Interleukin-4 Inhibits Both Paracrine and Autocrine Tumor Necrosis Factor-α-Induced Proliferation of B Chronic Lymphocytic Leukemia Cells

By Cees van Kooten, Irma Rensink, Lucien Aarden, and Rien van Oers

The proliferative response of purified malignant B cells from 26 patients with chronic lymphocytic leukemia (CLL) was investigated in vitro. In the majority of these patients, a proliferative response could be induced by the combination of tumor necrosis factor (TNF)-α and PMA. The concentration of PMA was found to be critical and showed a sharp optimum. In most cases maximal proliferation was obtained with as little as 0.1 ng/mL PMA. In all cases tested, TNF-α-induced proliferation could be inhibited completely by the addition of low doses of interleukin-4 (IL-4). Maximal inhibition was already found with 400 pg/mL IL-4. Inhibition by IL-4 was not caused by a downmodulation of TNF receptors. Apart from TNF-α, IL-2 was also in synergy with PMA able to induce proliferation in B-CLL cells of some patients. This IL-2-induced proliferation could be inhibited both by IL-4 and by neutralizing anti–TNF-α antibodies. This shows that TNF-α also can act as an autocrine growth factor. These data indicate that TNF-α is an important growth factor for neoplastic B-CLL cells and that IL-4 provides a tool to interfere with this TNF-α response.

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A ROLE FOR cytokines as paracrine and/or autocrine growth factors has been demonstrated in some B-cell malignancies. Unraveling the precise network of regulating cytokines opens the way to therapeutic intervention, eg, with neutralizing monoclonal antibodies (MoAbs) or with antagonistic cytokines. A recent example is the role of interleukin-6 (IL-6) as an autocrine or paracrine growth factor for neoplastic cells in multiple myeloma, where the first trials with MoAbs against IL-6, showing transient cytostatic effects, have already been performed.

B-chronic lymphocytic leukemia (B-CLL), a malignancy of relatively immature B cells, is the most common adult leukemia observed in Western societies. Patients are characterized by high numbers of circulating monoclonal B cells, with a restricted use of κ or λ light chains. Many cytokines, including tumor necrosis factor (TNF)-α, IL-2, interferon (IFN)-α, IL-4, and IL-7, have been suggested to be involved in the induction or regulation of B-CLL proliferation. However, both a paracrine and an autocrine role in malignant B-cell proliferation has been suggested for TNF-α only. TNF-α is a pleiotropic cytokine with multiple biologic activities, both in inflammation and in regulation of the immune response.

Materials and Methods

Patients. We studied 26 previously untreated patients with CLL. The diagnosis was based on clinical and clinical immunologic features. These 26 patients had a mean age of 66 years (range 42 to 87), and a male/female ratio of 19:7. Mononuclear cells (MNC) were isolated from heparinized peripheral blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. MNC were used for routine immunophenotyping and stored in liquid nitrogen until further use. For control experiments, lymphocytes from healthy donors were isolated by counterflow elutriation centrifugation and also stored in liquid nitrogen.

Indirect immunofluorescence. The phenotype of the cell populations studied was determined by fluorescence-activated cell sorter (FACS) analysis using the following antibodies in an indirect immunofluorescence technique: CD3 (CLB-CD3), CD14 (CLB-CD14), CD5 (CLB-CD5), CD19 (CLB-CD19), and CD23 (Blast-2). The membrane expression of IgG was determined by direct fluorescence using the following polyclonal rabbit antibodies: anti-κ and anti-λ light chain (DAKO), and anti-IgM (KH15), anti-IgG (KH16), anti-IgD (KH20) (all from the CLB, Amsterdam, The Netherlands). In most cases the Ig staining was also performed on cytospin preparations.

Separation of cells. Before culturing the malignant B cells, contaminating non-B cells were depleted by magnetic beads (Dynal-beads M450; Dynal, Oslo, Norway) using an appropriate cocktail of MoAbs. Cells were depleted of natural killer (NK) cells via CD16 (CLB-FcR gran), of monocytes via CD14 (CLB-CD14) and of T cells via CD2 (CLB-T11/1). Resulting populations contained no detectable T cells, NK cells, or monocytes as determined by FACS analysis. The absence of T cells was also checked by the activation with immobilized anti-CD3 antibodies, as described.

Cells used always showed a viability of greater than 90%, as determined by staining with fluorescein diacetate (FDA; Sigma Chemical Co, St Louis, MO).

