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Cytokine Expression in T-cell Lymphomas and Hodgkin's Disease

Its Possible Implication in Autocrine or Paracrine Production as a Potential Basis for Neoplastic Growth

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The detection of an increasing number of cytokines and the demonstration of autocrine and paracrine mechanisms perpetuating tumor growth prompted the investigation of the expression of the cytokines IL-2, IL-3, IL-4, IL-5, IL-6, IFN gamma, Tac, and GMCSF in primary lymph-node biopsies of patients with peripheral T-cell lymphoma (n = 11), Hodgkin's disease (n = 13), and large-cell anaplastic lymphoma (n = 6) by means of Northern blot analysis and in situ hybridization (ISH); 15 of 28 cases had IL-6 message, predominantly in cases of Hodgkin's disease (HD) and large-cell anaplastic lymphomas (LCAL). Interferon gamma was found in about 50% of the cases among all entities. Other cytokine expression was rare except two cases of HD with high amounts of IL-4 mRNA. These results indicate that large amounts of growth factor transcripts are present in a variety of malignant lymphomas. The meaning of this expression is still unclear. It may be a loss of physiologic regulation within the cytokine network which may thus influence neoplastic cell growth as some cases have a quantity of cytokine expression which is similar or even above that of stimulated T cells. ISH demonstrates in individual cases that the expression is at least in part due to malignant cells. (Am J Pathol 1991, 139:1173–1180)

Functionally heterogeneous activated T lymphocytes, especially helper/inducer T cells, are the major source of lymphokines. These soluble factors mediate at least in part activation, proliferation, and differentiation of T and B lymphocytes, monocytes, and other hematopoietic cells.[1]

In the B-cell system, the construction and composition of lymph-node follicles, the growth of follicular lymphomas with a neoplastic cell clone, and the constant coexistence of accessory cells give strong evidence of a cellular interaction mediated by soluble factors.[2,3]

The detection of autocrine or paracrine cytokine production in some B-cell lymphomas may be an additional ground for the hypothesis of a continuous neoplastic cell growth.[4,5]

Observations in B-cell lymphomas[2] prompted an investigation for possible cytokine expression within the different T-cell lymphoma entities and Hodgkin's disease (HD). The mixed cellular infiltrate, which is found in T-cell lymphomas and especially in HD, mirrors a cellular interaction between neoplastic and non-neoplastic cell compartments mediated by soluble factors. We investigated the expression of IL-2–IL-6 and of IFN gamma and granulocyte-monocyte stimulating factor (GMCSF) in total RNA and by means of *in situ* hybridization in tissue sections at the single-cell level.

Materials and Methods

Thirty sections from primary biopsies from untreated patients were investigated. One half of the lymph node was formalin fixed, the other half was snapfrozen in liquid nitrogen; 5-μm paraffin sections were stained for H&E, Giemsa, PAS (periodic acid Schiff-reaction), and for Gomori silver impregnation. Frozen sections were used for immunophenotyping with monoclonal antibodies (CD3, CD4, CD5, CD8, CD22, CD25, CD30) and for *in situ* hybridization. Cases of Hodgkin's disease were classified

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according to the Rye classification,[6] cases of T-cell lymphomas were classified according to the updated Kiel classification.[7,8] Eleven cases were diagnosed as peripheral T-cell lymphomas, 13 cases as HD and 6 cases as large-cell anaplastic lymphoma (LCAL), 3 of which were of T-cell type and 2 of B-cell type. One LCAL could not be classified concerning its cellular origin.

RNA Preparation and Northern Blotting

Frozen tumor material was homogenized and suspended in guanidine thiocyanate buffer according to the method of Chirgwin et al.,[9] followed by cesium-chloride gradient centrifugation.[10] 20 μg of total RNA per lane was size-fractionated by electrophoresis in 6% formaldehyde and 1.5% agarose gels.[11] Before RNA transfer to nylon membranes[12] (Hybond, Amersham, Braunschweig, FRG), the gels were stained with ethidium bromide to verify equal quantities and integrity of RNA. Equal amounts of total RNA of stimulated peripheral blood lymphocytes (PBL) phorbol-urgistate-A3 acetate 10 ng/ml, Sigma, Munich, FRG, Ca-ionophore-A 23187, Sigma, 100 ng/ml) were used as controls.

