Cell Membrane Expression and Functional Role of the p75 Subunit of Interleukin-2 Receptor in Lymphoproliferative Disease of Granular Lymphocytes

By Renato Zambello, Livio Trentin, Giovanni Pizzolo, Pietro Bulian, Maria Masciiarel, Cristina Fergugio, Carlo Agostini, Roberto Raimondi, Teodoro Chisesi, and Gianpietro Semenzato

The cell membrane expression and functional role of the interleukin-2 receptor (IL-2R) was analyzed in nine patients with lymphoproliferative disease of granular lymphocytes (LDGL) using monoclonal antibodies (MoAbs) specific for the p75 (TU27) and the p55 (anti-Tac) subunits of IL-2R. Four patients were characterized by the proliferation of CD3+CD8+ granular lymphocytes (GL) expressing the α/β T-cell receptor (Tαβ) and one case by the proliferation of CD3+CD4−CD8− GL expressing the γ/δ T-cell receptor (Tγδ); in four additional cases proliferating cells were CD3 negative GL. Consistent with data observed on normal GL, phenotypic analysis demonstrated that patients' GL lack the expression of the p55 IL-2R, whereas the p75 subunit is constitutionally expressed by expanding GL of both T-cell (either Tαβ and Tγδ) and natural killer (NK) origin in variable proportions (11% to 94% of cells). The analysis of the cytotoxic and proliferative activity demonstrated that the anti-p55 MoAb failed to inhibit IL-2-mediated activation, whereas a marked inhibition of both cytotoxicity and proliferation were obtained using the anti-p75 chain specific MoAb. These data indicate that the p75 chain of IL-2R is responsible for IL-2 signal transduction in both CD3+ and CD3− LDGL patients' GL.

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THE LYMPHOPROLIFERATIVE disease of granular lymphocytes (LDGL) is a rare disorder characterized by a chronic proliferation of lymphocytes with azurophilic granules in their cytoplasm (granular lymphocytes, GL). From an immunologic and molecular viewpoint, LDGL patients can be separated into two groups. The first is represented by the proliferation of CD3+GL, belonging to the T-cell lineage (CD3+ LDGL), and the other one by the proliferation of CD3− GL, which belong to the natural killer (NK) cell system (CD3− LDGL). However, the clinical pattern in both groups of patients is similar and usually indolent.

Recent data demonstrated that expanding cells in LDGL patients are highly responsive to interleukin-2 (IL-2) stimulation as they display cytotoxic activity and cell proliferation in vitro following the trigger with this lymphokine. Because in these cases GL do not spontaneously express the CD25/Tac antigen, ie, the p55(α) chain of IL-2 receptor (IL-2R), it has been suggested that the response to IL-2 is mediated by a different IL-2 binding protein. In fact, cross-linking experiments and schatchard plot analysis using labeled IL-2 provided indirect evidence for the presence of a 70/75 Kd protein structure with intermediate affinity for IL-2 on GL surface. Provided that fully functional high affinity IL-2R are generated by the association of p55 and p75 chains, the p75(β) subunit alone has been demonstrated to be able to transduce the activation signal. Recently, monoclonal antibodies (MoAbs) have been generated which recognize the p75 IL-2R, thus giving the opportunity to directly evaluate the role of this molecule on IL-2 mediated activation pathways. In this study, using the TU27 MoAb, we have investigated the presence of the p75 IL-2R on GL and its specific function on cell activation in a series of LDGL patients characterized by the proliferation of CD3+ GL, either expressing the T-cell receptor α/β (Tαβ) or the T-cell receptor γ/δ (Tγδ), or by the proliferation of CD3 negative GL.

MATERIALS AND METHODS

Patients. Nine patients with LDGL were studied. Diagnosis of LDGL was based on previously reported criteria. All patients were asymptomatic and untreated at the time of study, with the only exception being case no. 8, who suffered from autoimmune hemolytic anemia. In this patient, steroid therapy was withdrawn one month before this study was performed. Table 1 summarizes the clinical and immunological findings of cases under study.

