CONCISE REPORT

The Beta Subunit of the Interleukin-2 Receptor Mediates Interleukin-2 Induction of Anti-CD3 Redirected Cytotoxic Capability in Large Granular Lymphocytes

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The interleukin 2 (IL 2) receptor was studied in three cases of large granular lymphocyte (LGL) lymphocytosis. All cases were nonreactive with anti-Tac monoclonal antibody (MoAb; recognizing the p55 alpha subunit of the IL 2 receptor). Sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoretic analysis (PAGE) of cells to which radiolabeled rIL 2 had been chemically crosslinked revealed uniform expression of the p70/75 beta subunit of the IL 2 receptor in the absence of the alpha subunit. Stimulation of this receptor with 2 nmol/L rIL 2 for five days led to acquisition of anti-CD3 redirected cytotoxicity. This was accompanied by a fivefold to tenfold elevation in the activity of intracellular N-alpha-benzyloxycarbonyl-L-lysine thiobenzyl esterase, an LGL granule marker enzyme. These effects of IL 2 did not require induction of the Tac peptide.

INTERLEUKIN 2 (IL 2), via interaction with high-affinity IL 2 receptors, transduces a growth signal for mature T lymphocytes.[1-3] This high-affinity receptor has recently been shown to be a heterodimeric structure composed of an alpha subunit (p55), recognized by the monoclonal antibody (MoAb) anti-Tac, and a beta subunit (p70/75).[4-7] The sole expression of either subunit leads to receptors of lower affinity.[4-7] Although expression of the p55 alpha subunit alone is not capable of IL 2 stimulated signal transduction,[8] less is known about the functional properties of the p70/75 beta subunit. Indeed, the fact that the beta subunit protein is able to mediate internalization of surface bound IL 2 as fast as the alpha/beta, heterodimeric high-affinity receptor[9] suggests that p70/75 itself may function as a signal transducer in certain circumstances.

This protein is expressed on both normal and leukemic large granular lymphocytes (LGLs).[10,11] LGLs comprise approximately 15% of normal peripheral blood lymphocytes but are responsible for virtually all natural killer (NK) activity.[12-20] Several studies over the years have reported that IL 2 at concentrations much higher than necessary to support T cell growth, is capable of inducing cytolytic activity in LGLs.[12,13,21-23] This effect is not blocked by anti-Tac MoAb,[22,23] nor is it dependent on cell proliferation.[23,24]

Patients have been described in whom there occurs an LGL lymphocytosis that is typically nonprogressive over several years.[12] We have studied the occurrence and functional activity of the p70/75 protein in three cases of LGL lymphocytosis. Our findings indicate that this protein mediates IL 2-stimulated enhancement of LGL cytotoxicity as well as a marked elevation of intracellular granule-enzyme content.

MATERIALS AND METHODS

Cell preparation. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (FH) separation of heparinized blood from patients determined to have an LGL lymphocytosis based on clinical presentation, morphological, and/or surface phenotypic analysis. Cells were placed in culture at 1×10^6/mL in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin solution, with and without 100 U/mL (2 nmol/L) rIL 2 (a gift of Hoffman-La Roche, Nutley, NJ). Viability was assessed by trypan blue exclusion and remained above 90% during the course of the experiment. These cases were comprised of 67% to 74% Leu-7+ cells (see Table 1). However, following five days culture in rIL 2, 100% of the cells were Leu-7+ (data not shown).

Phenotypic analysis. Cell surface antigens were analyzed by indirect immunofluorescence using a FACS II flow cytometer (Becton Dickinson FACS Systems, Sunnyvale, CA).[22] The following antibodies were used: CD2 (Leu-5b, E rosette receptor), CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), CD16 (Leu-11), Leu-7 (HNK-1) all from Becton Dickinson; and CD25 (anti-Tac, a gift of Dr T. Waldmann, National Institutes of Health, Bethesda, MD). After incubation at 4°C with the primary antibody, cells were washed and reacted with fluorescein-labeled goat antimouse immunoglobulin (Kirkegaard and Perry, Gaithersburg, MD) at 4°C. A nonimmune mouse ascites preparation was used as a control for nonspecific reactivity of the primary antibody. After several washes, cells were analyzed on the FACS II by using the 488-nm line of an argon laser at 500 mW power. Data analysis was based on collection of 10^4 cells per sample.

