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Research Article

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HIV Infection–induced Posttranslational Modification of T Cell Signaling Molecules Associated with Disease Progression

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Abstract

In attempt to elucidate the mechanism of the HIV infection induced T cell unresponsiveness, we studied signal-transducing molecules proximal to the T cell receptor (TCR) in T lymphocytes of HIV-infected individuals. Total amounts of protein tyrosine kinases (PTKs) Lck, Fyn, and ZAP-70 and the ζ chain of the TCR were found significantly decreased in T cells of symptomatic/AIDS patients as well as in T cells of individuals in acute and early asymptomatic stages of HIV infection. Unexpectedly, the detection of Lck, Fyn, and ZAP-70 was reversed after the treatment of cell lysates with dithiothreitol. This suggests that PTKs Lck, Fyn, and ZAP-70 were modified by a mechanism altering the status of sulfhydryl groups. Moreover, this mechanism seems to affect selectively T cells of HIV infected patients since B cell PTKs Syk and Lyn were detected structurally and functionally intact. Interestingly, similar alterations of signaling molecules were not detected in T cells of HIV-infected long-term asymptomatic individuals. Modification of T cell PTKs may thus underlie the HIV-induced impairment of lymphocyte function and may potentially predict disease progression. (*J. Clin. Invest.* 1996. 98:1290–1297.) Key words: HIV • T cell receptor • tyrosine phosphorylation • signal transduction • protein tyrosine kinases

Introduction

Complex and progressive deterioration of T cell function is discernible at all stages of infection with human immunodeficiency virus-type 1(HIV-1) (1, 2). Impairment of the T cell re-ceptor (TCR)[1] signal transduction pathway is one of the early markers of progression of HIV infection to the acquired immunodeficiency syndrome (AIDS) (3). T cells from asymptomatic HIV-infected individuals have reduced proliferative capacity in response to recall antigens or to triggering by anti-CD3 monoclonal antibodies (mAbs) (2, 4, 5). Initial decline of response to recall antigen is followed by an impairment of reactivity to allogeneic MHC and mitogens (6). The function of CD4+ cells seems to be affected first (6). Early selective loss of CD4+ memory cells (4) is followed by a gradual deterioration of function of both naive and memory T cells (7) and in the later phase of infection involves also CD8+ cells (6). In the advanced stages of the HIV infection, profound immunodeficiency is associated with severe loss of CD4+ T cells (8, 9).

Gradual deterioration of T cell response in HIV-infected individuals is detectable in in vitro tests of T cell function as well as in delayed type hypersensitivity skin testing (6, 10) and seems to be an indicator of survival time and time for progression to AIDS regardless of number of CD4+ T cells (10). Although an increasing number of studies confirm a direct correlation between an impairment of T cell function and progression of HIV infection, the mechanism responsible for gradual deterioration of T cell function remains to be elucidated.

Signaling function of the TCR complex is critically dependent on its communication with intracellular signaling molecules. Those, critically involved in the initial phase of T cell signal transduction, are protein tyrosine kinases Lck, Fyn, and ZAP-70 (11–13). Their precisely orchestrated activation and interaction with individual chains of the TCR play a central part in T cell activation. Experimentally induced lack of Lck, Fyn, or ZAP-70 in mice have profound effect on downstream TCR signaling (14–17). Moreover, mutations in ZAP-70 kinase were found in patients with a severe combined immunodeficiency syndrome resulting in a TCR signal transduction defect in peripheral CD4+ T cells and CD8+ thymic selection (18–20).

Therefore, defects of T cell function of HIV infected patients could be potentially connected to a defect in the proximal TCR signaling cascade. To evaluate this presumption, we

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1. *Abbreviations used in this paper:* PTK, protein tyrosine kinase; TCR, T cell receptor.

analyzed expression and function of early signaling molecules in T cells isolated from HIV infected individuals in acute, early asymptomatic and symptomatic/AIDS phases of infection. Furthermore, to confirm a direct connection between the stage of T cell signaling molecules, T cell function and disease progression, we also analyzed signaling molecules in T cells of HIV infected long term asymptomatic individuals.

