EVALUATION OF THE REACTIVE T-CELL INFILTRATE IN UVEITIS AND INTRAOCULAR LYMPHOMA WITH FLOW CYTOMETRY OF VITREOUS FLUID (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

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ABSTRACT

Purpose: To describe the reactive T-cell infiltrate in uveitis and intraocular lymphoma using flow cytometry of clinical intraocular specimens acquired during diagnostic pars plana vitrectomy.

Methods: This was a retrospective review of diagnostic vitreous specimens (1992-2011) obtained at a university-based, tertiary care center. Seventy-eight patients with uveitis or lymphoma undergoing pars plana vitrectomy were selected for intraocular testing based on clinical diagnostic uncertainty. Pars plana vitrectomy with flow cytometry, gene rearrangement studies, and cytology was performed.

Results: T-cell infiltrates were found in all diagnostic categories with limited power to discriminate between uveitis and T-lymphocyte reactive infiltrates in response to intraocular lymphoma. Statistically significant differences by two-sample test of means between group means were found between 35 uveitis and 35 B-cell lymphoma cases for T-cell markers CD2, 3, 4, 5, and 7, but not for CD8. The CD4:CD8 ratio had a higher mean value in the uveitis group (P=.0113), and 8 T-cell lymphomas had a statistically greater number of CD3+ lymphocytes compared to uveitis (P=.0199) by two-sample test of means. Likelihood ratios were highest for CD2, CD5, CD7, CD4:CD8 ratio, CD20, and CD22.

Conclusions: Discrimination between uveitis and lymphoma based on cell identification by flow cytometry was limited because of the prevalence of T lymphocytes in all diagnostic categories, emphasizing the importance of a reactive T-cell infiltrate in B-cell lymphomas, which may impede diagnosis. Flow cytometry may allow identification of more cases of T-cell lymphoma than reported when it is combined with gene rearrangement and cytology.

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INTRODUCTION

The simplified classification of uveitis proposed by the International Uveitis Study Group recognizes only three types: noninfectious uveitis, infectious uveitis, and masquerade syndromes such as intraocular malignancy (primary vitreoretinal lymphoma, or PVRL).[1] Noninfectious uveitis is undoubtedly the most common, and all intraocular infiltrates regardless of etiology likely include a reactive noninfectious, nonmalignant lymphoid infiltrate; however, vitreous biopsy is typically performed only to exclude infectious uveitis or PVRL, rather than to confirm or elucidate a noninfectious uveitis.[2-4] This is due in part to limited diagnostic tools that are optimized for detection of pathogens[5-10] or abnormal cells[11-14] rather than immunologic description. It is unclear whether current testing strategies for diagnostic vitrectomy can provide enough positive information about noninfectious uveitis to be worthwhile performing when diagnostic uncertainty does not exist. Most of the research investigations in uveitis have focused on intraocular inflammatory mediators, such as cytokines,[15,16] or the cytokine polymorphisms of uveitis patients.[17] Studies of intraocular cellular profiles in uveitis are few.

Cytology is considered the "gold standard" for cellular characterization of diagnostic vitrectomy specimens[11,12] generally supported by phenotypic characterization with immunocytochemical staining for cell markers indicating B-cell lineage and light immunoglobulin chain expression, since most intraocular lymphomas are B cell.[18,19] The simplified rule of thumb for this approach is that B-lymphocyte predominance in a vitreous specimen indicates intraocular lymphoma, whereas T-lymphocyte predominance indicates a noninfectious uveitis. There are problems with this approach. Morphology of vitreous lymphocytes is often obscured by cell death or disruption. Some malignant B-cell lymphomas have lost the ability to express light chains. Infiltrating T cells may mask the malignant B cells by sheer number.[20] Finally, T-cell lymphomas and leukemias also infiltrate the eye.[21] Molecular characterization and biomarkers are now favored to supplement this basic strategy.[13,14,22,23]

Flow cytometry is an alternative technique to gain information regarding cellular constituents in diagnostic vitrectomy specimens.[3,24-26] Flow cytometry identifies cell lineage by cell surface markers using specific antibodies directed against cell surface markers in much the same way as immunocytochemical staining of slides but uses cell suspensions in liquid, which is well suited to vitreous specimens. It detects multiple cell surface markers simultaneously in a single sample and is quantitative with adequate precision to permit statistical analysis.[3] A case series describing flow cytometric results from diagnostic vitrectomy performed for 50 cases of suspected intraocular infection and 28 cases of suspected intraocular lymphoma reported a positive predictive value of 70% for a CD4:CD8 ratio >4.0 for the 11 patients who were ultimately diagnosed as having noninfectious uveitis rather than infectious uveitis or lymphoma.[3]

The purpose of this report is to characterize T-cell vitreous infiltrates using flow cytometry in a series of 78 patients who were ultimately diagnosed as having either uveitis or lymphoma. In addition, the report proposes a protocol for the examination of vitreous specimens using the molecular diagnostic techniques of flow cytometry and polymerase chain reaction (PCR) detection of gene rearrangement to discriminate between noninfectious uveitis and intraocular lymphoma.

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METHODS

The University of Miami Medical Institutional Review Board approved review of existing medical records with waiver of informed consent and authorization under Protocol 20057666 "Evaluation of Diagnostic Tests for Intraocular Lymphoma" on February 24, 2006, with expiration on May 29, 2012. Patients with surgery dates after April 27, 2010, signed informed consent and authorization to review their records retrospectively after completion of clinical care.

Setting

The research was conducted in a university-based tertiary care center. Clinical care and surgeries took place in the Anne Bates Leach Eye Hospital, Bascom Palmer Eye Institute, under the direction of a single surgeon (J.D.). Clinical care occurred from November 6, 1992, until January 5, 2011.

Study Population

Compiled operative notes identified consecutive patients undergoing diagnostic vitrectomy during the study years. Patients were included if the preoperative differential diagnosis included intraocular lymphoma and the postoperative diagnosis included uveitis (infectious or noninfectious) or lymphoma. No routine vitrectomy specimens from noninfectious uveitis patients undergoing surgery for vitreoretinal complications were included. No patients underwent vitrectomy for the primary indication of suspected intraocular infection. In some cases, lymphoid malignancy had been previously diagnosed in an extraocular site or in the central nervous system (CNS). A primary indication for vitrectomy of suspected infectious uveitis or endophthalmitis excluded participation from the record review unless there was a prior history of lymphoid malignancy. Seventeen of the included lymphoma patients and four of the included uveitis patients were part of a previously published case series of diagnostic vitrectomy.[3]

INTERVENTIONS

Vitrectomy

Vitrectomies were performed for diagnostic and/or therapeutic indications. Specimens were selected for intraocular diagnostic studies based on a differential diagnosis that included lymphoma. Some patients underwent vitrectomy in both eyes, usually to alleviate visual symptoms related to vitreous opacities, but also to improve diagnostic accuracy. All patients gave informed consent for the surgical procedure. Either 20-gauge or 23-gauge 3-port vitrectomy was performed with manual aspiration of both undiluted and diluted vitreous as previously described.[3] Undiluted vitreous was submitted for cytology and the diluted vitreous for flow cytometry and gene rearrangement.

Cytology

Cytology was performed at Jackson Memorial Hospital, Department of Cytopathology. Two slides were prepared using cytocentrifugation and Papanicolaou stain. A cytopathologist assigned to the Pathology service read the slides. Results were usually reported as "atypical lymphoreticular process," "malignant cells not identified," or "cellular evidence of malignant lympho-reticular process."

