Expression and Functional Role of the p75 Interleukin 2 Receptor Chain on Leukemic Hairy Cells

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ABSTRACT

Hairy cell leukemia is a chronic lymphoproliferative disorder characterized by the expansion of neoplastic B-cells expressing the p55 chain of the interleukin 2 receptor (IL-2R) system that is recognized by anti-CD25 monoclonal antibodies (mAb) and binds interleukin 2 (IL-2) with low affinity. In the present study we investigated leukemic hairy cells (HC) for the presence of the p75 IL-2R chain which binds IL-2 with intermediate affinity and plays a crucial role in transducing the message to the cell. For this purpose, we tested highly enriched leukemic HC from six hairy cell leukemia patients for the IL-2R expression and for the expression of the p55 and p75 IL-2R chains on their surface membrane by flow cytometry and immunoprecipitation analyses. The functional role of IL-2R in the regulation of HC proliferation was also investigated.

Our results indicate that freshly isolated HC express detectable messages for both the p75 IL-2R and the p55 IL-2R. Flow cytometry analysis demonstrated detectable levels of p75 IL-2R on the HC from all patients tested. A mixture of two specific mAb was able to immunoprecipitate detectable amounts of p75 IL-2R from leukemic HC. When leukemic HC were cultured in the presence of several concentrations of IL-2 a low proliferative response was observed. Moreover, the IL-2-driven proliferation of HC was markedly inhibited by anti-p75 IL-2R mAb and to a lesser extent by anti-p55 IL-2R mAb. These findings provide direct evidence for the expression of different IL-2 receptors on leukemic HC and suggest that these molecules might play a role in leukemic cell growth.

INTRODUCTION

HCL is a chronic lymphoproliferative disorder of B-cell lineage characterized by discrete morphological, cytochemical, and immunological features. A peculiarity of leukemic HC is the constitutive expression of the p55 chain of IL-2R (4), which is recognized by mAb belonging to CD25.

Two forms of the IL-2R have been demonstrated on different cell types (5-7): a M, 55,000 glycoprotein (p55 chain), which binds IL-2 with low affinity, and a M, 70,000–75,000 glycoprotein (p75 chain), which binds IL-2 with an intermediate affinity. Both molecules form a noncovalently linked heterodimer that binds IL-2 with high affinity. The effects of IL-2 on cells equipped with specific receptors seem to be mediated by the p75 chain of the IL-2R rather than through the p55 chain (8, 9).

It has been demonstrated that IL-2, via specific receptors, induces the proliferation of normal B cells that were previously activated in vitro. In spite of the expression of the p55 chain of IL-2R by leukemic HC and although binding studies using 125I-labeled IL-2 suggested the presence of a small number of high-affinity IL-2-binding sites on their surface membrane (10), HC appear to be marginally responsive to IL-2 (11). Recently, the generation of mAb specifically recognizing the p75 IL-2R (12, 13) allows the definition of the expression of discrete IL-2R on different cell types, thus providing new insights into the IL-2-mediated activation mechanisms. Moreover, these antibodies permitted the isolation and cloning of a cDNA encoding the p75 chain (14). To provide direct evidence for the expression of p75 IL-2R on HC and thus elucidate the discrepancies quoted above in terms of IL-2-induced proliferation, in this study we tested purified HC for the expression of both p55 and p75 IL-2R chains on their surface membrane and for the presence of IL-2R transcripts.

MATERIALS AND METHODS

Patients. Six patients (5 males and 1 female, age 43 to 64 years) with HCL were studied. The diagnosis of HCL was based on clinical, morphological, and cytochemical criteria (1–3). None of the patients had undergone splenectomy or had received previous treatment. All patients had leukemic hairy cells in the peripheral blood with WBC ranging from 2,300 to 18,700/mm3.

Cell Preparation. Peripheral blood lymphocytes were obtained from freshly heparinized blood by centrifugation on a Ficoll-Hypaque gradient. The cells were then washed three times with phosphate-buffered saline and resuspended in RPMI 1640 (Gibco, Paisley, Scotland).

