American Journal of Pathology, Vol. 136, No. 2, February 1990

IL-2 mRNA Expression in Tac-positive Malignant Lymphomas

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Expression of the IL-2 receptor (Tac antigen/CD25) is documented in malignant lymphomas. Because IL-2 is a major lymphocyte growth factor, an IL-2-dependent growth could be involved in the proliferation of Tac-positive lymphomas. Indeed such a mechanism has been demonstrated experimentally for the growth of T-cell lines. To investigate this point in human lymphomas, we used in situ ***hybridization to analyze the expression of the IL-2 gene in 20 non-Hodgkin's lymphomas, among which 12 expressed the IL-2 receptor. Nine of these were anaplastic large cell lymphomas expressing the Ki-1-related antigen. We here show that IL-2-producing cells are present in all the lymphomas we analyzed. As a mean, there is no significant difference in the percentage of IL-2-producing cells between Tac-positive and -negative lymphomas. However, the level of IL-2 production is highly heterogeneous in both groups, and the highest density of IL-2-producing cells was observed in 2 Tac-positive lymphomas. Simultaneous detection of cellular antigens and of IL-2 mRNA demonstrates that IL-2 is produced by reactive T cells rather than by tumor cells. These results suggest that if IL-2 is involved in the growth of Tac-positive lymphomas, it acts as a paracrine, rather than an autocrine, factor. (Am J Pathol 1990, 136:383–390)***

Interleukin 2 (IL-2) is a T-cell growth factor stimulating the proliferation of activated T cells that possess IL-2-specific receptors. IL-2 and IL-2 receptor (IL-2R) (Tac antigen, CD25) genes are not transcribed in normal resting T cells but are induced after antigenic or mitogenic stimulation.[1] Constitutive expression of IL-2 and IL-2R genes (ie, in the absence of inducing stimulus) has been implicated in the *in vivo* growth of mouse[2,3] or gibbon[4] T-cell lines. In humans such an autocrine loop was shown to be involved in the *in vitro* growth of a T-cell line derived from a T lymphoma[5] and of an HTLV-1-infected T-cell line.[6] The responsibility of this autocrine mechanism is suspected but not definitely established for human HTLV-1-associated leukemias or lymphomas.[7–10]

IL-2R expression by malignant lymphomatous cells is not restricted to HTLV-1-related lymphomas.[11–16] Thus an IL-2-dependent growth could be involved in these cases. To investigate this point we analyzed the activation of the IL-2 gene in 20 non-Hodgkin's lymphomas, 12 of which expressed the IL-2R. Nine of the IL-2R-positive malignant lymphomas were anaplastic large cell lymphomas (ALCL), a recently described high-grade lymphoma in which the neoplastic cells express the Ki-1 (CD30)-related antigen.[17]

In situ hybridization allows the visualization of cells producing IL-2 in frozen tissue sections. These cells can be further characterized by combining this technique with immunohistochemical study of specific membrane antigens. By this approach we show that IL-2-producing cells are present in every studied lymphoma without significant difference between Tac-positive or Tac-negative lymphomas. We demonstrate that IL-2 is not produced by tumor cells but arises from reactive T cells.

Materials and Methods

Tissue

Lymph nodes from 20 patients were obtained at surgical biopsy. A portion of each specimen was immediately snap frozen in liquid nitrogen, and stored at −80 C.

Diagnosis of lymphoma was established according to the criteria of the International Working Formulation[18] and the updated Kiel Classification.[19] ALCLs were defined according to the cytohistologic pattern and the expression of Ki-1 by all the tumor cells.[17,20–22] Histologic and phenotypic characterizations of the 20 lymphomas are summarized in Table 1.

This work was supported by Faculté de Médecine Kremlin-Bicêtre and by the Fond d'Etude du Corps Médical des Hôpitaux de Paris.

Accepted for publication October 5, 1989.