Crosslinking methods and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). This method was performed as described previously.[4] Briefly, 1×10^7 cells were washed twice in RPMI 1640/1% bovine serum albumin (BSA) and incubated with 10 nmol/L ^{125}I-rIL 2 for one hour at 4°C. Blocking experiments were performed by incubating the cells with a 500-fold molar excess of unlabeled rIL 2 or 200-fold molar excess of anti-Tac prior to incubation with radiolabeled IL 2. After washing, the cells were resuspended in 800 μL of PBS/1mmol/L $MgCl_2$, pH 8.3, crosslinked with 100 μg/mL disuccinimidyl suberate (DSS) for one hour at 4°C, lysed in 80 μL of 1% NP-40, 300 mmol/L NaCl, 50 mmol/L Tris, pH 7.4, and analyzed in an 8% SDS-polyacrylamide gel under reducing conditions. Gels were dried and exposed to

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Table 1. Surface Phenotype of Patient Peripheral Blood

	Case 1	Case 2	Case 3
CS2	74	93	93
CD3	64	92	93
CD4	16	0	32
CD8	20	80	65
LEU-7	74	67	74
CD16	18	70	0
CD25 (anti-Tac)	0	0	0

Data are expressed as percent of mononuclear cells positive for each antigen.

XAR-2 film (Kodak) for five to ten days. The apparent mol wts of visualized bands correspond to the summation of the weight of one IL 2 molecule (15 kd) with the weights of the alpha and beta receptor subunits.[4]

Cytotoxicity assay. The NK-resistant–acute-lymphoblastic leukemia cell lines CEM and 8402 were used as targets for study of anti-CD3 redirected cytotoxicity in a standard four-hour ^{51}Cr-release assay.[25] CEM or 8402 target cells were coated with TNP and plated with an anti-CD3–anti-TNP antibody heteroconjugate as described.[26]

Granule enzyme secretion from LGL cells. For quantitation of the cytolytic granule enzymne N-alpha-benzyloxycarbonyl-L-lysine thiobenzyl esterase (BLT-esterase), cells were washed twice in phosphate-buffered saline (PBS), centrifuged, and the pellets frozen, thawed, and dissolved in 200 μL of 1% Triton X-100 in 1 N NaCl, 10 mmol/L HEPES, pH 7.4. The assay used was modified from the original[27] to allow 250 μL reaction volumes in 96-well flat bottom microtiter plates with the results read in a Titertek Multiscan ELISA reader using a 414-nm filter. The final reaction volume in the wells contained 0.15 mol/L NaCl, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.2 mmol/L N-alpha-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), and 0.2 mmol/L 5,5'-dithiobis-(2-nitrobenzoic acid [DNBT]), pH 7.4. The reaction was initiated by addition of substrate to the appropriate wells and incubations were carried out for 30 minutes at 22°C. In all cases the optical density (OD) of a blank well containing sample but lacking BLT was subtracted from the OD obtained from sample wells containing substrate. We define 1 U/mL as that final concentration of enzyme in the well that gives an OD of 1 (hydrolysis of 220 pmol of substrate per minute).

RESULTS

Surface expression of the beta subunit of the IL 2 receptor. Table 1 contains the results of the flow cytometric analysis of these cases. Each case expressed the T cell antigens CD2, CD3, CD8, and the NK antigen Leu-7. The cells of case 2 also expressed CD16 (Leu-11). The MoAb anti-Tac was negative in all cases. Crosslinking studies using ^{125}I-rIL 2 were performed to determine the structure of the IL 2 receptor expressed in these cases. Figure 1 shows that rIL 2 bound to the beta subunit (p70/75) of the IL 2 receptor in all three cases (Fig 1a, lanes 1 and 4; and Fig 1B, lane 2). No alpha subunit (p55) was detected in the LGL proliferations as compared to HUT-102, a T cell line that expresses the high-affinity, alpha/beta, heterodimeric form of the receptor (Fig 1a, lane 7; Fig 1b, lane 1; and Fig 1c, lane 7). The binding of ^{125}I-rIL 2 to the beta subunit on the LGL cells and to the alpha and beta subunits on HUT-102 cells was completely inhibited by prior incubation with a 500-fold

