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Identification of a Granulocytotropic *Ehrlichia* Species as the Etiologic Agent of Human Disease

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Six patients from northern Minnesota and Wisconsin with a febrile illness accompanied by granulocytic cytoplasmic morulae suggestive of ehrlichial infection were identified. Two patients died, and splenic granulocytes of one patient contained cytoplasmic vacuoles with organisms ultrastructurally characteristic of ehrlichiae. From one patient, a 1.5-kb DNA product was amplified by PCR with universal eubacterial primers of 16S rDNA. Analysis of the nucleotide sequence of the amplified product revealed 99.9 and 99.8% similarities with *E. phagocytophila* and *E. equi*, respectively, neither of which has previously been known to infect humans. From the variable regions of the determined sequence, a forward primer specific for three organisms (human granulocytic ehrlichia, *E. phagocytophila*, and *E. equi*) and a reverse primer for these ehrlichiae and *E. platys* were designed. By nested PCR with amplification by the universal primers and then reamplification with the specific primers described above, the expected 919-bp product was generated from the blood of the index patient and three additional patients. Blood from these four patients and two more patients with granulocytic morulae contained DNA which was amplified by nested PCR involving a combination of a universal primer and the human granulocytic ehrlichia-*E. phagocytophila-E. equi-E. platys* group-specific primer. This apparently vector-borne human granulocytic ehrlichia has only 92.5% 16S rDNA homology with *E. chaffeensis*. Nested PCR with group-specific primers did not amplify *E. chaffeensis* DNA, and *E. chaffeensis*-specific primers did not amplify DNAs of the human granulocytic ehrlichia. Thus, six patients were shown to be infected by an *Ehrlichia* species never previously reported to infect humans.

Ehrlichiae are obligately intracellular bacteria which reside in a phagosome within the host's hematopoietic cells. An *Ehrlichia* species was first recognized in 1935 as the etiologic agent of canine ehrlichiosis (8). Subsequently, other veterinary diseases which are caused by ehrlichiae transmitted by tick bite have been described (4, 23). The ehrlichiae that infect humans include *E. sennetsu*, which was documented in Japan in the 1950s (19), and *E. chaffeensis*, which was visualized in a patient's blood in 1986 (17) and characterized in 1991 (1, 7). The main target cells of both human pathogens, *E. sennetsu* and *E. chaffeensis*, are monocytes (9, 10, 23). *E. phagocytophila*, *E. equi*, and *E. ewingii* infect granulocytes and are known only as veterinary pathogens (11–13). Genomic analysis on the basis of 16S rDNA sequence similarity has demonstrated that *Ehrlichia* species form three distinct groups (1, 2). By this criterion, *E. chaffeensis*, *E. canis*, and *E. ewingii* are more closely related to *E. phagocytophila* and *E. equi* (92.4 to 92.8%) than to *E. sennetsu* and *E. risticii* (84.2 to 84.4%). Granulocytic vacuoles resembling morulae (from the Latin for mulberry, referring to their form) containing ehrlichiae were observed in a series of patients with acute febrile illness in Minnesota and Wisconsin between 1990 and 1993. Because we were unable to cultivate these organisms in cell culture, PCR of patient's blood by using eubacterial universal primers was undertaken to detect a potential etiologic agent. The course of investigation was to sequence the DNA product and to design PCR primers to diagnose further cases. This report documents the fact that these six patients, two of whom died, were infected with a granulocytotropic *Ehrlichia* species that is closely related to *E. phagocytophila* and *E. equi*.

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MATERIALS AND METHODS

Case presentation. In June 1992, a 78-year-old male county maintenance worker (patient 1) from Gordon, Wis., was hospitalized with a flu-like illness. He presented with fever, headache, myalgias, leukopenia, thrombocytopenia, and pulmonary interstitial infiltrates. Unusual inclusions were observed within granulocytes in the patient's peripheral blood smear (Fig. 1), suggesting possible ehrlichial infection. A remarkable feature was the restriction of the infectious organisms to granulocytes, a finding previously observed in several other patients from this geographic region but rarely in humans with *E. chaffeensis* infection. Patients 2, 3, 4, 5, and 6 were also found to have similar inclusions within their granulocytes. All attempts thus far to cultivate this agent have failed. All cultures of blood for other infectious agents were sterile. A full clinical description of the disease will be published elsewhere.

