We investigated the mRNA content for tumor necrosis factor (TNF)/cachectin and lymphotoxin (LT) in tumoral tissues of a prospective series of 35 non-Hodgkin's (NHL) and 23 Hodgkin's (HL) lymphomas, to assess their postulated contribution to systemic symptoms. Total RNAs were extracted from diagnostic tissue specimens and submitted to Northern blot analysis, using specific TNF and LT cRNA probes. High amounts of TNF mRNA were found exclusively in NHL (12/35). The majority (9/12) of these were low grade B-cell NHL, which contained a uniform population of malignant cells. In contrast, abundant LT mRNA production was detected in most HL (21/23) and in 19 of 35 NHL. The highest LT mRNA levels were observed in high grade NHL and in lymphocytic predominant subtypes of HL.

Fever, asthenia, and weight loss are characteristic clinical features; when present at diagnosis, they are known to adversely influence responses to therapy and survival, but their origin remains undetermined. Over the past few years, evidence has accumulated suggesting that invasive diseases might induce metabolic abnormalities through the activation of the immune system. Indeed, monocytes/macrophages and lymphocytes synthesize and secrete a variety of cytokines that can affect host metabolism, such as interleukin-1 (IL-1), γ-interferon, tumor necrosis factor (TNF), and lymphotoxin (LT). The structural and functional characteristics of TNF have revealed its pleotropic effects and led to the recognition of its identity with cachectin, a macrophage-secreted protein capable of altering lipid biosynthesis and believed to be responsible for the wasting associated with some parasitosis. LT, a molecule distantly related to TNF and produced mainly by lymphocytes, has simultaneously been identified: it acts on target cells via the same binding sites and is considered to exert a similar spectrum of biologic activities. TNF and LT are therefore attractive candidate effectors for tumoral fever and cachexia. However, TNF participation in cancer cachexia is not yet firmly established. Recent studies evaluating the systemic production of TNF, using bioassays or immunoenzymatic assays, have provided conflicting data in cancer patients, whereas, to our knowledge, LT's production has not yet been determined. TNF and LT are secreted proteins with multiple deleterious properties: they are likely to be controlled by specific inhibitors and degradative enzymatic processes. Their estimation at the protein level might then be hampered by the presence of such regulatory mechanisms, which could explain the apparent discrepancies obtained so far in the published series. Nucleic acid analysis offers a way to circumvent the limitations of current biochemical assessments and might help in evaluating the synthetic potential for given cytokines in different tissues. We therefore determined the mRNA content for TNF, LT, and IL-1B in diagnostic specimens of 35 non-Hodgkin's lymphomas (NHL) and 23 Hodgkin's lymphomas (HL), using RNA probes complementary and specific for these mRNAs. The results of Northern blot hybridizations were correlated with histologic and clinical criteria, namely the presence of fever and weight loss.

MATERIALS AND METHODS

Tissue Sampling

Tissues were collected immediately after surgical removal. Portions free of fat or connective tissue were dissected, washed in buffered saline, immersed in liquid nitrogen, and kept at −80°C until analyzed. In each case, one corresponding cryopreserved adjacent portion was used for immunophenotyping and one was fixed in 10% formalin and paraffin embedded for conventional histologic examination.

Histopathological and Clinical Assessment

Non-Hodgkin's lymphomas. In all cases, the diagnosis was made on lymph node specimens. Lymphomas were classified according to the Kiel classification. 23 were considered as low-grade and 12 as high-grade lymphomas. All lymphomas were phenotyped as B-cell. The degree of neo-vascularization, necrosis, hyalnosis, and stromal reaction was assessed semiquantitatively by one of us (M.F.P.). Significant foci of necrosis were apparent in a single specimen, whereas neovascularization, hyalnosis, and stromal reactions varied marginally from one specimen to another. Twelve cases were associated with >10% weight loss and unexplained fever above 38°C, for 2 to 4 months before the diagnosis; one case had a history of unexplained fever alone.

