Genistein, a Tyrosine Kinase Inhibitor, Decreased the Affinity of $p56^{lck}$ to β-Chain of Interleukin-2 Receptor in Human Natural Killer (NK)-Rich Cells and Decreased NK-Mediated Cytotoxicity (43811)

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Abstract. **Intracellular signal transduction has been reported to be triggered by phosphorylation of interleukin-2 (IL-2) receptor by IL-2. In order to clarify the effect of tyrosine phosphorylation of IL-2 receptors on cell-mediated cytotoxicity by natural killer (NK) cells, we studied the effect of a tyrosine kinase inhibitor, genistein, on the lethal effects of NK-rich cells for K562 cells. Exposure of NK-rich cells to IL-2 (100 U/ml) for 3 days increased their cytotoxicities against K562 cells. The effect was reduced in the presence of 10 μg/ml of genistein.**

Samples immunoprecipitated by anti-IL-2Rβ antibodies were prepared from NK-rich fractions with or without exposure to IL-2 and/or genistein. Coprecipitated proteins with 75, 65, and 56 kDa were detected with an antiphosphotyrosine antibody. The amount of phosphorylated tyrosine residues of 56-kDa protein, which was predominantly detected in NK-rich cells, was remarkably increased by IL-2 treatment. The enhanced phosphorylation of 56-kDa protein was reduced by the presence of genistein. These results suggested that IL-2 increased tyrosine phosphorylation and the affinity to IL-2Rβ of the 56-kDa proteins in NK-rich cells. Immunoprecipitated samples by anti-IL-2Rβ were reblotted with anti-$p56^{lck}$ antibody and revealed that the 56-kDa protein was identified to be $p56^{lck}$. The increase of coprecipitated $p56^{lck}$ with anti-IL-2Rβ antibody by the treatment with IL-2 suggested that the affinity of $p56^{lck}$ to IL-2Rβ was increased by IL-2 in NK-rich cells. The amount of coprecipitated $p56^{lck}$ with IL-2Rβ was reduced in the sample exposed to genistein. The affinity of $p56^{lck}$ to IL-2Rβ was considered to be regulated by IL-2-induced tyrosine phosphorylation. Our results demonstrated a potential role for tyrosine kinase, $p56^{lck}$, in the signaling events that regulate the cytotoxicity by NK-rich cells. [P.S.E.B.M. 1994, Vol 207]

Natural killer (NK) cells have been identified as a subpopulation of lymphocytes with specialized functions, including the ability to kill malignant cells without prior sensitization and without restriction of major histocompatibility complex (MHC) (1). The NK cells represent a small subset of CD3-negative ($CD3^-$) peripheral blood lymphocytes (PBL) that neither express nor rearrange T-cell receptor (TCR) genes, but do mediate non-MHC-restricted killing of tumor cells (2, 3). Interleukin-2 (IL-2) exerts powerful regulatory effects on NK cell function. IL-2 has been shown to increase the non-MHC-restricted cytotoxicity of human peripheral lymphocytes. The majority of the cells involved in this response are CD3-positive ($CD3^+$) and -negative ($CD3^-$) NK cells, although $CD3^+$ T lymphocytes, that express TCRs also participate in this process (4, 5). The IL-2 receptor (IL-2R) comprises at least three distinct polypeptides,

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namely the α- (IL-2Rα:p55), β-(IL-2Rβ:p70–75) and γ-(IL-2Rγ:p64) IL-2R chains, which bind IL-2 with different affinities. The biological/physiological effects of IL-2 are believed to be mediated via IL-2Rβ (p70–75), which is expressed constitutively on NK cells that lack the p55 (Tac) component (6, 7). Thus, IL-2Rβ, unlike IL-2Rα and IL-2Rγ, can transduce the IL-2 signals that lead to proliferation and enhancement of cytotoxic activity (8, 9). However, the biochemical mechanism underlying IL-2Rβ-mediated signaling by IL-2, particularly in NK cells, has yet to be elucidated, as has the relationship between signaling by IL-2 and NK-cell-mediated cytotoxicity against tumor cells.