Nucleic acid preparation. DNA was extracted from the acute-phase blood collected on day 5 of illness from patient 1 by previously described methods (14). Cells in the whole blood were lysed by the addition of an equal volume of lysing buffer (320 mM sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, and 1% Triton X-100). The lysate was centrifuged at 13,000 × *g* for 20 s. The supernatant was removed, and the pellet was again subjected to lysis and centrifugation. The pellets were digested with PCR lysis buffer containing proteinase K (60 μg/ml), 0.45% Nonidet P-40, and 0.45% Tween 20 at 56°C for 1 h. Protease was inactivated by heating at 95°C for 10 min. The

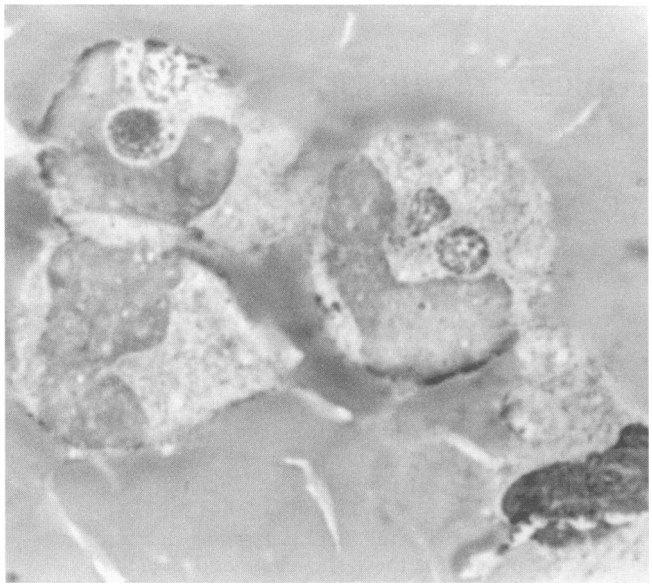

FIG. 1. Ehrlichial morulae in band neutrophils from peripheral blood of patient 3 with fatal granulocytic ehrlichiosis (Wright stain). Magnification, ×1,250.

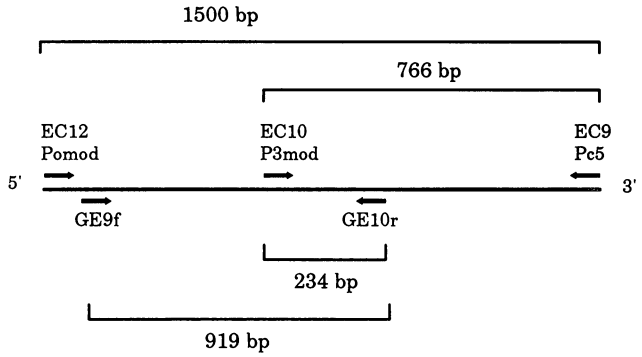

FIG. 2. Diagram of locations of primers on the 16S rDNA of human granulocytic ehrlichia. The designations 5′ and 3′ refer to the sense strand. Arrows indicate the sense or antisense orientation of the primers. The sizes of PCR products defined by the primers are given. Pomod, POmod; Pc5, PC5.

DNA lysate was stored at −20°C prior to use for PCR amplification.

Acute-phase blood was collected from patients 2, 3, 4, 5, and 6 as clotted blood or as EDTA-, citrate-, or heparin-anticoagulated blood on days 13, 5, 6, 5, and 21, respectively, after the onset of illness. DNA for PCR was extracted by different methods. Briefly, nucleic acids were liberated from a homogenized blood clot, anticoagulated blood, or leukocyte-rich plasma either by lysis with proteinase K (final concentration, 200 μg/ml) and sodium dodecyl sulfate (final concentration, 1%) in 50 mM Tris-HCl–1 mM EDTA buffer after overnight incubation at 55°C or by cellular lysis with four volumes of blood and one volume of 20% Chelex 100 in water at 100°C for 10 min as described previously (25). The DNA in the lysates was purified by extraction with phenol-chloroform and was precipitated with sodium acetate and ethanol.

The DNA of *E. chaffeensis* (Arkansas strain; kindly provided by Jacqueline Dawson, Viral and Rickettsial Diseases Division, Centers for Disease Control and Prevention, Atlanta, Ga.) (7) was prepared from infected DH82 cells. When 100% of the DH82 cells in culture had become infected, the cells were harvested and centrifuged at 13,000 × *g* for 5 min. The pelleted cells were washed twice in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 [pH 7.0]) by suspension in sterile PBS and centrifugation at 13,000 × *g* for 5 min. Then the infected cells were lysed in the PCR lysis buffer as described above. The DNAs of uninfected cells were prepared by the same method for use as a negative control.

PCR amplification and purification of 16S rDNA for sequencing. A pair of universal eubacterial primers, POmod and PC5, which recognize conserved sequences of the 5′ end and the 3′ end of the 16S rDNA, respectively, and which amplify nearly the entire gene (27), was used to prime the DNA lysate (Fig. 2). A 20-μl volume of DNA sample from patient 1 was amplified in a reaction volume of 100 μl by using a thermal cycler and GenAmp reagents (Perkin-Elmer Cetus, Norwalk, Conn.). The cycling program for patient 1 consisted of 3 min at 95°C and then 25 cycles, each of 30 s at 94°C, 1 min at 52°C, and 2 min at 72°C, and an additional cycle with an extension step of 3 min at 72°C. The DNA lysate prepared from *E. chaffeensis*-infected DH82 cells was used as a positive control of 16S rDNA amplification.

