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Expression of Perforin in Nasal Lymphoma

Additional Evidence of Its Natural Killer Cell Derivation

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Eight patients with nasal lymphoma in whom fresh-frozen tissues were available were studied to elucidate the nature of the lymphoma cells. Two cases were diagnosed as diffuse, large cell lymphoma, and the remaining six cases as diffuse, mixed cell types. Immunohistochemical studies revealed that all of the cases were positive for perforin, which is a specific marker for cytotoxic T or natural killer (NK) cells. As all of the cases were CD8 negative, the perforin-positive finding further confirmed the concept that nasal lymphoma is a distinct neoplastic entity derived from NK or NK-related cells. Light microscopic immunohistochemical studies revealed that these nasal lymphoma cases could be classified into Leu19(CD56)$^+$Leu4(CD3)$^+$ (two cases) and Leu19(CD56)$^+$Leu4(CD3)$^-$ (six cases) types according to the phenotypes of the proliferating cells. However, simultaneous staining for perforin and Leu4 (CD3) using immunoelectron microscopy on the Leu19$^+$Leu4$^+$ cases showed that the perforin-positive cells were different from the Leu4-positive cells. This finding suggests that the Leu4-positive cells are not neoplastic NK cells but reactive T cells. Six cases were positive for EBER-1 by* in situ *hybridization analysis. This finding reconfirms the previous studies that Epstein-Barr virus plays a significant role in the pathogenesis of nasal lymphoma. (Am J Pathol 1996, 149:699–705)

Lymphoproliferative disorders occurring in the sinonasal regions have been called various names such as lethal midline granuloma[1–5] or polymorphic reticulosis.[6–9] It has been made clear that these lymphoproliferative disorders are the T-cell-related lymphomas.[6–10] Recent studies have suggested that these cases are natural killer (NK)-cell-derived lymphomas.[7,11–14] This malignancy occurs at a relatively high frequency among people in Southeast Asia, including those in Japan and China[15,16] as well as those in Peru.[17] Although the reason is totally unknown, NK or NK-cell-related lymphomas were concentrated in some unusual extranodal sites including sino-nasal tissues.[18–20]

This lymphoma also appears to be associated with Epstein-Barr virus (EBV) infection.[5,12,16,21,22] It is therefore believed that this lymphoma can be categorized as a distinct, unique entity. In this study, we investigated eight patients with nasal lymphoma morphologically and immunohistochemically, and we have particularly focused on perforin[23,24] expression in the neoplastic cells, which is a specific marker of NK cells and cytotoxic T cells. We also discuss the lineage derivation of these cells.

Materials and Methods

Eight cases with nasal lymphoma in our laboratory, in which fresh-frozen tissues were available, were studied. The tumors of these cases were localized in nasal lesions, except the one who had subsequent cervical lymph node involvement. An additional two cases clinically diagnosed as nasal lymphoma were excluded from our current study because these cases had widespread necrosis and were difficult to evaluate immunohistochemically. A case with nasal plasmacytoma was also excluded.

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Paraffin Tissues

All specimens that were obtained from the tumor tissues of the patients were fixed in a 10% formaldehyde solution and embedded in paraffin. Sections 2 to 4 μm thick were prepared and stained with hematoxylin and eosin (H&E). The peroxidase-antiperoxidase method[25] was used for the polyclonal antibody staining (CD3; DAKO, Copenhagen, Denmark). The avidin-biotin-peroxidase complex method[26] was used for monoclonal antibody staining (L26 (CD20), LMP-1, DAKO; MT1, Bioscience, Emmerbruecke, Switzerland; and UCHL-1, Nichirei Corp., Tokyo, Japan).

Frozen Tissues

Fresh specimens were fixed in periodate-lysine-paraformaldehyde fixative,[27] frozen, cut with a cryostat to a thickness of 6 to 8 μm, fixed with acetone for 10 minutes, reacted with the primary antibodies (Leu1, Leu2, Leu3, Leu4, Leu5b, Leu7, Leu9, Leu11b, and Leu19, Becton Dickinson Monoclonal Center, Mountain View, CA; B1 and B4, Coulter Immunology, Hialeah, FL; EBNA-2, DAKO; βF1 and TCRδ1, T Cell Sciences, Cambridge, MA; perforin, T Cell Diagnostics, Cambridge, MA; and OKT10, Ortho Diagnostic Systems, Raritan, NJ), and then processed for the immunoperoxidase reaction (avidin-biotin-peroxidase complex method).

