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Tumor Necrosis Factor α and Lymphotoxin Production in Hodgkin's Disease

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It is likely that the characteristic histologic features of Hodgkin's disease reflect cytokine production by the tumor cell population. Tumor necrosis factor α (TNF-α) and lymphotoxin (tumor necrosis factor beta [TNF-β]) are important inflammatory mediators with wide-ranging effects within the lymphoreticular system. The aim of the present study was to investigate TNF-α and lymphotoxin production in the Hodgkin's disease-derived cell lines L428 and L540. At the product level, both cytokines could be demonstrated by immunostaining with specific monoclonal antibodies. TNF-α could be demonstrated by means of an enzyme-linked immunosorbent assay in culture supernatants from both cell lines as well as in cell lysates of L428 and L540 cells. Cytotoxic activity could be achieved only in L428 supernatants. This cytotoxic activity could not be blocked by the addition of a polyclonal antibody against TNF-α, but was partially inhibited with the monoclonal antibody against lymphotoxin. Synthesis of TNF-α and lymphotoxin in both L428 and L540 was confirmed by demonstrating the intracellular-specific messenger RNA (mRNA) using specific cDNA clones in Northern blot analysis. In situ ***hybridization studies with the TNF-α cDNA probe gave positive hybridization signals in L428 and in L540. These results demonstrate the transcription, translation, and export of TNF-α and lymphotoxin in cultured Hodgkin's disease-derived cell lines. In addition, results of preliminary experiments are presented in which we demonstrate Reed–Sternberg cells positive for TNF-α protein and mRNA in different Hodgkin's disease tissue biopsies, indicating that, at least for TNF-α, our cell line data are relevant to the neoplastic population present in Hodgkin's disease tissue. (Am J Pathol 1990, 137:341–351)***

The histopathologic picture characteristic of Hodgkin's disease (HD) consists of a sparse population of Reed–Sternberg cells and their mononuclear counterparts representing the neoplastic component admixed with many reactive inflammatory cells and often associated with collagen deposition and sclerosis (for recent reviews see Jones,[1] Stein and Gerdes,[2] and Kadin[3]). These characteristic histopathologic features seen in HD may be due to the production and release of cytokines by Hodgkin and Reed–Sternberg (H&RS) cells, a working hypothesis that was proposed originally by Newcom and O'Rourke,[4] who described an uncharacterized fibroblast-stimulating factor in supernatants derived from primary HD-derived cell cultures, and Ford et al,[5] who observed IL-1–like and fibroblast-stimulating activities in supernatants of involved HD spleen cell cultures. The identity and mode of production of cytokines or other biologically active mediators in HD may, therefore, be central to our understanding of the disease process.

Within the complex cell mixture found in HD-involved lymphoid tissue, it is possible that active mediators are produced either by cellular constituents of the reactive component or by the H&RS cells. The low frequency and consequent difficulty in extraction and purification of neoplastic cells from HD tissue biopsies complicates the direct investigation of cytokine production by the malignant cells.

Several cell lines have been derived from HD that show abnormal kariotype and exhibit phenotypic and genotypic features that parallel those seen in H&RS cells.[6–8] These cell lines provide good models for the investigation of the behavior of the neoplastic cell population in HD. The body of published work from Stein and others[1,2,8,9] suggests that H&RS cells originate from either activated B or T lymphocytes. Therefore we have used two cell lines, L428

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and L540,[6] with phenotypic and genotypic characteristics of either B or T cells[8] to investigate potential biologically active mediator production in HD. In this study we have concentrated on so-called tumor necrosis factors, TNF-α (TNFα) and lymphotoxin (TNF-β), which are known to be potent inflammatory mediators and to promote fibroblast proliferation.[10,11] Our data show that both L428 and L540 produce TNF-α and lymphotoxin. In addition, we present preliminary data showing TNF-α protein and messenger RNA (mRNA) in H&RS cells in routine diagnostic biopsies, demonstrating the validity of the results obtained on the cell lines.

Materials and Methods

Cells

The HD-derived cell lines L428 and L540 (courtesy of V. Diehl, Köln, FRG) were maintained under standard culture conditions in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal calf serum for L428 and 20% fetal calf serum for L540, respectively, L-glutamine, and antibiotics.

Both cell lines were negative for mycoplasma infection as determined by 4-6-diamidine-2-phenylindol-dihydrochloride (DAPI) staining performed according to the guidelines of the manufacturer (Boehringer Mannheim, Mannheim, FRG).

For the supernatant assay, washed L428 cells were grown for 2 days before assay in serum-free medium. Because L540 cannot be maintained under serum-free conditions, this cell line was cultured in 10% fetal calf serum for the supernatant assays. Supernatants were harvested at 24, 48, and 72 hours by centrifugation, filtered through 0.22-μm Millipore membranes, and stored frozen at −20°C until required for assay.