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Table 1. *Histologic and Phenotypic Characterizations of the 20 Malignant Lymphomas*

Case number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Age	26	28	20	9	39	25	61	77	64	66	17	26	20	27	49	74	77	60	22	89
Sex	M	M	F	M	F	F	M	M	M	M	F	F	F	F	M	F	F	F	M	F
HTLV-1 serology	−*	−	N.A.	−*	N.A.	N.A.	N.A.	N.A.	−	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
CD30 (DAKO RSC1)	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
CD30 (BER H2)	+	+	+	+	+	+	+	+	+	−	−	+/−†	−	−	−	+/−†	−	−	+/−†	−
CD25 (ANTI-IL2R)	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
EMA	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
HLA-DR	+	−	+	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	+	−
CD22	−	−	−	−	−	−	+	−	−	+	+	+	−	+	+	+	+	+	+	+
CD19	−	−	−	−	−	+	+	−	−	+	+	+	−	+	+	+	+	+	+	+
CD2	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CD3	−	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
CD5	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CD7	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CD4	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CD8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Phenotype	T	T	T	T	T	B	B	O	O	B	B	B	T	B	B	B	B	B	B	B
Histology	ALCL	ALCL	ALCL	ALCL	ALCL	ALCL	ALCL	ALCL	ALCL	DLCL	DLCL	DLCL	DLCL	DLCL	DLCL	DLCL	DLCL	DLCL	DLCL	DLCL
IL-2-producing cells‡	0.35	8.79	0.86	0.90	12.93	0.93	0.40	1.34	0.15	0.25	0.37	0.42	0.53	1.06	0.71	0.42	1.03	0.15	0.44	0.27

The phenotype of tumor cells was determined. All lymphomas were negative for macrophage-associated antigens and cytokeratin (data not shown). Cases 1 to 12 are Tac-positive lymphomas. Cases 13 to 20 are Tac-negative lymphomas.
ALCL: Anaplastic large-cell lymphoma.
DLCL: Diffuse large-cell lymphoma.
N.A.: Not analyzed.
* Patients of Caribbean Origin.
† Only a minority (<20%) of the tumor cells express the Ki-1 antigen recognized by the Ber-H2 antibody.
‡ Number of IL-2-producing cells per 10^4 cells (see methods).

Among the 12 Tac-positive lymphomas, 2 cases (1 and 4) were of Caribbean origin. No anti-HTLV-1 antibodies were detected in these patients using an ELISA assay (DuPont de Nemours, Wilmington, DE). No clinical data suggested an HTLV-1 infection in the 10 remaining cases. HTLV-1 serologic analysis performed for two of them was negative (cases 2 and 9).

In Situ *Hybridization*

IL-2 Probe

The Xba-1 - Stu-1 fragment of the human IL-2 cDNA extending from bp281 to bp540 of the cDNA was cloned in the Eco RV restriction site of T3-T7 Bluescript-KS plasmid (Stratagen, La Jolla, CA). Two ^{35}S-labeled RNA probes were synthesized according to manufacturer recommendations (Amersham-France, Les Ullis, France).

The anti-sens probe was obtained after linearization of the recombinant plasmid with Bam H1 and RNA synthesis with T3 polymerase (Stratagen) and ^{35}S-UTP (more than 1000 mCi/ml, Amersham-France). The sens probe, used as a negative control, was obtained after linearization with Cla-1 and RNA synthesis with T7 polymerase (Stratagen) and ^{35}S-UTP. Alkalin hydrolysis of the 259 bp RNA probe was performed to obtain fragments ranging in length from 40 to 150 bp.

