We studied the expression of the two tumor necrosis factor (TNF) receptors, p55 and p75, on B cells from patients with chronic lymphocytic leukemia (CLL), and the presence of soluble TNF receptors in serum. Expression of membrane-associated receptors was quantified by double labeling of peripheral blood mononuclear cells (PBMC) with monoclonal antibodies against CD19 and p55/p75 TNF receptors and flow cytometry. A high fraction of the CD19+ cells expressed the p55 receptor (44% ± 34% [SD]) and p75 receptor (61% ± 31%). In healthy controls, 0% to 1% of the CD19+ cells expressed the p55 receptor and 0% to 10% expressed the p75 receptor. Incubation of CD19+ cells with 10 ng/mL of TNF increased the incorporation of thymidine in 11 patients, and this was decreased to 65% (P < .05) by antibodies to the p55 receptor or the p75 receptor, and to 35% ± 7% (P < .001) when both antibodies were combined. With an enzyme-linked immunoassay, we measured soluble TNF receptors in serum from CLL patients. The mean level of p55 receptors was increased to 12.9 ± 8.9 ng/mL (P < .00001 v normal). The mean level of p75 receptors was increased to 13 ± 24 ng/mL (P = .01 v normal). The membrane expression of the two receptors was positively correlated (r = .97, P < .01); however, there was no correlation between membrane expression and serum concentration of either receptor. Autologous serum containing high levels of soluble TNF receptors inhibited TNF-induced proliferation of CD19+ cells. In conclusion, we have demonstrated that neoplastic cells from patients with CLL have increased expression of p55 and p75 TNF receptors, and that both receptors mediate signal to proliferation. Furthermore, serum from CLL patients has elevated levels of soluble TNF receptors, which may counteract the proliferative effect of TNF.

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were purchased from Boehringer Mannheim (Mannheim, Germany) and coated with 10 pg/mL of 44E (anti-p55) or utr 4 (anti-p75) that binds to the receptor without blocking TNF binding was developed in our laboratory (Lien, Liabakk, and Espevik, manuscript in preparation) and used for detection of p55 receptor in serum. Biotinylated anti-C19 monoclonal antibody was purchased from Becton Dickinson (Mountain View, CA). Biotinylated anti-TNF receptor antibodies and rTNF was performed as previously described.* Bound biotinylated utr 1 was detected with digoxigenin (DIG) and rabbit anti-DIG were purchased from Boehringer Mannheim (Mannheim, Germany). TNF was labeled with DIG according to the instruction of the manufacturer.

Flow cytometric quantification of TNF receptor antibody binding. Binding of TNF receptor antibodies or rTNF to cells was performed by adding 10 μg/mL of biotinylated antibodies or 50 ng/mL of biotinylated rTNF diluted in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA; Sigma Chemical, St Louis, MO) to 10⁶ cells for 45 minutes at 0 to 4°C. The cells were washed twice in PBS/BSA and stained with 20 μL of streptavidin-phycocerythrin for 45 minutes at 0 to 4°C. In two-color flow cytometry, fluorosecin isothiocyanate (FITC)-labeled anti-C19 was added. The cells were subsequently washed once in PBS and fixed in 2% formalin (Merck, Darmstadt, Germany) and stored at 0 to 4°C until they were analyzed using a FACScan flow cytometer (Becton Dickinson). Blocking experiments were performed by pretreating cells with 50 μg/mL of rTNF for 45 minutes at 0 to 4°C before addition of the biotinylated reagent.

Flow cytometric measurements were based on 5,000 cells and displayed as single-cell scattergrams when the cells were double-labeled, or as frequency distribution histograms. For each patient, clustering of cells without specific antibody labeling was evaluated and limited by borders that included 90% of the cells. After double labeling, cells outside these borders were counted as positive for TNF receptor (upper left and right quadrant), CD19 (upper right and lower right quadrant), or both TNF receptor and CD19 (upper right quadrant) (see Fig 2).