Recent studies have demonstrated that IL-2 provokes a rapid increase in tyrosine phosphorylation of cellular substrates, including the IL-2Rβ chain itself (10, 11), and have provided evidence for the coupling of protein tyrosine kinase activity with the IL-2R (12). These observations suggest that activation of one or more protein tyrosine kinases occurs during the early phase of intracellular signal transduction that is triggered by the interaction of IL-2 with IL-2R.

Among the protein tyrosine kinases, those of the *src*-family are particularly likely candidates for IL-2R signal mediators. They are plasma membrane-associated proteins comprising eight well-characterized members, including $p56^{lck}$ (13, 14), and have been shown to participate in lymphocyte signaling in four well-defined systems: $p56^{lck}$ with CD4 and CD8 (15, 16); $p56^{fyn}$ with the TCR-CD3 complex (17, 18); $p56^{lyn}$ with membrane-bound immunoglobulin M (IgM) (19); and $p56^{lck}$ with IL-2Rβ (20). Thus, the *src* family kinases can couple with lymphocyte receptors, and $p56^{lck}$ (21, 22) is a potentially interesting signaling molecule for IL-2 because it is expressed predominantly in those cells that respond physiologically to IL-2, such as T and NK cells (13). Although $p56^{lck}$ has been implicated in CD4- and CD8-mediated signal transduction (23), there is no obligate relationship between the expression of $p56^{lck}$ and expression of CD4 or CD8 coreceptor molecules, which suggests that $p56^{lck}$ may have additional functions in other signal transduction systems.

We have demonstrated that IL-2Rβ interacts with $p56^{lck}$ in NK cells and adduced evidence which lends support to the hypothesis that the NK-mediated cytotoxicity is regulated by the signal transduction systems.

Materials and Methods

Chemical Reagents and Antibodies. All the chemicals and drugs used, unless stated otherwise, were obtained from Sigma Chemical Co. (St. Louis, MO). A mouse monoclonal specific antibody against phosphotyrosine residues, PY20, and a rabbit monoclonal antibody for human lck kinase (CT) were obtained from ICN (Cleveland, OH) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively.

Target Cells. The susceptibility of a human chronic myelogenous leukemia cell line (K562) to natural killer and lymphokine-activated killer cell-mediated cytotoxicity was determined. The cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% (v/v) of heat-inactivated fetal calf serum (FCS, Bocknek Lab. Inc., Toronto, Canada), penicillin G 10 IU/ml, and streptomycin 1 μg/ml (RPMI-FCS).

Effector Cells. Venous blood was obtained from healthy volunteers, diluted with RPMI-FCS layered on top of Ficoll-Conray, and centrifuged at 1,800*g* for 30 min, to obtain mononuclear cells, which were collected and washed twice with RPMI-FCS. This mononuclear cell suspension was incubated at 37°C for 1 hr in a plastic dish to remove the adherent cells, and the nonadherent cells were collected by repeated extensive washing with RPMI-FCS and centrifugation. The pellets were resuspended in RPMI-FCS, incubated in a nylon wool column for 45 min and then passed through it to remove the B cells. The remaining cells were layered on top of Percoll discontinuous-gradients and spun at 550*g* for 30 min. Cells from the large granular lymphocyte (LGL) and small lymphocyte fractions were collected as NK-rich and T cell-rich fractions, respectively, and were both washed twice with fresh RPMI-FCS medium. The purity of LGL and T cell-rich fractions was more than 95%. These cells were preincubated with genistein 10 μg/ml at 37°C for 30 min, after which 100 U/ml IL-2 (Shionogi Pharm., Osaka, Japan) was added and they were incubated at 37°C for 72 hr, and washed once with RPMI-FCS medium and used as effector cells or protein sources. The viability of genistein-treated cells was more than 95% after 72 hr-incubation determined by dye exclusion assay.

