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Flow Cytometry in the Diagnosis of Lymphoproliferative Lesions of the Orbit and Eye Adnexa in Fine Needle Aspiration Biopsy*

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The goal of the investigation was to evaluate the validity of routine cytology and flow cytometry in the differential diagnosis of lymphoproliferative disorders of the orbit and eye adnexa. The investigations were carried out on materials originating from fine needle aspiration biopsy performed in 14 patients, including 9 females and 5 males aged 31 - 81 years. Apart from routine cytology, cytometric studies were also performed. Based on cytology, non-Hodgkin's lymphomas were diagnosed in six patients, while one was suspected of NHL. In seven patients the diagnosis was ambivalent, since based on cytology it was impossible to conclusively determine the biological character of the lesion, i.e. state whether it was benign or malignant. Flow cytometry was performed in 14 patients, but ultimately the results were available in 12 individuals, since in two cases the material was scant enough to exclude any assessment. Thanks to using a panel of monoclonal antibodies against light chains κ and λ, as well as against CD surface antigens, the authors demonstrated clonality in 90% (9/10) of NHL cases; of this number, in 7 instances the test confirmed the preliminary diagnosis and in 2 cases rendered the diagnosis more precise. On the other hand, in 3 cases no clonality was noted; of this number, in two instances the diagnosis was specified as a benign lesion (BLPL) and in one case the assessment of clonality had no impact on the final diagnosis. Out of 12 investigated aspirates, in 11 cases the result concerning clonality affected the final diagnosis. The evaluation of cellular phenotype in flow cytometry in materials obtained in the course of FNAB is a fast and sensitive method and in many cases allows for avoiding a surgical biopsy.

Introduction

Benign and malignant lymphoproliferative lesions and pseudotumors are among the most common primary tumors of the orbit or the ocular adnexa [3, 13, 14, 22]. These diseases have a very similar clinical course. The patients do not differ as to their age, sex, complaints, duration of symptoms and the ocular lesions themselves [5, 6, 17]. Microscopically, the lesions are also similar and hence it is difficult to differentiate between these conditions based on the morphology, especially while differentiating between benign lymphoproliferative lesions and chronic lymphomas composed of small lymphocytes [5].

Despite the fact that fine needle aspiration biopsy (FNAB) is a commonly employed method in modern oncological diagnostic management, it has found no extensive use in diagnosing lymphoproliferative lesions of the eye and ocular adnexa. Apart from several publications prepared by the Szczecin center [7, 20, 21], where FNAB was employed to diagnose lesions of the eye as early as in the beginning of the eighties, there are almost no reports on the subject in the Polish literature. Although Gierek et al. [16] mentioned the use of FNAB in ten patients with orbital tumors, but the authors provided no information as to the character of such lesions. Only in four reports [4, 12, 24, 25] were data obtained by FNAB used for further flow cytometry studies. Dunphy et al. [12] collected 73 aspirates, including 68 samples aspirated from extranodal sites of lymphocyte proliferation, all of them resulting from biopsies of 15 variously situated sites, while Meda et al. [24] investigated 290 aspirates, including 165 collected from variously located extranodal lymphoproliferative infiltration sites. In both reports the authors failed to present specific data as to the number of cases involving the orbit and the orbital adnexa. Char et al. [4] performed 49 biopsies, including 31 procedures targeted to orbital lymphoproliferative lesions. Nassar et al. [25] evaluated the immunophenotype of lymphoid cells using a flow cytometer only in a single case of 43 investigated samples.

The present authors have resolved to assess the validity of routine cytology and flow cytometry in the differential diagnosis of lymphoproliferative diseases of the ocular adnexa.

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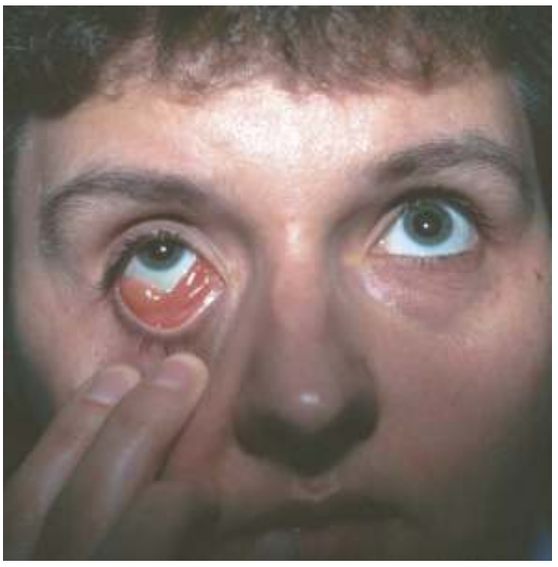

Fig. 1. Benign lymphoproliferative lesion in the inferior fornix of the left eye (N.W., patient No 1).

