Korean J Biol Sci 8: 125-133, 2004

TNFβ Induces Cytotoxicity of Antibody-Activated CD4+ T-lymphocytes Against Herpes Virus-Infected Target Cells

Sang Hoon Choi*

Department of Marine Biomedical Science, College of Ocean Science and Technology, Kunsan National University, Kunsan 573-400, Korea

Key Words:
p56^{lck}
Signal transduction
Cross-linking
In vitro kinase assay

We have extended our previous work that cross-linking CD4 molecules using specific MAb induced antigen nonspecific, MHC unrestricted killing of virally infected target cells by CD4+ T cells. The killing activity of antibody activated CD4+ T cells was completely blocked by herbimycin A, a protein tyrosine kinase (PTK) inhibitor, but not by bisindolylamaleimide, a protein kinase C (PKC) inhibitor. Herbimycin A treated human or bovine peripheral blood CD4+ T cells lacked PTK activity and failed to kill virally infected target cells even after cross-linking of CD4 molecules. The CD4 cross-linking failed to induce effector cell proliferation or the transcription of TNFβ. Upregulation of TNFβ was induced by incubating the antibody activated effector cells with BHV-1 infected D17 target cells for 10 h. Anti-TNFβ antibody partially abolished (13-44%) the direct effector cell-mediated antiviral cytotoxicity. However, this antibody neutralized 70 to 100% of antiviral activity of effector and target cell culture supernatants against BHV-1 infected D17 cells. The inhibition level of the antiviral activity by the antibody was dependent on the effector and target cell ratio. These results support the hypothesis that increased p56^{lck} enzyme activity in effector cells transduces a signal critical for effector cell recognition of viral glycoproteins expressed on the target cells. Following target cell recognition, lytic cytokines known to participate in target cell killing were produced. A better understanding of the killing activity displayed by CD4+ T lymphocytes following surface receptor cross-linking will provide insight into the mechanisms of cytotoxic activity directed toward virally-infected cells.

Killing of virus or bacteria infected cells or tumor cells by various kinds of effector cells is a well described self-defense phenomenon of the immune system. Cytotoxic CD4+ T cells are effector cells that kill bacteria or virus infected target cells in a MHC-restricted fashion. However, the killing mechanism of antigen specific CD4+ T cells has not been well characterized.

Resting or cloned CD4+ T cells can be activated by cross-linking of CD3 (Ting and Hargrove et al., 1991; Go et al., 1993; Fragoso et al., 2003) or CD4 molecules (Choi and Splitter, 1994), which results in killing certain target cells in an antigen non-specific and MHC-unrestricted fashion. The CD4 molecule is an integral membrane glycoprotein of 55 kD and a member of the immunoglobulin supergene family (Maddon et al., 1986). The CD4 molecule has two important functions in immmune responses. First, it is a cell adhesion molecule having specific affinity for class II MHC molecules (Biddison et al., 1982). The binding of CD4 to class II MHC molecules stabilizes the interaction of T cell recepter (TCR) and class II MHC-associated antigen. The adhesive role of CD4 may be critical when the TCR affinity is low. Second, CD4 may transduce signals or facilitate the TCR:CD3 mediated signal transduction upon binding class II molecules (Veillette et al., 1989; Sophie et al., 2000). Also, anti-CD4 antibodies induce IL-2 production and proliferation of resting CD4+ peripheral blood T lymphocytes in the absence of additional signals (Carrel et al., 1991; Bernd et al., 2002).

Signal transduction by receptors of the Ig superfamily involves activation of protein tyrosive kinase (PTK). For example, CD4, CD8 (Veillette et al., 1988) and FcγRIIIA of NK cells (Vivier et al., 1991; O'Shea et al., 1991; Ting et al., 1991; Salcedo et al., 1993) are tightly associated with p56^{lck}PTK. While the TCR and FcεRII are associated with p59^{fyn} (Samelson et al., 1990; Sugie et al., 1991), the FcεRI receptor is associated with p56^{lyn} or p62$^{c\text{-}yes}$ (Eiseman and Bolen, 1990). p56^{lck}, a member of the *src* family of tyrosine kinases, is expressed only in lymphoid

*Tel: 82-63-469-1886, Fax: 82-63-463-9493
E-mail: shchoi@kunsan.ac.kr

cells, particularly in T cells where it is associated with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain (Veillette et al., 1998). Anti-CD4 mAb induces a net increase in kinase activity (Veillette et al., 1989 and Luo and Sefton, 1990). Therefore, $p56^{lck}$ likely plays an important role in transducing an extracellular signal through CD4 receptors. Cross-linking CD4 receptors with antibody can activate $p56^{lck}$ associated with the cytoplasmic portion of the CD4 molecule (Veillette et al., 1989 and Luo and Sefton, 1990), resulting in T-cell activation. If antibodies against receptors induce killing of virally infected target cells, the signal transduced via cross-linking of CD4 molecules may be different from that transduced via the MHC-CD4 complex, because the latter does not induce antigen non-specific cytotoxic T cells. Recently, the effect of $p56^{lck}$ on effector function has been reported. Specifically, $p56^{lck}$-deficient mice do not have significant anti-viral effector functions against two different viruses, LCMV and vaccinia virus (Molina et al., 1993), and a $p56^{lck}$ deficient cell line displays a profound reduction in TCR dependent cytolytic effector fucntions (Karnitz et al., 1992). Target cell recognition may transduce signals to the cytoplasm of the effector cells leading to cell activation and lytic function.

In the present study, we investigated the mechanism by which antibody activated $CD4^+$ T cells kill virally infected target cells. The killing activity was completely blocked by herbimycin A, a PTK inhibitor. Herbimycin A treated $CD4^+$ T cells failed to increase PTK activity and kill virally infected target cells, even after cross-linking of CD4 molecules. However, the CD4 cross-linking failed to induce effector cell DNA synthesis (Choi and Splitter, 1994) or TNFβ transcription. Interestingly, the production of TNFβ was induced by incubating the antibody activated effector cells with virally infected target cells. Anti-TNFβ neutralizing antibodies partially blocked the antiviral ability of the antibody activated effector cells during effector-target conjugation. On the other hand, effector and target cell culture supernatants pretreated with anti-TNFβ antibodies effectively abrogated the antiviral activity in these supernatants against BHV-1 infected target cells, suggesting that TNFβ is a major source of virus-specific cytotoxicity observed in the present study. These results support the hypothesis that augmented $p56^{lck}$ enzyme activity is central in the pathway of signal transduction for effector cell lysis of infected target cells.

