MOLECULAR PATHOLOGY OF PRIMARY INTRAOCULAR LYMPHOMA

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ABSTRACT

Purpose: To evaluate immunoglobulin heavy chain (IgH) gene rearrangements, cytokines and chemokines, and infectious agents in primary intraocular B-cell lymphoma (PIOL) cells, in order to better diagnose and understand PIOL.

Methods: We studied ocular specimens from 57 patients with PIOL at the National Eye Institute from 1991 to 2001. Specimens were analyzed for IgH gene rearrangements using microdissection and polymerase chain reaction (PCR). We measured vitreal interleukin (IL)-10 and IL-6 levels by enzyme-linked immunosorbent assay. IL-10 mRNA was studied in PIOL cells using microdissection and reverse transcribed (RT)-PCR. Chemokine and chemokine receptor expression was examined by using immunohistochemistry. Infectious DNA of human herpetic virus-8 (HHV-8), Epstein-Bar virus (EBV), and *Toxoplasma gondii* was detected by using microdissection and PCR and was confirmed with Southern blot hybridization.

Results: IgH rearrangement(s) were demonstrated in all 50 tested cases. Cytokine levels were measured in the vitreous of 39 patients. Thirty-one had measurable cytokine levels: 24 of 31 had elevation of IL-10 relative to that of IL-6, and, in contrast, only 7 of 31 had elevation of IL-6 relative to IL-10. IL-10 mRNA was abundant in lymphoma cells of 6 examined cases. Lymphoma cells expressed chemokine receptors of CXCR4 and CXCR5 in three tested cases. HHV-8 DNA was found in 6 of 32 cases (18.8%), EBV DNA in 2 of 21 (9.5%), and *T gondii* DNA in 2 of 16 (12.5%).

Conclusions: Molecular analyses detecting IgH rearrangements and vitreal levels of IL-10 and IL-6 are useful adjuncts for PIOL diagnosis. A role for specific infectious agents is hypothesized in the pathogenesis of some cases of PIOL. B-cell chemokine is likely involved in attracting PIOL cells into the eye.

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INTRODUCTION

Intraocular lymphoma has two distinct forms: (1) arising in the central nervous system (CNS) and/or the retina and (2) arising outside the CNS with intraocular metastasis.[1] The former, also called primary CNS lymphoma (PCNSL), has a much higher prevalence than the latter. Primary intraocular lymphoma (PIOL) is a subset of PCNSL that initially presents in the eye with or without simultaneous CNS involvement.[2,3] The majority of cases of PIOL/PCNSL are extranodal non-Hodgkin, diffuse large B-cell lymphomas.[4,5] The latter, non-PCNSL lymphomas are from systemic lymphomas that metastasize via blood circulation to the uvea. The choroid is the most common site for the metastatic systemic lymphomas.[6,7]

PIOL, previously known as reticulum cell sarcoma, is thought to have been first reported by Cooper and Ricker in 1951.[8] However, that case might represent a choroidal lymphoma arising outside the CNS with intraocular metastasis. Reticulum cell sarcoma was well accepted both clinically and pathologically a decade later.[9] Later, the term reticulum cell sarcoma was changed to lymphoma on account of its lymphoid origin.[10]

From the National Eye Institute, National Institutes of Health, Bethesda, Maryland

EPIDEMIOLOGY

According to National Cancer Institute data, the incidence of PCNSL rose from 0.027 to 0.075 in 100,000 from 1973 through 1983, and to 1 in 100,000 in the early 1990s.[11] The main cause of the increased incidence of PCNSL is the increased number of patients with immunodeficiency and immunosuppresion.[12] Thomas,[13] however, reported an apparently lower prevalence of acquired immunodeficiency syndrome (AIDS)-associated lymphoma in sub-Saharan Africa as compared with that in the developing world. The cause for the increased incidence in immunocompetent patients is unknown.[14] Based on the statistics of PCNSL,[15,16] we estimate conservatively that in the past 3 years there were at least 100 new cases of PIOL in the United States.

PIOL occurs mostly in middle-aged and older adults, although patients as young as 15 years have been reported.[1,17] The male-female ratio is 1.2 to 1.7:1, with a significant increase of PCNSL seen in men but not in women.[11] However, in a recent study of 44 patients with PIOL in France, Cassoux and colleagues[18] reported that 82% of their PIOL patients were female. Approximately 25% of PCNSL patients without ocular involvement at the time of diagnosis will develop intraocular lymphoma. Alternatively, 60% to 80% of PIOL patients will develop CNS lymphoma.[17,19]

CLINICAL MANIFESTATIONS

The most common ocular complaints are blurred vision and floaters; redness and pain are rare.[3,17,20-22] Although visual acuity may be decreased, it is usually better than would be expected from clinical examination. The disease is bilateral in 80% cases. Vitritis is the most common finding, present in most patients.[18,20,23-25] Anterior chamber cells and cream-colored subretinal yellow infiltrates are frequently reported (Figure 1). Sometimes PIOL may mimic a viral retinitis that is characterized by large areas of yellow creamy infiltrates, retinal hemorrhages, vasculitis, detachment, and necrosis.[25-27] The most common characteristics of the fluorescein angiograms show disturbances at the level of retinal pigment epithelium (RPE), including window defects that appear to correspond to tumor infiltrates at the level of RPE (Figure 2).[3,18,28] Both hyperfluorescent and hypofluorescent lesions can be observed.

Given that the ocular findings are nonspecific, in many patients with PIOL a chronic uveitis is initially diagnosed after an unremarkable work-up for causes of noninfectious and infectious uveitis with similar features.[2,24,25] These entities include sarcoidosis, intermediate uveitis, multifocal choroiditis, acute posterior multifocal placoid pigment epitheliopathy, birdshot chorioretinopathy, toxoplasmosis, ocular tuberculosis, and acute retinal necrosis. Initially, most patients improve with the initiation of corticosteroid therapy. Ultimately, the disease progresses and the patient will not respond to anti-inflammatory treatment.

In addition to ocular signs, CNS symptoms may be associated with PIOL. Behavioral and cognitive changes are the most common presenting symptoms.[29] Hemiparesis, aphasia, and seizures may develop.[4] Recently, PIOL was noted to associate with testicular lymphoma.[30] In our series of PIOL cases at the National Eye Institue (NEI), two patients also had a clinical history of treated testicular B-cell lymphoma (unpublished data).

DIAGNOSIS

Because appropriate treatment of PIOL usually involves systemic chemotherapy and/or radiation therapy, a definitive pathologic diagnosis is required. Once the diagnosis of PIOL is considered, the patient should undergo a thorough CNS evaluation.[2] CNS work-up includes brain computed tomography (CT) and/or magnetic resonance image (MRI) staging, and lumbar puncture for cerebrospinal fluid (CSF) evaluation, including cytology.[14,31] PCNSL can disseminate within the CSF, from which the

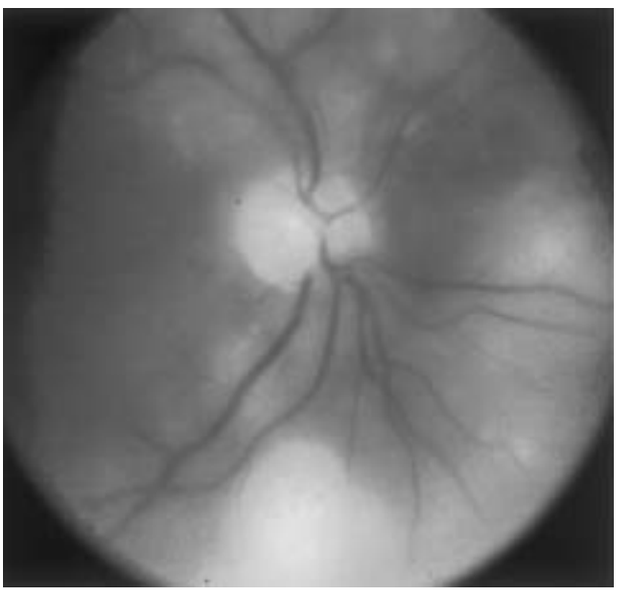

FIGURE 1
Fundus photograph showing multiple subretinal yellow creamy infiltrates that appears hazy due to infiltration of the vitreous in a patient with PIOL.

