In order to confirm the potency of the retroviral vectors, we transduced wild-type Jurkat cells and a Jurkat variant that harbors neither α nor β TCR ($\alpha\beta^-$ Jurkat) with these constructs, stained them with HLA tetramer, and analyzed them by flow cytometry (Fig. 1B). The $\alpha\beta^-$ Jurkat variant transduced with both α and β TCR 589 genes appeared to be stained by the HLA tetramer, whereas the Jurkat cells and the $\alpha\beta^-$ Jurkat variant transduced with the β gene alone were not stained by the HLA tetramer (Fig. 1B). These results clearly indicate that the TCR 589 complex was expressed on the surface of the transduced cells and that the TCR had antigen specificity identical to that of the parental CTL 589.

2.2 TCR gene transfer to primary human CD8 T cells

Primary human CD8 T cells prepared from an HIV-negative donor carrying *HLA-B**3501* were activated by plate-coated OKT3 mAb and sequentially transduced with α and β genes of TCR 589. The transduced CD8 T cells were selected for their expression of GFP and CD24 antigen using fluorescence-activated cell sorting and stimulated again with the plate-coated OKT3 mAb. A fraction representing 9.5% of the resultant CD8 T cells bound the HLA tetramer in complex with the Pol peptide, whereas only 0.05% of the cells were stained by the tetramer in the case of the mock-transduced cells (Fig. 2A). We then stimulated these cells with the Pol peptide and cultured them for 10 days in the presence of rIL-2. Of great interest, the tetramer$^+$ fraction increased to 35% of the TCR-transduced cells, whereas the level of GFP and CD24 expression remained comparable (Fig. 2A), suggesting preferential proliferation of the tetramer$^+$ phenotype in response to antigen stimulation.

We then tested the TCR-transduced cells for their cytotoxic activity toward HLA-B*3501-expressing cells either pulsed with the Pol peptide or infected with HIV-1. The TCR-transduced cells showed substantial cytotoxic activity toward the peptide-loaded cells even at a peptide concentration down to 0.01 nM (Fig. 2B). Also, the cells showed cytotoxic activity toward HIV-infected cells, whereas they were not cytotoxic toward uninfected cells (Fig. 2C), indicating the antigen-specific and high-avidity nature of the TCR-transduced T cells.

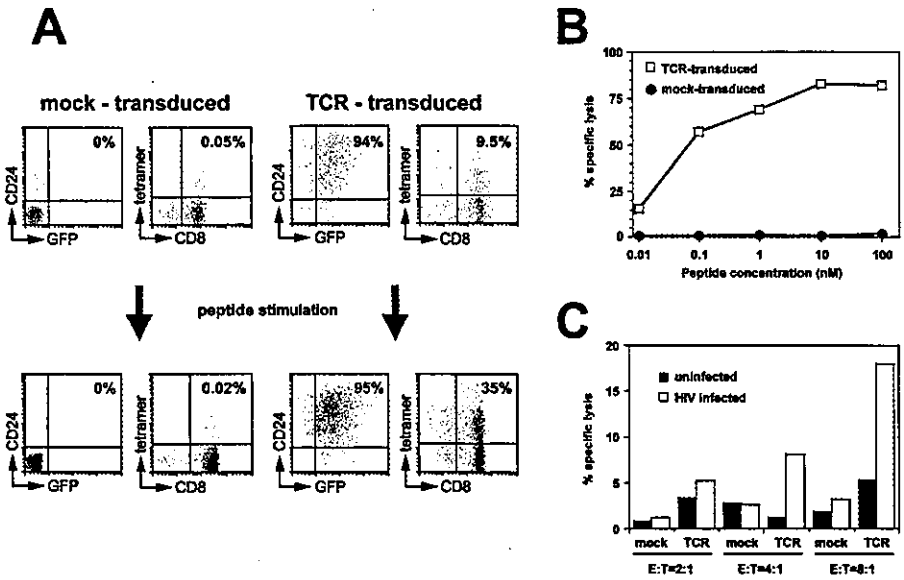

Fig. 2. Flow-cytometric and functional analysis of TCR-transduced primary CD8 T cells. (A) Primary human CD8 T cells that had been sorted into the GFP$^+$ CD24$^+$ fraction after transduction with TCR-encoded vectors were stained with anti-CD24-PE, anti-CD8-PerCP, and tetramer-allophycocyanin, and analyzed by flow cytometry before or after antigen stimulation. Mock-transduced cells were analyzed as above. Frequencies of GFP$^+$ CD24$^+$ or CD8$^+$ tetramer$^+$ fractions within the total cell population are indicated in the upper right corners of each dot plot. (B, C) The mock- or TCR-transduced cells were analyzed for their cytotoxic activity toward 221-CD4-B*3501 cells (2×10^4) either pulsed with the indicated concentrations of Pol peptide at an E/T ratio of 4:1 (B) or infected with HIV-1 LAI at the indicated E/T ratios (C). Data are shown as the means of duplicate assays in one experiment; an additional experiment gave similar results.

2.3 Maintenance of ligand specificity and functional avidity of TCR-transduced CD8 T cells

We next asked whether the ligand specificity and functional avidity of the parental CTL clone 589 would be preserved in the TCR-transduced T cells. The tetramer$^+$ subset of the TCR-transduced T cells was sorted and cultured with autologous feeder cells pulsed with the Pol peptide. A fraction comprising 82% of the resulting tetramer$^+$-sorted cells were CD8$^+$ tetramer$^+$ with a mean fluorescence intensity (MFI) value for the tetramer of 225; whereas >98% of the parental CTL 589 were CD8$^+$ tetramer$^+$ with an MFI for the tetramer of 411 (Fig. 3A).

Effector functions of the tetramer$^+$-sorted cells and parental CTL 589 appeared to be comparable as assessed by their cytokine production activity (Fig. 3B) and their cytotoxic activity (Fig. 3C) in response to the Pol peptide-pulsed target cells, indicating that the functional avidity of parental CTL 589 was maintained in the TCR-transduced T cells. In addition, the antigen specificity of both cells was tested using a series of naturally occurring mutations in the epitope region found in a database (http://www.hiv.lanl.gov/content/index). Both cells showed a similar pattern of reactivity toward the mutant peptides tested (Fig. 3C), indicating that the peptide fine-specificity of CTL 589 was preserved in the TCR-transduced T cells.