Hybridization Procedure

The method used for *in situ* hybridization was derived from Harper et al[23]: 5-μm cryostat sections of frozen tissue blocks were collected on RNase free slides, air dried for 4 hours, fixed in aceton for 10 minutes and stored at −80 C until used. The slides were postfixed for 20 minutes in paraformaldehyde (4% in PBS), rinsed in PBS, immersed in 0.1 M triethanolamine pH8 for 5 minutes at 4 C, rinsed in 0.1 M triethanolamine pH8 plus acetic anhydride 0.25% for 10 minutes at room temperature. Slides were then rinsed and dehydrated in ethanol. For each specimen three slides with two tissue sections per slide were hybridized with anti-sens probe and one slide with the sens probe. Hybridization was performed with 20 to 30 μl of the hybridization mixture containing 2×10^6 cpm ^{35}S UTP-labeled RNA probe. The slides were then covered with siliconized glass coverslips and incubated for 16 hours at 50 C in a moist box. After hybridization the slides were washed successively in 5×SSC, DTT 1 mM at 42 C for 30 minutes, in Formamide 50% 2×SSC DTT 1 mM at 60 C for 20 minutes, in NaCl 0.4 M EDTA 5 mM tris pH 7 10 mM at 37 C 10 minutes × 2. They were subsequently immersed in the latter solution containing RNase A (Sigma Chemical Co., St. Louis, MOl) 20 μg/ml at 37 C for 30 minutes, rinsed again in the same solution without RNase for 15 minutes, briefly rinsed in 2×SSC, and 0.1×SSC, dehydrated in ethanol 95 C containing 0.3 M ammonium acetate, and air dried. They were dipped into NTB-2 emulsion (Eastman Kodak, Rochester, NY) for autoradiographies, stored in absolute dark with desicant, and exposed at 4 C for various times (from 15 to 45 days). The slides were developed in Kodak D19, fixed in Kodak unifix, rinsed, counterstained with Harris hematoxylin, and coverslipped.

