Expression and Regulation of Tumor Necrosis Factor, Interleukin-2, and Hematopoietic Growth Factor Receptors in B-Cell Chronic Lymphocytic Leukemia

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Leukemic cells from patients with B-cell chronic lymphocytic leukemia (B-CLL) express tumor necrosis factor (TNF) and interleukin-2 (IL-2) receptors, but only a low proliferative response can be elicited in vitro by TNFα and IL-2. To investigate the functional properties of IL-2 and TNFα on leukemic B cells, we evaluated (1) the regulation of expression of TNF receptors (TNF-R) and IL-2 receptors on leukemic B cells after culture with TNFα and IL-2; (2) the effect of the combination of TNFα and IL-2 in a proliferative in vitro assay; and (3) the expression and regulation by these cytokines of receptors for hematopoietic factors, including IL-3, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Flow cytometry analysis showed that freshly isolated leukemic cells from B-CLL patients bear the 75-kD TNF-R and the 55-kD IL-2R; TNFα was able to upregulate the 55-kD IL-2R but not the 75-kD TNF-R. On the other hand, IL-2 was not able to modify the expression of the above-mentioned receptors. Although each cytokine alone was unable to induce a relevant proliferative response of leukemic cells, a synergistic proliferative effect was detected when these cytokines were used in combination. Leukemic B cells from B-CLL patients bear receptors for hematopoietic factors (IL-3, G-CSF, and GM-CSF) that were upregulated in vitro by IL-2 via the 55-kD IL-2R. On the contrary, TNFα was unable to affect the expression of the above-mentioned receptors. These results indicate (1) that IL-2 and TNF receptors are related to each other on leukemic cells in B-CLL and (2) that the IL-2R is involved in the regulation of other structures, i.e., CSF receptors, thus pointing to another functional role of this receptor complex and the related cytokine in leukemic cells.

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Leukemic cells from patients with B-cell chronic lymphocytic leukemia (B-CLL) express cytokine receptors, including interleukin-2 and tumor necrosis factor receptors (IL-2R and TNF-R). In particular, the 75-kD TNF-R and the low-affinity IL-2R (55-kD IL-2R) have been found on freshly isolated and cultured leukemic B cells. Recently, IL-2 and TNF-R have been observed to be related. In particular, IL-2 upregulates the expression of TNF-R on in vitro cultured peripheral blood T cells, and TNFα upregulates the expression of IL-2R, thus indicating that the regulation of these receptors (both IL-2R and TNF-R) and the effects of relevant cytokines are likely to be closely related on T cells. Furthermore, besides the well-known proliferative effect displayed in response to IL-2 by different cell types, this molecule is able to regulate the production of different cytokines. In particular, the separate triggering of the 55-kD IL-2R (CD25) or the 75-kD IL-2R (CD122) results in the production of interferon-γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) in T and natural killer (NK) cells, thus pointing to the role of IL-2R in the transmission of other functional properties that are unrelated to the proliferation effect. Although TNFα and IL-2 are able to induce a slight proliferative effect on leukemic cells from B-CLL patients, the relationships between IL-2R and TNF-R and between these two receptorial structures and other cytokine receptors in response to their specific cytokines have not been investigated in this disorder.

Other cytokines, including colony-stimulating factors (CSFs) and, in particular, IL-3 have been reported to act as growth factors for normal B cells, inducing the in vitro proliferation and differentiation of plasma cell precursors and leukemic B-cell precursors. CSFs represent a family of glycoproteins initially identified by their capacity to support the proliferation of hematopoietic progenitor cells in vitro. These factors are also capable of functionally activating differentiated cells in a lineage-specific fashion. Activated normal B lymphocytes have been shown to produce molecules with colony-stimulating activities. Furthermore, neoplastic B cells are able to synthesize GM-CSF.

Little is known on the role of these molecules and their own receptors on neoplastic B lymphocytes of patients with B-CLL.

