CYTOKINE EXPRESSION IN B-CELL NON-HODGKIN LYMPHOMAS

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SUMMARY

Cytokine expression of potentially active B-cell growth factors (IL-2, IL-4, IL-6) was investigated by means of mRNA-expression in 29 cases of malignant non-Hodgkin B-cell lymphomas. Strong signals were found for IL-4 and IL-6 in two and five cases, respectively, whereas the majority of the remaining cases did not show any detectable mRNA-signals for these growth factors. Comparing these results with those of the immunohistochemistry, it could be demonstrated that at least in some of these cases the signal was due to an autocrine or paracrine phenomenon of the tumour cells.

KEY WORDS   Cytokines   Malignant lymphoma   *In situ* hybridization

INTRODUCTION

Normal proliferation, differentiation and activation of hematopoietic and lymphoid cell compartments are regulated by a complex network of interacting cytokines frequently produced by helper/ inducer T-cells. Most of these extremely potent factors have been shown to have stimulatory effects, while others can act as downregulatory agents. Intriguing new data indicate that a cytokine secreted by tumour infiltrating cells or by the tumour cells themselves as an autocrine or paracrine phenomenon may have antitumoural as well as growth factor activity (Biondi *et al.*, 1989; Tepper *et al.*, 1989; Kupper *et al.*, 1987).

The clinical course of malignant lymphomas shows a broad range of behaviour with respect to tumour growth and therapy response. Differences can be found not only between high and low-grade malignant lymphomas but even within the same entity with identical phenotypic properties. This variation in behaviour raises the question as to the influence of cytokines, which may be produced by infiltrating cells in a malignant lymphoma or by the lymphoma cells themselves.

Since some cytokines are known to exert a strong influence on B-cell proliferation and differentiation (Wörmann *et al.*, 1987; Balkwill and Burke, 1989) we investigated a number of small lymphocytic and large cell (low and high grade) malignant B-cell lymphomas to determine their basic capacity for cytokine production and possible differences between the various lymphoma entities.

MATERIAL AND METHODS

Primary biopsies from 29 untreated patients with malignant lymphomas of nodal origin were investigated. A portion of the lymph node was fixed in 4 per cent formaldehyde and a portion snap

frozen in liquid nitrogen within 1 h after biopsy. The formalin-fixed material was embedded in paraffin and 5 μm paraffin sections were cut and stained for H and E, Giemsa and PAS. Cryostat sections were prepared from the frozen material and immunostained with monoclonal antibodies for CD2, CD3, CD5, CD19, CD22, CD23, CD25, Ki-M4 (dendritic reticulum cells) using an immunoperoxidase method.

RNA preparation and Northern blotting

Frozen tumour material was disrupted with an Ultraturrax tissuehomogenizer and suspended in a guanidine thiocyanate buffer (Chirgwin *et al.*, 1979) followed by caesium-chloride gradient centrifugation (Glisin *et al.*, 1974). Twenty μg of total RNA per lane was size-fractionated by electrophoresis in 6 per cent formaldehyde/1·5 per cent agarose gels (Lehrach *et al.*, 1977). Before RNA-transfer to nylon membranes (Hybond, Amersham), the gels were stained with ethidium bromide to verify equal quantities of RNA (Thomas, 1980). Equal amounts of total RNA of stimulated PBL (PMA 10 ng/ml Ca-ionophore-A23187 100 ng/ml) were used as controls.

Hybridization

Prehybridization was performed in 50 per cent formamide, 2 × SSC, 0·1 per cent SDS, 20 mM vanadyl-ribonucleoside-complex, 200 μg/ml salmon sperm DNA, 200 μg/ml yeast-tRNA, 5 × Denhardts solution, 10 per cent dextran sulfate for 4 to 6 h at 58°C. Five ng/ml of [^{32}]P-labelled anti-sense-cRNA probe was added and hybridization was performed for 12 to 16 h. Filters were washed 2 × 5 min 2 × SSC, 0·1 per cent SDS at room temperature, 2 × 30 min 2 × SSC, 0·1 per cent SDS at 65°C, 2 × 10 min 0·2 per cent SSC, 0·1 per cent SDS at 65°C to 70°C. Filters were exposed to Kodak-X-omat film (Kodak, Rochester, U.S.A.) for 8 to 72 h.