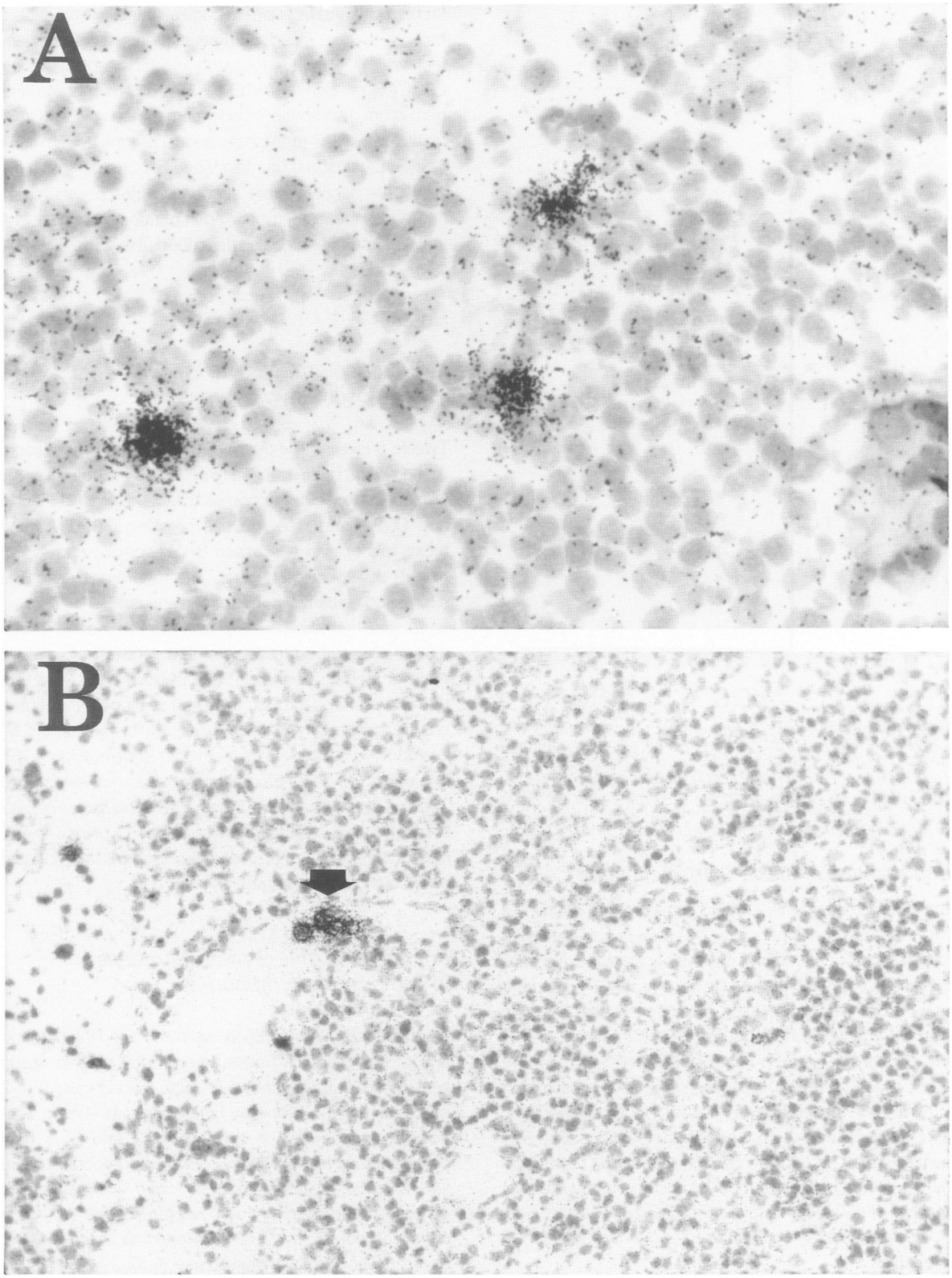

Figure 1. *IL-2 production in Tac-positive anaplastic large-cell lymphoma.* In situ *hybridization was performed using an IL-2-specific RNA antisens probe and slides were exposed for 20 days. The figure corresponds to case 2.* A *(original magnification* × *500) shows several IL-2-producing cells scattered among nonlabeled tumor cells.* B *(original magnification* × *200) shows exceptional intrasinusoidal IL-2 producing cells (arrow) in the same section.*

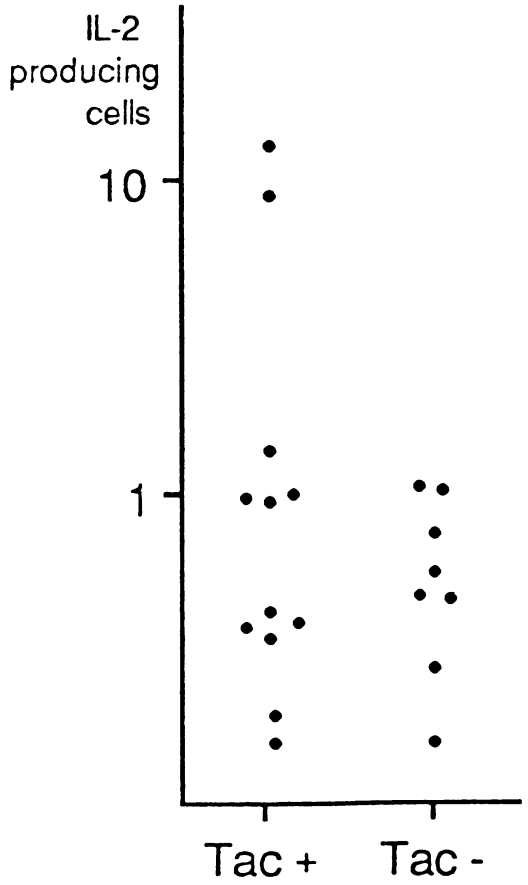

Figure 2. *Enumeration of IL-2-producing cells in 20 malignant lymphomas. The number of IL-2-producing cells was estimated per 10^4 cells (see Methods). Two Tac-positive lymphomas (cases 2 and 5) expressed a high level of IL-2-producing cells (8.79 and 12.93/10^4, respectively), as compared to the 10 remaining Tac-positive lymphomas (mean 0.59± 0.12/10^4) and to the eight Tac-negative lymphomas (mean 0.57± 0.11/10^4).*

Enumeration of IL-2 mRNA-containing Cells

Cells were scored as IL-2 mRNA positive when containing more than 20 grains per cells. This always corresponded to at least more than four times the background. No positive cells were detected with the sens probe. The percentage of IL-2 mRNA-containing cells in each tissue section was determined using a grid according to Garcia et al.[24] We counted the total number of IL-2 mRNA-containing cells per tissue section. Using the same grid we estimated in parallel the total cell count (TCC) in five high powerfields and the surface of the corresponding sections. The percentage of IL-2-producing cells corresponded to the ratio between the absolute number of such cells and the total number of cells per tissue section. Tac-positive and -negative lymphomas were hybridized and autoradiographed in parallel.