Detection of soluble TNF receptors in serum. Immunoplates were coated with 10 μg/mL of 44E (anti-p55) or utr 4 (anti-p75) antibodies diluted in PBS for 12 hours at 4°C. After blocking with 0.5% BSA (RIPA grade; Sigma) for 1 hour at 37°C and washing with PBS containing 0.1% Tween 20 (Sigma), 100 μL of different dilutions of serum was added and incubated for 12 hours at 4°C. After washing, bound p55 TNF receptor was detected by adding DIG-TNF (50 ng/mL) at 37°C for 1 hour. Bound DIG-TNF was detected by adding peroxidase rabbit anti-DIG Ig. Bound p75 TNF receptor was detected by adding biotinylated utr 1 (5 ng/mL) as previously described.* Bound biotinylated utr 1 was detected with streptavidin-biotin-peroxidase complexes using o-phenylenediamine as substrate (Dakopatts, Glostrup, Denmark), and the reaction was stopped with 2N H₂SO₄. The absorbance at 492 nm was measured with a Titertek Multiskan Plus MKII (Flow, Bioggio, Switzerland). Recombinant p55 and p75 (Hoffmann-LaRoche) were used as standards. The detection limit of the assays was 0.3 ng/mL for both receptors.

Cell culture experiments. In proliferation experiments, 100 μL of 10⁶ cells/mL in complete medium was dispensed in wells in flat-bottomed microtiter plates (Costar, Cambridge, MA), and reagents were added in a volume of 100 μL. Cells were incubated for 72 hours, and 1 μCi methyl-[3H]thymidine (Amersham Corp, Amersham, UK) was added to each well 18 hours before harvest of the cells with a Micromate 196 (Packard, Meriden, CT) cell harvester. Radioactivity was measured in samples in a Matrix 96, Direct Beta Counter (Packard).

Purity of cell populations. The B-cell population purified with anti-C19-coated magnetic beads contained less than 0.5% T cells (CD3) and monocytes (CD14), and less than 0.1% natural killer (NK) cells (CD56) as estimated by flow cytometry (data not shown). Similar purity has been reported by others.17 In double-labeling experiments, we used PBMC. To verify the purity of the CD19* cell population in these experiments, we double-labeled PBMC with anti-C19 and anti-CD3/CD14/CD56, which demonstrated two distinctly different populations in all cases. Accordingly, the CD19* cells contained less than 1% T cells and monocytes and less than 0.5% NK cells.

Statistics. Significance of differences were tested with two-sided Student's t-test. Summary statistics are given as the mean ± SD.

Table 1. Clinical Characteristics and Expression of CD19 and the p55 and p75 TNF Receptors in 15 Patients With CLL

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Duration (yrs)</th>
<th>Stage</th>
<th>Therapy</th>
<th>Lymphocyte Count (10⁶/L)</th>
<th>CD19</th>
<th>p55</th>
<th>p75</th>
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Mean ± SD: 58 ± 28 for CD19, 44 ± 34 for p55, and 61 ± 31 for p75

Abbreviations: *Phenotyping is based on double labeling of PBMC with anti-CD19 and anti-p55/p75. Numbers given for CD19 are percentages of PBMC and numbers for p55 and p75 are percentages of CD19* cells.
RESULTS

Expression of the p55 and p75 TNF receptors on CD19+ cells from CLL patients. PBMC from CLL patients were incubated with either biotinylated htr 9 or utr 1 antibodies as described in Materials and Methods and analyzed with flow cytometry. Figure 1A and B shows experiments from one patient and demonstrates that both antibodies bind to the surface of the cells. The fluorescence was higher when cells were incubated with utr 1 antibodies than with htr 9 antibodies, indicating that the expression of p75 receptor was increased compared with that of the p55 receptor. In the control experiments (dotted line), PBMC were incubated with TNF (50 ng/mL), which completely blocked the binding of the TNF receptor antibodies, confirming previous results that TNF and the receptor antibodies htr 9 and utr 1 compete for the same binding site. Figure 1C and D shows similar experiments in a healthy volunteer who expresses small amounts of p75 receptor and no detectable p55 receptor.