Hybridization

Prehybridization was performed in 50% formamide, 2 × SSC, 0.1% sodium dodecyl sulfate, 20 mM vanadyl-ribonucleosid-complex, 200 μg/ml salmon sperm DNA (Boehringer, Mannhein, FRG), 200 μg/ml yeast-tRNA (Boehringer), 5 × Denhardts solution, 10% dextran sulfate for 4 hours to 6 hours at 58°C; 5 ng/ml of alpha ^{32}p-labeled (Amersham) anti-sense-cRNA probe was added and hybridization was performed for 12 hours to 16 hours.[13]

Filters were washed 2 × 5 minutes, 2 × SSC, 0.1% SDS at room temperature, 2 × 30 minutes, 2 × SSC, 0.1% SDS at 65°C, 2 × 10 minutes, 0.2% SSC, 0.1% SDS at 65°C to 70°C.

Filters were exposed to Kodak-X-omat film (Kodak, Rochester, USA) for 8 hours to 72 at −70°C.

After exposure, RNA-blots were stripped of ^{32}p-labeled probes by incubation in boiling solution of 0.1% SDS and subsequent cooling to room temperature. Blots were reused 4 to 5 times.

In Situ *Hybridization*

Five-μm cryostat sections were placed on 2% 3-(Triethoxysilyl)-propylamine (Merck, Darmstadt, FRG) activated slides and fixed in 4% paraformaldehyde (Merck), 0.1% glutaraldehyde (Merck) in PBS (ph 7.5) for 60 minutes at room temperature and stored in 70% ethanol.

Rehydrated slides were acetylated in 0.1% triethanolamine (ph 8.0) (Sigma), 0.25% acetic anhydride (Sigma) for 10 minutes, and postfixed in 4% paraformaldehyde for 10 minutes, dehydrated in a series of graded ethanol and finally air dried.

Hybridization was performed in a solution containing 4 × SSC (1 × SSC = 0.15 NaCl, 0.015 M trisodium citrate), 10% dextran sulfate (Sigma), 20 mM vanadyl-ribonucleoside-complex, 200 μg/ml yeast-tRNA (Boehringer), 200 μg/ml salmon-sperm-DNA (Boehringer), 500 μg/ml bovine serum albumin (Sigma), 50% deionized formamide (BRL), 10mM Tris-HCL (pH 8,0), 1 mM EDTA, 5 ng alpha ^{35}S-UTP (Amersham) labeled antisense or sense riboprobe (specific activity: 1–2 × 10^8 cpm/μg) were added to 20 μl hybridization solution. Incubation time was 4 hours to 16 hours in a humidified chamber at 50°C.

Washing was performed in 4 × SSC at room temperature for 10 minutes, 2 × SSC at 52° for 30 minutes, 2 × SSC, 20 μg/ml RNase A (Boehringer) at 37°C for 30 minutes, 0.2 × SSC, 10 mM DTT (Sigma) at 52°C for 30 minutes. Slides were then rinsed twice in 70% ethanol, 200 mM ammonium-acetate (ph 5.4) for 10 minutes, dehydrated, and air dried.

Slides were subsequently dipped in Ilford K2 Emulsion, stored for 3 days at 4°C, developed in Kodak D19 developer and Giemsa stained.

Probes

The cDNAs of IL-2,[14] IL-3,[15] IL-6,[16] GMCSF,[17] IFN gamma[18] and TAC[19] were cloned and sequenced by one of us (W.S.) and were identical to the published sequences. The cDNAs of IL-4[20] and IL-5[21] were originally obtained from British Biotechnology and were identical to the published sequences.

