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Cytotoxicity of Anti-CD4 Antibody Activated CD4$^+$ T-Lymphocytes against Herpesvirus-Infected Target Cells is Dependent on p56^{lck} and p59^{fyn} Protein Tyrosine Kinase Activity

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MHC unrestricted, antigen nonspecific killing by CD4$^+$ T-cells against virally-infected target cells was induced following cross-linking of CD4 molecules. The cytotoxicity of antibody-activated CD4$^+$ T-cells was abolished by genistein (4′,5,7-trihydroxyisoflavone), a protein tyrosine kinase (PTK) inhibitor, but not by H-7, a protein kinase C (PKC) inhibitor. Genistein-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 cross-linking of CD4 molecules did not induce effector cell proliferation or the transcription of TNF β. TNF β synthesis was up-regulated by incubating antibody activated effector cells with bovine herpesvirus type 1 (BHV-1) infected D17 target cells. Anti-TNF β antibody partially abrogated direct effector cell-mediated antiviral cytotoxicity. On the other hand, this antibody effectively neutralized 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 effector and target cell ratio. These findings have importance to define the mechanisms of how CD4 cytotoxic cells control viral infection

Keywords: *In vitro* kinase assay, p56^{lck}, p59^{fyn}, Signal transduction, TNF β.

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Introduction

In an immune response, cytotoxic T-lymphocytes (CTL) are generated that can lyse antigen-specific target cells in the context of the major histocompatibility complex (MHC). The process of T-cell-mediated cytotoxicity consists of a sequence of events starting with the recognition of target cells by CTL, leading to CTL-target cell adhesion, followed by T-cell activation. After these steps, the CTL–target cell conjugates dissociate, and the lytic process proceeds independently of the CTL.

It is now well established that T-lymphocyte activation is initiated through interactions with the T-cell antigen receptor complex (Haskins *at al.*, 1983; Weiss *et al.*, 1986). In the presence of accessory cells, cross-linking of this complex by monoclonal antibodies directed at either the antigen receptor heterodimer or the noncovalently associated CD3 molecule induces the proliferation of resting T-lymphocytes (Weiss *et al.*, 1986). Cytotoxic CD4$^+$ T-cells are one type of effector cell that kills bacteria- or virus-infected target cells in an MHC restricted fashion (Takayama *et al.*, 1991). 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 CD3 (Biddison *et al.*, 1982; Mentzer *et al.*, 1985; Jung *et al.*, 1987; Ting *et al.*, 1991; Go *et al.*, 1993) or CD4 molecules (Choi and Splitter, 1994), resulting in killing certain target cells in an antigen nonspecific and MHC unrestricted fashion. The CD4 molecule is an integral membrane glycoprotein of 55kD and a member of the immunoglobulin supergene family (Maddon *et al.*, 1986). The CD4 molecule has two important functions in immune responses. First, CD4 is a cell adhesion molecule showing its specific affinity to

MHC class II molecules (Biddison *et al.*, 1982). The binding of CD4 to MHC class II molecules stabilizes the interaction of the T-cell receptor and MHC class II-associated antigen. The adhesive role of CD4 may be most critical when the TCR affinity is low. Second, the CD4 molecule may transduce signals or facilitate TCR:CD3-mediated signal transduction upon binding class II molecules (Veillette *et al.*, 1989: Luo and Sefton, 1990). CD4 may be part of the T-cell antigen recognition complex, physically associated with CD3 and $\alpha\beta$ TCR. 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).