We also tested the TCR-transduced T cells for their ligand specificity toward HLA class I molecules, since CTL clone 589 was previously found to exhibit dual specificity, recognizing the Pol peptide presented by both HLA-B*3501 and HLA-B*5101 [26]. The TCR-transduced T cells showed a peptide-specific response to cells expressing either HLA-B*3501 or HLA-B*5101, and their cytotoxic activities were comparable to those of parental CTL 589 (Fig. 3D), indicating that the TCR-transduced

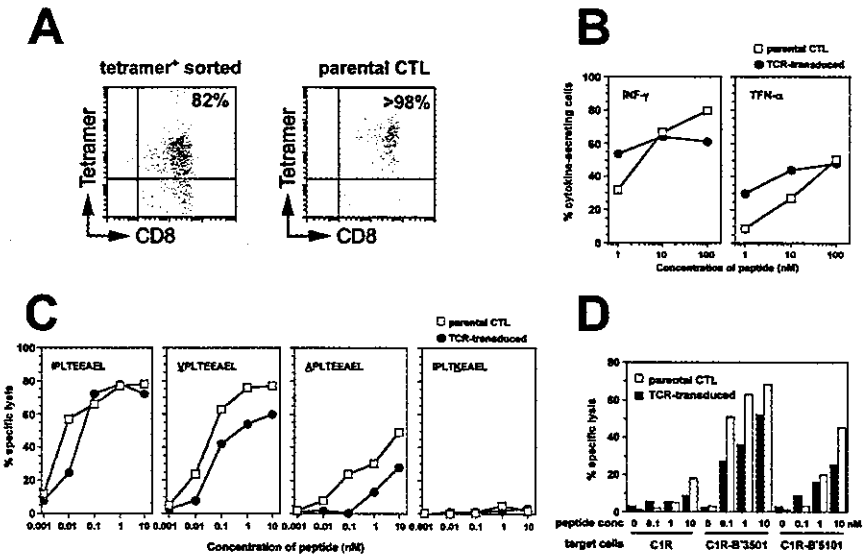

Fig. 3. Ligand specificity and functional avidity of TCR-transduced T cells compared with those of parental CTL 589. (A) The CD8$^+$ tetramer$^+$ subset of the TCR-transduced cells were sorted and cultured with autologous feeder cells pulsed with the Pol peptide. The resultant tetramer-sorted TCR-transduced cells and the parental CTL 589 were stained with anti-CD8-PerCP and tetramer-allophycocyanin and analyzed by flow cytometry. The frequency of the CD8$^+$ tetramer$^+$ subset within the total cell population is shown in the each dot plot. MFI values of tetramer-sorted TCR-transduced cells and the parental CTL 589 for the tetramer were 225 and 411, respectively. (B) Cytokine secretion activity of the tetramer-sorted TCR-transduced cells and the parental CTL 589 were analyzed in response to C1R-B*3501 cells pulsed with various concentrations of Pol peptide at an E/T ratio of 1:1. Percent cytokine-producing cells in response to C1R-B*3501 cells without pulsing the peptide was always <0.2%. Data shown are the means of duplicate assays. (C) Cytotoxic activity of the tetramer-sorted TCR-transduced cells and of the parental CTL 589 was analyzed in response to C1R-B*3501 cells pulsed with various concentrations of Pol peptide or a series of mutant peptides at an E/T ratio of 2:1. The sequences of the peptides tested are shown in each graph. The specific lysis activity of both cells in response to C1R-B*3501 cells without pulsing the peptide was always <5%. Data shown are the means of duplicate assays. (D) Cytotoxic activity of the tetramer-sorted TCR-transduced cells and of the parental CTL 589 was analyzed in response to C1R cells or transfectants expressing HLA-B*3501 or HLA-B*5101 pulsed with various concentrations of Pol peptide. The E/T ratio was 2:1. Data shown are the means of duplicate assays.

cells had promiscuous HLA restriction like their parental CTL 589. Moreover, there were virtually no signs of allogeneic responses in the TCR-transduced T cells toward target cells expressing these HLA molecules (Fig. 3D), despite the fact that the recipient T cells were derived from an individual who carries *HLA-B**3501 but not *HLA-B**5101.

These results indicate that the genetic transfer of HIV-specific TCR resulted in redirection of the antigen specificity of recipient T cells toward the HIV antigen, with activity of cytotoxicity and cytokine secretion (Fig. 3B, C) as well as specificity to peptides and HLA restriction elements (Fig. 3C, D) that were all comparable to those of the parental CTL clone. However, the tetramer binding level of the TCR-transduced T cells was slightly lower than that of the parental CTL clone (Fig. 3A).

2.4 Relationships between levels of tetramer binding and functional avidity of the TCR-transduced T cells

Ectopic expression of α and β chains of TCR in mature T cells will lead to the heterologous pairing of either protein with endogenous α and β chains of TCR. These 'hybrid' TCR would also result in decreased surface expression of the desired pair of TCR (*i.e.* ectopic chains) and may potentially lead to reduced avidity of the TCR-transduced T cells. In fact, TCR-transduced T cells obtained after sorting tetramer$^+$ fractions showed decreased tetramer binding activity compared to the parental CTL 589 (Fig. 3A). To examine this issue, we analyzed the relationships between HLA tetramer binding activity and functional capacity of the TCR-transduced T cells, since the tetramer binding activity of the TCR-transduced T cells could be dependent on the surface expression of the desired pair of TCR.