Mononuclear peripheral blood leukocytes were obtained from healthy donors by centrifugation through Ficoll/Hypaque gradients followed by washing.

Preparation of Cell Lysates

One million cells were pelleted by centrifugation and resuspended in 500 μl phosphate-buffered saline (PBS). Lysates were prepared by repeating freezing and thawing three times. After a further centrifugation to remove cell membranes, supernatants were used for tumor necrosis factor enzyme-linked immunosorbent assay (TNF-ELISA).

Tissue Biopsies

Frozen and neutral buffered formalin-fixed tissue biopsies were obtained from the diagnostic files of the University Department of Pathology, Southampton. All cases were confirmed as Hodgkin's disease on histopathologic review.

Immunostaining

Staining of cytocentrifuge slides and frozen sections was performed according to the alkaline phosphatase anti-alkaline phosphatase (APAAP) method described by Cordell et al[12] and developed using a New-Fuchsin technique.[13] The monoclonal antibodies TNF-E (against TNF-α) and LTX-21 (against lymphotoxin) were provided by Dr. G. R. Adolf, Ernst Boehringer Institute, Vienna, Austria.[14]

In situ *Hybridization in Cytocentrifuged Cell Lines*

Pretreatment of the Cells

Hodgkin's disease-derived cell-line cytocentrifuge slides were fixed for 10 minutes at room temperature in Zamboni's fixative[15] immediately after preparation. Cells were extensively washed in 70% ethanol and stored in 70% ethanol at 4°C until used.

Preparation of Sulphonated Probes

1–10 μg of plasmid DNA was sulphonated according to the guidelines of the Chemiprobe hybridization kit (Orgenics, Yavne, Israel), as originally described by Budowsky et al.[16] The efficiency of sulphonation was confirmed by dot-blot analysis of serial dilutions of labeled probes to ensure equivalent sensitivity of the specific and the control cDNA probes.

Prehybridization

Slides were transferred from 70% ethanol to RNase-free PBS containing 5 mmol/l (millimolar) $MgCl_2$ and incubated at room temperature for 10 minutes. This step was repeated once. Subsequently slides were incubated in freshly deionized formamide (50% in 5 × SSC) and then with 100 μl of prehybridization mixture for 1 hour at 37°C. This solution was prepared from 500 μl freshly deionized 100% formamide, to which was added 250 μl 20 × SSC, 50 μl tRNA (10 mg/ml), 5 μl ficoll (40 mg/ml), 5 μl polyvinylpyrolidone (40 mg/ml), 5 μl bovine serum albumin (40 mg/ml), 10 μl 5 M NaH_2PO_4, 75 μl H_2O, and 100 μl of freshly denatured herring sperm DNA (1 mg/ml).

Hybridization

After prehybridization, slides were washed briefly in freshly deionized formamide (50% in 5 × SSC). As hybridization solution we used a mixture of 500 μl freshly deionized formamide (100%), 150 μl 20 × SSC, and 25 μl tRNA (10 mg/ml). For each slide, 34 μl of this solution were mixed with 1 to 10 μl of sulphonated probe and the final volume adjusted to 50 μl by the addition of RNase-free water. This solution was boiled for 2 minutes, 1 μl of Denhardt's solution (50×) added, and immediately transferred to the cytocentrifuge preparations. Hybridization was allowed to proceed for 2 hours at 37°C. Subsequently slides were washed for 10 minutes in 1 × SSC, 50% freshly deionized formamide in 1 × SSC and 1 × SSC.

Visualization of the Hybridization Reaction

Slides were incubated with anti-sulphonated DNA antibody (Chemiprobe, Organics, Yarne, Israel) diluted 1:250 in the blocking solution provided for 30 minutes. Bound antibody was demonstrated by the APAAP technique,[12] as detailed above.

In Situ *Hybridization in Routinely Fixed Tissue Biopsies*

Preparation of Biotinylated Probes

Oligonucleotide DNA probes were synthesized, using published sequence data,[17] on an Applied Biosystems oligonucleotide synthesizer (Applied Biosystems, Model 380, Worrington, UK). Four separate regions of the TNF cDNA sequence, each 25 bases in length, were chosen because of their high percentage of guanine and cytosine residues. A cocktail of four probes increases the sensitivity of the assay. Both the antisense and the sense strands of the cDNA sequence were synthesized, the sense strands to act as a negative control in the hybridizations.

Probes were labeled at the 5′ terminus by chemical derivitization using Amino-link 2 (Applied Biosystems) and N-Hydroxysuccinimide Biotin (Sigma Chemical Co., St. Louis, MO). They were also labeled at the 3′ terminal using the enzyme Terminal Deoxynucleotidyl Transferase (Pharmacia Co., Piscataway, NJ) by a method adapted from Deng and Wu.[18] These labeling reactions added one biotin moiety at the 5′ terminal and approximately six biotin moieties at the 3′ terminal. Labeled and purified probes were then used at a concentration of 1 to 2 μg/ml in the hybridization reaction.

