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Primary malignant lymphoma of the brain: an immunohistochemical study of eight cases using a panel of monoclonal and heterologous antibodies

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Summary. Frozen sections of eight primary malignant lymphomas of the brain were examined by the immunohistochemical methods using a panel of monoclonal and heterologous antibodies to B lymphocyte (immunoglobulins, BA-1, Leu-12 and HLA-DR), T lymphocyte (OKT-11 and Leu-1) and monocyte (OKM-1) surface markers. Paraffin sections were also used in the examination of cytoplasmic immunoglobulins. Surface and/or cytoplasmic immunoglobulins (Ig) were observed in seven cases, four of which were shown to be distinctly monoclonal and the other three less so. The remaining 1 case showed no distinct staining for Ig. BA-1, Leu-12 and HLA-DR stainings were positive in four, four and five cases, respectively. The marker phenotypes of (BA-1, Leu-12, HLA-DR) were shown to be (+, +, +) in one lymphoma, (+, −, −) in three, (−, +, +,) in three, and (−, −, +) in one. Thus, it was demonstrated that the present lymphoma cases showed a marked immunological heterogeneity, and it was shown that all of them including the Ig-negative case revealed one or more of these three additional B cell markers, indicating B cell lineage of these cases. Examination of T cell and monocyte markers revealed positive staining in normal or reactive lymphoid cells distributed around blood vessels or sporadically in tumor tissues, but not in lymphoma cells. Epstein-Barr virus (EBV)-associated nuclear antigen was not demonstrated in the seven cases examined, making it unlikely that these lymphomas were related with EBV infection.

Key words: Malignant lymphoma – Brain tumor – Non-Hodgkin lymphoma – Burkitt's lymphoma – Immunohistochemistry

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Clinical and pathological studies have extensively been performed on the primary malignant lymphoma of the brain [17, 40]. Though less extensively, immunologic studies have also been carried out [6, 13, 16, 21, 22, 25, 27, 31, 36]. Among them, examinations of paraffin sections [13, 21, 31] have demonstrated cytoplasmic immunoglobulins in some of the lymphomas. Surface marker studies performed on cerebrospinal fluid (CSF) cells [6, 16, 22], cultured cells [25], single cells prepared from fresh tumor tissues [36] and fresh frozen sections [27] have also demonstrated immunoglobulins and some other B cell markers. These findings suggest that the primary malignant lymphomas of the brain are usually of B cell lineage. However, the findings hitherto obtained, especially those of surface markers in frozen tissue sections, are still scanty and more extensive studies are required for a better understanding of the immunological nature of this tumor.

In the present study, surface markers in a total of eight primary malignant lymphomas of the brain (diffuse large cell lymphoma) were examined by the immunohistochemical method using a panel of monoclonal and heterologous antibodies to B and T lymphocyte and monocyte antigens. Epstein-Barr virus associated nuclear antigen (EBNA) was also examined in seven cases.

Materials and methods

Tissue specimens

Both biopsied and autopsied lymphoma tissues were used. Each tissue had been stored at −80°C until used. Formalin-fixed, paraffin-embedded tissues were also used in some experiments. Pathological diagnosis was made on the basis of routinely stained paraffin sections. According to LSG [30] and WF [32] classifications, all the tumors were diagnosed as diffuse large cell lymphoma.

Antibodies

Antibodies used in surface marker studies are listed in Table 1. Their reactivity and sources are indicated. While antibodies to

Table 1. Antibodies and their reactivity

Antibody to	Antigen (molecular weight)	Main expression	Other expression
(Heterologous)			
μ[a]	μ heavy chain	Normal and neoplastic B lymphocyte	
γ[a]	γ heavy chain	Normal and neoplastic B lymphocyte	
δ[a]	δ heavy chain	Normal and neoplastic B lymphocyte	
κ[a]	κ light chain	Normal and neoplastic B lymphocyte	
λ[a]	λ light chain	Normal and neoplastic B lymphocyte	
(Monoclonal)			
BA-1[b] [1]	(30 kDa)	Normal and neoplastic B lymphocyte	granulocyte
Leu-12[c] [23]		Normal and neoplastic B lymphocyte	
HLA-DR[d]	(28 kDa, 34 kDa)	Normal and neoplastic B lymphocyte	monocyte, macrophage, activated T cell, some neoplastic T lymphocyte, some epithelial cell.
OKT-11[e] [38]	E rosette receptor (50 kDa)	Normal and neoplastic T lymphocyte	
Leu-1[c] [5]	(67 kDa)	Normal and neoplastic T lymphocyte	Some neoplastic B lymphocyte
OKM-1[e] [3]		Monocyte, acute myelomonocytic leukemia cell	Granulocyte