Methods

Cell isolation and activation. Between November 1993 and July 1995, a total of 28 HIV-1 positive symptomatic/AIDS patients accepted for various trials at the Clinical Center of the National Institutes of Health were enrolled onto a prospective study of T cell function. Patients were clinically classified according to the revised criteria from Center for Disease Control and Prevention (CDC) (21). Most symptomatic/AIDS patients were receiving anti-retroviral therapy including AZT and DDI at the time of analysis. HIV-infected individuals in an acute stage of infection were enrolled in the NIH protocol 0H93NCN011. Most of the long-term asymptomatic HIV-infected individuals were participants in the NIAID AVEG/ACTG protocol 101/205. All patients who entered this trial were asymptomatic with a mean CD4+ cell count above 600/mm^3 for all visits and with no single CD4+ cell count below 450/mm^3. All had normal complete blood counts and differentials. None of these individuals were on anti-viral or immunosupressive medications. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors or patients on a Ficoll-Hypaque gradient and monocytes/macrophages were depleted by adherence as described (22). B lymphocytes were isolated by positive selection on anti-CD19 covered magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's protocol. CD4 and CD8 lymphocytes were separated from peripheral blood lymphocytes by negative selection using CD8 and CD4 Dynabeads (Dynal, Oslo, Norway), respectively, according to the manufacturer's protocol. For immunofluorescence, cells were incubated with phycoerythrin (PE)-conjugated anti-CD3 antibody and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 or anti-CD8 antibody (Becton Dickinson, San Jose, CA) and analyzed on a FACScan® (Becton Dickinson, San Jose, CA). Cells were stimulated with the anti-CD3 mAb MEM-92 (IgM) (23) (50 μg/ml) for 2 min at 37°C and lysed in lysis buffer containing 1% NP-40, 10 mM Tris-HCl (pH 8.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, aprotinin (10 μg/ml), leupeptin (10 μg/ml), 0.1 mM quercetin, 0.1 mM TPCK, 0.1 mM TLCK, 0.1 mM ZPSK, and 1 mM Na$_3$VO$_4$ (Sigma Chemical Co., St. Louis, MO). Lysates were spun down by high speed centrifugation in an Eppendorf centrifuge for 5 min. Supernatants were mixed with non-reduced sample buffer and boiled for 5 min. TCR and TCR-CD4 stimulation was done by incubation of cells with magnetic beads covered with anti-mouse immunoglobulin (Dynal, Oslo, Norway) and anti-TCR mAb (T Cell Diagnostics, Cambridge, MA) or anti-TCR mAb and anti-CD4 antibody Leu-3a (Becton Dickinson, San Jose, CA) for 5 min at 37°C. Triggered cells were lysed in lysis buffer described above and lysates were mixed with non-reduced sample buffer and analyzed by 10% SDS-PAGE and immunoblotting. For dithiothreitol (DTT) treatment, freshly prepared DTT (Boehringer Mannheim, Mannheim, Germany) was added to the samples of lysates in SDS-sample buffer to a final concentration of 50 mM and boiled for 5 min.

Immunoblotting. Immunoblotting analysis was done as described (24). The following antibodies were used for detection: an anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), anti-Lck mAb 1F6 (25), rabbit anti-Lck antibody rAbNT (Upstate Biotechnology Inc.), anti-extracellular signal-regulated kinase-2 (ERK-2) (Santa Cruz Biotech., Inc., Santa Cruz, CA), anti-p85 subunit of phosphatidyl inositol kinase-3 (PI-3K) mAb (Upstate Biotechnology, Inc.), anti–β chain constant region of TCR mAb (T cell Diagnostics, Cambridge, MA), anti-Fyn mAb 5D3, rabbit anti-ZAP-70 antiserum, rabbit anti–ζ chain antiserum (26), rabbit anti-Lyn antise-

rum (27) and rabbit anti-Syk antiserum (26). Immunoblots were developed by enhanced chemiluminescence (Boehringer Mannheim, Mannheim, Germany). Lck immunoblots were further analyzed on a scanning densitometer (Hoefer, San Francisco, CA).

Characterization of anti-Lck antibodies. The epitope recognized by the 1F6 mAb is located in the N-terminal portion of the molecule (amino acid sequence [AAS] 39–64) (25). The rAbNT antibody (Upstate Biotechnology Inc.) was prepared against the peptide with AAS 22-51 of Lck. Neither 1F6 mAb nor rAbNT antibody recognized any protein in lysates of Lck negative cells including monocytes, B cells and T cells precleared with anti-Lck antibody 1F6 (data not shown).

PCR analysis. The PCR analysis was done according to the protocol recommended by Invitrogen. Total cellular RNA isolated with TRIsol reagent (Life Technologies, Inc., Gaithersburg, MD) was used as a template to prepare cDNA using Superscript II (Life Technologies, Inc.,). Oligonucleotides corresponding to the human lck cDNA sequence (28) were synthesized. Base pairs 61–79 as the 5′ primer, and base pairs 1201–1220 as 3′ antisense (template strand) were used to amplify the 5′ end of the lck cDNA. The products were analyzed on agarose gels containing ethidium bromide. RNA isolated from SK-OV-3 cell line was used as the negative control of the PCR analysis.

In vitro kinase assay. An in vitro kinase assay was performed on Lck immunoprecipitates with rAbNT antibody as described (24). Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

In vitro HIV infection. CD4+ T cells isolated from a healthy donor were cultivated for 12 h with phytohaemagglutinin (Murex Diagnostic Ltd, Dartford, England) (1 μg/ml). 1×10^8 cells in RPMI 1640 medium with 10% fetal calf serum were incubated with HIV$_{IIIB}$ (10,000 × TCID$_{50}$) (Advanced Biotechnologies Inc., Columbia, MD) for 2 h at 37°C. The infectious titer of HIV$_{IIIB}$ had previously been determined by limiting dilution in the H9 cell line as described (29, 30). The cells were then washed and cultured in medium with IL-2 (10 U/ml) (Cetus Corp.) at a concentration of 1×10^6 cells/ml. Cells were harvested at the indicated time points, washed with ice-cold PBS, pelleted, and frozen at $-70°C$. At the completion of the experiment (day 4), HIV p24 capsid antigen (gag) release into culture supernatant was quantified by a radioimmunoassay (Du Pont Co.) to confirm productive infection. Cells were lysed and analyzed by immunoblotting.

