Characterization of Vitreous B-Cell Infiltrates in Patients with Primary Ocular Lymphoma, Using CDR3 Size Polymorphism Analysis of Antibody Transcripts

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PURPOSE. Histopathology usually cannot be performed and cytology is unfortunately frequently insufficient to confirm a suspicion of primary intraocular lymphoma (PIOL). The purpose of this study was to evaluate the Immunoscope technique for the identification of ocular B-cell monoclonal infiltrates in patients with malignant or immune conditions.

METHODS. Polymorphism analysis of the size of the third complementarity-determining region (CDR3) of heavy chain antibody transcripts was used to differentiate between a polyclonal infiltrate and a monoclonal infiltrate within a clinical vitreous sample of two groups of patients. PIOL was confirmed in all patients of the first group (n = 6). Five patients with autoimmune uveitis or immune-recovery uveitis associated with AIDS were included in the control group. The level of IL-10 in the vitreous was determined in all patients.

RESULTS. In five cases of severe PIOL, CDR3 polymorphism analysis confirmed the presence of a dominant B-cell clone within the eye. In one case of confirmed PIOL presenting with mild vitritis, CDR3 polymorphism analysis was consistent with the existence of a polyclonal profile in the ocular sample studied. Conversely, it was shown that the detection of an intraocular monoclonal B-cell population does not necessarily imply ocular lymphoma. A clonal expansion was detected in a control patient who exhibited merely a nonmalignant response associated with immune-recovery uveitis.

CONCLUSIONS. This PCR-based technique can make an important contribution to the characterization of intraocular B cells, but it alone cannot confirm or exclude the existence of a malignant lymphocyte proliferation. In the evaluation of a patient with intraocular inflammation in whom PIOL is suspected, CDR3 polymorphism analysis is recommended to confirm clonality. In general, the information about lymphocyte diversity provided by this technology opens up new possibilities for the analysis of ocular infiltrates. (Invest Ophthalmol Vis Sci. 2003;44:5235–5241) DOI:10.1167/iovs.03-0035

Approximately 40% of all diffuse large B-cell lymphomas (DLCLs) are present at extranodal sites, most commonly the gastrointestinal tract. In comparison, the occurrence of primary lymphomas at the so-called immune-privileged sites such as the eye, the central nervous system (CNS), the testis, and the ovary is uncommon. Primary CNS lymphoma (PCNSL) is an uncommon cerebral tumor that accounts for approximately 0.5% of all primary brain neoplasms.1 PCNSL represents only 2% of all extranodal non-Hodgkin’s lymphomas.2 Ocular involvement in patients with PCNSL occurs in 25% of cases.3 However, there has been a dramatic increase in the diagnosis of primary lymphoma of the brain during the past decade, prompting speculation that it may become the most frequently diagnosed tumor of the CNS within the next few years.4 The incidence rates of brain lymphoma increased more than 10-fold, from 2.5 cases per 10 million people in 1973 to 30 in 1991 to 1992. Ocular involvement is often misdiagnosed, masquerading as posterior uveitis, vitritis, and retinitis. Because of the rarity of the disease and the difficulty of obtaining tissue for pathologic analysis, the diagnosis of primary or secondary ocular lymphomas has been particularly difficult and remains an important challenge.5,6 The clinical presentation is now better documented.7–12 The current diagnostic approach to patients in whom ocular lymphoma is suspected includes neurologic examination, cerebral magnetic resonance imaging (MRI), lumbar puncture, and diagnostic vitrectomy. The definitive diagnosis is based on the cytological analysis of a vitreous biopsy specimen.8,12,13 Malignant cells are often rare and degenerated, which makes their identification difficult.

Recently, new diagnostic approaches have improved the sensitivity of the vitrectomy.13,14 Immunologic and molecular tools can be of help to clinicians. An elevated interleukin (IL)-10 concentration in the vitreous is suggestive of lymphoma, even though false-positive cases have been reported.15–17 More recently, a test relying on PCR amplification of DNA was proposed to document the clonal expansion of intraocular B lymphocytes.5,20–21 The success of this approach could be limited, however, by the extremely small quantity of material obtained after anterior chamber paracentesis or vitrectomy. We speculate that RNA transcripts would have, over genomic DNA, the advantage of being present in multiple copies in B lymphocytes. Furthermore, an accurate description of the detected clone (CDR3 size and sequence) would be useful to evaluate the extent of the lymphoma. Finally, when a clonal expansion is detected, it would also be important to quantitate it over the polyclonal background. The latter issues were addressed in this study.