For the immunoelectron microscopic studies, after the immunoperoxidase protocol was performed, the specimens were fixed with 1.25% glutaraldehyde for 15 minutes and washed three times with cold phosphate-buffered saline. For simultaneous staining of perforin and Leu4 (CD3), equal amounts of primary antibodies were mixed, followed by the immunoperoxidase method. The specimens were then reacted with diaminobenzidine solution containing 0.01% hydrogen peroxide for 5 minutes at room temperature. They were post-fixed with osmium tetroxide, dehydrated, embedded, and observed under the electron microscope without any counterstain.

Details of the immunoelectron microscopy have been described elsewhere.[28]

In Situ *Hybridization Analysis*

After deparaffinization and digestion with proteinase K (Sigma Chemical Co., St. Louis, MO), the tissue sections were hybridized with digoxigenin-labeled oligonucleotide probes (30 bp) for EBER-1 RNA sense and antisense sequences. These probes were synthesized with an Applied Biosystems 392 DNA/RNA synthesizer (Perkin Elmer, Norwalk, CT). Stringently washed sections were reacted with anti-digoxigenin alkaline-phosphatase-conjugated antibody (Boehringer Mannheim, Bedford, MA) and visualized with 5-bromo-4-chloro-indolylphosphatase and nitroblue tetrazolium salt. Details of this *in situ* hybridization have been described elsewhere.[29–32]

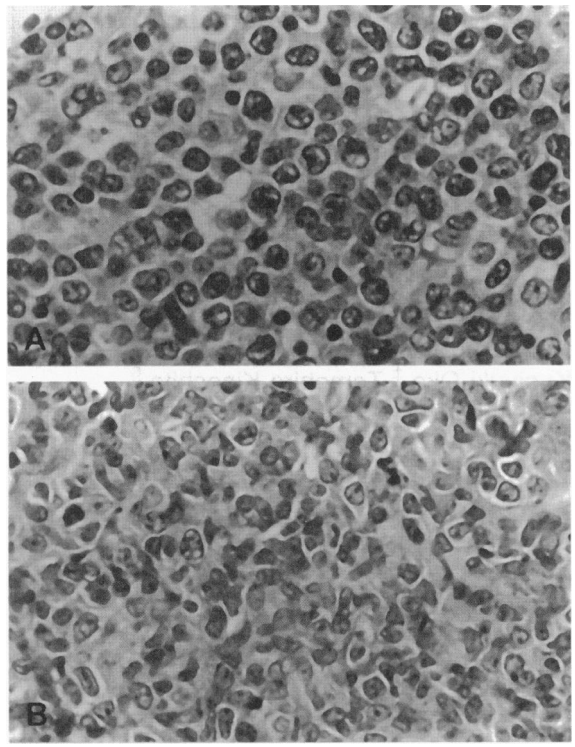

Figure 1. A: *H&E staining of a case with diffuse, large cell lymphoma (case 2), revealing mostly round, large blastic cells. Magnification, ×630.* **B:** *H&E staining of a case with diffuse, mixed cell lymphoma (case 5), revealing small cleaved-like cells as well as large, round blastic cells. Magnification, ×630.*

Results

Eight patients with nasal lymphoma were studied. All patients were men. The average age of the patients was 45 years old (range, 27 to 64). Two were diagnosed as having diffuse, large cell lymphoma (Figure 1A), and the remaining six had a diffuse, mixed cell type. In the patients with diffuse, mixed cell lymphoma, the proliferating cells consisted of round, large cells and medium-sized cells with small cleaved-like nuclei (Figure 1B). Widespread necrosis was observed in six cases, and angioinvasive or angiodestructive lesion was observed in two cases (Table 1).

Our present study revealed that all of the cases were positive for polyclonal CD3, except the one that was poorly fixed, on paraffin tissue sections (Table

Table 1. *Clinical and Histological Characteristics of the Eight Cases*

Case	Sex/age	Classification	Necrosis	Angio-invasion
1	M/64	Diffuse, large	+	−
2	M/52	Diffuse, large	+/−	+
3	M/32	Diffuse, mixed	+	−
4	M/27	Diffuse, mixed	+	−
5	M/37	Diffuse, mixed	+	−
6	M/47	Diffuse, mixed	+	−
7	M/44	Diffuse, mixed	+	−
8	M/59	Diffuse, mixed	−	+

2). On frozen tissue sections, our cases were classified immunohistochemically into two types (Table 3); 30 to 80% of the proliferating cells of six cases (the two with diffuse, large cell and four with diffuse, mixed cell) were Leu19(CD56)$^+$Leu4(CD3)$^-$, whereas the other two cases (diffuse, mixed cell) were Leu19(CD56)$^+$Leu4(CD3)$^+$ (Figure 2).