After exposure, RNA-blots were stripped of [^{32}]P-labelled probes by incubation in boiling solution of 0·1 per cent SDS and subsequent cooling to room temperature. Blots were reused 4 to 5 times.

In situ hybridization

Five μm cryostate sections were placed on 2 per cent 3-(Triethoxysilyl)-propylamine (Merck) activated slides and fixed in 4 per cent paraformaldehyde (Merck), 0·1 per cent glutaraldehyde (Merck) in PBS (pH 7·5) for 60 min at room temperature and stored in 70 per cent ethanol.

Rehydrated slides were acetylated in 0·1 per cent triethanolamine (pH 8·0) (Sigma), 0·25 per cent acetic anhydride (Sigma) for 10 min and postfixed in 4 per cent paraformaldehyde for 10 min, dehydrated in a series of graded ethanol and finally air-dried.

Hybridization was performed in a solution containing 4 × SSC (1 × SSC = 0·15 M NaCl, 0·015 M trisodium citrate), 10 per cent dextran sulfate (Sigma), vanadyl-ribonucleoside-complex 20 mM, yeast-tRNA 200 μg/ml (Boehringer), salmon-sperm-DNA 200 μg/ml (Boehringer), BSA 500 μ/ml (Sigma), 50 per cent deionized formamide (BRL), Tris-HCL (pH 8·0), 10 mM, EDTA 1 mM. Five ng [^{35}]S (Amersham) labelled anti-sense or sense riboprobe were added to 20 μl hybridization solution. Incubation time was 4 to 16 h in a humidified chamber at 50°C. Washing was performed in 4 × SSC at room temperature for 10 min, 2 × SSC at 52°C for 30 min, 2 × SSC, 20 μg/ml RNase A (Boehringer) at 37°C for 30 min, 0·2 × SSC, 10 mM DTT (Sigma) at 52°C for 30 min. Slides were then rinsed twice in 70 per cent ethanol, 200 mM ammonium acetate (pH 5·4) for 10 min, 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 cDNA of the coding region of IL-2 was cloned by W. Sebald and the sequence was identical to the published sequence of Taniguchi *et al.* (1983). The cDNA of the coding region of IL-4 was originally obtained from British Biotechnology and based on the published sequence of Yokota *et al.* (1986). The cDNA of IL-6 was cloned by one of us (W.S.) and is identical to the published sequence of Hirano *et al.* (1986). The cDNA of TAC, the p55 subunit of the IL-2 receptor, was cloned and sequenced by W.S. and is in accordance with the sequence published by Leonard *et al.* (1984).

The expected sizes of the mRNAs for IL-2, IL-4 and IL-6 are 800–900b, 700–800b, 1300b respectively and the transcript lengths for TAC are normally found to be 1500b and 3500b. The cDNAs of the coding regions of IL-2, IL-4, IL-6 and TAC were subcloned into transcription vectors (PSPT 18/19, Boehringer, F.R.G.).

The [^{32}P]- or [^{35}S]-labelled sense and anti-sense-cRNA probes were generated in *in vitro* transcription experiments with T7 RNA-polymerase to a specific activity of 1 to 5×10^8 cpm/μg cRNA (Zinn *et al.*, 1983).

RESULTS

All 29 cases were classified as B-cell lymphomas by morphology and immunohistochemistry. Twenty cases were classified as low-grade and 9 cases as high-grade malignant lymphomas.

The exact diagnosis according to the Kiel classification (Stansfield *et al.*, 1988) and the Working Formulation (non-Hodgkin's Lymphoma Pathologic Classification Project, 1982) as well as the immunophenotypes and the results of cytokine mRNA expression (IL-2, IL2-R, IL-4, IL-6) are detailed in Table 1. Cytokine mRNA of the lymphomas was quantified by comparison to stimulated PBL (12 h/36 h) signals. The maximum signal of PBL was quantified as + +, lower signals in the lymphomas were designated as +.

