The mechanisms leading to malignant cell proliferation may differ between the different histologic forms of high-grade non-Hodgkin’s lymphomas. To analyze the potential role of interleukin-6 (IL-6) as a growth factor for lymphomatous cells in these different forms, in the in situ production of this cytokine was analyzed in lymphomatous samples taken from 24 patients, 18 of whom were human immunodeficiency virus (HIV) infected. Eleven Burkitt’s lymphomas (BLs), seven diffuse large-cell lymphomas, and six immunoblastic lymphomas were studied. In situ hybridization experiments showed that the IL-6 gene was expressed in all tissues. The number of IL-6 gene-expressing cells was 7 times higher in the non-BLs than in the BLs, and it was 17 times higher than that of 14 control lymph nodes displaying a benign follicular hyperplasia. Analysis of individual cases indicated that the level of IL-6 gene expression was strongly correlated with the presence of immunoblasts within the malignant clone. In contrast, this level was not correlated with the presence of Epstein-Barr virus genome in the lymphoma or with the HIV status of patients. Immunohistochemical studies with an anti-IL-6 monoclonal antibody showed that IL-6 was produced in non-BLs, but not in BLs. In the former, IL-6 mainly originated from reactive, nonmalignant cells. Immunohistochemical analyses of non-BLs also showed that malignant cells produced the 80-Kd chain of the IL-6 receptor. Taken together, these results suggest that IL-6 may act as a growth factor in some forms of high-grade B lymphomas. The presence of immunoblasts may be an indicator of such forms.

INTERLEUKIN-6 (IL-6) is a pleiotropic cytokine with a number of effects on cells of the B-lymphocyte lineage. It is a major factor involved in the terminal differentiation of normal B lymphocytes. A number of reports have also outlined its role as a growth factor for Epstein-Barr virus (EBV)-infected and malignant lymphocytes. On the one hand, IL-6 is produced by lymphoblastoid cell lines and during anaplastic large-cell lymphomas, Lennert lymphomas, Hodgkin’s disease, and multiple myeloma. On the other hand, tumorigenicity of lymphoblastoid cells is correlated with their IL-6 production, and anti–IL-6 neutralizing antibodies or IL-6 antisense oligonucleotides inhibit the growth of transformed B cells. To our knowledge, the production of IL-6 in high-grade B lymphomas has not yet been reported.

This study was therefore undertaken to investigate the production of IL-6 in high-grade B lymphomas and to assess variations in IL-6 production according to the histologic type. We also asked whether the pattern of IL-6 gene expression differed in B lymphomas arising in human immunodeficiency virus (HIV)-infected patients as compared with HIV-seronegative patients. Indeed, HIV induces IL-6 production both in vitro and in vivo, and IL-6 is involved in the chronic stimulation of B lymphocytes from HIV-infected patients.

We show that the IL-6 gene is expressed in situ in all of the lymphomas tested. The level of IL-6 production is similar in lymphomas arising either in acquired immunodeficiency syndrome (AIDS) patients or in HIV-seronegative patients. In contrast, this level is strongly correlated with the histologic characteristics of the lymphoma.

MATERIALS AND METHODS

Tissues. Eighteen specimens from HIV-infected patients were provided through the French Study Group for Pathology of Human Immunodeficiency Virus-Associated Tumors. Six specimens were from patients seronegative for HIV-1. Fourteen samples displaying a reactive follicular hyperplasia without malignant cells were used as controls. Nine of them were from HIV-infected patients and five were from patients seronegative for HIV-1.

Tissues were obtained by surgical biopsy performed for a suspicion of lymphoma. Two blocks of each specimen were immediately frozen in liquid nitrogen and stored at −80°C until analyzed by in situ hybridization and immunohistochemistry. In all cases, samples of each tissue specimen were also fixed in neutral formalin and processed for histology.

Diagnosis of lymphoma. Non-Hodgkin’s lymphomas were classified according to the Working Formulation using standard histopathologic criteria. All morphologic features of the tissues were determined independently of the analysis of IL-6 production.

