Patterns of Cytokine Gene Expression in Peripheral T-Cell Lymphoma of Angioimmunoblastic Lymphadenopathy Type

By Hans-Dieter Foss, Ioannis Anagnostopoulos, Hermann Herbst, Maren Grebe, Katharina Ziemann, Michael Hummel, and Harald Stein

Peripheral T-cell lymphoma of angioimmunoblastic lymphadenopathy type (AILD-TCL) is histologically characterized by a mixed infiltrate of atypical T cells, B cells including B immunoblasts, and plasma cells, as well as eosinophilic granulocytes accompanied by proliferated high endothelial venules, while, clinically, fever and weight loss are often observed. These morphologic and clinical peculiarities are widely believed to reflect abnormal patterns of cytokine expression. To evaluate this hypothesis, 11 lymph nodes with AILD-TCL were studied for the presence of tumor necrosis factor-α (TNF), lymphotoxin (LT), interleukin-6 (IL-6), and IL-1β transcripts by in situ hybridization (ISH) using [35S]-labeled cytokine-specific RNA probes in seven cases subsequent to immunohistology for cell type characteristic antigens. Expression of all four cytokines was strongly enhanced in AILD-TCL when compared with the control groups of lymphoblastic lymphomas and peripheral T-cell lymphomas, other than AILD-TCL: TNF and LT transcripts were present in atypical T cells and in a variable proportion of B immunoblasts in all AILD-TCL cases, whereas IL-6 and IL-1β specific transcripts were mainly found in nonlymphoid cells.

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To verify a possible cytokine expression by Epstein-Barr virus (EBV)-infected cells, which are frequently present in AILD-TCL, the detection of EBV-encoded nuclear RNAs (EBER) was combined with ISH for cytokine transcripts. It became evident that expression of LT and TNF by EBV-infected cells was largely restricted to B immunoblasts, which were only infrequently present in most AILD-TCL cases, whereas the expression of IL-6 was very rare, and IL-1β was not found in EBV-infected cells. These data suggest that expression of TNF and LT genes may contribute to the characteristic histologic and clinical features of AILD-TCL, and that cytokine expression by EBV-infected cells does not, in most cases, contribute significantly to the overall cytokine expression. Because it has been shown that LT is an autocrine growth factor for EBV-infected B cells, expression of this cytokine could contribute to the proliferation of EBV-infected B cells in AILD-TCL and, in the setting of immunosuppression, may ultimately play a role in the development of B-immunoblastic lymphomas.

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IN THE EARLY 1970s, a new disease designated angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) or immunoblastic lymphadenopathy was recognized.1,2 The clinical presentation comprises lymphadenopathy, hepatosplenomegaly, skin rashes, and constitutional symptoms associated with a variety of laboratory features, including hypergammaglobulinemia and autoimmune hemolytic anemia.3 Histologically, affected lymph nodes show an effaced architecture caused by a proliferation of small to medium-sized lymphoid cells and high endothelial venules, intermingled with variable proportions of B immunoblasts, plasma cells, and eosinophils embedded in an extensive, irregularly defined network of follicular dendritic cells.4 Subsequent studies showed that the vast majority of proliferating cells in AILD show a T-cell immunophenotype.5,6 In addition, clonal chromosomal abnormalities characteristic for peripheral T-cell lymphomas7 as well as clonal rearrangements of the T-cell receptor genes and, in some instances, of the immunoglobulin genes or both8,9 were detected in those lesions. Based on these data, the majority of cases previously diagnosed as AILD are now considered by most investigators to represent a distinct entity of malignant lymphoma: peripheral T-cell lymphoma of AILD type (AILD-TCL). In addition, in up to 15% of cases, B-immunoblastic lymphoma develops subsequent to the diagnosis of AILD-TCL.10