In a series of B-CLL patients, we investigated the relationship between TNF-R and IL-2R and the expression of receptors for CSFs, including IL-3, granulocyte colony-stimulating factor (G-CSF), and GM-CSF, after in vitro culture of leukemic B cells in the presence of TNFα and IL-2. We observed that TNFα upregulates the expression of IL-2R and that these two cytokines display a synergistic proliferative effect when added together in vitro to leukemic cells. Leukemic B cells also express IL-3, G-CSF, and GM-CSF receptors; these receptors are upregulated by IL-2 but not by TNFα. Despite the expression of these receptors, CSFs exhibited no proliferative effect on CLL leukemic B cells, with the only exception being GM-CSF.

MATERIALS AND METHODS

Patients. Twelve patients with B-CLL (8 men and 4 women, aged from 48 to 66 years) were studied. All patients were studied from the Padua University School of Medicine, Department of Clinical Medicine, 1st Medical Clinic and Clinical Immunology Section, Padua; the Department of Internal Medicine, Rovigo Hospital; and the Department of Clinical Pathology, Cittadella Hospital, Padova, Italy.

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at the time of diagnosis and had never received any treatment before entering this study. The diagnosis was established on the basis of typical clinical, morphologic, histologic, and immunologic features, as previously described. B-CLL patients were graded according to the Rai staging system as follows: stage 0 (3 cases), stage I (5 cases), stage II (3 cases), and stage III (1 case). The total lymphocyte count ranged from 15,200 to 61,250/μL.

Cell separation and B-cell enrichment. Peripheral blood lymphocytes (PBL) from patients under study were obtained from freshly heparinized blood by centrifugation on Ficoll-Hypaque gradient. The cells were then washed three times with phosphate-buffered saline (PBS) and were resuspended in RPMI 1640 medium (GIBCO, Paisley, UK).

The samples were enriched for B lymphocytes using a modification of the method previously described. After removal of F-rosing cells by the neuraminidase- (Sigma Chemical Co, St Louis, MO) treated sheep red blood cell rosetting technique, the cell suspension was depleted of adherent cells by incubation for 45 minutes in plastic Petri dishes at 37°C in an atmosphere of 95% air and 5% CO2. Following the method of Lea et al.,25 cells were further purified by removing CD3+ and CD56+ lymphocytes using magnetic microspheres coated with antimouse IgG (Dynabeads, Dynoray, Norway), as previously described in detail.28 Brie-fly, after incubation (45 minutes at 4°C) of the cell suspension obtained as above with CD3+ and CD56+ lymphocytes were then isolated and removed applying a magnetic system on the outer wall of the test tubes for 2 minutes. After this multistep negative selection procedure, more than 97% of OKT3 (Ortho, Raritan, NJ) and CD56 (Leu 19; Becton Dickinson) for leukemic B cells. Cell suspensions were centrifuged on Ficoll-Hypaque gradient and posttraumatic splenectomy. After mechanic disruption of the spleen, magnetic system on the outer wall of the test tubes for 2 minutes.

Antibody-coated beads were then isolated and removed applying a continuous slow rotation. The CD3+ and CD56+ cells rosetting with protein was kindly provided by Dr Schlick (Knoll, Germany). Recombinant IL-2 with a specific activity of 5.8 X 10^6 Ulmg was obtained from Biogen (Cambridge, MA).