Normal B and T lymphocytes were isolated from peripheral blood as described before. Resulting populations contained greater than 90% B cells and greater than 95% T cells, respectively.

Culture conditions and proliferation assay. All cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal calf serum (FCS) and 2 mmol/L L-glutamine (GIBCO, Grand Island, NY).Cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 0.2 mmol/L L-glutamine, and 1% sodium pyruvate (GIBCO). B cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 0.2 mmol/L L-glutamine, and 1% sodium pyruvate (GIBCO). B cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 0.2 mmol/L L-glutamine, and 1% sodium pyruvate (GIBCO).

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were cultured at a concentration of $4 \times 10^5/200 \mu L$ in 96-well round-bottom plates (Greiner, Nürtingen, Germany). Purified cells were cultured at a concentration of $4 \times 10^5/200 \mu L$ in 96-well round-bottom plates (Greiner, Nürtingen, Germany). The following recombinant human cytokines were used: IL-1α at 5 ng/mL and IL-2 at 50 U/mL (both from Hoffman-La Roche, Nutley, NJ); IL-6 at 5 ng/mL (our institute); IFN-α (Boehringer, Ingelheim, Germany) at 2 ng/mL; IFN-γ (Genentech, San Francisco, CA) at 20 ng/mL; and TNF-α (a gift of A. Creasy, Cetus Corp, Oakland, CA) at 10 ng/mL unless indicated otherwise.

Cytokines were combined with varying concentrations of phorbol 12-myristate 13-acetate (PMA; Sigma). Antibodies were used at a concentration of 10 μg/mL: protein A purified sheep anti-TNF-α antisera, purified MoAb against TNF-α (10E11; a gift of A. Creasy), purified MoAb against IL-4 (CLB-IL4/6; T. van der Pouw Kraan: manuscript in preparation), purified MoAb against IL-6 (CLB-IL6/8), 29 anti-TNF-RA (Utr-1) and TNF-RB (Htr-9) antibodies (a gift of M. Brockhaus, Hoffman-La Roche, Basel, Switzerland).

Proliferation was determined on day 4 of culture: cells were labeled with 7.4 kBq [3H]-thymidine (74 GBq/mmol); 18 hours later cells were harvested and thymidine incorporation was measured.

Analysis of proliferating cells. For analysis of the proliferating cell population, cells were activated with a combination of PMA and TNF-α as indicated. At day 5 of culture cells were collected and stained via indirect immunofluorescence as described above. After staining cells were fixed and permeabilized with a cold mixture of 70% ethanol and 30% phosphate-buffered saline (PBS) for 30 minutes at 4°C. After three washes with cold PBS (PBS, 0.5% bovine serum albumin [BSA], and 0.1% azide), cells were incubated with RNase (50 μg/mL) for 30 minutes (4°C). Then cells were washed again with PBA and kept at 4°C. Propidium iodide was added at a final concentration of 5 μg/mL immediately before the two-color FACS analysis.

RESULTS

Proliferative response of CLL cells to different cytokines. Immunophenotyping of the unseparated MNC of the 26 patients with CLL showed that all suspensions contained more than 75% B cells (CD19+) with the exception of patients 2 (47%) and 18 (64%). Determination of the membrane-Ig phenotype showed that B-cell populations were monoclonal, IgM-positive, κ- or λ-restricted (17 κ-positive and 9 λ-positive), IgG-negative, and that 15 of 26 patients had greater than 10% IgD-positive cells. MNC of all 26 patients showed high expression of CD5 and CD23 as classical markers of B-CLL. To avoid possible effects of contaminating non-B cells, experiments were performed with purified B-cell populations, depleted of residual T cells (CD22+), monocytes (CD14+), or NK cells (CD16+). Furthermore experiments were performed at relatively low cell concentrations ($4 \times 10^5$ incubation). Under these conditions none of the patients showed a significant response when stimulated with either TNF-α, IL-2, IL-4, IFN-α, IL-1, IL-6, or IFN-γ alone, or by combinations of these cytokines (data not shown). Therefore, we performed a costimulation with low concentrations of the protein kinase C (PKC) activator PMA (0.2 ng/mL). The proliferative response to different cytokines after costimulation with PMA is shown in Table 1. Costimulation with low-dose PMA shows that TNF-α is an important growth factor for B-CLL cells. When TNF responsiveness was defined by a stimulation index higher than 5 (ratio of the proliferative response in the presence and absence of TNF-α) and a proliferative response higher than 1,500 cpm, B-CLL cells of 10 of 26 patients responded to TNF-α. Judged by the same criteria, 6 of 25 responded to IL-2, 3 of 26 responded to IL-4, and 1 of 26 responded to IFN-α. No significant proliferation was induced by combinations of PMA and IL-1, IL-6, or IFN-γ (data not shown). As a control for T-cell contamination, purified B-CLL cells were activated with immobilized anti-CD3 antibodies. Although no T cells were detected by FACS analysis, B-CLL cells of patients 2 and 6 showed proliferation after activation with anti-CD3 (8,400 and 2,700 cpm, respectively). Therefore, in these two cases proliferation induced by IL-2 might be caused by contaminating T cells.