Results

Evidence for posttranslational modification of the protein tyrosine kinase (PTK) Lck in T cells of HIV patients. Phosphorylation of several proteins on tyrosine is one of the earliest detectable responses after the T cell receptor engagement (31). Pattern of induced phosphoproteins marks the pathway of the activation signal in the cell and sequential involvement of individual signaling molecules. Therefore we initially analyzed tyrosine phosphorylation in T lymphocytes isolated from AIDS patients after CD3 cross-linking. Stimulation did elicit the expected tyrosine phosphorylation in both CD4+ and CD8+ T cells isolated from healthy donors. In contrast, no induction of tyrosine phosphorylation was detectable in CD4+ or CD8+ lymphocytes of AIDS patients (Fig. 1 *a*). The profile of tyrosine-phosphorylated proteins in resting T cells of AIDS patients before stimulation was comparable with that of healthy donors except for two additional proteins with molecular masses lower then 30 kD. The fact that the activation signal did not trigger protein tyrosine phosphorylation in T cells of AIDS patients, even though the CD3 antigen was expressed on their surface (Fig. 1 *b*), suggested a disconnection of the TCR from the signaling pathway or dysfunction of some of the initial members of the pathway. To elucidate this unresponsiveness, we initially analyzed expression of the p56^{lck}, one of

the protein tyrosine kinases essential for normal signal transduction through the TCR complex (32), in T cells of symptomatic HIV and AIDS patients. Cells isolated from patients were lysed and the Lck detected by immunoblotting with an anti-Lck mAb 1F6 (25) (Fig. 1 *c*). Surprisingly, Lck was almost undetectable in T cells of patients when compared to the cells isolated from healthy donors. To confirm that a comparable level of protein was present in all samples tested, the blots were also analyzed for the presence of the extracellular-signal regulated kinase-2 (ERK-2), the p85 subunit of phosphatidyl inositol-3 kinase (PI-3K) and TCR–β chain. All of these proteins were detected in the samples of cells isolated from HIV patients with an intensity that was comparable with those in healthy controls with the exception of a lowered level of PI-3K in patient 2 (Fig. 1 *c*). P56^{lck} was therefore apparently uniquely absent in T cells of HIV patients. This observation pointed to a possible defect in the early steps of the signaling cascade. Moreover, a recent study of Cayota et al. (33) showed defects in early tyrosine phosphorylation in CD4+ T cells of HIV patients and decreased levels of Lck.

To elucidate the apparent loss of the p56^{lck} in T cells of HIV patients, we first analyzed the presence of the mRNA encoding for the p56^{lck}. The DNA polymerase chain reaction (PCR) was used to amplify the lck transcript from cells isolated from HIV patients (Fig. 1 *d*). Amplified product of the lck cDNA of the expected size was detected in both control cells and cells of HIV patients but not in the Lck-non expressing SK-OV-3 cell line (Fig. 1 *d*). Therefore, the lack of the p56^{lck} could not be explained by the absence of mRNA.

To explain the apparent contradiction between the PCR data and failure to detect p56^{lck} with anti-Lck mAb 1F6, we tested reactivities of several different anti-Lck antibodies against the same samples of T cells of HIV patients. Using one of them, rabbit antibody rAbNT, we detected p56^{lck} in lysates of T cells of HIV patients with the same intensity as in T cells of healthy donor (Fig. 1 *e*). The 1F6 and rAbNT antibodies were prepared against different even if partially overlapping peptides (see Methods). An unusual discordance between reactivities of these two antibodies with Lck could be result of a change of conformation or a biochemical modification of the p56^{lck} in T cells of HIV patients which selectively affects 1F6 mAb binding site.