[a] Purchased from Behring (FRG)
[b] Purchased from Hybriteck (USA)
[c] Purchased from Becton Dickinson (USA)
[d] Purchased from Cappel (USA)
[e] Purchased from Ortho (USA)

human immunoglobulins (μ, γ, δ, κ, λ) were of rabbit origin, all the others (antibodies to BA-1, Leu-12, HLA-DR, OKT-11, Leu-1 and OKM-1) were mouse monoclonal antibodies. Reactivity of these antibodies had been examined by the indirect immunoperoxidase method using fresh frozen sections of normal lymph nodes.

Secondary antibodies used in the indirect immunoperoxidase method were horseradish peroxidase (HRP)-labeled goat anti-rabbit γ-globulin (EY Laboratory, San Mateo, USA) and HRP-labeled goat anti-mouse γ-globulin (EY Laboratory, San Mateo, USA). The secondary antibodies had been absorbed with normal human serum.

Immunohistochemical methods

Surface marker studies were performed by the indirect immunoperoxidase method [20]. Cryostat sections, 6 μm in thickness, were made from frozen lymphoma tissues, attached to 0.5% neoprene-coated slide glass [11] and fixed in acetone for 10 min. In the studies of surface immunoglobulins, the sections were incubated with rabbit anti-human immunoglobulins (μ, γ, δ, κ, λ) diluted 1:80 to 1:400 for 1 h in the primary reaction and with HRP-labeled goat anti-rabbit γ-globulin diluted 1:20 for 1 h in the secondary reaction. In the studies of other surface markers, the sections were incubated with mouse monoclonal antibodies (antibodies to BA-1, Leu-12, HLA-DR, OKT-11, Leu-1 and OKM-1) diluted 1:40 for 3 h in the primary and with HRP-labeled goat anti-mouse γ-globulin diluted 1:20 for 1 h in the secondary reaction. Each antibody was diluted with normal goat serum. Sections were then incubated in 3,3′-diaminobenzidine tetrahydrochloride-H_2O_2 (DAB-H_2O_2) solution [20] for 20 min to visualize peroxidase reaction. Each reaction was performed at room temperature. In the control, normal rabbit serum or normal mouse serum was used instead of the primary antibodies.

In some experiments, sections were also examined by the biotin-avidin-peroxidase method [14]. Sections reacted with the primary mouse antibodies described above were incubated with biotinylated-horse anti-mouse γ-globulin (Vector, Burlingame, USA) diluted 1:50 for 30 min and then with avidin-biotin-peroxidase complex (Vector, USA) diluted 1:300 for 30 min. Peroxidase reaction was as described above.

In addition to these surface marker studies, immunoglobulin stainings of paraffin sections were also examined. Deparaffinized sections were pretreated with 0.3% H_2O_2-methanol for 1 h and with normal goat serum for 16 h. The sections were then examined by the indirect immunoperoxidase method as described above.

Examination of EBNA

EBNA [29] was examined by the anticomplement immunoperoxidase method which was a modification of anticomplement immuno-fluorescence method [19, 29], differing in that HRP-labeled rabbit anti-human complement was used instead of a fluorescein-labeled one. Frozen lymphoma sections were fixed in carbon tetrachloride for 10 min, reacted with a mixture (1:1) of heat-inactivated anti-EBNA-positive human serum (Wako, Tokyo, Japan) and 1:5 diluted fresh anti-EBNA-negative human serum as a source of complement for 30 min at room temperature and for further 1 h at 37°C. The sections were then reacted with HRP-labeled rabbit anti-human C_3c (DAKO, Copenhagen, Denmark) diluted 1:20 for 1 h at room temperature. The sections were incubated in DAB-H_2O_2 solution for 20 min. Each dilution was performed with balanced salt solution, pH 6.9 (136.9 mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$-7 H_2O, 4.4 mM KH_2PO_4 and 3.4 mM Na_2HPO_4-12 H_2O).

In the control, heat-inactivated anti-EBNA-negative human serum was used in the primary incubation instead of anti-EBNA-positive serum. As an antigen control, EBNA slide (Wako, Japan) of Raji cells was used.