The cDNAs of the coding regions of IL-2 to IL-6, GMCSF, gamma-IFN, and TAC were subcloned into transcription vectors (PSPT 18/19, Boehringer)

The ^{32}P and ^{35}S labeled sense and antisense-cRNA probes were generated in *in vitro* transcription experiments with T7 RNA-Polymerase (Boehringer) to a specific activity of 1–5 × 10^8 cpm/μg cRNA.

Results

The detailed diagnoses of the investigated lymphomas based on morphology and immunophenotypic data as well as the mRNA cytokine expression are given in Table 1. Investigation of cytokine expression of total RNA con-

Table 1. *Immunophenotype and mRNA Expression in Peripheral T-cell Lymphoma, Hodgkin's Disease, and Large-cell Anaplastic Lymphoma*

No.	Diagnosis	IL-2	IL-3	IL-4	IL-6	GM-CSF	IFN g
1	T-pleom	–	nd	–	–	–	+
2	T-pleom	–	–	–	nd	–	nd
3	T-pleom	–	–	–	–	–	+
4	T-pleom	–	–	–	–	–	–
5	T-pleom	–	–	–	–	–	+
6	T-pleom	–	+	–	–	+	–
7	*T-AILD	–	–	–	+ +/+ +*	–	+
8	T-AILD	–	–	–	–	–	+ +
9	T-AILD	–	nd	–	–	–	–
10	T-AILD	–	nd	–	–	–	+
11	Lennert's	–	nd	–	–	–	–
12	LCAL-B-type	–	–	–	+ +	+	–
13	LCAL-B-type	–	–	–	–	–	–
14	LCAL-O-type	–	–	–	+	–	+
15	LCAL-T-type	–	–	–	+	–	+
16	*LCAL-T-type	–	nd	–/+*	+/+*	–	–
17	LCAL-T-type	nd	nd	–	+	nd	nd
18	HDLP	nd	nd	–	+ +	nd	nd
19	HDNS	–	–	–	+ +	–	+
20	HDNS	–	–	–	+ +	–	+
21	HDNS	–	–	–	–	–	–
22	*HDNS	–	–	–/+*	+/+*	–	+
23	HDNS	nd	nd	–	+ +	nd	nd
24	HDNS	nd	nd	–	–	nd	nd
25	HDNS	nd	nd	–	+ +	nd	nd
26	HDMC	nd	nd	+ +	+	nd	nd
27	HDMC	–	+	–	+	–	–
28	*HDMC	–	+	–/–*	+	–	–
29	HDMC	–	–	–	–	–	–
30	HDLD	–	–	+ +	–	–	–

* Additional investigation by ISH.
*Northern-mRNA expression***
–: no expression detectable.
+: detectable signal after 48h exposure.
+ +: strong or very strong signal after 48h exposure.
+, + +: refer to relative intensity of the message, with + + being comparable to PMA/PHA-stimulated PBI.
nd: not determined.
T-pleom: peripheral T-cell lymphoma pleomorphic.
T-AILD: T-cell lymphoma of angioimmunoblastic type.
Lennert's: Lennert's lymphoma.
LCAL: large-cell anaplastic lymphoma.
HDLP: Hodgkin's disease–lymphocyte predominant.
HDNS: Hodgkin's disease–nodular sclerosis.
HDMC: Hodgkin's disease–mixed cellularity.
HDLD: Hodgkin's disease–lymphocyte depleted.

tent in peripheral T-cell lymphomas (n = 11: AILD, pleomorphic T-cell lymphoma, Lennert's lymphoma) lacked positivity for IL-2 and IL-4. A single case had detectable amounts of IL-3. This case was, except for a single LCAL, the only one with a clear GMCSF message.

IL-4 mRNA was found in two cases (Hodgkin's disease, mixed cellularity; HDMC and Hodgkin's disease, lymphocyte depletion type; HDLD) of the whole series of Hodgkin's and non-Hodgkin's lymphomas (NHL) (Figure 1). A marked IL-6 message was found in 7 of 29 cases whereas 8 other cases had moderate amounts of this cytokine (Figure 2). The equal loading of the lanes as well as the integrity of RNA is documented in Figure 3.