Since the difference in reactivities of both anti-Lck Abs offered a new method for the detection of an alteration of the signaling molecule in AIDS patients, we used it for an analysis of the Lck in T cells of HIV-infected individuals in all stages of infection. The Lck modulation was calculated from a difference between reactivity of mAb 1F6 to the equal amounts of
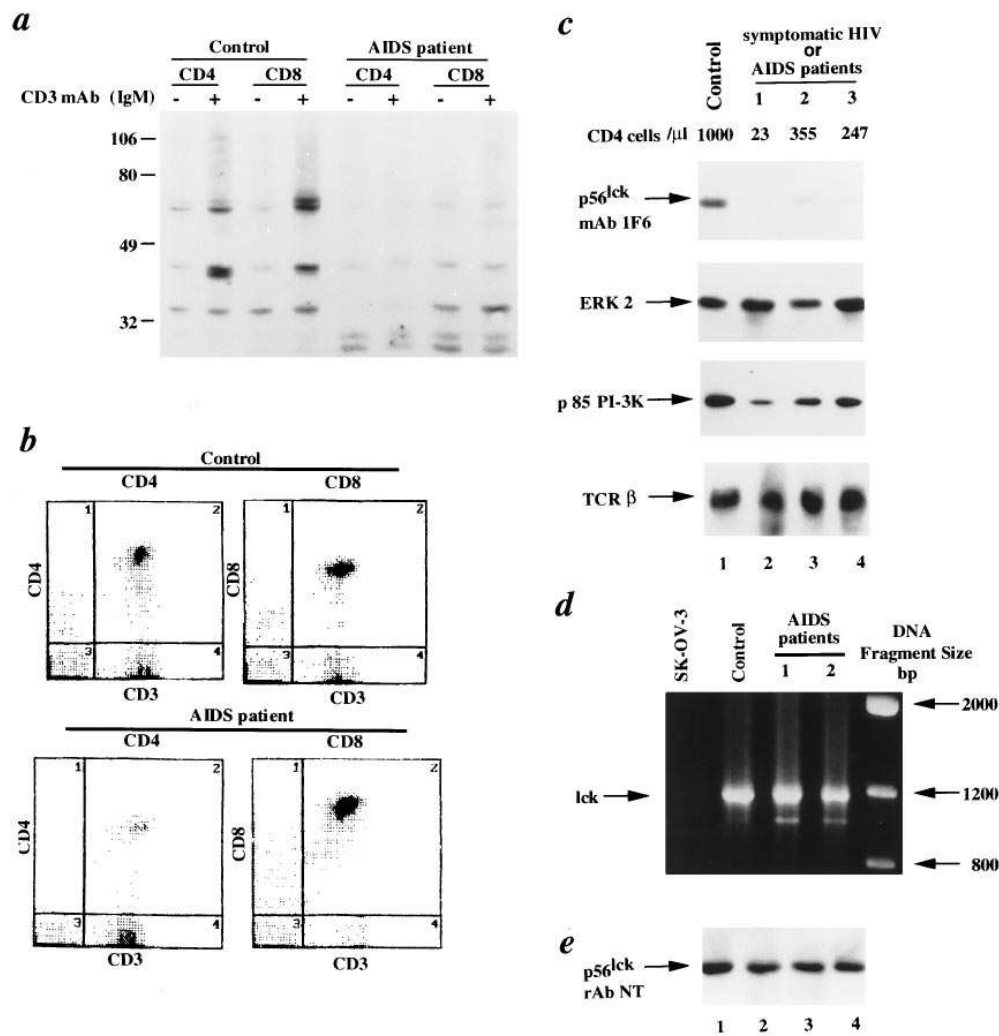

Figure 1. T cells of AIDS patients do not respond to CD3 stimulation and have altered PTK Lck. AIDS patients were clinically classified according to the CDC criteria (21) and numbers of their CD4+ T cells are indicated. T cells were isolated from peripheral blood as described in Methods and stimulated with anti-CD3 mAb for 2 min at 37°C. (*a*) Antiphosphotyrosine immunoblotting of cell lysates after CD3 triggering. Lysates were analyzed under nonreduced conditions. The profile of detected phosphoproteins was identical in presence of reducing agent DTT (data not shown). (*b*) Detection of CD3 expression on CD4/CD8 T-lymphocytes by immunofluorescence. (*c*) Detection of the p56^{lck} by mAb 1F6, ERK2, p85 PI-3K, and TCRβ on immunoblotting. (*d*) Detection of the lck transcripts by RT-PCR technique. (*e*) Detection of the p56^{lck} by rAbNT on immunoblotting.

Lck in T cells of healthy donors and patients determined by rAbNT. Surprisingly, T cells from 7 out of 7 individuals in the acute stage of HIV infection, i.e., in the first 40 d after infection, already contained modified Lck (Fig. 2, *a* and *b*). Similar data were obtained analysing T cells of individuals in the early stage, i.e., 75–332 d after seroconversion. In 10 out of 14 cases the Lck was found to be modulated. In the largest group tested, symptomatic HIV and AIDS patients, 20 out of 28 individuals had severely modulated Lck in their T cells, 4 out of 28 had moderately modulated Lck and in 4 out of 28 cases, the ratio of 1F6 to rAbNT reactivity was comparable to that of healthy donors. The difference between the 8 healthy controls and the 45 HIV-infected patients (all three stages of infection combined into one group) with respect to their degree of p56^{lck} modification is highly significant ($P < 0.00002$ by the Mantel Haenszel test for trends in ranks). In several symptomatic/AIDS patients, the CD4+ and CD8+ subpopulations of T cells were separated before analysis. The Lck modulation was observed in both CD4+ and CD8+ T cells, although in some cases the alteration was apparently more profound in CD4+ cells (Fig. 2 *a* and data not shown). The Lck was also precipitated by rAbNT antibody and analyzed for its kinase activity

in an in vitro kinase assay. The specific kinase activity of Lck was markedly reduced or completely abolished in cells of HIV infected individuals when compared to that of healthy controls (Fig. 2 *a*). Since Lck catalytic and regulatory domains are located on its COOH terminus (34), it is not obvious how the loss of kinase activity is connected to the modulation detected by Abs on the N-terminus, although some previously reported studies showed effects of distal domains on regulation of the Lck kinase activity (35, 36). The T cell Lck of a fraction of symptomatic/AIDS patients was detected as non-modified (Fig. 2 *b*). However, stimulation of these cells via TCR revealed significant changes in the pattern of tyrosine phosphorylation (data not shown). This suggests, that more subtle changes may precede Lck modification but still affect T cell signaling.