We first examined the effect of the expression level of transgenes on the surface expression of the desired TCR chains on the TCR-transduced T cells. The TCR-transduced T cells were gated in terms of tetramer$^+$ and tetramer$^-$ phenotypes, and each fraction was then analyzed for its expression levels of GFP and CD24 antigen (Fig. 4A). The tetramer$^+$ subset showed about twofold higher MFI values for both GFP and CD24 than the tetramer$^-$ subset (Fig. 4A), indicating that the difference in the levels of transgene expression contributed at some extent to the different tetramer binding activities of the TCR-transduced T cells. However, the tetramer$^+$ and tetramer$^-$ subsets showed markedly

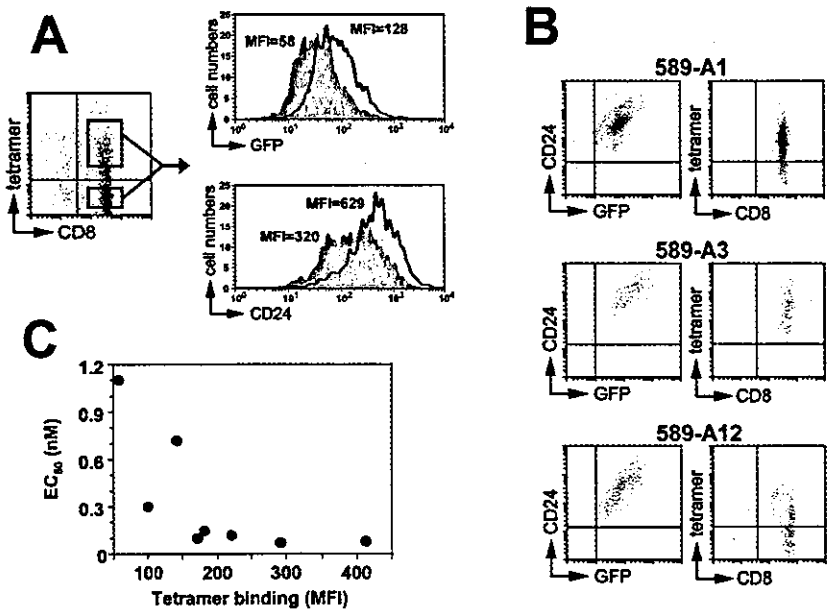

Fig. 4. Functional avidity versus tetramer binding activity of TCR-transduced cells. (A) The tetramer$^+$ (solid lines) and tetramer$^-$ (shaded areas) subsets of TCR-transduced, antigen-stimulated cells were gated and analyzed for their expression levels of GFP and CD24. The MFI values of each subset are indicated in the histograms. (B, C) The single-cell-sorted TCR-transduced cells were analyzed for their levels of tetramer binding and transgene expressions by flow cytometry. Representative dot plots are shown (B). Relationships between the tetramer binding levels and EC_{50} values of the TCR-transduced clones were analyzed (C). The EC_{50} values were determined from the cytolytic activities of these cells toward C1R-B*3501 cells pulsed with various concentrations of the Pol peptide at an E/T ratio of 2:1. The data are summarized in Table 1.

overlapped patterns in terms of both GFP and CD24 expression (Fig. 4A), suggesting that other factors, including heterodimer formation with endogenous TCR chains, influence the different surface expression of the desired TCR chains on the individual TCR-transduced T cells.

Next, the $CD8^+$ GFP^+ $CD24^+$ subset of transduced cells, including both $tetramer^+$ and $tetramer^-$ fractions, was single-cell sorted and cultured for 12 days in the presence of autologous feeder cells pulsed with the Pol peptide. Among the cells that had been positive for growth, eight clones were analyzed for their expression levels of CD8, GFP, and CD24 as well as for their levels of tetramer binding (Table 1, Fig. 4B). Tetramer binding by these cells resulted in MFI values ranging from approximately 50 to 400 (Table 1).

We then analyzed their cytotoxic activities toward C1R-B*3501 cells pulsed with various concentrations of the Pol peptide and determined their functional avidities as the 50% effective concentration of the Pol peptide (EC_{50}). The EC_{50} values of the TCR-transduced clones were also distributed widely, ranging from 0.07 to 1.1 nM (Table 1). A set of clones with the higher tetramer binding activity (MFI>200) showed relatively constant EC_{50} values (average EC_{50} of 0.09±0.026 nM), and these values were comparable to the value for the parental CTL 589 (EC_{50} of 0.07 nM) (Fig. 4C). In contrast, another set of clones with the lower tetramer binding activity (MFI<200) showed low functional avidity (average EC_{50} of 0.47±0.43 nM) and their EC_{50} values varied, mostly depending on their tetramer binding activity (Fig. 4C). These results are in good agreement with the data showing that the tetramer-sorted TCR-transduced T cells and the parental CTL 589 had comparable functional avidity (Fig. 3B, C) and yet had different tetramer binding activity (Fig. 3A).

Thus we concluded that although both the level of transgene expression and the extent of 'hybrid' TCR formation can differentially affect the surface expression levels of desired TCR chains in the individual TCR-transduced T cells, the fraction of TCR-transduced T cells with the surface expression of desired TCR chains above a certain threshold (*i.e.* MFI>200 for the tetramer under the assay condition tested in this study) had virtually identical functional avidities toward peptide-pulsed target cells.

2.5 Antiviral replication activity of TCR-transduced CD8 T cells

We further examined the ability of TCR-transduced cells to inhibit HIV-1 replication *in vitro*. CD4-expressing 221-B*3501 cells were infected with HIV-1 HXB2D and co-cultured with the TCR-transduced clones for 6 days. We determined the amounts of p24 Gag antigen production in the culture supernatant by an enzyme-linked immunosorbent assay (Fig. 5A), and the frequency of $p24^+$ cells, by flow cytometric analysis (Fig. 5B). In both assays, all of the TCR-transduced clones showed substantial suppression activity toward HIV-1 replication, whereas virtually no suppression activity was found in the case of mock-transduced cells (Fig. 5). It is of interest that all of the tested clones showed similar inhibitory activity against HIV replication (Fig. 5), although the functional avidity of these clones toward peptide-pulsed target cells showed a broad distribution, with EC_{50} values ranging

Table 1. Summary of functional and phenotypic characterization of mock- or TCR-transduced cells

	EC_{50} (nM)	MFI			
		Tetramer	CD8	GFP	CD24
A1	0.10	172	453	143	769
A3	0.08	412	584	286	1,405
A6	0.72	143	434	55.5	1,353
A11	0.15	182	311	220	161
A12	1.10	57.5	529	106	633
A14	0.07	292	453	288	809
A16	0.12	221	422	650	1,239
A23	0.30	100	331	50.7	1,101
Mock	>100	3.8	303	3.3	2.7