Signal transduction by receptors of the Ig superfamily involves activation of PTK. For example, CD4, CD8 (Veillette *et al.*, 1988), and the NK cell Fc γRIIIA (O'Shea *et al.*, 1991; Ting *et al.*, 1991; Vivier *et al.*, 1991; Salcedo *et al.*, 1993) are tightly associated with $p56^{lck}$ PTK. While the TCR and Fc εRII are associated with $p59^{fyn}$ (Samelson *et al.*, 1990; Sugie *et al.*, 1991), and the Fc εRI 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 only expressed in lymphoid 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.*, 1988). Anti-CD4 mAbs induce a net increase in kinase activity (Veillette *et al.*, 1989; Luo and Sefton, 1990). Therefore, $p56^{lck}$ likely plays an important role in transducing an extracellular signal through CD4 receptors. Cross-linking of CD4 receptors with antibody can activate the $p56^{lck}$ which is associated with the cytoplasmic portion of the CD4 molecule (Veillette *et al.*, 1989; Luo and Sefton, 1990), resulting in T-cell activation. If antibodies against receptors such as CD3 or TCR 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 nonspecific cytotoxic T-cells. Recently, the effect of $p56^{lck}$ on effector function has been reported. Specifically, $p56^{lck}$-deficient mice do not have significant antiviral 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 functions (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 this study, we report the mechanism by which antibody activated $CD4^+$ T-cells kill virally-infected target cells. The cytotoxicity of antibody activated $CD4^+$ T-cells was completely blocked by genistein (4′,5,7-trihydroxyisoflavone), a protein tyrosine kinase (PTK) inhibitor. Genistein-treated $CD4^+$ T-cells failed to increase PTK activity and kill virally-infected target cells, even after cross-linking of CD4 molecules. The cross-linking of CD4 molecules did not induce TNF β transcription. Interestingly, the production of TNF β was induced by incubating antibody activated effector cells with virally-infected target cells. During effector–target conjugation, anti-TNF β antibodies partially neutralized cytotoxicity of antibody activated effector cells. On the other hand, anti-TNF β antibody pretreated effector and target cell culture supernatants effectively abrogated the antiviral activity in these supernatants against BHV-1 infected target cells.

Materials and methods

Antibodies Human MAbs, anti-CD8 (leu2b), anti-CD16 (leu11b), anti-CD19 (leu12), anti-CD14 (leu-M4), and anti-TCR $\gamma\delta$-1 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, USA), and used in purifying $CD4^+$ T-cells. CLB159 (anti-human CD4) and goat anti-mouse IgG (G α mIgG) were purchased from ICN Immunobiologicals (Costa Mesa, USA) and Sigma (St. Louis, USA), respectively. CLB159 (IgG2b) ascites was passed through protein A affinity columns to purify IgG antibodies for activating effector cells. G α mIgG was heated for 30 min in a 45°C water bath to aggregate antibodies (Choi and Splitter, 1994). Anti-phosphotyrosine (4G10), anti-human *lck* and *fyn* kinase (N-terminal) antibodies were obtained from UBI (Lake Placid, USA). Polyclonal rabbit anti-human TNF β (EP-600) was obtained from Genzyme Corporation (Cambridge, USA).

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

^{51}Cr release assay Target cells contained in 15-ml conical tubes were labeled with ^{51}Cr-sodium chromate (10 μCi/ml) (Du Pont de Nemours, Boston, USA) for 60 min at 37°C. 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 dispensed into 96-well flat bottom plates (Costar, Cambridge, USA). Positively purified human $CD4^+$ T-cells or negatively purified $CD4^+$ T-cells with or without cross-linking of CD4 moleucles were added (100 μl/well) to the target cells and incubated for 18 h at 37°C. To examine whether anti-TNF β antibody could neutralize antiviral activity of antibody activated effector cells, anti-TNF β or isotype control antibodies (1 μg/ml) diluted in culture medium were applied to the mixture of effector and target cells. The cytotoxic activity of effector-target cell culture supernatant with or without anti-TNF β 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 protein tyrosine kinase (PTK) inhibitor genistein (1 μg/ml) (Gibco BRL,

Gaitherburg, USA) or protein kinase C (PKC) inhibitor, H-7 (1.5 μg/ml) (Calbiochem, La Jolla, USA) 14 h before isolating $CD4^+$ T-cells. The plates were centrifuged and ^{51}Cr-containing supernatant was measured by gamma-counter. Cytotoxicity was calculated by using the following formula:

$$\% \text{ cytotoxicity} = \frac{(\text{experimental cpm} - \text{spontaneous cpm}}{(\text{maximum cpm} - \text{spontaneous cpm})} \times 100$$