Out of a total of 19 cases, 2 cases of HD had a clear IL-3 message. Various amounts of IFN-gamma were found in 11 of 23 cases within all entities (Figure 4).

Four cases (2 HD: no. 22 and 28, 1 LCAL: no.16, 1 AILD: no.7) were additionally investigated by the *in situ* hybridization technique on cryostat sections with probes for IL-4, IL-5, and IL-6.

IL-4 message could be detected in one case of Hodgkin's disease, nodular sclerosis type (HDNS) in some small lymphoid cells. Hodgkin (H) and Reed–Sternberg (RS) cells were negative. IL-5 message was visible in both cases of HD with varying numbers of positive lymphocytes and additional positive H and SR cells. IL-6 was detectable in the examined cases of T-AILD (no.7) and LCAL (no.16) in the atypical T cells and in both cases of HD with moderate numbers of positive lymphocytes and positive H and SR cells in HD (Figure 5a). Using the sense probe for control, we achieved a nearly complete negative signal (Figure 5b).

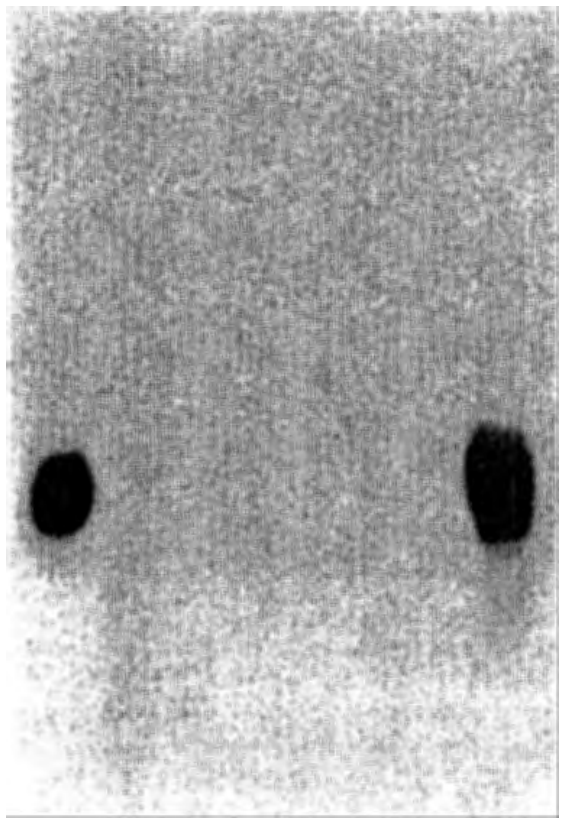

Figure 1. *IL-4 expression in cases of Hodgkin's disease. 1–3: Hodgkin's disease, nodular sclerosis[22,23,24]; 4: Hodgkin's disease, mixed cellularity[8]; 5: Hodgkin's disease, lymphocyte depletion[32]; c: control, RNA from PHA/PMA stimulated PBL; all lanes contained 10 μg of total cellular RNA; exposure time: 48 h.*

Discussion

Most cases of HD are characterized by a mixed cellular infiltrate of lymphocytes, plasma cells, eosinophils, macrophages and H and SR cells. Genotypic data gave evidence that in the majority of cases only a minor population, most probably comprised of H and SR cells, is confined to the clonally proliferating cell clone.[22,25] At least some of these were shown to express genotypic and phenotypic properties of the T-cell lineage.[26–31] Contrary to HD, peripheral T-cell lymphomas mostly consist of a homogeneous neoplastic cell population comprising the majority of the cellular infiltrate.[8,32] LCAL has some phenotypic and genotypic features of HD and may even be accompanied by a host cellular infiltrate of eosinophils and plasma cells.[33] Whether these different or related entities have similarities in their ability to produce effective cytokines which may be involved in the microenvironmental structure and in tumor growth is of interest. Moreover, clinical symptoms frequently prominent in HD may also be due to an uncontrolled or overwhelming cytokine production.