The approximately 1.5-kb PCR product of 16S rDNA from the blood of patient 1 was pooled and purified by Ultrafree-MC filter unit (Millipore Products Division, Bedford, Mass.) to remove the primers and nucleotides. The purified DNA was amplified again with the same primers and under the same conditions described above. The final PCR product was purified by using the Glass MAX DNA isolation system (GIBCO BRL, Grand Island, N.Y.), with the efficient recovery of a 1.5-kb DNA band from the agarose gel. The DNA in this band was used for sequencing of the gene.

Sequencing of 16S rDNA. To further characterize the phylogeny of the ehrlichia-like bacterium, the nucleotide sequence of the purified double-stranded PCR product of 16S rDNA from the bacterium in the blood of patient 1 was determined by the dideoxynucleotide method of cycle sequencing with *Taq* polymerase (15, 20, 22). The sequencing reaction was carried out by using a double-stranded DNA cycle sequencing system (GIBCO BRL) and was initiated with the universal primers POmod and PC5, which were end-labeled with [γ-^{32}P]ATP. Sequencing was continued in the forward and reverse orientations by synthesizing additional sequencing primers based on the emerging sequences. OLIGO primer analysis software (National Biosciences, Inc., Hamel, Minn.) was used for choosing optimal oligonucleotides as primers for DNA sequencing. Sequencing reactions were performed according to the manufacturer's protocol. The sequencing data were analyzed by using PC/Gene sequence analysis software (IntelliGenetics, Inc., Mountain View, Calif.). To confirm the sequencing data and to avoid possible errors of incorporation of nucleotides by *Taq* polymerase, both the sense and the antisense strands of the 16S rDNA of the amplified eubacterial DNA from the patient's blood were sequenced twice after independent PCR amplifications.

Nested PCR. In order to detect and identify the etiologic agent of human granulocytic ehrlichiosis in patients 2, 3, 4, 5, and 6, we used the approach of nested PCR with initial amplification using modified universal eubacterial primers and reamplification with primers that are specific for the *E. phagocytophila* group (Table 1; Fig. 2). The modified universal primers EC9, EC12, and EC10 (1), which recognize conserved

TABLE 1. Primers used for PCR amplification

Primer pair	Approx product size (bp)	Sequence range (bp)[a]
PC5-POmod (EC9-EC12)	1,500	Whole gene
PC5-P3mod (EC9-EC10)	766	734–1500
GE10r-GE9f	919	49–968
GE10r-EC10	234	734–968

[a] The approximate range of the 16S rDNA amplified relative to the sequence of the human granulocytic ehrlichia.

regions of the 3′ end, the 5′ end, and the middle of the gene, respectively, corresponding to the primers PC5, POmod, and P3mod, respectively, published by Wilson et al. (27), and modified at the 5′ end to contain the restriction enzyme cutting sites for subsequent cloning as described by Anderson et al. (1), were used for the initial PCR amplification. Two primers specific for the *E. phagocytophila* group, GE9f and GE10r, were constructed for the specific reamplification step. GE9f corresponds to nucleotides 49 to 74 on the sense strand, a region of the 16S rDNA near the 5′ end that has been reported to be highly variable in other ehrlichiae (1). The sequence of GE9f differs from that reported for other *Ehrlichia* species except *E. phagocytophila* and *E. equi* (Table 2). The reverse primer GE10r, which is complementary to nucleotides 943 to 968 of the sense strand, consists of the sequence 5′-GGAGAT TAGATCCTTCTTAACGGAA-3′. GE10r is identical to the corresponding sequences of *E. phagocytophila*, *E. equi*, and *E. platys* and differs from the reported sequences of other species of *Ehrlichia* (Table 2). Thus, the primer pair GE9f and GE10r defines a 919-bp PCR product that would be predicted to be specific for the phylogenetically closely related human granulocytic ehrlichia, *E. phagocytophila*, and *E. equi*.

The PCR conditions used for samples from the six patients for the initial amplification with the modified universal primers were 3 cycles at 94°C for 2 min, 48°C for 1 min, and 68°C for 4 min and then 37 cycles at 90°C for 2 min, 52°C for 1 min, and 68°C for 4 min. Amplified PCR products were detected by ethidium bromide staining after electrophoresis in 1 to 2% agarose gels. To ensure the absence of nonspecific amplification because of contamination of reagents with extraneous eubacterial rDNA, all amplifications included a control with no template DNA (water only), and all reagents (excluding template DNA) were premixed with 8-methoxypsoralen at a final concentration of 25 μg/ml and were irradiated for 4 min with a hand-held UV (360-nm) light source before aliquots were used in the PCR (18). The *E. chaffeensis*-specific primers HE1 and HE3 (3) were also used in PCR for each of the patients' extracted DNA.