The pathogenesis of AILD-TCL is not clear. Several studies using a variety of sensitive techniques disclosed a strong association of this disease entity with Epstein-Barr virus (EBV) infection. In situ hybridization (ISH) for the detection of EBV-encoded nuclear RNAs (EBER) combined with immunohistologic analysis for B/T-cell lineage showed that in the majority of cases, a proportion of atypical T cells and B immunoblasts are infected with EBV.11-13 In addition, latent gene expression of the EBV was further verified by the presence of EBV-encoded latent membrane protein in some of the infected cells.14 Therefore, it has been postulated that EBV may be involved in the pathogenesis of AILD-TCL. The B-immunoblastic lymphoma developing in the background of AILD-TCL has been shown to also be EBV-associated in one case examined.15 The biconality of this particular tumor was taken as evidence for the hypothesis that these lymphomas might develop in a way analogous to those arising in immunocompromised patients.10

Both the characteristic histomorphology and the clinical features of AILD-TCL suggest abnormal patterns of cytokine secretion. However, there are only a few, in part contradictory, reports on cytokine expression in isolated cases of AILD-TCL.15-18 To establish a characteristic cytokine expression pattern in AILD-TCL, we studied the expression of four cytokines (tumor necrosis factor-α [TNF], lymphotoxin [LT], interleukin-6 [IL-6], and IL-1β) in routinely processed biopitic specimens from 11 cases of diagnosed AILD-TCL by radioactive ISH using [35S]-labeled RNA probes in seven
cases combined with immunohistology using lineage specific/characteristic antibodies. Our particular interest concerned TNF and LT gene expression, as both cytokines are potent inflammatory mediators, B-cell growth factors, and activators of endothelial cells and are, thus, presumed to play an important role in the histopathogenesis of this lymphoma entity. We included investigation of IL-6 expression, because this cytokine is found in the tumor cells of Hodgkin's disease, in most instances. To determine whether cytokine expression is attributable to EBV-infected cells, a simultaneous radioactive/nonisotopic ISH procedure using [35S]-labeled cytokine probes and digoxigenin-labeled probes specific for EBER was used.

MATERIALS AND METHODS

Biopic material. Formal-fixed/paraffin-embedded tissues from 11 patients with the diagnosis of AILD-TCL (10 cases from the files of the Institute of Pathology, Berlin, Germany, and one case from the Department of Hematology, University of Oxford, United Kingdom (kindly provided by Dr K. Gatter)) were studied. All biopsies were obtained before the initiation of therapy. Histologically, they displayed the typical morphologic features of this lymphoma entity with proliferation of high endothelial venules, small to medium-sized neoplastic T cells, small B lymphocytes, plasma cells, B immunoblasts, and eosinophilic granulocytes leading to a complete destruction of the lymph node architecture. To compare the patterns of cytokine gene expression, we selected two control groups that do not exhibit a polymorphous infiltrate nor any tissue reaction like nodal sinus histiocytosis; as well as peripheral T-cell lymphoma (PTL) (21 cases). The PTL group comprised 7 cases of small cell PTL (5 cutaneous, 2 nodal), 7 cases of medium-sized PTL, 4 cases of medium-sized to large cell PTL, and 3 cases of large cell PTL. The diagnosis of these lymphoma cases was established according to the criteria of the updated Kiel Classification.

Immunohistology. Phenotype determination was performed using monoclonal antibodies against CD20 (L26), CD25 (ACT-1), CD30 (Ber-H2), CD45RO (UCHL1, A6), CD45RA (4KB5), CD68 (PGM1), CD31 (JC70A), and T-cell receptor β-chain (βF1). For the detection of EBV-encoded latent membrane protein (βC3, 3-4, a cocktail of four monoclonal antibodies, was used. All reagents were obtained from Dako (Glostrup, Denmark) except βF1, which was purchased from T-cell Sciences (Cambridge, MA). Four-micrometer sections of formal-fixed tissue-blocks were stained by means of the immunoalkaline phosphatase method. Affinity-purified mouse antirabbit immunoglobulin, rabbit-antimouse immunoglobulin antibodies, and the APAAP complex (diluted 1:20) were obtained from Dako. Formal-fixed sections required a proteolytic treatment with 1 mg/mL Streptomyces griseus protease (Sigma, St Louis, MO) for 10 minutes before incubation with the monoclonal antibodies βF1 and Ber-H2.