Combination of Immunohistochemistry and In Situ *Hybridization*

Simultaneous detection of cellular antigens and of IL-2 mRNA was assessed using a combination of immunohistochemistry and of *in situ* hybridization.[25] For immunohistochemistry we used a three-step immunoperoxidase technique[26] with the following monoclonal antibodies: CD30 (Ki-1 or BerH2) and EMA (Epithelial Membrane Antigen) from Dakopatts (Copenhagen, Denmark), CD25 (anti-IL-2R), CD3 (anti-Leu4) and CD2 (anti-Leu5b) from Becton Dickinson (Mountain View, CA). Aceton-fixed frozen-tissue sections were sequentially processed for immunohistochemistry, postfixed in paraformaldehyde (4% in PBS), and hybridized as described above.

Results

IL-2-producing Cells Are Present in Tac-positive Lymphomas

Twelve cases of Tac-positive lymphomas were studied. As shown in Table 1, these lymphomas included three B Diffuse Large Cell Lymphomas (DLCL) expressing CD19 and CD22, and nine ALCL expressing CD30. Among these two of nine expressed B-cell-specific and five of nine T-cell-specific antigens. The two remaining cases expressed neither B- nor T-related antigens.

In situ hybridization with anti-sens IL-2 RNA probe demonstrated the presence of IL-2 synthesizing cells in each of these 12 cases. These positive cells were randomly distributed throughout tissue sections without preferential localization (Figure 1A). In particular instances, IL-2-producing cells could be located in sinuses (Figure 1B). The presence of IL-2-producing cells was not restricted to Tac-positive lymphomas. Indeed we analyzed in parallel 8 Tac-negative DLCL-expressing B-cell- (7 of 8) or T-cell-related antigens (1 of 8). In all these cases, IL-2-producing cells could be demonstrated (Table 1).

Enumeration of IL-2-producing Cells in Tac-positive Lymphomas

To quantitate IL-2 production, we determined the percentage of IL-2-producing cells among total cells in tissue sections. Results are shown in Table 1 and Figure 2. IL-2 production level was highly heterogeneous among Tac-positive lymphomas. In two cases (2 and 5) a high percentage of IL-2-producing cells was detected (10.86 +/− 2.07 IL-2-producing cells/10^4 cells), contrasting with either the 10 remaining Tac-positive lymphomas, which contained only 0.59 +/− 0.12 producing cells/10^4 cells, or the 8 Tac-negative lymphomas (0.57 +/− 0.11 IL-2-producing cells/10^4 cells).

This amount of IL-2 synthesis did not correspond to the basal production by unstimulated T cells because an identical percentage of IL-2-producing cells was detected in 10 hyperplastic lymph nodes studied in parallel.[27] More-