The samples were enriched for HC using a modification of the method previously described (15). Following removal of E-rosetting cells with neuraminidase (Sigma Chemical Co., St. Louis, MO)-treated sheep RBC (15), the nonrosetting cell suspension was depleted of adherent cells by incubation for 45 min in plastic Petri dishes at 37°C in an atmosphere of 95% air and 5% CO2. Cells were further purified in B-cells by removing CD3+ and CD56+ lymphocytes using magnetic microspheres coated with anti-mouse IgG (Dynebeads; Dynal, Oslo, Norway), according to the method of Lea et al. (16). Briefly, after incubation (45 min at 4°C) of the cell suspension obtained as above with CD3 and CD56 mAb, 40 × 106 beads were incubated with 10 × 106 cells/ml for 30 min at 4°C under continuous slow rotation. The CD3+ and CD56+ cells rosetting with antibody-coated beads were then isolated and removed applying a magnetic system on the outer wall of the test tubes for 2 min. Following this multistep negative selection procedure, more than 96% of the cells were viable when evaluated by the trypan blue exclusion test and were found to be positive for tartrate-resistant acid phosphatase and for staining with CD19 and CD11c (SHCL3/LeuM5) mAb.

Northern Blot Analysis. Northern blots were performed as previously described in detail (17). Briefly, total cellular RNA was extracted from 107 HC after lysis with 4 M guanidine isothiocyanate and by centrifugation through a 5.7 M cesium chloride gradient. Equal amounts of RNA (10 μg) were size-fractionated in a 1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher &

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3 The abbreviations used are: HCL, hairy cell leukemia; HC, hairy cells; cDNA, complementary DNA; mAb, monoclonal antibodies; IL-2, interleukin 2; IL-2R, interleukin 2 receptor; SDS, sodium dodecyl sulfate; PGI, phosphoglycerate kinase; EBV, Epstein-Barr virus; PE, phycoerythrin; PBS, 0.01 M sodium phosphate 0.14 M NaCl, pH 7.4; NP40–, Nonidet P-40.
Schuell, Keene, NH). The messages for p55 IL-2R were detected as 3.5- and 1.5-kilobase mRNA by hybridization with a purified 0.9-kilobase cDNA fragment from the IL-2R cDNA obtained from the American Type Culture Collection. The transcripts for the p75 IL-2R were detected as 4.0-kilobase mRNA by hybridization with the cDNA fragment subcloned into pUC19 vector kindly provided by Dr. M. Hatakeyama (Osaka, Japan) (14). Constitutionally expressed PGK transcripts were detected as a 2-kilobase mRNA, by hybridization with the purified fragment (1.8-kilobase) from PGK, obtained from the American Type Culture Collection. Probes were labeled using the multiprime DNA labeling system (Amersham Corp., United Kingdom) with [32P]PdCTP. After hybridization, filters were washed, dried, and exposed to Kodak X-Omat X-ray films at ~70°C (Du Pont de Nemours International S.A., Regensdorf, Switzerland). Rehybridization of the filters with another probe was performed after washing the membrane for 2 h at 85°C in 20 mm Tris HCl 0.1% SDS.

Monoclonal Antibodies and Flow Cytometry Analysis. Enriched hairy cells obtained as previously described were studied for the expression of cell surface antigens with direct two-color analysis using fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated mAb, as previously described (18). Prior to performing the phenotypical study, cells were washed in 40 mm citrate containing 140 mm NaCl, pH 4, to remove cell-bound IL-2. The following mAb were used: anti-CD25 (Becton Dickinson, Sunnyvale, CA); anti-Tac mAb was a gift of Dr. T. Uchiyama (Kyoto, Japan); FITC-Mik/J1 mAb (Janssen Biochimica, Geel, Belgium); ascites containing TU27 and Mik/J1 mAb were gifts of Dr. K. Sugamura (Sendai, Japan), J. Hamuro (Kawasaki, Japan), and Dr. M. Taudo (Tokyo, Japan) (12, 13), respectively, who recognize the p75 chain of IL-2R.