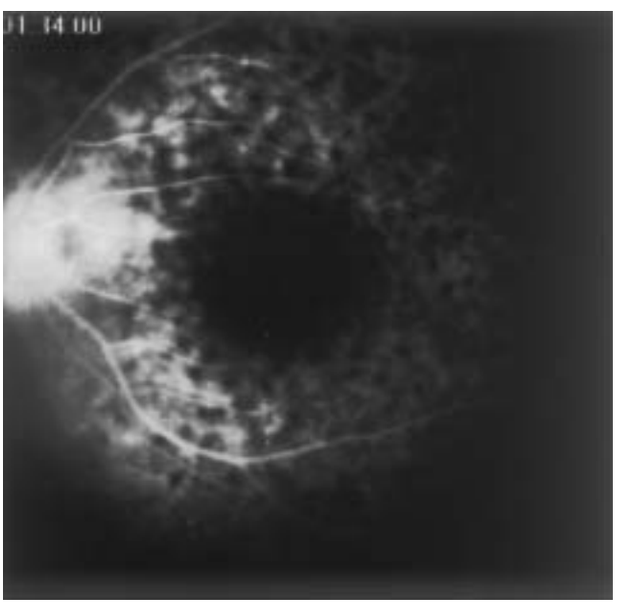

FIGURE 2
Fluorescence angiogram showing many punctate hyperfluorescent and hypofluorescent lesions in a patient with PIOL.

lymphoma cells can be identified in about 25% patients.[32] In contrast, if the diagnosis of metastatic lymphoma originating outside CNS is considered, the patients should undergo systemic work-up, including chest, abdominal, and pelvic CT scan and bone marrow biopsy.

When there is failure to demonstrate lymphoma cells in the CSF, a complete diagnostic vitrectomy on the eye with the most severe "vitritis" or poorest visual acuity should be performed. Because the lymphoma cells are often fragile and easily degenerate in the vitreous, it is critical to handle and process the specimen promptly and appropriately.[3,33,34] Typical lymphoma cells are large lymphoid cells with scanty basophilic cytoplasm and round, oval, clover, or bean-shaped nuclei with prominent nucleoli. Because the vitreous specimen usually contains many reactive T lymphocytes, necrotic cells, fibrin, and debris mixed with the lymphoma cells, examination of the specimen often requires an experienced cytologist and/or ophthalmic pathologist.

If the diagnostic vitrectomy fails to identify lymphoma cells, ocular tissue biopsy or diagnostic enucleation could be considered.[35-40] Fine-needle aspiration of intraocular mass lesions has been performed and can yield successful results.[41-43] Typically, chorioretinal biopsies and enucleated eyes demonstrate neoplastic cells between the RPE and Bruch's membrane. The tumor cells are large and hyperchromatic with prominent to conspicuous nucleoli. Necrosis is frequently observed. Lymphoma cells are often clustered in a perivascular arrangement in the retina and optic nerve head. Diffuse infiltration of the retina and vitreous, as well as retinal necrosis, may occur. Areas of RPE depigmentation, atrophy, and disciform scars may result from tumor detachment of the RPE.[28]

In addition to routine cytologic and histologic studies of the morphology of the lymphoma cells, immunohistochemistry and/or flow cytometry to characterize the lymphoma cell phenotype is needed to confirm monoclonality and the diagnosis.[44,45] From a molecular genetic point of view, lymphoma cells show monoclonal rearrangement of immunoglobulin heavy (IgH) and light (IgL) chain genes in B-cell lymphoma.[46,47] Immunoglobulin genes are rearranged in every B cell, and demonstration of IgH monoclonality is strong evidence for the diagnosis of PIOL.[48,49]

The majority of PIOLs are B-cell lymphoma; the tumor cells produce different interleukins (ILs), in particular IL-10.[50-53] IL-10 is described as a growth and differentiation factor for B lymphocytes that induce activated B cells to secrete large amounts of immunoglobulin.[54] We and others have found elevation of vitreal IL-10 level and an IL-10 to IL-6 ratio greater than 1 in many PIOL patients, suggesting that analysis of vitreal IL-10 and IL-6 may be helpful in the diagnosis of PIOL.[18,55-57] However, controversial data regarding vitreal IL-10 levels have been reported.[58] Two of the fundamental purposes of this study are to evaluate IgH gene rearrangements and ocular cytokines in PIOL to improve the understanding and diagnosis of the disorder.

PATHOGENESIS

The pathogenesis of PIOL is still an enigma. PIOL cells are usually monoclonal malignant cells, mostly composed of B cells expressing either kappa (κ) or lambda (λ) light chains on their surface.[23,40,43,48,59-61] In a few cases of T-cell PIOL, the tumor cells show T-cell receptor rearrangement(s) and expression of T-cell surface markers.[61]

Apoptosis (programmed cell death) plays an important role in tumor survival. Several genes have been implicated in the apoptosis process. A particular apoptotic family of bcl genes is closely linked to B-cell lymphoma. A survival protein of apoptosis, bcl-2 is expressed in the majority of PCNSL;[62-64] bcl-2 translocation is reported in some aggressive cases with PIOL.[48]

Infectious agents and various pathogens have been linked to malignancy. Epstein-Barr virus (EBV) is strongly associated with Burkitt's lymphoma.[65-67] In vitro, EBV efficiently transforms human B lymphocytes, causing them to proliferate continuously.[68] Human herpesvirus (HHV)-8 is another virus that has been considered to play a role in pathogenesis of certain lymphomas.[69,70] HHV-8 genome has been found in primary effusion lymphoma, Castleman's disease, and some PCNSL. However, the role of EBV and HHV-8 in PCNSL seems controversial, especially in immunocompetent patients.[71,72] We have also found a limited role of these two viruses in PIOL.[73] More recently, we reported a preliminary study on the association between *Toxoplasma gondii* and PIOL.[74] Although only a few cases of B-cell lymphomas are reported to be initiated by these microorganisms, the possible etiology of EBV, HHV-8, and *T gondii* requires further examination.

TREATMENT

Because lymphoma cells are sensitive to radiation, in the past, radiotherapy was the mainstay of treatment for PCNSL, resulting in a median survival of 12 to 18 months.[75] However, recurrences are common following radiation therapy. Currently, chemotherapy is recommended as the first treatment for all patients with PCNSL.[15,76,77] The best regimens include high-dose methotrexate (0.8 to 1.5 g/m^2) that can penetrate the blood-brain barrier and are associated with complete-response rates of 50% to 80%. When methotrexate-based regimens are used prior to cranial radiation, the median survival is increased to at least 40 months. Current treatment is directed toward using chemotherapy as the sole

modality in the treatment of PCNSL, with an optimal chemotherapy regimen; the role of radiotherapy remains to be determined.[14,78]

Optimal therapy for patients with PIOL without CNS involvement or recurrent PCNSL with ocular involvement remains undefined. Fewer patients are receiving ocular radiation as primary therapy because of the delayed toxic effects, including radiation retinopathy, optic neuropathy, dry eye, corneal epithelial defects, loss of limbal stem cells, cataracts, and glaucoma.[2,79] Intravitreal methotrexate of 400 mg/0.1 mL has shown encouraging results in a few patients with PIOL.[80-82] Multiple intravitreal injections seem to be required to achieve eradication of lymphoma cells in the eye. Larger studies will be needed to define and determine the effect of local therapy. Recently, promising results for PIOL were obtained with high-dose chemotherapy followed by autologous bone marrow transplantation.[83]

The present study focuses on molecular pathology of PIOL. Data of 57 patients collected at the NEI in the past 10 years (1991-2001) are presented. This large series of PIOL demonstrates IgH gene rearrangement(s), expression of cytokines and chemokine receptors, and DNA from certain infectious agents in the tumor cells. The results help us in improving the diagnosis of PIOL and understanding its pathogenesis.

MATERIALS AND METHODS

OCULAR SPECIMENS

From 1991 to 2001, the NEI received ocular specimens from 57 patients with PIOL (Table I). Two of the 57 patients also had a diagnosis of AIDS and provided one enucleated eye and a pair of autopsy eyes (patients 3 and 4, Table I). Fifty-five patients underwent diagnostic and/or therapeutic vitrectomies; 6 provided more than one vitreous sample. Of the 55 patients who underwent vitrectomy, 5 had diagnostic enucleation after inclusive diagnoses based on the vitrectomy samples, and 3 of 55 had retinal biopsies (two with diagnostic chorioretinal biopsies and one with diagnostic subretinal needle aspiration) combined with vitrectomy. Autopsy was performed on two patients. One (case 52) had been diagnosed by vitrectomy and treated for PIOL. The other (case 3) was an AIDS patient who had not been diagnosed as having PIOL. All samples and studies were under a clinical research protocol that was approved by the NEI institutional review board. The diagnosis of PIOL was based on clinical findings and pathological studies (see "Results" section).