Materials and Methods

Cells and virus

The adherent canine osteosarcoma, D17 cell line (ATCC no. CCL183, American Type Culture Collection, Rockville, MD, USA) was used as the target cell in ^{51}Cr release assays as previously described (Choi and Splitter, 1994). Human resting $CD4^+$ T cells were negatively or positively purified and bovine resting $CD4^+$ T cells were positively purified using magnetic beads as previously described (Choi and Splitter, 1994). Plaque-purified bovine herpes virus type 1 (BHV-1) Cooper strain (ATCC no. VR-864) was used for all virus experiments.

Antibodies

Human MAbs, anti-CD8 (leu2b), anti-CD16 (leu11b), anti-CD19 (leu12), anti-CD14 (leu-M4), and anti-TCRγδ-1 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA, USA) and used in purifying $CD4^+$ T cells. Bovine anti-CD2 (1E10) and anti-CD3 (MM1A) were provided by William Davis (Washington State University, Pullman, WA, USA). Anti-CD4 (IL-A11) and anti-CD8 (SBU-T8) were purchased from ATCC (no. CRL1879) and the University of Melbourne (Parkville, Victoria, Australia), respectively. Polyclonal rabbit anti-human TNFβ (EP-600) was obtained from Genzyme Corporation. Anti-phosphotyrosine (4G10) and anti-human *lck* kinase (N-terminal) antibodies were obtained from UBI. CLB159 (anti-human CD4) and goat anti-mouse IgG (GαmIgG) were purchased from ICN Immunobiologicals and Sigma, respectively. CLB159 (IgG2b) ascites was passed through a protein A affinity column to purify IgG antibodies for activating effecor cells. GαmIgG was heated for 30 min in a 45°C water bath to aggregate antibodies (Choi and Splitter, 1994).

Cytolytic assay

Chromium release assays were used to assess antibody activated effector cell mediated cytotoxicity. Target cells were incubated with ^{51}Cr-sodium chromate (10 μ Ci/ml)(Du Pont de Nemours) for 60 min at 37°C. Washed labeled cells were infected with BHV-1 for 1 h at a multiplicity of infection (MOI) of 10. Aliquots (10^4 cells/well) of virally infected and uninfected cells were pipetted into 96-well flat bottom plates (Costar). Magnetic cell separator purified human $CD4^+$ T cells or negatively purified $CD4^+$ T cells with or without cross-linking of CD4 moleucles were added (100 μl/well) and incubated for 18 h at 37°C. To examine whether anti-TNFβ antibody could neutralize antiviral ability of antibody activated effector cells, anti-TNFβ or isotype control antibodies (1 μg/ml) diluted in culture medium were applied to the mixture of effector and target cells. The cytotoxic ability of effector-target cell culture supernatant with or without anti-TNFβ neutralizing antibody (1 μg/ml) was investigated using BHV-1 (MOI of 10) infected D17 cells (10^4 cells/well) in the absence of effector cells. For blocking the effector cell cytolytic activity, peripheral blood mononuclear (PBM) cells were treated with the PTK inhibitor herbimycin A (1 μg/ml)(Gibco BRL) or the protein kinase C (PKC) inhibitor bisindolylamaleimide

(1.5 μg/ml)(Calbiochem) 14 h before isolation of $CD4^+$ T cells. The plates were centrifuged and the supernatant was measured for ^{51}Cr by gamma-spectrophotometry. Cytotoxicity was calculated using the formula: % cytotoxicity = (experimental cpm-spontaneous cpm)×100/(maximum cpm-spontaneous cpm).

Immunoblotting

Negatively purified $CD4^+$ T cells obtained by MCS were treated with anti-CD4 (CLB159)(0.3 mg/1×10^7 cells/ml) for 20 min on ice followed by washing cells in ice-cold PBS containing 400 mM $NaVO_3$, 5 mM EDTA, and 10 mM NaF. The washed cells were incubated for 10 min at room temperature with GαmIgG (10 μg/ml). Following washing, the cells were solubilized on ice at 10^8 cells/ml in lysis buffer with 50 mM Tris-HCl, pH 8.0, 2% Nonidet P-40, 10 μg/ml leupeptin, 1 mM PMSF $NaVO_3$, 5 mM EDTA, 10 mM idoacetamide, and 300 mM NaCl for 25 min. Cell lysates were centrifuged at 10,000×g and the postnuclear supernatant was mixed with an equal volume of 2x SDS sample buffer. Samples (equivalent to 5×10^6 cells) were resolved on 7.5% SDS-PAGE and transferred to Immobilon-P (Millipore). Membranes were blocked for 30 min with 0.2% Tween-20 in PBS, pH 7.4 and incubated with anti-phosphotyrosine or anti-$p56^{lck}$ antibodies (diluted to 1 μg/ml in blocking solution) for 1 h at room temperature. After five to six washes with 0.2% Tween-20 PBS, the membranes were exposed to alkaline phosphatase labeled GαmIgG (Promega) or goat anti-rabbit IgG (GαRIgG)(Sigma), washed again, and then subjected to substrate (NBT & BCIP)(Promega) for 10 min.

In vivo and in vitro kinase assays

For the *in vivo* kinase assay, negatively selected $CD4^+$ T cells were washed in phosphate free RPMI with 5% dialyzed FBS followed by incubation at 1×10^7 cells/ml for 30 min at 37°C in the same medium. After 30 min, 0.5 mCi [^{32}Pi] orthophosphoric acid (Du Pont)/ml was added. After a further 3 h incubation, the metabolically labeled $CD4^+$ T cells were treated with medium, anti-CD4 antibody alone or anti-CD4 plus GαmIgG at the indicated times and washed in medium 2 to 3 times followed by cell lysis. Lysates were precleared by incubation with an unrelated rabbit serum (0.5 ml) for 45 min. Samples were immunoprecipitated with a polyclonal anti-$p56^{lck}$ antibody bound to Sepharose 4B (Sigma) for 1 h Immunoprecipitates were resuspended in 2x sample buffer, boiled for 5 min in the presence of 5% 2-mercaptoethanol, and subjected to a 10% SDS-PAGE gel. For *in vitro* kinase assay, the antibody treated $CD4^+$ T cells were lysed in lysis buffer. Immunoprecipitation was performed as described above. After collection of the immune complexes with protein A Sepharose, the Sepharose containing complexes were extensively washed in lysis buffer. Immune complex kinase assays were carried out by adding kinase buffer (20 mM morpholinepropanesulfonic acid, pH 7.0 and 5 mM $MgCl_2$) containing 10 μCi γ-[^{32}P]-ATP (3000 μCi/mmole, Du Pont) and 3 μg of non-acid denatured rabbit muscle enolase (Sigma). The kinase reaction was stopped by adding 2x sample buffer. The samples were resolved on 10% SDS-PAGE gels and transferred to Immobilon-P, and radioactive bands were detected by autoradiography.