Analysis of the clonality of the lymphoma and of EBV infection. Total cellular and viral DNA was prepared by sodium dodecyl sulfate (SDS) cell lysis, proteinase K digestion, phenol/chloroform extraction, and ethanol preparation. Ten micrograms of DNA was digested with 50 IU of several appropriate endonucleases (BRL, Gaithersburg, MD). Digested DNAs were electrophoresed on a 0.7% to 0.8% agarose gel in Tris-Borate EDTA buffer, hydrolyzed for 15 minutes in 0.25 N HCl, denatured for 30 minutes, and restaturated before being transferred onto Hybond N filter (Amersham, Les Ulis, France) in NaCl 1.5 mol/L/NaOH 0.5 mol/L according to the method of Southern. Probes were 32P-labeled by multiple random priming system (Amersham) and hybridized with the filter according to the recommendations of the manufacturer.
Filters were washed at 60°C for 30 minutes in 2X SSC, 0.1% SDS and 15 to 30 minutes in 0.2X SSC, 0.1% SDS and autoradiographed using intensifying screens (Quanta III; Dupont, Paris, France).

The organization of the IgH locus was analyzed by hybridization to probes specific to the joining region (JH) and constant region (Cκ). The search for EBV DNA was performed using a probe specific for the BamHI W internal repeats of the virus (kindly provided by G. Bornkamm, Munich, Germany).

**Immunohistochemical analysis.** The anti-IL-6 monoclonal antibody (MoAb), used at a final dilution of 1/500, has been described previously.16 The MT18 MoAb recognizing the 80-Kd chain of the IL-6 receptor17 was used at a final dilution of 40 μg/mL. For both antibodies, the immunohistochemical analysis was performed with an immunoperoxidase assay using an Avidin-Biotin complex technique according to manufacturer's recommendations (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA).

**In situ hybridization.** The IL-6 probe used in in situ hybridization experiments has been previously described.15 It was an antisense riboprobe recognizing the coding sequence of the IL-6 messenger RNA (mRNA). It was labeled with 35S-UTP (> 1,000 Ci/mmol; Amersham). The sense probe was used as a control and gave no positive signals (data not shown).

In situ hybridization was performed as previously described.18 Cells were scored as positive when containing more than 20 grains. This corresponded to more than four times the background level in all cases. IL-6 gene expressing-cells were counted in a blind procedure, with the worker not knowing the pathologic type or the EBV status of the lymphoma. Results were expressed as the number of positive cells detected per square centimeter of tissue section. A Mann-Whitney U test was used for statistical analysis.

**RESULTS**

**Characteristics of lymphomatous tissues.** The main characteristics of lymphomatous tissues are shown in Table 1. All lymphomas exhibited phenotypic markers of B lymphocytes, as determined by immunohistochemical studies (data not shown).

Eleven of the lymphomas were Burkitt’s lymphomas (BLs), 10 of which displayed a typical pattern, characterized by a monomorphic infiltration of the tissue by small noncleaved malignant cells. In the remaining case (no. 4), an immunoblastic component with plasmacytic morphologic features was associated with typical small noncleaved cells. This lymphoma, which was from an HIV-infected patient, was undoubtly a BL as it fulfilled the main morphologic criteria of BL according to the World Health Organization description,19 namely, the cohesiveness of medium sized lymphomatous cells.

Of the non-BLs, seven were diffuse large-cell lymphomas (DLCLs) and six were immunoblastic lymphomas (IBs). Four of the DLCLs were monoclonal, whereas the three others were polymorphic, containing a component of immunoblasts, as described by Hui et al.20

**IL-6 gene expression in high-grade B-lymphoid malignancies.** IL-6 gene expression by single cells was analyzed by in situ hybridization in the 24 lymphomas. The level of IL-6 gene-expressing cells in these tissues was quantified by counting cells hybridizing the IL-6 probe. Four serial tissue sections were analyzed for each case. Results were compared with those from 14 lymph nodes displaying a benign follicular hyperplasia (control lymph nodes). IL-6 gene-expressing cells were detected in all lymphomas. However, their number was highly heterogeneous between cases. Interestingly, this number correlated with the pathologic findings.

Ten of the 11 BLs did not express the IL-6 gene at a higher level than control lymph nodes. The six IBs all contained particularly large numbers of IL-6 gene-expressing cells. Three of the seven DLCLs also contained numerous IL-6 gene-expressing cells (Fig 1). Overall, the number of IL-6 gene-expressing cells in the non-BLs was 17 times higher than in the control lymph nodes (P < .001), and was also higher (7 times) than in the BLs (P < .01). Similar findings were observed regardless of the HIV status of patients (Fig 1).

