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ORIGINAL ARTICLE

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Expression of genes coding for the tumor necrosis factor and lymphotoxin ligand-receptor system in non-Hodgkin's lymphomas

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Abstract Excessive production of the tumor necrosis factor (TNF) ligand-receptor system has been found to contribute to the severity of non-Hodgkin's lymphoma (NHL). We therefore investigated the expression of TNF, lymphotoxin α (LTα), lymphotoxin β (LTβ), and their receptor (p55, p75, LTβ-R) transcripts within the tumor tissue in different NHL histological subtypes. The constitutive expression of genes coding for TNF-related ligands and receptors was found in almost all 31 NHL samples studied. Semi-quantitative reverse transcription/polymerase chain reaction and computed densitometry assays revealed that the amounts of TNF, LTα, p55, and LTβ-R mRNA were higher in follicular NHL than in other histological entities. Therefore tumor cell immunopurification was performed in representative follicular NHL samples and consistent results were obtained. The pattern of LTβ gene expression was different from that of the other molecules, indicating the existence of distinct mechanisms of gene regulation. These results indicate that the transcription of genes coding for the TNF ligand-receptor system in NHL tumor tissue is more widespread than originally thought and that the heterogeneity of their expressions might be related to histological features. The expression of TNF-related ligands and receptors in tumor tissues is likely to contribute to the clinicopathological features of lymphoid-derived malignancies.

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Introduction

Tumor necrosis factor α (TNF) and its functionally and structurally related partner, lymphotoxin α (LTα), are immunoregulatory cytokines produced primarily by monocytes, macrophages and activated T lymphocytes in response to various stimuli. Excessive production of these cytokines can be deleterious, mediating severe inflammatory reactions, tissue injury, and shock. Slowly released TNF and LTα are thought to contribute to fever, anemia, wasting and bone resorption in chronic diseases [26]. Elevated levels of TNF and LTα have been found in the plasma of the majority of patients with non-Hodgkin's lymphoma (NHL) at the time of diagnosis, and are thereby associated with numerous adverse prognostic factors; they have also predicted shorter progression-free survival and overall survival [9, 20, 30].

LTα exists as a secreted molecule in a homotrimeric form but it may also accumulate on the cellular membrane when aggregated with LTβ, a type II transmembrane protein that belongs to the TNF ligand family [3]. LTβ signals exclusively through the LTβ receptor while TNF and LTα share the same cell-surface receptors, the TNF receptor type I or p55 and the TNF receptor type II or p75. These receptors are preferentially expressed on most cell types; p55 is constitutively expressed at low levels on all nucleated cells whereas p75 seems to be restricted to the hematopoietic lineage [21]. Both were found to be present at high levels in the sera of NHL patients. Interestingly, TNF and p75 values seemed to be associated with larger tumor burden while elevated p55 levels appeared to reflect the host response to tumor [30].

Earlier studies have suggested that tumor cells themselves are responsible for the excessive production of the ligands and receptors in the sera of patients [10] and that these cells have a greater tendency than non-malignant cells to produce and shed soluble forms

of their cell-surface proteins making them difficult to detect [1].

Given the importance of the TNF/LT ligand receptor in the immune response and its putative contribution to the clinicopathological features of lymphoid-derived malignancies, we addressed the question of their gene expression in different NHL histological subtypes. We developed a semiquantitative reverse transcription/polymerase chain reaction (RT-PCR) assay based on the coamplification of an internal standard not homologous to the cDNA target, in order to quantify the cytokine and the receptor gene transcripts in the tumor tissue and to be able to compare the expressions of the different molecules.

Materials and methods

Patients

A group of 31 newly diagnosed NHL patients were included in the study. The patients' median age was 62 years (range, 22–86 years); 15 were female and 16 male. The patients inclusion criteria and initial medical examination were as previously described [30]. Detailed characteristics of the patients enrolled in this study are presented in Table 1. Patients' lymph nodes, obtained before treatment initiation, were collected immediately after surgical removal and kept at the temperature of liquid nitrogen. The diagnosis was assessed by morphological and immunophenotyping analysis according to the Revised European-American Lymphoma (REAL) classification.