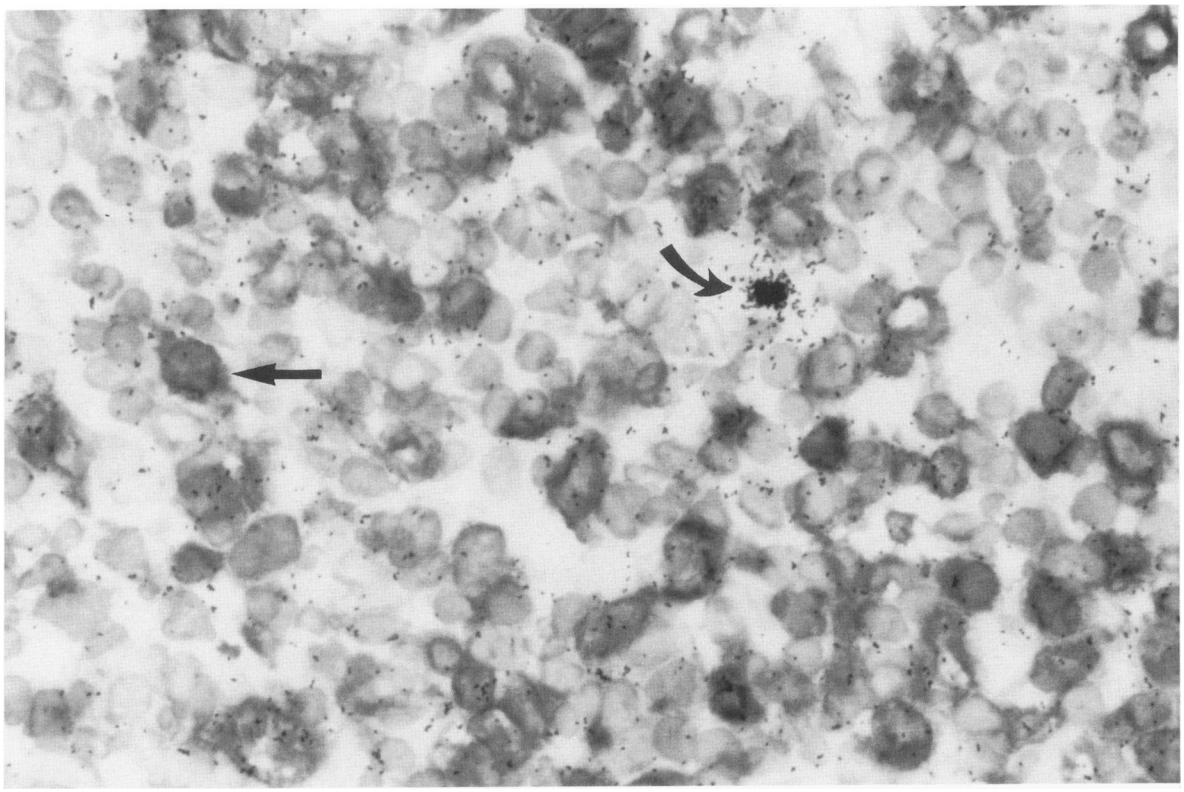

Figure 3. *Identification of IL-2-producing cells. Combination of immunohistochemistry using an anti-CD30 (Ki-1) antibody and of* in situ *hybridization: the IL-2-producing cell (curved arrow) is CD30 negative in contrast to a representative CD30-positive tumor cell (straight arrow; original magnification × 500).*

over, we found that less than one IL-2-producing cell/10^6 cells was detected in normal thymuses (data not shown).

Identification of IL-2-producing Cells in Tac-positive Lymphomas

Two points emerged from the previous results. 1) The number of IL-2-producing cells is low compared to that of malignant cells. 2) IL-2-producing cells can be demonstrated in B-cell lymphomas. Taken together this suggested that IL-2 production may be at least partly due to reactive T cells in malignant lymphomas. To definitely identify IL-2-producing cells in Tac-positive lymphomas, we performed in 5 cases (1, 5, 6, 8, and 10) simultaneous detection of cell-surface antigens and of IL-2 mRNA. We observed that IL-2-producing cells did not express CD30 (Figure 3), CD25 (Figure 4A), and EMA (data not shown). In contrast, IL-2-producing cells were CD3 positive (data not shown) and CD2 positive (Figure 4B), even in the case of CD3- or CD2-negative lymphomas.

These results show that IL-2 is not produced by malignant cells in Tac-positive lymphomas but rather by reactive T cells.

Discussion

The expression of IL-2R has recently been demonstrated in a number of malignant lymphoproliferative disorders, regardless of their expression of T-cell markers.[11-16] Because the major effect of IL-2 is the growth of CD25-bearing lymphocytes,[1] this spontaneous expression of CD25 raises the question of its functional involvement in the growth of malignant lymphoid cells. This hypothesis requires the demonstration of *in situ* IL-2 production in Tac-positive lymphomas. This interleukin synthesis could arise from either tumor cells or lymphoid stromal cells. The involvement of such an IL-2-dependent growth of lymphomatous cells has been recently demonstrated experimentally. When transfected with a constitutively expressed IL-2 gene, IL-2-dependent T-cell lines became highly tumorigenic when injected into recipient mice.[2,3] Similarly, IL-2 may be involved in the growth of a gibbon Tac-positive T-cell lymphoma related to a C-type retrovirus infection. Genomic analysis has shown in this case that the IL-2 gene is constitutively activated due to retroviral insertion.[4]

In vitro studies have demonstrated the possibility of an IL-2- dependent growth of human malignant lymphoid cells. The *in vitro* IL-2-dependent growth of a human cell line derived from a T-cell lymphoma has been reported.[5] Similarly normal T cells are immortalized when infected *in*