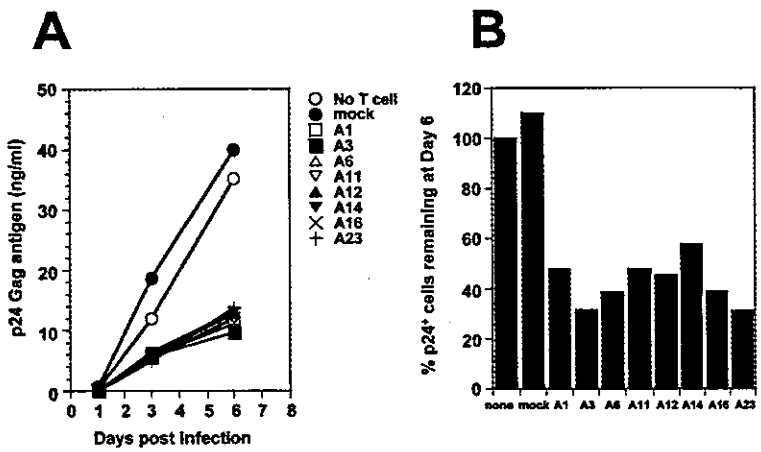

Fig. 5. Inhibitory potency of TCR-transduced clones toward HIV-1 replication *in vitro*. (A) The 221-CD4-B*3501 cells (2×10^4 cells/well) infected with HIV-1 HXB2D were co-cultured or not with the mock- or TCR-transduced clones at an E/T ratio of 0.5:1. On days 1, 3, and 6 post-infection, a portion of the culture supernatant was collected and the amounts of p24 antigen produced by the HIV-infected 221 cells were then determined. (B) The HIV-infected 221 cells were collected on day 6 and intracellularly stained with FITC-conjugated mAb specific for the p24 Gag antigen for flow cytometry. The relative frequency of $p24^+$ cells at day 6 in the co-cultures with the indicated TCR-transduced T cells is shown. The data are given as the means of duplicate assays in one experiment. These results are representative of those of two additional independent experiments.

from 0.07 to 1.1 nM (Table 1). Particularly, clone A12 showed potent suppression activity for HIV replication (Fig. 5) despite its modest functional avidity and tetramer binding activity (Table 1). This finding appears to be consistent with the recently reported study demonstrating that epitope specificity of CTL, rather than functional avidity of CTL, is a key factor in the ability of CTL to control HIV replication, and that the process of epitope presentation on HIV-infected cells greatly influences CTL efficiency *in vivo* [29].

It should be noted that we used an Epstein-Barr virus-transformed B cell line as an HIV-infected target cell. Since kinetics of HIV replication is generally variable dependent on cell types as well as HIV strains, it may be possible that inhibition potency of HIV-specific CD8 T cells toward HIV replication is also variable, dependent on host cell types both *in vitro* and *in vivo*. In this regard, suppression activity of HIV-specific CD8 T cells toward HIV replication is intriguing as assessed using primary human CD4 T cells and macrophages as HIV-infected target cells.

The introduction of a chimeric TCR containing human CD4 or HIV-specific immunoglobulin sequences linked to the signaling domain of the TCR ζ chain (universal TCR) into CD8 T cells for targeting of HIV-infected cells has been reported [30–32]. In comparison with chimeric receptor approaches, the transfer of native TCR may have distinct advantages. In a chimeric receptor approach, high-affinity receptor-ligand interaction will most likely result in incomplete T cell activation owing to omission of immunoreceptor tyrosine-based activation motifs normally present in the CD3 complex. More importantly, it is unclear whether or not chimeric receptor-ligand engagement results in functional maturation of transduced T cells to a fully differentiated effector phenotype, which character of CD8 T cells has been shown to be important to control HIV replication *in vivo* [16].

In summary, the data presented in this study suggest that the reconstitution of HIV-specific immunoreactive T cells engineered by genetic transfer of the native form of αβ TCR into primary CD8 T cells is a viable strategy to suppress HIV replication. This approach represents a potential alternative to other types of immunotherapy for HIV infection, although further studies to elucidate the phenotypic and functional properties of such engineered HIV-specific CD8 T cells *in vivo* are required.

3 Materials and methods

3.1 T cell culture

The Jurkat variant that lacked expression of either α or β TCR was kindly provided by Bent Rubin (Unite de Physiopathologie Cellulaire et Moleculaire, CNRS, France) and maintained in RPMI 1640 and 10% fetal bovine serum. CTL clone 589 cells were generated before [33] from an HIV-infected patient (*HLA-A**2402/*A**2601, *HLA-B**3501/*B**5101) following stimulation of peripheral lymphocytes with an HLA-B*3501-restricted and HIV Pol-derived epitope peptide (HIV $Pol_{448-456}$; IPLTEEAEL). CTL clones were maintained in

RPMI 1640 and 10% human serum supplemented with 200 U/ml rIL-2, and were stimulated weekly with irradiated C1R-B*3501 cells or autologous PBMC pulsed with 100 nM Pol peptide as needed.

3.2 Construction of retroviral vectors and gene transfer

The genes encoding full-length α and β TCR of CTL 589 (DDBJ accession numbers AB164620 and AB164621, respectively) were subcloned into the pGC-based retroviral vector pGCDNsap(MSCV) [28]. The genes encoding a mouse heat-stable antigen (CD24) or GFP were also incorporated into the constructs along with an IRES following the α or β TCR gene (Fig. 1A) to facilitate monitoring the expression of α or β TCR genes, respectively, in the transduced cells. Amphotropic retrovirus containing each of these constructs was then prepared, essentially as described previously [28].

Human primary CD8 T cells were isolated from PBMC of an HIV-negative healthy donor (*HLA-A*0201/A*2402, HLA-B*3501/B*4002*) using anti-CD8 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). The resultant CD8 T cells (>90% of the cells were $CD8^+$) were activated by plate-coated anti-CD3 mAb (OKT3) for 3 days, plated on recombinant fibronectin-coated plates (Takara Shuzo, Otsu, Japan), and incubated with the retrovirus supernatant containing the TCR 589 α-IRES-CD24 gene for 72 h, during which interval the cells were exposed to fresh retrovirus supernatant every 12 h. Transduced CD8 T cells expressing CD24 antigens were isolated by use of PE-labeled anti-CD24 mAb (PharMingen, San Diego, CA) and anti-PE magnetic beads (Miltenyi Biotech). The isolated cells were subsequently transduced by the construct containing TCR 589 β-IRES-GFP, as above. The fraction of transduced CD8 T cells that were positive for GFP, CD24, and CD8 molecules was obtained by sorting with a FacsVantage (BD Biosciences, San Jose, CA). The sorted T cells were propagated by stimulation with OKT3 mAb and subsequently by irradiated autologous PBMC pulsed with 100 nM Pol peptide. These T cells were maintained in RPMI 1640 and 10% human autologous serum supplemented with 200 U/ml rIL-2.