B-cell lymphomas usually express immunoglobulin (lg) transcripts. Immunoglobulins are composed of heavy (H) and light (L) chains, both encoded by rearranged globulin from sets of variable (V), diversity (D), joining (J), and constant (C) germline gene segments. Genetic diversity is in part generated

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by the junctions that occur between the V, D, and J or V and J segments, which are imprecise and can also result in addition of nucleotides. The junctional regions are also called the third complementarity determining region (CDR3) and encode a corresponding CDR3 loop that contacts the antigen. A similar recombination process occurs in T cells. Because the T-cell receptor (TCR) is composed of two chains, there are two different CDR3 loops at the tip of each receptor. Similarly, an antibody bears one CDR3 VH and one CDR3 VL at the tip of its variable antibody domains. T and B cells do not express similar CDR3 sequences. Moreover, the probability that two unrelated B cells would share the same CDR3 VH or VL is extremely low. Because of the recombination process, CDR3 VH sizes can vary by as many as 22 amino acids between two different heavy chains. Therefore, the probability distribution of the various VH (or VL) CDR3 sizes provides a low-resolution representation of the antibody repertoire. The Immunoscope method is based on a combination of RT-PCR and run-off reactions with fluorescent oligonucleotides. A subsequent software analysis allows the determination of the relative abundance of TCR or Ig transcripts bearing particular CDR3 sizes within a clinical sample. Until now, CDR3 length polymorphism analysis was mostly used to study the CDR3 regions of TCR genes.

We sought to determine whether such a sensitive method, relying on the detection of rearranged transcripts of immunoglobulin genes, could be used to detect intraocular clonal populations. We have reported the clinical features and laboratory investigations of a series of 44 patients with proven ocular and CNS lymphoma. Six similar consecutive cases were studied during the course of this work. The strategy that we adopted relies on heminested RT-PCR followed by polymorphism analysis of the size of the CDR3 of the amplified VDJ segments. To our knowledge, this is the first time that this strategy has been applied to the analysis of ocular infiltrates. We show that it is possible, using this approach, to differentiate between a polyclonal infiltrate and a monoclonal infiltrate within a clinical ocular sample. At the same time, the existence of monoclonal populations does not allow us to confirm the diagnosis of ocular lymphoma, because we show that clonal expansions are detectable in certain patients who exhibited merely a nonmalignant inflammatory response.

**Materials and Methods**

**Patients and Control Subjects**

We studied six patients (P1-P6) in whom a preoperative diagnosis of ocular lymphoma was confirmed by cytologic analysis of the vitreous humor (P1, P2, P4, P5, P6) or a cerebral biopsy specimen (P3). In this case, diagnostic vitrectomy was performed initially but remained noncontributory. The diagnostic evaluation performed to determine whether there were other sites of disease systematically included lumbar puncture and cytologic analysis of the cytocentrifuged cerebral fluid, CNS MRI, thoracoabdominal computed tomographic (CT) scan, osteomedullar biopsy, and serum levels of lactate dehydrogenase (LDH) and β2-microglobulin. This type of diagnostic evaluation was performed when primary intraocular lymphoma (PIOL) was suspected and before diagnostic vitrectomy. Similar vitreous samples were taken from five control patients (C1-C5) who exhibited a nonmalignant inflammatory response. Control subjects with clear-cut diagnoses were selected. Patients C1 to C4 presented with a corticosteroid form of uveitis associated with active ocular inflammatory lesions. The diagnosis of infectious uveitis or intraocular lymphoma was initially suspected in this group. Therefore, vitrectomy was proposed for diagnostic confirmation or for the treatment of a complication such as retinal detachment, which occurred in patient C5. The latter presented with a previous history of cytomegalovirus (CMV) retinitis and subsequent immune-recovery uveitis after the initiation of highly active antiretroviral therapy. CMV retinitis was inactive when retinal detachment occurred. The diagnostic evaluation described earlier was not performed in the control group, as there was no suspicion of PIOL in the subjects selected.

