of the appropriate molecular weight (451 bp). Blood samples from Patient 3, who had been treated previously with antibiotics, were negative (Fig. 2, lane 17) (sensitivity, <10 genomes; Fig. 2, lanes 9 to 11), but Southern blotting (sensitivity, 1 genome; data not shown) confirmed the identity of all positive signals as ehrlichial in origin and was positive for Patient 3 at an intensity of less than 10 genomes. In blood specimens obtained from Patients 1 and 2 one day after treatment was initiated, the intensity of bacteremia was already greatly diminished (Fig. 2, lanes 3 and 14). All control samples, including blood from a patient suspected of having ehrlichiosis but who actually had acute Epstein-Barr virus infection (Fig. 2, lane 6), uninfected HL60 cells (Fig. 2, lane 19), and water (water was processed instead of DNA) (Fig. 2, lanes 7, 8, and 20), were negative for ehrlichial DNA.

Analysis of HL60 cell cultures inoculated with blood from the three patients revealed that all were positive for ehrlichial DNA with the use of both the general ehrlichial primers (PER 1 and 2) (Fig. 2, lanes 4, 15, and 18) and the primers specific for granulocytic ehrlichia (GER 3 and 4) (Fig. 3, lanes 2, 3, and 4). In each case the intensity of the bands generated by PER 1 and 2 from the cultured samples was far greater than the intensity of the bands generated from the original blood samples (Fig. 2), despite the fact that the original inoculum had already been diluted by a factor of more than 1:500 during culture. The band in the lane showing results for HL60 cells inoculated with blood from Patient 3 (Fig. 3, lane 4) is faint because amplification was performed only five days after inoculation. Pericardial fluid from Patient 3 was strongly positive for granulocytic ehrlichial DNA (Fig. 3, lane 5). As expected, IDE8 and HL60 cells infected with *E. equi* yielded positive PCR