Therefore, the expression level of the IL-6 gene was highly heterogeneous among lymphomas, and this level was correlated with pathologic findings. Increased IL-6 gene expression mainly occurred in non-BLs.

**IL-6 gene expression in individual cases of high-grade B lymphomas.** Although the average level of IL-6 gene expression in high-grade lymphomas correlated with the Burkitt or non-Burkitt type of the tumor, analysis of individual cases showed some exceptions. We thus asked whether these latter displayed peculiar histologic features.

Only one case of BL was associated with an increased IL-6 gene expression as compared with benign control lymph nodes. This case (no. 4) differed histologically from the other 10 BLs as it was the only one to contain immunoblasts in association with small noncleaved cells (Table 1).
Of the non-BLs, the group of DLCLs appeared to be heterogeneous with respect to IL-6 gene expression. Only three of the seven cases expressed the IL-6 gene at a high level. These three cases (nos. 13, 15, and 18) were histologically different from the four others as they were polymorphic, associating immunoblasts and centroblasts (Table 1).

The presence of immunoblasts in the lymphomatous tissue may thus be an important pathologic parameter associated with an increased IL-6 gene expression. To formally demonstrate this finding, we assessed the level of IL-6 gene expression of lymphomas according to the presence of immunoblasts within the tumor. The IL-6 gene was expressed at a much higher level in the 10 lymphomas containing immunoblasts than in the 14 lymphomas containing no immunoblasts (Fig 2). On average (+SEM), 1,780 ± 277 IL-6 gene-expressing cells/cm² were present in the former as compared with 79 ± 11 in the latter (P < .001). This result confirmed the strong correlation linking the presence of immunoblasts to IL-6 gene overexpression in high-grade B-lymphoid malignancies.

Comparative analysis of IL-6 gene expression levels and EBV infection. We next asked whether the level of IL-6 gene expression could be correlated to the presence of the EBV genome in lymphomatous cells. Fifteen tissues, all from AIDS patients, were tested for the presence of EBV genome by Southern blot analysis. EBV was detected in 10 of them (Table 1). No correlation was found between the detection of EBV genome and the level of IL-6 gene expression. A mean (±SEM) of 739 ± 510 IL-6 gene-expressing cells/cm² was detected in the five EBV-negative lymphomas, as compared with 747 ± 289 in the 10 EBV-positive lymphomas. Similarly, the presence of EBV did not correlate with that of immunoblasts within the lymphoma (Table 1).

Production of the IL-6 protein in high-grade B lymphomas.

We next analyzed whether expression of the IL-6 gene, assessed in the preceding experiments by in situ hybridization, was associated with a parallel production of the IL-6 protein itself. This was performed by labeling IL-6-containing cells by an immunohistochemical technique using an anti-IL-6 MoAb. Nine lymphomas were studied: three BLs with low levels of IL-6 gene expression in situ hybridization experiments (cases no. 1, 9, and 10), the only BL with an increased IL-6 gene expression (case no. 4), one polymorphic DLCL expressing the IL-6 gene at a high level (case no. 13), and four IBs (cases no. 19, 20, 22, and 24).

The only three cases in which no IL-6-containing cells were detected were the three BLs in which the IL-6 gene was expressed at a low level in situ hybridization experiments. In contrast, IL-6-containing cells were detected in the six other cases, in which they were abundant (Fig 3D and E). Therefore, IL-6 mRNA production in high-grade B lymphomas was associated with the synthesis of the corresponding protein. Moreover, a good correlation was found between the number of IL-6 mRNA-containing cells and the detection of IL-6-containing cells.

Characterization of IL-6-producing cells. The characteristics of IL-6-producing cells in the lymphomas were then studied. We first analyzed the morphology and the location of cells hybridizing the IL-6 probe in situ hybridization experiments. In most cases, endothelial cells synthesized IL-6 mRNA, as shown by the positivity of elongated cells in an endothelial position (Fig 3A). However, such cells always accounted for only a minority of IL-6 gene-expressing cells, as most positive cells were located outside vessels (Fig 3B).
The morphology of IL-6–producing cells was then analyzed by immunohistochemistry in the six lymphomas in which positive cells were detected. Similar findings were observed regardless of the HIV status of patients. Virtually no malignant cells contained IL-6. Only a very few of them were unambiguously stained, and this labeling was weak (Fig 3C). In contrast, reactive cells accounted for the vast majority of IL-6–containing cells, and they belonged to distinct cell populations. Positive endothelial cells were evidenced in all instances. Their labeling was characterized by a granular cytoplasmic pattern, reflecting the presence of IL-6 in the Golgi apparatus (Fig 3D). IL-6–containing macrophages were also evidenced in all cases (Fig 3E). Cells with a morphology consistent with a benign lymphoid origin were occasionally detected.

