ABSTRACT

Several cytokines including γ-interferon, tumor necrosis factor α, interleukin 1β (IL-1β), and interleukin 6 (IL-6) are pyrogenic and can inhibit lipogenic processes. Because patients with lymphoma often suffer from fever, weight loss, and night sweats (B symptoms), the etiology of which is unknown, the authors investigated serum levels of these cytokines in normal volunteers and in patients with Hodgkin’s and non-Hodgkin’s lymphoma. Sixty serum samples from patients with Hodgkin’s disease (28 patients) or non-Hodgkin’s lymphoma (32 patients), as well as 20 samples from normal volunteers, were collected. The majority of patients had advanced (Stage III or IV) or relapsed disease. The assay for γ-interferon was a specific and sensitive radioimmunoassay (lower limit of detection = 0.1 unit/ml); the assays for tumor necrosis factor α, IL-1β, and IL-6 were enzyme-linked immunosorbent assays with lower limits of sensitivity of 10 pg/ml, 20 pg/ml, and 22 pg/ml, respectively. There were no statistically significant differences in γ-interferon, tumor necrosis factor α, or IL-1β levels between lymphoma patients and normal subjects. In contrast, 20 of 57 patients (35%) with lymphoma as compared with 0 of 19 normal volunteers (0%) had detectable serum IL-6 levels (P < 0.005, χ² test). Interestingly, 17 of 29 lymphoma patients with B symptoms (59%) as opposed to 3 of 28 lymphoma patients without B symptoms (11%) had detectable serum IL-6 levels (P < 0.001, χ² test); the median IL-6 level was 28.9 pg/ml (B symptoms present) versus undetectable (no B symptoms) (P < 0.005, Mann-Whitney U test). Analyzing Hodgkin’s and non-Hodgkin’s lymphoma groups separately revealed similar results. IL-6 levels showed no significant correlation with time from diagnosis, β₂-microglobulin, or lactate dehydrogenase levels. However, analysis by the method of Kaplan and Meier demonstrated that the median survival of Hodgkin’s disease patients with detectable IL-6 levels (≥22 pg/ml) was 10 mo, whereas the median survival has not been reached at a median follow-up time of 37.5 mo in those patients with lower values (Wilcoxon P value = 0.0012). There were too few patients in each subset of non-Hodgkin’s lymphoma to determine the correlation between IL-6 and survival but, considered as a single group, a statistically significant correlation was not found. We conclude that serum IL-6 levels are elevated in a substantial proportion of patients with lymphoma and that an association exists between endogenous overproduction of IL-6 and the presence of B symptoms. Furthermore, serum levels of IL-6 of ≥22 pg/ml are associated with a significantly shorter survival in patients with advanced or relapsed Hodgkin’s disease.

INTRODUCTION

Lymphoma patients frequently complain of unexplained fever, night sweats, and weight loss, and the presence of these clinical features generally indicates a poor prognosis. Several lines of evidence suggest that these B symptoms could be caused by aberrant production of endogenous cytokines, either by the tumor itself or by accessory elements. (a) B symptoms are strongly reminiscent of some of the side effects seen after the clinical administration of interferon, certain interleukins, and tumor necrosis factor (1–4). (b) Endogenous cytokines have been implicated in several disease processes which involve fever and weight loss. For example, increased serum levels of TNF-α have been found in patients with the acquired immunodeficiency syndrome (5), and serum levels of TNF-α, IL-1β, IFN-γ (6), and IL-6 (7) correlate with the severity of meningococcosis (8). Furthermore, (c) plasma TNF-α and IL-6 levels are high in septic shock (8, 9), and excess circulating IL-6 accompanies the inflammatory reaction and fever that develop in victims of severe burns (10). In gram-negative sepsis, IL-6 levels correlate inversely with the duration of survival (11). On the basis of these observations, we investigated the relationship between disease manifestations and serum levels of TNF-α, IL-1β, IFN-γ, and IL-6 in a group of patients with lymphomas.