Cytotoxicity Assay. The K562 target cells (1×10^5) were incubated with RPMI-FCS medium containing 2 μCi/ml $Na_2[^{51}Cr]O_4$ (3.68 mCi/mg of ^{51}Cr) at 37°C for 1 hr. The cells were washed with medium, centrifuged to produce pellets, and resuspended. One times 10^4 cells/0.1 ml were plated into each well of 96-well, U-bottomed plates. The required effector cells (0.1 ml), to produce the required effector/target (E/T) ratios, were added to the cells and the plates were incubated for 5 hr in a humidified atmosphere containing 5% CO_2 at 37°C. In order to attain the maximal ^{51}Cr release, target cells were incubated with 0.1 *N* HCl instead of a lymphocyte suspension. The supernatants were collected and their radioactivities were counted with an auto-gamma counter. The lytic activity was calculated as follows: percentage of lysis = (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100.

Immunoprecipitation and Immunoblotting. The NK- and T-cell fractions were lysed with TNE buffer (10 m*M* Tris-HCl, pH 7.6, 1% (w/v) Nonidet P-40 (Iwai Chemical Co., Tokyo, Japan), 0.15 *M* NaCl, 1 m*M* ethylenediamine tetraacetic acid (EDTA)) followed by freezing and thawing. Each lysate was centrifuged at 1,000*g* for 10 min, and the protein content of each supernatant assayed using the Lowry's method (24). The protein contents of same numbers of peripheral lymphocytes and NK-rich fraction were almost equivalent. One milliliter supernatant was transferred to a new tube, to which 30 μl protein G-Sepharose (4 Fast Flow; Pharmacia-LKB) was added, incubated overnight at 4°C followed by centrifugation at 1,500*g* for 10 min. The supernatants were incubated with anti-IL-2Rβ′ (Mik-β1) or anti-lck antisera for 1 hr at 4°C. Twenty microliters of protein G-Sepharose was added to the mixture, which was incubated for a further 2 hr at 4°C, and then centrifuged at 1,500*g* for 10 min followed by three washes and centrifugation with cold TNE buffer. The resulting pellets were mixed with equal volumes of 2× sample buffer (0.25 *M* Tris-HCl, 2% [w/v] sodium dodecyl sulfate [SDS], 30% [v/v] glycerol, 10% [w/v] 2-mercaptoethanol, 0.01% [w/v] 2-mercaptoethanol, 0.01% [w/v] bromophenol blue, pH 6.8), heated for 3 min at 95°C and subjected to electrophoresis using 10% SDS-polyacrylamide gel (SDS-PAGE PLATE 10; Daiichi Pure Chemicals, Tokyo, Japan). The gels were then equilibrated for 15 min with transfer buffer (25 m*M* Tris, 192 m*M* glycine, 0.02% [w/v] SDS, and 20% [v/v] methanol, pH 8.3) and transferred electrophoretically from the polyacrylamide gel to polyvinylidene difluoride membrane (0.45 μm; Millipore, CA). The membranes were blocked by incubation for 2 hr at room temperature in 5% (v/v) bovine serum albumin (BSA) in PBS, washed with PBS containing 0.1% (w/v) Tween 20 (PBS-T), incubated at 4°C for 2 hr with mouse antiphosphotyrosine (PY20) or anti-$p56^{lck}$ (CT) antibody which was diluted with PBS, and then rinsed with PBS-T. Next, the membranes were incubated with anti-rabbit immunoglobulin G (IgG) (Fc)-horseradish peroxidase conjugate diluted with PBS-T containing 1% (w/v) BSA for 1 hr at room temperature, and finally washed with PBS-T. The membranes were amplified using BLAST, a blotting amplification system (Du Pont NEN Research Product, Boston, MA), developed with a POD immunostaining kit (Wako Pure Chemical Co., Osaka, Japan), washed with distilled water and dried. Data was analyzed quantitatively by the densitometry (Image Master; Pharmacia Biotec, Tokyo, Japan).