In this study we show that peripheral T-cell lymphomas and cases of HD may express a variety of growth factors. Whereas expression of IL-2 was not found and IL-4 expression occurred in just two cases of HDLD, a marked heterogeneity in the expression of IL-6 was detected, especially in HD and LCAL. These data are in accordance with Tabibzadeh et al.,[34] who demonstrated a variable amount of IL-6 protein in different cases of HD and NHL by means of immunohistochemistry.

Besides a limited expression of GMCSF in 2 T-cell lymphomas and 1 HD, about 40% of the cases had different amounts of IFN-gamma. In previous studies both cytokines were detected in HD-cell lines.[35–37]

Surprisingly, not a single lymphoma could be demonstrated to express IL-2 message by Northern blotting. At least some cases of LCAL with T-cell properties were expected to express this cytokine as these lymphoma cells are known to carry markers of cell activation with expression of CD30, CD25, and HLA class II antigens. However, it was demonstrated that IL-2-R-(TAC)-

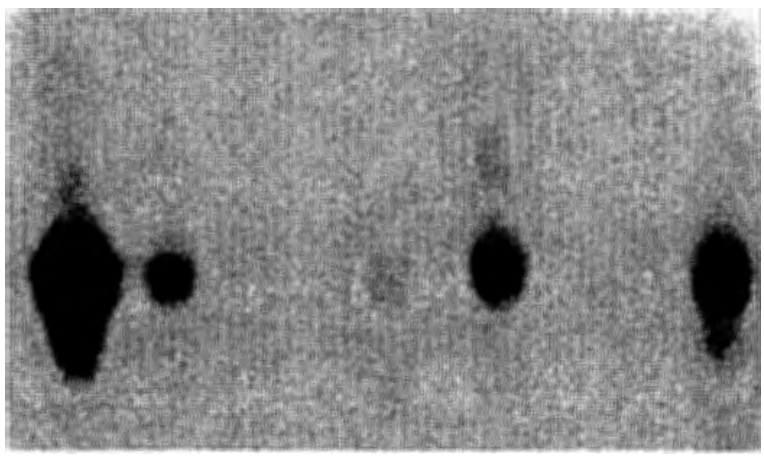

Figure 2. *IL-6 expression in cases of HD and LCAL. c: control, RNA from PHA/PMA activated PBL; NS: Hodgkin's disease, nodular sclerosis[26]; NS: Hodgkin's disease, nodular sclerosis[27];MC: Hodgkin's disease, mixed cellularity[29]; NS: Hodgkin's disease, nodular sclerosis[28]; LCAL: Large-cell anaplastic lymphoma 20; LPHD: Hodgkin's disease, lymphocyte predominant 21; all lanes contained 10 μg total RNA exposure time 48 h.*

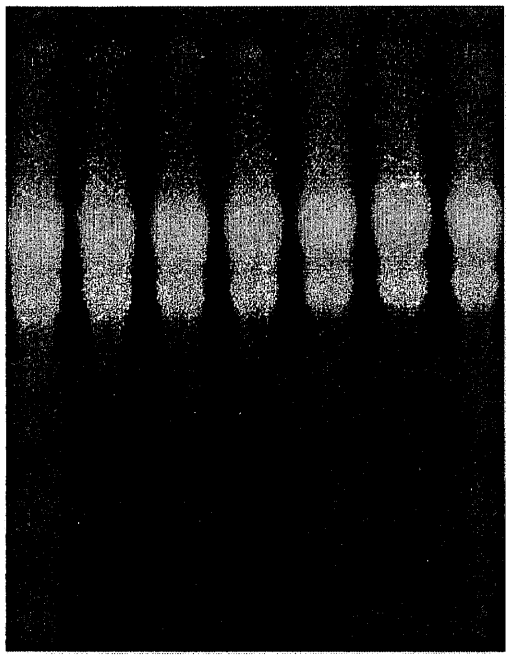

Figure 3. *Photograph of the ethidium bromide-stained gel. The corresponding blot is shown in Figure 2.*

expression is not necessarily coupled with a responsiveness for IL-2.[38]

