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LABORATORY STUDY

Cytopathological analysis of vitreous in intraocular lymphoma

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Abstract

Objective **To describe the cytopathological method used in the analysis of vitreous samples in the diagnosis of primary intraocular lymphoma (PIOL).**
Participants **Seven patients with refractory posterior uveitis referred to a regional ocular inflammatory service were diagnosed as having PIOL between 1999 and 2006.**
Methods **Clinical features of the uveitis and cytopathological preparation of the samples were described. All patients underwent vitrectomy and samples were placed in formal saline or prepared fresh. Following paraffin embedding generating a cell block, immunostaining, and polymerase chain reactions were performed.**
Results **Five women (71.4%) and two men (28.6%) (mean age 67.7 years) were included. Five patients had diagnostic vitrectomy performed within 6 months of presentation, but in two patients diagnosis was delayed up to 2 years. Uveitis was bilateral in two patients. Cytologic and immunohistochemical staining prepared from the vitreous specimens showed PIOL in all patients, and PCR displayed single band of immunoglobulin heavy chain rearrangement in five out of six samples tested.**
Conclusions **Diagnosis of PIOL is difficult due to small volume of sample with low number of malignant cells and inadequate preparation of samples. Our method of analysis with fresh samples together with immunohistochemistry and PCR analysis demonstrates a high yield of diagnosis reducing diagnostic delay.**

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Keywords: intraocular lymphoma; eye; vitrectomy; cytopathology; immunochemistry; polymerase chain reaction

Introduction

Malignant lymphoma is the generic term describing tumours of the lymphoid system and are divided into two major categories: Hodgkin's lymphoma and non-Hodgkin's lymphomas (NHL). Hodgkin's lymphomas are rare at extranodal sites such as eyes. Intraocular presentation in NHL, although more common, is estimated to represent only about 1% of cases,[1] either as (1) ocular involvement in primary central nervous system (CNS) lymphoma, or (2) systemic NHL with spread to the uveal tract, or (3) rarely as a primary intraocular event.

Primary CNS lymphoma is a high-grade non-Hodgkin's lymphoma restricted at presentation to the brain, spinal cord, or meninges. A proportion of these cases (17%) show eye involvement only, at initial presentation. About 60% of patients presenting with intraocular NHL will develop lymphomatous infiltrates of the brain, spinal cord, or meninges later.[1] Most intraocular lymphomas are of B-cell phenotype. Rare cases of T-cell lymphoma with intraocular involvement have been reported mostly representing a secondary manifestation of either a cutaneous or a systemic lymphoma. Intraocular lymphoma has a poor prognosis with a median survival of just over 3 years with treatment,[2] and once CNS involvement occurs, an untreated median survival is 1.8–3.3 months.[3] Aggressive modern-day treatments can prolong this median survival to 40 months,[4] with ocular management improving vision during this time. Primary ocular NHL often presents as a masquerade syndrome with features of posterior uveitis, which can result in diagnostic delays. The average interval from onset of ocular symptoms to diagnosis was still 11–21.4 months even in tertiary uveitis referral centres.[5,6] This interval can be decreased with an increased index of suspicion. Most significant cause of delay is inadequate

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cytopathological identification of tumour cells. Malignant cells are sparse and frequently accompanied by numerous inflammatory cells including macrophages, small mature lymphocytes, and cellular debris. The inflammatory component can often outnumber lymphoma cells hence increasing the difficulty in identifying malignant cells. Additionally, lymphoma cells are also more fragile so that mechanical trauma associated with vitrectomy and steroid therapy-induced tumour necrosis results in poor yield and difficulty with cytopathological interpretation. Here we describe our methods of cytopathological and molecular assessment of vitrectomy samples that yield a high diagnostic return.

Materials and methods

Clinical data

Between January 1999 and April 2006, 50 patients presented to a regional ocular inflammatory service with steroid-refractory posterior uveitis suspicious of masquerade syndrome. Forty-five (45) eyes underwent *trans*-pars plana vitreous biopsy; additionally, five had aspiration of subretinal fluid and two eyes underwent retinochoroidal biopsies. Of these, seven (14%) showed cytopathological evidence of lymphoproliferative disease. Of the remaining patients, biopsy samples showed chronic inflammatory cells but no evidence of lymphoproliferative disease on cytological analysis and polymerase chain reaction (PCR) analysis for immunoglobulin heavy-chain gene or T-cell receptor. None of these patients developed progressive signs of lymphoma and responded with varying success to conventional immunosuppression for non-infectious uveitis.