Therefore, IL-6 was produced mainly if not exclusively by reactive cells in high-grade B-lymphoid malignancies, and several cell populations were involved in this production.

Expression of the 80-Kd chain of the IL-6 receptor by malignant cells. The results reported above suggested that an in situ IL-6 production may stimulate the growth of malignant B cells in some pathologic forms of high-grade B-lymphoid malignancies. To support this hypothesis, expression of the IL-6 receptor by malignant cells was analyzed by testing for the presence of the 80-Kd chain of the receptor by immunohistochemistry. Four cases of lymphomas associated with an increased IL-6 production were studied: three IBs (cases no. 21, 22, and 24) and one DLCL (case no. 13).

Production of the 80-Kd chain of the IL-6 receptor was
evidenced in the four cases. Most malignant cells bound the
antibody and were intensively stained (Fig 4). Strikingly,
the anti-80-Kd chain antibody labeled the cytoplasm of
malignant cells as well as their membrane. Whether this
observation reflects the storage of the 80-Kd chain during
its synthesis or its internalization after the binding of IL-6
to its receptor remains to be determined. Nevertheless,
the synthesis of the 80-Kd chain of the IL-6 receptor by
malignant cells suggests that an in situ production of IL-6,
when occurring, participates in the growth of neoplastic
cells in high-grade B lymphomas.

DISCUSSION

The growth factor requirements of malignant B lympho-
cytes may differ according to their degree of maturation and
to their oncogene abnormalities. In this report, we investi-
gated whether IL-6, a potent growth factor for malignant B
lymphocytes, is produced in situ in high-grade B lympho-
mas. We show that IL-6 is produced at a high level in some
but not all of these lymphomas. Two different situations
were identified: non-BLs, in which IL-6–producing cells
were usually abundant, and BLs, in which they were less
numerous.

The IL-6 gene was expressed at a high level in most of the
13 non-BLs. The number of cells hybridizing the IL-6 probe
in these tissues was significantly increased as compared
with control lymph nodes. They also displayed a different
distribution. IL-6 gene-expressing cells were scattered
throughout tissue sections in non-BLs, whereas they were
mainly found in sinuses in control lymph nodes, as previ-
ously reported.

The increased level of IL-6 gene expression in non-BLs
was not related to the HIV status of patients. A similar
number of IL-6 gene-expressing cells was detected in the
nine non-BLs from HIV-infected patients as compared
with the four cases from patients seronegative for HIV.

IL-6 gene expression in lymphomas was associated with
the production of the corresponding protein, which could
be evidenced in the lymphomatous tissue by immunohisto-
chemistry. As malignant cells produced the 80-Kd chain of
the IL-6 receptor, this indicates that IL-6 may play a role in
the growth of malignant B cells in non-BLs.

The respective involvement of autocrine versus paracrine
production of IL-6 during B-lymphoid malignancies re-
mains a matter of debate. In the tissues we studied,
immunohistochemical experiments clearly showed that
the vast majority of malignant cells were not labeled
with the anti–IL-6 MoAb. Only rare positive lymphomatous
cells were shown to contain IL-6, and their labeling,
although unquestionable, was always weak. Whether this
labeling reflects a low level of IL-6 production by some
malignant cells or internalization of the cytokine in associa-
tion with the IL-6 receptor is unclear.

Regardless of the significance of the presence of IL-6 in
some malignant cells, the important point was that virtually
all cells containing IL-6 in the lymphomatous tissues were
reactive, nonmalignant cells. Therefore, even if IL-6 is
produced in an autocrine fashion by some malignant cells,
this would only account for a minute fraction of the IL-6
production in the lymphoma. Endothelial cells undoubt-
edly participated in the production of IL-6, as they were
labeled both in situ hybridization experiments using an IL-6–
specific probe and in immunohistochemical studies using an
anti–IL-6 MoAb. Similarly, tumor-infiltrating macrophages
played an important role in the production of IL-6, as a
number of IL-6–containing cells were morphologically
related to this cell population. In addition, some normal
lymphoid cells (displaying a morphology different from that
of lymphomatous cells) contained IL-6. Therefore, charac-
terization of IL-6–producing cells in non-BLs outlined the
important role of tumor-infiltrating reactive cells in IL-6
production as well as the heterogeneity of these IL-6–
producing cells.