Table 1 Clinicopathological features of the 31 lymphoma patients. Diagnosis was described according to the Revised European American Lymphoma (REAL) classification [24] (*LL* lymphocytic/lymphoplasmocytoid lymphoma, *FL* follicular cell lymphoma, *MCL* mantle cell lymphoma, *DLCL* diffuse large-cell lymphoma, *T-NHL* peripheral T cell non-Hodgkin's lymphoma). Stage was classified according to the Ann Arbor scale. Performance status (*PS*) was defined according to the ECOG scale. The lactate dehydrogenase (*LDH*) normal value is less than or equal to 450 UI/l

No.	Sex	Age (years)	Histology	Stage	PS	LDH (UI/l)
1	F	46	LL	IVA	2	440
2	F	70	LL	IVA	1	876
3	F	59	LL	IIB	1	934
4	M	39	LL	IVA	0	380
5	M	49	LL	IVA	0	382
6	M	36	FL	IIIA	0	453
7	F	63	FL	IVA	0	340
8	M	50	FL	IVA	0	362
9	F	71	FL	IVB	2	488
10	F	53	FL	IVB	1	481
11	F	22	FL	IVB	1	321
12	M	67	FL	IVA	1	214
13	M	49	FL	IVB	1	634
14	M	59	FL	IIA	0	527
15	M	62	FL	IVA	0	341
16	F	51	FL	IIA	0	401
17	F	73	FL	IVA	1	608
18	M	62	MCL	IVA	1	337
19	F	71	MCL	IVB	2	535
20	M	58	DLCL	IIIB	1	521
21	M	86	DLCL	IVB	2	590
22	F	62	DLCL	IVB	2	590
23	M	77	DLCL	IIB	3	1405
24	F	39	DLCL	IVB	1	277
25	F	86	DLCL	IVA	1	1362
26	F	69	DLCL	IIA	2	1200
27	M	64	DLCL	IVB	1	854
28	M	62	DLCL	IVB	1	346
29	M	72	DLCL	IA	0	303
30	M	65	T-NHL	IVB	2	501
31	F	34	T-NHL	IIA	0	321

Cells

The mononuclear cell fraction of a suspension of lymph node cells from 6 follicular NHL and 2 lymphocytic/lymphoplasmocytoid NHL patients was isolated by Ficoll-Hypaque (Gibco BRL, Grand Island, N.Y.) and E rosetting (BioMérieux, Marcy l'Etoile, France). The nonrosetting cells were incubated with anti-CD3 (OKT3; American Type Culture Collection, Rockeville, Md.) anti-CD14 and anti-CD-16 monoclonal antibodies (mAb) (Immunotech, Marseille, France), then with magnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with anti-(mouse IgG). The isolated cells were analyzed by flow-cytometry (FACScan, Becton Dickinson, Mountain View, Calif.) after staining with fluorescein-isothiocyanate-conjugated CD3, CD14 (Immunotech) and CD19 (Dako, Glostrup Denmark) and phycoerythrin-conjugated goat anti-(mouse immunoglobulin) (Dako) sIg and mAb light chains. All samples showed 98% cell purity and were monoclonal.

RNA extraction and cDNA synthesis

As a control for the expression of a given ligand/receptor gene, we used a panel of human cell lines representing B, T, and myeloid lineages, including B cell lymphoma (RL), Epstein-Barr-virus-transformed B cells (LAZ-388), pre-B-cell acute lymphoblastic leukemia (Nalm-6), T cell lymphoma (J-77), and promyelocytic leukemia (HL-60).

The total RNA from cell line suspensions, lymph node homogenates and purified cell suspensions, was isolated by a standard acid guanidium thiocyanate/phenol/chloroform extraction. Extracted RNA, dissolved in diethylpyrocarbonate-saturated water, was subjected to a genomic DNA decontamination procedure in a final reaction volume of 25 µl, consisting of 10 U RNase-free DNase I (Stratagene, La Jolla, Calif.), 25 U RNasin (Promega, Madison, Wis.), 100 mM $MgCl_2$, and 10 mM dithiothreitol, performed for 15 min at 37 °C. The reaction was stopped by adding an equal volume of DNase stop solution containing 50 mM EDTA, 1.5 M sodium acetate (pH 4), and 1% sodium dodecyl sulfate, Then the final phenol/chloroform/isoamyl alcohol (25/24/1) RNA extraction was performed. First-strand cDNA synthesis was performed in a total volume of 20 µl, consisting of 3 µg total RNA, 200 U recombinant Moloney murine leukemia virus reverse transcriptase and the recommended buffer (Gibco BRL Life Technologies, Gaithersburg, Md.), 10 pM oligo (dT), 500 µM each dNTP (Gibco BRL), 7.5 mM dithiothreitol, and 25 U RNasin (Promega). The reaction mixture was incubated in a water bath at 37 °C for 90 min and then heated at 95 °C for 3 min to inactivate reverse transcriptase.