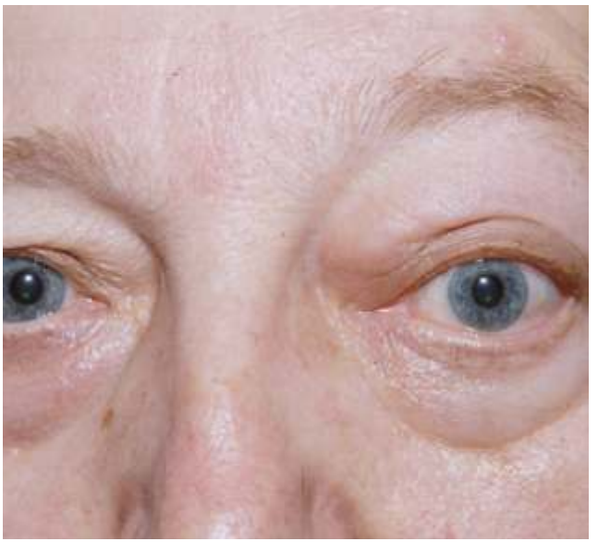

Fig. 2. A lymphoma involving the medial canthus of the left orbit (G.T., patient No 7).

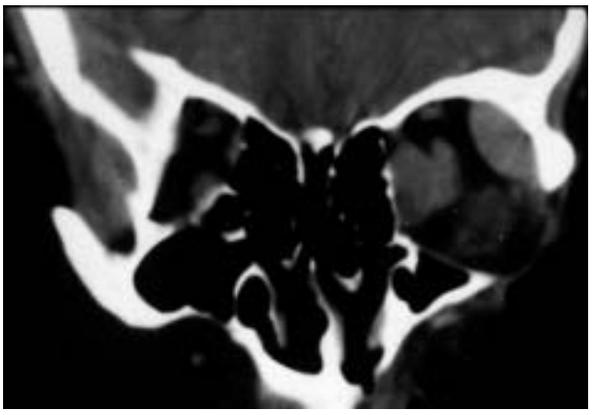

Fig. 3. CT scan - frontal plane: two lymphoma infiltration foci seen on the left, involving the superior temporal orbit (in the vicinity of the lacrimal gland) and in the medial part of the orbit (W.J., patient No 5).

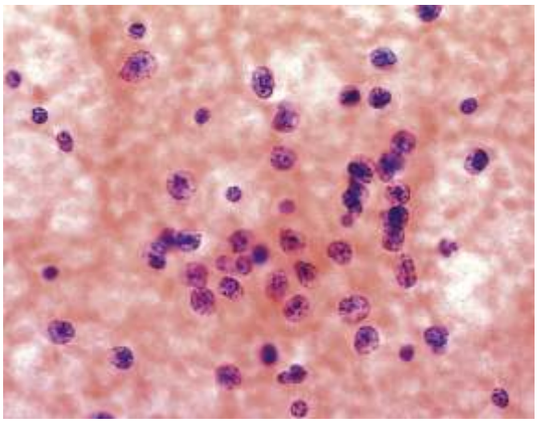

Fig. 4. Orbital tumor cytology (G.T. patient No 7, same as in Figure 2). HE.

Material and Methods

The study material consisted of cellular samples collected in the course of fine needle aspiration biopsies from 14 patients, including 9 females and 5 males aged 31 - 81 years of life, who were either treated or consulted in the Out-patient Department and Chair and Department of Ophthalmology, Pomeranian Medical University in Szczecin.