Immune-Complex Kinase Assays. The NK- and T cell-rich fractions were treated with 100 U/ml IL-2 with or without 10 μg/ml genistein and lysed on ice with NP-40 lysis buffer, which contained 50 m*M* Tris, 150 m*M* NaCl, 5 m*M* EDTA, 1% (v/v) Nonidet P-40, 100 μ*M* sodium orthovanadate, 100 μ*M* sodium molybdate, 500 μ*M* phenylmethanesulfonyl fluoride, 8 μg/ml aprotinin, 5 μg/ml leupeptin, pH 8.0, and cetrifuged for 10 min at 13,000*g* to remove the insoluble materials. The resulting samples were immunoprecipitated with antisera against $p56^{lck}$ and diluted 1:500, and the immune complexes were collected by incubation at 4°C for 4 hr with 15 μl protein G Sepharose beads, which were washed four times with NP-40 lysis buffer, followed by one wash with kinase buffer (20 m*M* Mops, pH 7, which contained 10 m*M* $MgCl_2$) and then resuspended in the latter buffer. The reactions were initiated by adding 3 μg acid-denatured rabbit muscle enolase (Sigma), 25 μCi of [γ-^{32}P] adenosine triphosphate (ATP) (5,000 Ci/mmol), and unlabeled ATP to a final concentration of 5 μ*M*. The reactions were terminated by boiling for 4 min in 2× SDS sample buffer, after which they were electrophoresed using 10% SDS-polyacrylamide gels and the radiolabeled proteins were detected using BAS 2000 the bioimage analyzer (Fuji Film Co., Tokyo, Japan).

Results

Effect of Genistein on the Cell-Mediated Cytotoxicities of Peripheral Lymphocytes and NK Cells. In order to evaluate the effect of tyrosine phosphorylation of the IL-2 receptor on cell-mediated cytotoxicity, we studied the effect of the tyrosine kinase inhibitor, genistein, on the cell-mediated cytotoxicities of peripheral lymphocytes and NK cells against human leukemia cells (K562) using a ^{51}Cr-release assay. In order to determine which of the cells responsible for the cytotoxic activity, were affected by genistein, we separated the lymphocytes using Percoll gradients and used the NK-rich cells from the LGL fraction. The cytotoxicities that had been exposed to 100 U/ml IL-2 alone for 3 days were 30% and 60% at E/T ratios of 1:1 and 1:4, respectively (Table I). In the presence of 10 μg/ml genistein, which did not affect the permeability of the cells, the cytotoxicities of the IL-2-treated NK fraction were reduced from 30%, 55%, and 60% to 12%, 22%, and 32% at E/T ratios of 1:1, 1:2, and 1:4, respectively. The cytotoxicity of NK-rich cells that had been exposed to IL-2 for 3 days was higher than

Table I. Cytotoxicity (%) of Peripheral Lymphocytes and NK-Rich Fraction for K562

	Peripheral lymphocytes			NK-rich fraction		
E/T ratio	1:6	1:15	1:30	1:1	1:2	1:4
IL-2[a]	65	75	76	30	55	60
IL-2 + genistein[b]	44	63	72	12	22	32

[a] Peripheral lymphocytes or NK-rich fraction were treated with 100 U/ml of IL-2.
[b] Peripheral lymphocytes or NK-rich fraction were incubated with 10 μg/ml of genistein for 30 min before the treatment with IL-2.

that of IL-2-pretreated whole lymphocytes (Table I). In the presence of 10 μg genistein, the cytotoxicity of the NK-rich cells exposed to IL-2 was reduced (Table I), to a greater extent than that of the IL-2-pretreated lymphocytes, which suggest that the cytotoxicity of IL-2-activated NK-rich cells was affected preferentially by genistein.

