Nasal T-Cell Lymphoma: A Clinicopathologic Entity Associated With Peculiar Phenotype and With Epstein-Barr Virus

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Recent evidence has shown that most nasal lymphomas (NL) are associated with a T-cell phenotype and are thus called nasal T-cell lymphomas (NTCL), but little information is available about the T-cell receptor (TCR) expression. The presence of Epstein-Barr virus (EBV) genome has been recently reported in NTCL in Oriental populations in which NL and EBV-associated tumors are more common and in occasional Occidental cases. This prompted us to investigate lymphoma biopsies from 7 non-Oriental patients with NTCL for the expression of natural killer (NK) and T-cell antigens, including TCR proteins, for the presence of EBV-encoded latent membrane protein (LMP) using immunohistochemistry and for the presence of EBV DNA and Epstein-Barr early region (EBER) RNA in situ hybridization (ISH). Six cases displayed a CD3+, TCRyδ+, CD8+, CD56+, phenotype, suggesting that these tumors may be peripheral T-cell lymphomas (PTCL) with extensive loss of T-cell antigens and expression of the NK-cell (CD56) antigen or, alternatively, NK-cell neoplasias. The remaining case was a γδ PTCL, as shown by the CD3+, TCRγδ+ phenotype and the biallelic γ and δ TCR gene rearrangements. Using ISH, EBER RNA transcripts were detected in tumor cells in all cases and EBV DNA was shown in the 6 tested cases. In all cases, tumor cells expressed LMP. These findings support the concept that NTCL constitute a distinct group of lymphomas that, in addition to their peculiar clinical features, exhibit an unusual TCR ‘‘silent’’ CD69+ or TCRγδ+ phenotype and harbor the EBV. In view of the LMP transforming potential, these data suggest that EBV may play a role in the pathogenesis of NTCL.

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grounds, involving predominantly or exclusively the nasal cavity, and in which patients had presenting symptoms related to nasal disease. According to this definition, 7 cases of NTCL, for which frozen tissue was available, were selected in 6 French patients and 1 Mauritanian patient and included in this study. A summary of clinical data is shown in Table 1. Clinical data of 1 patient (case 1) have been reported elsewhere, as well as part of the immunohistochemical and genomic results in this patient. One patient (case 4) had already received local radiotherapy 24 years before for a nasal "reticulosarcoma" that, after careful histologic and immunohistochemical review, was shown to be a pleomorphic large-cell lymphoma of T-cell type. Multiagent chemotherapy was administered in all cases, and was associated with local radiation therapy in cases 2 and 6. Persistent complete remission was observed in only 1 (case 6) of the 4 cases with significant follow-up.

Methods

Tissue specimens. Fresh tissue samples were obtained for diagnosis from the nasal cavity (6), from the skin (1), and from a lymph node (1). Fresh and/or paraffin-embedded tissues of sites of recurrence were also obtained in patients 2, 4, and 5. One portion was paraffin-embedded and the others were snap-frozen in liquid nitrogen after 30 minutes of incubation in gum-sucrose and stored at -70°C. In addition, frozen sections from 21 randomly selected nodal PTCL were also analyzed as a control for the expression of the EBV-encoded LMP protein.

Histologic studies. Paraffin-embedded tissue specimens were stained with hematoxylin-eosin, giemsa, and Gordon Sweet. Lymphomas were classified according to the Working Formulation (WF) and the updated Kiel classification.

Immunohistochemical methods. Cryostat sections were evaluated for T-, B-, and NK-cell differentiation antigens, for the activation CD25 and CD30 markers, as well as for the EBV-encoded LMP, using the alkaline phosphatase antialkaline phosphatase (APAAP) technique. The monoclonal antibodies (MoAbs) used in this study and their commercial sources are the following: Leu6/CD1, Leu5/CD2, Leu4/CD3, Leu3/CD4, Leu1/CD5, Leu9/CD7, Leu2/CD8, and ILR2/CD25 (Becton Dickinson, Mountain View, CA); B4/CD19 and NKH1/CD56 (Coulter, Hialeah, FL); BerH2/CD30, CD21, CD22, and anti-LMP-1/CS I-4 (Dako SA, Glostrup, Denmark); and HML1 (Immunotech SA, Marseille, France). LMP and CD30 antigens were also tested in paraffin-embedded sections using the same APAAP technique, because the corresponding MoAbs recognize epitopes resistant to fixation. Rabbit antimonus lgs and APAAP complexes were obtained from Dako. For LMP protein, optimum labeling required enhancement by twice repeating the bridge and APAAP complex incubation steps. As positive controls for LMP immunoreactivity, the Raji cell line and one case of infectious mononucleosis were used. The negative control consisted in substitution of the primary antibody with nonimmune serum.