For patients 4 and 5, no PCR product was obtained in the initial universal eubacterial amplification step with the primer pair EC12 and EC9. Thus, a pair of universal primers, EC10 and EC9, representing the 3′ half of the 16S rDNA gene was then used for the initial amplification step for DNA from all six patients (Fig. 2); EC10 and GE10r were used in the reamplification PCR (Table 1).

For nested PCR (6), the original PCR product obtained after amplification with the universal primer set EC9-EC12 for the entire 16S rDNA or primer set EC9-EC10 for the 3′ half of the 16S rDNA was used as the template. For PCR products originally obtained by the use of modified universal primers for the whole 16S rDNA (patients 1, 2, 3, and 6), 10 μl was added to a PCR with 1 μM GE9f and GE10r; for PCR products obtained from the use of modified universal primers for the 3′ half of the 16S rDNA (patients 1, 2, 3, 4, 5, and 6), 10 μl of the product of the first PCR was added to a PCR with 1 μM GE10r and EC10. Reamplification with the primer pair GE9f and GE10r was performed as described for patient 1 (see Fig. 3), and reamplification with primer pair GE10r and EC10 was performed for 3 cycles at 94°C for 1 min, 48°C for 2 min, 66°C for 1.5 min; this was followed by 37 cycles at 88°C for 1 min, 52°C for 2 min, and 68°C for 1.5 min. Controls included sequences derived by amplification of the entire and the 3′ half of the *E. chaffeensis* 16S rDNA and a no template (water) control. As an additional control, a simultaneous nested PCR was performed with the identical templates and PCR mixture, except that the *Taq* polymerase was omitted.

The PCR studies were performed at two different institutions (University of Texas Medical Branch, Galveston, and University of Maryland at Baltimore), and no veterinary granulocytic ehrlichial species or ticks likely to contain the ehrlichial agent have been previously studied at either location.

Electron microscopy. Because apparent ehrlichial morulae were observed microscopically in Giemsa-stained spleen tissues at necropsy, ultrastructural examination was undertaken. Postmortem splenic tissue from patient 3 was prepared by excising small fragments from paraffin-embedded spleen. These fragments were rehydrated and postfixed in 4% formaldehyde–1% glutaraldehyde in monophosphate buffer and then in 1% osmium tetroxide. The tissues were dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin. Semithin sections (1 μm) were stained with toluidine blue to select appropriate regions for ultrastructural examination. Ultrathin sections were stained with uranyl acetate and

TABLE 2. Nucleotide sequence alignment for the regions of the ehrlichial 16S rDNA corresponding to primers GE9f and GE10r[a]

Ehrlichia sp.	GE9f	GE10r(c)
Human granulocytic ehrlichia	AACGGATTATT-CTTTAT-AG-C-T-T-G-CT	GGAGATTAGATCCTTCTTAACGGAA
E. phagocytophila	..........-.....-..-.-.-.-.-..	.....
E. platys	.....-..-TG.CG.-..-.-.-.-.-..	.....
E. chaffeensis	.....CA...-GC....A.C-.T.T.G.-T.	.A..G.CGT....C..C...TA.-G
E. ewingii	....A.CA...C..AA..-..T.-.C.-.A..	.A..G.CGT....C.T.....A.-.
E. canis	.....CA...-A.....-..C.-.C.G.-..	.A..G.CGT....C..C....A.-G
E. sennetsu	.....-A-.--.-A-.--..-.-.-.-.-..	.TG...CGT....C...G..-..G.
E. risticii	.....-A-.--.-A-G--G.-.---.-.-..	.TG....GT....C...G..-..G.

[a] The sequences were aligned as described in the text. The sources for sequences used in the alignment were as follows: *E. phagocytophila*, *E. equi*, *E. chaffeensis*, *E. canis*, and *E. sennetsu*, reference 1; *E. platys* and *E. ewingii*, reference 2; and *E. risticii*, reference 24. All sequences are shown 5′ to 3′ with respect to the 16S rDNA. The complementary sequence of the reverse primer GE10r is shown as GE10r(c). The sequences of the human granulocytic ehrlichia are shown, and corresponding base substitutions for other ehrlichiae are shown. Conserved positions are indicated with periods, and gaps are indicated with hyphens. The 16S rDNA sequence of *E. equi* is identical to that of *E. phagocytophila* for the two regions and is not included here.