PROCESSING SPECIMENS

Vitreous

Fresh vitreous specimen, the first 5 to 10 mL, was immediately placed into a tube containing tissue culture medium (RPMI, Rosewell Park Memorial Institute, Mediatech Inc, Herndon, Va) for a total volume of 10 to 15 mL and centrifuged at 1,000 rpm (200*g* force) for 10 minutes at room temperature. The supernatant was collected for cytokine analysis. The sediment 1 to 2 mL was resuspended in 3 to 4 mL RPMI (a total volume of 5 mL) for cytocentrifuging with Cytopro (Wescor Inc, Logan, Utah). Each cytospin chamber contains 2 to 8 drops of the fluid (mixture of vitreous cells and RPMI) depending on the degree of cloudiness. Cytocentrifuge was spun for 5 minutes at 1,000 rpm. Cells were collected on the coated slides (usually 5 to 10 slides). The cytology slides were stained for Diff-Quick, Giemsa, and/or immunohistochemistry for CD20 (B cell), CD3 (T cell), CD68 (macrophage), and κ and λ immunoglobulin light chains using avidin-biotin complex immunoperoxidase (ABC) technique.[33]

Eye

The 5 enucleated eyes (cases 1, 4, 8, 13, and 35) were freshly sectioned through the lesions according to clinical examinations. Half of the eye was immediately embedded in optimal cutting compound (OCT, Miles Lab, Naperville, Ill) and snap-frozen in a mixture of dry ice and 2-methybutane. The other half was fixed in 10% formalin and embedded in paraffin. Paraffin or frozen sections were used for routine histological staining and ABC immunohistochemical staining as described previously.[38] Four autopsy eyes (cases 3 and 52) were received and fixed in formalin. They were embedded in paraffin and sectioned.

Chorioretinal and Needle Biopsies

Chorioretinal biopsy specimens (cases 2 and 17) and subretinal needle aspirate (case 31) were processed as reported previously.[40,43] Briefly, the specimens were handled and processed in the operating room. Under a dissection microscope, the specimen was divided in portions of fixation in 10% formalin (routine histology), in OCT (frozen sections), and in culture for microorganisms.

MICRODISSECTION

Microdissection was performed on ocular specimens obtained from 50 patients. Microdissection was carried out as described either manually or using laser capture microscopy (Arcturus, Mountain View, Calif).[48,84,85] Briefly, either 5-μm frozen or 10% buffered formalin fixed-paraffin sections were stained with hematoxylin-eosin. The paraffin sections required deparaffinization. At least 15 atypical cells were selected by visualization under the light microscope and microdissected. In manual microdissection, the lymphoma cells were gently scraped using a 30-

TABLE I: SUMMARY OF 57 PATIENTS WITH PIOL

CASE NO.	COUNTRY	CLINICAL PIOL	SPCIMENS	PATHOLOGIC PIOL	IGH GENE REARRANGEMENT	IL-10 : IL-6 VITREOUS	INFECTIOUS DNA HHV8	EBV	TG
1	USA	Y	V, E	Y	NT	N	N	N	NT
2	USA	N	V, R	N	Y	>1	N	N	NT
3*	USA	N	E†	Y	Y	NT	Y	N	NT
4*	France	Y	E	Y	Y	NT	Y	Y	NT
5	France	Y	V	N	Y	>1	N	N	NT
6	France	Y	V	N	Y	NT	N	N	NT
7	USA	R/O	V	N	Y	>1	Y	N	NT
8	USA	R/O	V, E	Y	Y	NT	N	N	NT
9	France	Y	V	?	Y	NT	Y	N	NT
10	USA	Y	V	Y	Y	>1	N	N	NT
11	France	R/O	V	N	Y	NT	N	N	NT
12	France	R/O	V	N	Y	NT	N	N	NT
13	France	Y	V, E	?	Y	NT	N	N	NT
14	France	R/O	V	Y	Y	NT	N	N	NT
15	France	Y	V	Y	Y	NT	N	N	NT
16	USA	Y	V	Y	Y	>1	N	N	NT
17	USA	N	V, R	Y	Y	<1	N	N	Y
18	France	R/O	V	Y	Y	NT	N	N	N
19	Canada	R/O	V	Y	Y	<1	Y	Y	N
20	Switzerland	N	V	Y	Y	<1	N	N	Y
21	Switzerland	Y	V	Y	Y	NDL	N	N	N
22	Netherlands	Y	V	Y	Y	>1	N	NT	N
23	Switzerland	R/O	V	?	Y	NDL	N	NT	N
24	USA	Y	V	N	Y	>1	N	NT	N
25	USA	N	V	Y	Y	>1	N	NT	N
26	Switzerland	R/O	V	Y	Y	NT	Y	NT	N
27	Saudi Arabia	Y	V	Y	Y	NT	N	NT	N
28	USA	R/O	V	Y	Y	>1	N	NT	N
29	USA	R/O	V	Y	Y	NDL	N	NT	N
30	USA	R/O	V	Y	Y	>1	N	NT	N
31	USA	Y	V, R	Y	Y	>1	N	NT	N
32	Japan	R/O	V	Y	Y	>1	N	NT	N
33	USA	Y	V	Y	Y	NDL	NT	NT	NT
34	USA	Y	V	Y	Y	>1	NT	NT	NT
35	USA	N	V, E	Y	Y	NDL	NT	NT	NT
36	France	Y	V	Y	Y	NT	NT	NT	NT
37	USA	R/O	V	N	Y	NT	NT	NT	NT
38	Japan	R/O	V	N	Y	>1	NT	NT	NT
39	USA	Y	V	Y	Y	>1	NT	NT	NT
40	USA	Y	V	?	Y	>1	NT	NT	NT
41	USA	Y	V	Y	Y	>1	NT	NT	NT
42	USA	R/O	V	N	Y	>1	NT	NT	NT
43	Japan	R/O	V	N	Y	NT	NT	NT	NT
44	USA	N	V	Y	Y	NDL	NT	NT	NT
45	Canada	Y	V	Y	Y	NDL	NT	NT	NT
46	USA	R/O	V	Y	Y	<1	NT	NT	NT
47	USA	Y	V	Y	Y	NDL	NT	NT	NT
48	Canada	R/O	V	Y	Y	<1	NT	NT	NT
49	USA	Y	V	Y	Y	<1	NT	NT	NT
50	USA	Y	V	Y	Y	NT	NT	NT	NT
51	Canada	R/O	V	Y	NT	>1	NT	NT	NT
52	USA	R/O	V, E‡	Y	NT	>1	NT	NT	NT
53	USA	R/O	V	Y	NT	>1	NT	NT	NT
54	USA	R/O	V	Y	NT	>1	NT	NT	NT
55	USA	Y	V	?	NT	>1	NT	NT	NT
56	USA	Y	V	N	NT	<1	NT	NT	NT
57	USA	R/O	V	?	NT	>1	NT	NT	NT

EBV, Epstein-Barr virus; E, eye; HHV8, human herpesvirus 8; N, no; NDL, below detection; NT, no data; R, biopsy including retina; R/O, rule out; TG, *Toxoplasma gondii*; V, vitreous; Y, yes.

*AIDS patient.

†Autopsy eyes.

‡Autopsy eyes that were treated for PIOL.

gauge needle. Soon the cells became detached from the tissue section. The loose cells were carefully picked up and transported by the needle. In laser capture microdissection (LCM), the lymphoma cells were captured using PixCell II (Arcturus). The PixCell II applies a low-power infrared laser to collect selected cells onto a LCM transfer film membrane (CapSure, Arcturus) located on the cap of a 1.5-mL eppendorf tube (Brinkmann Instruments, Inc, Westbury, NY). All removed cells were immediately placed in a single-step DNA extraction buffer containing 0.2 mg/mL proteinase K for 24 hours, which provides the starting point for polymerase chain reaction (PCR) amplification.

DETECTION OF IGH GENE REARRANGEMENTS

Lymphoma cells obtained from 50 patients (48 vitrectomy specimens, 7 eyes, 1 subretinal and 2 chorioretinal biopsies) were subjected to IgH gene analysis. Each immunoglobulin protein consists of two identical pairs of IgL and IgH chains. A variable (V_H) domain and a constant (C_H) domain form IgH. The variable region contains the antigen binding site and results from the recombination of different gene segments. The variability of the IgH gene is linked to three regions of complementarity determining region (CDR), which are separated by four relatively conserved framework regions (FW or FR).[86-88] Because rearrangement is always in the CDR3, the third CDR of the V_HDV_J region of the IgH gene, all samples were tested using primers termed FR3A (from FR3, the third framework of the V_H region of the IgH gene, to FR4 regions) and FR2A (from FR2, the second framework of the V_H region of the IgH gene, to FR4 regions). Additionally, 41 samples were tested using primers termed CDR3 (from CDR3 to FR4 regions). Therefore, all three primer sets included the CDR3 region, and all can detect rearrangement(s) in CDR3 (Figure 3). The extracted DNA from microdissected atypical cells was used for PCR amplification.