DNA and RNA in situ hybridization (DISH and RISH). EBV genomes were detected using ISH on cryostat sections with a biotinylated EBV DNA probe corresponding to the 3.1-kb BamHIW internal repeat fragment of the EBV genome (ENZO Diagnostics, New York, NY). The DISH was performed as described previously. Briefly, cryostat sections previously fixed in 10% formalin in phosphate-buffered saline (PBS), pH 7.6, were digested with pepsin, dehydrated, and air-dried. The probe was diluted in a mixture containing 50% deionized formamide, 25% dextran sulfate, 0.1 M SSC, and 250 mg/mL sonicated salmon sperm DNA. Sections were pretreated with streptavidin alkaline-phosphatase complex (Dako SA). Alkaline phosphatase activity was visualized by incubation in 50% formamide, the slides were incubated with streptavidin alkaline-phosphatase complex (Dako SA), and anti-LMP-I/CS 1-4 (Dako SA, Glostrup, Denmark); and HML1 (Immunotech SA, Marseille, France). LMP and CD30 antigens were also tested in paraffin-embedded sections using the same APAAP technique, because the corresponding MoAbs recognize epitopes resistant to fixation. Rabbit antimonus lgs and APAAP complexes were obtained from Dako. For LMP protein, optimum labeling required enhancement by twice repeating the bridge and APAAP complex incubation steps. As positive controls for LMP immunoreactivity, the Raji cell line and one case of infectious mononucleosis were used. The negative control consisted in substitution of the primary antibody with nonimmune serum.

Table 1. Clinical and Histologic Features in the Patients With NTCL

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Symptoms</th>
<th>Localization at Presentation</th>
<th>Classification (WF/Kiel)</th>
<th>Angiocentrity</th>
<th>Angioinvasion</th>
<th>Necrosis</th>
<th>Treatment, Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30/M</td>
<td>RMS</td>
<td>Nose (nasal septum, palate, cervical lymph node)</td>
<td>DM/PML</td>
<td></td>
<td></td>
<td>+</td>
<td>Chemotherapy (CHOP), hepatosplenomegaly, DOD after 6 mo (hepatic failure)</td>
</tr>
<tr>
<td>2</td>
<td>44/F</td>
<td>RMS</td>
<td>Nose, palate</td>
<td>DLC/PML</td>
<td></td>
<td>+</td>
<td>+</td>
<td>Chemotherapy (ACVB) + RT; local recurrence and skin nodules; DOD after 15 mo</td>
</tr>
<tr>
<td>3</td>
<td>34/F</td>
<td>RMS, fever, dyspnea</td>
<td>Nose, skin, lung</td>
<td>DLC/PML</td>
<td></td>
<td></td>
<td>+</td>
<td>Chemotherapy (MACOP-B); dead of pseudomonas septicemia after 3 mo</td>
</tr>
<tr>
<td>4</td>
<td>64/F</td>
<td>RMS, history of NTCL 24 yr before treated by RT</td>
<td>Nose (1962)</td>
<td>DLC/PML</td>
<td></td>
<td>+</td>
<td>+</td>
<td>Chemotherapy; alive with LED after 18 mo</td>
</tr>
<tr>
<td>5</td>
<td>17/M</td>
<td>RMS</td>
<td>Nose (NF), maxillary sinus, ethmoid node</td>
<td>DM/PML (NF)</td>
<td>+ + +</td>
<td></td>
<td></td>
<td>Chemotherapy (ACVB) + RT; local recurrence; alive with LED after 18 mo</td>
</tr>
<tr>
<td>6</td>
<td>35/M</td>
<td>RMS</td>
<td>Nose (NF)</td>
<td>DLC/PML</td>
<td></td>
<td></td>
<td>+</td>
<td>Chemotherapy + RT; NED after 15 mo</td>
</tr>
<tr>
<td>7</td>
<td>34/F</td>
<td>RMS</td>
<td>Nose (NF), maxillary sinus</td>
<td>DM/PML</td>
<td></td>
<td>+ + +</td>
<td></td>
<td>Chemotherapy (ACVB); alive with LED after 7 mo</td>
</tr>
</tbody>
</table>