Results

Examination of lymphocyte markers

The antigenic profiles of eight cases of primary malignant lymphoma of the brain are shown in Table 2 and

Table 2. Surface marker expression in eight primary malignant lymphomas of the brain. Frozen sections of lymphoma tissues were stained by the immunohistochemical methods using monoclonal and heterologous antibodies to B (Ig, BA-1, Leu-12, HLA-DR) and T (OKT-11, Leu-1) lymphocyte and monocyte (OKM-1) markers. Results of Epstein-Barr virus-associated nuclear antigen (EBNA) staining are also shown

Case No	Ig	Surface marker						EBNA
		BA-1	Leu-12	HLA-DR	OKT-11	Leu-1	OKM-1	
1	$\mu\,\lambda$ (sc)	+	+	+	−	−	−	−
2	$\mu\,\kappa$ (s)	+	−	−	−	−	−	nt
3	$\mu\,\kappa$ (s)	+	−	−	−	−	−	−
4	$\mu\,\kappa$ (c)	−	+	+	−	−	−	−
5	$\gamma\,\kappa^a$ (c)	+	−	−	−	−	−	−
6	$\mu\,\kappa^a$ (s)	−	+	+	−	−	−	−
7	$\mu\,\gamma\,\kappa\,\lambda^a$ (sc)	−	−	+	−	−	−	−
8	−	−	+	+	−	−	−	−

[a] Monoclonality was equivocal
+: Immunostaining was distinct; −: immunostaining was not distinct
Ig: Immunoglobulins; s: surface; c: cytoplasmic; nt: not tested