The PCR mixture contained the following components: microdissected DNA (minimal 0.1 ng), 400 nmol of ^{32}P-labeled sense primer, 400 nmol of antisense primer, 200 nmol of dATP, dCTP, dGTP, and dTTP, and 0.5 unit of Tag polymerase in a final volume of 10 mL of 1X Tag polymerase buffer containing 1.5 minimole of $MgCl_2$ (Perkin Elmer, Branchburg, NJ). The PCR was carried out for 35 cycles at 94ºC for 45 seconds, 56ºC for 1 minute, and 72ºC for 1 minute. Three primer sets, kindly provided by Dr Sherman Zheng, New York University Medical Center, were used.[89] They were: (1) FR3A, upstream (sense), 5′-ACA CGG CYS TGT ATT ACT GT-3′ and downstream (antisense) of the consensus JH region, 5′-GGA TGG TAT CAA GCT TTG AGG AGA CGG TGA CCA-3′; (2) FR2A, sense, 5′-TGG RTC CGM CAG CAG SCV YCN GG-3′ and antisense, 5′-ACC TGA GGA GAC GGT GAC C-3′; and (3) CDR3, sense, 5′-GTC CTG CAG GCY YCC GGR AAR RGT CTG GAG TGG-3′ and antisense, 5′-TAC AGG ATC CTG AGG AGA CGG TGA CC-3′ (mixed base: R=A/G, M=A/C, S=G/C, V=G/A/C, Y=C/T, N=A/T/C/G).

The ^{32}P-labeled amplified DNA was analyzed on 16% polyacrylamide gel for IgH gene rearrangement products. The resulting autoradiogram was developed using Kodak X-Omat film (Eastman Kodak, Rochester, NY). Positive control DNA was obtained from a monoclonal B lymphoma cell line GA-10 (ATCC, Manassas, Va). The negative control included DNA from microdissected normal retina, a polyclonal B-cell line 472 (Dr Zhengping Zhuang, National Cancer Institute, Bethesda, Md), or no DNA.

DETECTION OF EBV AND HHV-8 DNA

Examination for HHV-8 DNA was performed in 32 patients and for EBV in 21 patients with PIOL. An eye with herpetic enophthalmitis and without lymphoma was used as a control. The PCR-amplified mixture contained the microdissected DNA, sense and antisense primers of HHV-8 or EBV gene. The PCR was performed for 40 cycles at 94ºC for 45 seconds, 58ºC for 45 seconds, and 72ºC for 1 minute. The primers for HHV-8 were: sense, 5′-TCCG TGTT GTCT ACGT CCAG-3′, and antisense, 5′-AGCC GAAA GGAT TCCA CCAT-3′.[90] The primers for EBV were: sense, 5′-GACG AGGG GCCA GGTA CA-3′, and antisense, 5′-GCAG CCAA TGCT TCTT GGAC GT-3′.[91] Positive-control viral DNA was purchased from Advanced Biotechnologies Inc (Columbia, Md).

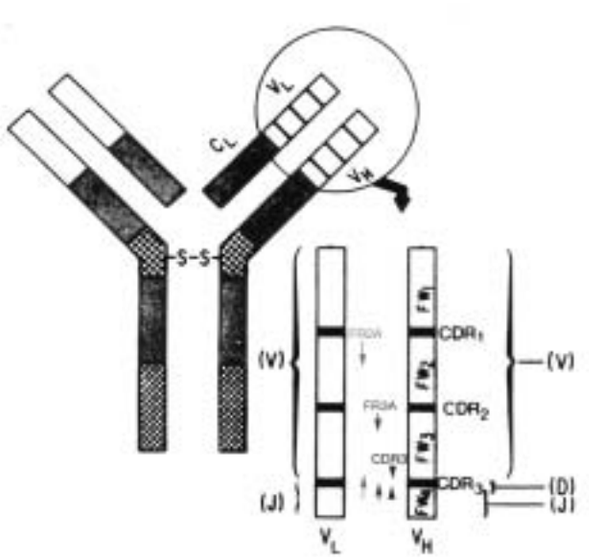

FIGURE 3
Schematic representation of IgH gene primers for the evaluation of IgH gene rearrangement(s) at the CDR3 region (- S – S -, bi-sulfa bond).

Southern hybridization was 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. Briefly, after PCR amplification, DNA was transblotted to nitrocellulose paper. The digoxigenin-labeled HHV-8 or EBV probe was hybridized with blotted DNA at 65ºC for 5 hours. After washing and binding with anti-digoxigenin antibody, the signals were visualized by chemiluminescent detection. Multiple primers, positive controls, and negative controls were used to confirm the results.

DETECTION OF *T GONDII* DNA

Sixteen PIOL patient samples and an eye with ocular toxoplasmosis were examined for *T gondii* gene using PCR and Southern hybridization. Briefly, the DNA extracted from the selected, microdissected cells was 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).[92] The PCR was performed for 40 cycles at 94ºC for 45 seconds, 58ºC for 45 seconds, and 72ºC 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 amplification, DNA was transferred to nitrocellulose membrane and hybridized at 65ºC for 5 hours. After washing and binding with anti-digoxigenin antibody, the signals were visualized by chemiluminescent detection. *T gondii* DNA for positive control was kindly provided by Dr Charles Egwuagu (NEI, Bethesda, Md).

CYTOKINE MEASUREMENT

Vitreous samples from 39 patients were assayed for IL-10 and IL-6. Levels of IL-10 and IL-6 were determined using a standard enzyme-linked immunosorbent assay (ELISA, Endogen, Cambridge, Mass). A minimal volume of 0.2 mL is required for each cytokine analysis. Briefly, high-binding sterile 96-well plates were precoated with the respective anticytokine antibodies. The plates were also preblocked with 10% bovine serum albumin. Samples of vitreous were diluted in the blocking buffer and incubated at room temperature for 2 hours to neutralize enzymatic inhibitory properties of the vitreous. Unbound material was washed, and the pertinent streptavidin–horseradish peroxidase reagent was added. Plates were then incubated at 37ºC for 30 minutes. After washing the unbound material, plates were developed using tetramethobenzidine and read at an absorbance of 450 nm. Concentrations of the cytokines were extrapolated from a standard curve constructed with standard samples of recombinant cytokine and reported as picograms per milliliter.

IL-10 MESSENGER EXPRESSION

IL-10 mRNA was detected in 6 patients using RT-PCR. RNA was isolated from microdissected PIOL cells and an IL-10 cell line (ATCC). The mRNA transcript of IL-10 gene was examined by nested PCR.[93] The sequences of outer primer were: forward, 5′-ATG CAC AGC TCA GCA CTG CTC TG-3′ and reverse, 5′-CAG CCT GAG GGT CTT CAG GTT CT-3′. The sequences of inner primer were: forward, 5′-TGG TCC TCC TGA CTG GGG TGA-3′ and reverse, 5′-GGG CAT CAC CTC CTC CAG GTA-3′, in which one primer was labeled with ^{32}P. RT-nest PCR products were visualized by polyacrylamide gel electrophoresis and radioautography. The positive control of IL-10 cell line shows a product of 260 base pair (bp) (IL-10 mRNA).

CHEMOKINE EXPRESSIONS

Chemokine expressions were demonstrated using standard immunohistochemical staining.[94] Chemokine receptors and chemokine were stained in the eyes of three patients. Briefly, frozen sections were fixed in acetone and absorbed in goat serum. The primary antibodies were monoclonal mouse anti-human antibodies (1 to 2 mg/mL) against B-cell chemokine receptors, CXCR4 and CXCR5 (R & D Systems, Minneapolis, Minn); and B-lymphocyte chemoattractant, BLC (R & D Systems).[95,96] The secondary antibody was biotin-labeled goat–anti-mouse antibody (Vector Lab, Burlingame, Calif). After amplification with avidin-biotin complex (Vector Lab), slides were developed in 3-3′ diaminbenzidine, nickel sulfate, and hydrogen peroxide.