Abbreviations: R, relapse; RMS, recurrent maxillary sinusitis; NF, nasal fossa; DM, diffuse mixed; DLC, diffuse large cell; PML, pleomorphic medium and large; DOD, dead of disease; RT, radiotherapy; NED, no evidence of disease; LED, local evolutive disease; WF, Working Formulation; Kiel, updated Kiel classification.
of the EBER 1 and 2 genes that are actively transcribed in latently infected cells were used. Deparaffinized sections were rehydrated and pretreated with proteinase K, dehydrated, air-dried, and hybridized for 2 hours at 37°C with the fluorescein isothiocyanate-conjugated (FITC) EBER oligonucleotides in hybridization solution consisting of 30% formamide, 10% dextran sulphate, 0.1% sodium pyrophosphate, 0.2% polyvinyl pyrrolidone, 0.2% ficoll, 5 mmol/L Na2 EDTA, and 50 mmol/L Tris/HCl, pH 7.5. After washing in TBS, pH 7.6, containing 0.1% Triton X-100, the following immunohistochemical detection system was used: mouse anti-FITC, rabbit antimouse Ig, and APAAP complexes (Dako SA). The visualization of the reaction was performed as for DISH using BCIP and NBT. As positive controls for DISH and RISH, the Raji cell line, 1 case of EBV-positive BL, 2 cases of EBV-positive B lymphoproliferations occurring in transplant patients, and 1 case of infectious mononucleosis were used. As negative controls, EBV-negative lymphoid tissues and the hybridization mixture without EBV probe (DISH) or EBER oligonucleotides (RISH) were used. Negative controls for each case were run in parallel in every experiment. The EBER signal was abolished after RNAsase treatment confirming the specificity of the reaction.

RESULTS

Histology

The histologic findings are summarized in Table 1. The lymphoid proliferations were classified as diffuse large cell lymphoma (cases 2, 3, 4, and 6) or diffuse mixed lymphoma (cases 1, 5, and 7) according to the WF. In Kiel classification, all cases were pleomorphic medium- and large-cell lymphomas. In all cases, histologic examination of biopsy specimens taken from the nasal cavity and other sites showed pleomorphic lymphoid proliferation composed mainly of large- and medium-sized lymphoid cells intermingled with variable numbers of small lymphoid cells, plasma cells, histiocytes, and polynuclear granulocytes. Areas of necrosis were observed in all cases. Angiocentricity and angioinvasion (Fig 1) were found in nasal biopsies in cases 1, 3, 5, and 6 and in the skin in case 2. Epitheliolymphoma was detected in the nasal biopsies in cases 1, 3, 4, and 5, and in the skin in case 2. In the two skin biopsies available (case 2 at relapse and case 3 at presentation), the atypical lymphoid infiltrate was identical to that observed in the nasal biopsies. It is noteworthy that in case 4 the patient had a past history of a nasal lymphoid tumor that was treated by local radiotherapy (Table 1) and had remained free of disease 24 years before she again developed a nasal lymphoid malignancy. Review of the histology showed that the initial tumor was a pleomorphic lymphoma, and paraffin section immunohistochemistry suggested a T-cell phenotype because tumor cells were UCHL1 (CD45RO) positive and L26 (CD20) negative.

Immunohistochemistry

Immunohistochemical staining results are recorded in Table 2. MoAbs recognizing the CD19 and CD22 B-cell antigens and Ig light chains labeled only a few, apparently reactive lymphocytes. By contrast, all cases expressed at least the CD2 and CD7 T-cell antigens. Of the 7 lymphomas, 6 showed an homogeneous phenotype, i.e., CD3+, TCRαβ+, TCRγδ+, CD2+, CD7+, CD5+, CD4+, CD8+. The remaining case (case 1) was a CD3+, TCRγδ+ lymphoma that also expressed CD2 and CD7 antigens, but was CD5+. The NKH1 (CD56) antigen was detected on tumor cells in all cases (Fig 2). The CD25 and CD30 antigens were expressed on a sizable proportion of tumor cells in all cases. The HML-1 antibody did not stain tumor cells in any case. CD21 antigen was expressed on a sizable proportion of tumor cells in case 6, whereas in the other cases, scattered, apparently nonneoplastic CD21+ cells were found.

