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Diagnosis of Toxoplasma Parasitemia in Patients with AIDS by Gene Detection after Amplification with Polymerase Chain Reaction

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We sought evidence of toxoplasma parasitemia among 37 people with active or dormant *Toxoplasma gondii* infection or no evidence of infection. DNA was extracted from erythrocyte-free portions of blood samples, and the *T. gondii* B1 gene was amplified by the polymerase chain reaction. Evidence of *T. gondii* parasitemia was found in six patients with severe immunosuppression from AIDS and clinical evidence suggestive of or compatible with toxoplasmosis. Results were negative for patients unlikely to have active toxoplasmosis. Gene detection after amplification with the polymerase chain reaction is a promising test for detection of parasitemia, and parasitemia should be tested for in patients with AIDS and unexplained fever or central nervous system abnormalities.

The epidemic of toxoplasmosis resulting from the worldwide spread of AIDS has added urgency to the need for better diagnostic tests. Definitive diagnosis of toxoplasmosis in immunosuppressed people currently depends on biopsy, often of the central nervous system (CNS). The occurrence of toxoplasma parasitemia in a patient implies active toxoplasma infection, and tests to demonstrate parasitemia represent a noninvasive way to diagnose active infection.

We have previously reported (9) detection of the *Toxoplasma gondii* B1 gene in blood samples from experimentally infected rabbits after amplification with the polymerase chain reaction (PCR). We now report studies of gene detection in human blood samples after amplification with PCR. The results demonstrate the usefulness of gene detection by PCR for detection of parasitemia and suggest that toxoplasmosis often appears atypically in patients with AIDS.

CASE REPORTS

Cases in which the B1 gene was detected. Case 1. A 38-year-old man with AIDS, immunoglobulin G (IgG) antibodies to *T. gondii*, and less than 200 $CD4^+$ lymphocytes per μl of blood had fever, lethargy, confusion, obstructive hydrocephalus, and multiple ring-enhancing cerebral masses typical of cerebral toxoplasmosis. Blood and ventriculostomy fluid samples obtained after single doses of sulfadiazine and pyrimethamine contained the B1 gene, and a portion of the same blood sample was positive by mouse inoculation. He improved with therapy.

Case 2. A 55-year-old man with AIDS, IgG antibodies to *T. gondii*, and 30 $CD4^+$ lymphocytes per μl of blood had fever, lethargy, and focal weakness. Computerized tomography and magnetic resonance imaging scans demonstrated an extensive area of edema containing two enhancing masses typical of cerebral toxoplasmosis. After 4 days of therapy with cotrimoxazole and clindamycin, an aspiration biopsy specimen from one mass demonstrated hyphae suggestive of a zygomycete in necrotic brain tissue. A blood sample obtained on that day contained the B1 gene, which prompted reexamination of the tissue after immunoperoxidase staining, and *T. gondii* tachyzoites were demonstrated.

Case 3. A 30-year-old man had AIDS, less than 50 $CD4^+$ lymphocytes per μl of blood, *Mycobacterium avium-M. intracellulare* infection, cytomegalovirus infection, and Kaposi's sarcoma. He had been diagnosed 6 months earlier with cerebral toxoplasmosis when he had antibodies to *T. gondii* and focal brain lesions that responded to therapy for toxoplasmosis. He could not tolerate sulfonamides, pyrimethamine, or clindamycin, and azithromycin was used to prevent recurrences. He had persistent fevers, which were attributed to one of his opportunistic conditions, and CNS dysfunction that was attributed to his prior toxoplasmosis and human immunodeficiency virus (HIV) infection. A blood specimen contained the B1 gene. He appeared 2 weeks later with a cerebral lymphoma which was proven by brain biopsy. He died, but permission for an autopsy was not granted.

Case 4. A 40-year-old man with AIDS, no IgG antibodies to *T. gondii*, and less than 50 $CD4^+$ lymphocytes per μl of blood had progressive dementia and a single ring-enhancing cerebral mass. A presumptive diagnosis of progressive multifocal leukoencephalopathy was made. A blood sample contained the B1 gene. A second blood sample, obtained 90 days later, was tested for the B1 gene by PCR, for live toxoplasma parasites by mouse inoculation, and for toxoplasma antibodies by the Sabin-Feldman dye test; all three tests were negative. He died 3 weeks later, and permission for an autopsy was not granted.

Case 5. A 30-year-old man with AIDS had an altered mental status and multiple ring-enhancing cerebral masses typical of cerebral toxoplasmosis. He had had 124 $CD4^+$ lymphocytes per μl of blood and IgG antibodies to *T. gondii* 2 months earlier. He improved with sulfadiazine and pyrimethamine therapy, and a blood sample obtained after 5 days of therapy contained the B1 gene.