Plasmids. cRNA probes were prepared by the subcloning of TNF, LT, IL-6, and IL-1β gene fragments in the run-off transcription vector pGEM1 (Promega Biotech, Madison, WI). The probe was the 500-bp EcoRI fragment of human LT kindly donated by Genentech Inc (South San Francisco, CA). The human TNF probe was the 600-bp Ava I-HindIII fragment of pAW711 (ATCC No. 39918), and the IL-6 probe was 600-bp EcoRI/PstI fragment of pXM309, generously provided by Genetics Institute (Boston, MA). The IL-1β was a 600-bp Smal/RanII fragment of YEpSEC1-bh1β (ATCC No. 67024). The nucleotide sequence of all cytokine probes was determined on the DNA sequencer 373A (Applied Biosystems, Foster City, CA) and proved to conform to published data. After linearization of the pGEM constructs with appropriate restriction enzymes, antisense and sense (control) RNA probes were generated by run-off transcription using T7 or SP6 RNA polymerases (Promega-Biotech, Madison, WI) with incorporation of [35S]-labeled nucleotides as previously described (average specific activity 1.3 x 10^5 cpm/µg). EBER1 and EBER2 specific plasmid-based vectors, prepared from plasmids pJ11 and pH2 were a gift of Dr Niedobitek (Birmingham, UK). EBER1- and EBER2-specific probes were combined to increase sensitivity and used as previously outlined.

ISH and double nonradioactive/radioactive ISH. The ISH procedures for the detection of cytokines or EBER, as well as the double ISH procedure for the simultaneous detection of both EBER and cytokine transcripts, were performed exactly as described. ISH experiments using sense probes for EBER or cytokine genes showed only weak nonspecific background. Cells containing more than 20

<table>
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<th>Case No.</th>
<th>LT</th>
<th>TNF</th>
<th>IL-6</th>
<th>IL-1β</th>
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<th>LT*</th>
<th>TNF*</th>
<th>IL-6*</th>
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<td>Single TC</td>
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<td>26</td>
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Abbreviations: TC, typical T cells; IC, small T cells; bC, small B-cells; IC, small lymphocytes; IB, B-immunoblasts; EC, endothelial cells; MP, macrophages; SH, sinus histiocytosis; U, strong signals on a large number of stromal cells in the capsule and surrounding the lymph node; ND, not done.

* Numbers indicated in the table signify the proportion of EBER-positive cells expressing the cytokine among all cells that express this cytokine.
grains were scored positive. This corresponded to more than four times background signal in all cases. Results were expressed in terms of the number of positive cells per mm² tissue section or the proportion of EBER positive cells expressing a particular cytokine among all cells expressing this cytokine (Table 1). Standard methods (comparison of means) were used for statistical analysis.

Combined immunohistology and ISH. The procedure was performed as reported previously using the APAAP method for immunohistology. In brief, dewaxed and rehydrated paraffin sections were incubated in 1 mg/mL Pronase. Antibodies were diluted in freshly prepared RPMI 1640 medium, pH 7.5, containing 10 mg/mL bovine serum albumin, 1.0 mg/mL yeast tRNA, and 5,000 U/mL heparin ammonium salt to inhibit RNA degradation. Each incubation step was performed for 30 minutes. After visualization of the immobilized antibodies, the slides were immediately subjected to the ISH procedure. Combined immunohistology and ISH for the detection of EBER was completed as reported.