MATERIALS AND METHODS

Over a period of 2 yr, serum samples from patients with a diagnosis of lymphoma and from normal volunteers were collected and stored at −70°C. Each person gave informed consent according to institutional guidelines. At the time that samples were obtained, newly diagnosed patients were not yet treated, and relapsed patients had been off therapy for at least 3 mo. Patients were evaluated in a prospective manner and had verification of their diagnosis by histopathological examination of tumor tissue. Complete staging evaluation was done in each individual, and patients with a history of unexplained fever (>38°C), night sweats, or weight loss (>10% of body weight) were considered to have B symptoms.

Each patient sample was aliquoted into separate vials containing 1 ml of serum; tests for each of the four cytokines examined (IFN-γ, TNF-α, IL-1β, and IL-6) were run on serum derived from separate vials to avoid freezing/thawing refreezing of individual samples (a procedure which could conceivably affect the level of cytokine measured).

Samples were assayed in duplicate, and the mean values were calculated. Each assay was performed with a commercially available RIA or ELISA kit in accordance with the manufacturer’s instructions. All sera were tested for an individual cytokine at the same time. Samples were assayed in a blinded fashion so that the person performing the cytokine RIA or ELISA did not have access to data revealing whether or not that patient had lymphoma with B symptoms, lymphoma without B symptoms, or was a normal volunteer. Each time samples were assayed, a standard curve was generated with the use of known amounts of cytokine material, and the amount of cytokine contained in the unknown samples was determined using this curve.

For IFN-γ, a RIA (Centocor, Malvern, PA) was utilized (lower limit of sensitivity = 0.1 unit/ml). This RIA uses a solid-phase system. Briefly, polystyrene beads coated with a mouse monoclonal anti-human IFN-γ antibody are incubated with 200 μl of the serum specimen. The IFN-γ in the serum is bound to the solid phase. Unbound material is removed by washing. Another monoclonal anti-IFN-γ antibody (labeled with 125I) is then incubated with the beads; the radiolabeled anti-IFN-γ will bind to the IFN-γ on the beads. Unbound antibody is removed by aspiration. The bound IFN-γ is determined by counting the beads in a gamma scintillation counter. A standard curve is obtained by plotting the IFN-γ concentrations of IFN-γ standards (measured by the RIA) on a curve. The IFN-γ concentration of the patients’ specimens, which have been run concurrently with the standards, can then be determined from the standard curve.

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IL-6, IL-1ß, and TNF-α were each measured by quantitative sandwich ELISAs (R & D Systems, Minneapolis, MN; for IL-6: Cistron Biotechnology, Pinebrook, NJ, for IL-1ß: T-Cell Sciences, Cambridge, MA, for TNF-α). The lower limit of sensitivity for each of these assays was as follows: 22 pg/ml for the IL-6 ELISA; 20 pg/ml, IL-1ß ELISA; and 10 pg/ml, TNF-α ELISA. Standard curves for serum cytokine detection below these levels did not maintain linearity; our findings for lower limits of sensitivity were identical to those proposed by each of the manufacturers of the individual ELISA kits. The methodology is similar for each of these assays. Briefly, a monoclonal antibody specific for IL-6 or IL-1ß has been coated onto a microtiter plate. Serum samples (200 μl for IL-6 and 100 μl for IL-1ß) are pipetted into the wells, and the IL-6 or IL-1ß in the samples is then bound by the immobilized antibody. After washing off unbound sample proteins, a polyclonal anti-IL-6 or anti-IL-1ß antibody (linked to horseradish peroxidase enzyme) is added to the wells and binds to any IL-6 or IL-1ß which was bound during the first incubation. The technique is very similar for the TNF-α ELISA except that the first monoclonal antibody is not coated onto the plates. After washing, a substrate solution [containing stabilized hydrogen peroxide and stabilized chromogen (tetramethylbenzidine for the IL-6 assay, o-phenylenediamine for the IL-1ß and TNF-α assays)] is added to the wells. If antibody-enzyme conjugate is present, the substrate will be oxidized, and the color that develops is proportional to the amount of bound IL-6, IL-1ß, or TNF-α. At the same time that serum samples are tested, a series of wells is prepared with the use of known concentrations of recombinant IL-6, TNF-α, or IL-1ß standards. A standard curve of absorbance (as measured with the use of a microtitration plate reader at a wavelength of 450 nm for the IL-6 assay and at 490 nm for the IL-1ß and TNF-α assays) versus the known IL-6, IL-1ß, or TNF-α concentrations in these wells is plotted. The amount of IL-6, IL-1ß, or TNF-α in the unknown serum samples can then be determined by comparing their absorbance to that of the points on the standard curve. The antiiserum in each kit is specific for the cytokine being measured. For example, the IL-6 antiseraum has no cross-reactivity with IL-1α, IL-1ß, IL-2, IL-3, IL-4, TNF-α, TNF-β, granulocyte-macrophage colony-stimulating factor, tumor growth factors β1 and β2, platelet-derived growth factor, or fibroblast growth factor.