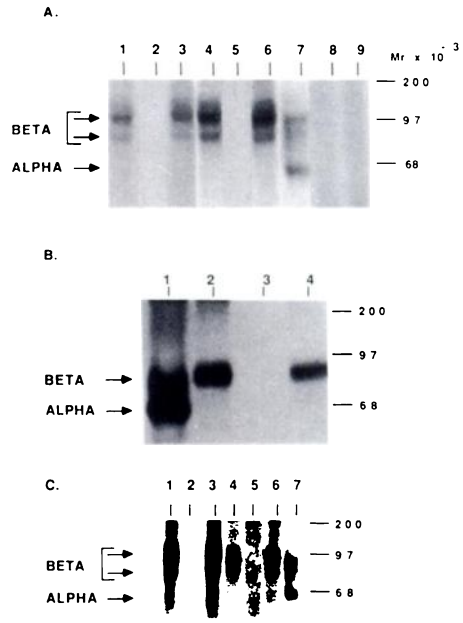

Fig 1. Analysis of IL 2 receptor expression in three cases of LGL lymphocytosis by crosslinking of ^{125}I-IL 2 to intact cells. (A) Case 1, lanes 1–3; case 2, lanes 4–6; HUT-102, lanes 7–9. Lanes 1, 4, 7: ^{125}I-IL 2; lanes 2, 5, 8: pretreatment with cold IL 2; lanes 3, 6, 9: pretreatment with anti-Tac. (B) HUT-102, lane 1; case 3, lanes 2–4. Lanes 1–2: ^{125}I-IL 2; lane 3: pretreatment with cold IL 2; lane 4: pretreatment with anti-Tac. (C) Following five days culture in rIL 2. Case 2, lanes 1–3; case 3, lanes 4–6; HUT-102, lane 7. Lanes 1, 4, 7: ^{125}I-IL 2; lanes 2 and 5: pretreatment with cold IL 2; lanes 3 and 6 pretreatment with anti-Tac. The apparent mol wts depicted represent summations of the mol wt of IL 2 (15 kd) and the respective mol wt of the alpha and beta receptor subunits.

molar excess of cold rIL 2 (Fig 1a, lanes 2, 5, and 8; Fig 1b, lane 3). However, a 200-fold molar excess of anti-Tac did not block the binding of ^{125}I-rIL 2 to the beta subunit of the IL 2R in LGL cells (Fig 1a, lanes 3 and 6; Fig 1b, lane 4) but did inhibit its binding to the high-affinity form of the receptor expressed in HUT-102 cells (Fig 1a, lane 9). Interestingly, in case 3 only one band corresponding to the beta subunit was detected. Although the reason for this is not known, it may be due to an altered pattern of glycosylation. Following culture with rIL 2, the cells of this case expressed the doublet characteristic of the beta subunit (see Fig 1c, lane 4).

LGL cells from the three cases were restudied after five days in culture with rIL 2 to determine whether the alpha subunit of the IL 2R was induced after treatment with 2 nmol/L rIL 2. In all three cases, treatment with rIL 2 did not result in expression of the IL 2 receptor epitope detected by anti-Tac as determined by flow cytometry (data not shown), and in cases 2 and 3 crosslinking studies confirmed this finding (Fig 1c, lanes 1 and 4). Again, a 500-fold molar excess of cold IL 2 blocked the binding of radiolabeled IL 2 (Fig 1c, lanes 2 and 5), but excess anti-Tac did not inhibit this binding (Fig 1c, lanes 3 and 6).

Recombinant IL 2 induces anti-CD3-redirected cytolysis and an elevation of granule enzyme content. To investigate the function of the beta subunit of the IL 2R expressed by the LGL cells, we placed the cells obtained from these three

