**CASE STUDY**

**Pyothorax-associated lymphoma: relationship with Epstein-Barr virus, human herpes virus-8 and body cavity-based high grade lymphomas**


Pyothorax-associated lymphoma (PAL) is a newly-described entity developing several decades after artificial pneumothorax treatment for pulmonary or pleural tuberculosis. It is known to be associated with Epstein-Barr virus (EBV) with constant expression of the two latent membrane proteins: latent membrane protein (LMP)-1 and EBV-associated nuclear antigen (EBNA)-2. We are reporting three new cases of PAL. All of the tumours were of B-cell lineage and classified as large-cell diffuse lymphomas according to the International Working Formulation for the Classification of Lymphomas. The EBV genome was detected in two of the cases with LMP-1 and EBNA-2 expression. No EBV could be detected in the third case suggesting that different mechanisms may be involved in the pathogenesis of the disease.

Body cavity-based high grade lymphomas (BCBL) represent a new disease, developing mainly in human immunodeficiency virus (HIV) infected patients: the tumoural cells often contain both human herpes virus (HHV)-8 (or Kaposi's sarcoma herpes virus) and EBV genomes, suggesting that these viruses might co-operate in the pathogenesis of the disease. The pleural location and the association of EBV have led to speculation that PAL could also be related to HHV-8 infection. However, no HHV-8 genome could be detected in any of the 14 tested cases already reported in the literature. Two different entities: PAL and BCBL have been detected already in the 14 reported cases already tested [19, 25]. The combined infection of tumoural cells by both HHV-8 and EBV genomes also suggests that both viruses might co-operate in the pathogenesis of the disease.

PAL and BCBL have different morphological and phenotypic features, but the pleural location and the association of EBV have led to speculation that PAL may also relate to HHV-8 infection [24]. However, no HHV-8 has been detected in the 14 reported cases already tested [19, 25]. We report three new cases of PAL from a Western country. We have studied the phenotype, the presence of EBV and also the presence of HHV-8.

**Patients and methods**

**Patients**

Three cases of non-Hodgkin's lymphoma (NHL) developing on pleural sequelae of therapeutic pneumothorax for tuberculosis were studied. The clinical data are summarized in table 1. Diagnosis in all cases was made on a surgical biopsy specimen. All patients had a clinical history of

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pneumothorax treatment for pulmonary tuberculosis 46, 48 and 49 yrs ago, prior to the discovery of pleural lymphoma. They presented with chest pain, dyspnoea and weight loss. They were all negative for HIV infection. Standard chest radiographs and computed tomography (CT) scans showed pleural sequelae lesions and a tumoural nodule of variable size connected to the chest wall in the area of the pyothorax. A biopsy revealed a lymphoma of diffuse large-cell type in all three cases. There was no evidence of recurrent tuberculous infection. Two patients received chemotherapy only (patients one and three) and one patient received combined chemotherapy and radiotherapy (patient two). Patients one and three both died a few months after diagnosis; no autopsy was performed. Patient two was still alive 15 months after diagnosis.

Histopathology and immunohistochemistry

Specimens obtained from the tumours were fixed in formalin or in aqueous Bouin's fixative and embedded in paraffin. Sections were prepared and stained with haematoxylin eosin saffron and Giemsa. The International Working Formulation for the Classification of Lymphomas was used for classification and diagnosis. Immunostains were performed according to the standard biotin-streptavidine (Dakopatts, Glostrup, Denmark) method. The monoclonal antibodies used are listed in table 2.