Eight patients reported with orbital or palpebral-orbital lesions, while four presented with palpebral lesions and two with lesions involving the conjunctiva (Fig. 1). In eight patients the lesion involved the orbit or orbital adnexa on the right side, while five patients showed such lesions on the left side (Fig. 2), and two had bilateral lesions. In six patients the disease had a sudden onset. All the patients were subjected to a fine needle aspiration biopsy; in four of them the procedure was performed under a CT control (Fig. 3).

From the FNAB-obtained material at least two smears were prepared and immediately fixed in 96% ethyl alcohol and subsequently stained with hematoxylin and eosin.

The cytological material for further cytometric studies was obtained by rinsing the aspiration needle following a puncture or punctures, and usually from an additional tap. The resultant material was placed in PBS and subsequently condensed through centrifuging for 5 minutes at 1500rpm. The material was subsequently transferred to test tubes and incubated with antibodies in a darkroom at ambient temperature for 30 minutes. To each 50µl of the cell suspension 10µl of the antibody were added in the case of single antibodies

TABLE 1

Tests results for the entire group of patients

Patient No and initials	Age/sex	Clinical presentation	Tests			Final diagnosis
			cytology	histopathology	flow cytometry	
1 N.W.	31/F	Lesion in the inferior conjunctival fornix, RE	NHL?, BLPL?	NHL s	no clonality	BLPL
2 A.S.	81/M	Small tumors involving the upper surface of LE, blepharoptosis, proptosis, double vision, abnormal LE mobility	NHL?, BLPL?	Pseudotumor	too scant material	Pseudotumor
3 N.Z.	69/F	A tumor involving the upper surface of RE	NHL?, BLPL?	BLPL	no clonality	BLPL
4 B.Z.	64/F	A tumor involving RE, proptosis (RE)	NHL?, BLPL?	NHL, MALT	no clonality	p-NHL-MALT
5 W.J.	82/M	Blepharoptosis (LE), double vision	NHL?, BLPL?		clonality	p-NHL-B-hg
6 P.A.	68/M	Tumors involving upper eyelids (RE and LE), eyelid edema	NHL-lg		clonality	p-NHL-B-lg
7 G.T.	62/F	A tumor involving the upper lid (LE), proptosis (LE)	NHL s		clonality	p-NHL-B-lg
8 G.Ł.	77/F	A conjunctival tumor surrounding the eyeball (RE)	NHL?, BLPL?	NHL-FL	clonality	p-NHL-FL
9 K.M.	76/M	A tumor involving the right eyelid and orbit (RE)	NHL (LP)		clonality	p-NHL-LP
10 J.M.	43/F	A tumor involving the lacrimal caruncle and conjunctival fornix (RE).	NHL?, BLPL?	NHL-MALT	clonality	p-NHL-MALT
11 B.K.	53/F	A tumor involving the internal eye canthus and lower eyelid (LE)	NHL		clonality	p-NHL-B-lg
12 N.Z.	63/M	Infiltration involving the left orbit and maxillary sinus (LE)	NHL	NHL-hg	too scant material	NHL-B-hg
13 G.B.	64/F	A tumor involving the internal eye canthus (RE)	NHL-hg		clonality	NHL-B-hg
14 J.M.	53/F	Infiltration involving all eyelids (RE and LE)	NHL		clonality	NHL-B

(e.g. CD22 PE), while 5µl of the antibody were added in the case of double antibodies (e.g. CD10FITC/CD5PE). Following the incubation, the cells were rinsed twice through the addition of 1ml PBS to remove the excess of antibodies, stirred and centrifuged for 5 minutes at 1500rpm. Subsequently, the supernatant was poured out and PBS was added to the cells. Following the final centrifuging, the cells were fixed in 0.5ml 1% buffered formalin solution. The thus prepared cells were stored at the temperature of 4˚C and analysed in a flow cytometer (FACS Calibur, BECTON DICKINSON) within 24 hours.

The following antibodies were employed: G1 FITC/G1 PE, KAPPA FITC/LAMBDA PE, CD3 FITC/CD19 PE, CD45 FITC/CD14 PE, CD10 FITC/CD5 PE, CD20 FITC/CD23 PE, CD4 FITC/CD8 PE, CD22 PE, CD2 FITC, CD16 + 56 PE.