The PTKs Fyn and ZAP-70 and the ζ chain of TCR are not detected in T cells of HIV patients. To further characterize the profound perturbation of the TCR signal transduction pathway caused by HIV infection, we analyzed the expression of other signaling molecules participating in the initial phase of T cell activation, the PTKs Fyn and ZAP-70 and the ζ chain of TCR (11). Antibodies used to detect these molecules on im-
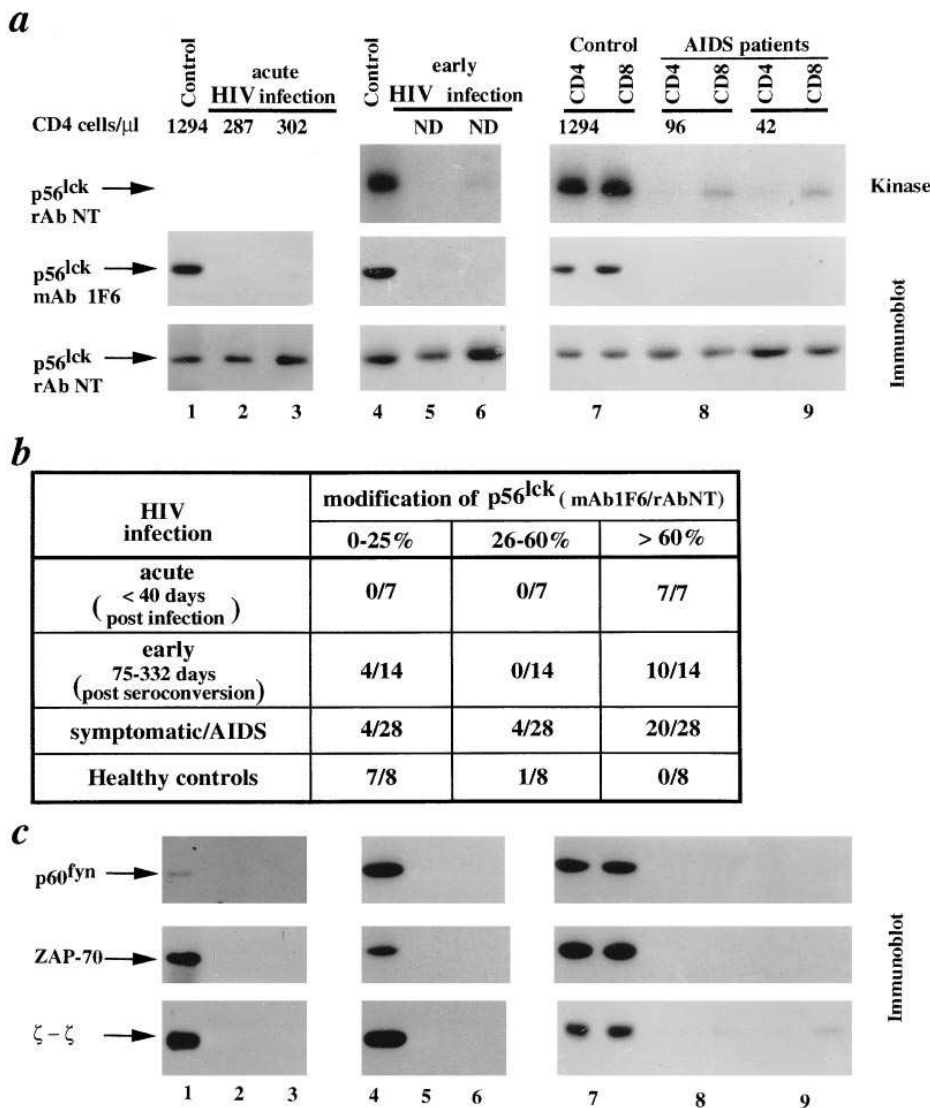

Figure 2. Lck is modified, its specific activity abolished and Fyn, ZAP-70, ζ chain of TCR are not detected in T cells of HIV positive individuals in all stages of infection. (*a*) Protein tyrosine kinase activity (*top panels*) and immunoblotting of the Lck in T cells of patients in acute and early stage of HIV infection and in CD4+ and CD8+ peripheral T cells of patients with symptomatic HIV infection and AIDS. Due to limited amount of cells from patients in acute stage of HIV infection, in vitro kinase assay on Lck immunoprecipitates was performed on only four samples of cells. Lck kinase activities were markedly decreased when compared to those of controls (data not shown). (*b*) Table summarizing the Lck modification as detected by anti-Lck Abs 1F6/rAbNT in T cells of all HIV patients tested. Modification of Lck is expressed as a percentage of Lck detected by 1F6 mAb when compared to Lck detected by rAbNT. (*c*) Immunoblotting detection of the Fyn, ZAP-70 and ζ chain of the TCR. ND means not determined.

HIV infection	modification of p56^{lck} (mAb1F6/rAbNT)		
	0-25%	26-60%	> 60%
acute (< 40 days post infection)	0/7	0/7	7/7
early (75-332 days post seroconversion)	4/14	0/14	10/14
symptomatic/AIDS	4/28	4/28	20/28
Healthy controls	7/8	1/8	0/8

munoblotting showed either very weak or no reactivity with lysates of CD4+ and CD8+ T cells isolated from symptomatic HIV and AIDS patients and individuals in both acute and early stages of HIV infection (Fig. 2 c). Even after using several antibodies raised against different parts of each signaling molecule, we were unable to find an antibody able to detect these proteins (data not shown). However, as for the Lck, PCR analysis revealed the presence of the mRNA encoding Fyn and ZAP-70 in several samples of HIV symptomatic/AIDS patients (data not shown). Based on these data, it was not possible to determine whether Fyn, ZAP-70, and ζ chain were absent in T cells, rapidly degraded after synthesis, or alternatively underwent a similar modification as the Lck and became inaccessible to the antibodies used.