Table 2. – Characteristics of the antibodies used in the immunophenotypic study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vendor</th>
<th>Dilution</th>
<th>Specificity</th>
</tr>
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<tbody>
<tr>
<td>KL1</td>
<td>ImmunoTech*</td>
<td>1/50</td>
<td>55–57-kda cytokeratins</td>
</tr>
<tr>
<td>EMA</td>
<td>ImmunoTech</td>
<td>1/100</td>
<td>Epithelial membrane antigen</td>
</tr>
<tr>
<td>L26 (CD20)</td>
<td>Dakopatts</td>
<td>1/100</td>
<td>B-lymphocytes</td>
</tr>
<tr>
<td>CD3 (polyclonal)</td>
<td>Dakopatts</td>
<td>1/100</td>
<td>T-lymphocytes</td>
</tr>
<tr>
<td>CD30 (BerH2)</td>
<td>Dakopatts</td>
<td>1/10</td>
<td>Reed-Sternberg cells</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Dakopatts</td>
<td>1/10</td>
<td>Immunoblasts</td>
</tr>
<tr>
<td>EBNA-2/R3</td>
<td>Dakopatts*</td>
<td>1/10</td>
<td>Large anaplastic cell lymphomas</td>
</tr>
<tr>
<td>LMP-1</td>
<td>Dakopatts</td>
<td>1/25</td>
<td>Latent nuclear protein of EBV</td>
</tr>
</tbody>
</table>

EM: epithelial membrane protein; EBNA: Epstein-Barr virus (EBV)-associated nuclear antigen; LMP: latent membrane protein. *: Marseille, France. †: Glostrup, Denmark. #: prepared by E Kremmer.

Results

Microscopic findings

The histological features were those of diffuse large cell lymphomas.

Case one had large cells with abundant and clear cytoplasm and irregular nuclei containing medium sized nucleoli. The tumoural cells were intermingled with numerous reactive

In situ hybridization analysis

EBV ribonucleic acid (RNA) *in situ* hybridization was performed on paraffin sections using oligonucleotides complementary to a portion of the EBV early RNAs (EBERs) as described [26] with the following modifications: the staining consisted of a first stage incubation with a monoclonal mouse antibody to digoxigenin (Dakopatts). After washing, a biotin-conjugated sheep anti-mouse antibody raised in goat (Dakopatts) was applied before a third stage of streptavidin-conjugated alkaline phosphatase. Finally, the antigen-antibody complex was visualized using a chromogenic peroxidase substrate solution (Dakopatts). The slides were counterstained with haematoxylin eosin saffron and mounted with glycergel.

Polymerase chain reaction (PCR)

To detect HHV-8 sequences, two different sets of primers were used. The first (KS81-KS82) amplified 601 base pairs (bp) while the second (KS84-KS85) corresponded to a fragment of 233 bp. The latter was identical to the KS330, as reported by CHANG et al. [15]. PCR was performed as described [16, 17], using 1–2 µg of deoxyribonucleic acid (DNA) with an initial denaturation step at 94°C and then 40 cycles for 1 min at 55°C and 2 min at 72°C (with time increased by 2° per cycle). DNA extracted from five KS cutaneous tumours or from peripheral blood mononuclear cells of HIV seronegative blood donors, were used as positive and negative controls, respectively. All DNA samples were also amplified by PCR for human β-globin sequences (primer pair PC04-GH20-size 268 bp to demonstrate the integrity of DNA specimen).

Results

Microscopic findings

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Case one had large cells with abundant and clear cytoplasm and irregular nuclei containing medium sized nucleoli. The tumoural cells were intermingled with numerous reactive

Fig. 1. – Case 3. Large cells with an abundant nonbasophilic cytoplasm and noncleaved nuclei containing prominent nucleoli. Mitosis are numerous. Haematoxylin eosin saffron stain; internal scale bar=20 µm.
Patients T
oural cells, and numerous mitoses and large areas of necrosis were noted. Case two presented with large cells with polylobulated nuclei containing prominent nucleoli. Mitoses were numerous and large areas of necrosis were noted (fig. 1).

Immunohistochemistry

Results are itemized in table 3. The B-cell lineage of the tumoural cells was confirmed in the three cases with a strong expression of CD20 (stained with L26 antibody) (fig. 2). The reactive cells described in case one were stained by CD3. There was no expression of epithelial membrane antigen (EMA).

