

Detection of *Toxoplasma Gondii* DNA in Primary Intraocular B-Cell Lymphoma

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Primary intraocular lymphoma, a variant of primary central nervous system lymphoma with ocular involvement, is a large B-cell non-Hodgkin's lymphoma. Some cases of primary intraocular lymphoma have been reported to be associated with microorganisms including Epstein-Barr virus (EBV) and human herpes virus-8 (HHV-8), but not parasites. We analyzed 10 cases of primary intraocular lymphoma using microdissection and PCR. Tumor and normal cells were microdissected from ocular tissue on slides and subjected to PCR for genes from *Toxoplasma gondii*, EBV, and HHV-8. We detected *Toxoplasma gondii*, not HHV-8 or EBV, DNA in the lymphoma but not in normal cells of two cases that resembled ocular toxoplasmosis clinically. We speculate that *Toxoplasma gondii* may play a role in some forms of primary intraocular B-cell lymphoma.

KEY WORDS: B-cell lymphoma, CNS lymphoma, Microdissection, Primary intraocular lymphoma, *Toxoplasma gondii*.

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Primary intraocular lymphoma is a primary central nervous system (CNS) non-Hodgkin's lymphoma initially involving the eye (1-5). The vast majority of primary intraocular lymphomas are diffuse large B-cell lymphomas (DLBCL) showing immunoglobulin heavy-chain (IgH) gene rearrangements (6-8). Recently, two molecularly distinct forms of DLBCL were identified: germinal center B-like and activated B-like DLBCL (9). The role of antigenic drive by self or infectious agents in malignant transformation has been raised in the latter form.

Toxoplasma gondii (*T. gondii*), a protozoan parasite, is responsible for ocular diseases in both immunosuppressed and immunocompetent individuals. For many years, *T. gondii* has been the most common recognizable cause of posterior uveitis (toxoplasmic retinochoroiditis) in immunocompetent individuals (10, 11). High anti-*T. gondii* IgG titers are often found in the eyes of patients with toxoplasmic retinochoroiditis because of polyclonal B lymphocyte activation (12). *Toxoplasma* seroprevalence and retinochoroiditis varies from country to country (20% in the United States and 80% in France). To the authors' knowledge, no previous published clinical, pathological, or epidemiological studies relating toxoplasma infection to primary intraocular lymphomas have been reported. In the present study, we show and speculate that *T. gondii* may be involved in 2 of 10 cases of primary intraocular B-cell lymphoma.

MATERIALS AND METHODS

Patient Tissues and Histological and Molecular Studies

Ten HIV-1-negative patients, aged 41 to 81 years, presented with moderate to severe vitritis in both eyes. Two patients also presented retinochoroidal lesions that were clinically similar to ocular toxoplasmosis. Written informed consent was obtained from each patient as approved by the institutional review board at the National Eye Institute, Bethesda, Maryland or by the University of Lausanne in Switzerland. Diagnostic vitrectomies in all patients were performed. In addition, one patient also underwent chorioretinal biopsy. All specimens were collected for study at the National Eye Institute.

All specimens were handled and processed as described previously (2). Briefly, vitrectomy samples were immediately transported and processed for cytological evaluation. The cytospin slides were

stained for Diff Quick, Giemsa, and immunohistochemistry. The chorioretinal tissue was fixed, embedded, sectioned, and stained with hematoxylin and eosin (13). Immunohistochemistry for CD20, CD3, and immunoglobulin κ and λ light chains was also conducted using avidin-biotin complex immunoperoxidase technique.

Neoplastic and normal cells from each specimen and normal lymphocytes from two nontoxoplasmic uveitic specimens were carefully microdissected as described previously (6, 14–16). Briefly, either frozen or 10% buffered formalin fixed-paraffin sections were stained with hematoxylin and eosin. Paraffin sections required deparaffinization. Cells of interest were selected by visualization under the light microscope and microdissected using a 30-gauge needle or by laser capture microscopy, PixCell II (Arcturus, Mountain View, CA). The PixCell II uses a low-power infrared laser to collect selected cells onto a membrane located on the cap of a 1.5-mL tube. In manual microdissection, the selected cells were gently scraped, detached, and removed from the slide using a 30-gauge needle.