To clarify whether the increased expression of TNF receptors could be attributed to the neoplastic cells, we double-labeled PBMC with htr 9 and anti-CD19 (Fig 2A) and utr 1 and anti-CD19 (Fig 2B). The percentage of cells positive for CD19 in this patient was 96%, and of the CD19+ cells, 59% and 67% expressed the p55 and p75 receptors, respectively (patient 6 in Table 1). Figure 2C represents a control experiment in which cells were labeled with streptavidin-phycoerythrin. When cells were incubated with biotinylated TNF instead of receptor antibodies, 66% of the cells bound TNF to the cell surface, confirming the existence of TNF-specific binding sites on the cell surface (Fig 2D). Such experiments were used as internal controls in every patient study. Control experiments with preincubation of cells with unlabeled TNF blocked the binding of the TNF receptor antibodies.

Fig 1. Flow cytometry analysis of PBMC from (A and B) a patient with CLL and (C and D) a healthy volunteer. (A and C) Labeling with anti-p55 antibodies (htr 9) for both individuals; (B and D) labeling with anti-p75 antibodies (utr 1). The antibody-labeled cells were compared with cells treated with TNF (50 ng/mL) to block the binding sites before incubation with the antibodies. Cells labeled with htr 9 or utr 1 antibodies (--), cells treated with TNF (--).

Similar scattergrams and calculations were obtained from 15 patients. Results based on double labeling of PBMC and calculation of the htr 9/utr 1-positive fraction of CD19+ cells are listed in Table 1. When the staining for CD19 was weak, the CD19+ population overlapped with the unlabeled cells, and, in such cases, the percentages given for CD19+ cells are low estimates. In all but one of the patients, binding of utr 1 and htr 9 was demonstrated, indicating expression of the p75 and the p55 receptors on the cell surface. In all cases, expression of utr 1 was higher than htr 9. The mean fractions of B cells that expressed the 55-Kd and 75-Kd receptor were, respectively, 44% ± 34% and 61% ± 31%. In contrast, in double-labeling experiments in 14 healthy controls, CD19+ cells had negligible expression of the p55 receptor (0% to 1% of the cells) and absent or low expression of the p75 receptor (0% to 10%). In some of the patients, we also examined expression of p55 and p75 receptors in B-cell populations purified by anti-CD19-coated beads and obtained results that were consistent with the double-labeling experiments.

Behavioral significance of p55 and p75 receptor. It is well known that TNF has a proliferative effect on lymphocytes from CLL patients. We confirmed this in studies on 11 patients in which the CD19+ cells were purified by anti-CD19-coated monodisperse particles and stimulated with TNF in the concentration range of 0.4 to 50 ng/mL for 3 days. A dose-dependent increase in thymidine incorporation was found in all experiments (data not shown). To test the functional significance of the two types of TNF receptors, we performed experiments in which the cells were
incubated with TNF and antibodies to one or both TNF receptors (Fig 3). All experiments showed the same pattern, i.e., antibodies to either the p55 and/or the p75 receptor decreased the thymidine incorporation to, respectively, 64% ± 16% and 65% ± 20% (P < .05) of control, and the effect was further enhanced when the two antibodies were combined (35% ± 7%, P = .001). As a control for the specificity of this effect, we performed experiments with a p55 antibody (htr 20) that does not block TNF binding to the receptor. This antibody had no effect on TNF-induced proliferation.

The results show that both types of TNF receptors are involved in TNF-mediated proliferation of CD19+ cells from CLL patients.

Soluble TNF receptors in serum. Serum samples from 18 patients were assayed for the presence of both types of receptors (Fig 4A and B). The p55 receptor was detected in 17 of the patients at a mean level of 12.9 ± 8.9 ng/mL (Fig 4A). In contrast, the mean concentration of soluble p55 receptor was 0.64 ± 0.76 ng/mL in 24 healthy subjects (P < .000001). The p75 receptor was detected in 13 of the patients (13.0 ± 24.0 ng/mL) and in none of the controls (P = .01) (Fig 4B).