EBV gene expression in tumoural cells

Results are detailed in table 3. In situ hybridization showed a nuclear expression of EBERs in numerous tumoural cells in cases one and two. No expression could be detected in case three. EBNA-2 and LMP-1 stainings were positive in cases one and two but were negative in case three.

PCR

No HHV-8 sequence was detected in the two cases tested (case one and case three). Case two could not be tested because no frozen tissue was available.

Discussion

PALs are high grade B lymphomas occurring several decades after artificial pneumothorax for pulmonary tuberculosis or tuberculous pleuritis. The most common symptoms presented are pain and various respiratory symptoms [27]. They may present with a tumoural mass in the chest or in the pleura, sometimes extending to adjacent structures and to regional or distant lymph nodes [27].

There is some clinical and biological evidence that the specific treatment by artificial pneumothorax represents a true risk factor for PAL. Indeed, comparing patients with PAL and patients with chronic pyothorax, OHSWA et al. [28] showed that the frequency of patients receiving artificial pneumothorax for the treatment of pulmonary tuberculosis was much higher in the first group (75%) than in the second group (19%), suggesting that this treatment represents a true risk factor. However, AOZASA [27] detected a p53 mutation in 15 of the 21 cases tested and showed that 16 of these 23 mutations were related to dipyrimidine sites. This type of mutation is observed with a high frequency in skin carcinomas and is known to be related to irradiation. These findings suggest that long-term radiation during the artificial pneumothorax or specific drug exposure may have caused specific mutations in the p53 gene [27].

Most PALs are of B phenotype, and are usually diffuse large cell types with frequent immunoblastic features (table 4); a few cases of diffuse lymphoplasmacytic types have been reported [27].

All cases of PALs already reported were shown to be associated with EBV. Actually, there is some evidence that EBV is involved in the pathogenesis. At first, when comparing chronic pyothorax and PAL for the presence of the EBV genome, OHSWA et al. [28] detected the virus in only one of the 16 cases of chronic pyothorax tested. This could suggest that neoplastic transformation of infiltrating lymphocytes in chronic pyothorax by the EBV is a late event in the lymphomagenesis, or that EBV-positive chronic pyothorax is predisposed to a malignant transformation which could imply that a second event is needed [28]. Moreover, all cases of PAL previously tested were shown to be associated with LMP-1 and EBNA-2 expression. The presence of these two proteins led to the hypothesis that PAL would occur in a context of immunodeficiency and also that EBV may be involved in the pathogenesis of the disease [13]. Indeed, LMP-1 and EBNA-2 are immunogenic proteins: they are known to induce a T-cell mediated reaction with destruction of the infected cells [29].

The presence of these two proteins in tumoural cells suggests a defect in the T-cell mediated immune response of the host. Indeed, the combined expression of these two proteins is exclusively detected in post-transplantation and HIV-related lymphoproliferative diseases [26, 30]. Patients with PAL have no generalized immune deficiency.
but FUKAYAMA et al. [31] have suggested that immunocompetent cells may not be able to enter the pleural cavity in the presence of chronic pyothorax, leading to a state of local immunodeficiency. Moreover, there is some evidence that the secretion of immunosuppressive cytokines such as interleukin (IL)-10 and the transforming growth factor (TGF)-1 in the context of a chronic inflammation could favour the transformation of the EBV-infected cells [32].

LMP-1 and EBNA-2 are also known to have oncogenic properties. LMP-1 induces the expression of the bcl-2 gene [33], which protects the cells from apoptosis and has a transforming activity in fibroblasts [34]; EBNA-2 is known to induce the expression of numerous cellular and viral oncogenes [29, 34–36]. The presence of these two proteins in tumoural cells suggests that EBV is not only a silent passenger, but could play a role in the pathogenesis of the disease. SAKADA et al. [13] pointed out that LMP-1 expression was weaker in PAL than in other lymphoid malignancies occurring in immunocompromised patients. KNÖPF et al. [37] also noted a very weak expression of LMP-1 in two cell-lines derived from PAL. This needs to be confirmed but the authors suggest that pathogenesis could be different in PAL.