Results

Routine cytological tests were performed in all the patients. Table 1 presents the clinical data, cytology and flow cytometry results. The final diagnosis was established based on cytology results, cell immunophenotype determined by flow cytometry and in some cases on histopathology. Routine cytology indicated a non-Hodgkin's lymphoma in six patients and a suspicion of non-Hodgkin's lymphoma in one (Fig. 4). In seven patients the diagnosis was ambivalent, since cytology did not allow for a firm diagnosis of a benign or malignant lesion. Material for histopathology was also collected from seven individuals. Of six non-Hodgkin's lymphoma cases diagnosed by routine cytology, only in a single was the type of lymphoma determined. The patient had lymphoplasmocytic lymphoma, and the presence of plasma cells and plasmablasts facilitated the diagnosis.

Flow cytometry was employed in 14 patients, but final results were obtained only in 12 individuals (Table 2). In two cases the material was too scant to allow for evaluation. In five patients the preliminary cytological diagnosis was inconclusive: non-Hodgkin's lymphoma (NHL?) or benign lymphoproliferative lesion (BLPL?), two other were suspected of non-Hodgkin's lymphomas. Due to a small amount of material available, in two individuals the sole tests performed were based on antibodies against λ and κ light chains of immunoglobulins. A greater number of reactions were performed in the remaining patients in order to determine the phenotype of lymphoid cells. In two individuals

TABLE 2

Cellular phenotype assessment in flow cytometry

Patient No and initials	Cytological diagnosis	κ %	λ %	κ:λ	CD2 %	CD3 %	CD4 %	CD5 %	CD8 %	CD10 %	CD19 %	CD20 %	CD22 %	Final diagnosis
1 N.W.	NHL?, BLPL?	27.7	12.2	2.27		21.9					33.6			BLPL
3 N.Z.	NHL?, BLPL?	3.16	1.66	2		18.21					6.58	1.47		BLPL
4 B.Z.	NHL?, BLPL?	0.09	0.60	0.15		0.12	0.06					0.10		p-NHL-MALT
5 W.J.	NHL s			14.53		4,23					92.28	36.1		p-NHL-B-hg
6 P.A.	NHL-lg	1.42	78.9	0.017	13.74	12.2					83.0	80.44		p-NHL-B-lg
7 G.T.	NHL s	2.08	0.29	7.17		2.41		2.54		0	2.01	3.37		p-NHL-B-lg
8 G.Ł.	NHL?, BLPL?	2.16	32.84	0.066		6.76	6.08	31.96	3.54	9.54	39.09	48.62		p-NHL-FL
9 K.M.	NHL-LP	0.18	18.53	0.097		4.23					92.28			p-NHL-LP
10 J.M.	NHL?, BLPL?	13.86	3.12	4.44		23.4		20.2			18.49			p-NHL-MALT
11 B.K.	NHL	16.99	1.5	11.32		6.49		18.88			49.93		59	p-NHL-B-lg
13 G.B.	NHL-hg	0.36	90.59	0.004		3.75	3.62	4.95	44.10	0.09	92.69	51.79		NHL-DLBCL
14 J.M.	NHL			7.03										NHL-B

TABLE 3

Cytological diagnosis vs. clonality assessment in flow cytometry

Cytological diagnosis	Clonality assessment		Final diagnosis
	+	–	
NHL	6		NHL
NHL s	1		NHL
NHL?, BLPL?	2	1*	NHL
NHL?, BLPL?		2	BLPL

* patient B.Z. - No 4

TABLE 4

The effect of clonality on final diagnosis

Cytological diagnosis	No of cases where the final diagnosis was affected by clonality assessment			Final diagnosis
	confirmed	specified	exerted no effect	
NHL	6			NHL
NHL s	1			NHL
NHL? BLPL?		2	1*	NHL
NHL? BLPL?		2		BLPL

*patient B.Z. - No 4

Abbreviations for Tables:
BLPL - benign lymphoproliferative lesion; NHL - non-Hodgkin's lymphoma; NHL s - non-Hodgkin's lymphoma suspected; p-NHL - primary non-Hodgkin's lymphoma; NHL-B - B-cell non-Hodgkin's lymphoma; NHL-lg - low-grade non-Hodgkin's lymphoma; NHL-hg - high-grade non-Hodgkin's lymphoma; NHL-FL - follicle center cell lymphoma; NHL-LP - lymphoplasmocytic lymphoma; LE - left eye; RE - right eye