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Hodgkin's lymphomas. All diagnoses were made on lymph node specimens. Fifteen cases were of sclerosing nodular type, four of mixed cellularity, and four of lymphocytic predominance. Clinical stages included two stage I, eight stage II, eight stage III, and five stage IV. Nine cases were associated with ≥10% weight loss and unexplained fever above 38°C, for 1 to 12 months before diagnosis.

Non-malignant lymph nodes. Five lymph node specimens displaying nonspecific reactive hyperplasia were analyzed as controls. Similarly, six specimens of lymph nodes from AIDS patients were analyzed, showing the characteristic prominent polymyal B-cell hyperplasia in the follicular zones, as assessed by immunohistochemistry.

Three lymph nodes were obtained from acquired immunodeficiency syndrome (AIDS) patients that presented with greater than 10% weight loss, but without fever, in the preceding 3 months.

RNA Extraction, RNA Probes, and Hybridizations

Total cellular RNA was extracted as previously described, using guanidium thiocyanate and CsCl gradient centrifugation. The following probes were transcribed in vitro using T7 or SP6 RNA polymerase, in the presence of 100 μCi of 32P-labeled uridine triphosphate (UTP; 400 Ci/mmole; Amersham, UK): pRS1-hTNF, containing the 330 bp HindIII-BamHI fragment isolated from 4W6; pG-hLT, containing the 490 bp EcoRI-BamHI fragment isolated from the pL-hLT 17; pSP64-hIL-1β.2, containing the 300 bp EcoRI-Pvu II isolated from the pSP64-hIL-1β.2 18; and pSP65-α-A-C, containing the 320 bp Ava II-Bgl II fragment corresponding to the coding region of rat α-smooth-muscle actin, which hybridizes to all actin mRNAs. 19

For Northern blot hybridizations, RNAs were denatured in glyoxal, electrophoresed in 1.2% agarose gels, and transferred overnight onto Biodyne nylon membranes ( Pall), as previously described. After transfer, the even loading and the integrity of RNAs were verified by staining membranes with methylene blue.

Prehybridizations and hybridizations were performed as described. Filters were submitted to two washes in 3 × SSC, 2 × Denhardt's solution for 15 minutes at 65°C, followed by three washes in 0.2 × SSC, 0.1% sodium dodecyl sulfate (SDS) for 15' at 78°C and autoradiographed using X-Omat AR film (Eastman Kodak, Rochester, NY) with an intensifying screen (DuPont Co, Wilmington, DE) for 72 hours (lymphomas) or 168 hours (non-malignant lymph nodes).

RESULTS

All positive samples expressed single TNF, LT, and IL-1β mRNA transcripts, of the expected sizes, respectively and approximately of 1.9, 1.4 and 1.8 kb, as reported for other tissues. 20,21

Non-Hodgkin's Lymphomas

Moderate to abundant levels of TNF mRNA were seen in 12 of 35 specimens: nine low grade and three high-grade lymphomas. The strongest signals (Fig 1, lanes 2, 10, 12, 18, and 29) were detected in specimens of lymphoplasmacytic lymphomas, characterized by a very homogeneous histologic appearance, without detectable macrophage accumulation or "starry sky" pattern. Variable amounts of elevated LT mRNA were observed in 19 of 35 specimens (Fig 1). Ten lymphomas were found to co-express TNF and LT mRNA (Fig 1). One high-grade NHL was associated with high amounts of IL-1β mRNA (Fig 1, lane 8).

No correlation could be demonstrated with the presence of necrosis, neo-angiogenesis, hyalinosis, or stromal reaction (macrophage, fibroblast, or "starry sky" pattern), as assessed histologically. Eight diagnostic samples from the 12 patients...
who presented with systemic symptoms were associated with high TNF mRNA levels, and 11 of 12 were associated with abundant LT mRNA expression. High levels of TNF gene expression (χ² test in two by two contingency table, \( P < .005 \)) and of LT gene expression (χ² test in two by two contingency table, \( P < .005 \)) correlated significantly with the presence of systemic symptoms.