Immunoblot analysis $CD4^+$ T-cells negatively purified by MCS were treated with anti-CD4 (CLB159)(0.3 μg/1 $\times$ 10^7 cells/ml) for 20 min on ice followed by washing cells in ice-cold PBS containing 400 mM $NaVO_3$, 5mM EDTA, and 10 mM NaF. The washed cells were incubated for 10 min at room temperature with the G α mIgG (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 each of leupeptin, 1 mM PMSF, 400 mM $NaVO_3$, 5 mM EDTA, 10 mM iodoacetamide, and 300 mM NaCl for 25 min. Cell lysates were centrifuged at 10,000 $\times$ g and the postnuclear supernatant was mixed with an equal volume of 2$\times$ SDS sample buffer. Samples (equivalent to 5 $\times$ 10^6 cells) were resolved on 7.5% SDS-PAGE and transferred to Immobilon-P (Millipore, Bedford, USA). Membranes were blocked for 30 min with 0.2% Tween-20 in 2% skim milk. The membranes were incubated with anti-phosphotyrosine, anti-$p56^{lck}$, or anti-$p59^{fyn}$ antibodies diluted to 1 μg/ml in blocking solution for 1 h at R.T. After five to six washes with 0.2% Tween-20 PBS, the membranes were exposed to alkaline phosphatase labeled G α mIgG (Promega, Madison, USA) or goat anti-rabbit IgG (G α rIgG) (Sigma), washed again, and then subjected to substrate (NBT & BCIP) (Promega) for 10 min.

***In vitro* kinase assays** For *in vitro* kinase assay, negatively purified $CD4^+$ T-cells were treated with medium, anti-CD4 antibody alone, or anti-CD4 plus G α mIgG with or without genistein at the indicated times and washed in medium 2 to 3 times followed by lysis of cells. Lysates were precleared by incubation with an unrelated rabbit serum (0.5 ml) for 45 min. Samples were immunoprecipitated with a polyclonal anti-$p56^{lck}$ or anti-$p59^{fyn}$ antibody bound to Sepharose 4B (Sigma) for 1 h at R.T. 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/mmol, Du Pont, Boston, USA) and 3 μg of nonacid denatured rabbit muscle enolase (Sigma, St. Louis, USA). The kinase reaction was stopped by adding 2$\times$ sample buffer. The samples with both phosphorylated $p56^{lck}$ and $p59^{fyn}$ were resolved on 10% SDS-PAGE gels and transferred to Immobilon-P, and radioactive bands were detected by autoradiography.

Reverse transcriptase polymerase chain reaction Effector cells, 10 h after incubation, with virally (BHV-1) or nonvirally infected D17 cells were separated from target cells on a stepwise Percoll (Pharmacia LKB, Uppsala, Sweden) density gradient (30 and 40%). RT-PCR was performed according to established methods (Park *et al.*, 1998). TRI REAGENT^{TM} (MRC, Cincinnati, USA) was used to prepare mRNA samples from cytoplasmic lysates. Each sample containing 1 μg of total RNA was incubated for 1–2 h at 37°C after adding 1 μl of RNasin (Promega, Madison, USA), 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, Palo Alto, USA), 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, Norwalk, USA) for a total of 30 cycles. Each cycle consisted of 1 min at 93°C, 2 min at 55 or 60°C, and 1 min at 72°C. Sense and antisense primers for IFN γ, TNF β, and β-actin were purchased from Clontech. Samples were analyzed after 30 cycles of amplification by agarose gel electrophoresis.

Results

Cytotoxic activity of anti-CD4 activated $CD4^+$ T-cells is inhibited by genistein, but not by H-7 Since $p56^{lck}$ and $p59^{fyn}$ PTKs are tightly associated with the CD4 and TCR molecules, respectively, 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 inhibitor, genistein. Effector cells pretreated with genistein failed to kill target cells even after cross-linking CD4 molecules (Fig. 1). Considering that the viability of effector cells treated with genistein was the same as that of nontreated effector cells (95%), the possibility of cytotoxicity by genistein was excluded. Cells can be stimulated by receptor-ligand binding with subsequent protein kinase C (PKC) activation through the phosphoinositide turnover process, although cell activation depends on the type of receptor-ligand binding. We investigated the effect of a PKC inhibitor on antibody activated $CD4^+$ T-cells. Figure 1 shows that the PKC inhibitor H-7 did not abolish the killing activity at a 20:1 effector:target (E:T) ratio. These results indicate that PTKs may play a critical role in inducing the killing activity of antibody activated $CD4^+$ T-cells. However, it cannot be excluded that PKC triggers, to some degree, the effector cytotoxicity. Three experiments using bovine $CD4^+$ T-cells (data not shown) produced results similar to those using human cells shown in Fig. 1.