The main characteristics of the patients studied are presented in Table 1 (lymphoma) and Table 2 (controls). The research was conducted according to the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. Healthy control subjects (seven men and nine women; mean age, 27.2 years; range, 22-51) were students from our university and members of our laboratory who gave their informed consent to participate to the study. Patients with chronic lymphocytic leukemia (CLL) were untreated at the time of analysis. It was determined by cytofluorometric analysis that all three patients with CLL presented with more than 80% of B cells (CD19+ CD20+) amon circulating PBMCs. All B cells were CD5+ and expressed a single light chain isotype. B-cell clonality was confirmed using standard multiplex PCR from DNA, as previously described.

**Collection of Vitreous Samples, Evaluation of IL-10 Concentrations, and Cytological Analysis**

A standard three-port vitrectomy was performed in all cases, with patients under peribulbar anesthesia. An initial 0.8-mL specimen of undiluted vitreous was collected and immediately taken to the Laboratory of Hematology to determine IL-10 level (Quantikine; RD Systems Europe Ltd., Oxon, UK) and for cytological analysis. Two hundred microliters were sent to the laboratory of Immunology for Immunoscope analysis. Standard cytological examination was performed on the vitreous sample after centrifugation on glass slides at 1000 rpm for 8 minutes at 4°C. The diluted vitreous was also collected and centrifuged (1000 rpm for 8 minutes at 4°C). The pellet was then suspended in 2 mL RPMI medium and cytocentrifuged at 450 rpm for 5 minutes. After resuspension, 200 μL of the cell suspension was placed drop-wise on gelatinized slides. The slides were either stained with May-Gruenwald-Giemsa (MGG) or air dried and fixed by immersion in acetone for 7 minutes. Immunocytochemical analysis was performed according to the alkaline anti-alkaline phosphatase (APAAP) method (Envision System-Alkaline Phosphatase; Dako, Trappes, France), by using monoclonal antibodies recognizing pan-T or T-cell subset-specific antigens (CD3, CD4, CD8) purchased from BD Biosciences (Rungis, France), B-cell antigen CD20 (Dako), and κ or λ light chains (Immunotech).
Beckman-Coulter, Marseille-Luminy, France). PIOLs are large cell B-lymphomas (CD20⁺, κ or λ⁺, CD4⁺, CD8⁺, CD3⁺).