Redox-state involved in modulation of signaling molecules of HIV patients. The Lck was the only molecule identified in T cells of HIV patients in an obviously modified form. The putative modification of the amino acid(s) in the 1F6 binding epitope or a possibility of an overall structural change of the protein were examined further. Surprisingly, a recovery of the epitope recognized by 1F6 mAb in vitro was observed after treatment of the cell lysates with dithiothreitol (DTT) (Fig. 3). In contrast, detection of Lck in T cells of several healthy donors was identical in the presence as in the absence of DTT (data not shown). The structure of the p56^{lck} in T cells of HIV patients thus seems to be altered by a process affecting the free -SH groups of the protein and thereby altering the molecule conformation and accessibility of antibodies. Assuming that similar modification could also change the conformation of other signaling molecules, we re-tested reactivity of anti-Fyn and ZAP-70 antibodies to lysates of T cells of HIV patients treated with DTT (Fig. 3). After the DTT treatment both anti-Fyn and anti-ZAP-70 antibodies detected these proteins in T cells of HIV patients. As in the case of the anti-Lck mAb 1F6, reactivity of both anti-Fyn and anti-ZAP-70 antibodies towards the Fyn and ZAP-70 in lysates of T cells of healthy donors were not dependent on DTT treatment (data not shown).

The PTKs Lyn and Syk are not altered in B cells of HIV patients. The T cell signaling molecules Lck, Fyn, and ZAP-70 have their structural and functional counterparts in early signaling molecules in B cells which transduce the activation signal from the B cell antigen receptor, which include the PTKs Lyn and Syk (11, 34). To determine, whether HIV-induced alterations of signaling molecules also involve B cells, we isolated B lymphocytes from the peripheral blood of AIDS pa-
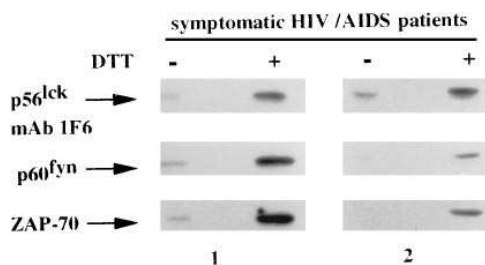

Figure 4. PTKs Lyn and Syk in B cells of AIDS patients are not altered. An in vitro kinase assay on Lyn and Syk immunoprecipitates (*top* and *middle panels*) and immunoblotting detection of Syk (*bottom panel*) in lysates of B cells isolated from symptomatic HIV/AIDS patients and healthy controls. AIDS patients were clinically classified according to the CDC criteria (21). Lck in T cells of these patients was modulated (data not shown).

tients and analyzed Lyn and Syk. The Lyn kinase activity in B cells of AIDS patients was quite comparable to that of control (Fig. 4). Furthermore, the specific kinase activity of Syk was also similar to that of control (Fig. 4). Thus the HIV-induced impairment of signaling molecules seems to affect T cells, but not B cells.

T cell signaling molecules are not altered after in vitro HIV infection. To further elucidate the process leading to alterations of T cell signaling molecules, we searched for a direct effect of the HIV-1 on T lymphocytes. CD4+ T cells isolated from a healthy donor were infected with HIV-1 and the Lck and ζ chain were analyzed in these cells after 1, 12, 48, and 72 h by immunoblotting (Fig. 5). No reduction of the reactivity of mAb 1F6 with Lck or changes in ζ chain detectability were noticed, even up to 72 h after infection. Moreover, no alterations of the Lck, Fyn, ZAP-70, and ζ chain of the TCR were detected either after the long term cultivation of PBMC from a healthy donor with HIV-1 or in the chronically HIV-1–infected T cell lines MOLT-4, CEM and H9 (data not shown). These data indicate that the presence of HIV alone does not seem to be directly responsible for modulation of the early T cell signaling pathway in HIV-infected individuals.

Figure 3. Lck, Fyn and ZAP-70 are detected in T cells of HIV patients after DTT treatment. Analysis of Lck, Fyn and ZAP-70 on immunoblotting in T cells of HIV patients before and following DTT addition. DTT was added to lysates of T cells in SDS sample buffer and boiled for 5 min. Identical result was obtained when DTT was present in the lysis buffer during solubilization (data not shown).
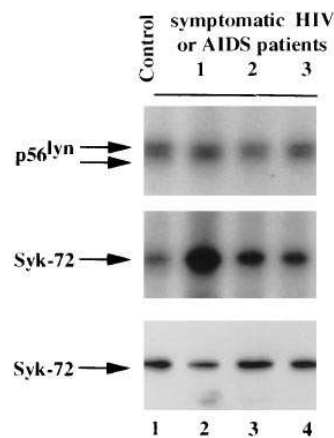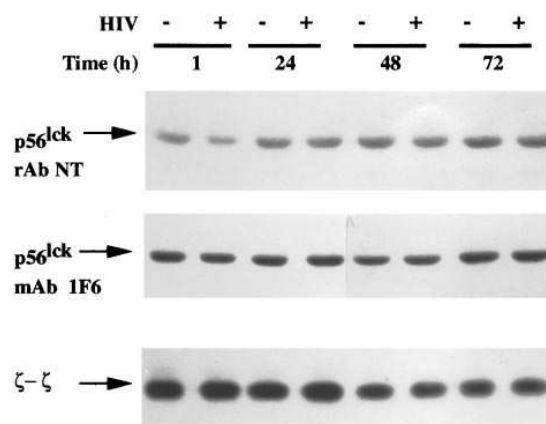

Figure 5. The Lck and ζ chain are not modulated in in vitro HIV-infected CD4+ T cells. CD4+ cells isolated from a healthy donor were stimulated with PHA and in vitro infected with the HIV-1 as described in Methods. Cells were lysed after indicated time and analyzed by immunoblotting for the presence of the Lck and ζ chain.