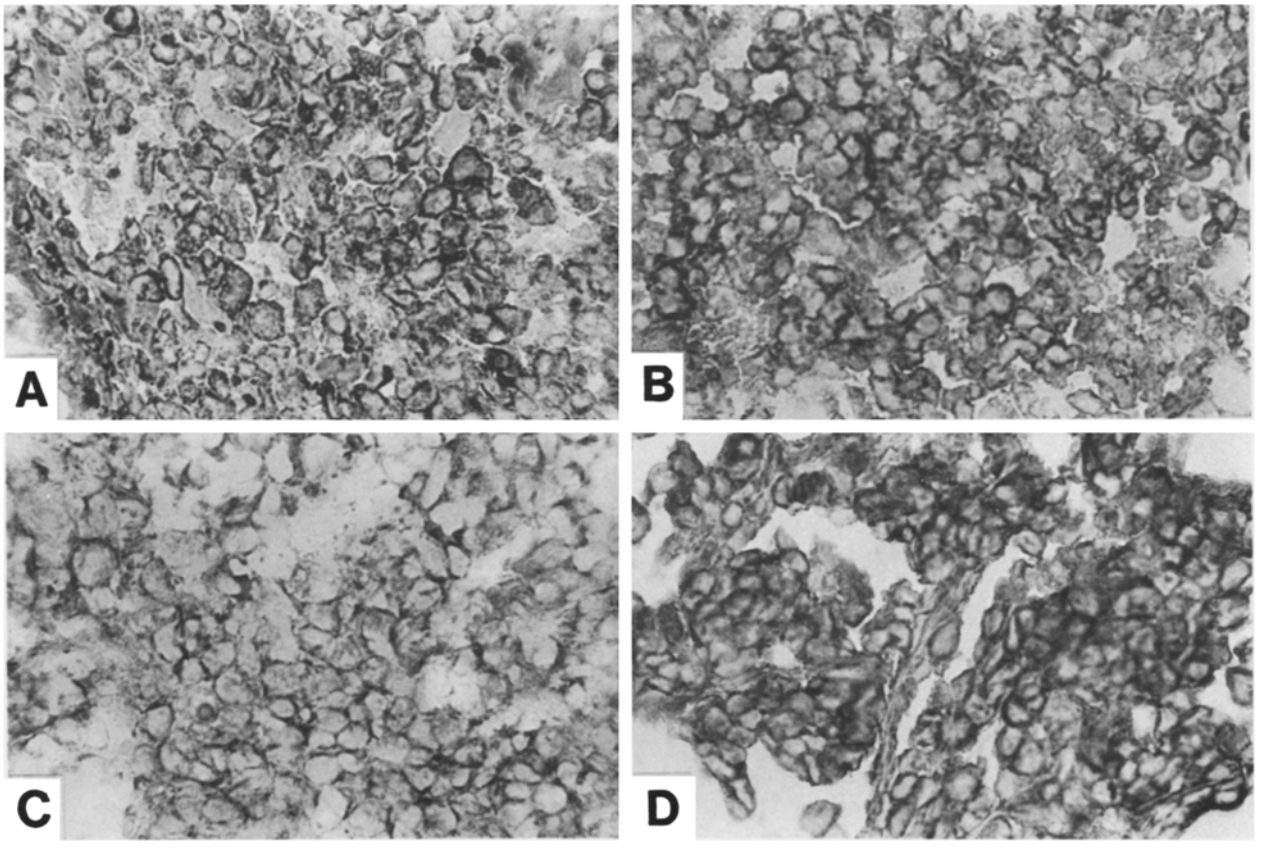

Fig. 1 A–D. Representative immunostainings for B cell surface markers in the primary malignant lymphomas of the brain. Note distinct staining mainly on the surface of neoplastic cells in each immunostaining. **A** Immunoglobulin (μ) staining (case 3). **B** BA-1 staining (case 3). **C** Leu-12 staining (case 8). **D** HLA-DR staining (case 1). Indirect immunoperoxidase method (**A**, **B** and **D**) and avidin-biotin-peroxidase method (**C**); no counterstaining. Frozen sections, × 500

representative immunostainings are depicted in Figs. 1 and 2.

Examination of immunoglobulins was performed by the use of both frozen and paraffin sections to compare the findings of surface immunoglobulins in the former with those of cytoplasmic immunoglobulins in the latter sections. Seven of the eight lymphomas revealed positive stainings for surface

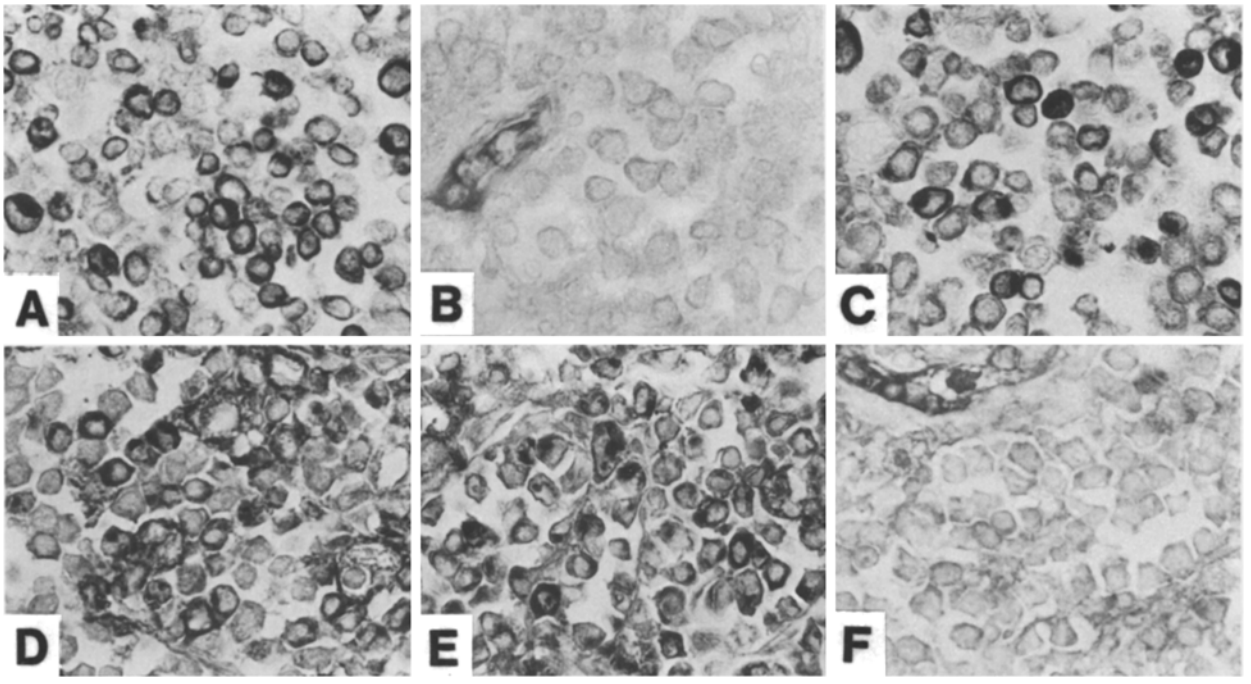

Fig. 2 A – F. Stainings for cytoplasmic immunoglobulins. **A – C** Cytoplasmic μ (**A**) and λ (**C**), but not κ (**B**), immunoglobulins are stained in one case (case 1) in which surface immunoglobulins are also μ λ (Table 2). **D – F** Cytoplasmic μ (**D**) and κ (**E**), but not λ (**F**), immunoglobulins are stained in the other case (case 4) in which surface immunoglobulins are not demonstrated (Table 2). In **B** and **F**, positive stainings are observed only in blood vessels. Indirect immunoperoxidase method using antibodies to immunoglobulins diluted 1:80 (**A – C**) or 1:400 (**D – F**); no counterstaining. Paraffin sections, × 500