Semiquantitative PCR

Samples comprising 2 µl cDNA were added to a final PCR reaction mixture of 50 µl, containing 2.5 U Taq polymerase and the recommended buffer (Gibco BRL), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, and 25 pM each of the specific sense and antisense primers (Table 2). PCR for p55, p75, and LTβ were performed in the presence of 5% dimethylsulfoxide (Sigma Chemical Co, St Louis, Mo.). Specific PCR primers for each ligand and receptor were design to span at least two exons to detect the amplification of any contaminating genomic DNA. To assess the specificity of PCR-amplified products, each of them was purified and digested

Table 2 List of primers and sizes of polymerase-chain-reaction (PCR)-amplified products. *TNF* tumor necrosis factor, *LTβ* lymphotoxin β, *LTβ-R* LTβ receptor

Molecule	5′-3′ primer sequence	Size (base pairs)	Restriction enzyme
G3PD	Forward: CCTCCTGCACCACCAACTGC Reverse: GGATACATGACAAGGTGCGGC	736	*Xba*I
TNF	Forward: ATGAGCACTGAAAGCATGATCCGG Reverse: GATAGATGGGCTCATACCAGGGC	586	*Pvu*II
p55	Forward: GTATCGCTACCAACGGTGGAAG Reverse: CTCGATGTCCTCCAGGCAGCC	618	*Pst*I
p75	Forward: GGAGTGGTGAACTGTGTCATC Reverse: CTTGGCTACGACACACAGCCCAC	589	*Pvu*II
LTα	Forward: CGTCTCTTCCTCCCAAGGGTG Reverse: GTGGTACATCGAGTGCAGCCAG	492	*Pvu*II
LTβ	Forward: GCTGGCCTTAGTGCCCCAGG Reverse: AGAGAGGCCCGTACCCTTGTC	473	*Pvu*II
LTβ-R	Forward: GCGTCGGAGAACCAGACCTGC Reverse: GGCCAGTGGCAGCAGAACGG	597	*Sac*I

by an appropriate restriction enzyme (Table 2). Each PCR amplification cycle consisted of a heat denaturation (95 °C for 1 min), annealing (64 °C for 1 min), and extension step (72 °C for 1 min). After the last cycle, the extension phase was prolonged for 9 min at 72 °C. As a PCR control, the same procedure was applied to the reaction mixture, which did not contain cDNA. The usual precautions to avoid cross-contamination of the samples were respected.

To normalize the amount of amplifiable cDNA in each sample, we used glyceraldehyde-3-phosphate dehydrogenase (G3PD) primers and primers specific for a given ligand/receptor sequence in the same PCR reaction mixture. The PCR was started with the primers specific for the transcript to be measured. After the first 11 cycles of amplification, primers specific for G3PD sequence were added and 24 additional cycles of amplification were carried out. The PCR products were resolved on ethidium-bromide-stained 1.5% agarose gel and photographed under UV light. Then the amounts of PCR-amplified products were evaluated according to the relative intensity of a given ligand/receptor band and G3PD by using the computed densitometry assay of the ImageMaster System (Pharmacia Biotech, Uppsala, Sweden).

PCR amplification, gel running, and densitometric assessment were performed twice and the results were expressed as the average with standard deviations, which were up to 10% for the analysis of the same sample on different occasions. The sensitivity of this RT-PCR protocol was estimated by serial dilutions of cDNA prepared from positive and negative samples. When 35 of such PCR cycles were performed with the TNF-specific primers, for example, an exponential phase of amplification was observed in a dilution range of $1.0–3 \times 10^{-3}$ (Fig. 1). The plateau phase was not reached after 35 cycles of PCR amplification (not shown). For reliable discrimination of differences in the extent of gene expression between different patients, the results were calculated as a mean of replicate determinations of the same sample from both experiments.

Statistical analysis

The correlations between the transcripts expression levels were evaluated using Pearson's rank correlation coefficient. Clinical and laboratory findings were compared by the paired Student's *t*-test. Statistical analysis was performed using the Statistica software (Statsoft Inc., Tulsa, Okla. USA).