Flow Cytometry

Flow cytometry was performed at the Department of Pathology Laboratory Special Services or the Transplantation Laboratory, Department of Surgery, University of Miami Miller School of Medicine, under the direction of Dr Philip Ruiz. The most recent method used has been reported[27]; there were minor differences in the earlier years of the study. In brief, red cells in the diluted vitreous specimen were lysed with ammonium chloride. Cell counts were assessed with a hemocytometer and cell viability with propidium iodide. Because of limited cell counts in most specimens, aliquots were stained with monoclonal antibodies conjugated to either fluorescein, phycoerythrin, peridinin chlorophyll, or allophycocyanin to permit multiple determinations from each aliquot. Antibodies against a panel of hematopoietic antigens were selected and included CD45 (panleukocyte); CD2, CD3, CD7 (T lymphocytes); CD5 (T cell and some B cell); CD4 (major histocompatibility complex [MHC] class II restricted coreceptor for the T-cell receptor [TCR] on T helper cells); CD8 (MHC class I restricted coreceptor for the TCR on cytotoxic T cells; TCR αβ; CD19, CD20, CD22 (B lymphocyte); kappa, lambda (light immunoglobulin chains); CD10 (germinal center B-cell lymphomas); CD11c (macrophages, monocytes); HLA-DR, CD25, CD69 (activation markers); and CD56 (natural killer cells). Stained cells were analyzed using a Becton-Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, California). Histograms gated to lymphocyte-sized cells were examined and converted to percentages of cells by a pathologist experienced in the interpretation of flow cytometric results (P.R.).

Gene Rearrangement

Gene rearrangement protocol and primers changed over the course of the study according to current published consensus methods.[28,29] Gene rearrangement was first performed in December 1995 and regularly performed after April 2003. Kappa gene rearrangement was added in selected cases in June 2004. In brief, a multiplex PCR reaction was performed for heavy and light (κ) chain immunoglobulin hypervariable complementarity determining region CDR3 and hypervariable regions of the T-cell receptor. Fragment analysis using GeneMapper v 3.7 software was used to detect fluorescently labeled amplified products producing a unique, single-sized peak with or without a polyclonal background.

Clinical Observations

Patients were evaluated for concomitant diseases relevant to their ocular condition, including primary CNS lymphoma and systemic leukemias and lymphomas. Patients suspected of having noninfectious uveitis were evaluated for known etiologies. Patients were followed for clinical outcomes within the limits achievable in a retrospective study. Late diagnosis of extraocular lymphoma was documented when possible, as well as dates of death. Although most patients received treatment directed at either infectious uveitis, noninfectious uveitis, or lymphoma after diagnostic vitrectomy, treatment outcomes were not assessed for this report. Patients were considered to have a final clinical diagnosis of either uveitis or lymphoma after evaluation of the results of the diagnostic vitrectomy and consultation with appropriate medical specialists in the case of lymphoma. Concomitant or sequential extraocular lymphoma, especially CNS lymphoma, was considered to have the highest specificity in confirming the diagnosis of an intraocular lymphoma.

RESULTS

OUTCOME MEASURES

The principal outcome measure was the characterization of the vitreal cellular infiltrate using flow cytometry. Gene rearrangement and cytology were used to support the diagnosis in the case of intraocular malignancy. Specimens consisting of atypical lymphocytes without a diagnosis of malignancy were noted as such and considered an equivocal result. Specimens originally considered cytologically negative were re-reviewed if possible by an ophthalmic pathologist and a hematopathologist (see "Acknowledgments" section). Cultures and PCR-specific infectious agents were used to support the diagnosis of the few cases of infectious uveitis in the series.

STATISTICAL ANALYSIS

Cases were grouped into lymphoid malignancy or nonlymphoid malignancy ("uveitis") based on the final clinical diagnosis. These two groups were further subdivided into B- and T-cell malignancies for some analyses. Data from the first biopsied eye was used for all analyses, except in one case of corticosteroid pretreatment of lymphoma in which the second eye was used. Mean and standard deviation were calculated using Microsoft Excel for Mac 2011 software for a panleukocyte marker, 4 pan-T-cell markers, CD4, CD8, T-cell receptor $\alpha\beta$, 3 B-cell markers, kappa light chain, lambda light chain, 2 macrophage/monocyte markers, CD56, and 3 activation markers. CD4:CD8 and kappa:lambda ratios were calculated individually for each case, then averaged. In cases of large excess of one light chain, or CD4 or CD8, with a zero value for the other parameter, the zero value was imputed to 1 in order to permit calculation of the kappa:lambda light chain and the CD4:CD8 ratios. For lambda restricted tumors the lambda:kappa ratio was used for these calculations. Smith's Statistical Package (SSP) version 2.80 (http://www.economics.pomona.edu/StatSite/framepg.html) was used to calculate 95% confidence intervals around the means of each of the flow cytometry parameters. Two-sample test of means was used to compare flow cytometry parameters. Two-sided *P* values of less than .05 were considered significant. Chi-square test (SSP) was used to compare the frequency of positive gene rearrangement tests between the lymphoma group and the uveitis group.

Sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood were calculated for 7 T-cell markers enumerated by flow cytometry according to standard formulas (http://www.infovoice.se/fou/epv/index.htm, accessed July 3, 2012). Useful flow cytometry tests for determining that the T-cell lymphocytes were disease-producing (uveitis) rather than reactive (B-cell lymphoma) were defined as having a positive predictive value of >60% and a positive likelihood ratio greater than 1.5, or a negative predictive value of >90% and a negative likelihood ratio of <0.67. For these calculations, values above the median for the entire study group were considered positive and those below the median were considered negative. Tests of 2 B-cell markers, CD20 and CD22, were similarly analyzed for their predictive value of detecting B-cell lymphoma vs uveitis. Receiver-operator curves (ROCs) were created for each variable and analyzed to see if other cut-off values would generate better discrimination between the binary categories of uveitis and B-cell lymphoma than the study group median. The area under the ROC and its statistical significance was calculated (SPSS Statistics v19, PSS Inc, an IBM Company, Armonk, New York).

DEMOGRAPHICS AND DIAGNOSES

Eighty patients underwent vitrectomy during the study years. Two patients were excluded in whom flow cytometry results were not available. Of the 78 remaining patients, 43 had a final diagnosis of a lymphoid intraocular malignancy, of which 35 were B-cell origin (2 T-cell-enriched B-cell lymphoma) and 8 were T-cell lymphomas. Thirty-five patients had a diagnosis of uveitis, of which 23 were noninfectious intermediate, posterior, or panuveitis; 10 were infectious uveitis; and 2 had other conditions (retained lens particles, possible amyloid). Thirteen patients with lymphoid malignancy and 2 patients with uveitis had the second eye operated on and diagnostic testing performed. In one case of intraocular lymphoma, the second eye was diagnostic, whereas the first eye was considered negative. In one other case, a second surgery was required in one eye to perform retinal biopsy for diagnosis. In these two lymphoma patients the data from the second eye surgery was used; otherwise first eye data was used. The second eye of one patient classified as uveitis showed B-cell predominance and kappa restriction; after consultation with the hematology-oncology service, the final diagnosis was panuveitis.