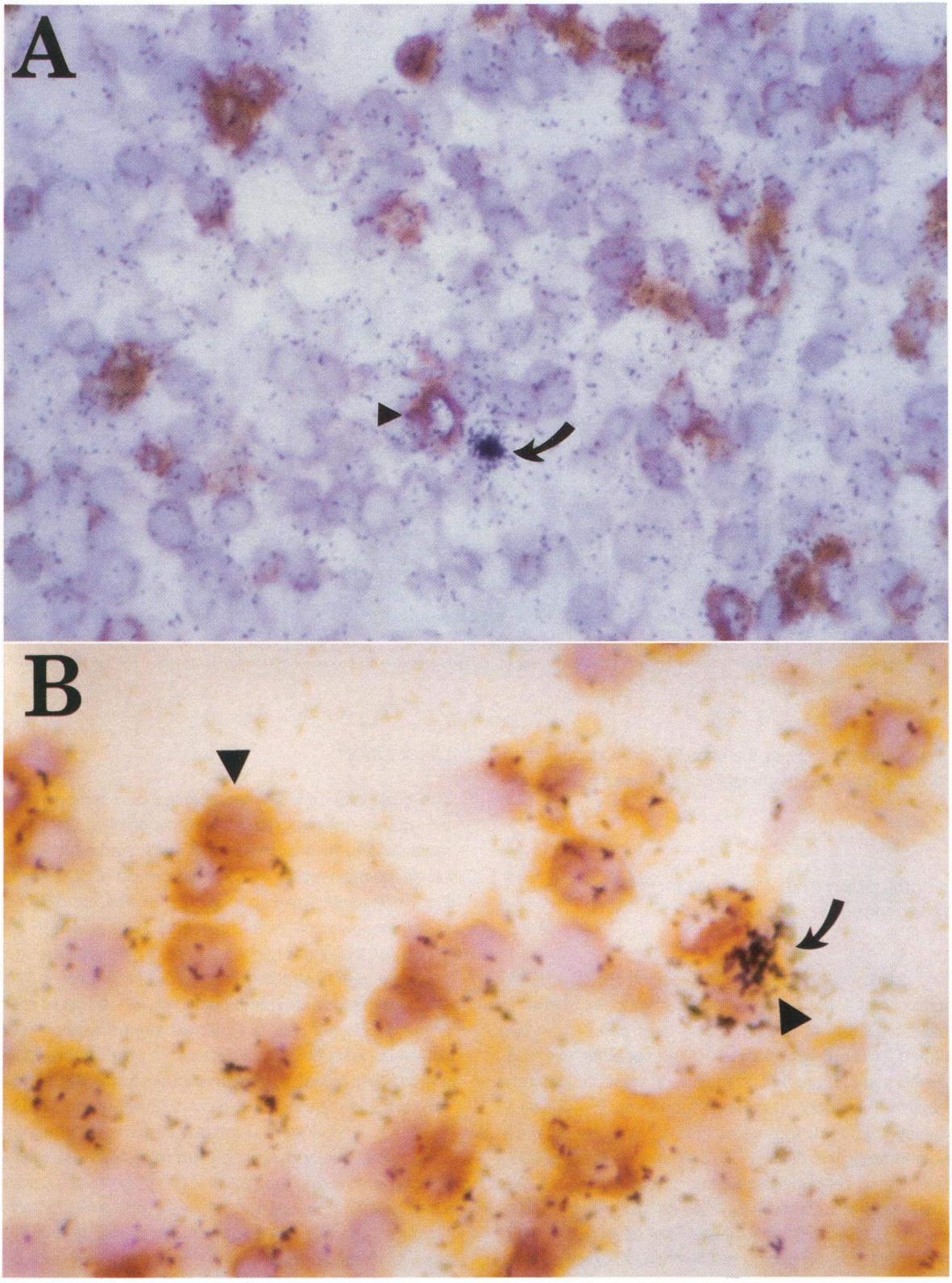

Figure 4. *Identification of IL-2-producing cells.* A: *Combination of immunohistochemistry with anti-CD25 antibody and of* in situ *hybridization (case 5): the IL-2-producing cell (curved arrow) is CD25 negative in contrast to a representative CD25 positive tumor cell (arrowhead) (original magnification × 500).* B: *Combination of anti-CD2 labeling and* in situ *hybridization: the IL-2-producing cell (curved arrow) is CD2 positive as well as an IL-2-nonproducing reactive T cell (arrowhead). In this case (number 5) tumor cells were CD2 negative (original magnification × 1250).*

vitro with HTLV-1, which induces the expression of both IL-2 and IL-2R genes.[6] Based on these observations, an autocrine loop has been suggested in some lymphoproliferative diseases. However the autocrine production of IL-2 by HTLV-1-induced lymphomatous cells remains controversial.[9]