3.3 HLA-tetramer analysis

The HLA-B*3501-tetramer complex with allophycocyanin-conjugated avidin (Molecular Probes, Inc., Eugene, OR) was prepared as previously described [26]. T cells were first stained by the tetramer for 15 min at 37°C, and then stained for 20 min at 4°C with other antibodies, such as anti-CD8 mAb and anti-mouse CD24 mAb conjugated to peridinin chlorophyll protein (PerCP) and PE (BD Biosciences), respectively. They were then washed twice with a washing buffer (2% newborn bovine serum in phosphate-buffered saline) and fixed in a 1% paraformaldehyde solution. The resultant cells were analyzed by flow cytometry.

3.4 Cytotoxicity assay

The cytotoxic activity of the CTL clone 589 and the TCR-transduced T cells was determined by a standard ^{51}Cr-release assay as described [26]. For peptide-pulsed target cells, ^{51}Cr-labeled C1R-B*3501 cells (2×10^3 cells/well) were pulsed with various concentrations of the peptide for 1 h and incubated with the effector T cells for an additional 4 h at 37°C. For virus-infected target cells, C1R-B*3501 cells or 221-B*3501 cells expressing human CD4 antigen (2×10^3 cells/well) were infected with HIV-1 HXB2D or HIV-1 LAI. These viruses have the same epitope sequence as was used for the synthetic Pol peptide. The cells were subsequently labeled with ^{51}Cr and incubated with the effector T cells for 6 h at 37°C. Note that >80% cells were positive for intracellular p24 Gag antigen when HIV-infected cells were used for CTL assays.

3.5 Cytokine secretion assay

CTL clone and TCR-transduced T cells were co-cultured with 221-CD4-B*3501 cells pulsed with various concentrations of the Pol peptide for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the cultures were continued for an additional 4 h. Cells were permeabilized and stained with anti-IFN-γ and anti-TNF-α mAb conjugated to PE and allophycocyanin, respectively (PharMingen), as previously described [34]. Frequencies of IFN-γ^+ and TNF-α^+ $CD8^+$ cells were determined by flow cytometry.

3.6 Antiviral replication assay

The 221-CD4-B*3501 cells were infected with HIV-1 HXB2D at 10 $TCID_{50}$/ml for 6 h at 37°C and washed with culture medium. The resultant infected cells were plated in a 96-well U-bottom plate at 2×10^4 cells/well, and then the mock- or TCR-transduced $CD8^+$ T cells were added to each well at an E/T ratio of 0.5:1. The culture medium contained RPMI 1640 and 10% human serum supplemented with 200 U/ml rIL-2. On days 1, 3, and 6 post-infection, a portion of the culture supernatant was collected and the amounts of p24 antigen produced by HIV-infected 221 cells were determined by use of an enzyme-linked immunosorbent assay (ZeptoMetrix Corporation, New York, NY). In addition, on day 6, cells were collected, intracellularly stained with FITC-conjugated mAb specific for p24 Gag antigen (KC57; Coulter Immunology, Hialeah, FL), and analyzed by flow cytometry.

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Spontaneous recovery of hemoglobin and neutrophil levels in Japanese patients on a long-term Combivir® containing regimen

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Abstract

Objective: In order to evaluate long-term toxicity of Combivir, we retrospectively reviewed clinical records of HIV-1 infected cases under treatment with Combivir-containing regimen and we analyzed the clinical data compared to other NRTIs-containing regimens.
Study design: A total of 55 patients who were on Combivir and 39 on a control regimen were examined.
Results: After starting treatment with Combivir-containing regimens viral load and $CD4^+$ T-cell count improved as well as the control group. Rates of adverse events in Combivir group and ZDV (400 mg/day) + 3TC group were 50.9% (28/55) and 60% (12/20), respectively. Some of these Japanese patients who started Combivir regimen as a first-line HAART (primary Combivir group) showed some decrease in hemoglobin levels or neutrophil counts within 6 months. However, a significant recovery of these indices of hematological toxicities occurred in patients who continued the regimen for 18–24 months.
Conclusion: Our findings suggest that the safety of 600 mg of ZDV is similar to 400 mg/day of ZDV and the existence of mechanisms that compensate for anemia and for the neutropenia associated with long-term use of Combivir.

Keywords: Combivir; Zidovudine; Lamivudine; Hemoglobin; Neutrophil; Long-term treatment

1. Introduction

Prognosis of HIV infections dramatically improved after introduction of highly active anti-retro viral therapy (HAART). However, the occurrence of adverse events and drug resistance during long-term use of anti-retrovirals are now big issues (Yeni et al., 2002; Dieleman et al., 2002). Present HAART also has a problem to maintain a high adherence because of the pill burden and patients' quality of life is affected. Combivir® is a fixed dose combination tablet containing zidovudine (ZDV) and lamivudine (3TC) (Eron et al., 2000). Each tablet contains 300 mg of ZDV and 150 mg of 3TC and has been widely used as a nucleoside reverse transcriptase inhibitor (NRTI) component of HAART against HIV-1 infection.

HIV infection and AIDS are known to be associated with a significant hematological toxicity, including anemia, neutropenia, and thrombocytopenia (Moses et al., 1998). In addition, studies with zidovudine have shown that this drug may compound the hematological toxicity of HIV and lead to an independent development of anemia and neutropenia (Wilde and Langtry, 1993). Consistent with these observations, the incidence of anemia or neutropenia in mildly or asymptomatic adults treated with zidovudine was between 1.1% and 9.7%, whereas in adults with AIDS or the AIDS related complex it ranged from 15% to as high as 61% (Wilde

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and Langtry, 1993). In Japan, many physicians prescribe low dose ZDV such as 400 mg/day to avoid drug-induced anemia and neutropenia even though the standard dose of ZDV is 500–600 mg/day in United States (Kimura et al., 1992, 1998). Given the dose-dependent nature of these adverse effects, they are concerned about increased risk of hematological toxicity using Combivir that contains 600 mg of ZDV as the daily dose for Japanese patients who have lower body weights compared to patients in United States. Moreover, long-term consequence of the hematological toxicity resulting from continuous use of Combivir has not been well defined. We retrospectively reviewed clinical records of HIV-1 infected cases under treatment of Combivir-containing regimen used in three hospitals in Japan and we analyzed clinical data cross-sectionally to evaluate long-term toxicity of Combivir.