IL-2 mRNA was found at low levels in only two cases of centroblastic–centrocytic lymphoma (follicular mixed, small cleaved and large cell). IL2-R mRNA patterns were found in 16 cases, with a strong signal in one case of hairy cell leukemia (HCL). Three cases expressed the TAC antigen in the tumour cells, whereas in the remaining cases expression was confined to the infiltrating 'host' T cells and macrophages as demonstrated by immunohistochemistry (CD25). In the one case of HCL exhibiting a strong mRNA signal the tumour cells showed complete positivity for CD25, whereas in the other two cases only partial positivity was found. An IL-4 signal was evident in only three cases, two of which had high levels (BCLL—small lymphocytic; cb—diffuse large cell), indicating that in these cases it was the tumour cells themselves that exhibited this autocrine expression. Immunohistochemistry revealed a T-cell content of less than 3 per cent in both cases. The other two cases were cb/cc (follicular mixed, small cleaved and large cell) lymphomas with a high content of T cells. The remaining cases (n = 25) were completely negative for expression of IL-4 mRNA. Five cases (2 cb—diffuse large cell; 3 cb/cc—follicular mixed, small cleaved and large cell) had high amounts of IL-6 transcripts (Figure 1), while all other cases were negative except for weak signals in one case of centrocytic lymphoma (diffuse, small cleaved cell) and one case of B-immunoblastic lymphoma.

Four cases with clear mRNA expression in Northern blots (case no. 11, 15, 21, 24) were additionally investigated by *in situ* hybridization (Figure 2a,b). All four cases showed positive signals for IL-6 mRNA in 20–60 per cent of the whole cellular infiltrate. In immunohistochemistry cases no. 21 and 24 were shown to have a non-neoplastic T-cell content of less than 10 per cent (Table 1).

Table 1. Detailed data of immunophenotypic properties and mRNA expression in human B-cell lymphomas

No.	Diagnosis	Immunophenotype* CD3 (%)	CD25	mRNA expression† IL2	IL2-R	IL4	IL6
1	BCLL	5	s	—	—	+ +	—
2	BCLL	10	s	—	(+)	—	—
3	IC	5	s	—	(+)	—	—
4	IC	10	—	—	(+)	—	—
5	BCLL/IC	10	s	—	(+)	—	—
6	HCL	25	+ +	—	+ +	—	—
7	HCL	25	+	—	+	—	—
8	cb cc	30	—	—	—	—	—
9	cb cc	20	—	—	—	—	—
10	cb cc	20	s	(+)	—	—	—
11	cb cc	15	+	(+)	+	—	+ +
12	cb cc	40	s	—	—	—	—
13	cb cc	30	+	—	+	—	+ +
14	cb cc	15	+	—	+	+	—
15	cb cc	20	s	—	—	—	—
16	cb cc	25	s	—	(+)	—	+ +
17	cc	20	—	—	—	—	—
18	cc	5	—	—	(+)	—	(+)
19	cc	10	s	—	+	—	—
20	cc	5	n.t.	—	—	—	—
21	cb	10	m+ +	—	+	—	+ +
22	cb	5	s.	—	—	+ +	—
23	cb	40	—	—	—	—	—
24	cb	5	m+	—	(+)	—	+ +
25	cb	10	m+	—	(+)	—	—
26	cb	15	m+	—	+	—	—
27	B ib	5	m+	—	—	—	—
28	B ib	5	—	—	—	—	(+)
29	B ib	10	—	—	—	—	—

*Immunophenotype: s: single cells (3 per cent); +: 3–10 per cent positive cells; + +: tumour cells positive; m: macrophages; n.t.: not tested.

†mRNA expression: —: no expression detectable; (+): just detectable; +: just above intensity of controls; + +: within or higher intensity compared to controls.

BCLL—chronic lymphocytic leukemia of B-type; IC—immunocytoma; HCL—hairy cell leukemia; cb cc—centroblastic-centrocytic lymphoma; cc—centrocytic lymphoma; cb—centroblastic lymphoma; B ib—B-immunoblastic lymphoma.

DISCUSSION

The factors influencing the proliferation and differentiation of malignant cells are poorly understood. Soluble growth factors acting in normal lymphoid cell development have been suggested as possible candidates (Touw et al., 1985; Wörmann et al., 1987) since malignant lymphatic cells are believed to originate from their normal counterparts arrested at defined stages of differentiation. Growth factors might stimulate the proliferation of malignant cells without inducing differentiation, thus leading to a self-sustaining growth (Greaves, 1986).