Relation between p55 and p75 receptors and other parameters. The levels of soluble p55 and p75 receptors were compared with other parameters of the disease, and correlation coefficients for these relations are shown in Table 2. Membrane expression of one type of TNF receptor was markedly positively correlated with expression of the other receptor (r = .97, P < .01). There were intermediate positive correlations (but not significant) between lymphocyte counts and expression of the two receptor types on the membrane (r = .44, r = .41), and between the concentrations of the two receptor types in serum (r = .58). There was no significant correlation between the concentrations of p55 or p75 receptors in serum and lymphocyte counts (r = -.29, r = -.18) or membrane expression of TNF receptors (r = -.28 to -.37). Also, when we compared the numbers of p55- and p75-positive lymphocytes with serum concentrations of p55 and p75, respectively, there was no correlation (not shown). Furthermore, we found no correlation between the stage of the disease and membrane expression or serum concentration of TNF receptors. However, the number of patients at each stage was low.

Effect of autologous serum on proliferation of neoplastic cells. The increased levels of TNF receptors in serum of CLL patients implicate a potential antiproliferative activity of endogenous origin and this was further evaluated by incubation of nonadherent PBMC with TNF and varying concentrations of autologous serum. Figure 5A shows experiments from a patient with high TNF receptor concentrations in serum (p55, 25.5 ng/mL; p75, 5.8 ng/mL) and demonstrates the presence of a marked TNF neutralizing

![Fig 3. Inhibition of proliferative effect of TNF by antibodies against the p55 and p75 receptors. CD19+ cells isolated by anti-CDlhoated monodisperse magnetic beads were grown in the presence of 10 ng/mL of rTNF and 10 µg/mL of htr 5 or utr 1 antibodies or both. Results are calculated as percent thymidine incorporation in relation to the wells with TNF without antibodies. Data are presented as the mean ± SD of four separate experiments (4 patients). The range of cpm without TNF was 54 to 2,834, and with TNF, 94 to 9,607.](www.bloodjournal.org)

![Fig 4. Soluble (A) p55 and (B) p75 receptors in serum from patients with CLL and healthy volunteers. Serum was frozen at -70°C until examination with an enzyme-linked immunoassay as described in Materials and Methods. Detection limits were 0.3 ng/mL and are marked with horizontal lines. Fourteen controls had p55 concentrations above the detection limit.](www.bloodjournal.org)

![Table 2. Correlation Coefficients of Comparisons Between Various Parameters](www.bloodjournal.org)

<table>
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<th>Parameters</th>
<th>Membrane</th>
<th>Soluble</th>
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<tr>
<td>p55</td>
<td>p55</td>
<td>p75</td>
</tr>
<tr>
<td>Membrane p75</td>
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<tr>
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<td>-.28</td>
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<tr>
<td>Lymphocyte count</td>
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<td>.41</td>
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</tbody>
</table>

*P < .01.
activity, which is still present at a dilution of 1/256. In contrast, a patient with low TNF receptor concentrations (p55, 6.2 ng/mL; p75, 0 ng/mL) had no TNF neutralizing effect in serum (Fig 5B).

The results show that the measured TNF receptor levels reflect biologically active TNF receptors that effectively block the proliferative activity of TNF.

**DISCUSSION**

This study shows that (1) CD19+ cells from CLL patients have increased expression of p55 and p75 TNF receptors on the surface; (2) signal to proliferation is mediated by both types of receptors; and (3) serum from CLL patients has increased concentrations of soluble p55 and p75 receptors that may block TNF-induced proliferation.