Our results differ from a recently published study in which IL-2 message was reported in 20 of 20 investigated cases of CD25 positive lymphoma, 9 of which were characterized as LCAL.[39] That investigation was performed with *in situ* hybridization technique. By combining immunohistochemistry with *in situ* hybridization Peuchmeur et al. could detect the IL-2 production only in reactive T-cells and suggest a paracrine IL-2 involvement. Thus Northern blot may not be sensitive enough to detect such small amounts of a cytokine.

IL-3, which is known to be a potent multilineage factor of early hematopoiesis,[40] was detected in 3 of 19 examined cases (2 HD, 1 T-cell lymphoma), which further documents the heterogeneity of these neoplasms.

Two cases with a strong IL-4 signal were diagnosed as HDMC and HDLD. The last case lacked any typical B- or T-cell antigen expression. However, compared with stimulated peripheral T-cells the intensity of the signal in the Northern blot and the homogeneity of the cellular infiltrate may indicate that this growth factor is produced by the tumor cells themselves (Figure 1).

In situ hybridization for IL-4, IL-5, and IL-6 in HD and LCAL demonstrates in individual cases that the expression is due to lymphocytes and H and SR cells. Demonstration of IL-4 in case no. 22 (HDNS) by *in situ* hybridization visualized some positive lymphocytes (up to 10%), whereas H and SR cells were invariably negative. In this case IL-4 mRNA could not be detected by means of Northern hybridization in whole tissue RNA.

The *in situ* hybridization results underline that IL-5 mRNA production is not only confined to H and SR cells[41] but may also be found in activated lymphocytes, which are known to be the main source of IL-5 production.[42].

The reliability of these results was documented in cases no. 7 (AILD) and no. 22 (HDNS) using a probe for IL-6; both cases had clear-cut signals for IL-6 in Northern blots. A positive signal was also found in both cases by *in situ* hybridization in the majority of atypical lymphocytes (case no. 7) in some small lymphocytes and more frequently in H and SR cells (case no. 22) (Figure 5).

These results demonstrate that even small amounts of highly effective cytokines could be detected by *in situ* hybridization even in those cases where Northern blot analysis gave negative results. It thus seems possible that the microenvironmental structure and probably even tumor growth can be modulated by locally acting cytokines.

The high number of individual cytokines found in the

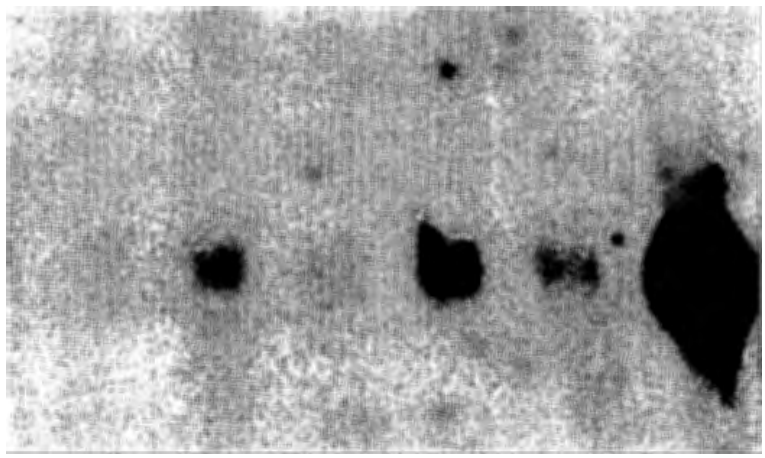

Figure 4. *Interferon gamma expression in cases of peripheral T-cell lymphoma, HD, and LCAL. 1: T-AILD[10]; 2: T-pleom[5]; 3: LCAL, T-type[18]; 4: T-AILD[13]; 5: Hodgkin's disease, nodular sclerosis[25]; c: control RNA of PHA/PMA stimulated PBL.*