Results

Expression of genes coding for TNF/LT ligand-receptor system in cell lines

PCR and restriction enzyme assays showed that all amplified products had the expected sizes and restriction enzyme digestion resulted in appropriate cleavage fragments. TNF-specific transcript was found in all but one (J-77) cell line, whereas LTα and both LTβ isoforms [29] were found in all lymphoid cell lines but were negative in HL-60 cells (Fig. 2, left panel). With respect to the receptors, the p55 transcript was detected in all cell lines while p75 and LTβ-R mRNAs were detected in all but one cell line (RL) (Fig. 3, left panel).

Expression of genes coding for TNF/LT ligand-receptor system in NHL tissues and in immunopurified lymphoma cells

The genes coding for TNF, LTα, and LTβ were found in all 31 NHL specimens (Fig. 2, right panel). Specific transcripts for each receptor were detected respectively in 29/31 (94%), 23/31 (74%), and 28/31 (90%) NHL tumor tissue samples (Fig. 3, right panel). Using the computed densitometry assay, the quantification of gene expression was performed by determining the relative intensity of the given ligand/receptor and the G3PD amplified product band, obtained from the same PCR reaction and separated on ethidium-bromide-stained agarose gel. We could detect the ligand/receptor bands in the samples diluted to 3×10^{-3}, with a linear decrease in the relative amount of a given gene's PCR product over the range of dilutions used (Fig. 1).

In a given tumor tissue sample, the levels of expression of the genes coding for TNF, LTα, p55, p75, and LTβ-R seemed to be correlated to each other, particularly TNF with LTα and p55, LTα with p55, p75 and LTβ-R, and p55 with p75 and LTβ-R. In the majority of samples, more than half of the LTβ mRNA was present in the larger-sized isoform, and the levels of expression of the two isoforms were closely correlated with each other. However, the expression pattern of the LTβ isoform was not associated with the expression of the remaining TNF/LT ligand-receptor transcripts (Fig. 4).

The amounts of TNF, LTα, p55, and LTβ-R specific transcripts were higher in the follicular type of NHL