The former cases also lacked various other T cell markers and were Leu1(CD5)$^-$Leu2(CD8)$^-$-Leu3(CD4)$^-$ and βF1$^-$. Three of six were positive for Leu5b (CD2). In contrast, the latter cases were positive for various T cell markers and were Leu1(CD5)$^+$Leu2(CD8)$^-$Leu3(CD4)$^+$ and βF1$^+$. In addition, all eight cases were strongly positive for perforin in the cytoplasm of the proliferating cells (Figure 3).

Immunoelectron microscopic studies with polyclonal CD3 on the cases with Leu19(CD56)$^+$Leu4(CD3)$^-$ (cases 1 to 6) revealed that positive reactivity was localized in the perinuclear space but was not on the surface membranes of the neoplastic cells (Figure 4). Immunoelectron microscopic studies with perforin revealed that positive reactivity was expressed as granules in the cytoplasm of the neoplastic cells (Figure 5). Simultaneous staining for perforin and Leu4 (CD3) using immunoelectron microscopy on the samples that were Leu19(CD56)$^+$Leu4(CD3)$^+$ (cases 7 and 8) light microscopically revealed that the perforin-positive cells were different from the Leu4 (CD3)-positive cells (Figure 6). These latter cells were mainly small lymphoid cells with positive reactivity for Leu4 (CD3) mostly on their surface membranes.

In situ hybridization using the EBER-1 probe revealed that positive reactivity was observed in most of the neoplastic cells in the six cases (Table 2).

Discussion

Chan et al[10] classified sino-nasal lymphoma into three types, namely, diffuse, small cleaved cell, mixed cell, and large cell types. They indicated that the appearance of small cleaved cells is a characteristic finding in sino-nasal lymphoma, although they stated that the term small cleaved cell is a bit confusing, because it indicates a B cell lineage of the cells. According to Chan et al,[10] small cleaved cells have elongated, angulated nuclei, moderately dense chromatin, and scanty cytoplasm. Although these cells have elongated nuclei similar to small cleaved cells of the B cell type, they have dense

Table 2. *Immunohistochemical Findings on Paraffin Tissue Sections*

Case	CD3	MT1 (CD43)	UCHL-1 (CD45RO)	Leu7 (CD57)	LMP-1	L26 (CD20)	EBER-1
1	+	−	+	−	+	−	+
2	+	−	+	−	+	−	+
3	+	+	+	−	−	−	+
4	+	+	−	−	+	−	+
5	+	+	−	−	+	−	+
6	ND	ND	ND	ND	ND	ND	+
7	+	+	+	−	+	−	−
8	+	−	−	−	−	−	−

ND, not done.

Table 3. *Immunohistochemical Findings on Frozen Tissue Sections*

Case	Leu1 (CD5)	Leu2 (CD8)	Leu3 (CD4)	Leu4 (CD3)	Leu5b (CD2)	Leu7 (CD57)	Leu9 (CD7)	Leu11b (CD16)	Leu19 (CD56)	βF1	TCR-δ1	PF	OKT10 (CD38)	EBNA-2	B1 (CD20)	B4 (CD19)
1	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−
2	−	−	−	−	+	−	−	−	+	−	−	+	+	−	−	−
3	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−
4	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−
5	−	−	+	−	+	−	+	+	+	−	−	+	+	+	−	−
6	−	−	−	−	+	−	+	−	+	−	−	+	−	−	−	−
7	+	−	+	+	+	−	+	−	+	+	−	+	−	+	−	−
8	+	−	+	+	−	−	−	−	+	+	−	+	+	−	−	−

PF, perforin.

chromatin in their nuclei, and this finding can differentiate them from B-cell-type small cleaved cells. However, these cells are rarely observed in the neoplastic cells of peripheral T cell lymphomas including adult T cell lymphoma.