Monoclonal antibodies. The following fluorescein (FITC) or phycoerythrin (PE)-conjugated MoAbs were used: anti-CD3 (OKT3), anti-CD4 (OKT4), anti-CD8 (OKT8) (Ortho Diagnostic, Raritan, NJ); anti-CD16 (Leu11a), anti-TCR1 (WT31), and anti-CD14 (Leu M3) and anti-CD22 (Leu 14) (Becton Dickinson Sunnyvale, CA); TCR81 and 5TC1 MoAbs (T Cell Science Cambridge, MA); BB3 MoAb, which reacts with an idiotopic determinant of the δ chain was a gift of Dr L. Moretta (Genova, Italy); ascitic fluid containing anti-CD25 (anti-Tac) MoAb that recognizes the p55 IL-2-R and blocks IL-2 binding to this subunit was a gift of Dr T. Uchiyama (Kyoto, Japan); ascitic fluid containing TU27 MoAb, which reacts with the p75 molecule of IL-2 receptor and blocks IL-2 binding to this subunit was a gift of Prof K. Sugamura (Tohoku, Japan) and Dr S. Taki (Kawasaki, Japan). In the functional studies, anti-Tac and TU27 MoAbs were used at 1:500 and 1:400 final dilution of ascitic fluid, respectively. In preliminary experiments, these doses were demonstrated to provide a 70% inhibition of phytohemagglutinin-mediated proliferation of normal peripheral blood lymphocytes.

Cell cultures. Peripheral blood mononuclear cells (PBMC) were isolated through a Ficoll-Hypaque gradient and washed twice in phosphate buffered saline. The resulting cell population was represented by 70% to 95% of GL, as evaluated by May-Grünewald Giemsa stained cytospins. Cells were cultured in RPMI 1640
GL-2R IN LDGL PATIENTS

Table 1. Hematologic and Immunologic Findings in LDGL Studied Patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age/Sex</th>
<th>WBC (x 10^3/L)</th>
<th>GL (mm²)</th>
<th>CD3 (%)</th>
<th>TCRαβ (%)</th>
<th>TCRγδ (%)</th>
<th>CD4 (%)</th>
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Controls (n = 10)

6.5 ± 2 225 ± 85 78 ± 5 72 ± 4 4 ± 3 44 ± 7 28 ± 6 13 ± 5 <2 10 ± 3

*The majority of cells (48%) reacted with the δTS1 MoAb, which recognizes the Cy2 associated δ chain determinant, while lacking the expression of the Cy1 associated ε chain determinant recognized by BB3 MoAb.

GIBCO, UK) with 10% fetal calf serum (FCS, Flow Laboratories) and 1% streptomycin/penicillin.

In some experiments, GL were further purified by using a complement-mediated lysis technique, as previously reported.11 In cases no. 2 and 4 (type CD3 + LDGL), 89% and 87% of peripheral blood lymphocytes, respectively, showed the typical GL morphology. Highly enriched GL (more than 97%) were obtained by eliminating the CD3 +, CD14 +, and CD22 + cells (residual normal T lymphocytes, monocytes and B lymphocytes, respectively). In case no. 8 (type CD3 + LDGL), 90% of peripheral blood lymphocytes were represented by GL. Two-color immunofluorescent flow cytometry analysis demonstrated that 98% of CD3 + cells coexpressed the CD16 marker, as proof that these cells belong to the abnormal GL population. GL were enriched in this patient by using complement mediated lysis of CD4 +, CD14 +, and CD22 + lymphocytes to obtain a final population consisting of more than 97% GL. The majority of cells obtained with these procedures (>95%) were viable when tested by trypan blue exclusion.

Cytotoxic assay. Cytotoxic activity was assessed by the lysis of 51Cr labeled NK sensitive K-562 targets or NK resistant Raji targets in a 4-hour assay, as previously reported in detail.13 Lytic function of GL was analyzed both at resting conditions and following in vitro activation of lymphocytes (10^6/mL) with 100 U/mL of rIL-2 (Glaxo Institute for Molecular Biology, Geneva, Switzerland) for 72 hours at 37°C in 5% CO₂ atmosphere. Results are expressed as lytic units (LU) times 10^9 effector cells for 30% specific lysis of 10^9 target cells.*

Proliferative activity. Cells at the concentration of 1 x 10^4 cells/mL were cultured in 96 round bottom well plates for 72 hours at 37°C in 5% CO₂ atmosphere with rIL-2 (100 U/mL). Each experiment was carried out in triplicate. For the last 18 hours of culture, plates were pulsed with 1 μCi/well of ³H-thymidine (CEA, IRE, Sorin, Italy); cells were then harvested and the radioactivity measured with a β counter. Results are expressed as counts per minute (cpm) ± standard error of the mean (SEM).