RESULTS

According to the quantity and quality of a particular specimen, selected procedures were performed on ocular tissues obtained from the 57 patients with PIOL (Table I). Because of limited tissue received from each case, a maximal number of experiment(s) were selected and performed. Specimens from 50 patients were subjected to microdissection and IgH gene analysis; all samples were tested with FR3A and FR2A primer sets but only 41 of 50 with the CDR3 primer set. These specimens included 43 vitreous samples, 7 eyes (5 surgical enucleation and a pair of autopsy AIDS eyes), and 3 biopsies involving the retina. Identical PCR products (IgH gene rearrangements) were yielded when two procedures (vitrectomy and enucleation/retinal biopsy) were

performed in the same patient.

PIOL cells selected from 36 patients were tested for HHV-8 DNA, 25 for EBV DNA, and 16 for *T gondii* DNA. Vitreous specimens from 39 patients were assayed for levels of IL-10 and IL-6. Of the 39 patients, 6 had repeated vitrectomies with multiple vitreous specimens. IL-10 mRNA was evaluated in all 6 patients. Selected chemokines and chemokine receptors were stained in three eyes of 3 non-AIDS patients.

All 57 patients had a clinical diagnosis of typical or "rule out" PIOL (Table I). Based on histological and cytological examination, typical large B-cell lymphoma cells were identified in 39 patients (31 of 54 with vitrectomy, 5 of 5 enucleation, 1 of 2 autopsy, 1 of 2 chorioretinal biopsy, 1 of 1 subretinal needle biopsy). Six vitrectomy samples showed suspicious malignant cells but were nonconclusive for the diagnosis of PIOL. Specimens obtained from 12 patients (12 vitreous and one chorioretinal biopsy [case 2]) were poorly prepared and processed, and no or less than 5 typical lymphoma cells were identified by cytological or histological examination. Of these 12 patients, 11 had IgH gene rearrangement and/or high IL-10 levels and an IL-10 to IL-6 ratio greater than 1 in vitreous and retinal biopsy, and the other patient (case 56) had brain lesions compatible with PCNSL. Those patients without a definitive pathological diagnosis of PIOL developed CNS lesions consistent with PCNSL at the time of or after their diagnostic procedures. Therefore, PIOL was confirmed.

MICRODISSECTION IS NEEDED FOR PIOL MOLECULAR ANALYSIS

A minimal of 15 atypical cells were carefully selected and microdissected for DNA extraction in each individual case (Figure 4). Selected DNA from each case was amplified successfully and yielded clean PCR products in each experiment. Without microdissection one could fail to detect molecular changes because the sample might consist of mostly ocular resident and infiltrating inflammatory cells but a relatively small number of malignant cells.[60]

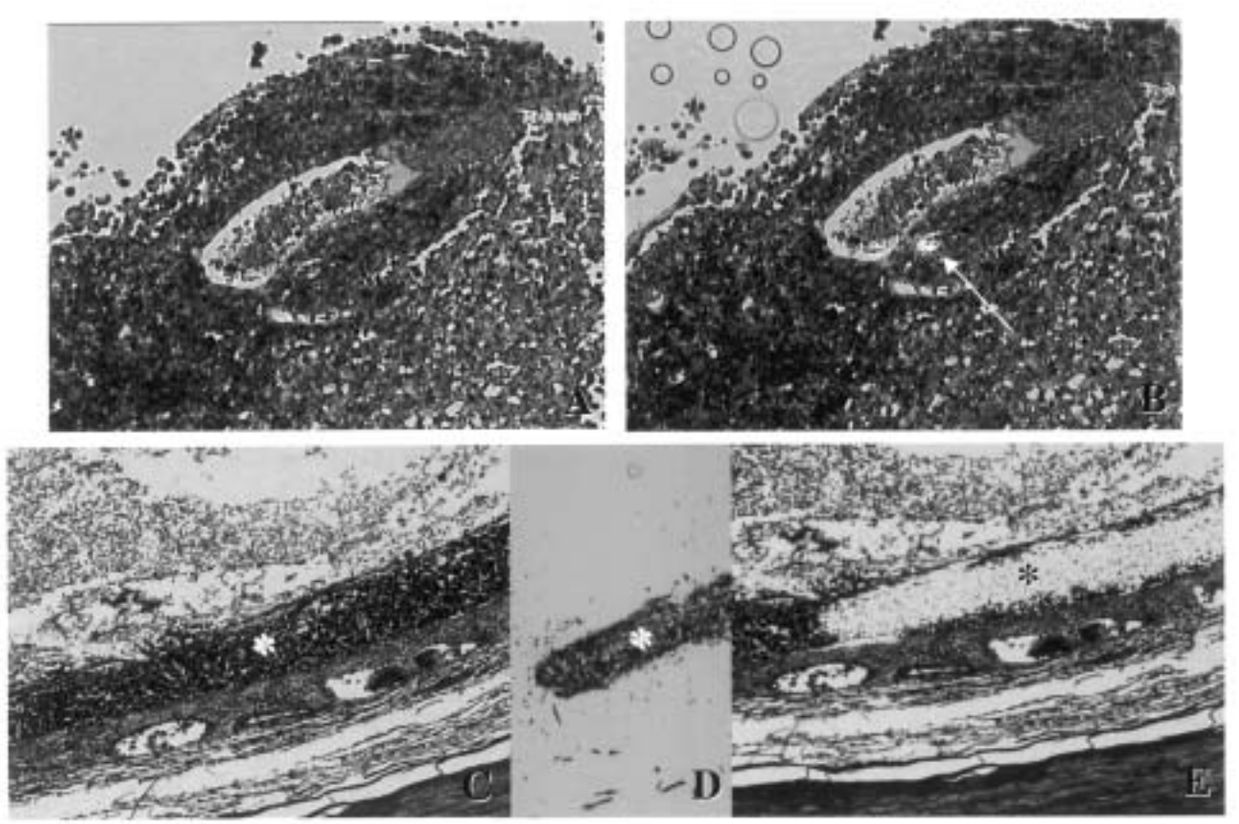

FIGURE 4

Microphotographs of laser capture microdissection (LCM) showing before (A) and during (B) microdissection of the lymphoma cells surrounding a retinal vessel in a PIOL case; and before (C), on LCM membrane (D), and after (E) microdissection of the lymphoma cells between retinal pigment epithelium and Bruch's membrane in another PIOL case (arrow, laser beam aiming on the PIOL cells; asterisks, area of microdissection; hematoxylin-eosin, ×125).

PIOL CELLS ARE MONOCLONAL

Atypical lymphoid cells microdissected from 50 tested samples showed positive IgH gene rearrangements (Figure 5, Table I). Using FR3A primer set, it was found in all 50 cases (100%). Using FR2A primer set, rearrangement was found positive in 22 patients (44%). Using CDR3 primer set, IgH rearrangement was positive in 37 of the 42 tested cases (88.1%). Of the 42 cases, IgH gene rearrangements were examined using three primer sets (FR3A, FR2A, and CDR3) of the variability (V), diversity (D), and joining (J) gene segments. Seventeen (40.5%) showed positive rearrangements with either one of the three primer sets, 20 (47.6%) showed positive with FR3A and CDR3 primer sets, 3 (7.1%) with FR3A and FR2A, and 2 (4.8%) with FR3A.

The above results are caused from use of different primer sets. FR3A primer set works better, because the region FR3 is more conserved among the many VH gene segments. The FR2 varies more, thus the primer set does not always work. A shorter length of bp of the CDR3 primer set may make rearrangement(s) located at the junction between FR3 and CDR3 impossible to detect. Additionally, immunoglobulin rearrangements may contain somatic mutations that could impair primer association.

PIOL IS ASSOCIATED WITH ELEVATION OF IL-10 EXPRESSION

Because of the lack of standardization for the concentration and volume of the vitreous collected during each vitrectomy, it is difficult to directly compare the absolute cytokine levels among the samples. However, it is reasonable to compare the ratio of cytokine levels in a single specimen.

IL-10 and IL-6 levels were assayed in the vitreous of 39 patients. Of 39 patients, 31 had measurable cytokine level (IL-10 and IL-6 both, IL-10 alone, or IL-6 alone). Both IL-10 and IL-6 levels were below the detectable level in 8 of 39 patients. In general, high IL-10 levels were detected in vitreous of patients with PIOL. Of the 31 patients with measurable cytokine levels, 24 (77.4%) had high IL-10 (12 to 38,000 pg/mL) and low IL-6 (0 to 284 pg/mL). Therefore, the ratio of IL-10 to IL-6 was greater than 1 in these 24 patients. In addition, IL-10 levels correlated to the number of malignant cells in the vitreous and disease severity in the 4 patients who had multiple vitreous samples and measurable cytokine levels. The other two patients with multiple vitrectomies did not have measurable IL-10 levels. Of 31 patients, 7 (22.6%) had measurable cytokine levels showing a low IL-10 (<11.7 to 37 pg/mL), a measurable IL-6 (37 to 294 pg/mL), and an IL-10 to IL-6 ratio lower than 1. All 7 patients had a clinical history of early suspicion of PIOL, showing absence of malignant cells in the vitreous and/or being treated with high dosage of systemic corticosteroids. Furthermore, PIOL cells from 6 randomly selected cases were analyzed for expression of IL-10 mRNA. They included two with high vitreal IL-10 levels (5,456 and 3,442 pg/mL), one with a low IL-10 (<23.4 pg/mL), and three with unknown IL-10 (not available to measure). All 6 samples demonstrated expression of IL-10 messengers in PIOL cells (Figure 6).