In our current study, 30 to 80% of the proliferating cells in all of the cases were positive for Leu19 (CD56), which is one of the markers of NK cells. In addition, our study also revealed perforin[23] expression in the proliferating cells of all of the cases examined. Recent studies indicated that perforin is expressed by cytotoxic T cells[33,34] or NK cells.[23] The former cells have CD8-positive reactivity. In our present study, all of the cases were negative for CD8. This further indicates that these neoplasms were derived from NK cells. Immunoelectron microscopy studies also confirmed that perforin is localized in the cytoplasm of the neoplastic cells in a granular fashion.[35]

In our study, on paraffin tissue sections, 30 to 80% of the proliferating cells of all of the cases except one, which was poorly fixed, were all positive for polyclonal CD3. However, in frozen tissue sections, the proliferating cells of six cases (cases 1 to 6) were negative for Leu4 (CD3). Our results were quite similar to Chan et al,[36] indicating a 60% discordant rate of CD3 expression (cases 1 to 5) between paraffin and frozen tissue sections. Similar results were also reported by other researchers.[13,37] Their explanation[13,36,37] for that discordance in the cases with T/NK cell lymphomas is as follows. Activated adult NK cells can express CD3 ϵ-chain transcript, which is recognized by polyclonal CD3 antibody. However, these cells cannot be recognized by Leu4 (CD3) antibody, which recognizes a conformational determinant of CD3$\gamma\epsilon$ or CD3$\delta\epsilon$ but cannot recognize CD3ϵ only. Our immunoelectron microscopy studies also revealed that the neoplastic cells were positive for polyclonal CD3 in their cytoplasm but not on the surface membranes. This finding further suggests that these lymphomas were derived from NK cells. We therefore consider that cases 1 to 6 belong to adult NK cell lineage, lacking Leu4 (CD3) antigen expression.

In the remaining two cases, the proliferating cells revealed Leu4(CD3)$^+$, in addition to Leu1(CD5)$^+$-

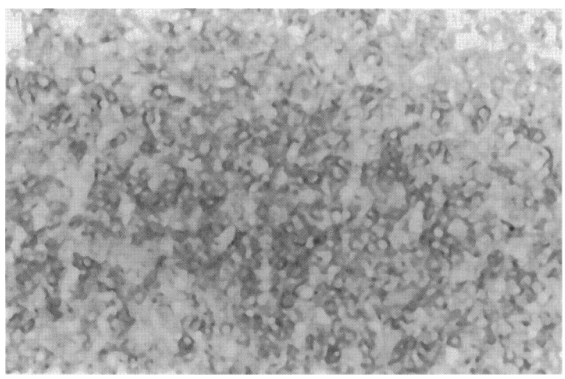

Figure 2. *Leu19 (CD56) staining revealed positive reactivity in most of the neoplastic cells (case 3). Magnification, ×315.*

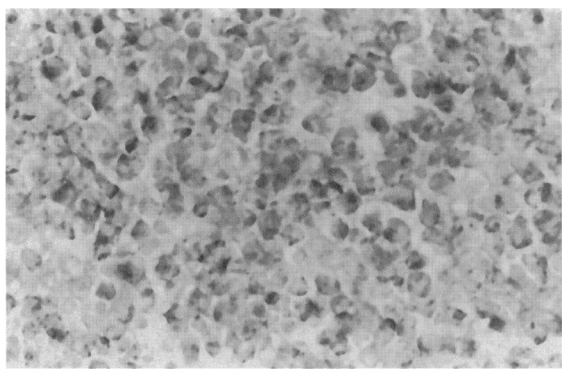

Figure 3. *Perforin staining revealed granular positive reactivity in the cytoplasm of the neoplastic cells (case 2). Magnification, ×630.*

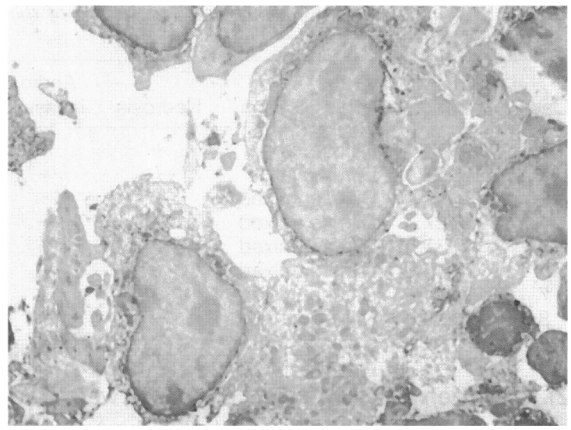

Figure 4. *Immunoelectron microscopy with polyclonal CD3 reveals intracytoplasmic (perinuclear) positive reactivity in the neoplastic cells (case 5). Magnification, ×8000.*