2. Patients and methods

HIV-1 positive Japanese patients were recruited from Kumamoto University Hospital, Osaka National Hospital and International Medical Center of Japan from June 1999 (after the Combivir launch) until June 2003. The clinical record was investigated in a retrospective manner. All collected cases were separated into four groups, as follows;

Primary Combivir Group (PCV): started Combivir as a first-line HAART.
Secondary Combivir Group (SCV): changed to Combivir from other NRTIs.
Primary Control Group (PCO): started NRTIs (except for Combivir) as a first-line HAART.
Secondary Control Group (SCO): changed to NRTIs except Combivir from other NRTIs.

We checked hemoglobin levels and neutrophil counts to examine the influence on hematological toxicity of ZDV every 6 months. We analyzed the data that could be followed over 18 months for removing various biases such as drop out cases with abnormal laboratory test values. Moreover, we also checked the HIV-RNA, $CD4^+$ T-cell counts and other laboratory test data every 6 months. We also checked any adverse events. This study was done under the approval of the Institutional Review Board of the Kumamoto University Hospital, Japan. All participants provided written informed consent.

3. Results

3.1. Patients' characteristics

Of the 94 data on subjects were 55 who were on Combivir (PCV: 27, SCV: 28) and 39 were on control regimens (PCO: 29, SCO: 10). The NRTIs used in the control group included of 20 cases of ZDV (400 mg/day) + 3TC, 18 cases of stavudine (d4T) + 3TC and one case of d4T + didanosine (ddI). Patients' characteristics are shown in Table 1. A couple of factors are statistically different such as the sex ($p < 0.01$: Fisher's exact test), weight ($p < 0.05$: Student's t-test) and Karnofsky score ($p = 0.0062$: Student's t-test) between Combivir group and control group. Combivir was likely to be used for patients with a higher baseline weight and the males. The mean viral load at baseline in Combivir group was $10^{3.9}$ copies/mL and for the control group was $10^{4.1}$ copies/mL. There was no statistical difference between the groups. The baseline $CD4^+$ T-cell counts in Combivir group were higher than in the control group significantly ($393/mm^3$ versus $263/mm^3$; $p = 0.0101$: Student's t-test). Most patients were prescribed efavirenz (EFV) or nelfinavir (NFV) as a concomitant drug. Fifty-two percent of all patients were on EFV and 16% were taking NFV. The Combivir group had more combination cases with EFV than did the control group, because these two drugs approved for use in Japan at the same period have similar characteristics such as small pill counts and frequency of ingestion.

3.2. Effects on hemoglobin levels

To avoid biases in the data resulting from inclusion of patients with a shorter time follow up, including drop out cases, we focused on the patients that could be followed for over 18 months. Mean hemoglobin levels at baseline of Combivir group (PCV group: 13.9 g/dL, SCV group: 14.2 g/dL) were higher than for the control group (PCO group: 13.1 g/dL, SCO group: 13.7 g/dL) (Fig. 1A). It seems Combivir was likely to give to those with a lesser risk of anemia. We divided patients in PCV group into two sub-groups such as hemoglobin level decreased (sub-group A; $n = 10$) and not changed or increased (sub-group B: $n = 8$) at 6 months after starting Combivir. Fig. 1B shows a trend of hemoglobin levels in sub-group A. Each hemoglobin level at 6, 12, 18 and 24 months after starting treatment decreased significantly compared to baseline ($p < 0.005$, $p < 0.005$, $p < 0.005$ and $p < 0.05$, respectively; Wilcoxon matched pairs signed rank test). However, the decreased hemoglobin levels at 6 months gradually recovered to the baseline level despite continuation of the same regimen. The hemoglobin level at 18, 24 months increased significantly compared to 6-month values ($p < 0.05$ and $p < 0.005$, respectively). On the other hand, the hemoglobin level of sub-group B did not decrease for 18–30 months of follow up period (data not shown). The difference of background between sub-groups A and B was baseline level of hemoglobin and hematocrit. These levels in sub-group A were higher than for sub-group B statistically (14.9 ± 1.2 versus 12.6 ± 0.7; $p < 0.001$, 44.4 ± 3.2 versus 37.4 ± 2.0; $p < 0.001$, Student's t-test).

3.3. Effects on neutrophil counts

The trend of mean neutrophil counts was similar to counts for hemoglobin levels. Mean neutrophil counts of all groups

Table 1
Baseline characteristics

	Combivir group (PCV+SCV) ($n=55$)	Control group (PCO+SCO) ($n=39$)	p-value
Sex (male/female)	54/1	32/7	0.00815[a]
Age	35.9±9.5 (22–68)	38.6±10.7 (23–78)	0.2117[b]
Weight (kg)	64.6±10.8 (47.0–91.6)	59.6±11.2 (36.4–81.0)	0.0303[b]
Hemophilia			
Non	48	32	0.562[a]
A	5	7	
B	2	0	
Baseline VL (log)			
<2.6	19	11	0.4432[b]
2.6–3	1	1	
3–4	6	4	
4–5	11	13	
>5	15	10	
Unknown	3	0	
Mean±S.D.	3.9±1.2	4.1±1.2	
Range	2.6–5.9	2.6–5.9	
Baseline CD4 count			
<200	14	14	0.0101[b]
200–500	25	19	
>500	13	5	
Unknown	3	1	
Mean±S.D.	393±265	263±179	
Range	1–1132	5–607	
CDC class			
A1	5	3	0.8064[c]
A2	22	17	
A3	6	13	
B1	2	0	
B2	3	0	
B3	2	5	
C1	3	0	
C3	12	11	
Karnofsky score			
20%	0	1	0.0062[b]
40%	0	2	
50%	0	1	
60%	1	0	
70%	0	1	
80%	4	6	
90%	11	8	
100%	39	20	
Mean±S.D.	95.8±7.9	87.7±19.4	