Of the 43 patients diagnosed as having intraocular lymphoid malignancy, 7 were known to have lymphoid malignancy before vitrectomy: 4 with cutaneous T-cell lymphoma (1 of whom had a B-cell intraocular lymphoma), 2 with primary CNS lymphoma, and 1 with systemic non-Hodgkin's lymphoma. Six were unknown to have any extraocular lymphoma at the time of last follow-up, and 31 had definite extraocular involvement diagnosed either as part of their evaluation at the time of vitreous biopsy or during follow-up. Of the 35 patients with uveitis, 3 had prior diagnoses of hematologic malignancy and 1 had cutaneous T-cell lymphoma, and 1 each had

multiple sclerosis, birdshot chorioretinopathy, or primary biliary cirrhosis.

Table 1 lists clinical characteristics of the two patient groups. Patients with uveitis were statistically younger, were less likely to have bilateral disease, had shorter duration of symptoms prior to biopsy, and were more likely to be alive at the end of follow-up. There was no difference in the proportion of females, time to death after surgery, time alive after surgery, or follow-up after surgery, which was a median of 12 to 13 months in each group.

TABLE 1. CLINICAL CHARACTERISTICS OF 78 PATIENTS UNDERGOING DIAGNOSTIC VITRECTOMY FOR SUSPECTED INTRAOCULAR LYMPHOMA OR UVEITIS ACCORDING TO FINAL DIAGNOSIS

CHARACTERISTIC	FINAL DIAGNOSIS		*P* VALUE
	INTRAOCULAR LYMPHOID MALIGNANCY (N= 43)	UVEITIS (N = 35)	
Age (yr)			$P = .0182^{*}$
Mean ± SD	67 ± 13.4	58 ± 18.4	
Median (range)	69 (39 – 88)	62 (12 – 82)	
Female	26 (59%)	21 (60%)	$P = .9667^{\dagger}$
White[‡]	42 (98%)	33 (94%)	$P = .7109^{\dagger}$
Hispanic origin	9 (21%)	8 (23%)	
African American	1 (2%)	2 (6%)	
Bilateral disease	34 (79.1%)	14 (40%)	$P = .0003^{\dagger}$
Symptoms before surgery			$P = .0493^{*}$
Mean ± SD	10.4 ± 15.4 months	5.3 ± 6.1	
Median (range)	5.5 (0.5 – 72)	3.5 (0 – 26)	
Deceased	32/42 (76%)	6/23 (26%)	$P < .0000^{\dagger}$
Alive	10/42 (24%)	17/23 (74%)	
Unknown	1/43	12/35	
Months alive after surgery[§]			$P = .4626^{*}$
Mean ± SD	64 ± 56.5	49.3 ± 31.0	
Median (range)	48 (11 – 189)	47 (13 – 105)	
Follow-up after surgery			$P = .8638^{*}$
Mean ± SD	26 ± 38.5	24.7 ± 28.6	
Median (range)	12 (0 – 190)	13.0 (0 – 103)	
Surgery of both involved eyes	13/34 (38%)	2/14 (14%)	$P = .1036^{\dagger}$
Etiologic diagnosis	33 B-cell lymphoma	23 Noninfectious	
	2 B-cell lymphoma, T cell enriched	10 Infectious	
	8 T-cell lymphoma	2 Other[¶]	

[*] Two-sample test of means (SSP).

[†] Chi-square test (SSP).

[‡] Demographics for the State of Florida: 78.5% white race; 22.9% Hispanic origin; 16.5% black race. (http://quickfacts.census.gov/qfd/states/12/12086.html, accessed June 30, 2012).

[§] Vital status unknown for 1 patient (2.3%) with lymphoid malignancy and 12 patients (34.2%) with noninfectious uveitis.

[¶] One case of inflammation due to retained lens fragments and one case of suspected amyloidosis.

FLOW CYTOMETRY RESULTS

Table 2 lists the cell surface markers analyzed for this study. T-cell and B-cell lymphomas are grouped separately to permit more accurate comparisons with uveitis. Confidence intervals for the group mean of the T-cell lymphomas are not displayed because of the small number of cases. Although group means are compared and probabilities reported, sample size of the T-cell group is small.

TABLE 2. FLOW CYTOMETRY RESULTS FOR VITRECTOMY SPECIMENS FROM 43 PATIENTS WITH INTRAOCULAR LYMPHOID MALIGNANCY AND 35 PATIENTS WITH INFECTIOUS OR NONINFECTIOUS UVEITIS

CELL SURFACE MARKER	UVEITIS MEAN ± SD (95% CI) NUMBER TESTED	B-CELL LYMPHOMA MEAN ± SD (95% CI) NUMBER TESTED	T-CELL LYMPHOMA MEAN ± SD MEDIAN[†] NUMBER TESTED	*P* VALUE[*] UVEITIS VS B CELL UVEITIS VS T CELL
CD45	48.3 ± 31.8 (CI 37.4 – 59.2) N = 35	55.1 ± 27.3 (CI 45.7 – 64.4) N = 35	78.2 ± 27.7 88.7 N = 8	*P*= .3428 ***P*=.0206**
CD2	41.4 ± 33.3 (CI 30.0 – 52.9) N = 35	18.6 ± 18.5 (CI 12.3 – 25.0) N = 35	55.5 ± 36.3 69.3 N = 8	***P*=.0008** *P*=.3384
CD3	36.9 ± 31.9 (CI 25.4 – 48.4) N = 32	19.5 ± 20.9 (CI 10.6 – 28.3) N = 24	71.9 ± 26.2 78.6 N = 6	***P*=.0090** ***P*=.0199**
CD5	39.4 ± 31.0 (CI 28.8 – 50.1) N = 35	16.5 ±18.3 (CI 10.1 – 22.9) N = 35	30.4 ± 34.9 12.6 N = 8	***P*=.0004** *P*=.5177
CD7	32.0 ± 27.2 (CI 22.4 – 41.7) N = 33	15.8 ± 18.5 (CI 8.3 – 23. 2) N = 26	68.3 ± 35.8 71.4 N = 6	***P*=.0087** *P*=.0870
CD4	28.0 ± 25.2 (CI 19.4 – 36.7) N = 35	9.5 ± 10.6 (CI 5.8 – 13.1) N = 34	13.7 ± 24.6 4.7 N = 8	***P*=.0002** *P*=.1688
CD8	12.9 ± 17.4 (CI 6.9 – 18.8) N = 35	8.2 ± 9.8 (CI 4.7 – 11.6) N = 33	36.2 ± 37.6 17.3 N = 8	*P*=.1716 *P*=.1269
CD4:CD8 ratio	5.63 ± 6.89 (CI 3.01– 8.25) N = 29	2.03 ± 2.20 (CI 1.20 – 2.87) N = 29	3.88 ± 4.58 2.50 N = 5	***P*=.0113** *P*=.4967
T-cell receptor alpha/beta	35.3 ± 30.2 (CI 22.8 – 47.7) N = 25	26.2 ± 21.9 (CI 16 – 36.5) N = 20	55.8 ± 43.2 76.4 N = 5[‡]	*P*=.2502 *P*=.3587
CD19 B-cell marker	1.7 ± 2.8 (CI 0.7 – 2.7) N = 33	35.1 ± 28.8 (CI 24.7 – 45.5) N = 32	12.7 ± 26.1 1.7 N = 7	***P*<.0000** *P*=.5600
CD20 B-cell marker	3.0 ± 7.7[§] (CI 0.4 – 5.7) N = 35	27.9 ± 26.6 (CI 18.6 – 37.2) N = 34	0.9 ± 1.5 0.0 N = 7	***P*<.0000** *P*=.1469
CD22 B-cell marker	5.3 ± 6.2 (CI 3.1 – 7.4) N = 35	29.7 ± 26.1 (CI 20.8 – 38.6) N = 35	3.6 ± 2.0 4.0 N = 7	***P*<.0000** *P*=.1979
Kappa[¶] Light immunoglobulin chain	1.52 ± 3.50 (CI 0.30 – 2.74) N = 34	15.8 ± 23.6 (CI 7.7 – 23.9) N = 35	0.8 ± 0.6 1.0 N = 7	***P*=.0012** *P*=.2801
Lambda[¶] Light immunoglobulin chain	1.47 ± 3.75 (CI 0.16 – 2.78) N = 35	10.4 ± 18.5 (CI 3.8 – 17.0) N = 35	1.4 ± 1.4 1.3 N = 7	***P*=.0082** *P*=.9226
Light chain ratio[#]	1.05± 0.39 (CI 0.85 – 1.25) N = 17	16.68 ± 16.47 (CI 10.43 – 24.94) N = 29	2.08 ± 2.70 0.95 N = 4	***P*<.0000** *P*=.4865
CD11c Macrophage Monocyte	8.9 ± 14.2 (CI 4.1 – 13.8) N = 35	6.9 ± 7.8 (CI 4.2 – 9.7) N = 34	30.9 ± 32.4 16.4 N = 7	*P*=.4744 *P*=.1248