**RT-PCR and CDR3 Size Polymorphism Analysis of Antibody Transcripts**

For CDR3 size polymorphism analysis we used the Immunoscope software package.²⁵ Immunoscope analysis couples fluorescence PCR and software analysis and allows the direct and accurate sizing of a clonal population of T or B cells within a polyclonal milieu. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation on single-density gradient (Ficoll Hypaque; Pharmacia, Upsala, Sweden) and processed for Immunoscope analysis, as previously described.²⁶ An Epstein-Barr virus (EBV)-transformed human B lymphoblastoid cell line (BLCL) was established in the laboratory and maintained as previously described.²⁶ The vitreous humor was obtained after vitrectomy. Approximately 200 μL of vitreous humor was diluted in a saline solution to a final volume of 1 mL, and the samples were centrifuged immediately (910g for 10 minutes at 4°C). Previous morphologic studies revealed that such amounts of vitreous humor obtained from PIOL patients contain, on average, 1000 cells (Merle-Beral H, unpublished results, 2003). The total RNA from the cells obtained by ocular sampling was immediately reverse-transcribed for RT-PCR analysis. Brieﬂy, total RNA was puriﬁed from the cell pellet according to the method of Chomczynski and Sacchi,²⁷ and RNA was reverse transcribed in a 50-μL reaction volume, using the reagents provided in an RT-PCR kit (ProSTAR First Strand; Strategene, La Jolla, CA), according to the manufacturer’s instructions. We designed three oligonucleotides that allowed us to perform a nested amplification of all the transcripts coding for a VH repertoire. The upstream degenerated primers hybridize with a conserved sequence coding for a part of the framework regions (FR2 or FR3) of the VH domains. The downstream primer hybridizes with a sequence shared by the various human JH segments. Two microliters of the RT reaction were used as a template in a ﬁrst round of PCR using the primers FR2 5′ TGGGTRCTCGMAGSNCYCGG-3′ and IgHJ 5′-TGARGAGACGGTGACCRK-3′ for 30 cycles of ampliﬁcation. One microliter of the previous reaction was used as a template in a second, semi-nested PCR, using the primers FR5 5′-ACAGCGCTSTGAT- TACTGT-3′ and IgH for 25 cycles of ampliﬁcation. Each cycle consisted of 95°C for 5 minutes, 94°C for 40 seconds, 48°C for 40 seconds, and 72°C for 90 seconds, with an additional extension interval of 5 minutes at 72°C after the last cycle. The PCR products were then ﬂuorescently labeled using a run-off procedure: Two microliters of PCR product was added to an 8-μL mixture containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl (pH 8.3), 0.2 mM of each dNTP, 0.2 U of Taq polymerase, and 0.1 μM of ﬂuorescently labeled IgH ROX 5′-GAGACGGTGACCRKKGTYCC-3′ oligonucleotide. Run-off products were then loaded on a 4% acrylamide, 4 μL urea sequencing gel and run on a DNA sequencer (model 577; Applied Biosystems, Foster City, CA). A mixture of dye-labeled size standards was also loaded on the sequencing gel to allow the precise determination of the sizes of the VH-JH run-off reaction products. The size and area of the peaks corresponding to antibody heavy chains were determined using the ImmuScope software.²⁸ The percentage of representation of each peak size among all VH-JH segments was subsequently calculated. The observed peaks are usually separated by 3 bases, and correspond to in-frame transcripts coding for antibody heavy chains. Windows of analysis were centered on expected sizes corresponding to VH transcripts encoding a 15-residue CDR3 region, defined according to Kabat et al.²⁹ Reference ImmunoScope VH proﬁles were established from the PBMCs of 16 healthy subjects. This study showed that the VH CDR3 size distribution proﬁle is made up of an average of 25 peaks. The size of the CDR3 loops ranged, according to this analysis, anywhere from 3 to 27 amino acids. The most common length for a human CDR3 VH loop is 15 amino acids (data not shown; Gorochov G, manuscript in preparation).

**DNA Sequencing**

VH amplions of interest were gel puriﬁed and directly ligated into a vector (PCR II; TA cloning kit; Invitrogen, San Diego, CA). After ampliﬁcation into Escherichia coli, plasmids were puriﬁed and their inserts sequenced on an automated sequencer, using M13R and T7 dye-labeled primers and DNA polymerase (AmphilTaq FS; Applied Biosystems Division).

**RESULTS**

Cytological analysis of the vitreous biopsy allowed the detection of intraocular malignant cells in all cases except P3 (Table 1). The detected cells were conﬁrmed to belong to the B-cell lineage (CD20⁺) by immunohistochemistry (data not shown). In the same patients, elevated IL-10 concentrations in the vitreous were suggestive of lymphoma (Table 1). In patient P3, diagnosis could be conﬁrmed only by histologic analysis of a CNS biopsy specimen (Table 1). The main characteristics of the control patients studied are presented Table 2. The proﬁles obtained by CDR3 length polymorphism analysis of antibody transcripts contained in the vitreous humor of ﬁve consecutive patients with ocular lymphoma and of ﬁve control patients are presented in Figure 1. In the lymphoma group, one can see that only three vitreous humor samples (P1, P2, and P4) appeared to be inﬁltrated by a purely monoclonal population of B lymphocytes. In patient P1, the CDR3 length polymorphism analysis of antibody transcripts revealed the existence of only one