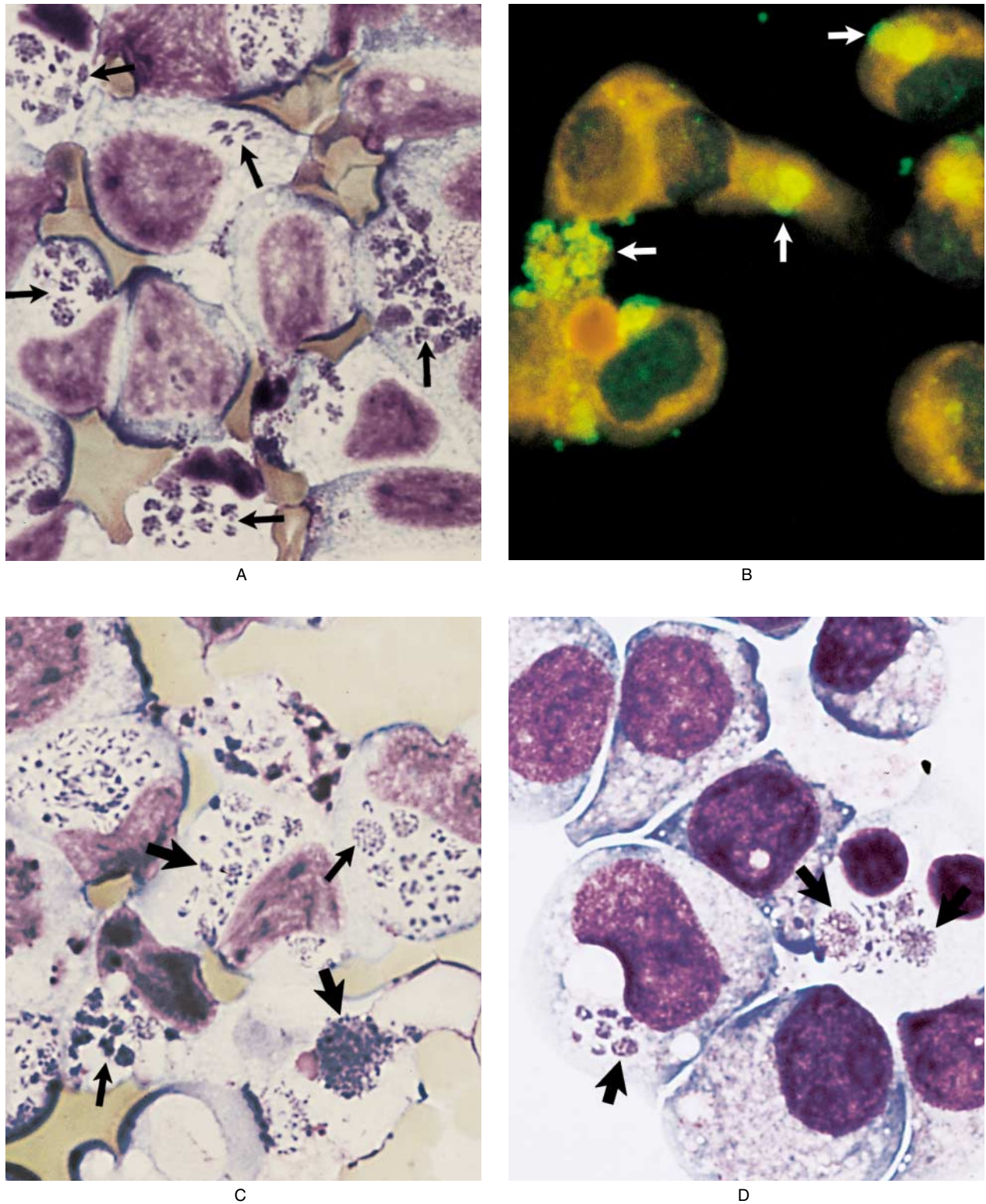


Figure 1. Photomicrographs of HL60 Cells Infected with Granulocytic Ehrlichia ($\times 750$).

Panel A shows Giemsa-stained cells infected by the agent of human granulocytic ehrlichiosis isolated from the blood of Patient 2. Panel B shows immunofluorescent staining of infected cells from Patient 2. Panel C shows Giemsa-stained cells infected by the agent of human granulocytic ehrlichiosis isolated from the blood of Patient 3. Panel D shows cells infected with *E. equi*. In each panel the arrows point to examples of various forms of the intracellular organisms.

results with both primer pairs, whereas IDE8 cells infected with *E. canis* were negative with the use of primers specific for granulocytic ehrlichia (GER 3 and 4). All control samples were negative.

Serologic Analysis

Serum obtained during the acute illness and follow-up was available from Patients 1 and 2, whereas a single sample from Patient 3 was obtained four weeks into his illness. Patient 1 had an initial *E. equi* antibody titer of 1:40 on immunofluorescence assay and a titer of 1:20 four weeks later. Patient 2 had an initial titer of 1:20 and a second titer of 1:5120. Patient 3 had a titer of 1:5120.

DNA Sequence Analysis of the Ehrlichial Isolates

The DNA sequence of both strands of the 113-bp 5' end of the 16S ribosomal DNA was determined from cultures of the isolates from all three patients and of *E. equi* (Table 1). All three sequences from the patients with human granulocytic ehrlichiosis were identical to each other and to the previously reported sequence.² However, all differed from *E. equi* at nucleotide 84, where *E. equi* had an adenine present, rather than a guanine, which is consistent with the previously identified sequences of *E. equi* and *E. phagocytophila* (GenBank accession numbers M73223 and M73220, respectively). However, the sequence of the MRK strain of *E. equi* that we used differed from that in the GenBank data base at base 33, which was previously described as the site of a second polymorphism between the agent causing human granulocytic ehrlichiosis and *E. equi*.² We found that *E. equi* and all three of the isolates from the patients had a thymine at this position. We also analyzed the DNA sequence of both strands from bases 818 to 929, surrounding base 886, the only other reported site of a 16S ribosomal sequence polymorphism between *E. equi* and *E. phagocytophila* and the agent of human granulocytic ehrlichiosis: the human agent was reported to have a guanine, whereas *E. equi* and *E. phagocytophila* were reported to have a gap of a single base.² We found that the DNA sequences were identical in all three cultured isolates from the patients and *E. equi* and that there was no gap in the *E. equi* sequence (Table 1).