Both $p56^{lck}$ and $p59^{fyn}$ activities are up-regulated following cross-linking CD4 molecules The relative activities of $p56^{lck}$ and $p59^{fyn}$ were augmented in cells activated by anti-CD4 plus G α mIgG compared with activation by anti-CD4 alone, or antibody nontreated cells (Fig. 2A). To determine whether the protein levels of $p56^{lck}$

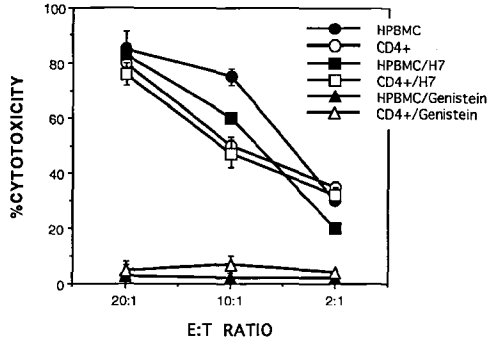

Fig. 1. The inhibition of cytolytic activity of antibody activated $CD4^+$ T-cells by genistein, but not by H-7. Human PBM cells were treated with genistein (PTK) (1 μg/ml) or H-7 (PKC) (1.5 μg/ml) 10 h prior to purifying $CD4^+$ T-cells. Negatively purified $CD4^+$ T-cells activated with anti-CD4 antibodies were incubated with BHV-1 infected D17 target cells (10^4 cells/well) for 18 h in a 37°C CO_2 incubator. The plates were centrifuged and ^{51}Cr-containing supernatant was measured by a gamma counter. The figure is representative of at least three experiments. Error bars represent SD from the mean of triplicate wells.

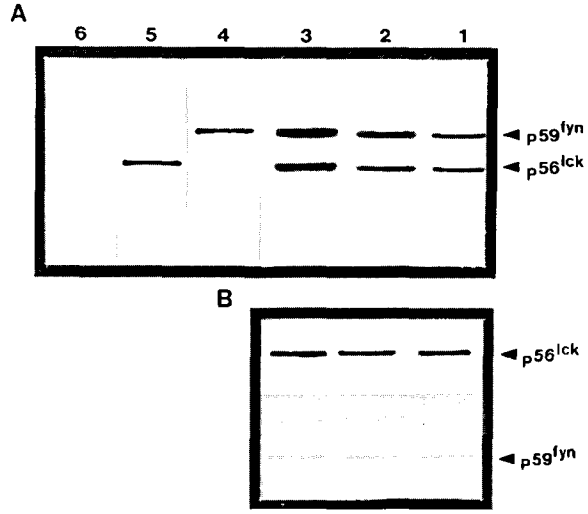

Fig. 2. Immunoblot analysis of the same cell lysates for $p56^{lck}$ and $p59^{fyn}$ phosphorylation activities (panel A) or $p56^{lck}$ and $p59^{fyn}$ protein level (panel B) after cross-linking CD4 molecules. Human $CD4^+$ cells were negatively purified by magnetic cell separation. Anti-pTyr antibody (4G10, 1 μg/ml) was applied to three samples prepared from nonactivated or activated (αCD4 alone and αCD4 + G αmIgG) $CD4^+$ cells to identify the tyrosine-phosphorylation (panel A: lanes 1, 2, and 3, respectively). Anti-*lck* and anti-*fyn* antibodies (1 μg/ml, respectively) were used to identify the $p56^{lck}$ and $p59^{fyn}$ enzymes (panel A: lanes 4 and 5) or measure the protein level of $p56^{lck}$ and $p59^{fyn}$ enzymes from the same samples as used in panel A (panel B: lanes 1, 2, and 3). Lane 6 shows control not treated with anti-phosphotyrosine antibodies. Lane 1: control; 2: anti-CD4 alone; 3: anti-CD4 + G αmIgG; 4: anti-$p59^{fyn}$; 5: anti-$p56^{lck}$; 6: w/o anti-pTyr Ab. Cells (5×10^6) were incubated with anti-CD4 (0.3 μg/ml) for 20 min and G αmIgG (10 μg/ml) for 10 min for further cross-linking of CD4 molecules. Cell lysate was resolved on 7.5% SDS-PAGE gels.