In our study, there were two men and five women. Mean age was 67.7 ± 9.8 years (range: 47–76 years). Floaters and blurring of vision were reported in all patients and two patients were found to have bilateral involvement.

In five cases, diagnostic vitrectomies were performed within 6 months of presentation, but in two cases, patients were referred for further management only 1–2 years after their initial presentation and unsuccessful immunosuppression. Final diagnosis was of high-grade B-cell NHL in all cases.

Cytology

Of the patients with diagnosis of primary intraocular lymphoma (PIOL), the cytopathology laboratory received 11 vitreous fluid samples from the seven patients. Three patients had repeated samples: patients 2 and 3 had two samples each and patient 5 had three samples. Most samples were of clear fluid between 0.5 to 7.5 ml in volume.

After macroscopic description, 0.5 ml from both fresh and formalin-fixed specimens was prepared as cytospin preparation (300 r.p.m. for 5 min) and stained with Giemsa or haematoxylin–eosin stains, respectively. After decanting the remainder of the samples into labelled tubes they were centrifuged for 10 min at 2000 r.p.m. If the specimen was received fresh the deposit was fixed in 10% formal saline for at least 1 hour. A cell block preparation was made by paraffin embedding the deposit. Thirty serial sections were cut at 2–3 μm intervals and the first 10th 20th, and 30th sections were stained for haematoxylin–eosin. A pathologist assessed cellularity and chose the appropriate level of serial sections for immunostaining. A standard range of immunostains was used including CD3, CD20, CD79a, CD68, S100, and Ki67. Eight samples had adequate cellularity and successful immunostaining. Depending on cellularity in some cases additional immunostaining was also possible (CyclinD1, CD5, CD10, Bcl6).

In six cases, enough material was available for DNA extraction. A 10-μm section was cut and subjected to DNA extraction. A semi-nested protocol was used for PCR using primers for FR2 and FR3 regions of the immunoglobulin heavy chain gene and T-cell receptor gene as described previously. These tests were done with known positive and negative controls.[7] In one case PCR was unsuccessful as significant amount of DNA could be extracted.

Results

The cytospin preparations showed variable cellularity but in all cases there was predominance of lymphoid cells. In six cases, there were numerous atypical medium-sized or large lymphoid cells seen. In case 7, only small lymphocytes and histiocytes were seen. Cellblock preparations also varied in cellularity and one sample in case 3 and two samples in case 5 were acellular. Further samples in these two cases were successful and data in Tables 1 and 2 refer to these specimens and the patient characteristics.

Immunostaining in cases 1–6 showed predominance of CD20 and CD79a positive B-cells with occasional CD3-positive T cells and CD68-positive histiocytes in the background. In case 7 the lymphoid population was predominantly CD3-positive T cells with a few CD20-positive B cells. PCR analysis showed a strong band with FR2/FR3 region primers indicating B-cell monoclonality in five cases, including case 7. All cases were reported as intraocular B-cell NHL. Case 2 had a previous history of testicular seminoma, which on subsequent review, following the diagnosis of NHL in the vitreous sample

Table 1 Clinical features of cases

Case	*Age*	*Gender*	*Laterality*	*Symptoms*	*VA (OD)*	*VA (OS)*	*Signs*	*Time to diagnosis (diagnostic vitrectomy)*
1	75	F	OS	Floaters, BOV	(6/9)	**6/18**	Vitritis	1 month
2	71	M	OU	Floaters, BOV (history of 'testicular seminoma' in 1999)	**6/9**	**6/36**	Vitritis	2 years
3	71	F	OU	Floaters, BOV	**6/24**	**6/24**	Vitritis	4 months
4	47	F	OD	BOV	**6/18**		Vitritis	2 months
5	68	F	OS	Floaters, BOV	(6/9)	**NLP**	Vitritis, exudative RD	1 week
6	76	F	OD	BOV	**LP**	(6/9)	Granulomatous uveitis	1 year
7	66	M	OD	BOV	**HM**	(6/7.5)	Haemorrhagic disc swelling, exudative RD	6 months

BOV, blurring of vision; LP, light perception only; NLP, no light perception; OD, right eye; OS, left eye; RD, retinal detachment; VA, visual acuity. Visual acuity in parenthesis and non bold refers to vision in fellow *unaffected* eye.