EBV Studies

The results of EBV studies are summarized in Table 3. Evidence of EBV was found by ISH and by immunohistochemistry in all cases. By DISH, the EBV genome was detected within the tumor cells in the 6 cases studied. Positive cells were numerous in 4 cases. Positive cells clearly surrounded residual negative mucous glands in most cases, whereas in case 2, positive intraepithelial tumor cells were present. By RISH, a large number of cells containing the EBV-EBER(1-2) mRNAs was identified in all cases (Fig 3). The positive cells included large neoplastic cells, sometimes showing multilobated nuclei. Intensively positive cells represented the great majority of the entire tumor cell population in 4 cases. The skin biopsy in case 3 displayed only a few positive cells surrounding vessels. A perivascular pattern of reactivity was also observed in case 7. By contrast, epithelial cells were negative, but occasional EBV-EBER-positive cells, consistent with infiltration of the epithelium by tumor cells, were found in epithelial structures in cases 1, 2, 4, 5, and 6. In the 2 patients (cases 2 and 5) with recurrent material, an identical large number of EBV-positive cells was observed at presentation and at relapse. Comparison of the DISH and RISH staining patterns disclosed, in most cases, that RISH gave stronger reactivity and that the RISH-positive cells outnumbered those detected by DISH, as well as those expressing the EBV-encoded LMP protein.

By frozen section immunohistochemistry, a substantial proportion of tumor cells were found to express LMP in cases 1, 2, 4, 6, and 7 (Fig 4), whereas in cases 3 and 5, a few cells were stained. Using paraffin section immunohistochemistry for LMP, 6 of the 7 cases were found to be positive, with the staining present exclusively in morphologically neoplastic cells. In addition, comparison of adjacent tissue sections disclosed an overlapping staining pattern for LMP and CD30. Interestingly, LMP was expressed in tumor cells in nasal biopsies at presentation and at relapse in cases 2 and 5. In addition, LMP was also found in tumor cells in skin specimens in cases 2 and 3 as well as in cervical lymph node in case 1. In the patient with a history of NTCL 24 years before (case 4), EBER mRNA and LMP were also found in tumor cells in the paraffin-embedded nasal biopsy that was retrospectively studied. By contrast, LMP was found in only 1 of the 21 nodal PTCL studied on frozen sections.

DISCUSSION

The 7 NTCL in our study had the clinical presentation of lethal midline granuloma and displayed histologic features of pleomorphic tumors. Angiocentricity, angioinvasion, and epitheliolymphoma were frequent although not constant findings, whereas areas of necrosis were constantly present. Dis-
Fig 1. Pleomorphic large-cell lymphoma with angioinvasion (case 6). Atypical lymphoid cells infiltrate the wall of a small artery (nasal biopsy with hematoxylin-eosin stain; original magnification × 200).

Fig 2. Immunohistology on cryostat sections of NTCL. Infiltrating lymphoid cells surrounding residual mucosal glands strongly express CD56 antigen (case 2, APAAP technique; original magnification × 320).

Fig 4. Detection of EBV LMP expression in NTCL (case 6). Immunohistology shows numerous neoplastic LMP-positive cells (cryostat section, APAAP technique; original magnification × 200).
Although most NTCL are characterized histologically by angiocentric polymorphous lymphoreticular infiltrates that involve primarily the midline structures and the lung, the term AIL has been proposed as a generic concept unifying entities previously defined as polymorphic reticulosis and lymphomatoid granulomatosis that are now considered to be similar if not closely associated to the NTCL. Indeed, CD2+, CD3-, TCR silent, CD56+ phenotype was much more frequently observed in the few cases studied in frozen section.30-33 Taken altogether, the above phenotypic data indicate that NTCL display distinct immunophenotypic profile independently of the racial/ geographical distribution.

Regardless of the cell origin of these tumors, this peculiar CD2+, CD3+, TCR silent, CD56+ phenotype seems to be closely associated to the NTCL. Indeed, CD2+, CD3+, TCRαβ+, CD56+ phenotype was much more frequently found in nasal lymphomas than in nodal lymphomas in Oriental populations.7,15 In Western populations, a CD2+, CD3+ phenotype was also frequently reported in the few cases of PTCL studied in frozen sections.14,28,30 The remaining case was a CD3+, TCRγδ+, CD56+ lymphoma. The clinical, histologic, immunohistochemical, and genotypic features of this case, along with those of other cases of γδ T-PTCL, have been extensively described previously.30,36,40 Detailed phenotypic analysis of the γδ TCR status in this case of nasal γδ T-PTCL showed that the tumor cells expressed the pan-TCR γδ marker TCR-δ1.29 These data were further substantiated by Southern blot analysis showing biallelic γ and δ TCR rearrangements involving the Jγ1 and the Jδ1 genes.30 Unfortunately, DNA analysis could not be performed in the other cases in which no sufficient material was available. Interestingly, δ-TCR rearrangement has been recently reported in a case of CD2+, CD3+, TCR silent “West-
EBR AND T-CELL RECEPTORS IN NASAL LYMPHOMA

EBR transcriptions were found in the majority of tumor cells in the nasal biopsy from 1990 (case 4, paraffin sections; original magnification ×200).