The expression of IL-2 receptors on leukemic HC was also investigated by evaluating the binding of PE-IL-2 on the cell surface using a flow cytometer. Briefly, 10 μl of PE-IL-2 (10 μg/ml) were added to 106 cells and the mixture was incubated for 60 min on ice. Cells were then washed twice and resuspended in 0.2 ml of PBS for flow cytometric analysis. As control for the fluorescence-activating cell sorting analysis, cells were incubated with avidin-PE. The lymphocytes were analyzed as indicated below. Blocking experiments were carried out by preincubating the cells for 1 h at 4°C with the following antibodies: 20 μg/ml anti-CD25; 20 μg/ml Mik/J1/TU27; and both antibodies. After this incubation the cells were washed twice and successively incubated with PE-CD25 (Becton Dickinson, Sunnyvale, CA); anti-Tac mAb was a gift of Dr. T. Uchiyama (Kyoto, Japan); FITC-Mik/J1 mAb (Janssen Biochimica, Geel, Belgium); ascites containing TU27 and Mik/J1 mAb were gifts of Dr. K. Sugamura (Sendai, Japan), J. Hamuro (Kawasaki, Japan), and Dr. M. Taudo (Tokyo, Japan) (12, 13), respectively, who recognize the p75 chain of IL-2R.

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External Cell Surface Radiolabeling. HC were surface radiolabeled with 125I by the lactoperoxidase-glucose oxidase-catalyzed iodination method originally described by Schenken et al. (19), with minor modifications, as previously reported (20). Briefly, 3 × 107 cells were washed first extensively in cold RPMI and then several times in PBS. Cells were then resuspended in 1 ml of PBS containing 11 mm glucose, and the following reagents were added: 20 μl of KI (5 × 10−3 m); 1 mCi of Na125I, carrier free (100 mCi/ml; Amersham Corp., Amersham, United Kingdom), and 50 μl of lactoperoxidase (1 mg/ml solution in PBS; Sigma). The reaction was started by the addition of 30 μl of glucose oxidase (19.55 units/ml; Sigma). The glucose oxidase step was repeated six times at 10-min intervals. To avoid cytoplasmic internalization of the labeling enzymes all reaction steps were carried out at 4°C. The reaction was stopped by adding 12 ml of cold RPMI supplemented with 5 mm L-cysteine hydrochloride (Sigma). Cells were then washed twice with cold RPMI and twice with cold PBS before lysis. Viability of the cells was higher than 99% in all experiments.

Cell Lysis and Immunoprecipitation. Cell lysates were prepared by incubating the cells for 60 min at 4°C in 1 ml of Tris-buffered saline, pH 8.2, containing 1% NP40 detergent, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml bovine serum albumin, according to the method of Owen et al. (21). Insoluble material was removed by centrifugation at 15,000 × g for 15 min. Cell extracts were incubated for 2 h at 4°C with killed Staphylococcus aureus Cowan I strain bacteria (Pansorbin; Calbiochem, La Jolla, CA) under rotation to remove material that could interfere with subsequent specific immunoprecipitates. This step was repeated three times. Detergent-solubilized cell extracts were reacted with specific mAb, and the resulting immunoprecipitate was recovered by incubation with Staphylococcus aureus Cowan I strain bacteria. The immunoprecipitated material was washed several times with 125 mS Tris-HCl buffer (pH 8.2) containing 0.5 m NaCl, 0.5% NP40, and 10 mS EDTA at 4°C. The specific immunoprecipitate, eluted from the packed immunoabsorbent by adding 50 ml of SDS-sample buffer (22) and boiling for 3 min, was analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis on 11% polyacrylamide gels, using the discontinuous buffer system according to the method of Laemmli (22).

Culture Conditions. Purified HC were cultured in round bottomed 96-well plates (Titertek, ICN, Oxnard, CA) in RPMI 1640 supplemented with 10% fetal calf serum (ICN), penicillin (100 units/ml), and streptomycin (50 μg/ml). Cultures were carried out in triplicate, with each well containing 1 × 105 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. Recombinant IL-2 was obtained from Biogen (Cambridge, MA) and was added at the beginning of the culture at different concentrations (10, 100, 1,000, and 10,000 units/ml). In order to block the IL-2-induced effects, leukemic HC were preincubated for 30 min with anti-Tac mAb (1:100 final dilution of ascitic fluid) and TU27 mAb (1:100 final dilution of ascitic fluid) or control isotype-matched IgG at the beginning of the culture at 4°C before adding the stimulus. Proliferation was determined by pulse-labeling plates with 1 μCi/well of [3H]thymidine (CEA Ire Sorin, Saluggia, Italy) for the last 12 h of culture; iImunline (CEA Ire Sorin) for the last 12 h of culture; [3H]thymidine incorporation was measured in a /3-scintillation counter.