Statistical Analysis The significance of cytokine levels in different subsets of patients was assessed by the Kruskal-Wallis test, the Mann-Whitney U test, and the χ² test. Relative relations were tested by the Spearman rank correlation coefficient (rₛ). Survival curves were plotted by the method of Kaplan and Meier. Wilcoxon P values were determined for differences in survival. All P values are two tailed.

RESULTS

A total of 60 samples from patients with Hodgkin’s disease (28 patients) or non-Hodgkin’s lymphoma (32 patients), as well as 20 serum samples from normal volunteers, were collected. The total number of patient samples run for each cytokine (76 for IL-6, 72 for IFN-γ, 56 for TNF-α, and 54 for IL-1ß) reflects the total number of previously unthawed serum vials available and the number of samples which could be assayed simultaneously for that particular cytokine.

Circulating Levels of IFN-γ, TNF-α, and IL-1ß. Testing results are summarized in Table 1 and Fig. 1. Serum IFN-γ levels were <1 unit/ml in all of 15 normal volunteers and in 56 of 57 lymphoma patients tested; one patient had a level of 2 units/ml. Serum TNF-α values ranged from undetectable to 20 pg/ml in all 15 normal volunteers and from 0 to 28 pg/ml in 41 lymphoma patients tested; the median TNF-α level was undetectable in both lymphoma patients with and without B symptoms and in normal volunteers. Eleven of 25 patients with B symptoms versus 4 of 16 without B symptoms had detectable (≥10 pg/ml) TNF-α levels (P > 0.1, χ² test). All of 16 normal volunteers and 35 of 38 lymphoma patients had IL-1ß levels below the lower limit of sensitivity of our assay (20 pg/ml). Three lymphoma patients had higher IL-1ß levels (30, 56, and 66 pg/ml). Two of these patients had Hodgkin’s disease, whereas the third had a diffuse mixed lymphoma. Only the individual with the highest IL-1ß level (66 pg/ml) had B symptoms.