Lck, Fyn, ZAP-70, and ζ chain of the TCR in T cells of HIV infected individuals with nonprogressive disease. To further elucidate the clinical significance of alterations of T cells signaling molecules for disease progression, we analyzed the T cells of healthy HIV infected individuals who had been HIV-infected for several years with stable CD4 counts > 450/mm³ and not treated with anti-retroviral therapy (Fig. 6). Date of seroconversion, range of CD4+ cell counts, and an average viral load of these individuals are summarized in Fig. 6. The T cells analyzed in Fig. 6 *a* were collected between July 1992 and January 1993. The level of Lck detected by 1F6 and rAbNT antibodies and Lck kinase activity were comparable with those in healthy controls (Fig. 6 *a*). In addition, levels of the other signaling molecules Fyn, ZAP-70, and the ζ chain of the TCR were also comparable with those in healthy donors. Decreased levels of ZAP-70 in samples P3 and P4 may be connected to a moderate degree of downmodulation. However, they may reflect a certain degree of variation of expression of these proteins observed also among healthy individuals (data not shown). Signaling molecules of patients 101868 and 101873 were analyzed again in samples collected in November and December 1995, i.e., 9 yr after the first detection of their HIV seropositivity. Lck protein and kinase activity and level of the ζ chain were found comparable to those of healthy donors. Levels of Fyn and ZAP-70 were decreased at that time (data not shown). Analysis of three other healthy HIV infected individuals infected for 11, 10, and 9 yr, respectively, revealed identical pattern of signaling molecules (data not shown). Differential decrease of Fyn and ZAP-70 in these HIV infected nonprogressors is not obvious and needs to be confirmed by testing of a larger panel of samples together with detailed analysis of TCR signaling.

In addition, functional status of PTKs in signal transduction pathway was tested in T cells isolated from HIV infected nonprogressor by cross-linking of CD3, TCR, and co-crosslinking of the TCR and CD4 antigens (Fig. 6 *b*). T cells of HIV nonprogressor did elicit tyrosine phosphorylation of several proteins on tyrosine with pattern comparable to those detected in T cells of healthy donors (Fig. 6 *b*) confirming thus the functional TCR signaling pathway. Reason for a more intensive tyrosine phosphorylation in cells of patient P5 is not obvious. However, similarly to a certain variation of expression of T cell signaling molecules, pattern of induced tyrosine phosphorylation varied among healthy donors and some of them elicited tyrosine phosphorylation comparable to patient P5 (data not shown). The biological consequences of these differences in tyrosine phosphorylation are not clear so far and need to be tested on a larger panel of patients and healthy controls.

Discussion

The profound changes of the T cell signaling molecules described here in the acute, early asymptomatic and symptomatic stages of HIV infection might at least partially explain the nearly irreversible impairment of T cell function observed through all stages of HIV infection (1, 2). The detection of the oxidation of -SH groups in the Lck, Fyn, and ZAP-70 molecules in T cells of HIV patients was unexpected. The -SH groups of these signaling proteins could be modified by binding of a small molecule or by forming of S-S bonds. However, S-S bonds are rarely, if ever formed in protein in cytosol because the high cytosolic concentration of -SH reducing agents breaks such bonds (37). Finding of the change in the status of sulfhydryl groups of several cytoplasmic signaling molecules in T cells of HIV patients might be related to a disbalanced reducing potential in these cells associated with HIV infection (38, 39), even though the exact nature of modifications remains to be determined.

Effect of redox balance on early signal transduction events has been suggested in previous studies (40, 41). One of the main regulators of cellular redox potential is the cysteine-con-
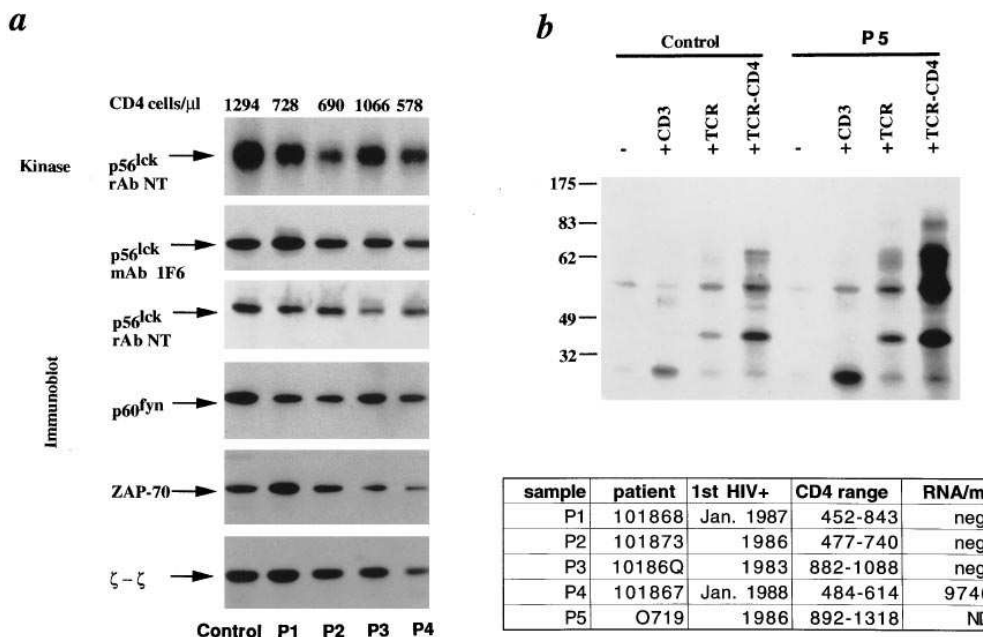