Case 6. A 36-year-old, 28-week-pregnant woman with

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AIDS, IgG antibodies to *T. gondii*, and 110 $CD4^+$ lymphocytes per μl of blood had fever, headache, and meningeal signs. She had been receiving cotrimoxazole to prevent *Pneumocystis carinii* pneumonia. She was diagnosed with active syphilis, pulmonary tuberculosis, and cryptococcal fungemia. A computerized tomography brain scan demonstrated diffuse cerebral edema. A cerebrospinal fluid sample contained no cells and normal glucose and protein, but culture of this specimen yielded *Cryptococcus neoformans*. A blood sample contained the B1 gene. She died on the same day, but permission for an autopsy was not granted.

MATERIALS AND METHODS

We tested heparinized blood samples from 37 people. Fourteen samples were tested with knowledge of clinical status (unblinded samples). Of these 14, 5 were from patients with AIDS and symptoms compatible with active opportunistic infections. Two others were from patients with AIDS who had had cerebral toxoplasmosis 1 or 3 years earlier and were receiving suppressive therapy for toxoplasmosis. Four were from patients seropositive for both *T. gondii* and HIV but without AIDS. One was from a congenitally infected infant whose mother was not infected with HIV. Two were from healthy people, one with and one without antibodies to *T. gondii*.

We studied 23 samples without knowledge of clinical information (blinded samples). Seven of these 23 were from patients with AIDS. Of these seven, one had typical CNS toxoplasmosis and had not received treatment for toxoplasmosis, five had had typical CNS toxoplasmosis and had been receiving therapy for toxoplasmosis for 5 days to several years, and one had advanced AIDS, symptoms of opportunistic infection, and antibodies to *T. gondii*. Fourteen of the 23 blinded samples were from neonates considered to be at risk for congenital toxoplasmosis because they were born to mothers with antibodies to both HIV and *T. gondii*. The remaining two were from healthy people, one with and one without antibodies to *T. gondii*.

Informed consent was obtained from all subjects. Tests were done weeks to months after samples were obtained, and results were not available to clinicians caring for patients in time for the results to influence clinical decisions.

Our strategy was to extract DNA from all sedimentable elements of blood, except for erythrocytes, which were discarded because of the inhibitory effects of hemoglobin on PCR. Blood was subjected to density gradient centrifugation with polysucrose-diatrizoate with a specific gravity of 1.019 (Sigma Chemical Co., St. Louis, Mo.) as previously described (9). Material from the interface up, containing granulocytes, mononuclear cells, platelets, and plasma, was collected and concentrated by centrifugation. Pellets were suspended in phosphate-buffered saline and divided. Portions were extracted immediately or frozen for extraction later.

DNA was extracted with phenol-chloroform after proteolysis with 78 U of proteinase K per ml as previously described (9, 22). Oligonucleotide primers used to initiate DNA amplification were complementary to previously reported (3) *T. gondii* B1 gene segments (5′-GGAACTGCATCCGTT CATGAG and 5′-TCTTTAAAGCGTTCGTGGTC). DNA was amplified in a solution containing 10 mM Tris (pH 8.3), 2.5 U of *Taq* polymerase (Perkins-Elmer Cetus, Norwalk, Conn.), 50 mM KCl, 1.5 mM $MgCl_2$, and each deoxynucleoside triphosphate at 200 μM. The reaction was carried through 30 cycles, each consisting of 60 s at 94°C, 90 s at 55°C, and 60 s at 72°C. Amplified DNA was separated by agarose or polyacrylamide gel electrophoresis and then transferred to nylon membranes. Membranes were exposed to an end-labeled 5′-^{32}P-labeled oligonucleotide (5′-GG CGACCAATCTGCGAATACACC) complementary to a unique segment of the B1 gene completely within the amplified segment. Membranes were washed, and hybridization was detected by autoradiography.

Strict steps that we used in our studies of rabbit infection (9, 13) to reduce contamination were taken here. Basically, PCR was done in a dedicated laboratory in which other material that contained or might contain the toxoplasma genome was excluded. Gloves and positive-displacement pipettes were employed routinely.

Results were accepted only if they were repeatable in accordance with previously published criteria (8, 9). Briefly, the sedimentable material that remained after polysucrose-diatrizoate separation was divided into separate portions and extracted separately, and each extracted DNA portion was tested on at least two separate occasions. Results were considered positive only if electrophoresis resulted in bands corresponding to the appropriate molecular size in at least two separate experiments. Positive and negative controls were included in each experiment, and results were considered valid only if control results were as expected.