and $p59^{fyn}$ increased following activation, we compared the $p56^{lck}$ and $p59^{fyn}$ enzyme turnover with $p56^{lck}$ and $p59^{fyn}$ activities by applying anti-$p56^{lck}$ or anti-$p59^{fyn}$ 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 $p56^{lck}$ and $p59^{fyn}$ from each sample was similar, whereas the level of phosphorylation of $p56^{lck}$ and $p59^{fyn}$ enzymes was different (Fig. 2A). These findings suggest that the cross-linking of CD4 molecules up-regulates both $p56^{lck}$ and $p59^{fyn}$ activities, but not their protein levels.

To further substantiate the above result, the effects of cross-linking CD4 by antibodies were analyzed by immune-complex kinase assays using $p56^{lck}$ and $p59^{fyn}$ specific antibodies. In Fig. 3, the immunoprecipitated $p56^{lck}$ and $p59^{fyn}$ enzymes from cells cross-linked with anti-CD4 plus G αmIgG show much higher increase, in relative tyrosine-specific protein kinase activity, than normal control or other samples with different activating conditions (Fig. 3). CD4 cross-linking for 5 min resulted in higher phosphorylation of substrates than for 60 min. Thus, phosphorylated substrates are dephosphorylated rapidly within 60 min. As expected, CD4 cross-linking of genistein pretreated effector cells failed to induce phosphorylation.

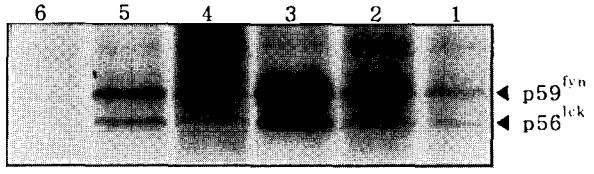

Fig. 3. Immune complex kinase assays following immunoprecipitation with anti-*lck* and anti-*fyn* antibodies. Negatively purified human $CD4^+$ T-lymphocytes were treated with the indicated agents for 5 or 60 min. After cross-linking, cells were lysed and immunoprecipitated by polyclonal $\alpha p56^{lck}$ (4G10) and $\alpha p59^{fyn}$ 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. Immunoprecipitate without $\alpha p56^{lck}$ and $\alpha p59^{fyn}$ was used as control. Lane 1: w/o antibodies; 2: αCD4; 3: αCD4 + G αmIgG (5′); 4: genistein + αCD4 + G αmIgG(5′); 5: αCD4 + G αmIgG (60′); 6: control. The reactions were stopped by adding 2× sample buffer, and the samples were resolved on 10% SDS-PAGE.

Incubation of antibody-activated effector cells with virally-infected target cells up-regulates TNF β transcription Reportedly, 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). Previously, we reported that the cross-linking of CD4 molecules induced

cytotoxicity but failed to induce T-cell DNA synthesis (Choi and Splitter, 1994). We examined whether some cytolytic cytokine is involved in inducing the cytolytic activity of effector cells against virally-infected target cells. The production of IFN γ and TNF β from antibody-activated effector cells was analyzed by RT–PCR after cross-linking CD4 molecules. However, no difference was observed between antibody-activated and nonactivated effector cells in producing IFN γ and TNF β (data not shown). This result suggests that the cross-linking of the CD4 molecule itself can trigger an up-regulation of $p56^{lck}$ and $p59^{fyn}$ activities, 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. 4, the level of TNF β was only up-regulated by incubating effector cells activated by anti-CD4 plus G α mIgG with virally-infected target cells.

Anti-TNF β antibodies block the selective cytotoxicity of effector and target cell culture supernatant We investigated whether TNF β was involved in the cytotoxicity against virally-infected cells because the level of TNF β gene transcription was augmented in antibody activated $CD4^+$ T-cells following incubation with virally-infected target cells. Supernatants collected 18 h after incubation of effector and target cells were pretreated with anti-TNF β antibodies, and then assayed for their cytotoxicity against virally-infected target cells. The antibody pretreatment dramatically reduced the antiviral activity in these supernatants (up to 79%), suggesting TNF β was a major mediator of this virus-specific cytotoxicity (Fig. 5). Other cytotoxic molecules, e.g., perforin, serine esterase, or granzymes may be involved in enhancing antiviral cytotoxicity since 18% cytotoxicity was retained. We tested whether these antibodies would neutralize any direct effector cell-mediated antiviral cytotoxicity. Anti-TNF β antibody, in a mixture of effector and virally-infected target cells, induced partial inhibition of antiviral cytotoxicity (16–43% inhibition at 0.05–1 μg/ml of antibodies E:T ratio of 20:1; Fig. 6).