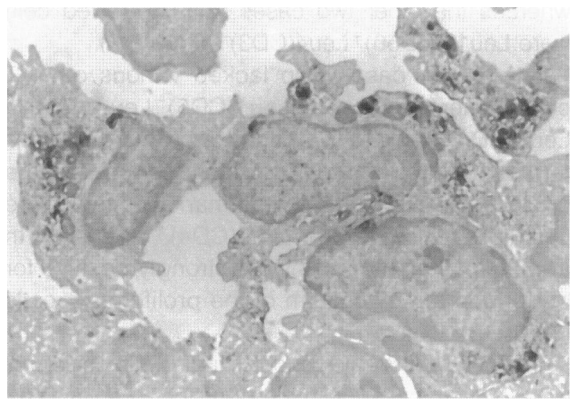

Figure 5. *Immunoelectron microscopy for perforin reveals granular positive reactivity in the cytoplasm of the neoplastic cells (case 5). Magnification, ×8000.*

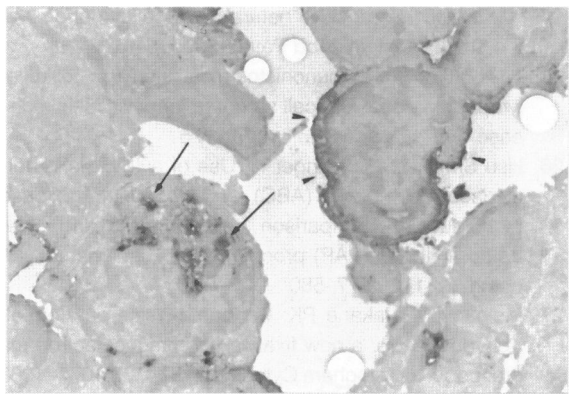

Figure 6. *Immunoelectron microscopy with simultaneous staining of perforin* (**arrows**) *and Leu4 (CD3;* **arrowheads**) *reveals positive reactivity in the different cells (case 7). Magnification, ×9000.*

Leu2(CD8)$^-$Leu3(CD4)$^+$ and βF1$^+$ phenotypes. Suzumiya et al[14] considered that Leu4$^+$(CD3)CD56$^+$ cases are derived from fetal NK cells, which have CD3γ, -δ, and -ϵ complexes in their cytoplasm[38] but not expressed on their surface membranes.[39,40]

With regard to the lineage derivation of sino-nasal lymphomas, Ho et al[7] have offered three possibilities: 1) a T cell lineage with variable aberrant expression of NK markers, 2) a T cell lineage with NK activity having T and NK markers, and 3) a NK lineage mixed with a large number of reactive T lymphocytes. Nasal lymphoma has been called polymorphic reticulosis.[6–9] This implies that the neoplastic cells are frequently admixed with a large number of reactive cells. Tao et al[41] studied clonal EBV integration in the neoplastic cells of nasal lymphomas to determine whether the CD3$^+$ cells were reactive or neoplastic. They found that 20 to 50% of the constituent cells were Leu4(CD3)$^+$. However, all of the Leu4$^+$ cells were EBV negative. In contrast, all of the EBV-positive cells were Leu4(CD3)$^-$. They therefore concluded that these Leu4-positive cells were background reactive T cells. Our immunoelectron microscopy study revealed that the perforin-positive cells were distinct from the Leu4(CD3)$^+$ cells in those specimens that were Leu4$^+$CD56$^+$ (cases 7 and 8). Furthermore, the Leu4(CD3)$^+$ cells showed mostly surface reactivity. Accordingly, we agree with Tao et al[41] that the Leu4(CD3)$^+$ cells are the background reactive T cells.

Although the number of cases in our present study was too small to draw definite conclusions in terms of lineage derivation, we believe that all of our cases were derived from adult-type NK cells.

Recently, it has been made clear that EBV plays a significant role in the occurrence of sino-nasal lymphoma.[5,12,16,21,22] Our present study also revealed that six out of eight cases were positive for EBV by *in situ* hybridization. This finding further suggests that EBV plays an important role in the pathogenesis of nasal lymphoma.

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