Previous studies have shown that neoplastic cells from CLL patients express receptors for TNF on their surface. The present study demonstrates that the increased expression of TNF receptors includes both the p55 and the p75 receptor. Unstimulated normal B cells express small amounts of the p75 receptor and negligible amounts of the p55 receptor. However, upon activation, the expression of the p75 receptor markedly increases, whereas the expression of the p55 receptor remains undetectable. In contrast, neoplastic B cells have a different pattern of expression of TNF receptors, as both receptor types are increased and the expression of p75 is higher than that of p55. The patients included in this study did not have fever or other clinical signs of bacterial or viral infection as a cause of activation of the B cells. The increase of both types of receptors thus appears to be characteristic of CLL.

Digel et al found that expression of surface TNF receptors was an in vitro observation that occurred after cultivation of the CLL cells. In contrast, our results were obtained on freshly isolated mononuclear cells, indicating that the receptors are also present in the in vivo situation. The explanation may be that different methods for detection of receptors have been used in the studies. Also, Heilig et al found expression of both the p55 and p75 receptors on freshly isolated neoplastic cells from three of six patients with CLL.

TNF has a proliferative effect on CLL cells, and the present demonstration of an increase of both types of TNF receptors naturally raises the question of the functional significance of the two receptor types. Our results clearly show that both receptors can independently mediate signal to proliferation. The involvement of both receptors in signal transduction is in accordance with results from cell lines and human endothelial cells and from rheumatoid synovial fibroblasts (prostaglandin production). However, it has also been reported that the two receptors may have different roles in the mediation of TNF effects on glycolysis, proliferation of thymocytes and cytotoxic T cells, and induction of manganese superoxide dismutase.

It has been shown that CLL cells may produce TNF, that the cells have increased expression of TNF receptors, and that TNF has proliferative effect on CLL cells. These observations have stimulated the idea that TNF is an autocrine growth factor for CLL cells and is potentially important in the pathogenesis and expansion of this disease. However, the present study demonstrates that two contradictory principles are present with respect to the effects of TNF. On one hand, the cells express increased numbers of TNF receptors that facilitate TNF-induced proliferation. On the other hand, the presence of soluble TNF receptors in serum implicates an endogenous TNF neutralizing activity that counteracts proliferation. The TNF neutralizing capacity in serum can be calculated to be in a range of approximately 2 ng/mL of rTNF in some patients. This exceeds most serum concentrations of TNF induced by gram-negative bacteremia, which probably generates the highest TNF serum levels encountered in...
humans. Soluble TNF receptors may thus create a very potent TNF neutralizing system in CLL patients.

What is the source of soluble TNF receptors in CLL patients? It is a reasonable, but still speculative idea that soluble TNF receptors are generated by shedding from neoplastic cells with increased expression of receptors. There was no correlation between serum level and membrane expression of TNF receptors (lymphocyte counts or percentage of cells with membrane-associated TNF receptors); however, such a comparison does not help to evaluate the hypothesis. Others have shown that activated granulo-
cytes, CSa, fMet-Leu-Phe,28 and elastase contained in azurophil granules27 can degrade the extracellular domain of the receptors. However, we do not know whether these mechanisms are important in CLL. Altogether, the evi-
dence of the source of the soluble TNF receptors is scarce.

Elevation of serum concentrations of soluble TNF recep-
tors is not unique to CLL patients. Aderka et al have re-
ported that patients with cancer in the gastrointestinal tract, breast, lung, and other locations have increased serum concentrations of TNF receptors.28 They also demon-
strated that patients with advanced stages of the disease had a tendency toward higher receptor concentrations. In CLL patients, they have found a similar association (Ader-
ka, personal communication, May 6, 1992). We were not able to confirm these observations in our material; however, we studied a relatively small number of patients.

In conclusion, the proliferative effect of TNF on CLL cells is mediated via two distinct membrane-associated receptors. However, the patients also have elevated serum levels of soluble receptors that neutralize the biological activity of TNF. In CLL it thus appears to be a proliferative/ antiproliferative balance that is governed by the presence of membrane-associated and soluble TNF receptors.

ACKNOWLEDGMENT

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p55 and p75 tumor necrosis factor receptors in patients with chronic lymphocytic leukemia

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