RESULTS

Application of isotopic ISH to tissue sections of AILD-TCL showed strongly increased specific signals for LT, TNF-α, IL-6, and IL-1β when compared with the control groups of lymphoblastic lymphoma cases and PTL, other than AILD-TCL (Fig 1). To elucidate the lineage allocation of the labeled cells in AILD-TCL, ISH results were compared with the staining patterns obtained by application of a panel of cell type characteristic/specific monoclonal and polyclonal antibodies on adjacent sections. In addition, a double labeling procedure for simultaneous detection of cell type characteristic/specific antigens and cytokine transcripts was applied in seven cases. The following patterns could be identified.

TNF- and LT-specific gene transcripts (Fig 2A and B) were detected in all AILD-TCL cases and were only found in lymphoid cells, except in three cases in which an additional labeling was observed over scarce macrophages with the TNF probe. As evidenced by simultaneous immunohistology

Fig 1. Enumeration of cytokine expressing cells in (A) AILD-TCL (n = 11), (B) PTL, other than AILD-TCL (n = 21), and (C) LB (n = 8). Results are expressed as mean number (+ standard deviation) of labeled cells. The differences between AILD-TCL and PTL or LB are significant (P < .0027) for LT, TNF, IL-6, and IL-1β. nd, not done.

Fig 2. AILD-TCL, detection of cytokine transcripts by ISH using [35S]-labeled antisense probe. Numerous lymphoid cells show intense LT-specific signals (A). The insert taken from an ISH experiment developed after shorter exposure time shows that these cells have enlarged nuclei with multiple nucleoli. Expression of TNF transcripts in enlarged lymphoid cells (B). Labeling of reactive cells (endothelial cells or perivascular fibroblasts) and of an endothelial cell (insert) with an IL-1β antisense probe (C). Paraffin section, hematoxylin, and eosin counterstaining, magnification × 250 (A and C), × 400 (A, insert), × 320 (B), × 500 (C, insert).
and ISH, these signals could be attributed to a variable proportion of B immunoblasts. However, most of the labeled lymphoid cells did not express CD20, showed clearing of the cytoplasm and atypical nuclei and were labeled with an antibody directed against CD45RO (Fig 3A through D). This suggests that most of the labeled cells represent neoplastic T cells.

The number of labeled cells was almost uniform displaying only minor variations in the steady state transcript levels when using the TNF probe. The number and distribution of LT-expressing cells, as well as the signal intensity, varied from case to case. Two cases contained few labeled T cells, whereas three cases displayed large numbers of labeled atypical T cells, often arranged in small clusters.

When compared with the level of expression of LT or TNF, the signal intensity for IL-6 was much weaker within the affected lymph nodes. The majority of cases exhibited labeled endothelial cells and sometimes macrophages scattered throughout the lymph nodes; in three cases, signals over macrophage accumulations in the sinus were identified. In one case, hybridization signals were observed over few B immunoblasts, whereas in two further cases, few atypical T cells were labeled. In addition, in three cases, strong IL-6-specific signals were observed over stromal cells in the surroundings and in the capsule of the affected lymph nodes.

IL-1β transcripts were detected in macrophages in all AILD-TCL cases (Fig 3E). In addition, IL-1β-specific signals were detected over fibroblasts, endothelial cells (Fig 2C), and rare lymphocytic cells in some cases.

To identify the number of cytokine gene expressing cells that were also infected by EBV, a double radioactive/nonisotopic ISH for the detection of cytokine gene transcripts and EBER was applied to the tissue sections. The findings were compared with those seen on adjacent sections subjected to combined immunohistology for cell line characteristic specific antigens and ISH for the detection of EBER. The results of these experiments, summarized in Table 1, are as follows: (1) EBER-positive small lymphocytes, as well as the majority of EBER positive atypical T cells, did not contain detectable cytokine transcripts; (2) a significant proportion of EBER-positive B immunoblasts expressed LT- (Fig 3F) and TNF-specific signals in some cases; (3) EBV-infected cells contributed significantly to the overall expression of LT and TNF only in two cases containing a large number of EBER-positive B immunoblasts; and (4) IL-6 (Fig 3H) was only rarely detected, and IL-1β was not detected in EBER-positive cells. Because in two cases, EBER-positive cells that also expressed IL-6 were very scarce, definite lineage allocation of these cells was not possible.