Prehybridization

Sections were washed in 0.2 N HCl at room temperature for 20 minutes followed by a 10-minute wash in 2 × SSC at 70°C. This was followed by a proteolytic treatment using Proteinase K (Boehringer Mannheim, Mannheim, FRG), concentration 1 to 20 μg/ml to determine optimum conditions for unmasking of mRNA targets. Subsequently sections were incubated in prehybridization solution for 1 hour at 37°C. This solution comprised 50% formamide, 600 mmol/l NaCl, 50 mmol/l TRIS HCl pH 7.6, 0.1% w/v sodium pyrophosphate, 0.2% w/v polyvinyl pyrrolidone MW 40000, 0.2% w/v ficoll MW 40000, 5 mmol/l ethylene-diamine tetraacetic acid (EDTA), 150 μg/ml sheared salmon sperm DNA, and 10% polyethyleneglycol 6000.[19]

Hybridization

After prehybridization, sections were drained and the hybridization mixture was added. This solution was prepared in the same way as the prehybridization solution, except this time the TNF probe was added at a concentration of 2 μg/ml. The slides were incubated overnight at 37°C. Sections were washed under stringent conditions to remove any unbound or partially bound probes.

Controls

Several controls were included in each experiment. These negative controls were a hybridization with sense probe cocktail biotinylated at the 5′ and the 3′ terminal in an identical manner to the antisense cocktail; a hybridization with a 3′ labeled poly-A probe of 25 to 30 bases in length; and a no-probe control and a ribonuclease control in which it was shown that all signals were sensitive to digestion with RNase type 1A.

A positive control was also incorporated by hybridizing with a 3′ labeled poly-T probe of 25 to 30 bases in length. This assay makes it possible to determine the integrity of the tissue biopsy as regards preservation of all mRNA.

Visualization of Hybrids

Detection of hybrids was carried out using the components of an sABC kit (Dakopatts, Copenhagen, Denmark) applied to the sections separately in four stages, and visualization was by a O-naphthol-AS-bi-phosphate (Sigma Chemical Co.) entrapment method.[19]

cDNA Probes

The specific TNF-α probe used for *in situ* hybridization of cytocentrifuge preparations and Northern blot analysis was an 0.8-kb Eco R1 fragment maintained in plasmid pSP64.[17] The lymphotoxin-specific cDNA was a 1.329-kb Eco R1 fragment maintained in plasmid pBR322.[20] Both probes were provided by Genentech Inc., San Francisco,

CA. We used a 2-kb Pst I cDNA fragment specific for c-beta actin maintained in pBr 322[21] as positive control for Northern blot analysis. The negative control for *in situ* hybridization was the plasmid PGEM2 obtained from Genofit, Heidelberg, FRG. PGEM2 was constructed from pSP65, which differs only marginally from pSP64.

Northern Blot Analysis

Total cellular RNA was isolated from routinely passaged cells in the presence of guanidinium isothiocyanate.[22] Twenty to thirty μg of each sample, determined photometrically, were size fractionated by formaldehyde/agarose gel electrophoresis and transferred to nitrocellulose. The filters were hybridized with specific cDNA labeled with ^{32}P by the random priming method.[23] Filters were washed at high stringency, and the hybridization reactions obtained were visualized by autoradiography against Kodak XAR5 with an exposure time of 1 to 3 days.

TNF-α ELISA

A sandwich ELISA for TNF-α was performed according to standard techniques,[24] using monoclonal anti-TNF-α antibodies provided by BASF/Knoll AG, Ludwigshafen, FRG. The assay was standardized against recombinant TNF-α. The sensitivity of this assay was more than 10 pg/ml. Values greater than 15 pg were considered TNF-α positive.

L929 Bioassay

The mouse fibrosarcoma line L929 was used as the target in the cytotoxicity assay, as described previously.[25,26] Briefly, 3-day-old L929 cells at a concentration of 1×10^6/ml were added in 100 μl aliquots to serially diluted test samples in 96-well flat-bottom microtiter plates in the presence of 1 μg/ml actinomycin D. After 19 hours incubation, viable cells remaining were quantified by the addition of 10-μl of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, 5 mg/ml in PBS, ph 7.4). After a further 2-hour incubation, the reaction was stopped by the addition of acidified isopropanol (0.04 N HCL in isopropanol). The MTT reaction was measured at 550 nm.[26]

Cytotoxicity levels corresponding to TNF-α values greater than 30 international units/ml (1 U/ml is equivalent to 0.025 ng TNF-α/ml) were considered positive (determined against recombinant human TNF-α (BASF/Knoll AG).

The specificity of this cytotoxic assay for TNF-α or lymphotoxin was determined by blocking with serial dilutions of rabbit anti-human recombinant TNF-α antiserum (BASF/Knoll AG) or a monoclonal anti-human lymphotoxin antibody (LTX-9, Dr. Adolf). One microliter (4.4 μg/ml) of LTX-21 neutralized the cytotoxic activity of 4.4 pg of human recombinant lymphotoxin (Dr. Adolf).