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>PMH</th>
<th>Evolution (mo)</th>
<th>Eye</th>
<th>Uveitis</th>
<th>IL-10 (pg/mL)</th>
<th>Vitreous</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>43</td>
<td>M</td>
<td>Retinal ischemia, neovascular glaucoma, Monophthalmus status</td>
<td>12</td>
<td>OU</td>
<td>Nongranulomatous, hypopion, V, retinal necrosis</td>
<td>5</td>
<td>T lymphocytes/ Macrophages</td>
<td>Behçet</td>
</tr>
<tr>
<td>C2</td>
<td>68</td>
<td>M</td>
<td>idiopathic CD4+ lymphocyte deficiency</td>
<td>4</td>
<td>OU</td>
<td>Retinochoroiditis, V, RD</td>
<td>5</td>
<td>T lymphocytes</td>
<td>Toxoplasmosis</td>
</tr>
<tr>
<td>C3</td>
<td>57</td>
<td>M</td>
<td>—</td>
<td>144</td>
<td>OU</td>
<td>V, retinitis, CME, papillitis, glaucoma</td>
<td>10</td>
<td>Polymorphonuclear</td>
<td>Idiopathic</td>
</tr>
<tr>
<td>C4</td>
<td>41</td>
<td>M</td>
<td>Buccal aphthosis</td>
<td>6</td>
<td>OS</td>
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<td>12</td>
<td>CME, RIU</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>42</td>
<td>M</td>
<td>AIDS, CMV retinitis</td>
<td>13</td>
<td>OU</td>
<td>RD, hypotonia</td>
<td>12</td>
<td>OOU</td>
<td>Nongranulomatous, V, RD 5 T lymphocytes</td>
</tr>
</tbody>
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PMH, past medical history; V, vitritis; RD, retinal detachment; CME, cystoid macular edema; IRU, immune recovery uveitis.
FIGURE 1. Immunoscope analysis of vitreous biopsies specimens in patients with PIOL (P1–P5) and nonmalignant uveitis (C1–C5).

VH CDR3 Length (Amino-Acids)
peak, corresponding to the transcripts coding for a VH domain carrying a CDR3 fragment measuring 15 amino acids. P1 had an abdominal lymph node removed after ocular biopsy and nodal lymphoma involvement was histologically confirmed. A peak of similar size was found in the eye (results not shown) and in the lymph node of P1. As shown, dominant intraocular clones were never detected in the blood of the patients (Fig. 1). The clonality of the infiltrate was further confirmed for P1 by DNA sequencing of the cloned PCR products. In patient P5, the Immunoscope analysis revealed the presence of an oligoclonal B-lymphocyte infiltrate. In the latter case, there was a dominant peak, but numerous transcripts were clearly detectable. In the sample (P3), a polyclonal population of B lymphocytes was detected. In this case, mild vitritis was associated with a cerebral tumor. Vitrectomy did not confirm the diagnosis, and the level of IL-10 was quite low. Finally, cerebral biopsy was necessary in this case to confirm the diagnosis of lymphoma.

The same CDR3 length polymorphism analysis of antibody transcripts was performed on the vitreous humor samples of five control patients, four of whom (C1–C4) had idiopathic uveitis. The fifth control sample was taken from an HIV-infected patient who had had CMV retinitis and who was not affected by HIV-associated lymphoma. This patient was on highly active retroviral therapy and presented with retinal detachment associated with immune-recovery uveitis. In patients C1 through C4, the size distribution profiles obtained showed the presence of an oligoclonal population of B lymphocytes in the infiltrate. In the sample taken from patient C5, the infiltrate was unexpectedly almost entirely monoclonal.

We applied the same approach to the study of healthy control subjects and blood samples obtained from three different patients who had documented CLL. As expected, polyclonal patterns of size distribution were present in the 16 healthy control subjects analyzed, whereas single peaks were detected in the patients with CLL (see representative Immunoscope control profiles in Fig. 1).