To determine the sensitivity of our methods for detection of toxoplasma in blood leukocytes, we spiked blood with monocytes that had been infected in vitro. Briefly, blood was obtained from *T. gondii*-seronegative volunteers, monocytes were separated from other blood elements, and the monocytes were cultured on glass slides. We infected the monocytes with five tachyzoites per monocyte for 1 h, washed off extracellular tachyzoites, and scraped cells off the glass, washed them, and counted them. We then added serial dilutions of this monocyte preparation to second blood samples collected from the same donors. Stained preparations of the monocytes were examined to determine the infection rate and the average number of tachyzoites per infected monocyte. The spiked blood samples were then processed as described above, and the B1 gene was tested for in erythrocyte-free fractions.

Mouse inoculation (9, 20) was performed in parallel for the four samples with sufficient fresh material. Two (from cases 1 and 4) had positive gene detection results, one was from a baby with congenital infection who had been treated for 5 days for toxoplasmosis, and one was from a healthy control subject. Briefly, blood was separated with polysucrose-diatrizoate as described above. Erythrocyte-free sedimentable material was resuspended in saline and divided into three portions which were injected intraperitoneally into Swiss mice. Mice that became ill were euthanized and examined for tachyzoites. Mice that remained well were bled after 1 month, and their sera were tested for antibodies to *T. gondii* with the Sabin-Feldman dye test (9, 21). Results were considered positive if tachyzoites were observed or if the mice produced antibodies to *T. gondii*.

RESULTS

In experiments to determine the sensitivity of gene detection for blood samples spiked with infected monocytes, we detected the B1 gene in samples with 0.2 to 2 infected monocytes per ml of blood. Each infected monocyte contained an average of five tachyzoites, and we tested DNA from approximately 45 μl of blood. Thus, we could detect 0.045 to 0.45 tachyzoite per test. Since each tachyzoite

TABLE 1. Gene detection for diagnosis of *T. gondii* parasitemia

Patient characteristic(s)	Antibodies to *T. gondii*	Gene detection results (samples positive/total samples)
AIDS, clinically suspected or proven toxoplasmosis	Present	3/5
AIDS, active febrile illness not suspected of being toxoplasmosis	Present	2/2
	Absent	1/2
AIDS, previous CNS toxoplasmosis quiescent on suppressive therapy	Present	0/5
Congenital toxoplasmosis, on therapy	Present	0/1
No evidence of active toxoplasmosis	Present	0/6
	Absent	0/16

contains approximately 35 copies of the B1 gene (3), we were able to detect 1.575 to 15.75 genes per test.

Four of the 14 unblinded samples (from cases 1 through 4) were positive. Of the 10 negative samples, 1 was from a person with AIDS, negative *T. gondii* serology results, and CNS disease with *Aspergillus* sp., cytomegalovirus, and lymphoma. One was from a 2-month-old infant with congenital toxoplasmosis born of an immunocompetent mother. This infant had received 5 days of pyrimethamine and sulfadiazine therapy. A parallel blood sample was also negative by mouse inoculation, but two cerebrospinal fluid samples from this infant were positive by gene amplification. Two negative samples were from asymptomatic persons with AIDS who had had cerebral toxoplasmosis 1 and 3 years earlier and were receiving suppressive therapy. Four were from HIV-infected people without AIDS but with antibodies to *T. gondii*. Two were from healthy individuals.

Two of the 23 blinded samples (from cases 5 and 6) were positive. Of the 21 blinded samples that were negative, one was from a patient with AIDS and untreated CNS toxoplasmosis. Four were from patients with AIDS who had had prior CNS toxoplasmosis and had been receiving primary or suppressive therapy for 3 weeks to several years. Fourteen were from children born to mothers dually infected with both HIV type 1 and *T. gondii*. All 14 children have since become seronegative and have no evidence of congenital toxoplasmosis. The remaining two samples were from healthy people.

Combining the results (Table 1), three of the six positive samples were from patients with AIDS, antibodies to *T. gondii*, and CNS disease typical of cerebral toxoplasmosis. The other three positive samples were from patients with advanced AIDS and active febrile illnesses which were not suspected of being from toxoplasmosis; two had antibodies to *T. gondii*, and one did not.

DISCUSSION

Evidence of parasitemia was common in profoundly immunosuppressed, *T. gondii*-seropositive patients with AIDS and febrile illnesses or CNS processes compatible with *T. gondii* infection. Three of the five patients with the most characteristic sign of CNS toxoplasmosis in this patient population, multiple brain abscesses, had positive tests. Of the two patients with multiple brain abscesses and negative results, one had been on therapy against *T. gondii* infection for 3 weeks and the other was untreated. Parasitemia may be intermittently undetectable in some patients by the current method.