Reagents. TNFα with a specific activity of 8.74 X 10^9 U/mg protein was kindly provided by Dr V. Schwenk and Dr E. Schlick (Knoy, Germany). Recombinant IL-2 with a specific activity of 5.8 X 10^9 U/mg was obtained from Biogen (Cambridge, MA). GM-CSF, G-CSF, and IL-3 (with specific activities of 1 x 10^10, 2 x 10^10, and 2 to 5 x 10^10 U/mg, respectively) were purchased from Genzyme Corp (Cambridge, MA). Phycoerythrin (PE)-conjugated IL-2, TNFα, GM-CSF, IL-3, and G-CSF were purchased from R & D Systems (Minneapolis, MN). The MoAbs utr-1 and htr-9 were kindly provided by Dr M. Brockhaus (Basel, Switzerland). utr-1 and htr-9 MoAbs recognize the 75-kD and 55-kD TNF-Rs, respectively.27 Anti-CD25 (anti-Tac) MoAb, which recognizes the 55-kD IL-2-R and blocks IL-2 binding to this subunit, was a gift from Dr T. Uchiyama (Kyoto, Japan);28 TU27 and Mikβ1 MoAbs were gifts from Dr K. Sugamura (Senday, Japan), J. Hamuro (Kawasaki, Japan), and Dr M. Tsudo (Tokyo, Japan), respectively; they recognize the 75-kD IL-2-R α chain and anti–GM-CSFR α chain MoAbs (S20 and S50; Santa Cruz Biotechnology) that recognize the 80-kD human GM-CSFR.

Flow cytometric analysis of TNF-receptors by MoAbs and cytokine binding. The analysis of the expression of cytokine receptors was performed with MoAbs recognizing TNF receptors (utr-1 and htr-9 for TNF-R1 and TNF-R2), IL-2 receptors (anti-Tac and Mikβ1) for 55-kD and 75-kD IL-2Rs, and CSF receptors (S12 for IL-3 receptor, S20 and S50 for GM-CSF receptor) and by using PE-conjugated cytokines (PE-TNFα, PE-IL-2, PE-GM-CSF, PE-G-CSF, and PE-IL-3), as previously described in detail. The analysis of the expression of the above-mentioned receptors was performed on freshly isolated normal and leukemic B cells and on neoplastic B lymphocytes cultured for 3 days in medium alone supplemented with 10% fetal calf serum (FCS) or in the presence of several cytokines, including TNFα (100 U/mL) and IL-2 (1,000 U/mL). Before staining, the cells were washed in 40 mmol/L citrate containing 140 mmol/L NaCl, pH 4, to remove possible bound TNFα. After this procedure, the viability of cells was higher than 90%, as evaluated with the trypan blue exclusion test. The cells were resuspended in PBS and were stained for indirect immunofluorescence analysis by incubating the cells with utr-1, htr-9, anti–IL-3, and anti–GM-CSFR receptor MoAbs followed by an incubation with Fe(γ), goat antimouse (Technogenetics, Turin, Italy), as previously described.27 Ten thousand cells bearing the typical lymphocyte scatter were scored. To analyze receptor expression with PE-conjugated cytokines, 10^6 cells were incubated with 10 μL of PE-conjugated cytokine (10 μg/mL) for 60 minutes on ice. Cells were then washed twice and resuspended in 0.2 mL of PBS for flow cytometric analysis. As control for the fluorescence-activated cell sorter (FACS) analysis, cells were incubated with avidin PE. The lymphocytes were analyzed as indicated below. In the analysis of TNFα, IL-2, IL-3, and GM-CSF receptors, blocking experiments were performed by pre treating the cells for 1 hour at 4°C with the following antibodies: 20 μg/mL of utr-1 MoAb, 20 μg/mL of htr-9 MoAb, 20 μg/mL of anti-CD25 MoAb, 100 μg/mL of TU27 MoAb, and 10 μg/mL of anti–IL-3 or anti–GM-CSF receptor MoAbs. After this incubation, the cells were washed twice and successively incubated with PE-TNFα, PE-IL-2, PE-IL-3, and PE-GM-CSF, as reported above. For flow cytometric analysis, 10^5 cells were acquired and the analysis was determined by overlaying the histograms of the samples stained with the different reagents. Cells were then analyzed using a FACSscan analyzer (Becton Dickinson) and data were processed by using Consort 30 Software programs (Becton Dickinson).