Table 1. *TNF-α and Lymphotoxin Production at the Product Level*

	L428	L540
TNF ELISA* of supernatants	+	+
Cytotoxic activity of supernatant†	+	–
Inhibition with anti-TNF	–	Not done
Inhibition with anti-LT	Partial	Not done
TNF ELISA of cell lysates	+	+
APAAP staining with anti-TNF	+	+
APAAP staining with anti-LT	+	+

* Values greater than 15 pg/ml were considered as TNF-α positive.
† Cell lysis of L929 induced by cytotoxic activity of supernatants greater than 750 pg/ml were considered positive.

Results

Cell-line Studies

TNF-α and Lymphotoxin Production at the Product Level

Table 1 summarizes our results relating to the production of TNF-α and lymphotoxin by L428 and L540. Culture supernatants from both cell lines were consistently positive in the TNF-α ELISA, demonstrating the presence of immunoreactive TNF-α. In the L929 bioassay, cytotoxic activity could be determined only in L428 supernatants. This activity could not be blocked by the addition of a polyclonal antibody against TNF-α but was partially (60%) inhibited with the monoclonal antibody against lymphotoxin.

Frozen-thawed L428 and L540 cell lysates were also positive in the TNF-ELISA. Both L428 and L540 stained for TNF-α and lymphotoxin, showing a marked similarity in staining pattern. In Figure 1 we illustrate the results of staining L428 (Fig. 1a) and L540 (Fig. 1b) with monoclonal antibodies directed against human TNF-α (1a) and lymphotoxin (Fig. 1b), respectively. The staining pattern seen with both cell lines and both antibodies was granular and cytoplasmic. A marked proportion of cells showed pronounced focal staining of the Golgi region. Figure 1c shows staining of L428 with Mac 387 as an inappropriate monoclonal antibody control. L540 was also negative after staining with Mac 387.

TNF-α and Lymphotoxin Production at the Level of mRNA

Synthesis of TNF-α and lymphotoxin in both L428 and L540 was confirmed in replicate experiments by the dem-