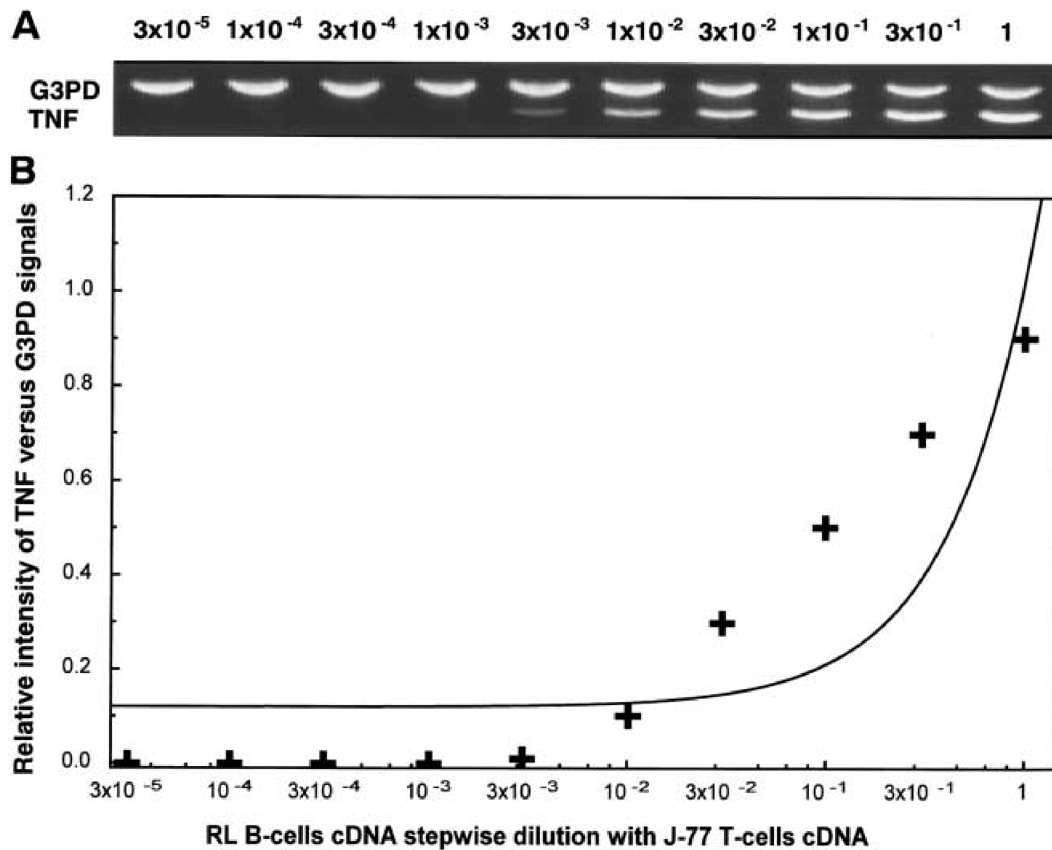


Fig. 1A, B Assessment of an exponential tumor necrosis factor (*TNF*) gene amplification by a semiquantitative reverse transcription/polymerase chain reaction (RT-PCR) assay. **A** RL B cell lymphoma cell (TNF-positive control) cDNA serially diluteds with cDNA of J-77 T-cell lymphoma line (TNF-negative control). An equal volume of each cDNA mixture was subjected to PCR. After the first 11 cycles of amplification, performed with primers specific for TNF, primers specific for the glyceraldehyde-3-phosphate dehydrogenase (*G3PD*) sequence were added, and 24 additional cycles of amplification were carried out. The PCR products were resolved on ethidium-bromide-stained 1.5% agarose gels, and photographed under UV light. The amounts of PCR-amplified products were then evaluated according to the relative intensity of each TNF and G3PD band, by the use of computed densitometry with the ImageMaster System (Pharmacia Biotech). The positions of the expected PCR products bands are indicated on the left side of the figure. The stepwise dilution of RL cDNA with J-77 cDNA is presented at the top of the figure. **B** Logarithmic plot stepwise dilutions of the RL B cells cDNA with cDNA of J-77 T cells (*x* axis) and the relative intensity of TNF versus G3PD bands in arbitrary units (*y* axis), obtained from the results presented in **A**

than in other histological subtypes ($P < 0.004$ for TNF, LTα, p55, and $P < 0.02$ for LTβ-R), although some histological subtypes were under-represented. Given this higher expression pattern among follicular NHL tissue, extended tumor cell purification was performed in 8 representative NHL samples, 6 follicular and 2 lymphocytic/lymphoplasmocytoid. Specific transcripts for TNF, p75, LTα and LTβ were found to be expressed in all samples. Transcript for p55 was detected in 2/6 follicular and 2/2 lymphocytic/lymphoplasmocytoid whereas LTβ-R was negative in all NHL samples tested (Table 3).

Discussion

The results of the present study indicate that genes coding for the TNF/LT ligand-receptor system are broadly expressed in tumor tissues obtained from NHL patients, which was confirmed on highly purified NHL cells for the majority of transcripts. However, in situ hybridization with TNF and LTα probes on B cell NHL was not able to detect their transcripts [6]. In another study, immunohistochemical staining showed the presence of many TNF-possessing cells in NHL samples, mainly of the lymphoplasmacytic/lymphoplasmocytoid hystological subtype [25], whereas another report revealed only 25% TNF weakly positive B cell NHL samples [18]. With respect to the TNF/LT receptors, only one immunohistochemistry study analyzed their expression but used a limited number of NHL samples and showed that only lymphoma cells with a high-grade malignant phenotype (6 of 14 B cell and 6 of 8 T cell NHL) expressed p75 but lacked p55 expression [19]. The reason for the discrepancies between our results and those of others remains unclear but might be attributed at least to the methodological variation of the experimental techniques. The use of RT-PCR, a very sensitive technique, probably explains the broad detection of the cytokines and receptor transcripts in the present study. Moreover, it appears that malignant cells are capable of shedding the expression of their surface molecules [1] and this may also mean that mRNA expression is detected while the