Costimulation with PMA shows a very sharp optimum. In initial experiments where we used both 0.2 and 2 ng/mL of PMA, it was found that the TNF-α response in some cases proliferation induced by IL-2 might be caused by contaminating T cells.

![Table 1. Effect of Cytokines on Proliferation of B-CLL Cells](#)

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B cells ($4 \times 10^6$ cells/200 μL) from 26 patients with CLL were incubated with PMA (0.2 ng/mL) in combination with several cytokines: TNF-α (10 ng/mL), IL-2 (50 U/mL), IL-4 (50 ng/mL), and IFN-α (2 ng/mL). After 4 days of culturing the proliferation was determined by the incorporation of [3H]-thymidine. Data presented are the median value of triplicate cultures in cpm.

Abbreviation: NT, not tested.
dependent responses are shown in Fig 1. Most TNF-α responders showed an optimum of proliferation after cosimulation with ± 0.1 ng/mL PMA. Surprisingly, some of the “non-responders” (see Table 1) could be activated by TNF-α at distinct PMA concentrations. This is best illustrated by Fig 1B (patient 13), which shows a strong proliferation at 0.04 ng/mL PMA but no proliferation at 0.3 ng/mL. We tested seven randomly selected TNF-α nonresponders and found that three remained negative (patients 9, 16, and 19), whereas three showed a response at low PMA concentrations (patients 4, 6, and 13) and one responded at 1 ng/mL PMA (patient 26). These data indicate that, depending on the PMA concentration, the vast majority of CLL cells can be activated by exogenously added TNF-α.

Titration of TNF-α showed that high concentrations were necessary to induce a maximal response. Optimal proliferation was found with at least 10 ng/mL TNF-α (data not shown). Concentrations of TNF-α up to 50 ng/mL showed no inhibition of the proliferative response. TNF-α-induced proliferation could be inhibited completely by purified anti-TNF-α antibodies, but not by purified anti-IL-4 or anti-IL-6 antibodies as a control (Fig 2).

Proliferation is not caused by contaminating cells. Although B-CLL patients have almost exclusively monoclonal B cells in the peripheral blood, we removed residual non-B cells by depletion with magnetic beads. To investigate the possibility that proliferation was caused by contaminating nonmalignant B cells or T cells, we performed two kinds of experiments. Firstly, we applied the same activation protocol for normal B and T cells from three healthy donors. As shown in Fig 3, TNF-α showed no significant effect on PMA-induced proliferation of normal B or T cells under these conditions. In contrast to what was found with B-CLL cells, the addition of PMA alone already induced strong proliferation of normal B lymphocytes. This PMA-induced proliferation of normal B lymphocytes could not be inhibited by the addition of neutralizing anti-TNF-α antibodies, but was enhanced by the addition of IL-2 or IL-4 (data not shown).

Secondly, we determined the immunophenotype of the proliferating cell fraction. After activation with low-dose PMA and TNF-α, cells were harvested at day 5 of culturing. After staining of membrane antigens via indirect immunofluorescence, cells were fixed and permeabilized, treated with RNase, and the DNA was stained with propidium iodide. Analysis of the cell fraction with more than 2N DNA (proliferating cells) is shown in Fig 4. Proliferating cells expressed no T-cell markers (CD3), were all CD19-positive, and, most importantly, expressed the phenotype of the B-CLL cells used.

Proliferation is inhibited by IL-4. We tested the effect of IL-4 on the TNF-α-induced proliferation of B-CLL cells. Figure 5 shows a representative example, demonstrating that TNF-α-induced proliferation is completely inhibited by IL-4. This complete inhibition by IL-4 of the TNF-α-induced proliferation of neoplastic B cells was found in all
nine patients tested and was independent of the magnitude of proliferation (Fig 6).