Immunofluorescence and flow cytometry. A panel of FITC and PE conjugated MoAbs was used for direct one- and two-color analysis, as previously reported in detail.13 Because the expression of the p75 IL-2R on cell surface is faint, an amplification system consisting of a biotinilated second antibody (goat anti-mouse IgG1), Boehringer Mannheim, FRG) and PE-conjugated streptavidin (Becton Dickinson) was used. When double staining experiments were performed, a FITC-conjugated MoAb as a third step or isotype matched control IgG was added together with PE streptavidin. When the third antibody belonged to the IgG1 subclass, free ligand sites of biotinilated goat anti-mouse were blocked using mouse serum (Dakopatts, Denmark). Cells were analyzed using a FACSScan analyzer (Becton Dickinson, Sunnyvale, CA) and data were processed by using the Consort 30 program. Ten thousand cells bearing the typical lymphocyte scatter were scored.

Statistical analysis. Data are expressed as mean ± SEM and comparisons between values made using the Cochran-Cox analysis. A value of P < .05 was accepted as significant.

RESULTS

Phenotypic analysis. Phenotypic evaluation of GL from our patients are summarized in Table 1. Patients no. 1 to 4 showed a proliferation of CD16+ GL. In these cases, two-color analysis demonstrated that CD16+ cells lacked the expression of CD3 marker, thus pointing to the NK lineage origin of these cells. In patient no. 2, CD16+ GL expressed the CD8 antigen at low density (CD8 "dim"). In the remaining five cases (no. 5 to 9), proliferating GL expressed the CD3 antigen. In four cases CD3+ GL coexpressed the T-cell receptor αβ (WT31 MoAb) and the CD8 antigen. In case no. 9, nearly 50% of cells were CD3+CD4−CD8+ (WT31)− and expressed the T-cell receptor γδ (TCRγδ1 MoAb). Interestingly, TCRγδ+ GL expressed the unusual Cy2 gene product, recognized by the δTS1 MoAb, but lacked the more common Cy1 gene product, recognized by the BB3 MoAb.8

Expression of p75 IL-2R on GL of LDGL patients. Although in all patients cells lacked the expression of the p55 IL-2R, both CD3+ and CD3− GL expressed the p75 IL-2R (range 11% to 94% of cells). The expression of TU27 MoAb showed different patterns of positivity (Fig 1), possibly related to the different densities of the p75 IL-2R on GL surface. Examination of the contour plots (Fig 2), demonstrated the coexpression on cell surface of the p75 IL-2R and CD16+ in CD3− LDGL patients and of p75 IL-2R and CD8+ on CD3+ Tαβ+ LDGL patients. In both groups, CD4+ cells were TU27 negative. In case no. 9, TCRγδ+CD4−CD8− GL were shown to express the p75 IL-2R, which was not detected on CD4+ and CD8+ cells.

Effect of anti-p75 IL-2R MoAb on activation of patients' GL. As shown in Fig 3A, CD3+ LDGL patients demon-
strated low baseline cytotoxicity against the K-562 target cells, which could be boosted by rIL-2. While anti-Tac MoAb did not provide any effect on cytotoxic function, the addition of TU27 MoAb dramatically inhibited the IL-2 induced cytotoxicity in these cases. Since GL of CD3–LDGL patients expressed high basal values of cytotoxicity against the K562 target cells (275 LU, 711 LU, 147 LU and 73 LU for patients no. 1, 2, 3, and 4, respectively), the NK-resistant Raji targets were used to test the cytotoxicity induced by IL-2. At baseline conditions, three cases showed low levels of lysis against the Raji targets, whereas in case no. 3, GL were spontaneously cytotoxic for these targets (81 LU). The addition of TU27 MoAb to the culture markedly inhibited the IL-2 induced lytic activity also in these CD3−cases. Neither anti-p75 IL-2R nor anti-p55 IL-2R MoAb affected the cytotoxicity against target cells mediated by...
resting lymphocytes (data not shown). Proliferation of either CD3+ and CD3—GL sustained by IL-2 was slightly inhibited by anti-Tac MoAb alone (Fig 3B) and, to a greater extent, by TU27 MoAb. Although anti-p55 IL-2R MoAb alone did not significantly modify the IL-2 induced proliferative activity in both CD3+ and CD3—LDGL patients, in the majority of cases the combination of anti-p55 and anti-p75 IL-2R MoAbs provided a synergistic effect in inhibiting the 3H-Thymidine incorporation, the phenomenon being more evident in CD3—cases.