**DISCUSSION**

The present study showed characteristic expression patterns of four cytokines (TNF, LT, IL-6, and IL-1β) in lymph node specimens with AILD-TCL. The application of sensitive RNA ISH to routinely processed biopsic material, in seven cases combined with immunohistology for cell type specific/characteristic markers, allowed the estimation of number, distribution, and lineage allocation of the cytokine expressing cells. By this approach, TNF- and LT-specific gene transcripts could be demonstrated in a proportion of atypical cells expressing T-cell antigens in all 11 cases investigated. Expression of these cytokines was also observed in B immunoblasts, the relative number of which, however, was smaller when compared with T cells. In contrast, IL-6 and IL-1β specific transcripts were absent in most cases from atypical T cells and only expressed by endothelial and histiocytic cells.

The constant finding of LT- and TNF-specific gene expression in the mixed cellular lesion of AILD-TCL and the comparatively low expression of these genes in the monomorphic infiltrate of the control groups of lymphoblastic lymphoma and peripheral T-cell lymphoma, other than AILD-TCL, suggests that these cytokines may be involved in the evolution of the histologic picture characteristic for AILD-TCL. These differences are apparently not due to the mere cellular composition of these lesions, because all of the cellular elements present in AILD-TCL are also observed in normal lymphoid tissues, however cytokine expression in latter tissues is generally very low. The different expression of cytokines is probably due to the activated state of cells present in AILD-TCL as demonstrated by the expression of activation antigens (eg, CD30). The above mentioned hypothesis is supported by the known properties of TNFs as activators of endothelial cells and macrophages and induction of high endothelial venule morphology.17,18,21 Furthermore, TNFs induce proliferation of B cells.19,20 Thus, the histologic picture of AILD-TCL with the proliferated high endothelial venules and the mixed cellular infiltrate with the accumulation of B cells might result from elevated production of TNFs by T and B cells. The demonstration of both B-cell growth factor and B-cell activating properties, which are well known functions of TNFs in culture supernatants of peripheral T cells of AILD-TCL patients and in a AILD-TCL–derived T-cell line, fits well within this scenario.12 The proposed concept is also compatible with the finding of preferential expression of IL-6 in endothelial cells and macrophages in several AILD-TCL cases, as it is known that TNFs induce IL-6 expression in many cell types, including endothelial cells.33,34 In addition, this hypothesis would explain the elevated expression of IL-6 in reactive cells in AILD-TCL when compared with the expression of the control groups. Our results support the immunohistologic findings of a previous report demonstrating IL-6 expression in histiocytes of all three and in tumor cells in only one of three cases of AILD-TCL.16

Our in situ findings of elevated levels of cytokine transcripts in AILD-TCL might also have implications for the clinical presentation of AILD-TCL patients. TNF and LT are mediators of systemic symptoms such as fever or weight loss,18,35 which are present in more than 50% of the AILD-TCL patients.3 In addition, a recently published study showed a significant correlation between TNF and LT RNA levels in tissue extracts and the presence of systemic symptoms in non-Hodgkin’s lymphomas.36

In this context, it is of interest that elevated transcript levels of both TNF and LT have also been detected in the
tumor cells of Hodgkin’s disease, which, like AILD-TCL, is characterized by an abundant admixture of reactive cells and, in a proportion of cases, by the presence of systemic symptoms. Elevated transcript levels of LT and TNF have also been obtained in infectious mononucleosis, which shares some histologic (proliferation of B cells, prominent vascularization) and clinical features (fever) with AILD-TCL. At variance with AILD-TCL, however, the tumor cells in Hodgkin’s disease also express IL-6 transcripts in a high proportion of cases. This latter finding may suggest that characteristic cytokine expression patterns of different lymphoma entities do exist.