The microdissected cells were immediately placed in proteinase K–enriched DNA extraction buffer. PCR amplification was performed with a mixture of 1 μ L of extracted DNA, 3.0 pmol of 32 P-labeled sense primer, 3.0 pmol of antisense primer, 4.0 nmol of each dNTP, 1 \times GeneAmp buffer, 1.0 U of AmpliTag Gold Polymerase (Perkin-Elmer, Hayward, CA) and 1.5 mM MgCl₂. PCR cycle conditions included a hot start at 94°C for 9 minutes, 40 cycles of denaturing at 94°C for 45 seconds, annealing at 58°C (for IgH primers) or 63°C (for TCR γ primers) for 60 seconds, and extension at 72°C for 120 seconds. The IgH FR3A gene (sense: 5'-ACA CGG CYS TGT ATT ACT GT-3' and antisense: 5'-CGA TGG TAC CAA GCT TTG AGG AGA CGG TGA CCA-3') was chosen for detection of B-cell malignancy. The TCR γ gene (sense: 5'-AGG GAT GTG TTG GAA TCA GG-3' and antisense: 5'-CGT CGA CAA CAA GTG TTG TTC CAC-3') was used for detection of T-cell malignancy.

Detection of *T. Gondii* and Viral Genes

DNA isolated from above microdissected lymphoma cells was also subjected to PCR amplification using *T. gondii* B1 gene-specific PCR primers (5'-GGA ACT GCA TCC GTT CAT GAG-3' for the sense and 5'-TCT TTA AAG CGT TCG TGG TC-3' for the antisense; 17). The PCR reaction was performed for 40 cycles at 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 1 minute. Authenticity of the amplified fragments was verified by Southern blot hybridization using a *T. gondii* antisense probe B1 (5'-GGC GAC TAT GCG AAT ACA CC-3') end-labeled with digoxigenin. Briefly, after PCR ampli-

fication, DNA was transferred to nitrocellulose membrane and hybridized at 65°C for 5 hours. After washing and binding with anti-digoxigenin antibody, the signals were visualized by chemiluminescent detection.

In addition, nonmalignant cells from those two cases that demonstrated positive *T. gondii* B1 gene in the lymphoma cells were carefully microdissected. DNA was extracted from normal cells in each case and subjected for *T. gondii* B1 gene analysis as described. The DNA amount obtained from each sample (minimum 20 cells per sample) was standardized and compared with the expression of a housekeeping gene, β -actin.

The microdissected DNA of each case was also analyzed by PCR for human herpes virus-8 (HHV-8) or Epstein-Barr virus (EBV) genome as described previously (18). The primers for HHV-8 were (sense) 5'-TCC GTG TT G TCT ACG TCC AG-3' and (antisense) 5'-AGC CGA AAG GAT TCC ACC AT-3'. The primers for EBV were (sense) 5'-GAC GAG GGG CCA GGT ACA-3' and (antisense) 5'-GCA GCC AAT GCT TCT TGG ACG T-3'. Southern hybridization was also performed to confirm the amplified DNA sequences using specific hybridization probes for HHV-8 or EBV. The probes were: 5'-TGC AGC AGC TGT TGG TGT ACC ACA TCT ACT CCA AA-3' for HHV-8 and 5'-CGT CCT CGT CCT CTT CCC CGT CCA CGT CCA CGT CCA TGG-3' for EBV.

RESULTS

Diagnosis of primary intraocular B-cell lymphoma was made in 10 cases based on clinical and pathological evaluation. Large, pleomorphic cells with round, oval, or hypersegmented nuclei with fingerlike projections, prominent nucleoli, and scanty basophilic cytoplasm were identified in 8/10 vitreous or chorioretinal biopsy specimens (Fig. 1A,B). These cells were CD20 positive and either κ or λ positive. The other two cases showed necrotic cells and suspicious atypical lymphocytes in the vitrectomy samples. Both malignant lymphoma cells and suspicious atypical cells in all 10 specimens were microdissected and demonstrated rearrangement of immunoglobulin heavy-chain, but not T-cell receptor γ genes (Fig. 2), confirming the diagnosis of primary intraocular B-cell lymphoma in these 10 patients (8).

The presence of EBV, HHV-8, and *T. gondii* DNA was examined in the microdissected primary intraocular lymphoma cells. The *T. gondii* B1 gene was detected in two cases with primary intraocular B-cell lymphoma, including one from Switzerland with typical clinical features of ocular toxoplasmosis and a positive serum IgG anti-*T. gondii* titer of 18 U/mL. The other case had a large pigmented