TABLE 2.(CONTINUED)

CD14	8.6 ± 13.5	3.4 ± 3.5	1.3 ± 1.9	*P*=.3750
Macrophage	(CI 1.0 – 10.2)	(CI 2.2 – 4.6)	0.0	***P*=.0041**
Monocyte	N = 35	N = 34	N = 7	
HLA-DR	16.3 ± 18.1	22.8 ± 23.4	36.7 ± 32.2	*P*=.2066
Activation marker	(CI 10.0 – 22.6)	(CI 14.6 – 30.9)	33.1	*P*=.1218
Antigen presentation	N = 34	N = 34	N = 8	
CD25	9.3 ± 11.0	11.9 ± 17.4	29.5 ± 23.8	*P*=.5082
Activation marker	(CI 5.4 – 13.2)	(CI 4.9 – 18.9)	27.5	*P*=.0928
Interleukin 2 receptor	N = 33	N = 26	N = 6	
CD69	27.8 ± 26.2	29.0 ± 24.8	62.5 ± 31.8	*P*=.8594
Activation marker	(CI 18.2 – 37.4)	(CI 18.6 – 39.5)	59.7	*P*=.0692
	N = 31	N = 24	N = 5	
CD56	5.0 ± 4.9	4.9 ± 5.4	26.2 ± 37	*P*=.9434
Natural killer cells	(CI 3.2 – 6.8)	(CI 2.6 – 7.2)	12.5	*P*=.2197
	N = 32	N = 24	N = 6	

CI, confidence interval (95% CI means there is 95% probability that the true mean lies between these two values); SD, standard deviation.

[*]Two-sample test of means (SSP).

[†]Median reported rather than 95% CI because of small patient numbers.

[‡]One case expressed T-cell receptor gamma/delta and is excluded from this analysis. See reference 27.

[§]Three B-cell lymphomas expressed ≤3% CD20 marker despite CD22 present on 13%, 26%, and 78% of cells.

[¶]Includes all values. Kappa and lambda bearing tumors not separated for these analyses.

[#]Ratio not calculated if both values zero, or 0 and 1. Ratios of lambda light chain restricted tumors inverted and expressed as positive rather than negative values. Six lymphomas expressed no surface light chains.

Panleukocyte and Activation Markers

The specimens from uveitis and B-cell lymphoma averaged around 50% expression of the panleukocyte marker CD45+. T-cell lymphomas had statistically more hematopoietic cells (*P*=.0206) by two-sample test of means. The increase in activation markers, HLA-DR, CD25, and CD69, approaches statistical significance for the T-cell lymphoma group, likely due to the large number of T lymphocytes present in these specimens. Activation markers, likely candidates for expression in inflammatory disease, were not elevated in the uveitis group compared to lymphoma.

T-cell Markers

Statistically significant differences between group means were found between uveitis and B-cell lymphoma for T-cell markers CD2, 3, 4, 5, and 7, but not for CD8, a marker associated with cytotoxic T cells. The CD4:CD8 ratio had a higher mean value in the uveitis group (*P*=.0113 by two-sample test of means) with no overlap between the 95% confidence interval for the expected true means, which clustered above 3.0 in the uveitis group and below 3.0 in the lymphoma group.

Comparisons between T-cell lymphoma and uveitis showed a statistically significant increase in CD3 antigen expression. CD7 antigen expression approached statistical significance in part because of the influence of two cases of mycosis fungoides. Two other patients with high CD7 expression had presumed primary T-cell lymphoma, one with brain involvement. CD4, CD8, and the CD4:CD8 ratio did not differ statistically, but CD4+ cells were fewer, CD8+ cells more numerous, and the CD4:CD8 ratio lower in the patients with T-cell lymphoma.

The T-cell receptor αβ paralleled the percentage of T cells in the specimen in each group. The group mean is high in the B-cell lymphoma group because of the influence of 4 patients with percentages of CD2+, CD3+, and CD5+ cells above the 95% confidence range of the group mean. Two were designated T-cell-enriched B-cell lymphoma[20] based on absent B-cell markers, low CD4:CD8 ratio on vitreous flow cytometry, and demonstration of cytologically abnormal CD20+ cells on subretinal aspirates. Two others were not considered to have T-cell-enriched B-cell lymphoma: one had an equal percentage of CD22+ cells (negative for CD19 and CD20) as T-cell markers, and one had metastatic systemic non-Hodgkin lymphoma with diffuse involvement at the level of the retinal pigment epithelium.

B-cell Markers

All B-cell markers, CD19, 20, 22, and kappa and lambda were significantly lower in the uveitis group than in the B-cell lymphoma group; the T-cell lymphoma group did not differ from the uveitis groupThe light chain ratio was also significantly lower in the uveitis group with no overlap between the 95% confidence interval around group means. Three of the 35 B-cell lymphomas (8.6%) had CD20+ staining of 1% to 3%, which was negligible compared to CD22+ staining of 13%, 26%, and 78%.

Other Cell Surface Markers

CD10 antigen was present in negligible amounts except for one case of kappa-restricted B-cell lymphoma in which 15% of cells bore CD10 vs 18% CD20+, suspicious for a germinal center origin, and one case of endophthalmitis in a patient with mycosis fungoides in which the 16% CD10+ staining cells likely represent neutrophils.

There was no detectable difference between macrophages or monocytes between uveitis and B-cell lymphoma, and no difference between these cell types in infectious vs noninfectious uveitis, or infectious uveitis vs lymphoma (data not shown). CD14+ macrophages/monocytes were less numerous (*P*=.0041 by two-sample test of means) in T-cell lymphoma than in uveitis.