and/or cytoplasmic immunoglobulins as shown in Table 2. Among them, four revealed distinct monoclonality concerning heavy and light chains, of which two showed surface μ κ, one showed surface and cytoplasmic μ λ and one showed cytoplasmic μ κ immunoglobulins. Two other lymphomas also showed stainings for immunoglobulins; γ κ mainly in the cytoplasm in one and μ κ mainly on the cell surface in the other. However, though less intensely, these cases showed staining for another light chain (λ) as well. The remaining case showed μ γ κ λ stainings on the cell surface and in the cytoplasm. In addition to these immunoglobulin-positive cases, there was one case without distinct staining for any of the heavy and light immunoglobulin chains (Table 2).

Examination of other B cell surface makers was performed with monoclonal antibodies to BA-1, Leu-12 and HLA-DR, which are known to recognize different antigens present in most B cells except plasma cells (Table 1). Positive stainings for BA-1, Leu-12 and HLA-DR were observed in four, four and five cases, respectively (Table 2). The marker phenotypes of (BA-1, Leu-12, HLA-DR) were (+, +, +) in one, (+, −, −) in three, (−, +, +) in three and (−, −, +) in one lymphomas.

Examination of T cell markers was performed with antibodies to OKT-11 and Leu-1. No positive lymphoma was observed with these antibodies (Table 2). However, positive staining was observed in most cases in some of the small lymphoid cells which were distributed either around blood vessels or sporadically in neoplastic fields (data not shown). The number and the distribution pattern of the positive lymphoid cells were similar for OKT-11 and Leu-1. Morphologically,

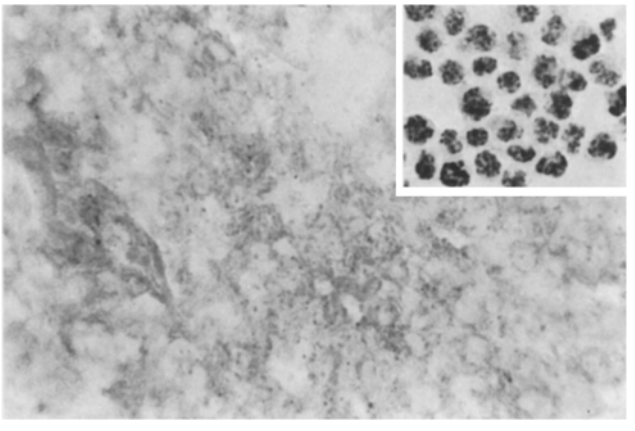

Fig. 3. Epstein-Barr virus-associated nuclear antigen (EBNA) staining. No positive staining is seen in lymphoma tissue (case 5), whereas intranuclear granular staining is distinct in Raji cells used for positive control (*insert*). Anticomplement immunoperoxidase method; no counterstaining. Frozen sections, × 500

these lymphoid cells were different from neoplastic cells in size and shape, suggesting that these cells were non-neoplastic and probably reactive in nature.

OKM-1 antibody also showed no positive staining in lymphoma cells in each case (Table 2), although a few positive lymphoid cells were observed in two cases (data not shown).

Examination of EBNA

None of the lymphoma tissues showed positive staining for EBNA (Table 2, Fig. 3). Under the same experimental conditions, intense intranuclear staining was observed in almost all Raji cells in the EBNA slide used as a positive control (Fig. 3).

Discussion

As outlined in the introduction, immunological studies of the primary malignant lymphomas of the brain have already been carried out. Cytoplasmic immunoglobulins have been demonstrated in paraffin sections [13, 21, 31]. Surface immunoglobulins and some other B lymphocyte markers have also been demonstrated in fresh specimens [6, 16, 22, 25, 27, 36]. Among them, Varadachari et al. [36] have revealed surface and cytoplasmic immunoglobulins (μ κ) and complement receptor in single cells prepared from a fresh lymphoma tissue. Using frozen sections of a single needle biopsy, Pearl et al. [27] have observed positive stainings with antibodies to immunoglobulin (λ), HLA-DR, T29/33 (hematopoietic cell marker) and Leu-14 (pan B marker), but not with anti-T-11 (T cell marker). All of these previous observations suggested B lymphocyte lineage of the primary malignant lymphomas of the brain.