[a] Fisher' exact test.
[b] Student's t-test.
[c] Wilcoxon 2-sample test.

were over 2000/mm^3 and did not have statistically change from the baseline during the follow up period (Fig. 1C). We separated subjects in the PCV group into two sub-groups as well as for hemoglobin levels to examine the toxicity of Combivir to neutrophils. In the sub-group C ($n=10$) those with mean neutrophil counts decreased and the sub-group D ($n=7$) included subjects with no changes or increased neutrophil counts at 6 months after being on Combivir. Fig. 1D shows the trend of the neutrophil counts in sub-group C. Neutrophil counts at 6, 12, 18 and 24 months after starting the treatment decreased significantly compared to baseline ($p<0.005$, $p<0.05$, $p<0.05$ and $p<0.05$, respectively; Wilcoxon matched pairs signed rank test). However, the decreased neutrophil counts gradually recovered as did hemoglobin levels. The mean neutrophil counts at 18 months increased significantly compared to data at 6 months ($p<0.05$; Wilcoxon matched pairs signed rank test).

3.4. Effects on other laboratory test value

MCV values at baseline for the secondary treatment group such as SCV group and SCO group were higher than for pri-

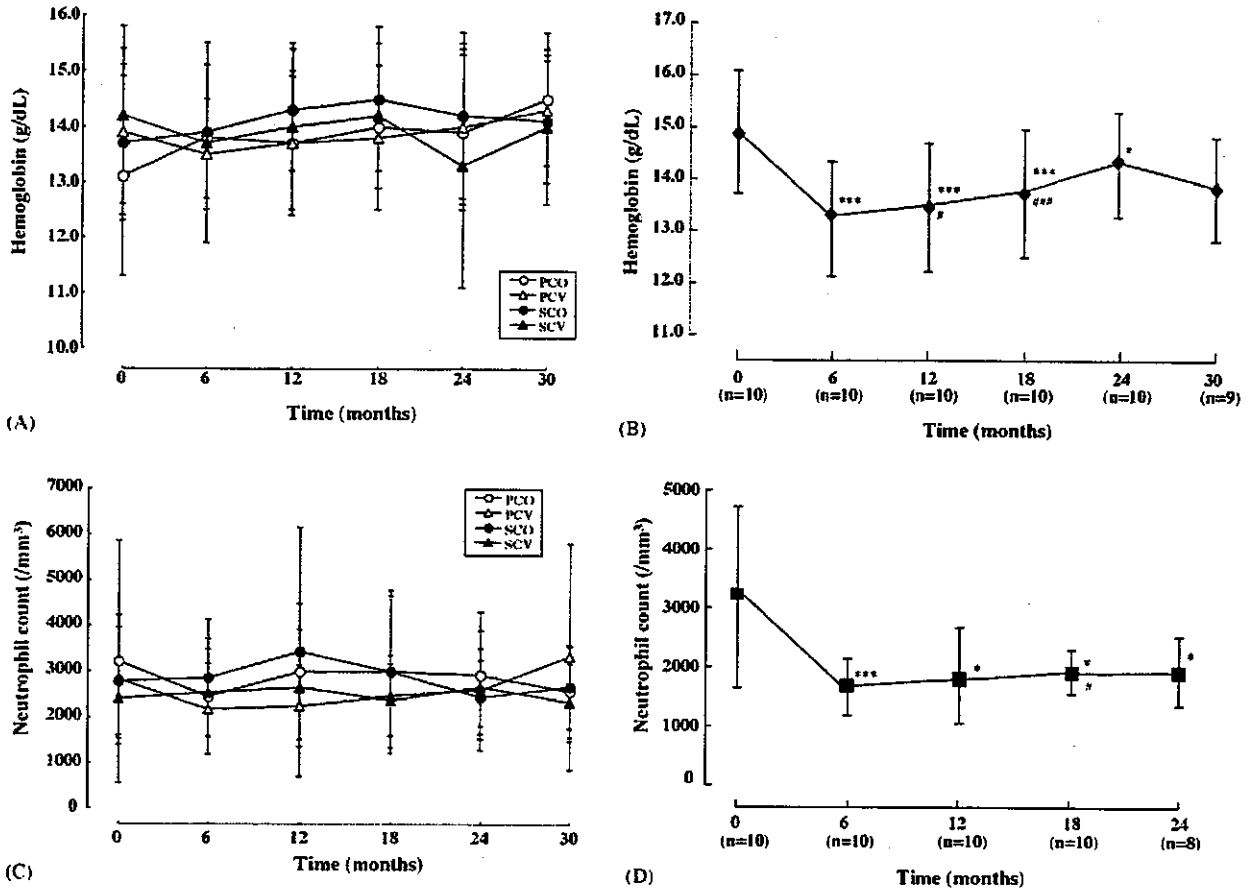

Fig. 1. Recovery after transient suppression of hemoglobin and neutrophil levels in patients with long-term use of Combivir. (A) Mean hemoglobin levels did not change significantly in all groups during each treatment. The baseline hemoglobin level in the Combivir group (PCV + SCV) was higher than in controls (PCO + SCO). (B) Mean hemoglobin levels at 6, 12, 18 and 24 months after start of treatment decreased significantly compared to baseline in the subgroup A of PCV group ($n = 10$). However the decreased hemoglobin level gradually reverted to the baseline levels despite continuation of the same regimen. Hemoglobin levels at 12 and 18 months were significantly high compared to findings at 6 months. (Wilcoxon matched pairs signed rank test; $^{*,\#}p < 0.05$, $^{***,\#\#\#}p < 0.005$). (C) Mean neutrophil counts did not change significantly in all groups during each treatment. (D) Mean neutrophil counts at 6, 12, 18 and 24 months after beginning treatment decreased significantly compared to baseline in sub-group C of PCV group ($n = 10$). However, the neutrophil counts gradually reverted to baseline levels despite continuation of the same regimen. The neutrophil counts at 18 months was significantly high compared to that of 6 months (Wilcoxon matched pairs signed rank test; $^{*,\#}p < 0.05$, $^{***}p < 0.005$).

mary treatment groups such as PCV group and PCO group. It seems ZDV or d4T in the secondary treatment group affected red blood cell counts. However, after starting each treatment, MCV values increased and became high at around 110/mm^3 in all groups (Fig. 2A). Other laboratory test values did showed no notable changes (data not shown).