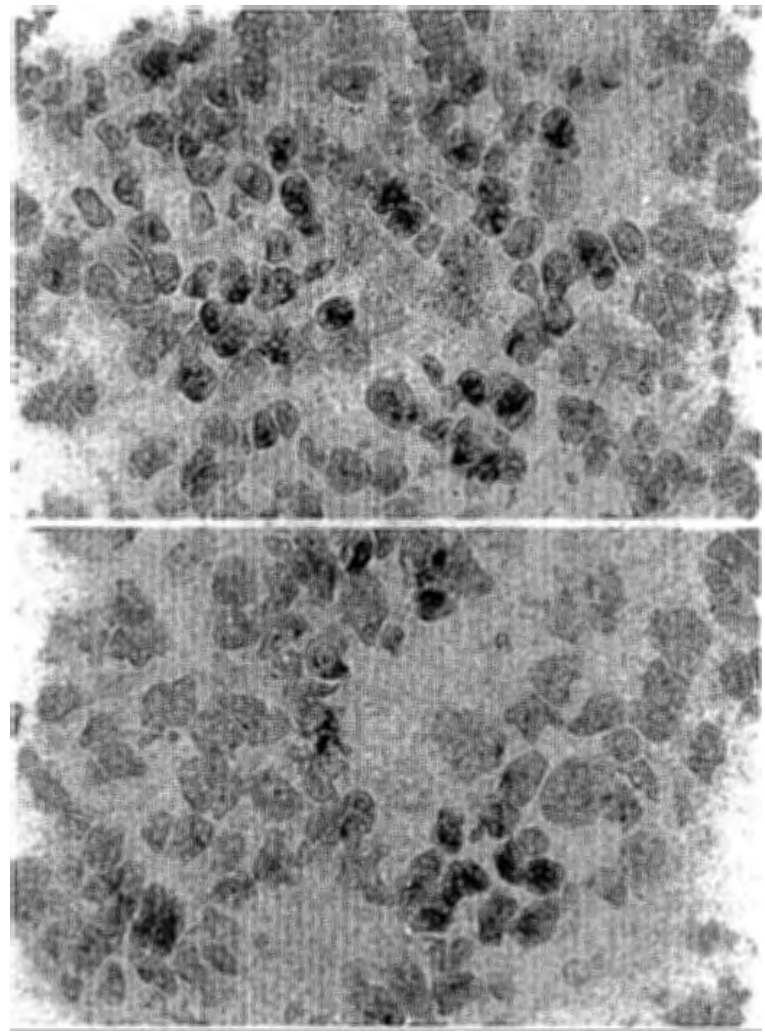

Figure 5. a,b: In situ *hybridization for IL-6 in cases of Hodgkin's disease[25] positive signal is found predominantly over large Hodgkin-like cells;* **(b)** *shows the same case as* **(a)** *using a sense probe as control.*

total RNA of the whole cellular content can be interpreted as due to a loss of the physiologic regulation within the cytokine network and thus may be a sign of neoplastic cell growth. However, it is known that even small amounts of cytokines can act as potential stimulators or inhibitors.[43] Investigations on cell lines of HD and some T-cell lymphomas especially have demonstrated the expression of a number of growth factors like IL-1,[44] IL-2,[45] IL-5,[42] IL-6,[34,46,47] GMCSF,[36] TGFβ,[48] and TNF/LT.[49] It is still a matter of speculation whether these cell lines use these factors as autocrine growth promoters or whether they may even grow independently.

The present results document a strong heterogeneity in cytokine expression even among lymphoma types that were morphologically homogeneous and referred to as distinct lymphoma entities. However, cytogenetic studies in T-cell lymphomas and HD describe an enormous number of different chromosomal aberrations.[50–52] It is thus interesting to speculate on a lymphoma-specific cytokine expression which may not only be responsible for defined clinical symptoms but may also influence the individual cellular composition of malignant lymphomas and their growth behavior.

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