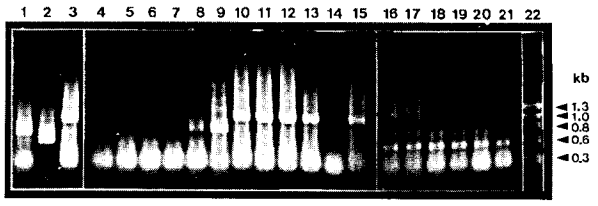

Fig. 4. RT-PCR analysis of cytokine transcription in antibody-activated effector cells in the presence of virally-infected target cells. Antibody activated or nonactivated human $CD4^+$ T-lymphocytes 10 h after incubation with BHV-1 infected or noninfected D17 target cells were separated from target 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 IFN γ, TNF β, 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. Standards: lane 1, TNF β; 2, IFN γ; 3, β-actin. Lanes 4–9, TNF β primer; lanes 10–15, β-actin primer; lanes 16–21, IFN γ; lane 22; marker standard. Lanes 4, 10, 16: $CD4^+$ T-cells from D17 cells w/o virus; Lanes 5, 11, 17: $CD4^+$ T-cells cross-linked with anti-CD4 from D17 cells w/o virus; lanes 6, 12, 18: $CD4^+$ T-cells cross-linked with anti-CD4 plus G α mIgG from D17 cells w/o virus; lanes 7, 13, 19: $CD4^+$ T-cells from D17 w/ virus; lane 8, 14, 20: $CD4^+$ T-cells cross-linked with anti-CD4 from D17 cells w/ virus; lanes 9, 15, 21: $CD4^+$ T-cells cross-linked with anti-CD4 plus G α mIgG from D17 cells w/ virus. The lack of a band in lane 14 of the β-actin reaction is due to a failure to add RT to the reaction.

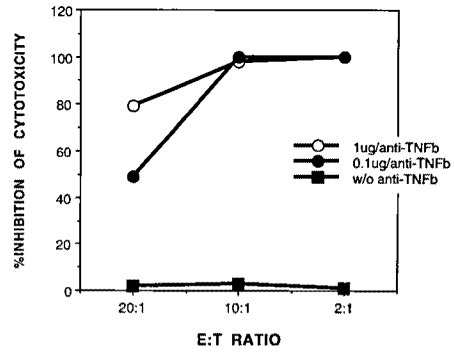

Fig. 5. Inhibitory effect of anti-TNF β against antiviral activity in effector-target cell culture supernatants. Effector-target cell culture supernatants harvested 18 h after incubation were applied to BHV-1 (MOI of 10) infected D17 cells (10^4 cells/well) in the absence of effector cells. The supernatants were pretreated with anti-TNF β at varying amounts as indicated (μg/ml). The percentage of inhibition was calculated relative to the control treated with 1 μg/ml of normal rabbit IgG. Data represent the mean values of two experiments.

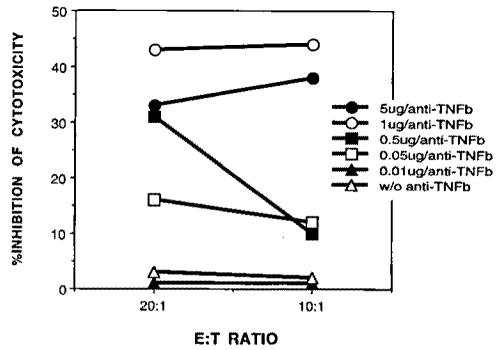

Fig. 6. Neutralization of effector antiviral activity by anit-TNF β. Antibody activated human $CD4^+$ effector cells were incubated with anti-TNF β at varying dilutions, as indicated by the antibody concentration (μg/ml). BHV-1-infected and ^{51}Cr-labeled D17 cells were then added as targets at the indicated E:T ratio. The percentage of inhibition was calculated relative to the control in which 1 μg/ml of normal rabbit IgG was used. Data represent the mean values of two experiments.