Effect of IL-2 and/or Genistein on Phosphorylation of IL-2 Receptors Tyrosine Residues. After treatment with IL-2, intracellular signal transduction has been reported to be triggered by IL-2Rβ. Therefore, we studied the effect of IL-2 on phosphorylation of the tyrosine residues in IL-2Rβ by reblotting followed by immunoprecipitation using an anti-IL-2Rβ antibody. Samples immunoprecipitated by anti-IL-2Rβ were prepared from NK- and T-rich cells (LGL and small lymphocyte fractions, respectively) with or without exposure to 100 U/ml IL-2 and 10 μg/ml genistein. A band of about 75 kDa, which was reblotted with the anti-IL-2Rβ antibody, was detected among the phosphorylated tyrosine residues (Fig. 1). Other coprecipitated proteins with band of about 65 and 56 kDa were also detected by silver staining (Fig. 2) or reblotting with the antiphosphotyrosine antibody (PY20) (Fig. 1), which suggests that the tyrosine residues of the proteins that formed complexes with IL-2Rβ were also phosphorylated. The most remarkable result was that the 56-kDa bands were observed in the NK-rich but not the T-rich cell fractions (Fig. 1 and 2: arrows). This suggests that this protein has a high affinity for IL-2Rβ, at least in the NK-rich cells. In the NK-rich cells, the number of phosphorylated tyrosine residues in the 56-kDa protein was increased markedly by IL-2 treatment compared with that of untreated cells. Furthermore, this enhanced phosphorylation was reduced by genistein, which suggests that IL-2 increases tyrosine phosphorylation of the 56-kDa proteins in NK cells.

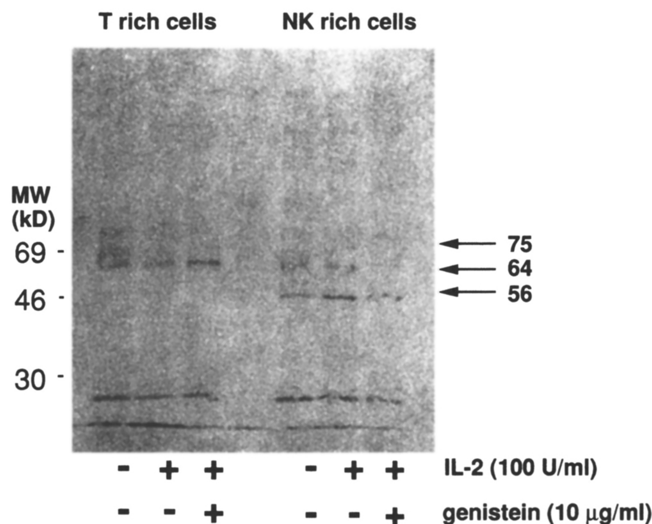

Figure 1. Phosphorylation of tyrosine residues in samples coprecipitated with IL-2Rβ in NK- and T-rich cells. Each sample was obtained from the cells after exposure to IL-2 (100 U/ml) with or without genistein (10 μg/ml), as indicated in the figure. Samples immunoprecipitated by anti-IL-2β were fractionated by 10% SDS-PAGE, transferred to a PVDF filter, and immunoblotted with a mouse antibody against phosphotyrosine (PY20). The arrows indicates 75-, 64-, 56-kDa bands, respectively.

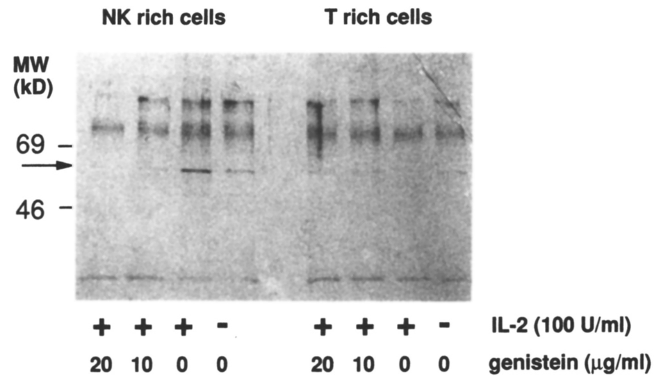

Figure 2. Proteins that coprecipitated with IL-2Rβ in the NK- and T-rich cells. Cell lysates were obtained from the cells after exposure to IL-2 (100 U/ml) with or without genistein (10 μg/ml) as indicated in the figure. The immunoprecipitated samples were analyzed by 10% SDS-PAGE and visualized by silver staining. Arrow indicates the 56-kDa band.