In dose-response experiments, inhibition was found at low concentrations of IL-4; even 400 pg/mL of IL-4 resulted in a complete inhibition of the TNF-α-induced proliferation (data not shown). The inhibition by IL-4 could be reversed completely by the addition of a neutralizing MoAb against human IL-4. The addition of IL-4 gave no shift in the kinetics of proliferation, because the inhibiting effect of IL-4 was also found when the [3H]-thymidine incorporation was determined at days 2, 6, or 8 (data not shown).

**IL-2 can induce proliferation via an autocrine TNF-α pathway.** Apart from TNF-α, other cytokines also have been implicated as growth factors for B-CLL cells. Several groups reported IL-2 as a growth factor for B-CLL. We investigated whether TNF-α might be involved in the IL-2-driven proliferation of B-CLL cells. Purified B-CLL cells of four patients showed a proliferative response to IL-2 (without additional T-cell contamination). In all of these cases, IL-2-induced proliferation could be inhibited by IL-4 (Fig 7). Addition of both polyclonal or monoclonal neutralizing anti–TNF-α antibodies showed a 50% inhibition of IL-2-induced proliferation. No inhibition was found with control antibodies. In addition, activation of purified B-CLL cells by PMA plus IL-2 resulted in production of TNF-α in the supernatant, as measured by enzyme-linked immunosorbent assay (ELISA) (data not shown). These data indicate that, at least in some donors, IL-2 can activate an autocrine–, TNF-α-driven proliferation, and also that under these conditions IL-4 is able to inhibit the proliferation.

**PMA but not IL-4 modulates TNF-R expression.** We investigated the expression of both TNF-α receptors,
TNF-R (p75) and TNF-R (p55), on B-CLL cells to see whether IL-4 inhibits TNF-α-induced proliferation via a direct downmodulation of the TNF-R. In agreement with previous reports, freshly isolated B-CLL cells were found to lack receptors for TNF-α (data not shown). After 3 days of culturing without stimulus, B-CLL cells only expressed TNF-RA, as determined by indirect fluorescence with MoAbs Utr-1 (TNF-RA) and Htr-9 (TNF-RB) (a kind gift of Manfred Brockhaus) (Fig 8A). Culturing in the presence of low-dose PMA (0.1 ng/mL) did not change the expression of TNF-RA. However, high-dose PMA (1 ng/mL) showed a marked downregulation of TNF-RA expression on B-CLL cells. Under none of these conditions was TNF-R expression downmodulated by the addition of IL-4 (Fig 8B).

DISCUSSION

In this study we have demonstrated that, in the presence of low doses of PMA, recombinant TNF-α can induce a proliferative response in purified neoplastic B-cell populations from the majority of B-CLL patients. Moreover, we have provided evidence that the IL-2 responsiveness, present in a minority of the patients tested, is mediated via an autocrine TNF-α pathway.

Titration of PMA showed a very sharp concentration optimum (Fig 1). Optimal proliferation was observed at low concentrations of PMA (0.1 ng/mL). This concentration is at least 10 times lower than the concentrations generally used for the activation of malignant B cells. The lower proliferation at higher PMA concentrations is probably partially caused by the downmodulation of TNF receptors (Fig 8B). From our experiments it is clear that the PMA concentration resulting in optimal growth stimulation by TNF-α is different for individual patients (Fig 1). Because in all experiments we have used a broad range of PMA concentrations as a costimulus, we found higher response rates than reported by investigators using only one stimulus. Differences in PMA optimum might reflect differences in in vivo activation stage. We did not find a correlation between TNF-α responsiveness and disease stage of these patients. Because the results were largely reproducible when individual patients were tested on separate occasions (using different batches of reagents), it is unlikely that the different activation stages were induced in vitro.

The relatively high concentrations of TNF-α (10 ng/mL) required to induce a maximal proliferative response are in agreement with data of previous reports. TNF-α-induced proliferations could be inhibited completely by neutralizing anti-TNF-α antibodies. Studies with normal B cells showed that TNF-α can induce IL-6 production via an autocrine loop. We observed that in B-CLL cells IL-6 could not...
replace TNF-α and that the TNF-α–induced proliferation was not inhibited by anti–IL-6 antibodies.