(G.B., No 13 and G.Ł., No 8) a total of 9 determinations could have been performed, while in the remaining patients the number ranged between 3 and 7. In two patients (No 1 and 3), in whom no firm diagnosis had been established by routine cytology, the ratio of κ/λ was found to be 2.27 and 2.0, respectively, what allowed for diagnosing a benign lesion (Figs. 5 and 6). In lymphoma cells originating from the remaining patients the κ/λ ratio ranged from 0.004 to 14.53. In the majority of cases these were primary B-cell non-Hodgkin's lymphomas of the eye, including two cases of MALT-type lymphomas, one - follicle center cell lymphoma and one lymphoplasmocytic lymphoma. Here, particular attention should be paid to the case of large B-cell lymphoma (a female patient G.B., No 13). The analysis of the FSC and SSC graphs, which constitute cellular morphology determinants (size and granularity), allowed for identifying a clearly separate population of cells that were significantly larger than lymphocytes, but their granularity was similar (Fig. 7, region R1); these cells accounted only for approximately 1.5% of the investigated cells. A further analysis of cellular material from this region demonstrated that these were almost exclusively CD19+, CD20+ and CD22+ lymphocytes (Fig. 8), and the clonality analysis of their capability of light chain production confirmed the neoplastic character of these cells, since cells with λ chains constituted 90.2% (Fig. 9).

Table 3 lists preliminary cytological diagnoses and clonality assessment achieved through flow cytometry. Table 4 presents the effect of clonality assessment on the final diagnosis. Based on flow cytometry, in four cases the diagnostics was rendered more precise, in seven patients it was confirmed, while in one case the result did not affect the ultimate diagnosis.

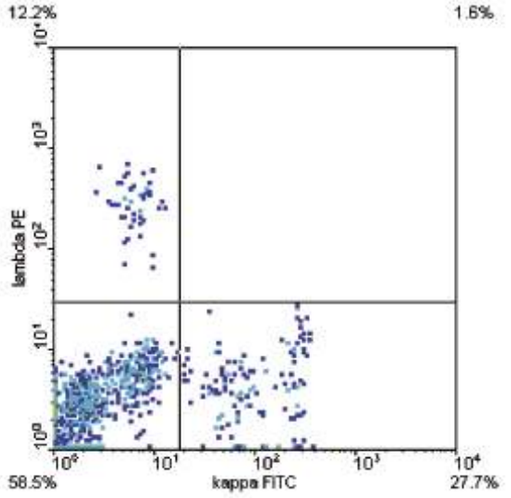

Fig. 5. Clonality assessment in flow cytometry, κ chains = 27.7%, λ chains = 12.2%, ratio κ/λ=2.27 (N.W., patient No 1, same as in Fig. 1).

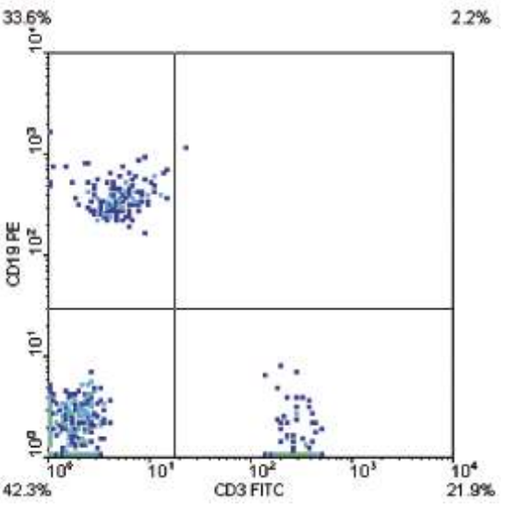

Fig. 6. Immunophenotype in flow cytometry. CD19 cells = 33.6%, CD3 cells = 21.9% (N.W. Patients No 1, same as in Figures 1 and 5).