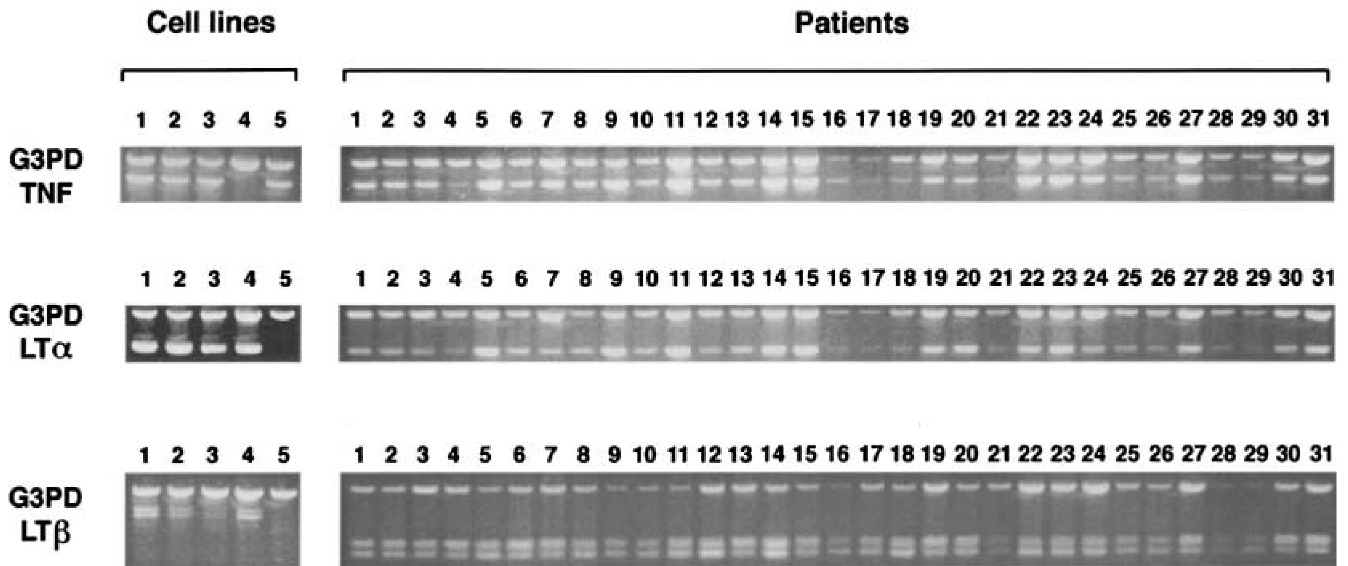
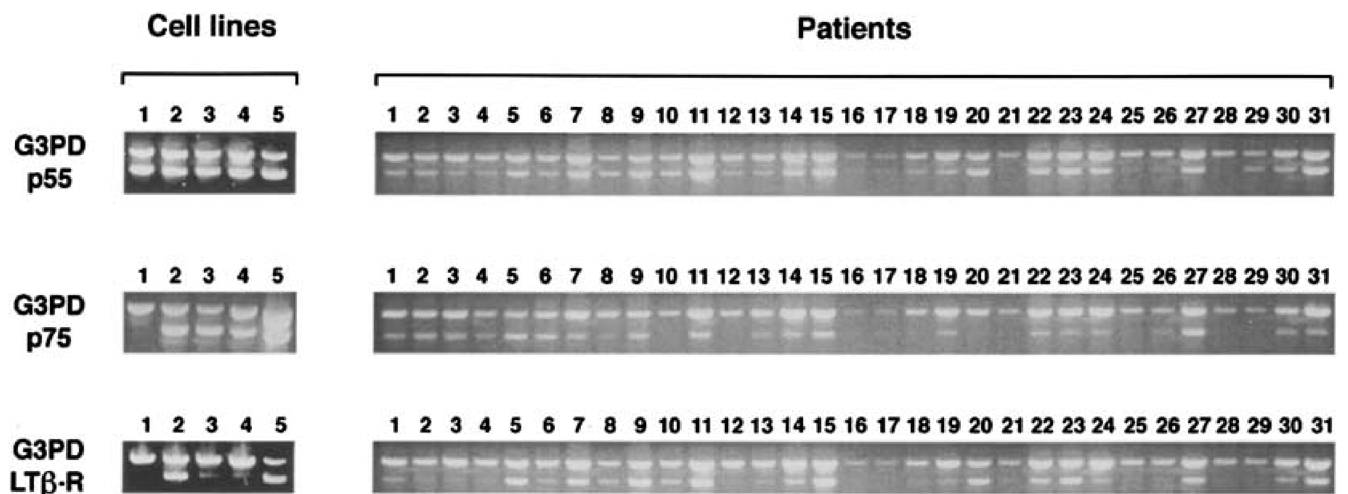


Fig. 2 RT-PCR analysis of the TNF, lymphotoxin α (*LT*α) and LTβ gene expression in human cell lines of B, T, and myeloid lineages (*left panel*), including B cell lymphoma (RL; *lane 1*), Epstein-Barr-virus (EBV)-transformed B cells (LAZ-388; *lane 2*), pre-B-cell acute lymphoblastic leukemia (Nalm-6; *lane 3*), T cell lymphoma (J-77; *lane 4*), and promyelocytic leukemia (HL-60; *lane 5*), and in the tumor tissues obtained from 31 patients with non-Hodgkin's lymphoma (NHL; *right panel*). The cases numbered *1–5* refer to lymphocytic/lymphoplasmocytoid NHL patients, *6–17* to follicular NHL, *18* and *19* to mantle cell NHL, *20–29* to diffuse large-cell NHL, and finally *30* and *31* to T cell NHL. The positions of expected PCR product bands are indicated on the left side of the figure

cell-surface proteins may be missing. Our flow-cytometry analysis of the same highly purified samples also failed to detect the surface expression of TNF/LT ligands and receptor proteins (unpublished data).