Mediating a diverse range of biomodulatory activities on B-cells IL-2, IL-4 and IL-6 represent candidate growth factors which may play an important role in the regulation of the proliferative activity of malignant B-cell lymphomas (Balkwill and Burke, 1989; Abbas, 1988).
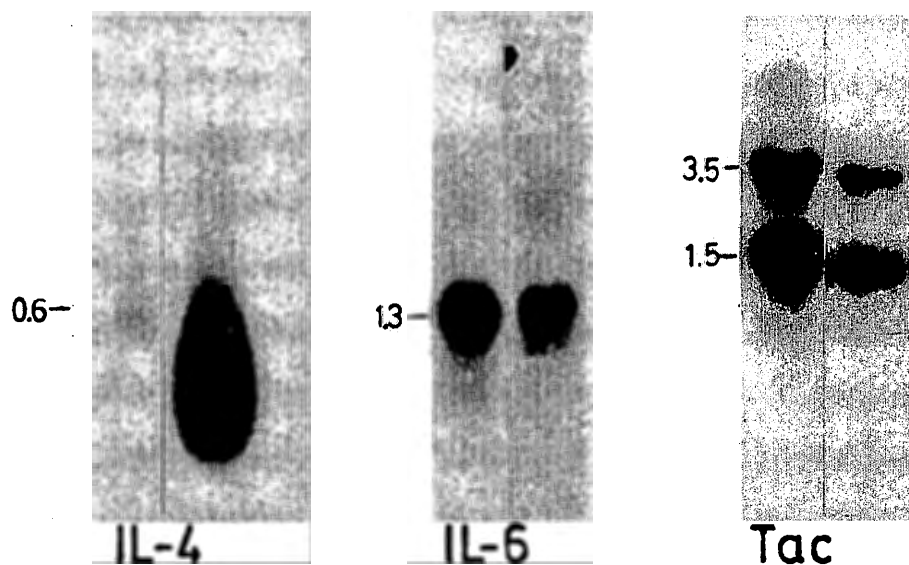
Figure 1. Northern blot analysis of stimulated peripheral T cells (lane 1) and B-cell lymphomas (lane 2). (a) IL-4—lane 1: 12 h stimulation, lane 2: BCLL (case 1). (b) IL-6—lane 1: 36 h stimulation, lane 2: cb (case 21). (c) Tac—lane 1: 12 h stimulation, lane 2: HCL (case 6).

In some lymphoma cell lines a dependency, an enhanced, or even an independent growth on exogenous added growth factors such as IL-2, IL-4, IL-6 and GMCSF or a combination of these has been demonstrated (Tweeddale *et al.*, 1989; Uchiyama *et al.*, 1988; Yee *et al.*, 1989; Freeman *et al.*, 1989).

However, it must be born in mind that *in vitro* activities of cytokines on cell lines are detected in artificial systems. Therefore the evaluation of cytokine-activity in malignant lymphomas may provide a better insight into *in vivo* activities.

Our results demonstrate that high amounts of transcripts of growth factors do indeed occur in some malignant lymphomas.

Five cases of B-cell lymphoma showed strong signals for IL-6 mRNA. Comparison with immunohistochemistry demonstrated low numbers of infiltrating T cells and monocytes in these cases. *In situ* hybridization in four cases clearly demonstrated a positive IL-6 mRNA signal in 20–60 per cent of the infiltrating cells, two of which had a T-cell content of less than 10 per cent. It can therefore be concluded that the IL-6 signal was produced at least in part by the tumour cells themselves. The same was suggested by Biondi *et al.* (1989). Only three of our cases had transcripts for IL-4, with high levels in two cases (BCLL—small lymphocytic; cb—diffuse large cell). Two other cases showed weak IL-2 expression, whereas all other cases had no detectable signals.

Comparing the immunohistochemical results with the mRNA expression of IL-2-R (TAC), it is clear that the signals in some cases, especially in those with weak signals, were due to the infiltrating T cells or histiocytes. On the other hand, this comparison also demonstrates that the IL-2 receptor, especially in one case of HCL, was expressed by the tumour cells.