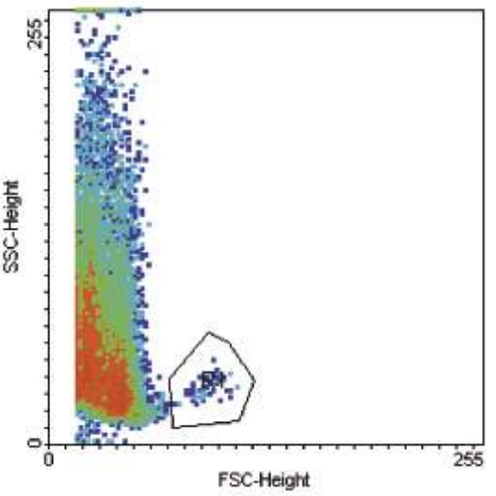

Fig. 7. The FSC/SSC graph in flow cytometry. Note the clearly separated population of lymphoma cells accounting for approximately 1.5% of all the analyzed cells (R1), (G.B., patient No 13).

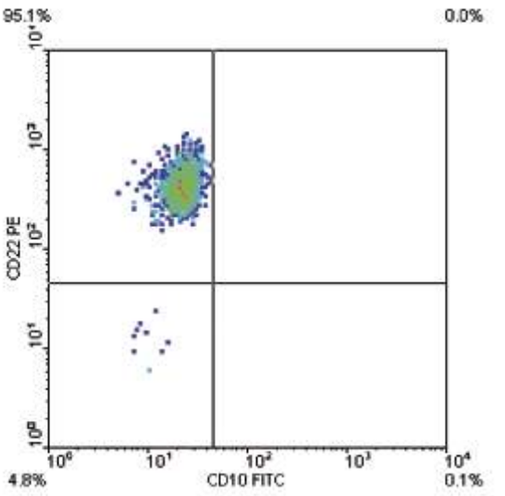

Fig. 8. Immunophenotype assessment in flow cytometry. CD22 cells = 95.1%, CD10 cells = 0.1% (G. B. patient, same as Figure 7).

Discussion

The results presented in the report confirm the opinion that in the diagnostic management of lymphoproliferative lesions routine cytology is not sufficient in establishing a firm diagnosis, especially in primary lesions of the ocular adnexa. In spite of the fact that in benign lymphocyte proliferation smears usually reveal cellular polymorphism associated with the presence of cells originating from germinal centers, while non-Hodgkin's lymphomas usually are monomorphic, with one cell type predominant in the entire cellular population [15, 19, 22], in many instances the morphological similarity of benign lymphoproliferative lesions and non-Hodgkin's lymphomas, especially when the latter are low-grade, is high enough to trigger diagnostic problems

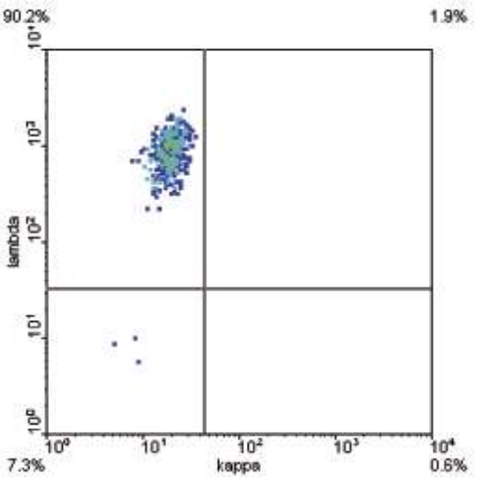

Fig. 9. Clonality assessment in flow cytometry. κ chains = 0.6%, λ chains = 90.2% (G.B., patient No 13, same as Figures 7 and 8).

not only in cytology, but also in some percentage of histopathological examinations.

The assessment of the possibility of establishing the final diagnosis by routine cytology has varied from author to author. Steel et al. [29[estimated the accuracy of the method as 72%, Zeppa et al. [33] - as 83%, Zajdela et al. [32] - as 87%, while Dey et al. [9] claimed a success rate of as much as 94%.

In our material routine cytology allowed for a preliminary diagnosis of non-Hodgkin's lymphoma in 6/11 cases (54.54%), with one patient being suspected of the disease. In seven patients (7/14 - 50%) the cytology was inconclusive (NHL? BLPL?). Although routine cytology is fast and simple, it is associated with quite a high percentage of inconclusive diagnoses. In addition, in our material only in one patient with non-Hodgkin's lymphoma we have been able to determine lymphoma type on the basis of cytology.