Ocular samples are always very limited in size, one potential limitation to studying them is that the number of cells analyzed varies greatly from one sample to the next. In most cases presented, the sample volume (and its gel-like quality) was not sufficient to allow a reliable measurement of cellular concentration. To test whether this level of uncertainty has a detrimental effect on the accuracy of the analysis presented, we performed a control experiment. We mixed a human B-lymphocyte clone (B LCL transformed by EBV) with a polyclonal population of human B lymphocytes purified from a healthy control subject. Transcripts from this concentrated repertoire (2 \times 10^{10} human B cells) were reverse transcribed. We then performed serial dilutions of the cDNA before Immunoscope analysis (Fig. 2A). The Immunoscope profiles presented therefore correspond to an analysis that would have been performed on the same cellular population at different concentrations. The first study point corresponds to a clinical sample of 2 \times 10^{10} human B cells, a quantity far greater than has ever been obtained by ocular sampling. At the other end of the spectrum, the least concentrated sample (shown on the right) corresponds to the analysis of only 2 \times 10^7 B cells. One can see that the general appearance of the Immunoscope profile is remarkably well conserved. In the spiking experiment presented, the B LCL clone (CDR3 size: 16, indicated by an arrow) provided 27.23% of the VH transcripts analyzed in the nondiluted sample. The same issue was addressed using a clinical sample (P6). We

![Image of Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932917/ on 01/20/2019)
compared by Immunoscope analysis the crude vitreous aspirate and the dilute vitreous (diluted four times during sampling) of a sixth patient with PIOL (P6). A single monoclonal peak was detected in both samples (Fig. 2, bottom).

**DISCUSSION**

The CDR3 regions of the heavy and light chains are the most polymorphic elements of the antibodies and are the regions that establish the strongest contacts with the antigen. There are not only diversities in the genetic sequence, but also in the size of the CDR3 segments. Therefore, analysis of the diversity of the CDR3 sequences found in a clinical sample allows one to define the characteristics (mono-, oligo-, or polyclonal) of the corresponding B-lymphocyte infiltrate.

In this study we show that it is possible to study the diversity of B-lymphocyte infiltrate from samples of vitreous humor. Our goal was to determine whether this type of analysis could contribute to the differentiation of inflammatory infiltrates and malignant monoclonal proliferation.

CDR3 size polymorphism analysis (also called Immunoscope analysis or spectratyping) was first used to study TCR transcripts. More recently, a strategy similar to the size polymorphism, allowing the comparison of patient and control repertoires and malignant monoclonal proliferation. The latter approach has been applied very recently to study the diversity of human B-cells in the peripheral blood. Besides its relative simplicity, the main advantage of this global approach is that it allows direct and accurate sizing of clonal population within a polyclonal milieu. Like Immunoscope, spectratyping has been used mostly to study T-cell repertoires. The murine B-cell repertoire has already been studied using this approach. We modified this analysis in a way that gives a quantitative measure of each size polymorphism, allowing the comparison of patient and control repertoires. More recently, a strategy similar to the one we have applied for the study of TCR repertoires has been used to study the diversity of human B-cells infiltrating the cerebral lesions of patients who have multiple sclerosis (MS).

This work relied on a collection of several VH-specific primers used in separate PCR reactions generating as many CDR3 length distribution profiles. In this context, when two (or more) clones are detected in separate distribution profiles, it cannot be directly determined whether a single clone is actually dominant or not. We present herein an approach based on a lower resolution analysis, because most of the B-cell repertoire is depicted as a single profile. Besides its relative simplicity, the main advantage of this global approach is that it allows direct quantitative comparisons between the various peaks detected. The latter approach has been applied very recently to the study of a case of liver B-cell lymphoma and to the study of a circulating lymphoma. To our knowledge, we are reporting the first intraocular B-cell repertoire analysis.

The robustness of Immunoscope analysis for the study of samples with varying concentrations was tested (Fig. 2). We attempted to mimic extreme conditions that would frame the cell count range expected in clinical samples. Of note was that the shape of the Immunoscope profile curve showed little variation as a function of the absolute amount of B-cell RNA analyzed (Fig. 2). In fact, its versatility with respect to cell count is among the advantages that Immunoscope has over other techniques, such as cytology. It is also clear from that experiment that a large quantity of polyclonal cells coming from a concentrated sample does not mask the detection of a minority clone.