RT-PCR

Cytokine profiles were performed with antibody activated $CD4^+$ T cells or effector cells 10 h after incubation with virally (BHV-1) or nonvirally infected D17 cells. Effector cells (>40%) were separated from target cells on a stepwise Percoll (Pharmacia LKB) density gradient (30% and 40%). Cytoplasmic lysates were prepared for mRNA analysis using TRI REAGENT™ (MRC) as recommended by the manufacturer. Each sample containing 1 mg of total RNA was incubated for 1-2 h at 37°C after adding 1 μl of RNasin (Promega), 4 μg oligo (dT)(Promega), 1.5 mM deoxynucleoside triphosphates (dNTPs)(Promega), 400 U reverse transcriptase (murine molony leukemia virus, Promega), and 8 μl reverse transcriptase buffer (Promega).

Amplification of synthesized cDNA from each sample was done using RT-PCR. cDNA (5 μl) was added to a reaction mixture containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), between 1.0 and 3.0 mM $MgCl_2$ (titrated to produce optimal conditions for each primer pair), 0.2 mM dNTPs, 1 mM of each primer (Clontech), and 2.5 U Taq polymerase (Promega). Each 100 μl sample was overlayed with 50 μl of mineral oil (Sigma) and incubated in a DNA thermal cycler (Perkin-Elmer) for a total of 30 cycles. Each cycle consisted of 1 min at 93°C, 2 mn at 55 or 60°C, and 1 min at 72°C. Sense, and antisense primers for IFNγ, TNFβ, and β-actin were purchased from Clontech. Samples were analyzed after 30 cycles of amplification by agarose gel electrophoresis.

Results

Inhibition of cytolytic activity of antibody activated $CD4^+$ cells by PTK inhibitor, but not by PKC inhibitor

Because the CD4 molecule is tightly associated with $p56^{lck}$ PTK, we investigated the role of PTK activity in the induction of cytolytic activity of antibody activated $CD4^+$ T cells by reacting the cells with the PTK inhibitors herbimycin A or genistein. Fig. 1 shows that effector cells pretreated with the PTK inhibitor failed to kill target cells even after cross-linking CD4 molecules. Since the viability of effector cells treated with the PTK inhibitor was the same as that of non-treated effector cells (95%), the possibility of toxicity by the inhibitors was excluded.

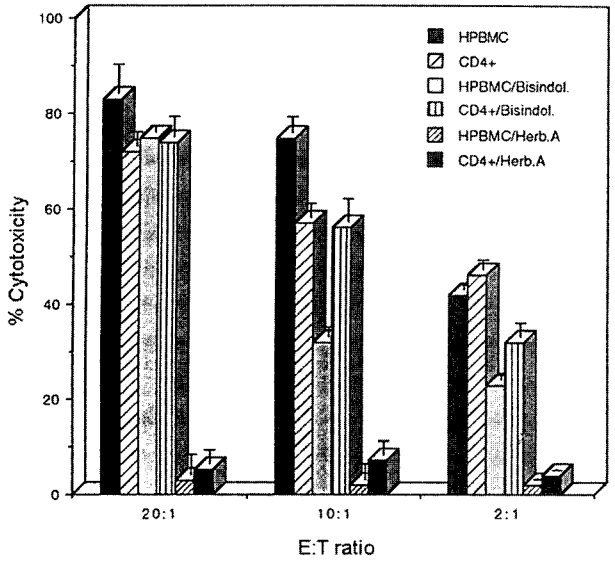

Fig. 1. Inhibition of cytolytic activity of antibody activated CD4⁺ T cells by PTK inhibitor, but not by PKC inhibitor. Human PBM cells (HPBMC) were treated with herbimycin A (PTK) (1 μg/ml) or bisindolylamaleimide (PKC) (1.5 μg) 14 h prior to purifying CD4⁺ T cells. Negatively purified CD4⁺ T cells with CD4 cross-linking were mixed with BHV-1 infected D17 target cells (10^4 cells/well) and incubated for 18 h in a 37°C CO_2 incubator. The plates were centrifuged and ^{51}Cr containing supernatant was measured by γ-spectrophotometer. The figure is a representative of at least 3 experiments. Error bars represent SD from the mean of triplicate wells.

Cells can be stimulated by receptor-ligand binding with subsequent PKC activation through the phosphoinositide turnover process, although cell activation depends on the type of receptor-ligand binding. We tested the impact of a PKC inhibitor on antibody activated CD4⁺ T cells. As shown in Fig. 1, the PKC inhibitior bisindolylamaleimide did not abolish the killing activity at 20:1 E:T ratio, but it did reduce the activity at 10:1 and 2:1 E:T ratio. These results indicate that PTK may play a critical role in inducing the killing activity of antibody activated CD4⁺ T cells. However, it can not be excluded that PKC triggers, to some degree, the effector cytotoxicity. Three experiments using bovine CD4⁺ T cells produced results similar to those using human cells shown in Fig. 1.

Upregulation of p56^{lck} activity after cross-linking CD4 molecules

Using immunoblotting, the relative activity of p56^{lck} was increased in cells activated by anti-CD4 plus GαmIgG compared with activation by anti-CD4 alone, or untreated cells (Fig. 2A). To determine if the protein level of p56^{lck} increased following activation, we compared the p56^{lck} enzyme turnover with p56^{lck} activity by applying anti-p56^{lck} antibodies and anti-phosphotyrosine (αpTyr) antibodies, respectively, to samples transferred to nitrocellulose membranes. As shown in Fig. 2B, using an aliquot of the same cell lysate used in Fig. 2A, the concentration of p56^{lck} from each sample was similar, whereas the level of phosphorylation of p56^{lck} enzyme and other cytoplasmic proteins was different (Fig. 2A). These findings suggest that the cross-linking of CD4 molecules upregulates p56^{lck} activity, but not p56^{lck} protein levels.