Results of the cultures of HC in the presence of different concentrations of IL-2 are expressed as stimulation index according to the formula

Mean cpm cultured cells
Mean cpm stimulated cells

The effect of anti-IL-2R mAb is expressed as a percentage of inhibition of IL-2-induced proliferation.

RESULTS

The overall results of the Northern blot, flow cytometry, and immunoprecipitation analyses are summarized in Table 1.
Evaluation of mRNA Transcripts for IL-2R. Enriched HC from patients 1, 3, 4, and 5 were evaluated for the expression of p75 and p55 IL-2 receptor mRNA using Northern blot analysis. As shown in Fig. 1, HC from all patients tested contained detectable amounts of p75 mRNA with a size of 4.0 kilobases. When the same Northern blot was hybridized with a specific probe for the p55 IL-2R, p55 3.5- and 1.5-kilobase mRNA was detected in all patients. The amount of RNA loaded on the gel is represented by the constitutionally expressed PGK 2.0 kilobase mRNA and is reported at the bottom of the figure.

Flow Cytometry Analysis of p55 and p75 IL-2R Subunits on Leukemic HC Cell Surface. To determine whether the p55 and p75 mRNA are translated into stably expressed protein products, we examined enriched HC using flow cytometry. Leukemic HC from all patients were more than 96% CD11c+ and CD19+. The results of the flow cytometric analysis related to two representative patients (cases 4 and 5) are shown in Fig. 2.

The majority of cells (98 and 95%) in the considered cases reacted with anti-p55 chain IL-2R mAb (Fig. 2). The evaluation of the p75 IL-2R chain with Mik61 mAb demonstrated detectable levels of p75 chain on HC from all patients (Table 1), as reported in Fig. 2. This finding was documented by a statistically significant shift of the fluorescence histogram to the right as compared to the histogram of control.

Since anti-p55 and anti-p75 IL-2R-chain mAb bind leukemic B-cells, blocking experiments of PE-IL-2 binding with these antibodies were performed. As illustrated in Fig. 3, anti-CD25 mAb blocked the binding of IL-2 to leukemic B-cells while anti-p75 IL-2R chain mAb poorly affected the binding of IL-2 to these cells when used singularly (data not shown). The histogram obtained following the block with both mAb was significantly shifted with respect to the one obtained with the anti-CD25 alone.

Immunoprecipitation of p55 and p75 IL-2R Subunits from Leukemic HC. To confirm the results provided by flow cytometry analysis, a mixture of specific anti-p75 IL-2R mAb (to mimic a polyclonal situation) was used to immunoprecipitate the p75 molecule of the high affinity receptor for IL-2. NP40 extracts from surface 125I-lactoperoxidase-glucose oxidase-labeled cells were obtained from purified HC from peripheral blood lymphocytes of one patient (n. 1 of Table 1). Extracts were immunoprecipitated with a mixture of Mik61 (10 μg) and TU27 (10 μg) mAb and analyzed under reducing conditions by SDS-polyacrylamide gel electrophoresis. The anti-p75 mAb mixture immunoprecipitated the band of the p75 IL-2R chain.
from HC (Fig. 4A); the band is characterized by a molecular weight of 75,000 (13). Tac antigen was immunoprecipitated from labeled HC extracts by the MAR 108 mAb (23) (Fig. 4B). As previously described (24), this antigen migrated with an apparent molecular weight of 64,000 under reducing conditions due to the disruption of intrachain disulfide links.

**Biological Effect of IL-2 on HC and Effects of Anti-IL-2R Antibodies on Leukemic Cell Growth.** The proliferative effect of IL-2 on HC recovered from all HCL patients is illustrated in Fig. 5. When HC of patients under study were cultured in the presence of different concentrations of IL-2 a low proliferative effect was demonstrated. The stimulation index ranged from 1.2 to 1.92, from 1.61 to 2.6, from 1.9 to 3.2, and from 2.3 to 4.7 at the following IL-2 concentrations: 10, 100, 1,000, and 10,000 units/ml, respectively. The [3H]-thymidine incorporation of leukemic HC cultured in medium alone ranged from 522 to 893 cpm.

When leukemic HC were cultured in the presence of different concentrations of IL-2 following the preincubation with anti-p55 and anti-p75 IL-2R subunit mAb (Fig. 6), we observed a marked inhibition of the proliferation only when the cells were cultured in the presence of TU27 mAb either alone or in combination with anti-Tac mAb. Moreover, anti-CD25 mAb was not able to consistently affect the IL-2-driven proliferation when added independently.