Although the specific transcripts for TNF/LT ligands and receptors were found in almost all NHL samples, considerable differences between their expression levels existed among the cases. The level of expression of TNF, LTα, p55 and LTβ-R genes in follicular NHL seemed to be higher than that in lymphocytic/lymphoplasmocytoid, mantle cell, and diffuse large-cell NHL. The discrepancy found for p55 and LTβ-R transcript expression between tumor tissue and purified lymphoma cells might indicate that bystander reactive cells contribute to their production. In keeping with this, it is of interest that follicular NHL is characterized by the accumulation of germinal-center-like lymphocytes associated with follicular dendritic cells (FDC) that remain localized to the lymphoid system for most of the course of the disease. In vitro, FDC and lymphoma cells spontaneously form cellular

Fig. 3 RT-PCR analysis of the p55, p75 and LTβ receptor (*LT*β*-R*) gene expression in human cell lines of B, T, and myeloid lineages (*left panel*), including B-cell lymphoma (RL; *lane 1*), EBV-transformed B cells (LAZ-388; *lane 2*), pre-B-cell acute lymphoblastic leukemia (Nalm-6; *lane 3*), T cell lymphoma (J-77; *lane 4*), and promyelocytic leukemia (HL-60; *lane 5*), and in the tumor tissues obtained from 31 patients with NHL (*right panel*). The cases numbers *1–5* refer to lymphocytic/lymphoplasmocytoid NHL patients, *6–17* to follicular NHL, *18* and *19* to mantle cell NHL, *20–29* to diffuse large-cell NHL, and finally *30* and *31* to T cell NHL. The positions of expected PCR product bands are indicated on the left side of the figure