PIOL CELLS EXPRESS CHEMOKINE RECEPTORS, CXCR4, AND CXCR5

Immunohistochemistry was performed on the three non-AIDS, enucleated eyes. PIOL cells were stained positively for chemokine receptors, CXCR4, and CXCR5 (Figure 7, A through C). CXCR4 was also expressed on some other inflammatory cells. Of note, only the RPE but not other nonmalignant cells in the eye were stained mildly positively for chemokine BLC, the ligand of CXCR5 (Figure 7D).

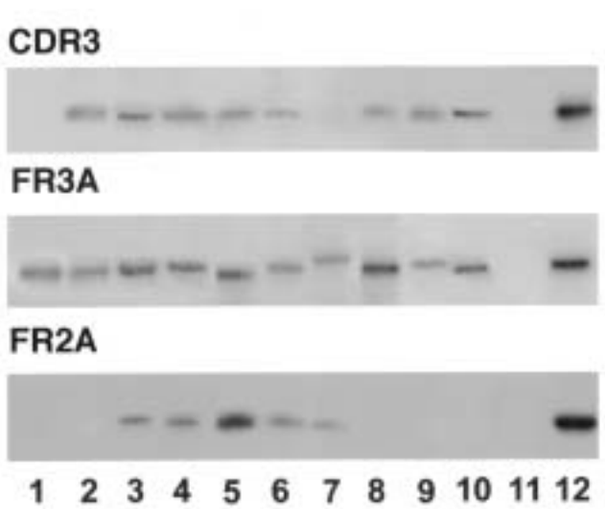

FIGURE 5

Polymerase chain reaction amplification showing IgH gene rearrangements of CDR3 region with three different primer sets in 10 patients with PIOL (lanes 1-10, cases 15-24; lane 11, negative control; lane 12, positive control of a B-cell lymphoma cell line DNA).

FIGURE 6

Reverse transcriptase polymerase chain reaction products showing IL-10 mRNA in PIOL cells of 6 cases (lanes 1-6, cases 1, 2, 4, 5, 8 and 52; lane 7, negative control of normal retina; lane 8, another negative control of normal lymphocyte; lane 9, positive control of an IL-10 cell line).

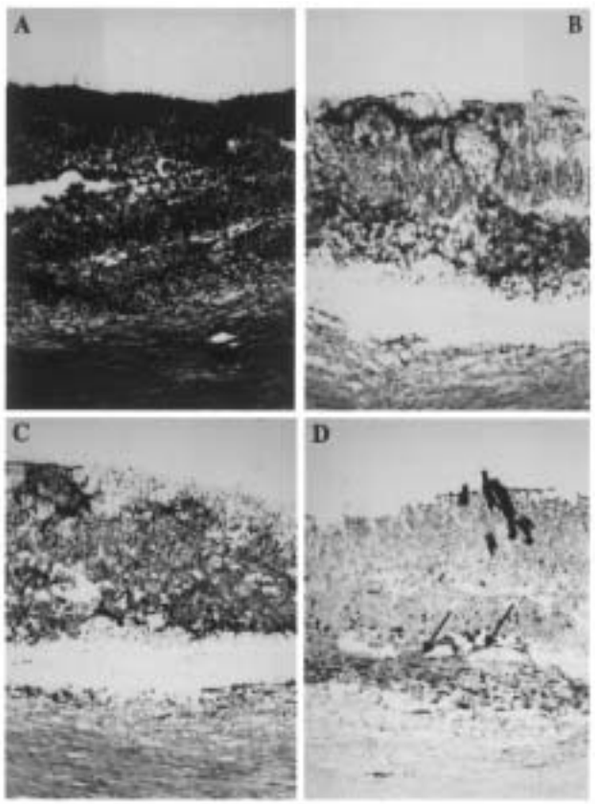

FIGURE 7
Microphotographs of immunohistochemistry showing a case of PIOL (A) with intense expression of (B) CXCR4 and (C) CXCR5 on PIOL cells in the retina and subretinal area but not the lymphocytes infiltrating in the choroid, and (D) weak expression of B-lymphocyte chemoattractant on retinal pigment epithelium (A, hematoxylin-eosin; B through D, ABC immunoperoxidase staining, ×200).

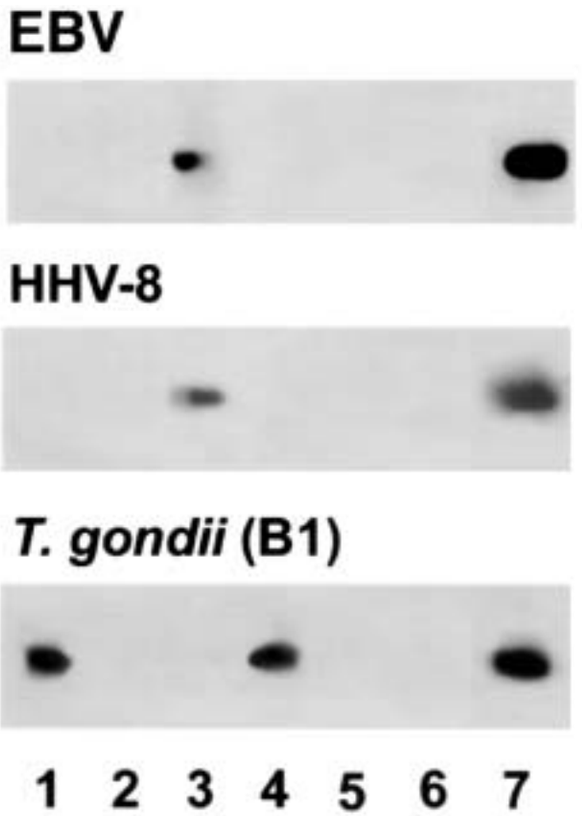

FIGURE 8
Southern blot showing detection of Epstein-Barr virus, human herpesvirus 8, and *Toxoplasma gondii* DNA in some PIOL cells (lanes 1-5, cases 18-22; lane 6, negative control; lane 7, positive control of three microorganisms DNA).

PIOL CELLS CONTAIN DNA FROM INFECTIOUS MICROORGANISMS

Microdissected DNA from available samples was subjected to the detection of HHV-8, EBV, and *T gondii* gene using PCR amplification and Southern hybridization (Figure 8, Table I). HHV-8 DNA was detected in 6 (18.8%) of 32 randomly chosen cases. Three patients were Northern Americans and 3 were Europeans. Two were diagnosed with AIDS (one American and one European). Two patients had classic morphology of PIOL cells. The other 4 patients were diagnosed clinically and demonstrated suspicious atypical lymphoid cells and positive IgH gene rearrangements by molecular analysis.

EBV DNA was detected positively in only 2 (9.5%) of 21 randomly chosen cases, one AIDS eye from France and one vitreous sample from Canada. Of the 16 patients whose specimens were chosen for testing *T gondii* DNA, two were positive (12.5%). One was from Europe and the other was from the Middle East. Importantly, all infectious DNA was detected in PIOL cells only. Other cells, including normal ocular and inflammatory cells that were microdissected from the same slides, were negative for the three microorganism genes. One AIDS eye contained both HHV-8 and EBV genes in PIOL cells.

Infiltrating lymphocytes microdissected from the two control infectious eyes (one with herpes and one with toxoplasmosis) were free of HHV-8 and *T gondii* DNA. Microorganisms were identified in the retina of these two eyes.

DISCUSSION

The current study presents molecular pathology in a large number of ocular specimens with PIOL. PIOL is aggressive, and most patients with the disease die within 5 years of diagnosis.[97] Recent data suggest that prompt therapy can improve prognosis.[76] Since initiation of therapy requires an accurate diagnosis, it is essential to identify lymphoma cells in the eye. One of the elements to improve the yield of diagnosis is prompt and appropriate handling and processing of specimens, particularly vitreous specimens.[3] Our experience has shown that the

addition of cell culture medium such as RPMI in the container of vitreous sample helps prolong the integrity and survival of PIOL cells (Figure 9). Unfortunately, sometimes there may be only necrotic malignant cells in vitreous samples, and in such cases even the experienced ocular pathologist and cytopathologist could have difficulties identifying the PIOL cells. Although immunophenotyping studies such as immunohistochemistry and/or flow cytometry can confirm the diagnosis by demonstrating monoclonality, these techniques usually require a relatively large quantity of samples.[10,45,98] Therefore, better alternative tests to assist the diagnosis are necessary.