Expression of LT and TNF in lymphocytes of T and B phenotype is well in line with in vitro data. However, it was surprising that macrophages were labeled with the TNF-probe in only 3 of 11 cases, because these cells on stimulation are able to produce large amounts of this cytokine in vitro. Using the same batch of reagents in parallel experiments, tumor cells of ovarian serous carcinoma samples serving as positive controls were labeled with this probe. Also histiocytes present in other disease entities, such as Langerhans’ cell histiocytosis and Rosai-Dorfman disease constantly proved to express high levels of TNF transcripts (Foss HD, submitted). Lack of sufficient amounts of preserved RNA in our AILD-TCL cases can also be excluded, as strong specific signals were obtained in lymphoid cells with the TNF probe. Finally, the probe was sequenced, and the results proved to conform with published data. Taking all these facts into consideration, we conclude that macrophages in many AILD-TCL cases do not contain TNF transcripts or contain very low levels which are below the threshold of sensitivity of our in situ hybridization procedure.

Morphologic demonstration of cytokine transcripts does not answer the question as to which mechanism induces these cytokines. In vitro and in vivo investigations have shown that EBV-infected B cells may express cytokine genes, including LT and TNF. The recent demonstration of EBV-infection of a significant proportion of atypical T cells and B cells in AILD-TCL raises the question as to whether EBV is involved in the induction of cytokines in this lymphoma entity. By simultaneous double labeling for cytokine and EBER transcripts, however, only a relatively small proportion of EBV-infected cells proved to express LT, TNF, or IL-6 transcripts. By morphology and immunophenotype, these cells corresponded predominantly to B immunoblasts, whereas the vast majority of infected atypical T cells and small lymphocytes did not contain TNF, LT, and IL-6 transcripts. It is of interest that isomorphous EBV-positive B immunoblasts, but not small EBV-positive B lymphocytes, were found to express these cytokine transcripts in infectious mononucleosis. In AILD-TCL and infectious mononucleosis, EBV-infected B immunoblasts, but not EBV-infected small lymphoid cells, proved to express the activation molecule CD30 and EBV-encoded latent membrane protein on adjacent sections. Therefore, it appears that TNF and LT expression in EBV-infected cells in vivo is frequently associated with blastic morphology and expression of a characteristic antigenic profile, raising the possibility that induction of these cytokines related to cellular activation might be, in part, due to the action of certain EBV-encoded proteins.

Continuous proliferation of EBV-infected B cells is thought to be related to the secretion of autocrine growth factors. In particular, for both LT and IL-6 such an activity has been demonstrated in vitro in EBV-infected lymphoblastoid cell lines. Therefore, expression of LT in EBV-infected B immunoblasts could contribute to the proliferation of these cells and ultimately result in the development of B-immunoblastic lymphoma, given the frequent immunosuppression found in patients with AILD-TCL. This hypothesis is supported by the finding that one such tumor examined so far has been shown to be EBV-associated. Interestingly, this tumor was biclonal, and this has been taken as evidence for the hypothesis that the development of these B-immunoblastic lymphomas might be similar to the pathogenesis of lymphomas found in immunocompromised patients.

In conclusion, characteristic cytokine gene expression patterns in AILD-TCL may contribute to the well-established histologic and clinical features of this disease. Cytokine expression also occurred in EBV-infected B immunoblasts, although the amount of these transcripts represented only a minor percentage of the total amount of cytokine expression. Expression of LT in EBV-infected B immunoblasts may contribute to the proliferation of these cells in AILD-TCL and possibly to the development of B-immunoblastic lymphoma in the setting of immunosuppression. However, studies on the protein level are required to substantiate our interpretations because expression of some cytokines, such as TNF, is regulated at the level of translation and secretion. Comparison of the pattern established here with the patterns of other EBV-associated entities may lead to the elucidation of the influence of EBV-infected cells in the histologic composition and possibly the clinical presentation of these entities.
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Patterns of cytokine gene expression in peripheral T-cell lymphoma of angioimmunoblastic lymphadenopathy type

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