In order to identify the 56-kDa protein that coupled to IL-2Rβ, the samples that were immunoprecipitated by IL-2Rβ were reblotted with an anti-$p56^{lck}$ antibody, which showed that this protein was $p56^{lck}$ (Fig. 3). The amount of $p56^{lck}$ coprecipitated with anti-IL-2Rβ antibody was increased by IL-2 treatment which suggests that the affinity of $p56^{lck}$ for IL-2Rβ in NK-rich cells was increased by IL-2Rβ treatment. In addition, the amount of coprecipitated $p56^{lck}$ and IL-2Rβ was reduced in the sample exposed to both IL-2 and genistein compared with IL-2 alone (Fig. 2). These results suggest that the affinity of $p56^{lck}$ for IL-2Rβ is regulated by IL-2-induced tyrosine phosphorylation. We speculate that the phosphorylation of $p56^{lck}$ on the tyrosine residues increased the affinity of $p56^{lck}$ for IL-2Rβ, which is supported by the increase in tyrosine phosphorylation of the 56-kDa protein observed (Fig. 1, 2, and 3).

In order to confirm that exposure to IL-2 or genistein affect the affinity of $p56^{lck}$ for IL-2Rβ and not the amount of $p56^{lck}$, NK-rich cells were immunoprecipitated with an anti-$p56^{lck}$ antibody and analyzed by reblotting (Fig. 4a). The amount of $p56^{lck}$ was not affected by IL-2 with or without genistein (Fig. 4b). On the other hand, the increased phosphorylation of $p56^{lck}$ by IL-2 was significantly inhibited by genistein ($P < 0.003$ by Student's *t* test).

Effect of IL-2 and or Genistein on the Enzymatic Activity of $p56^{lck}$ in NK-Rich Cells. Next, we evaluated the effects of IL-2 and/or genistein on the enzymatic activity of $p56^{lck}$ in NK-rich cells using immune-complex kinase assays. The lysate from the NK-rich

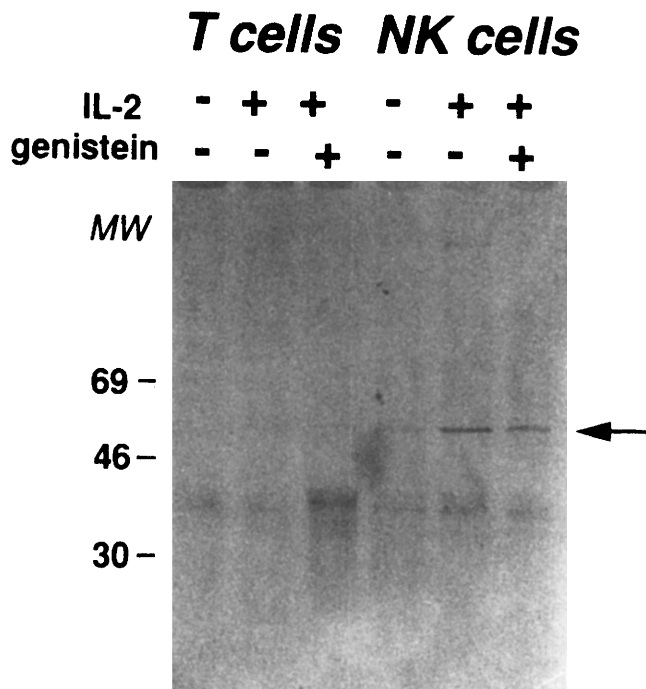

Figure 3. Coprecipitation of p56^{lck} and IL-2Rβ in the NK- and T-rich cells. The samples were obtained from the cells after exposure to IL-2 (100 U/ml) with or without genistein (10 μg/ml), as indicated in the figure. Samples immunoprecipitated by anti-IL-2β were fractionated by 10% SDS-PAGE, transferred to a PVDF filter, and then immunoblotted with a rabbit antibody against human lck kinase. The arrows indicate the position of p56^{lck}.