Circulating Levels of IL-6. Sera from a total of 76 people were tested for IL-6 levels (Fig. 2). Twenty-six had Hodgkin’s disease, and 31 had non-Hodgkin’s (B-lineage) lymphoma (intermediate grade, diffuse large-cell, diffuse mixed, or follicular large-cell, 28 patients; low-grade, follicular mixed or small cleaved cell, 3 patients) (Table 2). Nineteen of the samples came from normal volunteers. Twenty of 57 lymphoma patients (35%) had detectable (≥22 pg/ml) levels of IL-6. IL-6 was discerned in 17 of 29 patients (59%) with one or more B symptoms but in only 3 of 28 patients (11%) without B symptoms and 0 of 19 normal volunteers (0%) (P < 0.001, χ² test) (Table 1. Fig. 2). Of the 26 patients with Hodgkin’s disease, 15 had B symptoms and 11 did not. Nine of the 15 patients with B symptoms (60%) and one of the 11 without B symptoms (9%) had detectable IL-6 levels (P < 0.01, χ² test). In the group with non-Hodgkin’s lymphoma, 8 of 14 patients with B symptoms (57%) and 2 of 17 patients without B symptoms (12%) had detectable IL-6 levels (P < 0.01, χ² test). The median IL-6 level for patients with B symptoms was 28.9 pg/ml (range, 0 to 173 pg/ml) whereas the median level for those without B symptoms and for normal volunteers was below the limits of assay sensitivity (Table 3). Statistical analysis (using the Mann-Whitney U test) revealed significantly higher IL-6 values in lymphoma patients as compared with normal volunteers (P < 0.025) and in lymphoma patients with B symptoms as compared with those without B symptoms (P < 0.005) and in lymphoma patients with B symptoms as compared with normal volunteers (P < 0.005). There was no statistically significant difference between IL-6 levels in normal volunteers as compared with lymphoma patients without B symptoms (P = 0.9, Mann-Whitney U test). Similarly, analyzing Hodgkin’s and non-Hodgkin’s lymphoma groups separately revealed a median IL-6 level of 37 pg/ml (B symptoms present) versus undetectable (no B symptoms) in Hodgkin’s disease and of 25 pg/ml (B symptoms present) versus undetectable (no B symptoms) in non-Hodgkin’s lymphoma. The Mann-Whitney U test again revealed that these differences were significant at P values <0.005 and <0.03 for the Hodgkin’s and non-Hodgkin’s disease patients, respectively.

Since only eight patients had Stage I or II disease, and since all but four Hodgkin’s lymphoma patients had advanced (Stage III or IV) or relapsed disease, a comparison of early versus advanced disease was not feasible in either the entire cohort or the Hodgkin’s subgroup. However, there was no correlation between IL-6 levels and time from diagnosis (rₛ = 0.08, P = 0.6). There was also no correlation between IL-6 and lactate dehydrogenase levels (rₛ = 0.026, P = 0.19) or IL-6 and β2-microglobulin levels (rₛ = 0.108, P = 0.5) (Fig. 3).

The median survival of the Hodgkin’s disease patients with IL-6 levels ≥22 pg/ml was 10 mo; the median survival of those with lower levels has not been reached at a median follow-up time of 37.5 mo. (Survival was calculated from the date that the serum sample for IL-6 was obtained.) Analysis by the method of Kaplan and Meier (Fig. 4) revealed these differences to be statistically significant (Wilcoxon P...
IL-6 IN LYMPHOMA

Fig. 1. Serum levels of TNF-α, IL-1β, and IFN-γ in patients with lymphoma (with or without B symptoms) and in normal volunteers. The lower limits of reproducible detection for the assays are 10 pg/ml (TNF-α), 20 pg/ml (IL-1β), and 0.1 unit/ml (IFN-γ) shaded area. No significant differences between groups were seen.

Fig. 2. Serum levels of IL-6 in patients with lymphoma (with or without B symptoms) and in normal volunteers. The lower limit of reproducible detection for the assay is 22 pg/ml shaded area. The horizontal lines represent median IL-6 values for each group. Differences are significant with a P value <0.001 (Kruskal-Wallis test).

Table 2 Serum IL-6 levels in patients with Hodgkin's disease, non-Hodgkin's lymphoma, and in normal volunteers

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients assayed</th>
<th>No. of patients with IL-6 levels</th>
<th>χ² significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin's disease</td>
<td>26</td>
<td>10 (38)</td>
<td></td>
</tr>
<tr>
<td>Non-Hodgkin's lymphoma</td>
<td>31</td>
<td>10 (32)</td>
<td></td>
</tr>
<tr>
<td>Low grade (follicular mixed or small cleaved cell)</td>
<td>3</td>
<td>0 (0)</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Intermediate grade (diffuse large cell, diffuse mixed, or follicular large cell)</td>
<td>28</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Normal volunteers</td>
<td>19</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses, percentage.

value = 0.0012). In non-Hodgkin's lymphoma, the small number of patients precluded assessing statistical correlations for survival in each disease subset. However, if taken as a single group, the association between IL-6 levels and survival did not attain statistical significance.