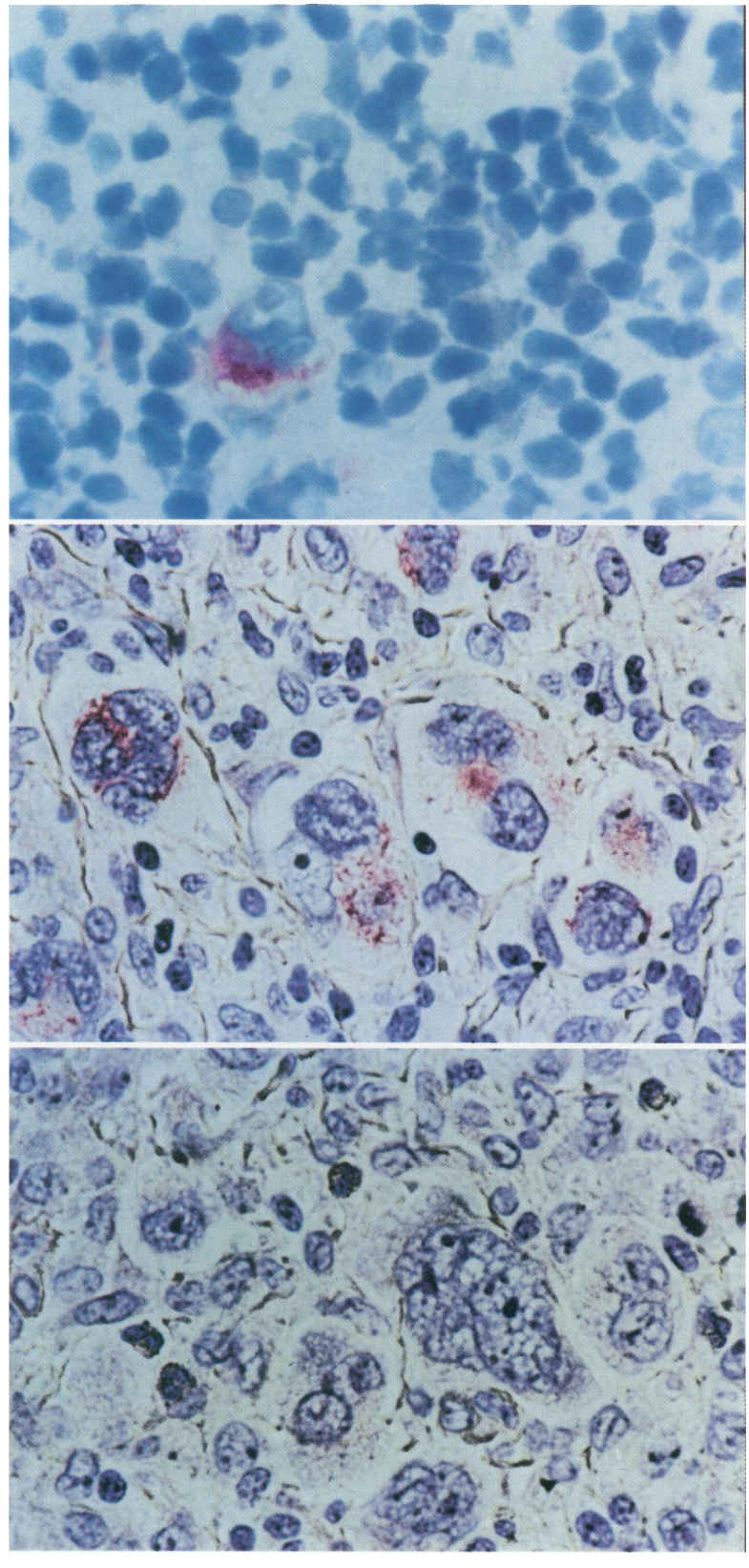

Figure 1. *Immunostaining for tumor necrosis factor alpha and lymphotoxin of Hodgkin's disease-derived cell lines L428 and L540.* **a:** *APAAP staining with anti-TNF-α antibody of L428. Staining with anti-TNF-α of L540 was very similar.* **b:** *APAAP staining of L540 with monoclonal antibody against lymphotoxin. Staining with anti-lymphotoxin of L428 was merely identical.* **c:** *APAAP staining of L428 with Mac387 as an inappropriate monoclonal antibody control. Staining of L540 with Mac387 was negative, too.*

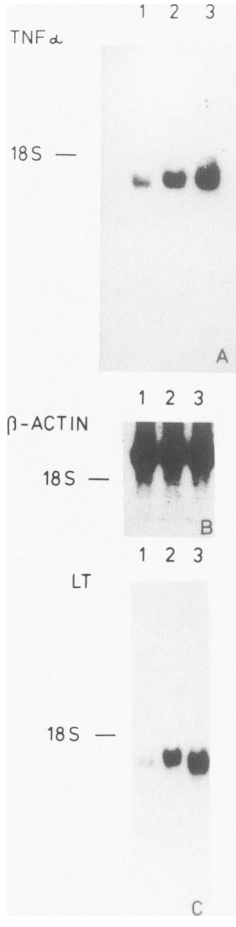

Figure 2. *Northern blot analysis. mRNA prepared from mononucleated peripheral blood leukocytes (lane 1), L428 (lane 2), and L540 (lane 3) were probed with the TNF-α-specific cDNA probe* (a), *with the beta actin cDNA probe* (b), *and with the lymphotoxin cDNA probe* (c).

onstration of intracellular specific mRNA using specific cDNA clones.

Northern blot analysis of total cellular RNA prepared from routinely maintained cell cultures of L428 and L540 gave strong hybridization signals close to the position of 18S rRNA consistent with the presence of TNF-α mRNA, which is 1.67 kb (Figure 2a), and consistent with the presence of lymphotoxin mRNA, which is 1.33 kb (Figure 2c). A weak signal of equivalent size was seen with both probes with mRNA prepared from peripheral blood mononuclear cells. Hybridization with a probe specific for beta-actin gave hybridization signals of equivalent intensity with all mRNA preparations (Figure 2b).

In addition, we were able to conduct *in situ* hybridization studies with the TNF-α cDNA probe. Zamboni's fixed cytocentrifuge preparations of L428 and L540 (Figures 3a, b) gave a positive granular cytoplasmic signal when hybridized with the specific TNF-α probe. The intensity of the hybridization reaction varied between individual cells. Reactivity was absent in cell preparations hybridized with the control plasmid PGEM2 (Figure 3c).

Tissue Biopsy Studies

In a preliminary set of experiments 10 different frozen HD tissue biopsies were immunostained with the antibody TNF-E specific for TNF-α and LTX-21 specific for lymphotoxin. In three cases TNF-α positive H&RS cells were seen (Figure 4a). Immunostaining with anti-lymphotoxin could not be evaluated due to the large amount of immunoreactive material present in these sections.

A further eight different HD biopsies were investigated using an oligonucleotide-based *in situ* hybridization technique developed for use in formalin-fixed paraffin-embedded tissue. In six of these cases a strong cytoplasmic signal was seen with the specific probe cocktail for TNF-α (Figure 4b). The anti-complementary control oligonucleotides were negative (Figure 4c). The positive signal was sensitive to RNAse digestion.

Discussion

The aim of the present study was to investigate the working hypothesis originally proposed by Newcom and O'Rourke[4] and by Ford et al[5] that the histologic pattern characteristic of HD may be caused by cytokine production by H&RS cells. In this study we concentrated on investigations on the role of the pleotropic cytokines TNF-α and lymphotoxin as possible pathophysiologic mediators in HD because these cytokines are regarded to be of central importance in cytokine interactions.