TABLE 3. Nucleotide differences among the 16S rDNA sequences of the human granulocytic ehrlichia, *E. phagocytophila*, and *E. equi*

Ehrlichia sp.	Nucleotide difference at position[a]:		
	33	84	886
Human granulocytic ehrlichia	T	G	G
E. phagocytophila	T	A	—
E. equi	C	A	—

[a] The position of nucleotides relative to the sequence of the human granulocytic ehrlichia. —, no nucleotide corresponds to human granulocytic ehrlichia nucleotide 886. A gap was required at this position to align the adjacent sequences.

lead citrate and were examined on a JEOL 1200 EX1 transmission electron microscope.

RESULTS

Amplification of 16S rDNA of the granulocytic agent and its identification by sequencing of the PCR product. The eubacterial universal primers POmod and PC5 amplified 16S rDNA of the expected size (1.5 kb) from the blood of patient 1 and from *E. chaffeensis* in DH82 cell culture. No nucleic acids were amplified from uninfected DH82 cells or a mixture of all reagents except template DNA (water only), confirming the specificity of the reaction and the absence of environmental rDNA in the reagents. The *E. chaffeensis*-specific primers HE1 and HE3 did not amplify any nucleic acid from the patient's blood, confirming that *E. chaffeensis* is not the causative agent of the disease.

The sequence data for 1,433 bp of the 16S rDNA of the human granulocytic ehrlichia (GenBank accession number U02521) were aligned for maximal homology with the 16S rDNA reported for all identified *Ehrlichia* species. On the basis of these results, the phylogenetically most closely related species are *E. phagocytophila* (99.9%) and *E. equi* (99.8%), the granulocytic ehrlichiae which cause tick-borne fever of sheep and cattle and equine granulocytic ehrlichiosis, respectively. The 16S rDNA sequence of the human granulocytic ehrlichia showed only two nucleotide differences from *E. phagocytophila* and three nucleotide differences from *E. equi* (Table 3). *E. platys* is slightly less closely (98.2%) related to the human granulocytic ehrlichia. *E. chaffeensis* (92.5%) and the closely related species *E. canis* (92.5%) and *E. ewingii* (92.2%) are distantly related to this new human pathogen. *E. sennetsu* (85.3%) and *E. risticii* (85.1%) make up the most distantly related group of ehrlichial species.

Validation of the specificity of the PCR primers for the human granulocytic ehrlichia. The purified 1,500-bp PCR products from patient 1 and *E. chaffeensis* were used as templates for reamplification by GE9f and GE10r. As shown in Fig. 3, lanes d and e, GE9f and GE10r generated the predicted 919-bp DNA fragment from the 16S rDNA amplified from the blood of patient 1 and did not amplify any band from *E. chaffeensis* DNA. *E. chaffeensis*-specific primers HE1 and HE3 produced the expected 389-bp PCR product from *E. chaffeensis* DNA but failed to amplify any product from the PCR-amplified DNA of the human granulocytic ehrlichia. Thus, it may be concluded that the human granulocytic ehrlichia differs from *E. chaffeensis*.

Detection of human granulocytic ehrlichial DNA in the blood of additional patients. When the modified universal primers EC9-EC12 were applied to DNA extracted from the blood of all six patients, the entire 1,500-bp 16S rDNA was amplified from samples from patients 1, 2, 3, and 6. The samples from patients 4 and 5 failed to produce PCR products with the primers EC9-EC12, but the universal primers EC9-EC10 amplified the expected 766-bp 3′ half of the 16S rDNA for all six patients. *E. chaffeensis*-specific primers did not amplify nucleic acids from any of the patients' DNA extracts. *E. chaffeensis* was used as a positive control for the universal primer pairs EC9-EC12 and EC9-EC10 and gave the expected bands of 1,500 and 766 bp upon PCR amplification. The negative control containing no template (water only) did not generate any DNA band in the PCR.