Predictive Values and Likelihood Ratios of Selected Cell Surface Markers

Table 3 reports the positive predictive values and likelihood ratios for test results above a certain arbitrary cut-off value assigned as the median value for the test among all study participants. Prevalence of disease minimally affected these calculations because the study group was approximately 50% lymphoma and 50% nonlymphoma. T-cell markers were analyzed for the likelihood of indicating uveitis; B-cell markers were analyzed for the likelihood of indicating B-cell lymphoma. No tests were specifically analyzed for T-cell lymphoma because of the small number of cases. Higher levels of CD2, CD5, and the CD4:CD8 ratio appear useful for the diagnosis of uveitis by the standard of a positive predictive value greater than 60% and a likelihood ratio of greater than 1.5. Higher levels of CD20 and CD22 markers appear useful for the diagnosis of B-cell lymphoma using similar criteria. No flow cytometric tests were useful according to their negative predictive value and negative likelihood ratios.

Table 4 reports the areas under ROCs constructed for each of these markers. The CD2, CD4, CD5, CD7 T-cell markers, the CD4:CD8 ratio, and the CD20 and CD22 B-cell markers produce a statistically significant discrimination between the binary disease states of uveitis and B-cell lymphoma.

TABLE 3.POSITIVE AND NEGATIVE PREDICTIVE VALUES AND LIKELIHOOD RATIOS FOR SELECTED CELL SURFACE MARKERS IDENTIFIED BY FLOW CYTOMETRY IN UVEITIS AND B-CELL LYMPHOMA

CELL SURFACE MARKER POSITIVE AND NEGATIVE CUT-OFF VALUES	B-CELL LYMPHOMA NUMBER OF TESTS	UVEITIS NUMBER OF TESTS	SENSITIVITY*†	SPECIFICITY*‡	POSITIVE PREDICATIVE VALUE§	NEGATIVE PREDICTIVE VALUE¶	POSITIVE LIKELIHOOD RATIO#
T-cell markers							
Uveitis vs B-cell lymphoma							
CD2**			0.60	0.71	**0.68**	0.63	**2.0**
>22.8	10	21					
≤22.8	24	14					
CD3			0.56	0.45	0.72	0.55	1.0
>22	7	18					
≤22	17	14					
CD4			0.60	0.60	0.62	0.60	1.5
>10	13	21					
≤10	21	14					
CD5**			0.63	0.65	**0.65**	0.63	**1.8**
>17	12	22					
≤17	22	13					
CD7**			0.48	0.73	**0.70**	0.53	**1.8**
>16.6	7	16					
≤16.6	19	17					
CD8			0.46	0.58	0.53	0.50	1.1
>5.5	14	16					
≤5.5	19	19					
CD4:CD8 ratio**			0.60	0.66	**0.64**	0.61	**1.7**
>1.8	10	18					
≤1.8	19	12					

TABLE 3 (CONTINUED).

B-cell markers							
B-cell lymphoma vs uveitis							
CD20[**]			0.79	0.77	**0.77**	0.79	**3.5**
>3	27	8					
≤3	7	27					
CD22[**]			0.77	0.76	**0.73**	0.76	**3.2**
>6.1	27	10					
≤6.1	8	25					

[*]A test was considered positive if a larger percentage of cells bore the marker of interest than the median percentage for the entire study group and negative if it was lower than this value.
[†]Sensitivity was defined as the number of positive tests in the reference group divided by the number of positive and negative tests in that group.
[‡]Specificity was defined as the number of negative tests in the comparison group divided by the number of negative and positive tests in that group.
[§]Positive predictive value was defined as the number of positive tests in the reference group divided by the number of positive tests in reference and comparison groups. Values greater than 60% are considered clinically useful.
[¶]Negative predictive value was defined as the number of negative tests in the comparison group divided by the number of positive tests in the comparison and reference groups. Values greater than 90% are considered clinically useful.
[#]Likelihood ratio was defined as the sensitivity of the factor divided by 1 – the specificity. Values equal to 1 are equivalent to coin tosses. Values greater than 1.5 or less than 0.67 are considered clinically useful.
[**]Test names in bold indicate that quality measures were met. See text.

TABLE 4. AREA UNDER RECEIVER-OPERATOR CURVES (ROC) GENERATED FOR BINARY COMPARISONS BETWEEN UVEITIS AND B-CELL LYMPHOMA FOR SELECTED T-CELL AND B-CELL SURFACE MARKERS DETERMINED BY FLOW CYTOMETRY

CELL SURFACE MARKER	AREA UNDER ROC[*]	*P* VALUE[†]
T-cell surface markers predictive of uveitis[‡]		
CD2	0.688	.007
CD3	0.648	.060
CD4	0.723	.001
CD5	0.714	.002
CD7	0.674	.022
CD8	0.538	.59
CD4:CD8 ratio	0.750	.001
B-cell surface markers predictive of B-cell lymphoma[‡]		
CD20	0.880	<.001
CD22	0.884	<.001

[*]Larger areas under the curve indicate less likelihood that the results are related to chance.
†Calculation of ROC and statistical significance by SPSS v19, SPSS Inc, an IBM Company, Armonk, NY.
[‡]T-cell lymphoma not included in these analyses because of small numbers.

GENE REARRANGEMENT RESULTS

Table 5 reports the frequencies of positive gene rearrangements for B-cell lymphomas, T-cell lymphomas, and uveitis. False-positives for IgH and TCR were unusual. The IgH rearrangement occurred in a patient with known B-cell chronic lymphocytic lymphoma, who also had one of the 2 positive kappa PCRs. Both TCR and kappa gene rearrangements were positive in another patient suspected of

intraocular lymphoma who was not confirmed by hematology-oncology consultation; a specimen from the fellow eye was negative for gene rearrangements. For B-cell lymphomas, kappa PCR appeared the most efficient but was performed in few samples. Overall 16 of 25 cases tested (64%) had a gene rearrangement of some type. All 5 T-cell lymphomas tested had rearrangement of the TCR with evidence of clonality. Of 2 cases of T-cell-enriched B-cell lymphoma, 1 had a TCR and kappa rearrangement and 1 had kappa rearrangement.

TABLE 5. GENE REARRANGEMENT RESULTS FOR 30 CASES OF LYMPHOID MALIGNANCY AND 21 CASES OF INFECTIOUS AND NONINFECTIOUS UVEITIS

GENE REARRANGEMENT LOCUS	INTRAOCULAR LYMPHOID MALIGNANCY N POSITIVE/N TESTED (%)	UVEITIS N POSITIVE/N TESTED (%)	*P* VALUE[*]
	B-cell lymphomas		
IgH	13/25 (52%)	1/21 (4.8%)[§]	***P*=.0053**
Kappa	6/7 (86%)[†]	2/3 (67%)[§#]	*P*=.4901
TCR	9/22 (41%)[‡]	1/19 (5.2%)[#]	***P*=.0079**
Any	16/25 (64%)	2/21 (9.5%)[§#]	***P*<.0001**
	T-cell lymphomas		
IgH	0/5 (0%)		*P*=.6271
Kappa	…		
TCR	5/5 (100%)		***P*<.0000**
Any	5/5 (100%)		***P*<.0000**

IgH, immunoglobulin H; TCR, T-cell receptor.

[*]Chi-square test comparing lymphoma to uveitis.

[†]Three of 6 kappa gene rearrangements were in patients with negative IgH rearrangements; two of these also had TCR rearrangements.