We show for the first time that IL-2 is indeed produced *in vivo* in the microenvironment of human Tac-positive lymphomatous cells. Using *in situ* hydridization, we analyzed the presence of IL-2-producing cells in a variety of malignant lymphomas. We studied 12 Tac-positive lymphomas including 3 DLCL and 9 ALCL. ALCL is a recently described entity[17,20–22] in which lymphomatous cells express the CD30 (Ki-1) antigen.[28,29] They are thought to be derived from an activated lymphocyte of either T or B origin.[17] As controls, we studied eight Tac-negative DLCL. We observed an IL-2 production in each of the 20 lymphomas we analyzed. In two cases of Tac-positive lymphomas the number of IL-2-producing cells was dramatically higher than in the other 18 cases. Interestingly these two cases were the only ones in which tumor cells expressed simultaneously Tac and CD3 antigens.

Having established that IL-2 was produced in Tac-positive lymphomas, we wished to determine whether this production arose from tumoral or reactive T cells. It should be pointed out that even in the two cases displaying the highest level of IL-2-producing cells, their number stayed far below that of tumor cells. Two hypothesis could explain this result: 1) IL-2 may be produced by only a minority of tumor cells due to functional heterogeneity or to a cell–cycle-dependent IL-2 production.[30,31] 2) IL-2 may be produced by reactive T cells. This latter possibility was supported by the fact that we observed similar amounts of IL-2-producing cells in T-cell and B-cell lymphomas.

To directly examine this question we combined immunohistochemistry and *in situ* hybridization. This allowed us to demonstrate that IL-2-producing cells were devoid of antigens expressed by malignant cells: Ki-1, CD25, EMA. In contrast, IL-2-producing cells expressed CD3 or CD2 even in cases where malignant cells were CD3 or CD2 negative. Although we cannot exclude that lymphomatous T cells may also produce IL-2, this production, if present, should be very low. Indeed no labeling of tumor cells was detected after a 45-day exposure (data not shown), whereas reactive cells were strongly positive after a 15-day exposure. Thus IL-2 production in Tac-positive lymphomas arises from reactive T cells.

Most IL-2-producing cells were scattered within lymphomatous tissue, and some of them were found inside the sinuses. As infiltration of the sinuses by lymphomatous cells is a feature of anaplastic large cell lymphomas,[17,20–22] this raises the question of the nature of such intrasinusoidal IL-2-producing cells. However, we found similarly located IL-2-producing cells in hyperplastic lymph nodes,[27] indicating that reactive T-cells present in the sinuses are also involved in IL-2 production.

Although the number of IL-2 responsive T cells may be low among reactive T cells infiltrating lymphomatous lymph nodes,[32] the *in situ* production of IL-2 here demonstrated may play a role in the activation of antitumoral killer cells and thus in antitumor defense mechanisms.[33,34] This effect of IL-2 constitutes the basis of its therapeutic use in some disseminated cancers.[35,36] However, IL-2 may also stimulate the growth of IL-2R-expressing lymphomatous cells. Although Tac expression is not sufficient for an optimal IL-2 responsiveness,[37] this molecule plays a major role in the constitution of the high affinity IL-2 receptor. Thus the inhibition of IL-2 binding by anti-CD25 antibodies may provide a clue to determine the functional significance of IL-2 receptor expression by lymphomatous cells. Indeed Waldman et al[9] have recently shown that remission can be obtained in some cases of HTLV-1-induced leukemias after anti-Tac monoclonal antibody therapy. After this study, we successfully treated one of the presently reported patient with anti-Tac monoclonal antibody (manuscript submitted). Therefore, IL-2 receptors detected on lymphomatous cells may be functional, and *in situ*-produced IL-2 may play a role in the tumor growth.

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Acknowledgment

The authors thank R. Falkoff for the gift of IL-2 probe.