DISCUSSION

We chose to measure TNF-α, IL-1β, IFN-γ, and IL-6 levels in lymphoma patients with fever, night sweats, and/or weight loss, because these molecules are pyrogenic (1–4, 12), they mediate inhibition of lipogenic enzymes and/or development of anorexia, both of which may lead to weight loss (13–17), and many of the side effects associated with their administration to human beings and/or animals are highly similar to B symptoms (1–4, 12, 15).

In agreement with the results of Lahdevirta et al. (5) who demonstrated that the normal serum levels of TNF-α are <28 pg/ml (mean + 2 SD = 8.1 + 20 pg/ml), we found that all of 15 normal volunteers had ≤20 pg/ml of TNF-α in their serum; although four patients (three of whom had B symptoms) had higher values, the elevations were modest (highest level = 28 pg/ml) and not statistically significant. Similarly, serum IFN-γ levels were not elevated in our lymphoma patients. As in normal individuals, serum IL-1β levels were less than 20 pg/ml in 35 of 38 lymphoma patients (92%). Three patients had slightly higher levels, but only the one with the highest value (66 pg/ml) had B symptoms. In contrast, serum IL-6 values were significantly higher in lymphoma patients with B symptoms (median level = 28.9 pg/ml) than in those without B symptoms (median level = undetectable) (Table 3). These statistically significant differences persisted when patients with Hodgkin's and non-Hodgkin's lymphoma were analyzed separately.

Previous investigators have examined IL-1β and TNF expression in tumor tissue derived from lymphoma patients with constitutional symptoms. Ree et al. (18) showed that biopsy specimens from Hodgkin's disease patients may stain positive for IL-1β by immunohistochemical methods but that this reactivity was not associated with the presence of B symptoms. High tumor content of TNF-α and lymphotoxin mRNA in non-Hodgkin's lymphoma and of lymphotoxin mRNA in Hodgkin's lymphoma has been demonstrated by Sappino et al. (19), and correlation with the presence of constitutional symptoms was significant. Even so, we did not find statistically significant elevations of TNF-α protein in serum samples. Since IL-6 plasma levels increase following TNF-α infusion in humans (20), and since
accumulation of IL-6 transcripts occurs in response to TNF-α and lymphotoxin in vitro (21), it is conceivable that localized production of TNF within malignant tissue induces autocrine or paracrine release of IL-6 in amounts sufficient to be discerned in the serum.

IL-6 has been previously termed B-cell stimulatory factor 2 (22), β2-interferon, (23), hybridoma growth factor (24), or hepatocyte stimulating factor (25). This molecule is produced by protean cell types [activated B-cells, T-cells, monocytes, fibroblasts, keratinocytes, endothelial cells, and mesangium cells (reviewed in Ref. 26)]. It is a pleiotropic cytokine which is involved in multiple lymphopoietic processes including the final steps in the maturation of B-cells into antibody-producing cells (27), the growth of plasmacytoma/myeloma cells (28), and the proliferation of Epstein-Barr-infected B-cells (29) and activated T-cells (30).

Several lines of evidence from recent publications have suggested a pathogenic role for IL-6 in some lymphoid disorders. Large quantities of IL-6 are produced by the germinal centers of hyperplastic lymph nodes from patients with Castleman’s disease and have been implicated in the clinical manifestations of this syndrome (giant lymph node hyperplasia with plasma cell infiltration, fever, anemia, hypergamaglobulinemia, and increased acute phase proteins) (31). Cultured supernatants of neoplastic cells from a patient with advanced K1-positive lymphoma associated with fever and bone destruction have been shown to produce high levels of IL-6 (32), and expression of both IL-6 and IL-6 receptors has been demonstrated in Hodgkin’s disease (33, 34). Furthermore, an autocrine role for IL-6 in two lymphoma cell lines has been suggested by Yee et al. (35), and this molecule may act as a growth factor for a Lennert's lymphoma-derived T-cell line (36). Finally, patients with multiple myeloma have previously been shown to have elevated serum IL-6 levels (37), though only those individuals with very advanced disease (plasma cell leukemia) have median levels similar to those found in our lymphoma patients with B symptoms (38). It is of interest that IL-6 is a growth factor for myeloma cells (28, 39, 40). Although initial studies indicated that this molecule may act in an autocrine capacity in this disease (28), subsequent investigations have suggested a paracrine role (41, 42); Nilsson et al. (39) have claimed that, while autocrine IL-6 stimulation of myeloma cells is uncommon, it may develop as a consequence of tumor progression.