Discussion

T-cell activation by cross-linking the CD3-TCR complex induced the production of specific intracellular second messengers associated with a signal transduction (Schwab *et al.*, 1985; Weiss *et al.*, 1986). These signals generated a new gene transcription, followed by protein synthesis, cell proliferation, soluble cytokine secretion, and enhanced cytotoxicity (Hoffman *et al.*, 1985; Leeuwenberg *et al.*, 1985; Manger *et al.*, Schwab *et al.*, 1985; June *et al.*, 1990). We previously reported 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). Since $p56^{lck}$ and $p59^{fyn}$ have been implicated in T-cell activation after TCR engagement (June *et al.*, 1990; Glaichenhaus *et al.*, 1991; Klausner and Samelson, 1991), we studied on the possibility that augmented $p56^{lck}$ or $p59^{fyn}$ activity following cross-linking of CD4 molecules plays a key role in inducing effector cell cytotoxicity against virally-infected target cells. The association of CD4 with *lck* is crucial for Ag-specific T-cell responses (Abraham *et al.*, 1991), and *lck* can act as a signal amplifier (Uehara *et al.*, 1989). Furthermore, cross-linking the CD4 molecule induces a rapid increase in $p56^{lck}$ tyrosine-specific protein kinase activity (Veillette *et al.*, 1989; Luo and Sefton, 1990). It is also reported 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 did not exhibit the TCR-dependent cytolytic responses of both factor-deprived and IL-2 stimulated effector cytotoxicity (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 unclear.

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, we have used the selective PTK inhibitor, genistein. Herbimycin A or genistein inhibits several *src*-family PTKs including *src* (Uehara *et al.*, 1989), *lck*, and *fyn* (Klausner and Samelson, 1991) but does not significantly effect protein-serine/threonine kinases *c-raf* and PKC (Klausner and Samelson, 1991). In the present study, we showed that genistein blocked the cytotoxicity of antibody-activated $CD4^+$ T-cells and correspondingly down-regulated $p56^{lck}$ and $p59^{fyn}$ activities. In contrast, PKC inhibitor, H-7, failed to abrogate effector cell cytotoxicity. These results suggest that up-regulation of the $p56^{lck}$ and $p59^{fyn}$ activities 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 G α mIgG resulted in the rapid (i.e., within 5 min) increase in tyrosine phosphorylation of $p56^{lck}$ and $p59^{fyn}$. Cells treated with anti-CD4 plus G α mIgG showed higher $p56^{lck}$ and $p59^{fyn}$ activities than nonactivated cells. In fact, $p59^{fyn}$ is associated with the TCR complex (Glaichenhaus *et al.*, 1991). In this regard, we speculated that the signal pathway through cross-linking of CD4 molecules is dependent of the TCR complex. To further substantiate the up-regulation of both $p56^{lck}$ and $p59^{fyn}$ PTK activities, immune-complex kinase assays were performed. The level of tyrosine autophosphorylation of both PTKs was observed in immunoblotting and immune-complex kinase assays. This result supports the hypothesis that the augmented $p56^{lck}$ and $p59^{fyn}$ PTK activities by cross-linking CD4 molecules play a major role in inducing effector cell cytotoxicity.

The mechanism by which the antibody-activated $CD4^+$ T-cells kill target cells expressing viral glycoproteins is presently unknown. The cytotoxicity by Th1 clones, but not CD8 CTL, is inhibited by actinomycin D and cycloheximide (Tite and Janeway, 1984; Tite, 1990). Thus, we hypothesized that the antibody-activated CD4 effector cells need *de novo* synthesis of cytotoxic machinery for lytic function. IFN γ and TNF β were considered as the most likely candidates of soluble factors since those are indeed secreted by $CD4^+$ killer cells (Golding *et al.*, 1985; Tite *et al.*, 1985; Ju *et al.*, 1990). Unexpectedly, however, cross-linking of CD4 molecules failed to elicit transcription of IFN γ and TNF β from antibody-activated $CD4^+$ T-cells although the effector cells were treated with anti-CD4 plus G α mIgG for 10–18 h. This result demonstrates that the cross-linking of the CD4 molecule itself is capable of inducing increased $p56^{lck}$ and $p59^{fyn}$ PTK activities but are not sufficient for the induction of effector cytokines involved in killing target cells.