TNF-α and lymphotoxin have been variously shown to enhance the proliferation of T cells,[27] modulate T-cell receptor expression,[28] enhance natural killer cell activity,[29,30] and to modulate human B-cell function.[31,32] With regard to inflammation, both cytokines have a marked effect on neutrophil[33] and eosinophil recruitment, macrophage activation,[34] and endothelial cell/leukocyte interactions.[10,35,36] Stimulation of fibroblast growth is also well documented.[11] These cytokines also act synergistically as part of the cytokine cascade and regulate prostaglandin production.[10,37-39] In HD the reactive tissue component is large and contains many activated neutrophils, macrophages, lymphocytes, and eosinophils.[1-3] Furthermore the nodular sclerosis form of HD (NS) is characterized by extensive fibroblast proliferation.[1,4,40] The low frequency and consequent difficulty in extraction and purification of neoplastic cells from HD tissue biopsies complicates the direct investigation of cytokine production by HD tumor cells.

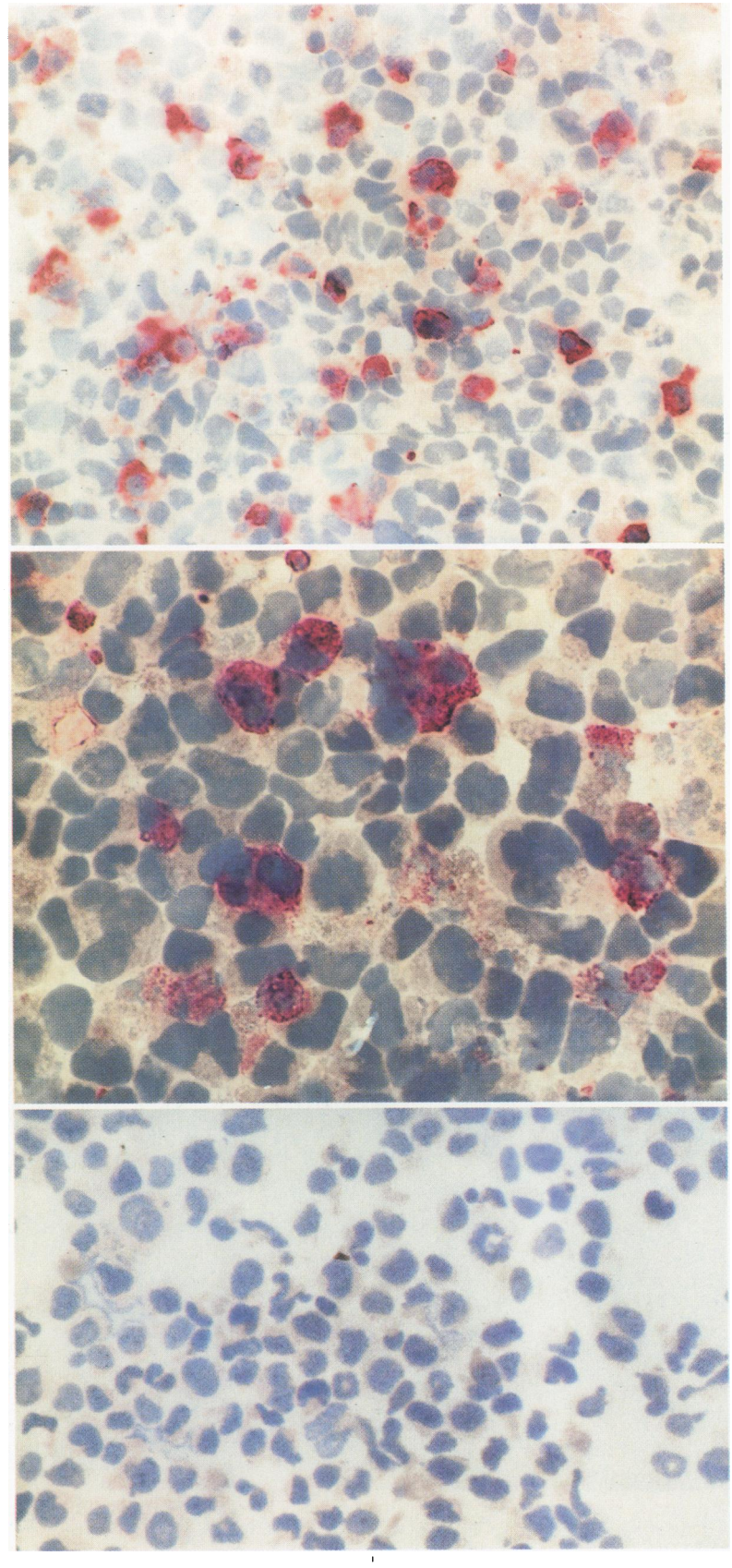

Figure 3. *Immunoenzymatic detection of* in situ *hybridization reaction for TNF-α in Hodgkin's disease-derived cell lines L428 and L540.* **a**: *L428 hybridized with TNF-α–specific cDNA probe.* **b**: *L540 hybridized with TNF-α cDNA probe.* **c**: *L540 hybridized with the control plasmid PGEM2. The control hybridization with L428 was also negative.*