Culture conditions. Purified B-CLL leukemic cells were cultured in round-bottom 96-well plates (Tirtetek ICN, Oxnard, CA) in RPMI 1640 supplemented with 10% FCS (ICN), penicillin (100 U/mL), and streptomycin (50 mg/mL). Cultures were performed in triplicate, with each well containing 1 x 10^5 cells in 0.2 mL of medium and were incubated for 5 days at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cultures were added at the beginning of the culture in different concentrations. TNFα was used at 20, 200, and 500 U/mL; IL-2 at 10, 100, and 500 U/mL; GM-CSF at 10, 100, and 500 ng/mL; G-CSF at 10, 100, and 500 U/mL; and IL-3 at 1, 10, and 100 U/mL. To investigate a cooperative effect of the above-mentioned cytokines on leukemic cells, different combinations of these molecules were used. Proliferation was determined by pulsing plates with 1 mCi/well of 3H-thymidine (CEA Ie Sorin, Saluggia, Italy) for the last 12 hours of culture; cells were then harvested and 3H-thymidine incorporation was measured in a β-scintillation counter.

Results are expressed as stimulation index (SI) according to the formula: mean cpm stimulated cells/mean cpm cultured cells in medium alone; and as a percentage of inhibition according to the following formula: (1 – cpm in the presence of the stimulus and antibody cpm in the presence of the stimulus) x 100.

RESULTS

Expression and function of TNF-R and IL-2R on leukemic B cells. To investigate the relationship between TNF-R and IL-2R, leukemic B cells were analyzed for the expression of the above-mentioned receptors after culture in medium

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Fig 1. Expression of IL-2R and TNF-R on leukemic B cells obtained from 1 representative B-CLL patient. Cells were cultured in medium alone, IL-2, TNFα, and a combination of both cytokines. They were successively stained with MoAbs recognizing different IL-2 and TNF receptors and with PE-conjugated cytokines (IL-2 and TNFα).

Fig 2. Proliferative assay of leukemic B lymphocytes cultured for 3 days in the presence of IL-2 (1,000 U/mL), TNFα (10, 100, and 500 U/mL), and the combination of both cytokines. The counts per minute (cpm) of cells cultured in medium alone was 282 ± 62.
Expression and function of hematopoietic growth factor receptors on leukemic B cells. Because other cytokines, including CSFs and, in particular, IL-3, have been reported to act as growth factors for normal B cells, we evaluated the role of hematopoietic growth factors and their receptors on leukemic B cells from B-CLL patients. Receptors for GM-CSF, G-CSF, and IL-3 were investigated by using PE-conjugated cytokines and antibodies that recognize the α chain of IL-3 and GM-CSF receptors. The analysis of CSF receptors on freshly isolated B cells from 2 patients with B-CLL and 2 normal subjects is reported in Fig 3. The histograms reported in Fig 3 show that freshly isolated leukemic B cells (Fig 3A and B) bind PE-conjugated IL-3, G-CSF, and GM-CSF, whereas normal B lymphocytes (Fig 3C and D) did not bind the above-mentioned reagents. Flow cytometry analysis of B cells using MoAbs that recognize the α chain of IL-3 and GM-CSF receptors showed a slight but significant shift on freshly isolated leukemic B cells (Fig 4A and B) but not on freshly isolated normal B lymphocytes (Fig 4C and D). To address the issue of whether the binding of these PE-conjugated cytokines is related to the actual presence of CSF receptors rather than to an aspecific binding, blocking experiments with specific MoAbs were performed. Figure 5A shows that the binding of PE-IL-3 in one representative patient is shifted to the left when leukemic cells were preincubated with anti-IL-3R MoAb. A similar finding was observed when GM-CSF receptor is considered (Fig 5B). In particular, the binding of PE-GM-CSF is shifted to the left when leukemic cells were preincubated with a mixture of two MoAbs against GM-CSF receptor. Because TNF-R and IL-2R are constitutively expressed on leukemic B cells from CLL patients and not on normal B lymphocytes, we investigated whether these cytokines (IL-2 and TNFα) regulate the expression of IL-3, G-CSF, and GM-CSF receptors and whether they induce proliferation in different combinations.