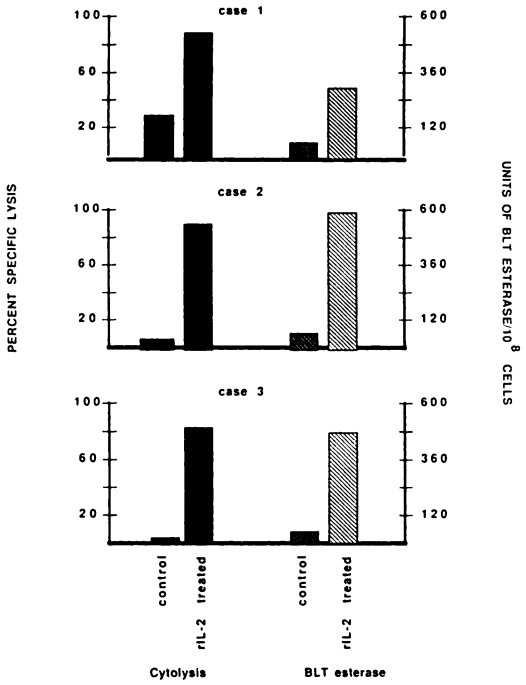

Fig 2. Recombinant IL 2 induces anti-CD3 redirected killing and enhancement of BLT-esterase content of LGL cells. Killing assays were performed at an effector:target ratio of 10:1.

cases in culture in the presence or absence of 100 U/mL (2 nmol/L) rIL 2. After the cells were cultured for five days their cytolytic activity against NK-resistant target cells was examined using the method of anti-CD3-redirected killing. Figure 2 shows that LGLs stimulated with 100 U/mL (2 nmol/L) rIL 2 for five days displayed high levels of cytotoxic activity against NK-resistant CEM cells, while the LGL cells cultured in the absence of rIL 2, freshly thawed (data not shown), exhibited very low (case 1) or no (cases 2 and 3) cytolytic activity. To further assess rIL 2 induced activation of cytolysis in these LGLs, we studied the induction of the granule marker enzyme BLT-esterase. This enzyme is present in LGL and T cell granules and is secreted from cytotoxic T lymphocytes during cytolysis.[28,29] The right panel of Fig 2 shows that the levels of BLT-esterase in homogenates were enhanced fivefold to tenfold after five days of rIL 2 treatment.

DISCUSSION

In the majority of LGL lymphocytoses reported, the cells express both CD3 and CD8 surface antigens as well as one or both of the NK antigens Leu-7 and Leu-11.[18,30] The three cases we have studied in this report conform to this description. Although freshly isolated LGLs seldom demonstrate significant cytotoxicity, culturing in the presence of rIL 2 for short periods invariably leads to acquisition of potent cytotoxic activity with no MHC restriction.[12,13,21-23] Since these cells are not recognized by the MoAb anti-Tac, and since anti-Tac cannot block IL 2-stimulated acquisition of MHC-unrestricted cytotoxicity, it has been suggested that this effect is through a different IL 2 receptor than that recognized by anti-Tac (p55).[22-24]

Recently expression of the beta subunit of the IL 2 receptor (p70/75) has been reported on the surface of normal and leukemic LGLs.[10,11] We have studied the LGLs from three patients with LGL expansions. These cells express the beta subunit (p70/75) of the IL 2 receptor without concomitant expression of the alpha subunit (p55). Culture with 100 U/mL (2 nmol/L) rIL 2 for five days induced anti-CD3 redirected killing and a fivefold to tenfold increase in the level of the granule marker enzyme BLT-esterase. These data are the first to demonstrate functional activity induced via the beta subunit of the IL 2 receptor. Interestingly, neither a significant increase of ^{3}H-thymidine incorporation over that seen in untreated cells (data not shown) nor induction of the alpha subunit (p55) studied by flow cytometry and crosslinking methods was found after five days of rIL 2 treatment. It has recently been reported that an IL 2 concentration higher than 5 nmol/L results in stimulation of ^{3}H-thymidine incorporation and induction of alpha subunit expression by LGLs.[11] Since neither cell proliferation[23,24] nor Tac protein expression[12,13,21-23] are required for IL 2-stimulated cytolysis by NK cells, the significance of these findings is unclear. Our data demonstrate that at an IL 2 concentration that induces cytolytic capability as well as a fivefold to tenfold elevation of granule enzyme content, neither cell proliferation nor alpha subunit (Tac) expression is necessary. The affinity of the beta subunit protein for IL 2 has been reported to be 0.6 to 1.0 nmol/L.[6,9] Thus the amount of rIL 2 we are adding in our experiments corresponds to approximately twice the kd value. Others have found rIL 2 stimulates NK activity at similar concentrations.[13,21,23]

We conclude that the beta subunit protein of the IL 2 receptor, expressed in the absence of the alpha subunit, mediates IL 2-induced elevation of cytolytic capability in LGLs. Activation of the beta subunit did not transduce a proliferative signal, suggesting that in the appropriate milieu IL 2 may function as a differentiative stimulus.

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