Chronic inflammation may also induce the secretion of cytokines which could play a role in the proliferation of EBV-infected cells [8]. For example, KNÖPF et al. [38] showed that IL-6 enhanced the proliferation of the lymphoid cells in some PAL derived cell lines. In conclusion, the lymphomagenesis of PAL could have many stages including EBV infection, genetic aberrations, and soluble factors supplied from chronic inflammation.

We were unable to detect the HHV-8 genome, or expression of LMP-1 or EBNA-2 in any of our three cases. This suggests that PAL could sometimes develop by mechanisms other than by EBV infection.

Both PAL and BCBL are high-grade lymphomas showing pleural location and EBV infection. However, they present some morphological and phenotypical differences [21, 22] (table 4). First of all, the tumoural cells in BCBL most often show features of large-cell immunoblastic or anaplastic large-cell lymphoma [21] with sometimes plasmacytoid differentiation [22]. Moreover, they often express the leukocyte common antigen (LCA) CD45 and sometimes epithelial membrane antigen (EMA) but usually do not express B-cell-associated antigens CD19, CD20 and CD22 neither T-cell-associated antigens CD2, CD3 and CD5. The B phenotype can often be assessed only by molecular studies, showing a clonal immunoglobulin heavy- and light-chain gene rearrangement. Furthermore, there is no c-myc rearrangement and no alteration in p53 or ras genes. All of these morphological and immunophenotypic characteristics are consistent with a late stage of B-cell activation following EBV infection [21].

We were unable to detect the HHV-8 genome in the two cases of PAL we tested. Our results are in agreement with the reported studies of CÉSAR MAN et al. [20] and SOCLEK et al. [19] who did not find the virus in the 12 and two cases analysed, respectively. It seems, therefore, that the pathogenesis of PAL and BCBL are also different.

The prognosis of PAL is poor. FUKAYAMA et al. [31] pointed out that anti-viral capsid antigen (VCA) and anti-EBNA immunoglobulin G serum antibodies were elevated in some of the PALs. This could be helpful in the monitoring of patients, allowing an earlier diagnosis. Administration of antiviral drugs might prevent the disease from progressing [9].

In conclusion, we have described three new cases of pyothorax-associated lymphoma from a Western country. Two of them were proved to be associated with Epstein-Barr virus infection, with latent membrane protein-1 and Epstein-Barr virus-associated nuclear antigen-2 expression. No viral genome could be detected in the third case, suggesting that different mechanisms may be involved in the pathogenesis of the disease. No human herpes virus-8 sequence could be detected in the two cases we tested, suggesting that pyothorax-associated lymphoma and body cavity-based high grade lymphoma are two different diseases as mentioned by CÉSAR MAN et al. [25] in a previous study.

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**References**


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**Table 4. Summary of pathological findings of pyothorax-associated lymphoma (PAL) and body cavity-based high grade lymphomas (BCBL)**

<table>
<thead>
<tr>
<th></th>
<th>Pyothorax</th>
<th>Tumoural mass</th>
<th>Morphology</th>
<th>Phenotype</th>
<th>HIV</th>
<th>EBV</th>
<th>HHV-8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAL</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Immunoblastic, noncleaved, cleaved large cell type</td>
<td>CD45+, CD20+, CD3-, EMA-</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>BCBL</strong></td>
<td>No</td>
<td>No</td>
<td>Immunoblastic/anaplastic large cell type</td>
<td>CD45+, CD20-, CD3-, EMA+/-</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

HIV: human immunodeficiency virus; EBV: Epstein-Barr virus; HHV-8: human herpes virus-8; EMA: epithelial membrane antigen.