By analysis of the proliferating CLL cell fraction, as well as by analysis of the TNF-α and PMA responsiveness of purified normal B and T lymphocytes, we could exclude the possibility that TNF-α–induced proliferation was caused by contaminating non-CLL cells. The proliferative responses of the malignant B-CLL cells differed from those of normal peripheral B cells in three respects: (1) CLL cells did not proliferate in response to PMA alone; (2) TNF-α only stimulated proliferation of B-CLL cells; and (3) only the proliferation of CLL cells was inhibited by IL-4. The absence of a stimulatory effect of TNF-α on the proliferation of normal B cells is in accordance with Digel et al.7 but in contrast with the report of Kehrl et al.19 A possible explanation for this controversy might be the source of the normal B lymphocytes: peripheral blood versus tonsil.

A special feature of the B-CLL cells is the expression of the CD5 antigen. CD5 is a pan-T cell marker that is expressed on a minor subpopulation of normal B lymphocytes. CD5-positive B cells are thought to be involved in the production of natural autoantibodies and probably represent a separate differentiation lineage of B cells.34 It could be argued that the responses of the CD5+ B-CLL cells are a reflection of the responsiveness of normal CD5+ B cells. Therefore, we investigated the TNF-α responsiveness of normal CD5+ and CD5− subpopulations, purified from peripheral blood by FACS. However, a stimulatory effect of TNF-α could not be obtained in any of these populations (data not shown). Therefore, TNF-α responsiveness seems not to be a feature of normal CD5-positive peripheral B cells, but a characteristic of B-CLL cells.

A second important observation in this study was that in all donors tested, TNF-α–induced proliferation could be inhibited by the addition of low doses of recombinant IL-4. Complete inhibition was found with as little as 400 pg/mL of IL-4, which is around 10 times less than the concentration required to give a maximal induction of IgE production.35 IL-4–induced inhibition was overcome by the addition of a neutralizing MoAb against IL-4. We investigated a possible mechanism of this IL-4 inhibition. Signaling of TNF-α is mediated via two different receptors: TNF-RA (p75) and TNF-RB (p55). Both binding studies with19 TFN-α and immunofluorescence studies with MoAbs indicated that there were virtually no TNF-α receptors on freshly isolated B-CLL cells.30,31 Culturing of B-CLL cells induced TNF-α binding and staining with MoAbs. We investigated whether there was a direct effect of IL-4 on the expression of TNF receptors. However, under conditions where a complete inhibition of the TNF-α–induced proliferation was found, there was no inhibitory effect of IL-4 on the expression of TNF receptors. These findings are compatible with binding studies showing no effect of IL-4 on TNF-α binding to B-CLL cells.30

In some individual cases (ie, patient 16) a positive effect of IL-4 on proliferation was observed. It has to be investigated whether this is another mechanism of B-CLL activation, or whether this is a reflection of the presence of normal B lymphocytes, which can be stimulated by IL-4.

Most interesting was the fact that IL-4 could also interfere with the IL-2–stimulated autocrine loop of TNF-α–driven proliferation. When purified B-CLL cells were stimulated with IL-2, the proliferative response could be inhibited by IL-4, as reported before,24 but also by neutralizing antibodies against TNF-α (Fig 7). This indicates that TNF-α can also act as an autocrine growth factor for B-CLL cells, as suggested by others.5,15 These reports even show that TNF-α itself can enhance the induction of TNF-α mRNA. In agreement with this possibility, in initial experiments we have found that both IL-2– and TNF-α–induced proliferation was inhibited by pentoxifylline, a drug that interferes with TNF-α production.36,37 Therefore, one possible mechanism of inhibition by IL-4 might be at the level of TNF-α production, but a definite proof of this requires mRNA studies. A similar mechanism has been shown for inhibition by IFN-α.15 Another possible mechanism of inhibition by IL-4 is a direct interference of IL-4–induced second messengers (ie, cyclic adenosine monophosphate...
[cAMP] with the TNF-α signaling pathway. This would be in concert with the recent experiments of Vazquez et al.\textsuperscript{23} showing the effect of CAMP on IL-2–induced activation of normal B lymphocytes.

In conclusion, our data demonstrate that TNF-α plays an important role in the growth of B lymphocytes. Several studies suggest that TNF-α also plays an important role in the growth of B cells in chronic lymphocytic leukemia.\textsuperscript{13,15,38,40} It might be worthwhile to include a group of CLL patients in clinical trials with IL-4 and to take the TNF-α responsiveness into consideration during the evaluation of this treatment.

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Interleukin-4 inhibits both paracrine and autocrine tumor necrosis factor-alpha-induced proliferation of B chronic lymphocytic leukemia cells

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