To further substantiate the above result, we performed *in vivo* ^{32}Pi labeling and *in vitro* kinase assays. Fig. 3 shows that autophosphorylation of the p56^{lck} enzyme was increased following anti-CD4 plus GαmIgG compared to anti-CD4 alone treated cells or untreated CD4⁺ T cells. The phosphorylation of p56^{lck} was higher at 5 min than 60 min following activation with GαmIgG (Fig. 3). To determine whether binding of ligand to the extracellular domain of CD4 results in an intracellular signal involving alterations of p56^{lck}, we examined the effects of antibody-mediated CD4 cross-linking on the enzymatic activity of p56^{lck}.

The effects of cross-linking CD4 with antibodies were analyzed by immune-complex kinase assays using p56^{lck} specific antibodies. In Fig. 4, the immunoprecipitated p56^{lck} from cells cross-linked with anti-CD4 plus GαmIgG

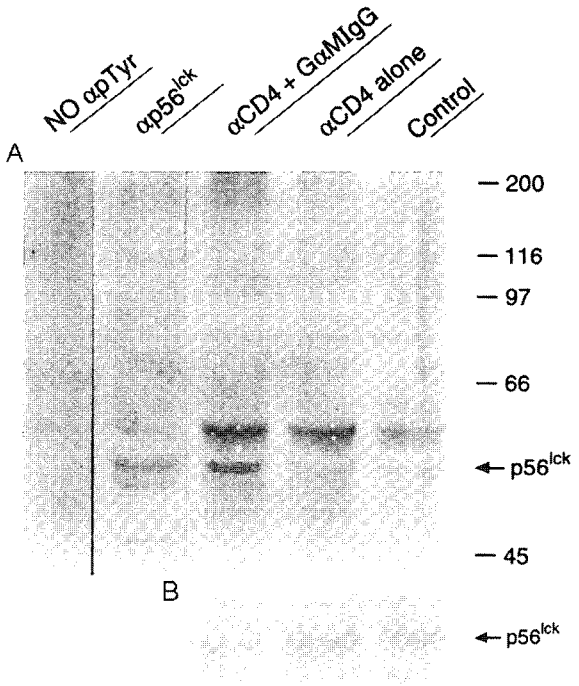

Fig. 2. Immunoblot analysis of the same cell lysates for p56^{lck} phosphorylation activity (panel A) or p56^{lck} protein level (panel B) after CD4 cross-linking. Human CD4⁺ cells were negatively purified by magnetic cell separation. Anti-pTyr antibody (4G10, 1 μg/ml) was applied to three samples prepared from non-activated (control) or activated (αCD4 alone and αCD4+GαmIgG) CD4⁺ cells to identify the tyrosine-phosphorylation (panel A). Anti-*lck* antibody (1 μg/ml) was used to identify the p56^{lck} enzyme (Fig. 2A, αp56^{lck}) or measure the protein level of p56^{lck} enzyme (Fig. 2B). Cells (5×10^6) were incubated with anti-CD4 (0.3 μg/ml) for 20 min and GαmIgG (10 μg/ml) for 10 min for further cross-linking of CD4 molecules. Cell lysate was resolved on 7.5% SDS-PAGE gels.

showed a five- to eight-fold increase in relative PTK activity, as measured by *in vitro* phosphorylation of enolase, an exogenous substrate (Fig. 4). CD4 cross-linking for 5 min resulted in higher phosphorylation of enolase than for 60 min. Thus, phosphorylated substrates are dephosphorylated rapidly within 60 min. As expected, CD4 cross-linking of herbimycin A-pretreated effector cells failed to induce phosphorylation.

TNF-β transcription by antibody activated effector cells following incubation with virally infected target cells

In our previous study, the cross-linking of CD4 molecules induced cytotoxicity but failed to induce T-cell DNA synthesis (Choi and Splitter, 1994). Others have reported that CD4 specific mAb B66 induced IL-2 production and proliferation of resting CD4$^+$ peripheral blood T lymphocytes in the absence of additional signals (Carrel et al., 1991).

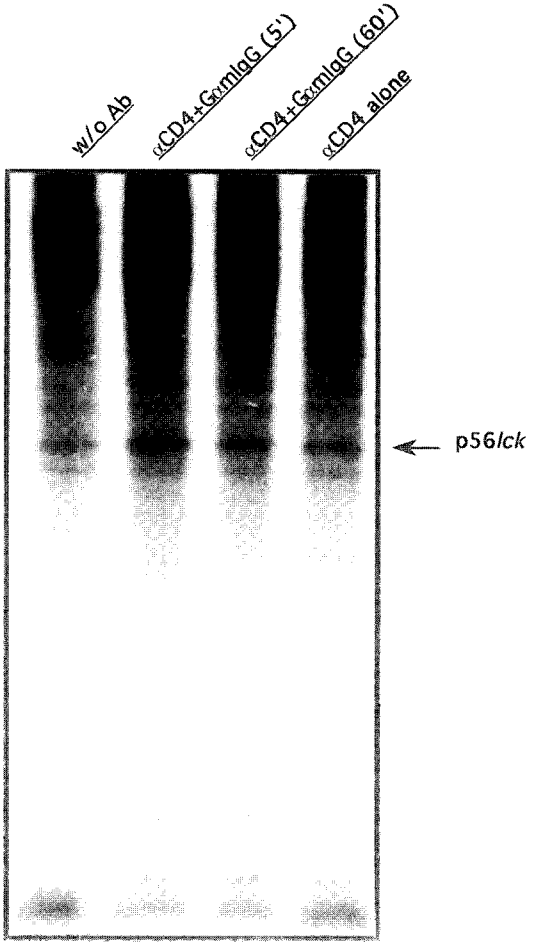

Fig. 3. Immunoprecipitation of p56^{lck} PTK after CD4 cross-linking. Negatively purified human CD4$^+$ T lymphocytes were labeled with 0.5 mCi/ml ^{32}Pi for 3 h and subsequently treated with anti-CD4 (30') and GαmIgG (5' or 60'). The cells were then lysed and p56^{lck} PTK was immunopredipitated as described in Materials and Methods.

We examined the possibility of cytolytic cytokine involvement in inducing the cytolytic activity of effector cells against virally infected target cells. The production of IFNγ and TNFβ from antibody activated effector cells was analyzed by RT-PCR after cross-linking CD4 molecules. However, no difference was observed between antibody activated and unactivated effector cells in production of IFNγ and TNFβ (data not shown). This result suggests that the cross-linking of the CD4 molecule itself can trigger an upregulation of p56^{lck} activity, but is not sufficient to induce the synthesis of cytokines involved in killing target cells. We examined whether activated effector cells incubated with virally infected target cells induce the transcription of lytic cytokines. As shown in Fig. 5, the level of TNFβ was upregulated by incubating effector cells activated by anti-CD4 plus GαmIgG with virally infected target cells, whereas the transcription of IFNγ was detected at the same level in all samples.