In five patients, the primary diagnosis of ocular lymphoma was directly determined by cytological analysis. In patient P3 although the clinical examination was consistent with PIOL and the patient had a brain biopsy confirming the presence of CNS lymphoma, the diagnosis of PIOL was not confirmed histologically. Among the six patients studied, only four exhibited a CDR3 length polymorphism analysis of antibody transcripts compatible with monoclonality. In the P5 sample there are at least three rearranged transcripts of the immunoglobulin heavy chain, two being dominant. This does not exclude the possibility that one of these dominant rearrangements remains unproductive and that one unique clone is responsible for the expression of these two transcripts. Because DNA rearrangements at one locus stop on both chromosomes only when a viable chain is produced, the two heavy chain loci can be rearranged in the same B cell. In that case, two transcripts can be produced, with one of them being out of frame or including a stop codon. In other words, the presence of several peaks (two of which are dominant) in P5 is not necessarily incompatible with the presence of a monoclonal infiltrate in the sample studied. In patient P3, the presence of numerous rearranged transcripts of the antibody heavy chain is compatible with the presence of a monoclonal infiltrate. However, it is interesting to note that vitritis was mild in this case, whereas it was severe in the other five patients. Therefore cytologic diagnosis of PIOL was difficult, and the level of IL-10 was quite low. An IL-10 concentration above 100 pg/mL in the vitreous is suggestive of lymphoma (Cassoux N, manuscript in preparation). A cerebral biopsy was performed a few months later and definitely confirmed the diagnosis of primary oculocerebral lymphoma.

CDR3 length polymorphism analysis of antibody transcripts is, above all, a qualitative test that allows in this case the description of the diversity of the repertoire of rearranged transcripts of the antibody heavy chain. This type of analysis does not allow the direct quantification of the transcripts analyzed nor the determination of the number of B lymphocytes present in the sample. As stated earlier, the observed peaks in each sample were not correlated between each other. The area under the curve of a particular peak gives direct information about the proportion of B lymphocytes within the total sample population that show the corresponding rearrangement. This is true if one assumes that a given B lymphocyte expresses mostly a single heavy chain transcript. It is possible, for example, to determine that in patient 5, 85% of the transcripts show a CDR3 region of the same length (coding for a CDR3 fragment of eight amino acids). Although it is theoretically possible that the same dominant peak corresponds to different sequences of the same length, it has been shown in T-cell studies that a dominant peak usually corresponds to a clonal expansion.

This small series is sufficient to illustrate that the detection of a monoclonal population of rearranged transcripts of the immunoglobulin heavy chain is not necessarily evidence of malignant lymphoma. Indeed, the CDR3 length polymorphism analysis of antibody transcripts of the sample taken from control patient C5, who had immune-recovery uveitis, shows the presence of a quasimonoclonal infiltrate. Conversely, a polyclonal infiltrate is detected in the sample from patient P3, whereas the diagnosis of lymphoma was nevertheless established by classic methods applied to a cerebral biopsy specimen. The latter results are not necessarily artifactual. One cannot exclude the possibility that in patient C5 the vitreous humor was infiltrated by a clone of B cells producing anti-CMV-specific antibodies. Again, this patient presented with immune-recovery uveitis and normalized levels of lymphocyte function. The results presented do not imply that in this case the predominant anti-CMV response would be humoral. As expected, oligoclonal T cells were also detected by Immunoscope analysis in the same sample (data not shown). Of interest, Onkosuwito et al. have reported on the presence of significant levels of IL-10 in herpetic acute retinal necrosis syndrome. This condition can masquerade as ocular lymphoma in some exceptional cases (data not shown). Recently, Mu-
timer et al.\textsuperscript{35} have identified an association between immune-recovery uveitis and a diverse intraocular CMV-specific T-cell response. Considering the negative results obtained in patient P3, the possibility that the malignant clone remains masked by a polyclonal reactionary infiltrate cannot be excluded. Overall, the presence or absence of a monoclonal infiltrate detected by PCR analysis does not per se allow the confirmation or exclusion of a malignant lymphocyte proliferation. We nevertheless recommend including the Immunoscope approach (or any other related technique) in the evaluation of a patient with intraocular inflammation with suspected PIOC to confirm clonality. In conclusion, the information about lymphocyte diversity provided by this technology opens up new possibilities for the analysis of ocular infiltrates. One can see the insights that would be offered by such an approach for the study of autoimmune diseases targeting the eye.

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