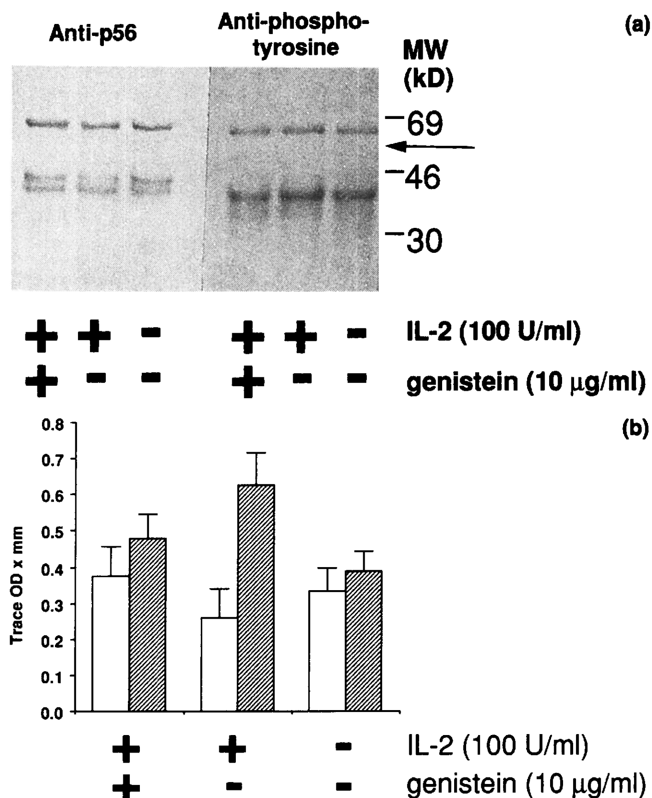

Figure 4. Immunoblotting of p56^{lck} and the NK-rich cells. (a) Lymphocyte lysates after exposure to IL-2 (100 U/ml) with or without genistein (10 μg/ml), as indicated, were immunoprecipitated with anti-human lck kinase, analyzed by 10% SDS-PAGE and anti-human lck kinase and antiphosphotyrosine immunoblotting. Arrow indicates p56^{lck}. (b) Densitometrical measurement of the p56^{lck} of the band of immunoblotting by anti-lck antibody (□), antiphosphotyrosine antibody (▨). SD was calculated by the three different experiments.

cells treated with IL-2 in the presence or absence of genistein were immunoprecipitated with antisera against p56^{lck}. The protein tyrosine kinase activity in the immunoprecipitates was determined by measuring the phosphorylation of acid-denatured rabbit muscle enolase, which was used as an exogenous substrate, and by the autophosphorylation of the p56^{lck} in the NK-rich cells (Fig. 5). Three-fold stimulation of tyrosine kinase activity was observed in the lysate of IL-2-treated cells compared with that of untreated cells. In the presence of genistein, the activity of tyrosine kinase was reduced to 70% of that of the IL-2-treated cells. This result was consistent with the changes in p56^{lck} phosphorylation observed in NK cells treated with IL-2 and genistein.

Discussion

The IL-2 receptor (IL-2R) consists of at least three chains. In this study, we have demonstrated that phosphorylation of the tyrosine residues of p56^{lck} was increased by IL-2 and that genistein decreased IL-2-enhanced this phosphorylation in NK-rich cells. The affinity of p56^{lck} for IL-2Rβ may be related to tyrosine phosphorylation. Therefore, we hypothesize the activation of NK-rich cells by IL-2 was regulated by p56^{lck}-mediated tyrosine phosphorylation.

We also demonstrated that genistein inhibited the cell-mediated cytotoxicity and tyrosine phosphorylation of p56^{lck}. Both of these results may be important for the clarification of the relationship between NK cell-mediated cytotoxicity and intracellular signaling.