IL-10 FAVORS B-CELL GROWTH AND PROTECTS PIOL CELLS FROM CYTOTOXIC T CELLS

IL-10 is a potent growth and differentiation factor for activated B cells.[54,99] During antigen-driven immune responses, antigen-specific naive B lymphocytes undergo a cascade of events, including activation, expansion, mutations, class switch, selection, and differentiation into either antibody-secreting plasma cells or memory B cells. B cells interact with antigens and other cells through numerous cell surface molecules and cytokines. IL-10 is also an immunosuppressive cytokine that inhibits cell-mediated immunity via inhibition of Th1 cytokine profiles and reduction of major histocompatibility complex class I molecule expression.[100-103] Therefore, IL-10 probably participates in proliferation of PIOL cells and protection of PIOL from the immune system.

The present study shows a significant association between cytokine levels and PIOL. These findings support the notion that elevation of IL-10 level in the vitreous relative to that of IL-6 is helpful in diagnosing PIOL (Table II).[56,104] However, in a few cases, this notion may not apply.[58,105,106] One possibility could be that in the early stage of the disease, the vitreous is infiltrated mainly with inflammatory cells (particularly T lymphocytes) and with very few or no PIOL cells. Therefore, a high IL-6 released by the inflammatory cells and a low IL-10 released by the B-lymphoma cells are found.[40] Another possibility is that inflammatory response and relative ratios of inflammatory cells to PIOL wax and wane, corresponding to different IL-10 to IL-6 ratios. In a preliminary study, we calculated geometric mean ratios of IL-10 and IL-6 levels in the vitreous of 32 patients with PIOL and 63 patients with uveitis (unpublished data). For PIOL, we found a geometric mean ratio of 5.64 (95% CI, 2.33-13.63). For uveitis, the geometric mean ratio was 0.23 (95% CI, 0.15-2.77). A statistically significant difference was observed ($P<.001$).

Other advantages for measuring cytokines include requirement of a small amount of fluids and availability of further molecular studies of the malignant cells. A minimum of 0.5 mL is needed for analyses of both cytokines. The relatively simple ELISA is often available in clinical immunological laboratories.

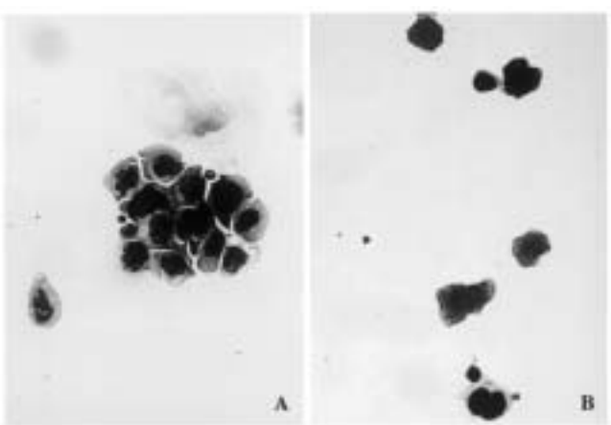

FIGURE 9

Microphotographs of PIOL cells showing (A) alive cells in a container with RPMI and (B) degenerating and early necrotic cells in a container without RPMI for 2 hours. PIOL cells in (A) and (B) were obtained from the same vitrectomy specimen of a patient with PIOL (Diff-Quick, ×640).

PIOLS ARE USUALLY MONOCLONAL AND MAY HAVE ENCOUNTERED ANTIGEN SELECTION

B cells are precursors of antibody-secreting cells and plasma cells. The proteins produced by plasma cells are antibodies or immunoglobulin molecules. Antibodies (immunoglobulins) consist of two heavy (H) polypeptide chains and two light (L) polypeptide chains.[86,87] The H and L chains are made up of a series of domains, each about 110 amino acids. The L chains, of which there are two major types (κ and λ), consist of two domains. The carboxyterminal domain is essentially identical among L (also H) chains of a given type and is referred to as the constant (C) region. The aminoterminal domain of L (also H) chains varies from antibody to antibody. This domain represents the L (also H) chain's contribution to the binding site of the antibody molecule. Because of its variability, it is referred to as the variable (V) domain. The variability of this domain is actually concentrated in three segments of the region, designated the hypervariable or complementarity determining regions (CDRs). The CDRs appear to contain the amino acids that line the antibody's combining site. The three CDRs are interspersed in four regions of much lower degrees of variability, designated framework regions (FR or FW, Figure 3).

The variable domains of IgL and IgH chains are produced from the combination of two (IgL) or three (IgH) gene segments. These gene segments are termed V (variability), D (diversity), and J (joining). Light chains do not have a D gene segment. The combination of the different germline elements occurs at the DNA level through a

TABLE II: STUDIES OF IL-10 AND IL-6 IN THE VITREOUS OF PATIENTS WITH PIOL

SOURCE	CASES (n)	IL-10	>1	<1	NDL
Current study	39	+	24	7	8
Chan et al[55]	3*	+	3	0	0
Whitcup et al[56]	5†	+	5	0	0
Tritten et al[57]	1	+	1	0	0
Buggage et al[40]	1‡	+	0	1	0
Akpek et al[58]	4	+	1§	ND	ND
Cassoux et al[18]	16	+	ND	ND	ND

ND, No data; NDL, both IL-10 and IL-6 are below the detectable level; +, measurable levels (IL-10 is not measurable in normal vitreous).
*Cases 25, 50, and 51 in current study.
†Cases 2, 25, 50, 51, and 56 in current study.
‡Case 17 in current study.
§Only 1 in 4 cases has data of ratio of IL-10 to IL-6.

cell-specific process that is called V_HDJ_H recombination.[107] During B-cell development in the bone marrow, the IgH gene segments are usually rearranged first. Then, the IgL κ chain gene segments are rearranged. If κ rearrangements are not productive, rearrangement of λ will take place. V(D)J recombination is a critical process in B-cell development and results in the generation of an unlimited number of antibodies.[108]

In addition to V(D)J recombination, the production of antibodies by B cells involves three main general processes: (1) allelic exclusion limiting B cells to the production of one heavy and one light chain, (2) class-switching, leading to antibodies with the same antigen-binding properties but different effector functions, and (3) somatic hypermutation, leading to antibodies with increased affinity for the antigen. Class-switching and somatic mutation usually occur in the periphery, in germinal centers in an antigen and T-cell dependent reaction.[49,109,110]

Every B cell has immunoglobulin rearrangement. Lymphomas usually derive from one B cell; therefore, the tumor is called monoclonal.[111] Consequently, DNA sequences at the junction of V, D, J segments are used as clone-specific markers in individual patients with B-cell lymphoma.[112-115] The analysis of gene segment can reveal eventual biases indicative of antigenic selection and somatic mutation of the lymphoma cells.[116-120] Immunoglobulin rearrangements have been sequenced from patients with PCNSL and shown a preferential utilization of the V_H4-34 gene segment.[121-123]

Analyses of immunoglobulin rearrangements have been limited because of the paucity and fragility of the PIOL cells (Table III). Using PCR without microdissection, White and colleagues[61] detected IgH gene rearrangement in the vitreous of 1 of 2 patients with PIOL. Using microdissection and PCR, we have reported IgH gene rearrangements in PIOL cells from more than 10 cases.[18,40,48,60,73] The present study accumulates a total of 50 cases, all demonstrating IgH gene rearrangements at

TABLE III: STUDIES OF IGH GENE REARRANGEMENTS IN B-CELL PIOL

SOURCE	YEAR	CASES (n)	IGH GENE REARRANGEMENTS	
			POSITIVE	NEGATIVE
Current study	2001	50	50	0
Chan et al[60]	1998	3*	3	0
Shen et al[48]	1998	4†	4	0
Chan et al[73]	1999	13‡	13	0
Buggage et al[40]	1999	1§	1	0
White et al[61]	1999	2	1	1
Cassoux et al[18]	2000	12¶	12	0
Levy-Clarke et al[43]	2001	1#	1	0

*Cases 1, 2, and 3 in the current study.
†Cases1, 2, 3, and 4 in the current study.
‡Cases 1 through 13 in the current study.
§Case 17 in the current study.
¶Cases 4, 5, 6, 9, 11 through 15, 18, and 36 (no illustration) in the current study.
#Case 31 in the current study.

the CDR3 region. These data confirm PIOL diagnosis of monoclonality. Furthermore, we detect IgH gene rearrangements in 17 of 57 cases without cytological diagnosis, and vitreous examination showed that 9 of the 17 had an IL-10 to IL-6 ratio of more than 1, 7 of the 17 had no vitreal cytokine assay, and only 1 of the 17 had an IL-10 to IL-6 ratio of less than 1. The findings help to diagnose PIOL, specifically in cases with poor quality of vitreous cytology. Therefore, the combination of microdissection and PCR is a powerful and useful tool for the detection of PIOL.