### Hodgkin's Lymphomas

All samples examined displayed very low amounts of TNF mRNA, whereas 21 of 23 specimens exhibited moderate to abundant amounts of LT mRNA (Fig 2). The strongest signals were observed in the four specimens from lymphocytic predominant subtypes (Fig 2, lanes 2, 13, 14, and 17). High levels of IL-1β mRNA were associated with 6 of 23 lymph nodes (Fig 2).

No correlation between LT mRNA expression, histologic pattern, and histologically-defined criteria of necrosis, neangiogenesis, hyalinosis, or stromal reaction could be observed. Though all samples (9/9) from the patients presenting with systemic symptoms were associated with LT mRNA expression, and three of nine were associated with IL-1β mRNA, no significant correlation was observed between LT and/or IL-1β gene expression and the presence of systemic symptoms.

### Non-Malignant Lymph Nodes

In all five samples containing aspecific reactive hyperplasia, and in all six lymph nodes from AIDS patients, TNF, LT and IL-1β mRNA levels were uniformly low and comparable with the levels detected in the lymphoma samples that were considered as low producers (Fig 3). Interestingly, no differences were noted between AIDS tissues obtained from patients with symptoms and from those without symptoms.

### DISCUSSION

The evaluation of systemic TNF production in a series of cancer patients has provided contradictory results. While some studies, using a bioassay or an enzyme-linked immunosorbent assay (ELISA), failed to demonstrate circulating levels of TNF in patients with neoplasia, others, using an ELISA, have found a serum factor immunologically related to TNF in 50% of cancer patients with active disease. These discrepancies have been attributed to the limitations of biochemical or biologic assays in estimating proteins that are susceptible targets for degradative enzymes or specific inhibitors. Using a method that evaluates the synthetic potential of tissue specimens for TNF and LT, we demonstrate in our study their heterogeneous production by lymphomatous tissues.

We show here that high amounts of TNF/cachectin mRNA are expressed exclusively in NHL, whereas elevated levels of LT mRNA are detected in the majority of NHL and HL; 34% of the specimens involved with NHL displayed high amounts of TNF mRNA, while elevated levels of LT were expressed by 91% of HL and 54% of NHL. These findings raise several questions of potential biologic significance. Despite recent progresses achieved in the phenotypic and genotypic characterizations of lymphomas, functional differences that may account for their variable clinical behavior have not been identified. The heterogeneous pattern of cytokine production observed in malignant lymphoid tissues differs clearly from non-malignant lymphoid tissues and reveals a functional diversity that is not apparent on conventional histopathologic assessments and that may influence the clinical course of lymphoproliferative diseases. Indeed, TNF/cachectin expression in involved lymphoid tissues correlated significantly with the presence of systemic symptoms.
**TNF/CACHECTIN AND LT IN LYMPHOMAS**

![Fig 3. Northern blot analysis of total RNAs extracted from non-malignant lymph nodes. All numbered lanes were loaded with 10 µg of RNA. Lanes 1 through 5 correspond to lymph nodes displaying specific reactive hyperplasias; lanes 6 through 11, to lymph nodes specimens from AIDS patients. C = 5 µg of total RNA extracted from PMA-stimulated U-937 cell line. All filters were hybridized as in Figs 1 and 2: they were exposed for 7 days. Arrows indicate the position of 18S RNAs. The lower pictures show the transferred RNAs stained with methylene blue before hybridizations.](image)