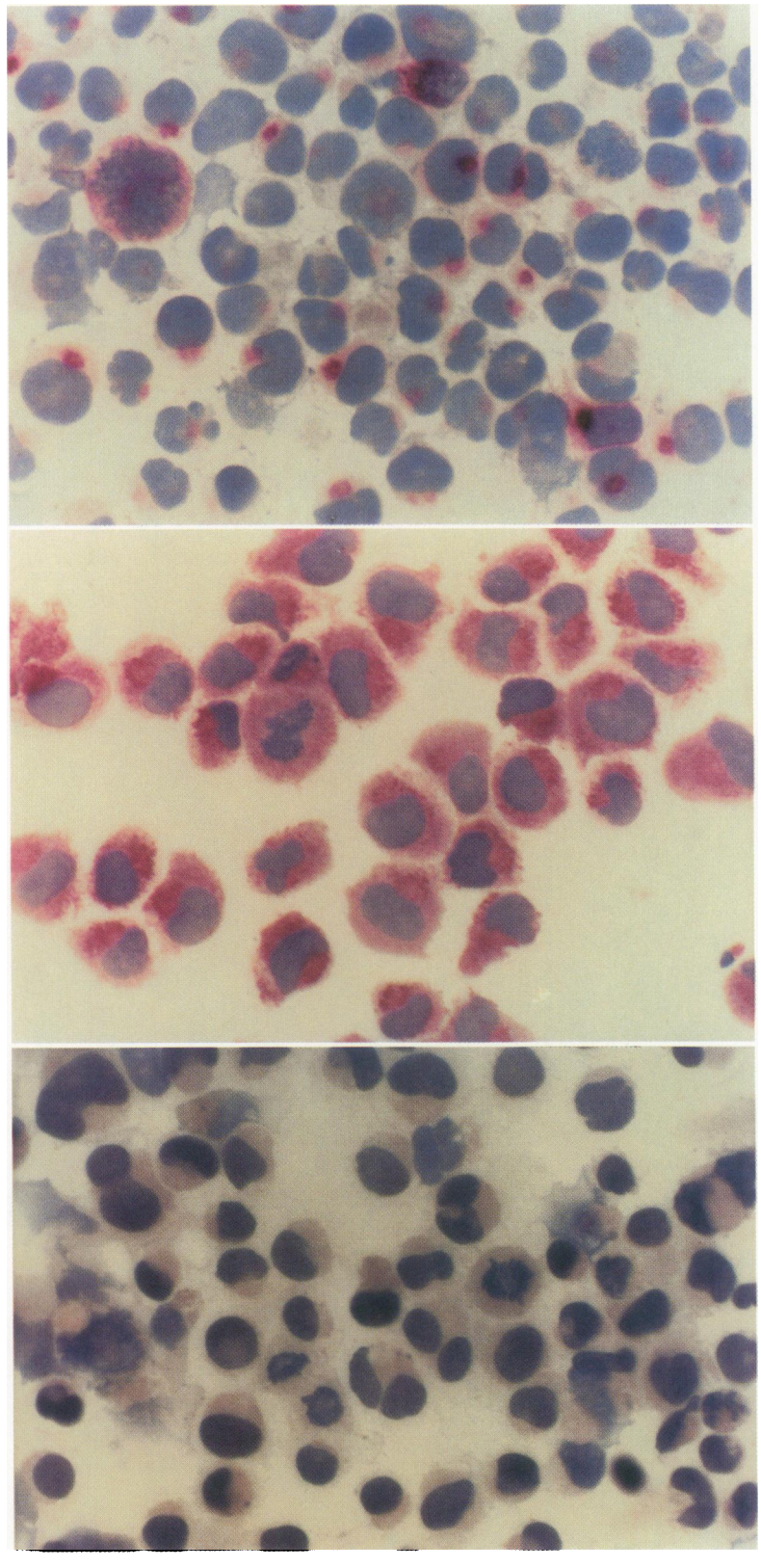

Figure 4. *Demonstration of TNF-α in Hodgkin's disease tissue biopsies at the product* (a) *and the mRNA* (b) *level.* a: *APAAP staining of a frozen section with anti-TNF-α–specific antibody.* b: *Formalin-fixed paraffin section of Hodgkin's disease biopsy hybridized with TNF-α–specific oligonucleotides.* c: *Formalin-fixed paraffin section of Hodgkin's disease biopsy hybridized with the anticomplimentary control oligonucleotide.*

The development of cell lines from HD patients with phenotypic and genotypic characteristics, which parallel those described for H&RS cells,[6–8] enables initial investigations to be conducted on stable models of the tumor-cell population.