INFECTIOUS AGENTS MAY PLAY A ROLE IN PIOL PATHOGENESIS AND HETEROGENEITY

Recently, an increasing number of pathogens have been discovered and linked to cancer. Several infectious agents are identified with strong association to lymphoma oncogenesis. In vitro, EBV efficiently transforms human B-lymphocytes, causing them to proliferate continuously.[68,124] LMP-1 (EBV latent membrane protein-1) is able to up-regulate anti-apoptosis (bcl-2, bcl-x) and cell cycle regulation (cyclin D2) proteins and inactivate the tumor suppressor gene p53. EBV is associated with endemic Burkitt's lymphoma, AIDS-related lymphoma, posttransplantation lymphoproliferative disease, Hodgkin's disease, and rare T-cell lymphomas.[71,125,126] However, it is rarely found in immunocompetent patients with CNS lymphoma.[72] This study suggests that EBV may play a role in the induction of PIOL in AIDS patients, not immunocompetent patients.

HHV-8 is linked to Kaposi's sarcoma.[127] This virus is also associated with lymphomagenesis in humans.[68] HHV-8 genes are reported in PCNSL and PIOL with and without AIDS.[70,73] However, it is found in only a few cases, including the present series. Therefore, HHV-8 may not play a major role in lymphomagenesis.[128] HHV-8 is a highly lymphotrophic virus to B cells, and its genome encodes homologues of cyclin D1 that involves cell cycle control, certain cytokines, regulators, receptors, and bcl-2.[129] Therefore, HHV-8 could affect cell growth and transformation through activation of cell cycle and thus may be involved in PIOL lymphomagenesis in some rare cases.

T gondii is one of the most common infectious agents that cause posterior uveitis in immunocompetent patients.[130,131] High anti–*T gondii* IgG titers are often detected in the eyes of patients with toxoplasmic retinochoroiditis because of B-cell activation.[132] *T gondii* has been documented to be involved in *T gondii*–induced lymphadenitis (Piringer's lymphadenopathy), a potentially malignant disease in which the monocytoid B cells may arise from polyclonal B-cell transformation.[133] The detection of *T gondii* gene in PIOL cells of rare cases suggests that *T gondii* may be able to transform B cells and induce their proliferation.[74] Investigations including molecular epidemiology are under way in our laboratory.

EBV, HHV-8, and *T gondii* are intracellular microorganisms. During the infection, the microorganism(s) may invade and transform the infiltrating cells in the eye by disrupting gene regulation and expression. Immunoglobulin sequence analysis suggests that the transformed B cell went through a selective process based on the surface immunoglobulins. The etiology and driving forces for clonal expansion are heterogeneous; that may explain the well-known clinical and pathological heterogeneity of DLBCL such as PCNSL and PIOL.[120] No infectious pathogenic DNA was identified in the lymphocytes of two eyes with infection and without malignancy in this study. However, our findings of infectious DNA in PIOL cells in a few cases must be interpreted with caution because of the relatively small number of PIOL and control cases examined and the possibility of incidental coinfection(s) in these PIOL cases. Additional studies are required to determine the mechanisms involved in B-cell transformation by these or other new infectious pathogens.

A close association between mucosa-associated lymphoid tissue (MALT) lymphoma and *Helicobacter pylori* has been studied and reported recently.[134-137] Gastric MALT is specifically acquired most commonly in response to infection of the gastric mucosa by *H pylori*. The lymphocytes within this MALT are therefore programmed to respond to this microorganism, and the neoplastic cells of the lymphoma that may develop within this acquired MALT retain the ability to respond to the immunological proliferative drive associated with the continued presence of the microorganism. B-cell monoclonality has been identified to precede the development of gastric MALT lymphoma in *H pylori*–associated chronic gastritis.[136] Following the removal of this immunological drive by eradication of *H pylori*, the lymphoma, in particular the low-grade MALT lymphoma, is reported to show clinical and histological regression.[138-142] Similarly, various infectious agents capable of inducing chronic ocular inflammation could play a role in the pathogenesis of PIOL.

PIOL has been reported with clinical features simulating various forms of ocular infections, including viral retinitis and ocular toxoplasmosis.[27,143-145] It is possible that in such cases the eyes were indeed infected and the infectious process recruited various inflammatory cells, including a few B lymphocytes, into the eye. The infectious DNA might incorporate in the B lymphocytes. These infectious genes could trigger the B-cell transformation and proliferation resulting in a particular B-cell clonal expansion and the development of PIOL. Proliferation of the PIOL cells can be driven by the presence of infectious

microorganisms or other specific antigens through a complex pathway of cellular interactions involving specific T cells. However, the present study cannot exclude the possibility of a concomitant infection (ocular toxoplasmosis or HHV-8 retinitis) and lymphoma. Since common infectious agents were absent in the majority of test cases, one must be cautious when stating the role of infectious agents in the pathogenesis of PIOL. Nevertheless, this presumptive speculation of infectious molecular signatures in certain lymphomas deserves our attention and needs additional exploration.

PIOL HOMING MAY BE RELATED TO EXPRESSION OF SURFACE CHEMOKINE RECEPTORS

Chemokines constitute a family of chemotactic cytokines that have been shown to direct the migration of leukocytes during inflammation and that may be involved in the constitutive homing of lymphocytes into follicles and T-cell zones. BLC, or B-cell attracting chemokine (BCA-1), is strongly expressed in the follicles of Peyer's patches, the spleen, and lymph nodes.[95] BLC is the first chemokine to be identified that is selective toward B cells. CXCR5 (or BLR1 in Burkitt's lymphoma cells and B lymphocytes) is the receptor for BLC and acts as a B-cell homing chemokine.[96,146] Interestingly, we showed that BLC is detected only in RPE, not other normal ocular cells in the choroid (Figure 7). CXCR4 is the ligand of chemokine SDF (stromal cell-derived factor-1).[147,148] CXCR4 is essential for viability of the embryo, B lymphopoiesis, bone marrow hematopoiesis, and cardiogenesis. It allows entry of HIV-1 into target cells with human CD4.

CXCR5 has been recently identified in MALT lymphoma and non-Hodgkin's lymphoma.[149] High expression of CXCR4 has also been shown in mantle cell lymphoma and B-cell acute lymphoblastic and chronic lymphocytic leukemia cells.[150] The present study shows strong expression of CXCR5 and CXCR4 on PIOL cells and of BLC on RPE cells. The findings of BLC that functions as a homing chemokine expressed in RPE and CXCR5 that is detected in PIOL cells may be one important mechanism for tumor cell homing and localizing between the RPE and Bruch's membrane in the eye. We may consider inhibition of B-cell chemoattractants as a new approach for the prevention and treatment of PIOL. Inevitably, additional experiments are required to delineate the role of B-cell chemokine/chemokine receptors on PIOL.

SUMMARY

This study investigates ocular tissues from 57 patients with PIOL. Molecular analyses of IgH gene rearrangements and IL-10 and IL-6 levels in the vitreous are beneficial adjuncts for the diagnosis of PIOL. Evaluation of IgH gene rearrangements and detection of cytokine and chemokine levels in PIOL have improved our understanding and diagnosis of the malignancy. A hypothesis that infectious microorganisms may possibly play a role in intraocular lymphoma oncogenesis for some PIOLs is suggested. Identification of potential pathogens may account for the heterogeneity of the disease. Anti-infectious and anti-inflammatory medications, as well as oligonucleotides and/or therapeutic and prophylactic vaccination, may be helpful in improving prevention and treatment of PIOL. Chemokine and chemokine receptors selective for B cells are identified in the RPE and PIOL cells, respectively. The fact of B-cell chemokine strongly attracting lymphoma cells may generate PIOL cellular homing between the RPE and Bruch's membrane in the eye. Inhibition of IL-10 in the eye, chemokine expression on RPE cells, and chemokine receptors on malignant B-cells may be useful in improving the management of PIOL.

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