The analysis of cells cultured in different experimental conditions showed that the above-quoted receptors are constitutively expressed on neoplastic B cells cultured in medium alone (Fig 6 shows the data obtained in 1 representative patient). All these receptors were upregulated by the in vitro culture of leukemic cells in the presence of IL-2. This finding was consistently observed in other patients studied and is detectable at almost 100 and 1,000 U/mL IL-2 concentrations, whereas the 10 U/mL IL-2 concentration did not affect the binding of PE-CSF on these cells. On the other hand, TNFα did not upregulate these receptors. In the representative patient shown in Fig 6, the histogram of G-CSF receptor binding was even shifted to the left with respect to control, indicating a downregulation of G-CSF receptor expression; this finding was observed in 2 of 12 patients studied and its meaning is, at present, elusive.

To verify whether the 55-kD IL-2R is involved in the mechanism leading to the upregulation of CSFs receptors, leukemic B cells were cultured for 48 hours in the presence of anti-CD25 MoAb and IL-2, as well as in the presence of medium containing control MoAb or IL-2 alone. When the 55-kD IL-2R subunit was blocked with specific MoAb before in vitro stimulation with IL-2, we did not find any upregulation of G-CSF and IL-3 receptors as shown after culture of leukemic cells with IL-2 alone (Fig 7), whereas a slight shift of the GM-CSF receptor histogram was observed. This indicates that CSF receptor upregulation is mediated via the 55-kD IL-2R.

Hematopoietic growth factors were assessed for their functional activity on leukemic B cells in a proliferative assay. The data reported in Fig 8 show the results of prolifer-
Results obtained from 2 B-CLL patients (A and B) and 2 normal subjects (C and D) by using MoAbs against the \( \alpha \) chain of IL-3 receptor (S121) and the \( \alpha \) chain of GM-CSF receptor (S20 and S50).

Fig 4. Flow cytometry analysis of the expression of GM-CSF and IL-3 receptors on freshly isolated B cells obtained from 2 B-CLL patients (A and B) and 2 normal subjects (C and D) by using MoAbs against the \( \alpha \) chain of IL-3 receptor (S121) and the \( \alpha \) chain of GM-CSF receptor (S20 and S50).

Results obtained from 2 B-CLL patients (A and B) and 2 normal subjects (C and D) by using MoAbs against the \( \alpha \) chain of IL-3 receptor (S121) and the \( \alpha \) chain of GM-CSF receptor (S20 and S50).

FIGURE 5. Effects of pretreatment with anti-IL-3 and anti-GM-CSF receptor antibodies on PE-conjugated cytokine binding. Leukemic B cells were pretreated with 10 \( \mu \)g/mL of S12 MoAb before staining with PE-IL-3 (A) or pretreated with S20 and S50 MoAbs before staining with PE-GM-CSF (B). The staining with PE-cytokines alone and with streptavidin-PE reagent alone is shown.

DISCUSSION

Data presented in this report show that the 55-kD IL-2R on leukemic B cells obtained from B-CLL patients is upregulated by TNF\( \alpha \) and that the combination of IL-2 and TNF\( \alpha \) exhibited a synergistic proliferative effect. Furthermore, evidence has been provided that leukemic B cells bear receptors for hematopoietic growth factors (GM-CSF, G-CSF, and IL-3) that may be upregulated by IL-2 via the 55-kD IL-2R.

The evidence that TNF\( \alpha \) is able to upregulate the expression of the 55-kD IL-2R indicates that these two receptorial structures (75-kD TNF-R and 55-kD IL-2R) are closely re-
Fig 6. Flow cytometry analysis of the expression of GM-CSF, G-CSF, and IL-3 receptors on leukemic cells obtained from 1 B-CLL patient by using PE-conjugated cytokines. Lymphocytes were cultured in medium alone and in the presence of IL-2 and TNFα.

