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 proliferation 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, ie, 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 type-B chronic lymphocytic leukemia (B-CLL) express cytokine receptors, including interleukin-2 and tumor necrosis factor receptors (IL-2 and TNF-R).1,4 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-2R and TNF-R have been observed to be related. In particular, IL-2R upregulates the expression of TNF-R on in vitro cultured peripheral blood T cells,4,6 and TNFα upregulates the expression of IL-2R,5,7 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,8,9 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,10-13 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,14 inducing the in vitro proliferation and differentiation of plasma cell precursors and leukemic B-cell precursors.25 CSFs represent a family of glycoproteins initially identified by their capacity to support the proliferation of hematopoietic progenitor cells in vitro.26 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.19-23 Furthermore, neoplastic B cells are able to synthesize GM-CSF.23 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, Citadella 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.1 B-CLL patients were graded according to the Rai staging system23 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 E-rosetting cells by the neuraminidase- (Sigma Chemical Co, St Louis, MO) treated sheep red blood cell rosetting technique, the cell suspension obtained as above with CD3+ and CD56+ lymphocytes by the neuraminidase- (Sigma Chemical Co, St Louis, MO) treated sheep red blood cell rosetting technique, the cell suspension obtained as above with CD3+ and CD56+ lymphocytes were incubated with biotinylated antimouse IgG (Dynebeads, Dynal, Norway), followed by an incubation with fluorescein isothiocyanate (FITC)-labeled streptavidin and successively incubated with PE-TNFα, PE-IL-2, PE-IL-3, and PE-GM-CSF. 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 anti-IL-1, anti-IL-6, and anti-GM-CSF receptor MoAbs followed by an incubation with FITC-goat antimouse (Technogenetics, Turin, Italy), as previously described.24 Ten thousand cells bearing the typical lymphocyte scatter were scored. The analysis of the expression of cytokine receptors by MoAbs and cytokine binding was performed with MoAbs recognizing TNF-R and IL-2 receptors by MoAbs and cytokine binding. The analysis of the expression of cytokine receptors was performed with MoAbs recognizing TNF-Rs, respectively. Anti-CD25 (anti-Tac) MoAb, which recognizes the 70-kD TNF-R1 and TNF-R2, and IL-2 (with specific activity of 8.74 X 10^8 U/mL, and 2 to 5 X 10^10 U/mL, respectively) 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, 10^6 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 (Tritetek; ICN, Oxford, CA) in RPMI 1640 medium supplemented with 10% FCS (ICN), penicillin (100 U/mL), and streptomycin (50 μg/mL). Cultures were performed in triplicate, with each well containing 1 X 10^6 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. Cytokines were added at the beginning of the culture in different concentrations. TNFα was used at 200, 500, 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 μg/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 Irc 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
alone and in the presence of IL-2, TNFα, and the combination of both cytokines. The data obtained by flow cytometry analysis with specific antibodies and PE-conjugated cytokines in 1 representative patient are reported in Fig 1. The histogram of cells stained with anti-IL-2R antibodies showed that TNFα upregulates the 55-kDa IL-2R (Fig 1B) subunit but not the 75-kDa IL-2R (Fig 1A) with respect to cells cultured in medium alone. On the contrary, IL-2 did not upregulate the expression of these receptors. The combination of both cytokines determined the same effect of TNFα alone. The binding assay of PE-IL-2 to leukemic cells showed an enhanced IL-2 binding (Fig 1C) only when cells were cultured in the presence of TNFα or in the presence of the combination of TNFα and IL-2. This was documented by the shift to the right of the IL-2 histogram and by the block provided by cold IL-2 (data not shown). These findings indicate that the 55-kDa IL-2R on leukemic B cells is upregulated by TNFα. When leukemic B cells were analyzed for the expression of TNF receptors, only the 75-kDa TNF-R was detectable on neoplastic B lymphocytes cultured in medium alone, thus confirming data previously reported. After culture of leukemic B cells with IL-2, TNFα, or in the presence of both cytokines, we did not observe any modification in the expression of the two different TNF-R (Fig 1D and E). These observations were further confirmed by the demonstration of the lack of any modification in the binding of the PE-TNFα (Fig 1F).

To verify whether the upregulation of IL-2R leads to a functional difference in B-cell response to these two cytokines, leukemic B cells were cultured in the presence of TNFα and IL-2 both individually and in the presence of a combination of these two molecules. The results obtained are illustrated in Fig 2. Both cytokines (IL-2 and TNFα) displayed a low proliferative activity on these neoplastic cells when used individually (IL-2 10 U/mL, 1.7 ± 0.5; IL-2 100 U/mL, 6.1 ± 3.2; IL-2 1,000 U/mL, 20.2 ± 5.6; TNFα 10 U/mL, 7.2 ± 4.7; TNFα 100 U/mL, 9.8 ± 5.5; TNFα 500 U/mL, 8.4 ± 2.8). A synergistic effect was observed by the combination of both cytokines, thus validating the results obtained with flow cytometry analysis. On the other hand, we did not observe any synergistic effect when leukemic cells were preincubated with TNFα or IL-2 and successively cultured in the presence of IL-2 or TNFα.

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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 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 proliferative activity on leukemic B cells.
ative assays after in vitro culture of leukemic B cells in the presence of different CSF, either alone or in combination with IL-2 and TNFα. When leukemic cells were cultured in the presence of CSF (Fig 8A), only GM-CSF exhibited a low proliferative activity on these cells in vitro at different concentrations (SI, 3.0 ± 1.7, 2.9 ± 1.1, and 5.6 ± 3.4 at 10, 100, and 500 U/mL, respectively), whereas neither G-CSF nor IL-3 induced the growth of leukemic B cells. To assess the role of the combination of CSFs, IL-2, and TNFα on leukemic cell proliferation, grading concentrations of CSFs and fixed IL-2 or TNFα concentrations were used. Figure 8B and C shows the results of these experiments. Neither G-CSF nor IL-3 enhanced the proliferation induced by IL-2 and TNFα. Only GM-CSF was shown to increase the IL-2 (SI, 28.2 ± 5.6, 32.0 ± 6.9, and 54.2 ± 18.3 at 10, 100, and 500 U/mL GM-CSF concentrations, respectively) and TNFα (SI, 15.1 ± 10.1, 22.2 ± 16.0, and 14.0 ± 6.4, at 10, 100, and 500 U/mL GM-CSF concentrations, respectively) mediated proliferation.

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 TNFα and that the combination of IL-2 and TNFα 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 TNFα 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.

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.
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Fig 8. In vitro effect of CSFs on leukemic B cells in proliferative assays. Leukemic lymphocytes were cultured in the presence of CSFs alone (A) and in combination with IL-2 (B) and TNFα (C). CSFs were used at different concentrations: GM-CSF at 0, 100, and 500 U/mL; G-CSF at 0, 100, and 500 U/mL; and IL-3 at 100, 100, and 500 U/mL. The results represent the mean ± SD from the mean of experiments performed in all patients. The cpm of cells cultured in medium alone was 282 ± 62.

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. Furthermore, the shift to the right of CSF receptor histograms induced by IL-2 is likely to be related to the increase of 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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