Table 2 Pathological features and clinical outcomes of cases.

Case	*Cytospin*	*Cell block*	*PCR*	*Immunocytochemical* ***Profile***	*Final VA* ***(OD)***	***Final VA (OS)***
1	Few atypical cells and histiocytes	Mononuclear lymphoid blasts	Not performed	CD20+, CD79a+, CD3−, CD5−, CD10−, CD23−, cyclin D1−, CD138−, CD68−	(6/12)	**6/12**
2	Numerous lymphoid cells, few histiocytes	Mononuclear lymphoid blasts	B-cell monoclonality	CD20+, CD3−, PLAP−, CD68−	**6/9**	**6/9**
3	Numerous lymphoid cells, few histiocytes	Sparsely cellular, medium-sized lymphoid cells	Unsuccessful	CD20+, CD3−	**6/9**	**6/6**
4	Numerous lymphoid cells and histiocytes	Medium-sized lymphoid cells	B-cell monoclonality	CD20+, CD79a+, CD3−	**6/9**	(6/12)
5	Mononuclear lymphoid blasts	Mononuclear lymphoid blasts	B-cell monoclonality	CD20+, ki67+, Bcl6−	(6/9)	NLP
6	Mononuclear lymphoid cells and histiocytes	Mononuclear lymphoid blasts	B-cell monoclonality	CD20+, ki67+, CD3−	CF	(6/9)
7	Small lymphocytes and histiocytes	Small lymphocytes and histiocytes	B-cell monoclonality	Mixture of CD3+ T-cells and CD20+ B-cells	**NA**[a]	NA[a]

CF, counting fingers only; NLP, no light perception.
[a]NA: not available. Patient deceased before review. Visual acuity in parenthesis and non bold refers to vision in fellow *unaffected* eye.

was found also to be high-grade B-cell NHL. This was therefore interpreted as involvement of vitreous in a systemic high-grade NHL.

Discussion

Primary intraocular lymphoma is one of the most difficult and challenging diagnosis clinically and pathologically. Diagnostic delay is due to limits of a cytological diagnosis. A high index of clinical suspicion and cytological examination of the vitreous fluid aided by PCR and immunohistochemistry is the gold standard for early and accurate diagnosis.

The most common ocular presentation of intraocular lymphoma is that of floaters and decreased vision, and the commonest mis-diagnosis is chronic vitritis (masquerade syndrome) that responds partially to steroid therapy. We describe a reproducible and sensitive cytopathological assessment facilitating diagnosis and reducing difficulties with interpretation. Although cerebrospinal fluid (CSF) and stereotactic brain biopsy can be useful, ocular onset occurs before CNS signs and 60–80% patients with PIOL develop CNS symptoms within a mean of 29 months.[8] CSF can provide clearer cytological assessment than vitreous biopsy but often provides fewer cells for examination. Intraocular lymphomas are rare and the vast majority of them are high grade and classified as diffuse large B-cell non-hodgkin's lymphoma.[9] Distinction from inflammatory conditions is difficult as the cellularity of the vitreous

fluid is increased in infections and chronic inflammatory conditions with mature lymphocytes and histiocytes. These are predominantly of T-cell lineage. Therefore identifying atypical lymphoid cells of B-cell lineage is crucial for the diagnosis of PIOL. Diagnostic vitrectomy is the method preferred for cytological diagnosis but its usage and cellular yield may be limited by previous steroid use, handling delays, and cytopathologist's skill.[10] Coupland *et al*[11] reported the use of specialized HOPE-fixative media (Herpes-glutamic acid buffer mediated organic solvent protection effect) to facilitate transport of the specimen while being able to preserve the cytomorphology, immunoreactivity, and DNA of the cells. In cases with a high index of suspicion for primary intraocular lymphoma, diagnostic vitrectomy should be performed early to avoid the lympholytic effects of prolonged steroid treatment.