DISCUSSION

We report the direct isolation from three patients of the causative agent

of human granulocytic ehrlichiosis, an emerging infection in the eastern and midwestern United States. The HL60 leukemia cell line was highly susceptible to infection with this agent, as indicated by the rapid development of cytopathic effects and the direct visualization of organisms within 5 to 12 days after inoculation. The identity of the organisms as the agent of human granulocytic ehrlichiosis was confirmed by immunofluorescence microscopy, PCR, and DNA-sequence analysis. The recovery of viable ehrlichia from Patient 3 (before chloramphenicol therapy), despite prior treatment with other antibiotics and an extremely low level of bacteremia, demonstrates the sensitivity of the culture system. Patient 3 appeared to respond rapidly to chloramphenicol, suggesting that it may be useful for patients with human granulocytic ehrlichiosis who cannot take tetracyclines, as has been suggested for patients with *E. chaffeensis*.¹⁰ This patient's course was also noteworthy because of the development of cardiac tamponade with an exudative pericardial effusion, which was strongly positive for the agent of human granulocytic ehrlichiosis on PCR. To our knowledge this manifestation has not previously been associated with this disease.

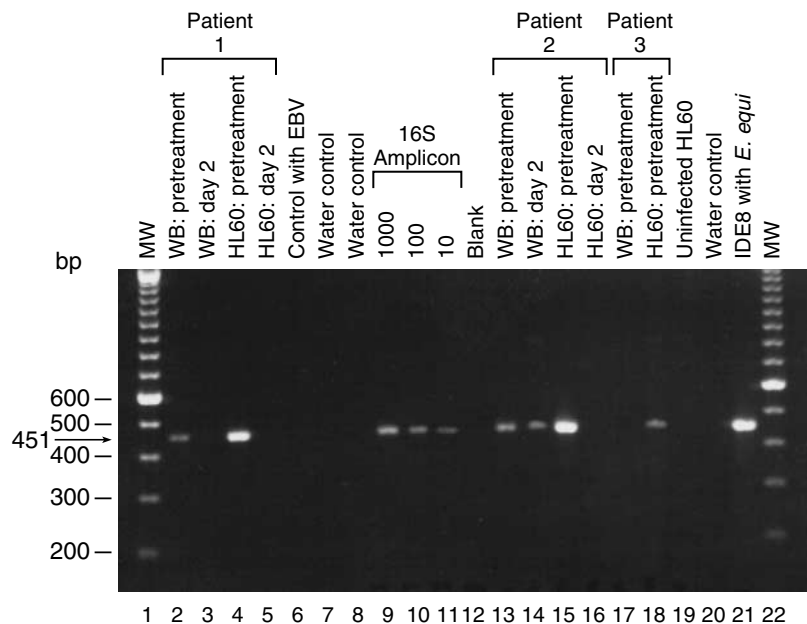


Figure 2. Identification of a 451-bp PCR Product of Ehrlichial DNA in Blood Samples from the Three Patients and in HL60 Cells Inoculated with the Blood Samples.

The primer pair PER 1 and 2 amplifies a 451-bp product of ehrlichial DNA (arrow). Lanes 1 and 22 show the molecular weight (MW) standard. Whole blood (WB) was analyzed before antibiotic treatment (lanes 2, 13, and 17) and after one day of treatment (day 2) (lanes 3 and 14). HL60 cells inoculated with the whole-blood samples obtained before treatment (lanes 4, 15, and 18) and after one day of treatment (lanes 5 and 16) were also examined. Negative controls included blood from a patient with Epstein-Barr virus (EBV) infection (lane 6), uninfected HL60 cells (lane 19), and samples in which water rather than blood was processed (lanes 7, 8, and 20). Positive controls included the 16S ribosomal DNA amplicon (in which 10 to 1000 molecules of the 451-bp amplicon were quantitated before it was used as a target for the PCR) (lanes 9, 10, and 11) and IDE8 tick cells infected with *E. equi* (lane 21).