[‡]Three of 9 TCR gene rearrangements were in patients with negative IgH rearrangements; two of these also had kappa rearrangements.

[§]One patient with culture-proven candidal endophthalmitis and prior B-cell chronic lymphocytic leukemia had gene rearrangements for IgH and kappa from the vitreous specimen.

[#]One patient classified as uveitis had both kappa and TCR rearrangements from the first eye after pretreatment with corticosteroids but negligible B cells. Her second untreated eye had no gene rearrangements, 47% CD20-positive cells, kappa:lambda ratio of 22. Lymphoma was denied by hematology-oncology consultants and the patient was treated with single intravitreal methotrexate injections in each eye and four courses of rituximab with resolution of the posterior lesions at the level of the retinal pigment epithelium. She developed B-cell central nervous system lymphoma 2.5 years after v

Cytology Results

No patient with uveitis had positive cytology. Of patients with intraocular lymphoid malignancy, 28 specimens were considered to show malignant lymphocytes (20) or to show atypical lymphocytes (8), including 10 specimens that were initially read as negative but later were read as positive on re-review by a hematopathologist and ophthalmic pathologist.

DISCUSSION

Intraocular lymphoma is a unique disease within ophthalmology. It is rare, with probably no more than 300 to 400 cases diagnosed each year in the United States, and many of these patients may have prior diagnoses of primary CNS lymphoma.[30] In Japan, it accounts for 21 per 100,000 patients with ocular disorders.[31] In the United States, it is an overwhelmingly Caucasian disease consistent with the demographics of our patients. Despite its rarity, it enters into the differential diagnosis of almost all cases of bilateral vitreous cellular infiltrates in individuals older than 50 years, and in patients of any age with posterior uveitis with disruption of the retinal pigment epithelial layer. Its rarity ensures that even specialists in ocular inflammatory diseases or vitreoretinal diseases may have little experience with it and may be uncomfortable suspecting or excluding it on clinical grounds. Uncertainty at the initial clinical encounter can lead to delay in diagnosis or, alternatively, large numbers of diagnostic vitrectomies performed relative to small numbers of confirmed diagnoses.[2]

High clinical suspicion of intraocular lymphoma by an experienced clinician meets subsequent challenges of adequately confirming it with diagnostic testing of vitreous or retinal biopsy to the satisfaction of the hematology-oncology or neuro-oncology colleagues who will ultimately be asked to treat the patient, in some cases without any evidence of primary CNS lymphoma other than ocular involvement with a diffuse large B-cell lymphoma.[30] Among potentially lethal eye diseases, intraocular lymphoma is more difficult to diagnose than choroidal metastases in a patient with known cancer elsewhere, or primary eye tumors such as malignant melanoma or retinoblastoma.

Despite these difficulties, the diagnosis of intraocular lymphoma is practiced as a high art in some centers, and large case series of advanced molecular diagnostic techniques have been published.[32] The most accepted protocol is based on cytologic studies with or without expert microdissection, gene rearrangement studies, and calculation of an IL-10 to IL-6 ratio.[32-34] With proper resources, very good tools therefore exist for the analysis of vitreous specimens in patients suspected of having lymphoma. The protocol is not ideal for all circumstances. It needs expert cytopathologic review, and it lacks customizability for other ocular infiltrates, such as those produced by infectious or noninfectious uveitis. The addition of TCR gene rearrangement to the protocol has improved the ability to diagnose these rare tumors.[32]

A protocol incorporating flow cytometry is generally criticized as requiring more cells than can be obtained from a single vitreous specimen. Compared to preparations of vitreous cells on glass slides for immunohistochemical staining, far more cells can be studied by flow cytometry. Analysis of the actual histograms and conversion of results to percentages of total cells, rather than receiving a report with a summary diagnosis from the pathologist, should provide a far larger sample for study than cells captured on glass slides and used for cytologic and immunohistochemical study. Multiparametric displays using different fluorescent channels can segregate relevant cell types such as CD3+CD4+ T lymphocytes more efficiently than immunohistochemical staining of individual glass slides. Nonetheless, there is some loss of the statistical advantage of flow cytometry if relatively small cell numbers are studied, because flow cytometry is designed to objectively quantify the phenotypes of large numbers of cells. The question of how few cells constitute a statistically valid vitreous sample for flow cytometry is unanswered. For peripheral blood samples, precision falls off as cell numbers or cell percentages in a sample decrease.[35] Interpretation of flow cytometric data from eye specimens relies largely on heuristics, or pattern recognition. Expert gating is needed to focus the display to reveal peaks corresponding to the cells with the phenotype of interest. Automated programs for unsupervised gating are being developed but are unlikely to be applicable to small cell numbers.[36,37] Despite these concerns about adequate sampling and sample size, flow cytometry certainly examines more cells than immunohistochemistry, since the entire vitreous specimen can be used. In addition, the same cells examined by flow cytometry are used in our protocol in molecular studies to detect gene rearrangement. Compared to microdissection techniques in which only a few cells are selected for amplification, use of a larger quantity of cells provides the polyclonal background, if one exists, to highlight the clonal peak diagnostic of lymphoma and to avoid a false-positive due to amplification of too few cells.

Flow cytometry also is a more efficient technique than immunohistochemical staining[19] to investigate large numbers of distinct cell surface markers.[3] The importance of this is demonstrated by the potential failure to characterize three cases of B-cell lymphoma in this series if only CD20+ antibodies had been used to assess cellular phenotype; each isolate bore the CD22 early B-cell marker only. As a tool to diagnose intraocular lymphoma, flow cytometry was highly efficient when combined with gene rearrangement and cytology and satisfactory for diagnostic purposes. As a tool to elucidate the role of reactive T lymphocytes in eyes with uveitis or lymphoma, which was the original intent of this report, little new information was gained. Even with the quantitative information from flow cytometry and the inclusion of more patients than in previous studies,[3] it is difficult to define a cut-off value of percentages of T lymphocytes, percentages of CD3+CD4+ T helper lymphocytes, or a value of the CD4:CD8 ratio that can be used to definitively diagnose uveitis and exclude lymphoma. Despite the lack of overlap between diagnostic groups in the 95% confidence intervals around group means, box plots (not shown) reveal substantial overlap if all cases are considered, because outliers exist for each diagnosis. Both lymphoma and uveitis are heterogeneous diagnostic categories. Larger sample sizes might reduce variability enough to make it easier to characterize these diseases quantitatively according to the phenotypes of the infiltrating vitreous cells.

Classic forms of test evaluation, such as sensitivity and specificity, positive and negative predictive values, and likelihood ratios, are often useful in assessing diagnostic tests. Flow cytometry provides objective quantitative data that permits this. When these measures are calculated for individual T-cell and B-cell surface markers using a cut-off value equivalent to the entire study group median, both T- and B-cell markers had fair discriminative power for uveitis and B-cell lymphoma. Predictive values are dependent on the prevalence of disease; in this data set the impact of this was minimal because of the equal distribution between B-cell lymphoma and uveitis. Clinicians who perform many diagnostic vitrectomies in patient groups with a low prevalence of lymphoma will have different results than reported here. An assumption is made that the pretest probability of disease will be high if a skilled clinician has screened the patient and decided that diagnostic vitrectomy is warranted. If diagnostic techniques such as flow cytometry can ultimately be used to better diagnose and manage uveitis, then a different population with more cases of uveitis and fewer cases of lymphoma might be appropriate. Otherwise, case selection remains an essential component of the diagnostic process.