Our results demonstrate that both L428 and L540 synthesize TNF-α and lymphotoxin. At the mRNA level TNF-α and lymphotoxin gene expression was seen in both cell lines with Northern blot analysis. The size of the hybridized mRNA was the same as described for TNF-α[17] and lymphotoxin,[20] respectively. The quantity of mRNA present in the lanes illustrated in Figure 2 is equivalent, as is shown by the beta-actin hybridization (Figure 2b). The weak signal with both cytokine probes seen with peripheral blood mononuclear cell mRNA presumably represents low levels of activation of this population during preparation.

The detection of cytokines at the level of protein is important, as at least the TNF-α gene transcription is not necessarily followed by translation.[10] Immunostaining of L428 and L540 with antibodies against TNF-α and lymphotoxin showed fine granular cytoplasmic and pronounced Golgi reactivity. Both reagents have been shown previously to have exclusive specificity for the individual cytokines.[14] Furthermore ELISA for TNF-α shows the presence of immunoreactive protein in cell lysates as well as in supernatants of both cell lines investigated. Thus in this study both cytokines could be demonstrated at the level of protein.

With regard to biologic reactivity, cytotoxic activity was present only in L428 supernatants, which could not be inhibited by an antiserum against TNF-α, but was inhibited by 60% with a monoclonal anti-lymphotoxin antibody. This demonstrates that at least part of the cytotoxic reactivity measured in L428 supernatants is caused by lymphotoxin. The remaining activity and the failure of the anti–TNF-α reagent to block may be due either to the production of an abnormal protein not bound by the antibodies used in this study or to another cytotoxic mediator as yet unidentified. In this respect Newcom et al[41] have described a transforming growth factor (TGF)-like activity in L428 cells, which does not show the characteristic molecular weight described for TGF. The absence of measurable cytotoxic activity in L540 supernatants in the presence of a positive ELISA result may also indicate the production of abnormal and/or unusual protein products.

Taken together, we conclude that our data clearly demonstrate the transcription, translation, and export of TNF-α and lymphotoxin in cultured HD-derived cell lines.

We do not suggest that TNF-α is the only cytokine produced in HD. Apart from early studies,[4,5,42–44] which predate specific antibodies and/or gene probes, and must therefore be interpreted with caution, other data exist in the literature, which suggest that cultured HD cells may produce cytokines. The TGF-like activity described by Newcom et al[41] has been proposed as a factor responsible for sclerosis in NS HD and may also modulate IL-2 receptor expression and act as an immunoregulator.[40,41] As cytokines clearly interact in the generation of a complex tissue pathology, we do not think that these data conflict with our observations on TNF-α and lymphotoxin.

Ellis et al[45] have shown that L428 exhibits marked accessory cell activity for CD3-driven T-cell proliferation and CD3-dependent production of IL-2 by Jurkat cells. These authors exclude the possibility that the genes for either IL-1-α or IL-1-β are transcribed by L428 cells in both functional and molecular biologic investigations. It is known that TNF-α can substitute for IL-1 in the mouse thymocyte proliferation assay.[27] It is therefore possible that TNF-α represents the IL-1–independent accessory cell activity of L428 cells described by Ellis et al.[45]

In view of the fact that TNF-α and lymphotoxin are known to be functionally active in the cyclooxygenase pathway[39] and at least TNF-α is a known potentiator of prostaglandin synthesis,[10] their production by two HD-derived cell lines is particularly interesting with regard to the elevated prostaglandin E_2 levels, which occur in HD patients.[46]

Clearly *in vitro* studies may not reflect the situation *in vivo*. Therefore we include preliminary investigations relating to cytokine content of H&RS cells in HD biopsy tissue. Initially we have established an oligonucleotide-based *in situ* hybridization method for the demonstration of TNF-α mRNA in formalin-fixed paraffin-embedded HD biopsy material.

In all cases in which mRNA preservation could be demonstrated by poly dT hybridization an RNAse-digestible TNF-α mRNA signal was present, whereas hybridization with the TNF-α sense control probes was consistently negative. These data in tissue parallel our results with sulphonated TNF-α–specific probes in L428 and L540. Furthermore immunostaining in frozen sections with the antibody TNF-E demonstrated positive H&RS cells. Thus, at least for TNF-α, we present evidence that our cell-line data are relevant to the *in vivo* situation in HD.

The data presented in this study coupled with earlier publications[4,5,40–45] are consistent with the hypothesis that cytokine production by H&RS cells contribute greatly to the complex histotopographic pattern seen in HD. Further studies are needed to correlate the production of individual cytokines to the different histologic subtypes of HD. As a first step in this direction a more detailed analysis of the production of TNF-α and lymphotoxin in a large number of HD patients' biopsies is now under way.

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