Although the receptor that initiates direct NK cell-mediated cytotoxicity has not been identified, NK cells can also use their FcR to trigger antibody-dependent cell-mediated cytotoxicity (Anegon *et al.*, 1988). FcR cross-linking can affect specific gene transcription, cytokine secretion, and cytotoxicity. In addition to NK cells, cytotoxic T-cells also generate cytolytic factors when stimulated by appropriate target cells (Liu *et al.*, 1989). However, the effector cells should conjugate first with target cells through receptor-ligand association prior to manufacturing cytolytic factors. In this regard, we hypothesized that the up-regulated $p56^{lck}$ activity by cross-linking the CD4 molecule triggers some unknown activation signals to mediate the effector and target cell conjugation through specific viral glycoproteins expressed on infected target cells with synthesis of cytolytic cytokines. It was investigated whether activated effector cells incubated (10 h) with BHV-1 infected target cells induce the production of IFN γ or TNF β. Interestingly,

augmented synthesis of TNF β, but not IFN γ, was observed in anti-CD4 plus G α mIgG treated effector cells incubated with virally-infected target cells. These results strongly suggest that the increased $p56^{lck}$ and $p59^{fyn}$ activities induced by cross-linking CD4 molecules may be central in the pathway of signal transduction for effector cell cytotoxicity by initiating the production of the lytic cytokine, TNF β. The mechanism of TNF β-induced cytotoxicity against virus-infected cells is not completely understood. However, the antiviral effects of the TNF β can be achieved through several mechanisms. One is to induce a protective effect similar to that induced by IFN. Uninfected cells, when treated with TNF β, become resistant to virus infection; this effect is not mediated through IFN α or IFN γ (Wong and Goeddel, 1986). Another complementary mechanism is the potential of TNF to selectively lyse virally-infected cells (Wong and Goeddel, 1986; Wong *et al.*, 1988). In our study, anti-TNF β antibodies failed to completely abrogate antiviral activity of TNF β in effector and target cell culture supernatant against BHV-1 infected target cells, suggesting that other cytotoxic molecules manufactured by antibody-activated effector cells might participate in the killing mechanism. The addition of anti-TNF β 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-TNF β may not have access to the cytokines produced by antibody-activated effector cells during effector-target conjugation or that other factors such as perforin, serine esterase, or granzymes are involved. This latter possibility is also supported by our finding that TNF β antibody blocked only 79% 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 nonvirally-infected $CD4^+$ cells leading to the death of HIV-infected cells, followed by apoptosis of activated $CD4^+$ effector cells. 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 augmented $p56^{lck}$ PTK activity was induced by gp120 and, to a greater extent, by cross-linked gp120 (Juszczak *et al.*, 1991; Hivroz *et al.*, 1993). gp120 plays an important role in HIV infection, i.e., virus binding to target cells and syncytium formation. Whether rapid activation of $p56^{lck}$ and $p59^{fyn}$ PTKs is involved in virus entry is not known. Our findings support the observation of others that the augmented $p56^{lck}$ PTK activity occurs by cross-linking CD4 molecules with gp120 plus anti-gp120 (Juszczak *et al.*, 1991; Hivroz *et al.*, 1993). It is conceivable that cells with augmented $p56^{lck}$ and $p59^{fyn}$ PTK activities conjugate to virally-infected target cells, resulting in the production of TNF β followed by apoptosis of activated $CD4^+$ T-cells.

Taken together, our studies demonstrate that T-cell activation by cross-linking CD4 molecules augments $p56^{lck}$ and $p59^{fyn}$ activities, and plays a central role in transducing a positive activation signal for effector cell lysis of target cells expressing viral glycoproteins. These activated CD4 lymphocytes require viral glycoproteins expressed on the target cells to produce the lytic cytokine, TNF β, indicating the importance of $p56^{lck}$ and $p59^{fyn}$ activities in eliciting the conjugation between effector and target cells expressing particular viral proteins. The information obtained in the study has significance to clarify the mechanisms of $CD4^+$ T-cell killing of virally-infected cells.

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