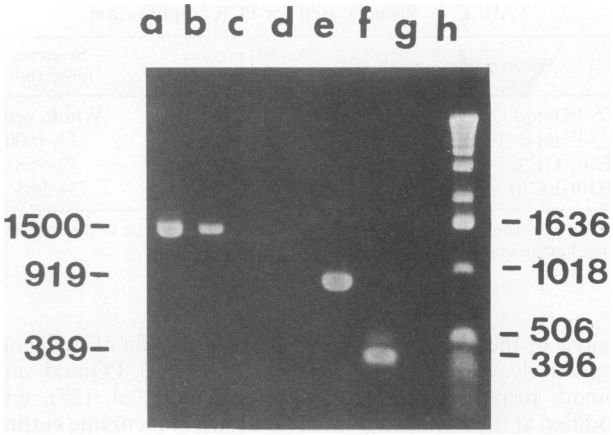

FIG. 3. PCR of 16S rDNA from patient 1 and *E. chaffeensis*. Universal primers POmod and PC5 were applied to amplify the entire gene of 16S rDNA from *E. chaffeensis* (lane a), patient 1 (lane b), and uninfected DH82 cells (lane c). PCR reamplification with primers GE9f and GE10r with the PCR product of initial amplification of the 16S rDNA of *E. chaffeensis* (lane d) and patient 1 (lane e) (0.25 μM each primer GE9f and GE10r in a 100-μl reaction mixture with 1 μl of purified 16S rDNA template; the cycling program was as follows: 95°C for 3 min and then 25 cycles each of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min and an extension step of 72°C for 3 min). The *E. chaffeensis*-specific primers HE1 and HE3 were used in the PCR reamplification of *E. chaffeensis* (lane f) and the DNA from patient 1 (lane g). Lane h contains molecular size markers (1-kb DNA ladder), as marked on the right of the gel (in base pairs). The approximate sizes of PCR products are marked on the left of the gel (in base pairs).

Nested PCR for detection of the human granulocytic agent performed by using the specific primers GE9f and GE10r after initial PCR amplification with the universal primers EC9 and EC12 produced a DNA band of the correct size (919 bp) for patients 1, 2, 3, and 6. The entire 1,500-bp 16S rDNA product of the initial amplification was still present in the sample (Fig. 4). The 766-bp PCR product of the 3′ half of the 16S rDNA gene was used as a template for reamplification with GE10r-EC10 and generated the predicted 234-bp product in all six patients (Fig. 4). Neither *E. chaffeensis* DNA nor the template (water) control yielded amplified DNA bands of 919 or 234 bp. The products of the original amplification were still present (1,500 and 766 bp). A simultaneous nested PCR was performed with the identical patient specimen templates described above, except that the *Taq* polymerase was omitted from the reamplification step. These reaction mixtures contained only the initial PCR products of 1,500 and 766 bp and did not yield the human granulocytic ehrlichia-specific DNA products, thus serving as a further specificity control.

Ultrastructural detection of the human granulocytic ehrlichia in human necropsy tissue. Examination by electron

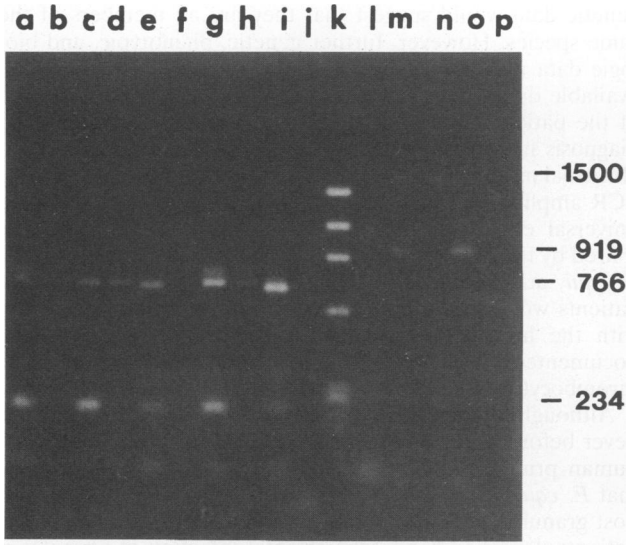

FIG. 4. Nested PCR (see text) for detection of the human granulocytic agent in the blood of infected patients. Shown in lanes a through j is the 3′ half of the 16S rDNA which was first amplified with primer pair EC9-EC10 and then reamplified with primer pair EC10-GE10r. Lanes a and b, blood from patient 2 with and without *Taq* polymerase, respectively; lanes c and d, blood from patient 3 with and without *Taq* polymerase, respectively; lanes e and f, blood from patient 4 with and without *Taq* polymerase, respectively; lanes g and h, blood from patient 5 with and without *Taq* polymerase, respectively; lanes i and j, blood from patient 1 with and without *Taq* polymerase, respectively; lane l, no template (water only) control with *Taq* polymerase. Shown in lanes m through p are the whole 16S rDNAs first amplified with EC9-EC12 and then reamplified with GE9-GE10r. Lanes m and n, blood from patient 2 with and without *Taq* polymerase, respectively; lanes o and p, blood from patient 3 with and without *Taq* polymerase, respectively (similar PCR data for patient 6 are not shown); lane k, *Hae*III-digested ϕX174 DNA molecular size standards (molecular sizes from top to bottom are 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp). The approximate sizes of the PCR products are marked on the right of the gel (in base pairs).