lated, similar to what is reported in the literature in terms of normal T cells in vitro. In fact, IL-2 enhanced the TNFR expression and TNFα increased the IL-2R expression, indicating that the functional effects shown by these cytokines are closely related. In our study, IL-2 poorly influenced or had no effect on TNF-R expression, whereas TNFα upregulated the expression of the 55-kD IL-2R. The partial shift of the 55-kD IL-2R histogram after TNFα stimulation might suggest that the receptor enhancement occurred in a subpopulation of CLL cells. The fact that IL-2 did not induce any modification of TNF receptors in our patients is not in agreement with the data reported by Digel et al, who showed an upregulation of TNF receptors by IL-2. These discrepancies might be the consequence of different approaches in assessing the receptors on cell surface membrane. This finding indicates that TNFα, in terms of receptor expression, displays a more pronounced effect on leukemic B cells from these patients than does TNF-α. Leukemic B lymphocytes obtained from B-CLL patients slightly proliferated to IL-2 and TNFα when used independently, but a high proliferative rate was commonly documented when both cytokines were combined. The proliferative rate displayed by the combination of these two cytokines on leukemic B cells is likely to be the consequence of a synergistic rather than a cumulative effect. In fact, the SI obtained after culture of leukemic cells in the presence of both TNFα and IL-2 increased 5 to 10 times with respect to the SI obtained after culture with TNFα alone and from 2 to 4 times as compared with the in vitro stimulation with IL-2. Whether this effect is discretely related to the upregulation of 55-kD IL-2R by TNFα remains unclear.

Fig 7. Flow cytometry analysis of the expression of CSF receptors in 1 representative patient after culture of leukemic cells in medium alone and in the presence of IL-2 alone as well as after blocking the 55-kD IL-2R with specific MoAb.
The evidence that the expression of CSF receptors can be upregulated after culture of leukemic B cells in the presence of IL-2 represents a new interesting observation, documenting another functional property of IL-2. In other words, IL-2 not only delivers a proliferative signal to leukemic B lymphocytes from B-CLL patients but also provides other regulatory properties, i.e., the upregulation of different cytokine receptors, including GM-CSF, G-CSF, and IL-3 receptors. This new effect might play a role in the physiopathology of leukemic cells because IL-2 can display a functional role not only directly by inducing B-cell proliferation but also indirectly by modifying the expression of receptors, as observed here, or by regulating the production of other cytokines, as shown in other cell systems.14 Furthermore, the shift to the right of CSF receptor histograms induced by IL-2 is likely to be related to the increase in receptor expression or affinity rather than to the increased number of cells constitutively expressing the receptors. The fact that the upregulation of CSF receptors by IL-2 is inhibited by the blocking of the 55-kD IL-2R (Fig 4) indicates that the effect we observed is in fact mediated by IL-2 and specifically via the 55-kD IL-2R. These findings point out a new functional activity of these structures on leukemic B cells, which may ultimately take place in vivo in these patients.

The observations that leukemic B cells bear hematopoietic growth factors and that their expression is regulated by IL-2 represent new areas of investigation and discussion. The fact that GM-CSF, IL-3, and G-CSF receptors are constitutionally expressed on neoplastic B lymphocytes obtained from patients with B-CLL might suggest new pathogenetic views to explain the physiopathologic events leading to the decrease of other cell types in the peripheral blood of B-CLL patients. In particular, the in vivo binding of these cytokines to leukemic B cells might result in a starvation of these factors for the relevant targets, with a consequent depletion of neutrophils in these patients.

To investigate the role of CSF in leukemic growth, we performed in vitro proliferative assays with these cytokines. The evidence that neither G-CSF nor IL-3 induced leukemic cells to proliferate when used singularly or in association with IL-2 and TNFα, even though their receptors are upregulated by IL-2, indicates that these molecules do not behave as autocrine factors in this disorder. Because these receptors can be upregulated by IL-2 but do not transduce any proliferative signal, this upregulation in terms of functional properties remains to be clarified. Furthermore, only GM-CSF evokes a detectable proliferative response in B-CLL. This functional property, coupled to the demonstration of GM-CSF receptors on leukemic cells, might indicate that this molecule plays a functional role in leukemic cell growth in vitro and possibly in vivo.

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