The flow cytometry data presented does emphasize that T lymphocytes are constituents in all types of uveitis considered in its broadest sense.[1] Infiltration of vitreous by reactive T lymphocytes in CNS lymphoma was recognized in 1975.[38] With flow cytometry, it is the *number* of reactive lymphocytes that overwhelms, rather than the visual confusion they produce on a cytology slide. Phenotypic characterization of cell lineage and subset may in fact not be an adequate approach to understanding vitreous cellular infiltrates. Studies in the characterization of peripheral blood lymphocytes in patients with Behçet uveitis document changes in T-cell types during disease activation.[39] Aqueous-humor-infiltrating cells also demonstrate phenotypic changes with disease activity in Behçet.[40] Both approaches use easily obtained aqueous specimens in a changing disease. Any characterization of vitreous infiltrating cells is likely a one-time event; the ocular microenvironment may be dramatically changed by the intervention of vitrectomy. Resampling may not reduce heterogeneity in the data and may increase it. Flow cytometry does allow for customization to investigate specific hypotheses. A basic example is the assessment of the T-cell receptor as alpha/beta or gamma/delta type, as was done in this study. However, to truly characterize a cell, molecular techniques are necessary. Sequencing of the somatic hypermutations of intraocular B-cell lymphomas indicates that they are likely derived from B lymphocytes that have already undergone a germinal center reaction with exposure to antigen.[41,42] Corresponding flow cytometry data presented here merely indicates that most cases do not

express CD10, a cell surface marker associated with germinal center lymphomas. The obvious extension beyond cellular phenotype and flow cytometry is to use molecular biologic techniques to investigate the ontogeny and pathobiology of the infiltrating cells. Molecular techniques may eventually elucidate a specific homing mechanism for lymphoma cells to the brain and eye via expression of selective vascular adhesion molecules.[43] Similar studies in uveitis are possible but will likely be molecular rather than focused solely on cell surface markers.

This series is unusual in the number of T-cell lymphomas presented, 8 of 44, or 18.2%. In the series of Wang and colleagues,[32] with 114 cases of PVRL, there were only 5 cases of T-cell lymphoma, or 4.4%. Three of our 8 cases had prior diagnoses of mycoses fungoides, and the vitreous infiltrates were assumed to be metastatic. One woman had undiagnosed systemic T-cell chronic lymphocytic leukemia that was discovered during the course of her workup for intraocular lymphoma.[44] Two others had rearrangements of the T-cell receptor and compatible cytology. Cases in doubt are therefore two diagnosed before the use of gene rearrangement, one of whom had positive cytology and the other of whom was HIV positive and later diagnosed with CNS lymphoma on the basis of brain biopsy. The cases therefore seem to be well supported by other data, but it is possible that the last two cases were actually two additional examples of T-cell-enriched B-cell lymphoma that were not recognized as such.

Experimental work by Touitou and associates[45] provides a potential model for understanding infiltrating T lymphocytes in B-cell lymphoma. In mice with experimentally induced intraocular B-cell lymphoma, tumorigenesis recruits natural and adaptive regulatory T cells. The reactive lymphocytes may therefore play an important role in tumor control. Analysis of intraocular specimens as a guide to prognosis has not been attempted. It is conceivable that lymphoma patients with many infiltrating T cells have better tumor control and survive longer. The ability to quantitate numbers of reactive T lymphocytes with flow cytometry would be helpful in the investigation of such a hypothesis. More data regarding intraocular cells and inflammatory mediators in uveitis is also potentially useful. If numbers of T lymphocytes and the CD4:CD8 ratio are only partially successful as predictors of uveitis, flow cytometry provides an easily customizable tool that could be designed to analyze vitreous specimens from uveitis patients more productively. This, combined with cytokine determinations, may lead to better understanding of the mechanism of uveitis through the study of clinical specimens.

Treatment of lymphoma goes beyond the scope of this thesis. For patients with ocular-only disease, consultants may be reluctant to treat with systemic chemotherapy.[30] Combining a baseline IL-10 determination from an anterior chamber tap at the time of vitreous surgery for future monitoring, performing a complete core vitrectomy for diagnostic studies, and injecting a single dose of intravitreal methotrexate, followed by serial observation, might be a highly efficient means of stabilizing eyes harboring diffuse large B-cell lymphoma and improving vision, an adequate goal for the ophthalmologist if consulting oncologists decline to treat. Eyes with retinochoroidal infiltration have an imminently sight-threatening disorder that will warrant chorioretinal biopsy in many cases. Diagnosis and identification of cases remains the first step in managing this potentially fatal disease.

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REFERENCES

1. Deschenes J, Murray PI, Rao NA, Nussenblatt RB; International Uveitis Study Group. International Uveitis Study Group (IUSG): clinical classification of uveitis. *Ocul Immunol Inflamm* 2008;16(1):1-2.
2. Mruthyunjaya P, Jumper JM, McCallum R, Patel DJ, Cox TA, Jaffe GJ. Diagnostic yield of vitrectomy in eyes with suspected posterior segment infection or malignancy. *Ophthalmology* 2002;109(6):1123-1129.
3. Davis JL, Miller DM, Ruiz P. Diagnostic testing of vitrectomy specimens. *Am J Ophthalmol* 2005;140(5):822-829.
4. Margolis R, Brasil OF, Lowder CY, et al. Vitrectomy for the diagnosis and management of uveitis of unknown cause. *Ophthalmology* 2007;114(10):1893-1897.
5. Sugita S, Shimizu N, Watanabe K, et al. Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR. *Br J Ophthalmol* 2011;95(3):345-349.
6. Sugita S, Kamoi K, Ogawa M, Watanabe K, Shimizu N, Mochizuki M. Detection of *Candida* and *Aspergillus* species DNA using broad-range real-time PCR for fungal endophthalmitis. *Graefes Arch Clin Exp Ophthalmol* 2012;250(3):391-398.
7. Rothova A, de Boer JH, Ten Dam-van Loon NH, et al. Usefulness of aqueous humor analysis for the diagnosis of posterior uveitis. *Ophthalmology* 2008;115(2):306-311.