**DISCUSSION**

Our study demonstrates that purified HC express detectable messages for the p75 chain of IL-2R. The corresponding protein product at cell membrane level was detected in all cases by
cytfluorimetric analysis with Mikß1 mAb and could be revealed by immunoprecipitation. At a functional level, this pattern was associated with a low proliferative rate to different concentrations of IL-2. The IL-2-driven proliferation was greatly inhibited by the anti-p75 IL-2R chain mAb rather than by the anti-p55 subunit mAb.

The presence of the p55 IL-2R chain both at RNA level and on the cell surface of leukemic HC recovered from our patients is in line with previous reports (4, 25). With regard to the p75 IL-2R, the observation that leukemic HC bind anti-p75 IL-2R subunit mAb coupled with the demonstration of mRNA messages for this subunit provides direct evidence that leukemic HC are equipped not only with the low-affinity IL-2R but also with intermediate and high-affinity IL-2 binding sites. The finding that leukemic HC express a complete IL-2R system (p55 and p75 subunits) is in line with the data reported by Begley et al. (10) who demonstrated, using cross-linking studies, that HC bind IL-2 with intermediate and high affinity. Whether the p75 IL-2R chain represents a peculiar finding of this disorder needs to be further investigated in a great number of patients with other chronic lymphoproliferative disorders. Preliminary data from patients with B-cell chronic lymphocytic leukemia indicated that leukemic cells lack the p75 IL-2R (26), thus supporting the above-mentioned suggestion.

Because of the expression of different IL-2R subunits on leukemic HC, we focused our attention on the biological relevance of IL-2 in the in vitro cell proliferation. Our results showed that IL-2 is able to induce the proliferation of HC to a slight rate, thus confirming the findings reported by Ford et al. (11). The low proliferative rate we observed was strictly related to leukemic HC. In particular, we did not observe any increase in the frequency of contaminating non-B-cells (i.e., T- and granulocyte lymphocytes, stained with anti-CD3 and CD56 mAb, respectively) at the time of harvesting the culture with respect to the beginning. In fact, the presence of both types of contaminating cells was less than 3% and the frequency of cells displaying hairy cell features (CD19+/CD1 lc+) was more than 96% both at the beginning and at the end of the culture. Our functional data regarding the blocking effect of anti-p75 IL-2R mAb on IL-2-driven HC proliferation suggest that the p75 subunit represents the major structure involved in the transduction of the proliferative signal by IL-2 to leukemic HC. The low proliferative rate we observed at the different IL-2 concentrations might be due to a low number of high-affinity IL-2 binding sites per cell, as previously demonstrated by cross-linking studies (10) or, alternatively, to the involvement in this process of other receptorial structures which may regulate the binding of IL-2 to its receptor (27).

Although the IL-2R is able to transduce a low proliferative signal in leukemic HC, it is not known why the p55 chain is abnormally expressed on their surface membrane and whether IL-2R (both p55 and p75 chains) have some other specific roles in the biology of tumor cell growth. In this regard, the expression of IL-2R on B cells is regulated by several cytokines. Some agents, and in particular the EBV, have been claimed to play a role in the regulation of the expression of p75 IL-2R (28). Interestingly enough, it has been observed that EBV-negative cell lines express the p55 IL-2R only, while in EBV-positive cell lines the p55 IL-2R is associated with the p75 IL-2R, in this way configuring a high-affinity IL-2R. Moreover, leukemic cells recovered from some HCL patients (29) have been shown to harbor the EBV thus suggesting that this virus might play a role in the regulation of the expression of the p75 IL-2R. Studies are in progress to validate this hypothesis. Finally, other biological properties are likely to be mediated by IL-2 through different IL-2R (30, 31). In particular, recent observations demonstrated that different T- or natural killer cell-derived cytokines can be triggered by IL-2 and that the type of cytokine produced (notably, granulocyte-macrophage colony-stimulating factor and γ-interferon) is related to the type of IL-2R chain involved in the transduction of the signal. In this view, it is tempting to speculate that IL-2 receptors on leukemic HC play a role in the leukemic cell growth not only in transducing a proliferative signal but also in regulating the expression of some cytokines which might act in an autocrine way.

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REFERENCES


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