Figure 6. Lck, Fyn, ZAP-70, and the ζ chain in T cells of long term asymptomatic HIV positive individuals and their response to the TCR triggering. (*a*) Protein tyrosine kinase activity (*top panel*) of the Lck in cells isolated from a healthy donor and HIV infected long term asymptomatic individuals and immunoblotting detection of the Lck, Fyn, ZAP-70, and the ζ chain of the TCR. (*b*) Antiphosphotyrosine immunoblotting of cell lysates after CD3, TCR, and TCR-CD4 triggering of peripheral cells isolated from a healthy donor and HIV nonprogressor. The levels of Lck, Fyn, ZAP-70, and the ζ chain in T cells of patient P5 were comparable with those of healthy donors (data not shown). Data on patients are summarized in the Table. Average viral load was determined by Amplicor™ HIV Monitor assay (56). ND, not determined.

sample	patient	1st HIV+	CD4 range	RNA/ml
P1	101868	Jan. 1987	452–843	neg.
P2	101873	1986	477–740	neg.
P3	10186Q	1983	882–1088	neg.
P4	101867	Jan. 1988	484–614	9746
P5	O719	1986	892–1318	ND

taining tripeptide glutathione (GSH). Decrease of the GSH level could completely inhibit T cell activation and has been reported to have a profound effect on immune function (42–45). It has been proposed that HIV infection is associated with systemic deficiency of the GSH at all stages of infection (38, 46, 47). Alternatively, increased levels of oxidized GSH rather than GSH deficiency may be responsible for disturbed redox balance in CD4+ T cells of HIV patients (39). Interestingly, intracellular GSH depletion seems to affect predominantly T cells of HIV patients, whereas B cells do not manifest a significant decrease of GSH concentration (48). Striking correlation between the different functional status of the early signaling molecules in T cells versus B cells observed in this study and T cell–specific depletion of GSH in HIV patients further supports a close connection between the deregulated redox control mechanism and impairment of T cell signaling function.

However, an in vitro induced decrease in the level of intracellular glutathione in normal T cells by treatment with buthionine sulfoximine, an inhibitor of glutathione synthesis, is not sufficient to induce the modulation of signaling molecules identical to those detected in T cells of HIV patients (data not shown). This suggests that conditions of an in vitro experiment can not mimic entirely an effect of a chronic oxidative stress induced by an excessive antigenic stimulation (37) or/and participation of an additional mechanism on an alteration of the TCR signaling in vivo.

Furthermore, the lost of the ζ chain detected in T cells of HIV infected patients can not be directly explained by an alteration of redox potential in T cells since the ζ chain does not contain intracellular cysteine residues. The mechanism underlying the ζ chain down-modulation remains to be defined.

HIV alone does not seem to be directly responsible for modulation of the early T cell signaling pathway since in vitro infection of healthy CD4+ T cells failed to induce any alterations similar to those detected in patients. However, in vitro infected T cells can not recapitulate the complex interactions between peripheral T cells and follicular dendritic cells in HIV infected lymph nodes or other secondary lymphoid organs (49–51).

A small fraction of HIV infected individuals remain immunologically competent with a stable CD4+ count over many years without anti-retroviral therapy (52–54). The reason for an apparent resistance of immune system of these individuals to the disease progression (55) is not understood. However, a contrast between the profound alterations of signaling molecules in T cells of HIV patients (Fig. 2), and the obviously normal pattern of these proteins in T cells of HIV infected long term asymptomatic individuals (Fig. 6 *a*) suggests a direct connection between the status of signaling molecules in T cells and deterioration of immune function. Based on these data, it is however not possible to determine if the alteration of T cell signaling molecules is due to a differential viral burden in HIV infected individuals.

Nevertheless, alterations of signaling molecules seem to be one of the earliest signs of progressive disease and may help to identify individuals at high risk for rapid progression towards AIDS at the early stage of their infection. A prospective study of HIV infected individuals with stable versus progressive disease will be needed to provide a paradigm of value in understanding the processes leading to T cell immunosuppression.

Study of events leading to the alteration of signaling molecules associated with T cell dysfunction during HIV infection may also help to uncover the process, which regulates a deli-cate balance of the immune system function. Therapeutic intervention, targeted specifically to abrogate altered T cell signaling transduction pathway(s) in HIV patients may open new avenues to the reconstitution of the impaired immune system.

Acknowledgments

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