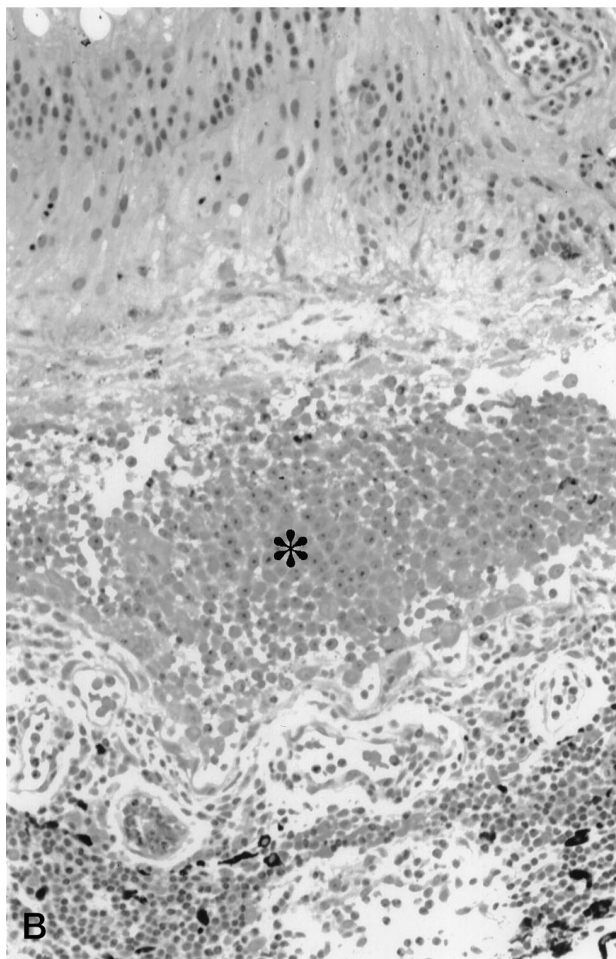
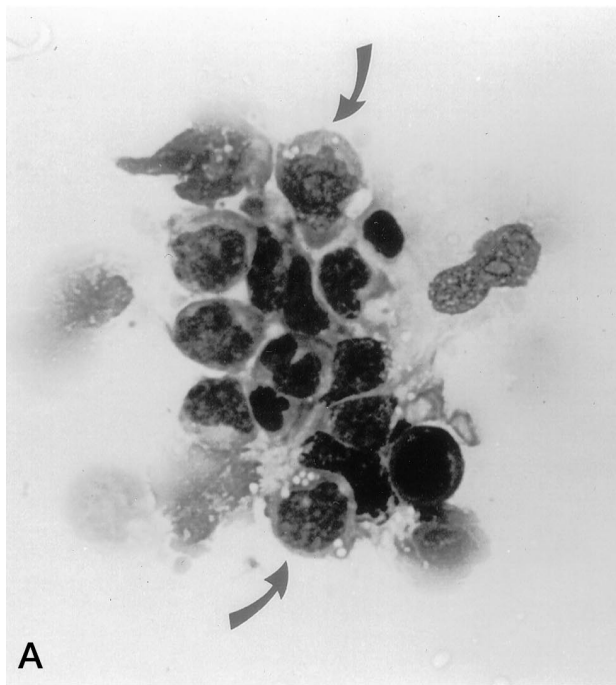


FIGURE 1. Microphotograph showing (A) typical intraocular lymphoma cells in the vitreous (arrows) and (B) tumor cells (asterisk) located between the retina and choroid of the second patient whose lymphoma cells contained *T. gondii* B1 DNA (Lane 1 in Fig. 2). Nonmalignant T cells are, however, present in the choroid. (A, Diff-Quick, original magnification 400 \times ; B, avidin-biotin-immunoperoxidase, original magnification, 200 \times).

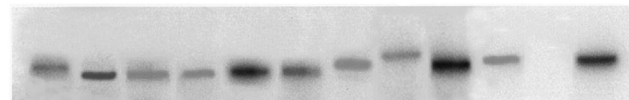
***T. gondii* (B1)**



HHV-8



IgH(FR3A)



TCR- γ

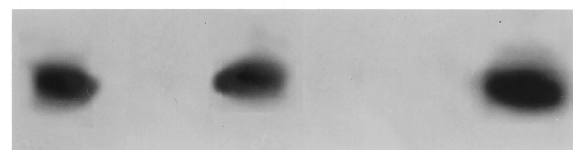


1 2 3 4 5 6 7 8 9 10 11 12

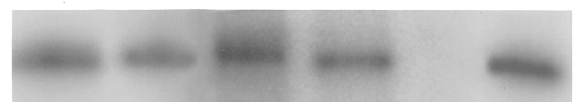
FIGURE 2. PCR amplification showing immunoglobulin heavy-chain clonality in lymphoma cells of all 10 patients, *T. gondii* B1 gene in two patients, and HHV-8 DNA in two other patients. Lanes 1 to 10: Cases 1–10, respectively; Lane 11, negative control; Lane 12, positive control. The positive controls are as follows: *T. gondii*-infected cell line DNA (Advanced Biotechnologies, Inc., Columbia, MD) for *T. gondii*; HHV-8 infected cell line (Advanced Biotechnologies) for HHV-8; tumor cell DNA of a patient with central nervous system B-cell lymphoma for IgH FR3A; and tumor cell DNA of a patient with acute T-cell leukemia for TCR- γ . The negative controls are amplifications without DNA.