In this study, IL-2 did not change the phosphorylation status of IL-2Rβ, although exposure of IL-2Rβ to IL-2 has been demonstrated to induce phosphorylation of its tyrosine residues (11, 25). However, there are possibilities that T cells are less sensitive than NK cells by IL-2. This result also leads support to the suggestion that the cytotoxic effect, which was inhibited to genistein, was attributable to NK-rich cells. It is noteworthy that the 56-kDa protein that coprecipitated with the anti-IL-2Rβ antibody was reblotted with the anti-p56^{lck} antibody only in NK-rich and not in T-rich cells (Fig. 2). This result suggests that the affinity of p56^{lck} for IL-2Rβ is greater in NK-rich than T-rich cells and that the affinity in NK-rich cells was increased by IL-2 treatment. However, the possibility that phosphorylation of p56^{lck} by IL-2 is also coupled to IL-2Rβ in T cells cannot be excluded.

We conclude that phosphorylated p56^{lck} increases its affinity for IL-2Rβ and that phosphorylated p56^{lck}

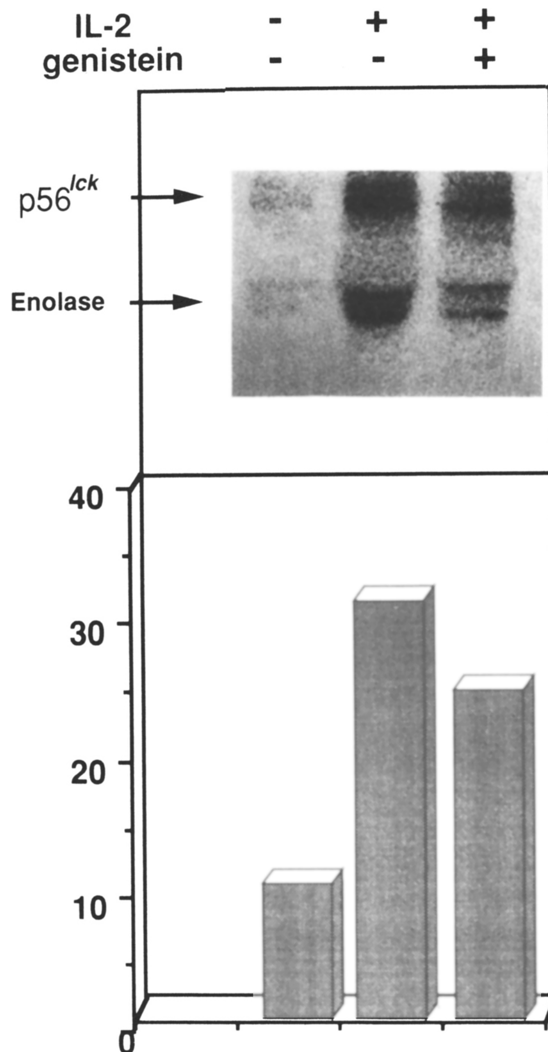

Figure 5. Effect of IL-2 and/or genistein on the enzymatic activity of p56^{lck} protein tyrosine kinase in NK-rich cells. Immune-complex kinase assays were performed on NK-rich cells, and the proteins labeled by [γ-^{32}P]ATP were analyzed by autoradiography after SDS-PAGE. The phosphorylated p56^{lck} and protein tyrosine kinase substrate, enolase, that were detected are indicated. The protein tyrosine kinase activity was quantified using BAS 2000 (Fuji).

is an activated form that phosphorylates IL-2Rβ. Recent studies have demonstrated that p56^{lck} possesses tyrosine kinase activity and phosphorylates IL-2Rβ (23) and, in the light of these results, it was suggested that the tyrosine phosphorylation of p56^{lck} occurs further upstream in the IL-2 signaling process than phosphorylation of IL-2Rβ.

Therefore, our results have demonstrated a potential role for tyrosine kinase, p56^{lck}, in the signaling events that regulate NK cell-mediated cytotoxicity.

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