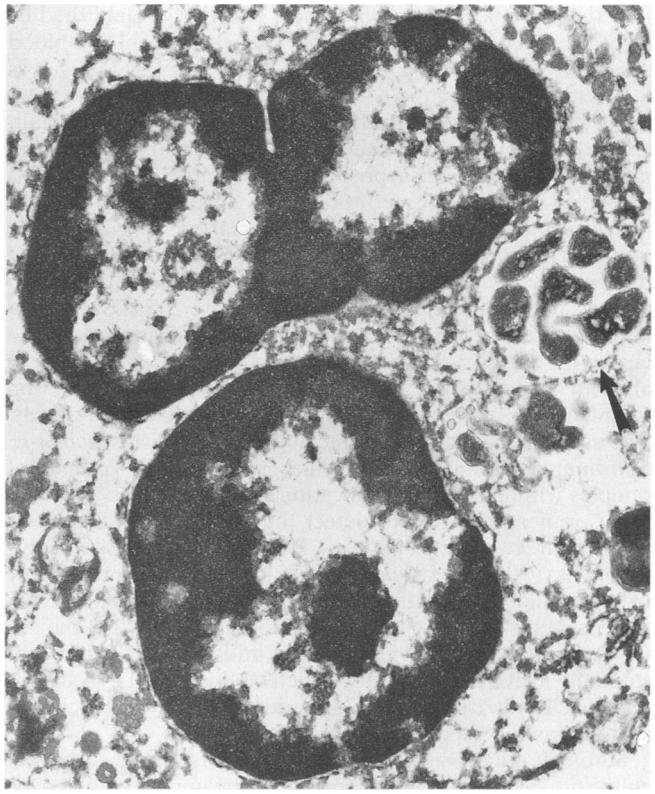

FIG. 5. Electron photomicrograph of an ehrlichial morula with pleomorphic, gram-negative bacteria in a membrane-bound vacuole of a neutrophil identified by the presence of a multilobed nucleus and typical cytoplasmic lysosomes in the spleen of patient 3 with fatal granulocytic ehrlichiosis. Magnification, ×15,000.

microscopy of splenic tissue of patient 3 obtained at the time of autopsy revealed clusters of small, pleomorphic gram-negative cocci within cytoplasmic membrane-bound vacuoles in polymorphonuclear leukocytes (Fig. 5). These organisms exhibited the characteristic morphology of ehrlichiae.

DISCUSSION

The studies described here documented the existence of a previously unknown ehrlichial infectious disease of humans. The definitive identification of the organisms was achieved by PCR amplification and sequencing of the eubacterial 16S rDNA (26). The sequence was virtually identical to those reported for *E. phagocytophila* and *E. equi*. Thus, a human granulocytic ehrlichiosis exists, and it is caused by an *Ehrlichia* species closely related to obligate intracellular bacteria which cause granulocytic infections of sheep, cattle, and horses. Data further supporting the documentation of this novel human infection are visualization of the ehrlichial morulae in the patients' circulating neutrophils, ultrastructural identification of bacteria with morphologies characteristic of ehrlichiae in splenic polymorphonuclear leukocytes in a patient who died, and the presence of antibodies reactive with *E. phagocytophila* in convalescent-phase sera from three of the four patients who survived the illness. The details will be reported in a separate clinical description of the disease.

The clinical illness manifests as fever, myalgia, and headache and sometimes nausea, cough, or confusion. The patients usually have pancytopenia and mildly elevated concentrations of hepatic transaminases in their sera. Two patients with the illness died. The seasonality (June to October) and a history of outdoor exposure with arthropod bite in most of these patients suggest a vector-borne zoonosis. Several patients reported bites from ticks, some with descriptions suggestive of *Ixodes scapularis* and others resembling *Dermacentor variabilis*. Indeed, *Ixodes ricinus* is the vector of *E. phagocytophila* infection of sheep, cattle, and goats in Europe, the only continent where this disease, tick-borne fever, has been documented. Because *I. ricinus* is related to *I. scapularis* (*Ixodes dammini*), it would be a reasonable hypothesis that the latter might be the vector of human granulocytic ehrlichiosis in northern Minnesota and Wisconsin. Although all of the patients were diagnosed in northern Minnesota and northwestern Wisconsin, the actual geographic limits of the infection are yet to be determined. The illness appears to respond favorably to treatment with doxycycline.

Unlike human ehrlichiosis caused by *E. chaffeensis*, in which the main target cells are mononuclear phagocytes and rarely lymphocytes or polymorphonuclear leukocytes (9, 10, 17), the only cells observed to contain ehrlichial morulae in the present study were polymorphonuclear leukocytes. Thus, for now, "human granulocytic ehrlichiosis" is a reasonable name to apply to the disease.