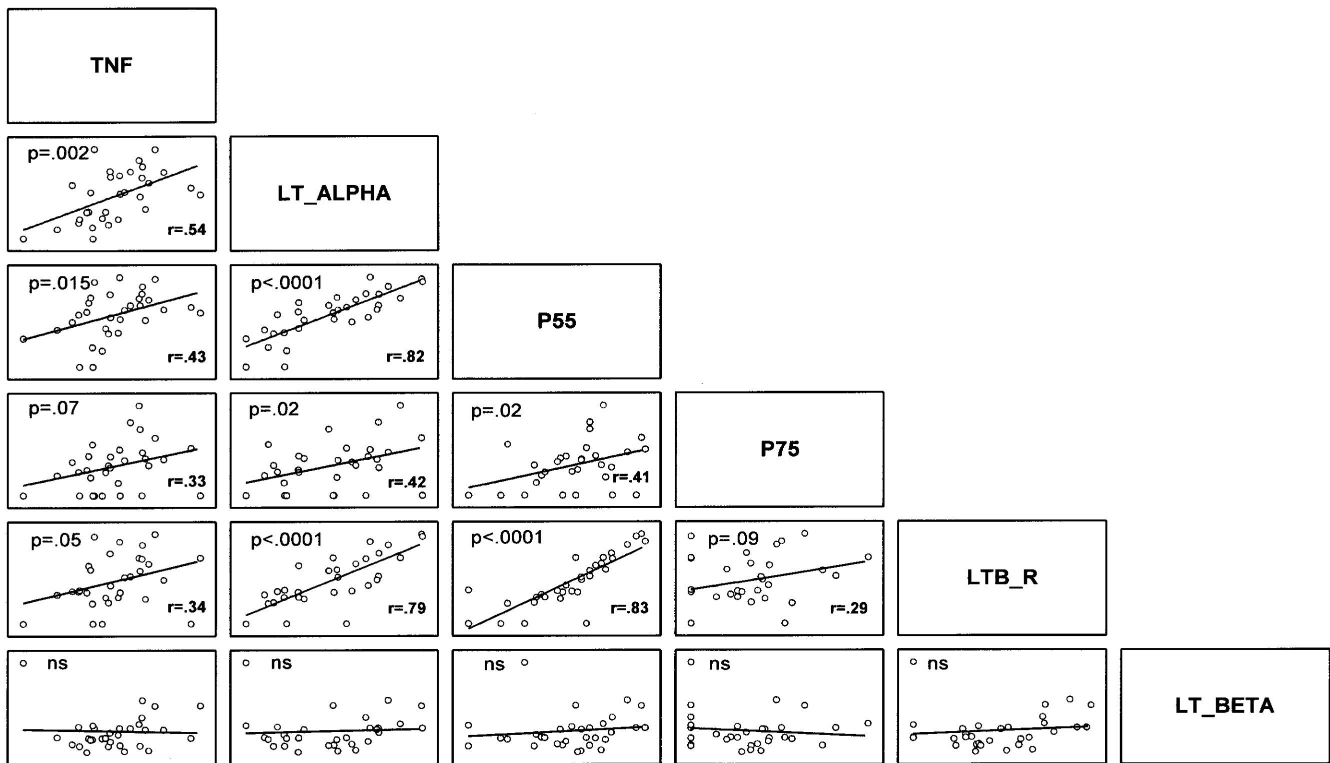


Fig. 4 Correlations of the TNF, LTα, p55, p75, LTβ-R, and LTβ transcript expression levels in 31 NHL tumor tissue samples using Pearson's rank correlation coefficient. The amounts of PCR-amplified products were semiquantified by determining the relative intensity of a given ligand/receptor band and G3PD signal using the computed densitometric assays. PCR amplification, gel runs, and densitometric assessment were performed twice, and the results were calculated as the means of replicate determinations of the same sample from both experiments (arbitrary units). Each symbol represents a pair of means of relative intensity of a given ligand/receptor and G3PD bands from two separate experiments. Curve fits were done by the linear-regression analysis. The line is the best linear fit

Table 3 Reverse transcription/PCR analysis of the expression of the genes for TNF-related ligands and receptors in tumor cells obtained by cell immunopurification from 6 follicular (*FL*) and 2 small lymphocytic/lymphoplasmocytoid (*SLL*) NHL samples

Sample	TNF	p55	p75	LTα	LTβ	LTβ-R
FL 1	+	+	+	−	+	−
FL 2	+	−	+	+	+	−
FL 3	+	+	+	+	+	−
FL 4	+	−	+	+	+	−
FL 5	+	−	+	+	+	−
FL 6	+	−	+	+	+	−
SLL 1	+	+	+	+	+	−
SLL 2	+	+	+	+	+	−

aggregates, via interactions between a number of adhesion molecules expressed on the surface of these cells, and lymphoma cells are rescued from apoptosis when they aggregate with FDC [7, 17]. TNF, involved in the development and control of FDC viability and function [4, 16, 22, 28], stimulate the expression of the adhesion molecules and induce proliferation of normal lymphocytes [2, 8, 24]. Moreover TNF, LTα, p55 and LTβ ligand-receptor pairs are critically involved in the generation of germinal centers within lymphoid organs [5, 12, 14, 16]. Thus, the results of the present study showing higher transcription of genes coding for TNF, LTα, p55, and LTβ-R in follicular NHL than in the other NHL histological subtypes may suggest their functional relevance in germinal-center-derived malignant lymphomas.

It is of interest that TNF, LTα, p55, p75, and LTβ-R shared similar gene expression patterns in the tumor samples studied while LTβ expression had a discordant pattern. Since all the results were quantified to the same internal standard, the differences in the amount and quality of cDNA in the different samples can not account for these results. Several features distinguish the LTβ gene from the other two genes in the TNF/LTα locus, which may explain their distinct expression patterns. The LTβ gene is transcribed in the opposite direction to TNF and LTα. The TATT repeats, which are known to play an important role in mRNA stability and are found in the 3′ untranslated region to TNF and LTα, are lacking in the LTβ gene, and finally the LTβ promoter lacks a consensus SP1 site, which is present in each of the other two genes [3, 11, 23, 27]. Altogether, these results indicate the presence of important differences in molecular mechanisms involved in the transcription regulation of TNF, LTα, and LTβ genes, and confirm the general concept of more compound evolutionary events associated with LTβ than with the TNF/LTα genomic loci [13, 15, 29].

In summary, the results of the present study indicate that the transcription of genes coding for the TNF-

related ligands and receptors in NHL tumor tissues is more widespread than originally thought, and suggest that the heterogeneity of their expression pattern might be related to the histological features of the tumor. The distinct pattern of LTβ gene expression suggests that its transcription is regulated differently from that of the TNF and LTα genes.

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