8. Harper TW, Miller D, Schiffman JC, Davis JL. Polymerase chain reaction analysis of aqueous and vitreous specimens in the diagnosis of posterior segment infectious uveitis. *Am J Ophthalmol* 2009;147(1):140-147.
9. Errera MH, Goldschmidt P, Batellier L, et al. Real-time polymerase chain reaction and intraocular antibody production for the diagnosis of viral versus toxoplasmic infectious posterior uveitis. *Graefes Arch Clin Exp Ophthalmol* 2011;249(12):1837-1846.
10. Cornut PL, Sobas CR, Perard L, et al. Detection of *Treponema pallidum* in aqueous humor by real-time polymerase chain reaction. *Ocul Immunol Inflamm* 2011;19(2):127-128.
11. Zaldivar RA, Martin DF, Holden JT, Grossniklaus HE. Primary intraocular lymphoma: clinical, cytologic, and flow cytometric analysis. *Ophthalmology* 2004;111(9):1762-1767.
12. Farkas T, Harbour JW, Dávila RM. Cytologic diagnosis of intraocular lymphoma in vitreous aspirates. *Acta Cytol* 2004;48(4):487-491.
13. Shen DF, Zhuang Z, LeHoang P, et al. Utility of microdissection and polymerase chain reaction for the detection of immunoglobulin gene rearrangement and translocation in primary intraocular lymphoma. *Ophthalmology* 1998;105(9):1664-1669.
14. Gorochov G, Parizot C, Bodaghi B, et al. Characterization of vitreous B-cell infiltrates in patients with primary ocular lymphoma, using CDR3 size polymorphism analysis of antibody transcripts. *Invest Ophthalmol Vis Sci* 2003;44(12):5235-5241.
15. Banerjee S, Savant V, Scott RA, Curnow SJ, Wallace GR, Murray PI. Multiplex bead analysis of vitreous humor of patients with vitreoretinal disorders. *Invest Ophthalmol Vis Sci* 2007;48(5):2203-2207.
16. Curnow SJ, Falciani F, Durrani OM, et al. Multiplex bead immunoassay analysis of aqueous humor reveals distinct cytokine profiles in uveitis. *Invest Ophthalmol Vis Sci* 2005;46(11):4251-4259.
17. Atan D, Fraser-Bell S, Plskova J, et al. Cytokine polymorphism in noninfectious uveitis. *Invest Ophthalmol Vis Sci* 2010;51(8):4133-4142.
18. Faia LJ, Chan CC. Primary intraocular lymphoma. *Arch Pathol Lab Med* 2009;133(8):1228-1232.
19. Davis JL, Solomon D, Nussenblatt RB, Palestine AG, Chan CC. Immunocytochemical staining of vitreous cells. Indications, techniques, and results. *Ophthalmology* 1992;99(2):250-256.
20. Cummings TJ, Stenzel TT, Klintworth G, Jaffe GJ. Primary intraocular T-cell-rich large B-cell lymphoma. *Arch Pathol Lab Med* 2005;129(8):1050-1053.
21. Coupland SE, Anastassiou G, Bornfeld N, Hummel M, Stein H. Primary intraocular lymphoma of T-cell type: report of a case and review of the literature. *Graefes Arch Clin Exp Ophthalmol* 2005;243(3):189-197.
22. Chan CC, Whitcup SM, Solomon D, Nussenblatt RB. Interleukin-10 in the vitreous of patients with primary intraocular lymphoma. *Am J Ophthalmol* 1995;120(5):671-673.
23. Cassoux N, Giron A, Bodaghi B, et al. IL-10 measurement in aqueous humor for screening patients with suspicion of primary intraocular lymphoma. *Invest Ophthalmol Vis Sci* 2007;48(7):3253-3259.
24. Davis JL, Viciana AL, Ruiz P. Diagnosis of intraocular lymphoma by flow cytometry. *Am J Ophthalmol* 1997;124(3):362-372.
25. Wilson DJ, Braziel R, Rosenbaum JT. Intraocular lymphoma. Immunopathologic analysis of vitreous biopsy specimens. *Arch Ophthalmol* 1992;110(10):1455-1458.
26. Raparia K, Chang CC, Chévez-Barrios P. Intraocular lymphoma: diagnostic approach and immunophenotypic findings in vitrectomy specimens. *Arch Pathol Lab Med* 2009;133(8):1233-1237.
27. Saenz AD, Amador A, Ruiz BM, Davis J, Ruiz P. Cytofluorographic and molecular identification of a CD8-positive, TCR-alpha/beta-negative intraocular T cell lymphoma: a case report and review of the literature. *J Med Case Rep* 2007 Oct 26;1:114.
28. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003;17(12):2257-2317.
29. Lehmann U, Bock O, Länger F, Kreipe H. Demonstration of light chain restricted clonal B-lymphoid infiltrates in archival bone marrow trephines by quantitative real-time polymerase chain reaction. *Am J Pathol* 2001;159(6):2023-2029.
30. Chan CC, Rubenstein JL, Coupland SE, et al. Primary vitreoretinal lymphoma: a report from an international primary central nervous system lymphoma collaborative group symposium. *Oncologist* 2011;16(11):1589-1599.
31. Mochizuki M, Singh AD. Epidemiology and clinical features of intraocular lymphoma. *Ocul Immunol Inflamm* 2009;17(2):69-72.
32. Wang Y, Shen D, Wang VM, Sen HN, Chan CC. Molecular biomarkers for the diagnosis of primary vitreoretinal lymphoma. *Int J Mol Sci* 2011;12(9):5684-5697.
33. Ohta K, Sano K, Imai H, Kikuchi T. Cytokine and molecular analyses of intraocular lymphoma. *Ocul Immunol Inflamm* 2009;17(3):142-147.
34. Sugita S, Takase H, Sugamoto Y, Arai A, Miura O, Mochizuki M. Diagnosis of intraocular lymphoma by polymerase chain reaction analysis and cytokine profiling of the vitreous fluid. *Jpn J Ophthalmol* 2009;53(3):209-214.
35. Hübl W, Tlustos L, Bayer PM. Use of precision profiles to evaluate precision of the automated leukocyte differential. *Clin Chem* 1996;42(7):1068-1073.
36. Zeng QT, Pratt JP, Pak J, Ravnic D, Huss H, Mentzer SJ. Feature-guided clustering of multi-dimensional flow cytometry datasets. *J Biomed Inform* 2007;40(3):325-331.
37. Lugli E, Roederer M, Cossarizza A. Data analysis in flow cytometry: the future just started. *Cytometry A* 2010;77(7):705-713.

38. Kennerdell JS, Johnson BL, Wisotzkey HM. Vitreous cellular reaction. Association with reticulum cell sarcoma of brain. *Arch Ophthalmol* 1975;93(12):1341-1345.
39. Yasuoka H, Yamaguchi Y, Mizuki N, Nishida T, Kawakami Y, Kuwana M. Preferential activation of circulating CD8+ and gammadelta T cells in patients with active Behçet's disease and HLA-B51. *Clin Exp Rheumatol* 2008;26(4 Suppl 50):S59-63.
40. Yu HG, Lee DS, Seo JM, et al. The number of CD8+ T cells and NKT cells increases in the aqueous humor of patients with Behçet's uveitis. *Clin Exp Immunol* 2004;137(2):437-443.
41. Malumbres R, Davis J, Ruiz P, Lossos IS. Somatically mutated immunoglobulin IGHV@ genes without intraclonal heterogeneity indicate a postgerminal centre origin of primary intraocular diffuse large B-cell lymphomas. *Br J Haematol* 2007;138(6):749-755.
42. Coupland SE, Hummel M, Müller HH, Stein H. Molecular analysis of immunoglobulin genes in primary intraocular lymphoma. *Invest Ophthalmol Vis Sci* 2005;46(10):3507-3514.
43. McCann KJ, Ashton-Key M, Smith K, Stevenson FK, Ottensmeier CH. Primary central nervous system lymphoma: tumor-related clones exist in the blood and bone marrow with evidence for separate development. *Blood* 2009;113(19):4677-4680.
44. Calfa CJ, Lossos IS, Ruiz P, Davis JL. Ocular involvement as the initial manifestation of T-cell chronic lymphocytic leukemia. *Am J Ophthalmol* 2007;144(2):326-329.
45. Touitou V, Daussy C, Bodaghi B, et al. Impaired th1/tc1 cytokine production of tumor-infiltrating lymphocytes in a model of primary intraocular B-cell lymphoma. *Invest Ophthalmol Vis Sci* 2007;48(7):3223-3229.