To rule out the possibility that normal residual lymphocytes and not the abnormal GL population may account for the findings associated with IL-2 mediated activation, highly purified GL were studied in three representative patients (two CD3— and one CD3+ LDGL) in the same experimental conditions. As shown in Fig 4, the patterns observed for the cytotoxic and proliferative abilities were consistent with those shown using PBMC, with the exception of patient no. 8, in whom a relevant inhibition of proliferative activity of purified CD3+ GL was demonstrated by anti-p55 IL-2R MoAb.

**DISCUSSION**

In this study we demonstrated that in patients with LDGL proliferating GL constitutionally express the p75 IL-2R. Both CD3+ (either Tαβ or Tγδ) and CD3—GL are equipped with p75 IL-2R, whereas detectable levels of surface p55 IL-2R were not found on cell surface. Using MoAb to the p75 IL-2R, we provided direct evidence that the initial events leading to the IL-2 activation of GL in LDGL patients are mediated by the p75 chain of IL-2R.

Previous reports on LDGL patients had already suggested that the IL-2 activation was mediated through the p75 IL-2R.4,5 These results were based on an indirect demonstration that made use of radiolabeled IL-2. Together with the p75 IL-2R, however, other proteins could cross-link with 125I-IL-2 on the membrane of cells, such as the not well defined 70 Kd protein16 or MHC class I antigens.17 The
evidence we provided that anti-p75 IL-2R MoAb greatly reduces the effect of IL-2 as a definitive demonstration supporting the crucial role of the p75 IL-2R in the signal transduction.

Recent studies on the expression of the p75 IL-2R using MoAbs have demonstrated that the majority of normal CD16+ circulating lymphocytes (NK cells) and a variable percentage of CD3+ T lymphocytes (mostly CD8+ or CD4−CD8−γδ T cells) constitutionally expressed the p75 IL-2R, although this point is still controversial.2 Because these cell populations represent discrete subsets with low frequency in peripheral blood, further studies are needed to precisely characterize these cells for the presence and the kinetic of expression of membrane p75 IL-2R either in the blood or in other compartments. To explain the findings that CD8+ cells do not express the p75 IL-2R, an alternative hypothesis supports the fact that residual T cells in this disorder are to some extent abnormal. Taken together, our data indicate that the expression and the functional properties of the p75 IL-2R are maintained in the pathologic counterparts of cells with which we are dealing, pointing to a possible role for the p75 IL-2R on the abnormal proliferation of GL. In this regard, although LDGL encompasses a wide spectrum of clinical, immunologic and biologic conditions, the expression of the p75 IL-2R represents a consistent marker of all different types of proliferating GL in this disorder.

Interestingly enough, although anti-p55 IL-2R MoAb did not significantly inhibit the IL-2-induced cytotoxicity against the targets used and exerted only a minimal effect on the IL-2 mediated proliferative activity, the combination of anti-p75 and anti-p55 IL-2R MoAbs showed slightly higher levels of inhibition than those detected in experiments using anti-p75 IL-2R MoAb alone, particularly in terms of induction of the proliferative activity of CD3− cases (Fig 3). As to cytotoxic function, the pattern is more evident following enrichment of GL (Fig 4B). The differences in blocking experiments using anti-p75 IL-2R MoAb as compared with the combination are not statistically significant, perhaps because of the low number of cases studied. However, it is tempting to suggest that low levels of the p55 IL-2R subunit, possibly not detectable by flow cytometry, are expressed and functional on GL of patients, irrespective of their T-cell or NK-cell origin. According to this interpretation we have recently shown in LDGL patients that IL-2 is able to stimulate the synthesis of mRNA for p55 IL-2R in CD3+ GL even in the absence of a detectable CD25 antigen on cell surface of cells under study (manuscript in preparation).

In conclusion, the results obtained from the present study directly implicate the p75 IL-2R as the structure responsible...
for IL-2-mediated activation of GL in patients with LDGL, irrespective of the lineage (T or NK) of proliferating cells.

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