Although the diagnosis of toxoplasmosis was considered for all of the patients with samples that were eventually found to be positive, the diagnosis was actually made during life for only two. In the others, the clinical findings were not considered typical enough or the diagnostic tests used by the clinicians failed to yield evidence of toxoplasmosis. The case histories illustrate the problems that arise in these complex cases and how useful a readily available test for parasitemia would be. In case 2, fungal hyphae led the pathologist and clinicians astray, and *T. gondii* organisms were recognized long after death, when the tissue was reexamined after immunoperoxidase staining. In case 3, the patient with unexplained fevers was known to have had cerebral toxoplasmosis months earlier but a recurrent brain abscess was not apparent and recurrent toxoplasmosis was not considered likely. In case 6, the patient had fevers and multiple opportunistic infections. She had diffuse cerebral edema, and her cerebrospinal fluid contained cryptococci but no evidence of inflammation. *T. gondii* infection may have been active in the brain, contributing to the edema, or may have been active elsewhere in her body.

Although most patients with AIDS and toxoplasmosis have multiple cerebral abscesses, cerebral toxoplasmosis also appears as diffuse encephalitis (15). In fact, before AIDS and before the use of intense immunosuppression to allow for organ transplantation, diffuse encephalitis was the most commonly recognized sign of CNS toxoplasmosis (16). Toxoplasmosis also involves other organs in people with AIDS (25), especially the lungs (18). Since serological tests are usually not helpful, and other diagnostic tests are not widely available or used, many of these less typical cases probably go undiagnosed.

One problem with this study was that a tissue diagnosis of toxoplasmosis was made for only one of our six patients with positive tests for parasitemia. In part, this reflects the widespread clinical practice of treating patients with AIDS and suspected cerebral toxoplasmosis empirically. Although one or more positive results may have been false, they were clustered among patients with advanced AIDS, fever, and CNS disease. Five of these six patients had antibodies to *T. gondii*. One did not, but in a recent study (19), 4 of 18 patients with AIDS and pathologically proved cerebral toxoplasmosis had negative tests for antibodies. Another problem was that because of the retrospective, referral nature of our study, mouse inoculation could be done for only 4 of the 37 samples.

Support for the specificity of our gene detection test was provided by our experiments with infected rabbits (9). In

that study, 12 of 32 blood samples from infected rabbits were positive and all 12 were confirmed by parallel mouse inoculations. None of 32 samples from uninfected rabbits were positive.

Although detection of *T. gondii* genes after amplification with PCR has been reported before, detection in human blood samples has been reported in only three cases (11, 12). Many more cases of detection in amniotic fluid (4, 6, 8), cerebrospinal fluid (6, 7, 14, 17, 26), aqueous humor (2), or tissues (1, 7, 12, 26) have been reported, but none of these specimens offer the advantages of blood for diagnosis of active disease in immunosuppressed patients. Amniotic fluid is uniquely suited to congenital cases. Cerebrospinal or eye fluid is useful only for brain or eye disease and is unlikely to be positive for deep brain lesions. Lumbar puncture is dangerous in patients with CNS mass lesions. Detection of *T. gondii* genes in tissues does not differentiate between the tachyzoite and the tissue cyst, which persists for life in infected people. Detection of *T. gondii* genes in blood probably reflects active infection.

Mouse inoculation, the "gold standard" for diagnosis of parasitemia, is cumbersome and requires the use of live animals. Tissue culture has been used to detect parasitemia in several cases (5, 10, 23, 24), but we found that it was less sensitive than mouse inoculation and gene detection (9). Both mouse inoculation and tissue culture must be done with specimens containing viable *T. gondii*. Gene detection after amplification by PCR is technically more difficult, and rigorous measures must be used to prevent contamination, but it can be done on frozen, erythrocyte-free pellets and it is more rapid. Further studies of this technique, preferably with concurrent mouse inoculation, are needed to confirm and expand our findings. Such a study with pregnant women with HIV infection and asymptomatic *T. gondii* infection is under way.

Data from this and other studies (15, 18) indicate that many cases of toxoplasmosis in patients with AIDS are probably being missed. These cases could be easily diagnosed if tests for parasitemia were more widely available. On the basis of our findings, we suggest that parasitemia should be tested for in severely immunosuppressed patients with AIDS, antibodies to *T. gondii*, and febrile illnesses, especially if they have CNS disease or if they have had toxoplasmosis before. If results can be obtained quickly enough, tests for parasitemia should be used instead of brain biopsy to confirm the diagnosis in patients with typical CNS toxoplasmosis. The incidence of toxoplasmosis in patients with AIDS varies substantially in those from different parts of the world. In patients with advanced AIDS from areas with a greater incidence (e.g., Haiti, France, Germany, and southern Florida), tests for parasitemia should be used to diagnose unexplained febrile illnesses or CNS lesions, even in the absence of antibodies to *T. gondii*.

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