in NHL patients. Though we do not know if the detected mRNAs correspond to the level of translation into bioactive molecules, our results are in agreement with the TNF immunoreactivity found in the serum of 6 of 23 lymphoma patients recently reported. In addition to TNF, our analysis suggests that LT should be considered among the cytokines susceptible to contributing to systemic symptoms, as a significant correlation was also found between LT gene expression and the presence of systemic symptoms in NHL. However, we were not able to detect elevated amounts of TNF mRNA in HL and found no correlation between high LT production and systemic symptoms in this type of lymphoma. Our results, however, are based on a limited number of cases, and Northern blot analysis provides only semiquantitative estimations. In addition, LT and TNF bioactivities might be regulated, locally as well as systemically, by the presence of specific inhibitors, or degradative enzymes, or modulations in the number of binding sites on target cells. It should be pointed out that direct estimations of LT and TNF in tumoral tissues might not be appropriate: they do not take into account the possible participation of adjacent or nonaffected tissues in the synthesis of LT and TNF.

Total RNA analysis of mixed cell populations does not allow the identification of the cellular site(s) for LT and TNF synthesis; it therefore remains to be determined if these cytokines are expressed by the malignant clones on whether they merely represent a host reaction in response to the neoplastic proliferation. TNF production has been ascribed mainly to cells of the monocyte/macrophage lineage, although circulating lymphocytes, human B-cell lines, and tumor cell lines of epithelial origin have been found to synthesize and release TNF. The strongest signals were observed in specimens composed of a predominant population of malignant lymphoplasmacytic cells, suggesting that some transformed B cells are capable of constitutive TNF production, as it has been shown in vitro. LT is considered to be predominantly a lymphocyte product, and myeloma cell lines have been shown to express LT. The results obtained in HL, where malignant cells constitute a minor fraction of involved tissues, favor the dominant reactive cell populations as probable sources of LT production, while LT expression in NHL might be attributed to the malignant cells. However, even if inflammatory and not malignant cells are the main source of cytokine production, high amounts of TNF and LT transcripts appear to be a characteristic feature of some lymphoma tissues, since non-malignant, though activated, lymph nodes are associated with very low levels of cytokine mRNAs.

TNF and LT are pleotropic cytokines that can modulate the proliferation and differentiation capacities of various cell types: they are expected to be involved in a wide range of physiopathologic processes such as tissue necrosis, neoangiogenesis, and fibroblast proliferations. In our series, a single specimen of NHL exhibited areas of marked necrosis and concomitant detectable TNF mRNA and LT mRNA. In all other cases, no correlation was noticed between TNF and LT mRNA expression and manifest necrosis at the histological level. This observation does not formally exclude TNF or LT participation in tissue necrosis, but could also result from posttranslational regulatory mechanisms such as mentioned above. In addition to its cytotoxic properties, TNF has been shown to mediate macrophage-induced angiogenesis and to exert mitogenic activity on fibroblast cells. However, we observed no correlation between TNF and/or LT mRNA expression and the degree of vascularization or fibroblast proliferation as assessed histologically.

Numerous biologic activities mediated by TNF and LT can be elicited by IL-1, a cytokine synthesized mainly by monocytes/macrophages. We therefore also evaluated IL-1ß synthesis and found high amounts of IL-1ß mRNA in only one NHL and in six HL. These results do not support a role for IL-1ß in symptoms of disease and are in agreement with protein estimations obtained by others, who described IL-1 immunoreactivity mainly in HL and found no correlation with manifestation of systemic symptoms.

Our analysis shows that an elevated expression of the LT gene is common in lymphomatous tissues, whereas high TNF/cachectin gene expression is unfrequent and seems to be restricted to a subset of NHL. Further investigations are clearly needed to establish the postulated roles for TNF/cachectin and LT in the causation of systemic symptoms and to clarify their potential implications on tumor growth control. Our findings reveal a highly heterogeneous pattern of cytokine gene expression in lymphomatous tissues and suggest that the evaluation of TNF/cachectin and LT in lymphomas might help to elucidate the mechanisms underlying systemic symptoms in neoplasia.
REFERENCES