Contrasting with the high level of IL-6 production in
non-BLs, the IL-6 gene was expressed at a low level in most
BLs. In this situation, IL-6 gene-expressing cells were
detected in all cases by in situ hybridization, but they were
much less abundant than in the non-BLs. Their number was
in the range of that found in control lymph nodes. Immuno-
histochemical experiments confirmed this finding, as no
cells were labeled with the anti–IL-6 MoAb in the three
typical BLs studied. This finding suggests that production of
IL-6 is not involved in the growth of lymphomatous cells
during BLs.

A significant association was thus observed between the
pathologic characteristics of the lymphoma according to the
Working Formulation and the level of IL-6 production in
the microenvironment of malignant cells. Analysis of indi-
vidual cases showed, however, that the best parameter
associated with increased IL-6 production was the presence
of immunoblastic cells within the malignant clone. DLCLs
in its polymorphic form can include an immunoblastic
component within malignant cells. Similarly, BLs may
display immunoblasts associated with small noncleaved
cells, and particularly in HIV-related BLs. We ob-

Fig 4. Production of the 80-Kd chain of the IL-6 receptor by
malignant cells. The presence of the 80-Kd chain of the IL-6 receptor
was analyzed in three IBs and in one DLCL. Malignant cells were
labeled by the anti–80-Kd chain antibody in all cases. No labeling was
detected when the anti–80-Kd chain MoAb was not added during the
first step of the reaction (not shown). Results shown are from case no.
22 (original magnification ×400).
served that the level of IL-6 production in the BLs and in the DLCLs containing immunoblasts was similar to that of typical immunoblastic lymphomas. This level was 22 times higher than in the lymphomas that did not contain immunoblasts. To our knowledge, this is the first reported correlation between the in situ amount of a cytokine and the pathologic characteristics of lymphomatous cells.

Several mechanisms could account for the clearcut association between an increased IL-6 production and the presence of malignant immunoblasts. EBV may play a role in this phenomenon, as EBV infection of normal B lymphocytes upregulates their IL-6 gene expression. Moreover, IL-6 is one of the factors required for the growth of lymphoblastoid cell lines, and injection of such lines in immunodeficient mice induces lymphomas with an immunoblastic phenotype. However, the presence of EBV in the lymphomas we studied did not correlate with the level of IL-6 production, indicating that there must be other mechanisms responsible for this process.

IL-6 production by macrophages and endothelial cells is induced by interferon-γ (IFN-γ), and we previously showed that the IFN-γ gene is expressed by T lymphocytes infiltrating lymphomatous tissues. Whether the expression level of the IFN-γ gene in the various forms of lymphomas is correlated to that of the IL-6 gene remains to be determined. The low level of expression of adhesion molecules by Burkitt's lymphomatous cells may prevent their recognition by infiltrating T lymphocytes.

Another interesting hypothesis is that malignant cells directly stimulate IL-6 production by stroma cells. In this case, only malignant B lymphocytes at a particular stage of differentiation along the B-cell lineage may be able to stimulate IL-6 production by reactive cells.

Finally, whatever the triggering mechanism of IL-6 production by stroma cells, this interleukin may be a critical factor responsible for the differentiation of malignant cells in immunoblasts. Indeed, the differentiating properties of IL-6 on normal B lymphocytes are well documented.

Our results have potential therapeutic applications. Drugs interfering with the growth factors of malignant cells could be of benefit in high-grade B lymphomas, and particularly MoAbs directed toward IL-6 or its receptor. Administration of an anti–IL-6 MoAb inhibits the in vivo growth of malignant B lymphocytes in advanced myelomas. A similar approach may be evaluated in AIDS lymphomas, which often respond poorly to chemotherapy. Our results show that such a treatment should be primarily considered in lymphomas in which immunoblasts are present.

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REFERENCES

Interleukin-6 production in high-grade B lymphomas: correlation with the presence of malignant immunoblasts in acquired immunodeficiency syndrome and in human immunodeficiency virus-seronegative patients

D Emilie, J Coumbaras, M Raphael, O Devergne, HJ Delecluse, C Gisselbrecht, JF Michiels, J Van Damme, T Taga and T Kishimoto