The first recognized case of ehrlichial infection in humans occurred in Japan and was due to *E. sennetsu*.¹¹ Subsequent cases were documented in the United States¹¹⁻¹⁴ as due to a new species, *E. chaffeensis*.¹⁵ *E. chaffeensis* is distributed throughout the southeastern and south central United States, infects mononuclear phagocytes, and causes disease manifestations similar to those of human granulocytic ehrlichiosis. The agent of human granulocytic ehrlichiosis is serologically and genetically distinct from both *E. chaffeensis* and *E. sennetsu* but closely related to *E. equi* and *E. phagocytophila*,^{2,16} pathogens of horses and ruminants, respectively. In infected horses, *E. equi* causes fever, thrombocytopenia, and edema of the legs. Like the agent of human granulocytic ehrlichiosis, *E. equi* infects neutrophils and appears to be transmitted by ixodes ticks. The diagnosis of human granulocytic ehrlichiosis has depended on the results of blood smears or PCR or on findings of serologic reactivity against *E. equi*. As in our patients, both serologic analysis and blood smears may be nondiagnostic when the patient is first seen. The diagnosis may be missed when there is simultaneous infection with *Borrelia burgdorferi*, causing a localized rash (which is not normally found in ehrlichiosis). In such cases, patients may receive treatment (e.g., penicillins, cephalosporins, or macrolides) that is not effective against ehrlichiosis.

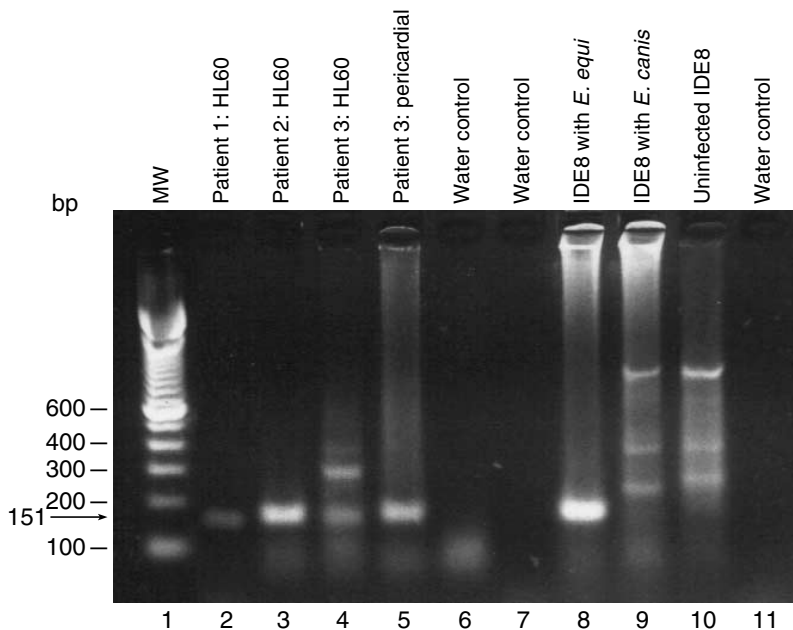


Figure 3. Identification of a 151-bp Segment of Granulocytic Ehrlichial DNA in Amplified DNA Obtained from HL60 Cells Inoculated with Patient Blood Samples and from Uncultured Pericardial Fluid from Patient 3.

The primers used for amplification (PER 3 and 4) are specific for a 151-bp segment (arrow) of granulocytic ehrlichial DNA. The 151-bp segment was identified in HL60 cells inoculated with blood from the three patients (lanes 2, 3, and 4) and in pericardial fluid from Patient 3 (lane 5). Lane 1 shows the molecular weight (MW) standard. Negative controls included samples in which water rather than cultured cells was processed (lanes 6, 7, and 11), IDE8 tick cells infected with *E. canis* (lane 9), and uninfected IDE8 cells (lane 10). The positive control consisted of IDE8 cells infected with *E. equi* (lane 8).

Table 1. Nucleotides at Key Positions in the 16S Ribosomal DNA of the Agent Causing Human Granulocytic Ehrlichiosis, *E. equi*, and *E. phagocytophila*.