Our data suggest a strong correlation between serum IL-6 levels and the presence of B symptoms. Based on the established pyrogenic and metabolic effects of IL-6 (12, 15), this molecule alone could be causing fever, night sweats, and weight loss. Even so, some patients without B symptoms, albeit a minority, have elevated IL-6 levels, and some patients with B symptoms do not have detectable amounts of circulating IL-6. Several explanations are possible. It may be that, in order for B symptoms to manifest, the presence of another cytokine in addition to IL-6 or perhaps induced by IL-6 is necessary. Second, the symptoms experienced by different individuals in response to similar doses of exogenously administered cytokines can vary greatly (1), and it may therefore be reasonable to assume that individual tolerance to circulating endogenous cytokines is also variable. In regard to the small number of patients with B symptoms who had undetectable levels of IL-6, it is possible that another cytokine was responsible for their fever, night sweats, or weight loss. Indeed, in one such patient, IL-1β was elevated. Alternatively, an even more sensitive IL-6 assay might show increased serum IL-6 levels in additional patients.

It has been suggested that circulating IL-6 levels are an important prognostic factor for multiple myeloma (38) and for renal cell carcinoma (43). In our study, the limited number of patients in each subgroup of non-Hodgkin’s lymphoma did not permit a reasonable statistical analysis of the prognostic value of IL-6. In Hodgkin’s disease, however, the median survival of patients with detectable IL-6 levels (>22 pg/ml) was only 10 mo, whereas the median survival of

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Patients</th>
<th>Median IL-6 levels (pg/ml)</th>
<th>Range of IL-6 levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B symptoms</td>
<td>29</td>
<td>28.9</td>
<td>0–173</td>
</tr>
<tr>
<td>No B symptoms</td>
<td>28</td>
<td>Undetectable</td>
<td>0–54</td>
</tr>
<tr>
<td>Normal volunteer</td>
<td>19</td>
<td>Undetectable</td>
<td>0–21</td>
</tr>
</tbody>
</table>

* A significant difference between the three patient subtypes is seen at P < 0.001 as determined by the Kruskal-Wallis test.
those patients with lower levels has not been reached after a median follow-up exceeding 3 yr (p = 0.0012) (Fig. 4). Our observations differ from those of Gause et al. (44), who also found high IL-6 levels in Hodgkin’s disease patients, but did not note a correlation with prognosis. The reasons for this discrepancy are unclear, but could be due to variations in assay methodology. Both groups used the same ELISA kit for IL-6, but Gause et al. (44) felt that the lower limit of assay sensitivity was 10 pg/ml, whereas we found that the assay was not consistent for serum IL-6 levels below 22 pg/ml. An alternative explanation is that Gause et al. (44) assessed previously untreated patients, whereas the vast majority of our patients had disease which had relapsed. We are currently studying larger cohorts of patients with both Hodgkin’s and non-Hodgkin’s lymphoma in order to more accurately delineate the prognostic significance of serum IL-6 levels in these diseases. In addition, considering the critical role that IL-6 plays in the differentiation and/or proliferation of normal and neoplastic B-cells, further investigation of the biological impact of high levels of this cytokine on the pathogenesis or progression of lymphomas is warranted.

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Serum Interleukin 6 Levels Are Elevated in Lymphoma Patients and Correlate with Survival in Advanced Hodgkin's Disease and with B Symptoms

Razelle Kurzrock, John Redman, Fernando Cabanillas, et al.


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