Immunophenotyping using a flow cytometer and monoclonal antibodies that is carried out simultaneously with cytology definitely increases the accuracy of a diagnosis in non-Hodgkin's lymphomas and lymphoproliferative lesions [1, 8, 12, 24]. To evaluate cells in flow cytometry a small amount of material is required and this is why collecting a sample by fine needle aspiration biopsy is sufficient. In our material only in two cases of 14 did we fail to obtain an adequate amount of material for cytometry. The method also allows for identifying small populations of abnormal cells that would be difficult to pinpoint by cytology alone [10, 11]. In our patient G.B. (No 13) it was possible to identify a population of neoplastic cells that constituted only 1.5% of all investigated cells. To immunophenotype cells using a flow cytometer one employs a panel of antibodies against the κ and λ light chains of immunoglobulins and against CD surface antigens which are specific for B or T cells [2]. The most often employed monoclonal antibodies against B-cell line surface antigens include: CD19, CD20, CD23, CD5, CD10, CD45, while antibodies against T-cell line antigens encompass CD2, CD3, CD4 and CD8 [2, 24, 26, 30, 31]. In accordance with the recommendations of the Clinical Cytological Society formulated during the ISAC 2000 Congress, the fundamental panel of antibodies for immunophenotyping lymphomas should include at least nine antibodies [2].

In view of the fact that the most common lymphomas of the eye and orbital adnexa include MALT-type lymphomas, followed by follicle center cell lymphomas (FC), diffuse large B-cell lymphomas (DLBCL), as well as mantle cell lymphomas (MCL) and small lymphocyte lymphomas (SLL) [6, 18, 23, 27], the most important antibodies for differential diagnosis are as follows: CD19, CD20, CD23, CD5, CD10, CD3, anti-κ and anti-λ.

Dunphy et al. [12] used the FNAB method to collect material from lymph nodes and various other extranodal sites in 73 cases of lymphoproliferative lesions and assessed the samples both by cytology and by flow cytometry. In 71% of cases they determined the final diagnosis and primary lymphoma type based on determining cellular phenotype in flow cytometry. In the remaining cases it was necessary to collect a specimen for histopathology. In secondary lymphomas the diagnosis was established in 80% of the cases. Dong et al. [10] investigated material obtained in the course of 139 fine needle aspiration biopsies of primary and relapsed lymphomas. The cytology-based diagnosis was established in 67% of cases, while in combination with flow cytometry, the percentage increased to 75.5% (105/139), the number included 82% cases of non-Hodgkin's lymphomas. In five patients with NHL flow cytometry yielded negative results. Young et al. [31] investigated 100 aspirates collected from 87 patients. In 80% of the cases the diagnosis was established based on FNAB and flow cytometry, without any need to obtain a specimen for histopathology. The latter two reports provided no information on whether the investigations have been carried out in lesions involving the eye. Davis et al. [8] employed flow cytometry in material obtained from fine needle aspiration biopsies of the eye. In seven cases of ten the lymphoma could have been diagnosed when routine cytology was combined with the assessment of cellular phenotype in flow cytometry, while only in 3/10 cases were the patients diagnosed based on cytology alone. Sharara et al. [28], who collected tissue samples from 43 patient with lymphoproliferative lesions of the eye, subjected these materials to histopathology combined with immunophenotype evaluation using flow cytometry and to PCR and found that while assessing clonality, the method of flow cytometry was proven to be of singular validity.

In our material flow cytometry was performed in 12 cases since in two cases the amount of material was insufficient to permit the assessment. These two patients suffered from a pseudotumor and BLPL, respectively. Thanks to employing a panel of monoclonal antibodies against light chains (κ and λ) and against CD surface antigens, clonality was demonstrated in 90% (9/10) of NHL cases; of this number in seven cases the preliminary diagnosis was confirmed and in two cases it was rendered more precise. In three cases no clonality was observed; of the three, in two instances the diagnosis was specified as a benign lesion (BLPL), while in one the assessment of clonality had no bearing upon the final diagnosis (histopathology revealed a MALT-type lymphoma in the patient). Out of 12 investigated aspirates, in 11 the result describing their clonality affected the final diagnosis.

Summing up, one may say that the evaluation of cell phenotype by flow cytometry in materials originating from fine needle aspiration biopsy is a fast and sensitive method, which in many cases may allow for avoiding a surgical biopsy. The usability of the method may be limited by inadequately scant amount of material, as it happened in two of 14 investigated cases.

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