In the present immunohistochemical study, all of the eight primary intracranial malignant lymphomas revealed two or more of four B cell markers, but no T cell or monocyte markers (Table 2). Even when one excludes the findings of immunoglobulin stainings, which were equivocal as to monoclonality in some cases, and those of HLA-DR staining, which might also be seen in some cells of T and other cell lineages, seven of the eight cases still revealed the remaining BA-1 and/or Leu-12 surface markers (Table 2), both of which are almost exclusively present in B cell lymphocytes. These findings indicated that at least seven, or probably all, of the eight lymphomas examined were of B cell lineage, supporting the previous works.

On examination of immunoglobulins, distinct monoclonality was observed in four cases, of which two showed surface μ κ, one showed surface and cytoplasmic μ λ and one showed cytoplasmic μ κ immunoglobulins. Whether or not cytoplasmic immunoglobulins were stained might be interpreted in connection with the difference in the stage of B cell differentiation among these lymphoma cases. However, monoclonality was rather equivocal in the other three immunoglobulin-positive cases. While two showed predominant κ and less predominant λ chain stainings, one showed both κ and λ chain stainings in similar intensities. The reason for these dual light chain stainings is unknown. A possibility that the patient's serum immunoglobulins in tissues, which might have been adsorbed to lymphoma cells through cellular Fc receptors, disturbed the present analyses of these cases is conceivable. Another possibility is that transformed cells in some lymphomas may express two different light chains, as postulated by Taylor et al. [31]. No direct evidence for these possibilities is available. To prove or disprove these possibilities, it might be worthwhile to carry out further studies including those using in situ nucleic acid hybridization technique as in recent work with other peptides [37].

The BA-1 [1, 34, 35], Leu-12 [23] and HLA-DR [2, 8, 12, 28, 35, 39] antigens have been demonstrated in many lymphomas of B cell lineage, though HLA-DR has also been found in some of T and other cell lineages [4, 12, 28]. The correlation of the expression of BA-1, Leu-12 or HLA-DR antigens with that of surface immunoglobulins has been studied in generalized B cell lymphomas [1, 2, 4, 8, 12, 23, 28, 34, 35, 39]. In the present study, coincidence of all four B cell markers (Ig, BA-1, Leu-12, HLA-DR) was observed in one case only. In the other seven cases, combinations of these marker stainings were considerably variable as shown in Table 2.

Negative immunostaining does not necessarily imply the absence of marker antigens. It is possible that we have overlooked weak immunostainings in some of the present cases and, therefore, our observation of heterogeneity of lymphocyte marker expressions might be merely of a quantitative sense.

Practically, however, the difference between positive and negative immunostainings was distinct in each surface marker study and immunological heterogeneity, if not in the strict sense, was apparent in the present intracerebral lymphoma cases. Using different combinations of antibodies, surface marker heterogeneity has already been observed in generalized large cell lymphomas [4, 7, 12, 35, 39]. Considering the present findings together with these previous works, it seemed likely that immunological heterogeneity might be a common feature to both intracranial and generalized large cell lymphomas.

In connection with these observations, it was also suggested that none of the antibodies to lymphocyte

markers are completely reliable in immunological classification of lymphomas. This should be taken into consideration in surface marker studies of lymphomas which are devoid of reaction with some particular antibodies.

Although neoplastic cells of each lymphoma were unstained with OKT-11 or Leu-1 antibodies, a variable number of small lymphoid cells showed positive stainings in most cases. The existence of T lymphocytes in B cell lymphomas has already been observed in generalized cases and its significance has been discussed [4, 28, 34, 35]. In the present study, however, these cells were not extensively analysed. Further studies using antibodies to other T cell subsets seemed to be worthwhile for a full understanding of these cells.

Non-African Burkitt-type lymphomas have been observed in many countries including Japan [24, 26]. Primary Burkitt-type lymphomas of the brain have also been reported [9, 15, 18, 33]. Hochberg et al. [10] have demonstrated Epstein-Barr virus DNA in a primary intracerebral lymphoma by DNA hybridization technique. The present examination of seven cases, however, failed to demonstrate EBNA which is closely related with EB virus infection [29]. A cultured cell line derived from a primary intracerebral lymphoma has also been reported to be negative for EBNA by Miyoshi et al. [25].

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