Selective cytotoxicity of effector and target cell culture supernatant is neutralized by anti-TNFβ antibodies

Since TNFβ gene transcription was upregulated in the antibody activated CD4$^+$ T cells following incubation with virally infected target cells, we tested whether this cytokine was involved in the cytotoxicity against virally infected cells. Supernatants collected 18 h after incubation of effector and target cells were pretreated with antibodies to TNFβ, and then assayed for their cytotoxicity against BHV-1 infected D17 cells. This antibody pretreatment dramatically reduced (70%) the antiviral activity in these supernatants, suggesting TNFβ

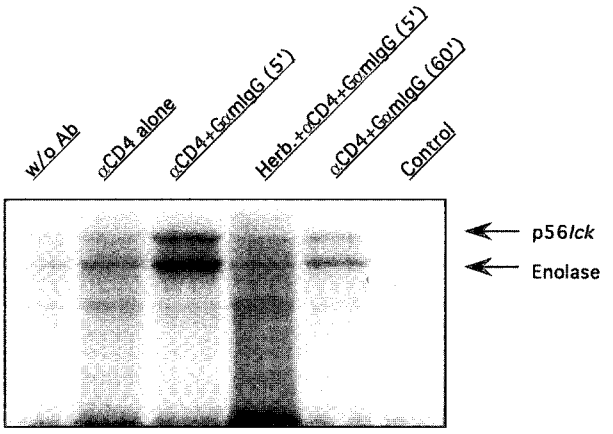

Fig. 4. Immune complex kinase assays following immunoprecipitation with anti-*lck* antibodies. Negatively purified human CD4$^+$ T lymphocytes were treated with the indicated agents at 5 or 60 min. After cross-linking, cells were lysed and immunoprecipitated by polyclonal anti-p56^{lck} (4G10) bound to Sepharose 4B. Immune complex kinase assays were performed on washed immunoprecipitates in the presence of kinase buffer containing 10 μCi γ-[^{32}P]-ATP and non-acid denatured rabbit muscle enolase. Immunoprecipitate without αp56^{lck} was used as control. The reactions were stopped by the addition of 2x sample buffer and the samples were resolved by 10% SDS-PAGE.

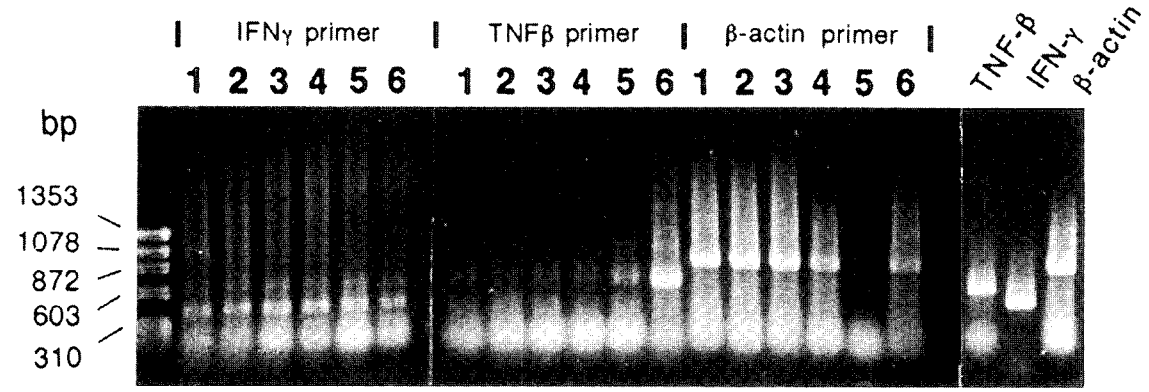

Fig. 5. RT-PCR analysis of cytokine transcription in antibody activated effector cells in the presence of virally infected target cells. Antibody activated or non-activated human $CD4^+$ T lymphocytes 10 h after incubation with BHV-1 infected or non-infected D17 target cells were separated from target cells on a stepwise Percoll density gradient (30 and 40%). Effector cells were harvested from the bottom fraction of the gradient (>40%). Sense, and antisense primers for IFNγ, TNFβ, and β-actin were used for amplification of synthesized cDNA from each sample. Samples were analyzed after 30 cycles (1 min at 93°C, 2 min at 55 or 60°C, and 1 min at 72°C) of amplification by 2% agarose gel electrophoresis. Lanes: 1, $CD4^+$ T cells from D17 cells w/o virus; 2, $CD4^+$ T cells cross-linked with anti-CD4 from D17 cells w/o virus; 3, $CD4^+$ T cells cross-linked with anti-CD4 plus GαmIgG from D17 cells w/o virus; 4, $CD4^+$ T cells from D17 w/ virus; 5, $CD4^+$ T cells cross-linked with anti-CD4 from D17 cells w/ virus; 6, $CD4^+$ T cells cross-linked with anti-CD4 plus GαmIgG from D17 cells w/ virus. The lack of a band in lane 5 of the β-actin reaction is due to a failure to add RT to the reaction.

was the major mediator of this virus-specific cytotoxicity (Table 1). Other cytotoxic molecules, e.g., perforin, serine esterase, or granzymes may be involved in enhancing antiviral cytotoxicity, since 30% cytotoxicity was retained. Although we recognized that anti-TNFβ neutralizing antibodies might have limited access to the cytokines secreted by antibody activated effector cells during effector-target conjugation, we tested whether these antibodies would neutralize any direct effector cell-mediated antiviral cytotoxicity. The addition of anti-TNFβ antibody to a mixture of effector and BHV-1 infected D17 cells in 18 h ^{51}Cr release assay resulted in partial inhibition of antiviral cytotoxicity (13-44% inhibition at 0.05-1 μg/ml of antibodies at 20:1 E:T ratio) (Table 2).