SOURCE OF EHRLICHIAL DNA	NUCLEOTIDE AT INDICATED POSITION*		
	33	84	886
Human granulocytic ehrlichiosis agent			
Isolate from Patient 1	T	G	G
Isolate from Patient 2	T	G	G
Isolate from Patient 3	T	G	G
GenBank accession no. U02521	T	G	G
<i>E. equi</i>			
MRK strain (this study)	T	A	G
GenBank accession no. M73223	C	A	—
<i>E. phagocytophila</i> , GenBank accession no. M73220	T	A	—

*Position relative to the consensus sequence for the agent causing human granulocytic ehrlichiosis that is in the GenBank data base. Dashes indicate a gap in the sequence. Boldface type indicates nucleotides that differ from those in the consensus sequence for the agent causing human granulocytic ehrlichiosis.

On the basis of similarities in their 16S ribosomal DNA sequences and biologic properties,² it is possible that a granulocytic ehrlichia causing disease in animals (e.g., *E. equi* or *E. phagocytophila*) is identical to the species causing human granulocytic ehrlichiosis.

Recent studies have shown that the agent of human granulocytic ehrlichiosis can infect and cause disease in horses and that infected horses are immune to infection with *E. equi*.^{17,18} Some of our results suggest that the agent of human granulocytic ehrlichiosis differs from the *E. equi* isolate studied. Tick-cell-derived *E. equi* grew quite slowly in HL60 cells, causing incomplete cytopathic effects even after eight weeks, whereas all three human isolates lysed HL60 cells within two weeks of inoculation. This difference may, however, reflect changes occurring as a result of passage in tick cells, given the observation that *E. equi* obtained directly from an infected horse rapidly infected HL60 cells. *E. equi* grew rapidly in IDE8 tick cells (unpublished data), whereas only one of three human isolates grew at all in IDE8 cells and, in that case, extremely slowly. Finally, at the genetic level, the sequences of all three human isolates differed by one nucleotide from those of *E. equi* and *E. phagocytophila* in the 16S ribosomal regions studied and were identical to the sequence of the agent causing human granulocytic ehrlichiosis registered in the GenBank data base, suggesting that

this difference is conserved among temporally and geographically distinct isolates.² The potential biologic or taxonomic importance of this difference is uncertain. Our *E. equi* isolate did not have two other previously reported DNA-sequence changes distinguishing it from the agent causing human granulocytic ehrlichiosis. In addition, it was recently reported that granulocytic ehrlichia from dogs and horses in Sweden¹⁹ and a horse in the northeastern United States²⁰ have 16S ribosomal sequences identical to those of the agent causing human granulocytic ehrlichiosis. Further studies of the biology and genetics of this agent and related granulocytic ehrlichia causing infection in animals will be needed before it can be determined whether the agent is a unique species or a zoonosis caused by one or more animal pathogens.

The HL60 cell line consists of myeloid precursors similar to those in human bone marrow. Like human myeloid precursors, HL60 cells can attain the functional properties of neutrophils, including phagocytosis. Thus, studies of interactions between pathogens and this cell line are relevant to human infection. The HL60 cell line may also be useful for the isolation of other granulocytotropic pathogens of humans and animals.

With our techniques, antigen specific to the agent causing human granulocytic ehrlichiosis can be prepared that may lead to improved diagnostic assays. The ability to propagate the infectious agent in culture should help further the understanding of the epidemiology, genetics, pathogenesis, and treatment of human granulocytic ehrlichiosis, which is an emerging public health problem.

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CORRECTION

Direct Cultivation of the Causative Agent of Human Granulocytic Ehrlichiosis

Direct Cultivation of the Causative Agent of Human Granulocytic Ehrlichiosis . On page 210, 18 lines from the bottom of the right-hand column, the sequence for the primer GER 3 should have read, "5'TAGATCCTTCTTAACGGAAGGGCG3'," not "5'TAGATCCTTAACGGAAGGGCG3'," as printed. Also, on page 214, in the legend to Figure 3, the primers used for amplification should have read "GER 3 and 4," not "PER 3 and 4," as printed.