The most striking result of this investigation is the fact that certain cases of well defined malignant B-cell lymphomas expressed high amounts of B-cell growth factors such as IL-4 or IL-6, whereas other cases of the same entity had no signal for these cytokines. It is thus provocative to speculate on a possible biological activity within those cases with strong signals influencing tumour proliferation and accessory cell activity.
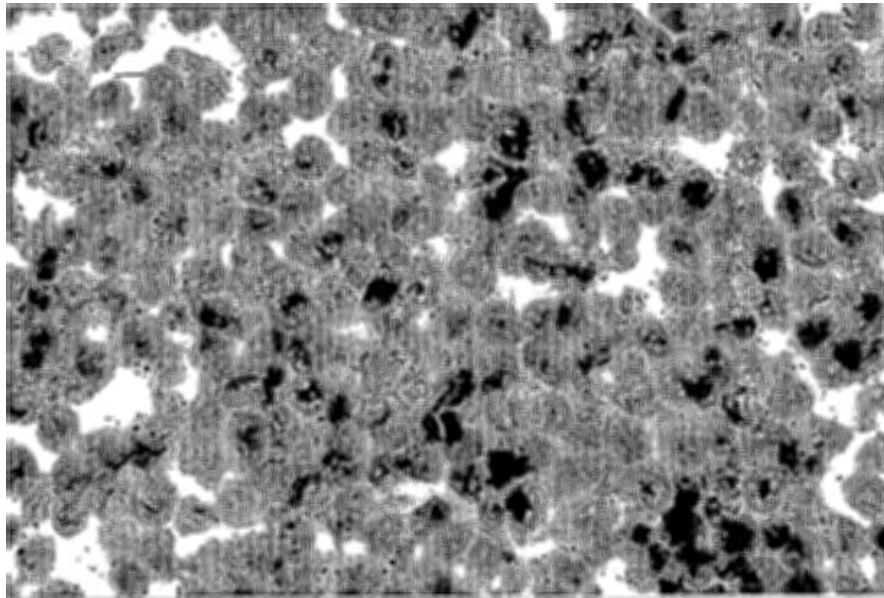
(a)
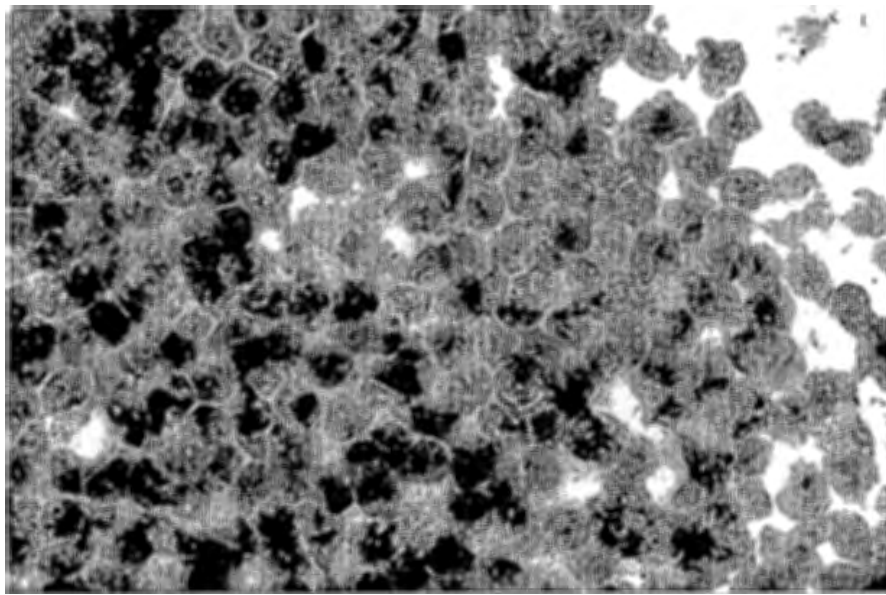
(b)

Figure 2. (a) IL-6 mRNA expression in a high grade B-cell lymphoma (case no. 24—centroblastic) detected by *in situ* hybridization with antisense IL-6 cRNA. (b) Control section treated with sense IL-6 cRNA in the same case. 5 μm cryostate sections, Giemsa stained, × 820.

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