Table 1. Inhibitory effect of anti-TNFβ against antiviral activity in E:T culture supernatants

No of experiment	E:T ratio	% inhibition of cytotoxicity			RIgG 1(μg/ml)
		Anti-TNFβ			
		0	1	0.1	
1	20:1	0	70	40	0
	10:1	0	100	100	0
	2:1	0	100	100	0
2	20:1	0	65	ND	0
	10:1	0	95	100	0
	2:1	0	100	100	0

[a]Lysis was calculated using the formula: (exerimental cpm-spontaneous cpm) x 100/(maximum cpm-spontaneous cpm). Effector-target cell culture supernatants harvested 18 h after incubation were applied to BHV-1 (MOI of 10) infected D17 cells (10^4 cells/well) in the absence of effector cells. The supernatants were pretreated with anti-TNFβ at varying amounts as indicated (μg/ml). The percentage of inhibition was calculated relative to control treated with 1 μg/ml of normal rabbit IgG. ND: not determined.

Discussion

Earlier models of T cell activation suggested that cross-linking of the CD3-TCR complex led to the generation of specific intracellular second messengers (Schwab et al., 1985). The induction of these signals resulted in new gene transcription, followed by protein synthesis, cell proliferation, soluble cytokine secretion, and enhanced cytotoxicity (Leeuwenberg et al., 1985; June et al., 1990). We previously showed that cross-linking of CD4 molecules using specific mAbs induced antigen nonspecific, MHC unrestricted killing by $CD4^+$ T cells and allospecific Th1 clones against virally infected (BHV-1 and HSV-1) target cells (Choi and Splitter, 1994). We hypothesized that increased $p56^{lck}$ activity following cross-linking of CD4 molecules was involved in the induction of effector cell cytotoxicity against virally infected target cells, since $p56^{lck}$ has been implicated in T cell activation after TCR

Table 2. Neutralization of effector antiviral activity by anit-TNFβ

No of experiment	E:T ratio	% inhibition of cytotoxicity						RIgG 1(μg/ml)
		Anti-TNFβ						
		0	5	1	0.5	0.05	0.01	
1	20:1	0	33	44	31	13	0	0
	10:1	0	38	46	8	13	0	0
2	20:1	0	32	41	33	ND	0	0
	10:1	ND	ND	ND	10	11	0	0

Lysis was calculated using the formula: (experimental cpm-spontaneous cpm) x 100/(maximum cpm-spontaneous cpm). Antibody activated human $CD4^+$ effector cells were incubated with anti-TNFβ at varying dilutions, as indicated by the antibody concentration (μg/ml). BHV-1 infected and ^{51}Cr labeled D17 cells were then added as targets at the indicated E:T ratio. The percentage of inhibition was calculated relative to control in which 1 μg/ml of normal rabbit IgG was used. ND: not determined.

engagement (Klausner and Samelson, 1991). Moreover, the association of CD4 with *lck* is crucial for antigen-specific T cell responses (Abraham et al., 1991) and *lck* can act as a signal amplifier (Uehara et al., 1989). Several investigators also reported that cross-linking the CD4 molecule induced a rapid increase in $p56^{lck}$ PTK activity (Veillette et al., 1989 and Luo and Sefton, 1990). It is also known that $p56^{lck}$-deficient mice failed to generate CTL against two different viruses, LCMV and vaccinia virus (Molina et al., 1993) and $p56^{lck}$-deficient CTLL-2 cells failed to exhibit the TCR-dependent cytolytic responses of both factor-deprived and IL-2 stimulated effector cytotoxocity (Karnitz et al., 1992). Although these studies suggest that $p56^{lck}$ participates in signal transduction related to the recruitment of cytotoxic effector cells or the activation of $CD4^+$ T cells by cross-linking CD4 molecules, the actual roles of endogenous $p56^{lck}$ activity in the regulation of T-cell activation and/or growth responses are not yet clear.

In the present study, we have used the selective PTK inhibitors herbimycin A and genistein to investigate the effect of PTK activity on the induction of effector cell cytotoxicity following cross-linking of CD4 molecules on human $CD4^+$ T cells. Herbimycin A non-competitively inhibits several *src*-family PTK including *src* (Nakamura et al., 1986), *lck*, and *fyn* (Klausner and Samelson, 1991), but does not significantly affect protein-serine/threonine kinases, *c-raf* and PKC (Klausner and Samelson). In our study, we showed that herbimycin A blocked the killing activity of antibody activated $CD4^+$ T cells and correspondingly reduced $p56^{lck}$ activity. In contrast, the PKC inhibitor bisindolylmaleimide failed to abrogate effector cell cytotoxicity. These results suggest that upregulation of the $p56^{lck}$ enzyme activity may play a critical role in inducing T cell activation, resulting in cytotoxicity against virally infected target cells.

Cross-linking of CD4 molecules by anti-CD4 alone or anti-CD4 plus GαmIgG resulted in an rapid (i.e., within 5 min) increase in tyrosine phosphorylation of 60 kD and 50 kD cellular substrates and $p56^{lck}$. Also, cells treated with anti-CD4 plus GαmIgG showed three- to four-fold higher $p56^{lck}$ activity than non-activated cells. To examine whether the 60 kD cellular substrate is $p59^{fyn}$, we performed immunoprecipitation using anti-$p59^{fyn}$ and anti-$p56^{lck}$ antibodies after lysing the antibody activated $CD4^+$ T cells. However, $p56^{lck}$ (Fig. 3) but not $p59^{fyn}$ (data not shown) was detected. This result suggests that $p59^{fyn}$ is not involved in protein tyrosine phosphorylation after cross-linking CD4 molecules. In fact, $p59^{fyn}$ is associated with TCR complex (Glaichenhaus et al., 1991). In this regard, we speculated that the signal pathway mediating the cross-linking of CD4 molecules was independent of the TCR complex. To further substantiate the upregulation of $p56^{lck}$ PTK activity, immune-complex kinase assays were performed. The level of tyrosine phosphorylation of enolase, an exogenous substrate, was consistent with autophosphorylation of $p56^{lck}$ PTK observed in immunoblotting and immune-complex kinase assays. This result supports the hypothesis that the augmented $p56^{lck}$ PTK activity after CD4 cross-linking plays a major role in inducing effector cell cytotoxicity.

How the antibody activated $CD4^+$ T cells kill target cells expressing viral glycoproteins is presently unknown. Murine CD8 CTL, but not CD4 Th1 clones, are equipped with perforin, which induces osmotic injury (Henkart, 1985; Young and Cohn, 1986; Strack et al., 1990). Moreover, the cytotoxicity by Th1 clones, but not CD8 CTL, is inhibited by actinomycin D and cycloheximide (Tite, 1990; Tite and Janeway, 1984). Thus, we hypothesized that the antibody activated CD4 effector cells need *de novo* synthesis of cytotoxic machinery for lytic function. The most likely candidates of soluble factors considered were IFNγ and TNFβ, which indeed are secreted by $CD4^+$ killer cells (Tite et al., 1985; Golding et al., 1985; Ju et al., 1990). Unexpectedly, the cross-linking of CD4 molecules failed to elicit transcription of IFNγ and TNFβ from the antibody activated $CD4^+$ T cells, although the effector cells were treated with anti-CD4 plus GαmIgG for 10-18 h. This result demonstrates that CD4 cross-linking itself induces increased $p56^{lck}$ PTK activity, but is not sufficient for induction of the effector cytokines involved in killing target cells.