Fig 3. RNA ISH in NTCL. EBER transcripts were found in the majority of tumor cells in the nasal biopsy from 1990 (case 4, paraffin sections; original magnification ×200).

ern” NTCL, and we have previously observed that, in a CD2+, CD3+, TCRγδ+, CD56+ lymphoma, tumor progression was associated with phenotypic changes resulting in a CD2-, CD3-, TCR silent, CD56+ lymphoma with the same δ gene rearrangement as initially. Based on these data, we could speculate that some NTCL with the above phenotype could correspond to γδ tumors that have lost CD3 and TCRγδ expression during the course of the disease.

In the present study, by using DNA and RNA ISH, the EBR DNA and the EBR-encoded EBER mRNA, respectively, were detected in tumor cells in all tested cases of NTCL. This is in keeping with previous findings in Oriental and Western NTCL. In the present study, EBR genomes were also shown at distant sites of spread and detected independently of the presence of angiocentric lesions. Taken together, these data show a close association between EBR and NTCL and indicate that this association is not restricted to the racial/geographical distribution. These data were further substantiated by our immunohistochemical detection of LMP in all NTCL, irrespective of tumor site. Interestingly, using paraffin sections, LMP was demonstrated morphologically in tumor cells. These results show that EBR genomes in NTCL may be transcribed and translated to proteins, indicating that EBR is not just a “silent” passenger in the tumor cells. Furthermore, the expression of LMP by tumor cells is an important finding because LMP is thought to play a crucial role in EBR-induced in vitro transformation and has been detected in human malignancies. LMP has transforming activities when introduced into rodent fibroblastoid cell lines and renders these cells tumorigenic in nude mice. In epithelial cells, LMP induces inhibition of differentiation and morphologic transformation. In B-cell lines, LMP transfection induces gene deregulation and morphologic changes, and prevents programmed cell death by inducing the expression of bel-2 gene product. In human malignancies, LMP has been found in undifferentiated nasopharyngeal carcinomas, B-cell lymphoproliferations in immunosuppressed individuals, in HD, in CD30 positive anaplastic and nonanaplastic large-cell NHL, and in angio-immunoblastic lymphadenopathy. By contrast, we observed LMP expression in only 1 of the 21 nodal PTCL. As a result of the present study and of a previous Oriental report, NTCL must be added to this series of lymphoid malignancies associated with LMP expression.

The present findings in NTCL support recent data that expression of LMP correlates with CD30 expression in NHL. Indeed, the pattern of CD30 expression was found to overlap with that of LMP in paraffin sections in all 7 NTCL. It could be suggested that EBR induces the expression of the “activation” molecule CD30 and LMP may play a crucial role in this process. This can be further supported by the findings that LMP has been detected in Reed-Sternberg cells HD, which characteristically express the CD30 antigen.

The mechanism by which EBR can gain entry into tumor cells of NTCL is not clear. Indeed, the CR2 EBR receptor (CD21), which is expressed in about 30% of human peripheral blood T-lymphocytes, was detected on tumor cells in 1 of the 7 NTCL in this study and has been so far reported in only 1 T-NHL. Thus, it is possible that in most EBR-harboring T-NHL, the EBR infection has occurred during transient CD21 expression before clonal expansion of the transformed cells. Alternatively, molecules other than CD21 may also play the role of EBR receptor on T cells.

The clinical significance of the unusual T-cell phenotype of NTCL as well as of their close association with EBR is currently unclear. It has been shown that patients with NTCL have a relatively short median survival. This could fit with our data because 3 of the 7 patients with NTCL died of disease within 3 to 15 months after diagnosis. Further studies are...
necessary to evaluate whether the presence of EBV and/or LMP antigen in tumor cells has an unfavorable prognostic significance. In this respect, PTCL containing EBV DNA have been recently reported as clinically aggressive lymphomas.22

Our immunohistologic and ISH findings provide further evidence that the so-called NTCL can be regarded as a distinct group of lymphomas that, in addition to their peculiar clinical and histologic features, exhibit an unusual TCR “silent” CD56+ or TCRγδ+ phenotype and are closely associated with EBV. The detection of LMP in tumor cells of NTCL indicates that EBV is not a “silent passenger” and suggests that the virus may play a role in the pathogenesis of these lymphomas.

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