A timely clinical diagnosis is most effectively established by identification of the granulocytic morulae in a peripheral blood smear. Until cultivation is accomplished, serologic diagnosis will remain problematic. Amplification of larger DNA fragments from minute quantities of template DNA is difficult, especially when the template DNA is not in optimal condition initially. Thus, it is not surprising that the entire 16S rDNA was amplified from samples from only four of the six patients. In fact, by using an alternative set of universal eubacterial primers designed to amplify the 5′ end of the 16S rDNA, both halves of that gene were amplified separately from the blood of the two patients for whom amplification of the entire 16S rDNA of the infecting isolate was not accomplished, indicating that the entire 16S rDNA was present (data not shown). Anderson et al. (3) have used primers specific for amplification of a 389-bp fragment of *E. chaffeensis*-16S rDNA from clinical specimens. The use of similar specific primers for the *E. phagocytophila-E. equi*-human granulocytic ehrlichia group directly on clinical samples without prior amplification with the universal eubacterial primers was able to detect the presence of the nucleic acids in three of our six patients. Possibly, the large size of this fragment (919 bp) restricted our ability to amplify it effectively from the limited quantities present in the whole blood from some patients. Because of this situation, it is likely that the use of universal eubacterial primers for initial amplification substantially increases the ability to detect ehrlichial DNA amplified specifically from minute quantities present in the clinical samples, as reported previously (24).

Serologic diagnosis is impaired by the fact that successful cultivation has never been reported for the human granulocytic ehrlichia, *E. phagocytophila*, or *E. equi*, and thus, the antigens are not readily available for developing serologic assays. Antibodies to *E. phagocytophila* were detected in convalescent-phase sera from several of the patients by an immunofluorescent-antibody assay with bovine peripheral blood granulocytes infected with *E. phagocytophila*. Similarly, *E. canis* was formerly used as a surrogate antigen for the diagnosis of human (monocytic) ehrlichiosis prior to the isolation of *E. chaffeensis*. Subsequently, it was recognized that some patients with human monocytic ehrlichiosis produce antibodies that react with *E. chaffeensis* but not with *E. canis* in the immunofluorescence assay. It is possible that *E. phagocytophila* does not contain all of the major immunodominant antigens of the human granulocytic ehrlichia. Although serologic cross-reactions have been used to detect the presence of antibodies to *Ehrlichia* species, this approach has its drawbacks. The *E. phagocytophila* group is not closely related genetically or antigenically to the *E. chaffeensis-E. canis-E. ewingii* group, as shown by the absence of antibodies to *E. chaffeensis* by an immunofluorescent-antibody assay in the convalescent-phase sera of the patients with human granulocytic ehrlichiosis. The *E. sennetsu* group is even less related genetically and antigenically to the *E. phagocytophila* group. Antigenic cross-reactions between ehrlichial groups often fall in the nonspecific titer range of 10 to 40 when measured by immunofluorescence assay. In contrast, Western immunoblotting with high-titer late-convalescent-phase serum may detect antibodies reactive with ehrlichial polypeptides containing shared antigens (5, 21). Although such studies have shown the antigenic relatedness of some ehrlichial polypeptides, Western immunoblotting has not been evaluated as a diagnostic method for the ehrlichioses.

Although the DNA sequence analysis indicates that the human granulocytic ehrlichia is very closely related to *E. phagocytophila* and *E. equi*, it is unclear whether all of these organisms are variant strains of a single species or whether they represent two or three distinct *Ehrlichia* species. The genetic data would suggest that they are all members of the same species. However, further genetic, phenotypic, and biologic data may reveal important distinguishing features. The available data do not exclude the unlikely possibility that two of the patients could have been infected with *E. platys*. The diagnosis in those two patients was based on visualization of ehrlichial morulae in peripheral blood granulocytes and nested PCR amplification of DNA with a combination that included universal eubacterial primers and a primer with sequences shared by the human granulocytic ehrlichia, *E. phagocytophila*, *E. equi*, and *E. platys*. It would seem unlikely that the two patients would have been infected with *E. platys* rather than with the human granulocytic ehrlichia. *E. platys* has been documented to infect only the platelets of dogs with cyclic thrombocytopenia (23).

Although human infection with a granulocytic ehrlichia has never before been reported, experimental inoculation of nonhuman primates and other non-equine mammals has shown that *E. equi* is capable of initiating a productive infection of host granulocytes (16). Unlike the clinical presentation in the patients described here, experimental infection of primates is usually associated with only mild fever and anemia which resolve spontaneously within 1 week. Ultimately, cultivation of the organisms or even construction of genomic libraries from ehrlichial DNAs purified from the blood of animals with equine granulocytic ehrlichiosis and tick-borne fever and patients with human granulocytic ehrlichiosis would be required to provide the opportunities for the required comparative studies of these ehrlichiae. The possibility of cultivating granulocytic ehrlichiae in several hematopoietic cell lines is under intense investigation.

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