Cytotoxic T cells release cytolytic factors when stimulated by appropriate target cells (Liu et al., 1989). However, the effector cells require binding of physical association with target cells. We therefore hypothesized that the activated effector cells recognize specific viral glycoproteins expressed on infected target cells and that this results in the release of cytolytic cytokines. Increased synthesis of TNFβ, but not IFNγ, was found in anti-CD4 plus GαmIgG treated effector cells incubated with virally infected target cells. These results suggest that the augmented $p56^{lck}$ activity induced by CD4 cross-linking may result in the synthesis of the lytic cytokine, TNFβ. The mechanism of TNFβ-induced cytotoxicity against virus-infected cells is not completely understood. However, the antiviral effects of the TNFβ can be achieved through several mechanisms. One is to induce a protective effect similar to that induced by IFNα or IFNβ. Uninfected cells, when treated with TNFβ, become resistant to virus infection. This effect is not mediated through IFNα or IFNγ (Wong and Goeddel, 1986). Another complementary mechanism is the potential for TNFβ to selectively lyse virally infected cells (Wong and Goeddel, 1986; Wong et al., 1988). In our study, anti-TNFβ in effector and target cell culture supernatant against BHV-1 infected target cells, suggesting other cytotoxic molecules manufactured by the antibody activated effector cells can participate in the killing mechanism. The addition of anti-TNFβ antibody to a mixture of effector and BHV-1 infected D17 cells partially blocked the cytolytic activity of effector cells. These results suggest either that anti-

TNFβ may not have access to the cytokines produced by the antibody activated effector cells during effector-target conjugation or that other factors such as perforin, serine esterase, or granzymes may also be involved. This latter possibility is also supported by our finding that TNFβ antibody blocked only 70% of the activity of supernatants.

We have previously suggested that the cross-linking of CD4 molecules by soluble gp120 plus anti-gp120 antibody may induce viral specific cytotoxicity in non-virally infected $CD4^+$ cells leading to the death of HIV-infected cells followed by apoptosis of activated $CD4^+$ effector cells (Choi and Splitter, 1994). There have been conflicting reports on the capacity of gp120 to activate CD4-associated $p56^{lck}$. Some workers reported that binding of HIV-1 or gp120 to $CD4^+$ human T cells failed to elicit detectable $p56^{lck}$ PTK activation and signalling (Horak et al., 1990). Others demonstrated that the augmentation of $p56^{lck}$ PTK activity was induced by gp120 and, to a greater extent, by cross-linked gp120 (Juszczak et al., Hivroz et al., 1993). gp120 plays an important role in HIV infection, i.e., virus binding to target cells and syncytium formation. Our findings support the observation of others that the augmentation of $p56^{lck}$ PTK activity occurs by cross-linking CD4 molecules with gp120 plus anti-gp120 (Juszczak et al., Hivroz et al., 1993). It is conceivable that cells with augmented $p56^{lck}$ PTK activity conjugate to virally infected target cells, resulting in the production of TNFβ followed by apoptosis of the activated $CD4^+$ T cells.

In conclusion, our studies demonstrated that CD4 cross-linking augmented $p56^{lck}$ activity, suggesting that $p56^{lck}$ played an important role in transducing a positive activation signal for effector cell lysis of target cells expressing viral glycoproteins.

Acknowledgments

This research was supported by the grant of Fisheries Science Institute in Kunsan National University in 2004.

References

Abraham N, Miceli MC, Parnes JR, and Veillette A (1991) Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase $p56^{lck}$. *Nature* 350: 62-64.

Bernard M, Spriewald J, Billing S, Ensminger SM, Morris PJ, and Wood KJ (2002) Linked unresponsiveness: early cytokine gene expression profiles in cardiac allografts following pretreatment of recipients with bone marrow cells expressing conor MHC alloantigen. *Cytokine* 19: 6-13.

Biddison WE, Rao PE, Talle MA, Goldstein G, and Shaw S (1982) Possible involvement of the OKT4 molecule in T cell recognition of class II HLA antigens. *J Exp Med* 156: 1065-1076.

Carrel S, Salvi S, Gallay P, Rapin C, and Sekaly RP (1991) Positive signal transduction via surface CD4 molecules does not need coexpression of the CD3/TcR complex. *Res Immunol* 142: 97-102.

Choi SH and Splitter GA (1994) Induction of MHC-unrestricted cytolytic $CD4^+$ T cells against virally infected target cells by cross-linking CD4 molecules. *J Immunol* 153: 3874-3881.

Eiseman E and Bolen JB (1990) *Src*-related tyrosine protein kinases as signaling components in hematopoietic cells. *Cancer Cells* 2: 303-308.

Fragoso R, Ren D, Zhang X, Su MWC, Burakoff SJ, and Jin YJ (2003) Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. *J Immunol* 170: 913-921.

Glaichenhaus N, Shastri N, Littman DR, and Turner JM (1991) Requirement for association of $p56^{lck}$ with CD4 in antigen specific signal transuction in T cells. *Cell* 64: 511-520.

Go C, Lanky DW, Fitch FW, and Miller J (1993) Anergized T cell clones retain their cytolytic ability. *J Immunol* 150: 367-376.

Golding H, Munitz TI, and Singer A (1985) Characterization of antigen-specific, Ia-restricted, $L3T4^+$ cytolytic T lymphocytes and assessment of thymic influence on their self specificity. *J Exp Med* 162: 943-961.

Gratton S, Haughn L, Sekaly RP, and Julius M (2000) The extracellular domain of CD4 regulates the initiation of T cell activation. *Mol Immunol* 37: 213-219.

Henkart PA (1985) Mechanism of lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* 3: 31-58.

Hivroz C, Mazerolles F, Soula M, Fagard R, Graton S, Meloche S, Sekaly RP, and Fischer A (1993) Human immunodeficiency virus gp120 and derived peptides activate protein tyrosine kinase $p56^{lck}$ in human $CD4^+$ T lymphocytes. *Eur J Immunol* 23: 600-605.