retinal lesion adjacent to the subretinal infiltrate in one eye that resembled ocular toxoplasmosis, but his serum antibody against *T. gondii* was not tested. In addition, the normal cells from these two patients were negative for *T. gondii* DNA (Fig. 3). These results indicated that *T. gondii* DNA was only

***T. gondii* (B1)**



Beta-Actin



1 2 3 4 5 6

FIGURE 3. PCR amplification showing *T. gondii* B1 gene in the lymphoma, not normal cells of two patients. Lane 1, lymphoma cells from Case 1; Lane 2, inflammatory cells from Case 1; Lane 3, lymphoma cells from Case 4; Lane 4, normal cells from the choroid of Case 4; Lane 5, negative control; and Lane 6, positive control.

present in the B-lymphoma cells in these two patients.

HHV-8 and EBV genomes were not detected in the lymphoma cells of these two patients, although HHV-8 DNA was detected in two other patients with primary intraocular lymphoma. HHV-8 DNA and EBV DNA have been previously reported to be associated with primary intraocular lymphoma (18). Normal lymphocytes obtained from the eyes of two other patients with uveitis were negative for immunoglobulin clonality and microorganism genomes (data not shown).

DISCUSSION

In this study we detected *T. gondii* DNA in primary intraocular B-cell lymphoma cells but not in normal cells from two patients using microdissection and molecular amplification technique. Because the cell subpopulation of our interest (lymphoma cells) only constitutes a tiny fraction of the total tissue volume, analysis of critical gene expression requires the microdissection and extraction of a microscopic homogeneous cellular subpopulation (lymphoma) from its complex tissue milieu (eye tissue; 6, 14–16). This subpopulation can then be compared with adjacent interacting subpopulations of cells (normal or infiltrating inflammatory cells) in the same tissue. Laser capture microdissection is a technique developed in the past few years to provide a rapid and reliable means to procure a pure population of cells from specific microscopic regions of tissue sections; in one step, under visualization (15, 16). The cells of interest are transferred to a polymer membrane that is activated by laser pulses. The exact morphology of the procured cells with intact DNA, RNA, and proteins is retained and held on the transfer membrane.

T. gondii has been documented to be involved in toxoplasma lymphadenitis (Piringer's lymphadenopathy), a potentially malignant disease in which the monocytoid B cells may arise by transformation of polyclonal B cells (19). The B cells undergoing a monocytoid B-cell transformation are, in the majority (74%), unmutated naive B cells, and only a minority (26%) disclose VH mutations compatible with a derivation from nonantigen-selected post-germinal center B cells. Given that *T. gondii* has recently been shown to have T-cell superantigen activity, it has therefore been suggested that *T. gondii* could also possess B-cell superantigen activity (20).

Using nested PCR techniques, Tachikawa and associates (21) detected *T. gondii* genome in cerebrospinal fluid of 1/5 AIDS patients with CNS B-cell lymphoma. Toxoplasmic encephalitis is one of most common opportunistic infections in ad-

vanced AIDS patients. Additionally, EBV DNA was detected in all five patients. As EBV has been linked to lymphoma in AIDS patients (22, 23), it is unclear what role *T. gondii* may have played in the malignant transformation of the CNS lymphoma of the AIDS patient in Tachikawa and colleagues' report. Furthermore, in that study, it was not clarified whether *T. gondii* DNA was present in lymphoma cells or whether lymphoma cells were present in the tested CSF.

Although primary intraocular lymphoma is a component of primary CNS lymphoma (3, 4), it may also represent a subgroup of relatively heterogeneous DLBCL (9). Using microdissection and PCR techniques, we have detected *T. gondii* DNA in two cases of primary intraocular lymphoma cells. In both cases, toxoplasmosis could be present in adjacent ocular tissue; however, we did not detect *T. gondii* DNA in cells from the normal tissue. We speculate that *T. gondii* may play a role in lymphomagenesis. This study raises the question of whether primary intraocular lymphoma associated with *T. gondii* is distinct from other cases of primary intraocular lymphoma. Our results must be interpreted with caution because of the relatively small number of cases investigated. Additional studies are required to determine the mechanisms involved in B-cell transformation by *T. gondii*. Further investigation of *T. gondii*-associated malignancies and identification of new infectious pathogens will continue to provide clues to the complicated process of oncogenesis and bring us closer to a more effective treatment for lymphoma.

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