Tissue collection is safe via both vitreous tap and vitrectomy, although the latter is preferred because cellular yields are higher and less vitreoretinal traction is exerted.[12,13] Char *et al*[14] showed a greater loss of cellular detail with vitrectomy than vitreous tap. However, it was unclear if this was due to the cutting instrument or the duration spent in the cassette in basic salt solution (BSS). An experimental model by Conlon *et al*[15] showed that vitrectomy does not cause any more cellular degradation compared to simple aspiration. Our methods involve a vitrectomy cutter attached to a 10 ml syringe to extract the initial undiluted sample. This, plus the subsequent 30 min of the cassette aspiration are sent to the laboratory where a preinformed cytology technician awaits. Although Whitcup *et al*[5] reported that the addition of tissue culture medium into both the syringe and the vitrectomy machine reservoir assisted preservation of cytological detail compared with the use of BSS alone. We find that modern techniques of immunocytochemistry no longer require fresh tissue media, and therefore prefer immediate placement in normal saline as soon as the sample is removed from the eye to prevent cell degradation. Importantly, alcohol fixation may jeopardize the identification of PIOL cells in the vitreous sample.[15]

We have designed a standardized method to process vitreous fluid specimens. Processing of fluid samples as a cellblock is now a well-established technique in cytology laboratories. The main advantage of the cellblock technique is that it can be used for special stains, immunocytochemical studies, and PCR. This technique to our knowledge has not been used for the vitreous samples, which by nature are of low volume and low cellularity.

Malignant lymphoid cells are usually discohesive, large, pleomorphic cells with increased nucleo-cytoplasmic ratios and prominent nucleoli (Figure 1a). Despite these fairly characteristic HE/Giemsa appearances the paucity of these malignant cells can make the diagnosis very difficult, especially when reactive lymphocytes, histiocytes, and necrotic debris predominate the picture. In these cases, accurate assessment on morphological grounds (HE/Giemsa) alone can be very difficult even for an experienced cytopathologist. Immunocytochemistry is a widely available adjunct to the diagnostic repertoire in vitreous samples. Immunocytochemistry for markers of B-cell phenotype (CD20, CD79a) is very useful. Atypical lymphoid cells of B lineage are almost diagnostic of intraocular lymphoma (Figure 1b). Positive staining for Ki67 also contributes to a positive diagnosis. Most samples are graded as high-grade based on blastic morphology and Ki67 expression. Diagnosis of metastatic carcinoma and malignant melanoma was routinely excluded by staining for pan cytokeratin and melanoma cocktail.

Demonstration of monoclonality in lymphomas is of great importance in distinguishing low-grade lymphomas from reactive lymphoid lesions. Flow cytometry and DNA extraction have been performed in CSF samples for diagnosis of lymphoma.[16] Other workers, such as White *et al*[17,] have also used similar methodology and extracted DNA from vitreous samples for PCR analysis of gene rearrangements in PIOL for diagnosis of T-cell NHL. Lobo *et al*[18] also reported that the use of the combination of primers for FR2, FR3, and t(14;18) translocation of the bcl-2 gene was more powerful than conventional cytopathological methods in differentiating between lymphomatous and inflammatory cells.

Diagnosis of lymphoma and its accurate classification according to the WHO guidelines requires detail morphological, immunohistochemical, and molecular analysis.[19,20] This is challenging in a vitreous sample and significance and clinical implications of PIOL diagnosis is immense. We therefore undertake this analytical approach for diagnosis to increase our chance of providing a reliable diagnosis.

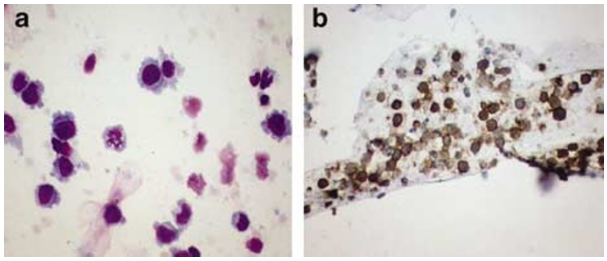

Figure 1 (a) Numerous large atypical lymphoid cells. Giemsa stain $\times 400$. (b) Predominance of large CD20-positive B cells. CD20 immunostaining $\times 400$.

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