Horak ID, Popovic M, Horak EM, Lucas PJ, Gress RE, June CH, and Bolen JB (1990) No T-cell tyrosine protein kinase signalling or calcium mobilization after CD4 association with HIV-1 or HIV-1 gp120. *Nature* 348: 557-560.

June CH, Fletcher MC, Ledbetter JA, and Samelson LE (1990) Increase in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J Immunol* 144: 1591-1599.

Ju S-T, Ruddle NH, Strack PI, Dorf ME, and Dekruyff RH (1990) Expression of two distinct cytolytic mechanisms among murine CD4 subsets. *J Immunol* 144: 23-31.

Juszczak RJ, Turchin H, Trun A, Cuhp H, and Kassis S (1991) Effect of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase $p56^{lck}$ with CD4 in human T lymphocytes. *J Biol Chem* 266: 11176-11181.

Karnitz L, Sutor SL, Torigol T, Reed JC, Bell MP, McKean DJ, Leibson PJ, and Abraham RT (1992) Effects of $p56^{lck}$ deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. *Mol Cell Biol* 12: 4521-4530.

Klausner RD and Samelson LE (1991) T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell* 64: 875-878.

Leeuwenberg JFM, Spits H, Tax WJM, and Capel PJA (1985) Induction of nonspecific cytotoxicity by monoclonal anti-T3 antibodies. *J Immunol* 134: 3770-3775.

Liu C-C, Rafii S, Granelli-Piperno A, Trapani JA, and Young JDE (1989) Perforin and serine esterase gene expression in stimulated human T cells: kinetics, mitogen requirements, and effects of cyclosporin A. *J Exp Med* 170: 2105-2118.

Luo K and Sefton BM (1990) Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the *lck*

tyrosine protein kinase at the autophosphorylation site. *Mol Cell Biol* 10: 5305-5309.

Maddon PJ, Dalgleish AG, McDougal SJ, Glapham PR, Weiss RA, and Axel R (1986) The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 47: 333-348.

Molina TJ, Bachmann MF, Kundig TM, Zinkernagel RM, and Mak TW (1993) Peripheral T cells in mice lacking $p56^{lck}$ do not express significant antiviral effector functions. *J Immunol* 151: 699-706.

Nakamura M, Ross DT, Briner TJ, and Gefter ML (1986) Cytolytic activity of antigen-specific T cells with helper phenotype. *J Immunol* 136: 44-47.

O'Shea JJ, Weissman AM, Kennedy ICS, and Ortalo FR (1991) Engagement of the natural killer cell IgG Fc receptor results in tyrosine phosphorylation of the ζ chain. *Proc Natl Acad Sci USA* 88: 350-354.

Salcedo TW, Kurosaki T, Kanakaraj P, Ravetch JV, and Perussia B (1993) Physical and functional association of $p56^{lck}$ with FcγRIIIa (CD16) in NK cells. *J Exp Med* 177: 1475-1480.

Samelson LE, Phillips AF, Luong ET, and Klausner RD (1990) Association of the fyn protein-tyrosine kinase with the T cell antigen receptor. *Proc Natl Acad Sci USA* 87: 4358-4362.

Schwab R, Crow MK, Russo C, and Weksler ME (1985) Requirements of T cell activation by OKT3 monoclonal antibody: role of modulation of T3 molecules and interleukin 1. *J Immunol* 135: 1714-1718.

Strack P, Martin C, Satio S, Dekruyff RH, and Ju S-T (1990) Metabolic inhibitors distinguish cytolytic activity of CD4 and CD8 clones. *Eur J Immunol* 20: 179-184.

Sugie K, Kawakami T, Maeda Y, Kawabe T, Uchida A, and Yodoi J (1991) Fyn tyrosine kinase associated with FcεRII/CD23. Possible multiple roles in lymphocyte activation. *Proc Natl Acad Sci USA* 88: 9132-9135.

Ting AT, Einspahr KJ, Abraham RT, and Leibson PJ (1991) Fcγ receptor signal transduction in natural killer cells: coupling to phospholipase C via a G protein-independent, but tyrosine kinase dependent pathway. *J Immunol* 147: 3122-3127.

Ting CC and Hargrove ME (1991) Anti-CD3 antibody-induced activated killer cells: cytokines as the additional signals for activation of killer cells in effector phase to mediated slow lysis. *Cell Immunol* 135: 273-284.

Tite JP (1990) Differential requirement for protein synthesis and cytolysis mediated by class I and class II MHC-restricted cytotoxic T cells. *Immunology* 70: 440-445.

Tite JP and Janeway CA (1984) Cloned helper T cells can kill B lymphoma cells in the presence of specific antigen: Ia restriction and cognate vs no cognate interaction in cytolysis. *Eur J Immunol* 14: 878-886.

Tite JP, Powell MB, and Ruddle NH (1985) Protein-antigen specific Ia-restricted cytolytic T cell: analysis of frequency, target cell susceptibility, and mechanism of cytolysis. *J Immunol* 135: 25-33.

Uehara Y, Fukazawa H, Murakami Y, and Mizuno S (1989) Irreversible inhibition of *v-src* tyrosine kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. *Biochem Biophys Res Commun* 163: 803-808.

Veillette A, Bookman MA, Horak EM, Samelson LE, and Bolen JB (1989) Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. *Nature* 338: 257-259.

Veillette A, Bookman MA, Horak EM, and Bolen JB (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell* 55: 301-308.

Vivier E, Morin P, O'Brien C, Druker B, Schlossman SF, and Anderson P (1991) Tyrosine phosphorylation of the FcγRIII (CD16): ζ complex in human natural killer cells. Induction by antibody-dependent cytotoxicity bur not by natural killing. *J Immunol* 146: 206-210.

Weiss A, Imboden J, Hardy K, Manger B, Terhorst C, and Stobo T (1986) The role of the T3/antigen receptor complex in T-cell activation. *Annu Rev Immunol* 4: 593-619.

Wong GHW and Goeddel (1986) Tumor necrosis factor α and β inhibit virus replication and synergize with interferons. *Nature* 323: 819-822.

Wong GHW, Krowka JF, Stites DP, and Goeddel DV (1988) *In vitro* anti-human immunodeficiency virus activities of tumor necrosis factor α and interferon-γ. *J Immunol* 140: 120-124.

Young JD and Cohn ZA (1986) Role of granule proteins in lymphocyte-mediated killing. *J Cell Biochem* 32: 1510-1156.

[Received February 23, 2004; accepted May 13, 2004]