3.5. Adverse events

The most common adverse events in each group were nausea/vomiting, dizziness and malaise. Anemia was observed in two in the Combivir group and one in the control group. Discontinuing each treatment led to elimination of these adverse effects. The anemia in two in the Combivir group was observed 2 months after their starting treatment, and that in one in the control group was evident as early as the eighth day. The occurrence of anemia in the control group was on ZDV 400 mg/day + 3TC. The frequency of anemia in the Combivir group was 3.6% (2/55) and similar to that in the control group {2.6% (1/39)}. The 20 in the control group on ZDV + 3TC regimen were on a ZDV 400 mg/day. We compared the safety profile of ZDV 600 mg/day to ZDV 400 mg/day. Adverse events rate of Combivir was 50.9% (28/55) and 60.0% (12/20) of AZT + 3TC group. Moreover, the number who discontinued Combivir group was 7 (12.7%) and that in ZDV + 3TC group was 5 (25.0%). In the SCV group, nineteen were changed to Combivir from ZDV 400 mg/day + 3TC. There were six with some adverse events and these were similar to other groups' events. These observations suggest that increasing the ZDV dose to 600 mg/day does not affect the incidence of adverse events. In addition there were no concomitantly used drugs that could affect pharmacokinetic parameters of ZDV and enhance its toxicity.

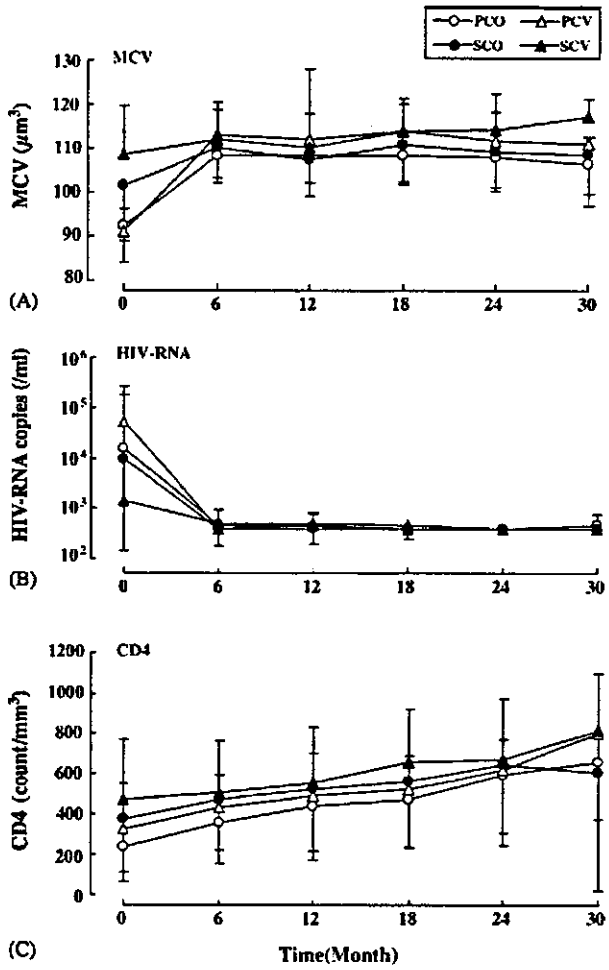

Fig. 2. Changes in MCV values, HIV RNA level and CD4⁺ T cell counts in each group of patients. (A) MCV values for the secondary treatment group such as SCV and SCO group were higher than for primary treatment groups such as PCV and PCO group at baseline. However, after starting each treatment, MCV values increased and became high at around 110/mm³ in all groups. (B) Mean HIV RNA level in all groups of treatment decreased compared to baseline significantly ($p<0.05$–$p<0.001$; Wilcoxon matched pairs signed rank test). (C) Mean CD4⁺ T cell counts in all groups of treatment increased significantly compared to the baseline ($p<0.05$–$p<0.001$; Wilcoxon matched pairs signed rank test).

3.6. *Effects on viral load and CD4⁺ T-cell counts*

Baseline viral load in the primary treatment group (PCV + PCO) was higher than in the secondary treatment group (SCV + SCO). Mean baseline viral loads of each group were $10^{4.6}$ copies/mL (PCV), $10^{4.0}$ copies/mL (PCO), $10^{3.0}$ copies/mL (SCV) and $10^{3.7}$ copies/mL (SCO), respectively. However, after starting each treatment, HIV RNA was not detectable in serum samples from in each group (VL < 50 or <400 copies/mL) (Fig. 2B). Baseline CD4⁺ T-cell count in the SCV group was 518/mm³ and higher than other groups (PCV: 304/mm³, SCO: 345/mm³, PCO: 277/mm³) significantly ($p<0.001$; Student's t-test) (Fig. 2C). This result suggests effective treatment with the previous combination for the SCV group. CD4⁺ T-cell counts during each treatment increased significantly ($p<0.05$–$p<0.001$; Wilcoxon matched pairs signed rank test) and reached over 600/mm³ at 30 months in all groups (Fig. 2C).

4. Discussion

The nucleoside reverse transcriptase inhibitor (NRTI) was first developed as an anti-HIV drug. However, the appropriate dosage was unclear because this type of drug is only active after being phosphorylated inside cells. A daily dose of 400 mg of ZDV has been widely used in Japan because anemia and neutropenia occurred frequently in cases of ingesting a higher dose (800 mg/day) than did 400 mg/day of ZDV in a clinical trial conducted in Japan (Kimura et al., 1992). Bone marrow toxicity associated with AZT such as macrocytic anemia and neutropenia has been frequently reported for the patients treated with a higher dose of ZDV mono therapy (Richman et al., 1987). Given the dose-dependent nature of these adverse effects, Japanese health care providers have some hesitance to prescribe Combivir that contains 600 mg of ZDV, as the daily dose. Data on four patients with severe anemia associated with Combivir have also been reported (Sibery et al., 2003). To evaluate the long-term toxicity of Combivir, we reviewed clinical records of HIV-1 infected Japanese patients on treatment with Combivir-containing regimen.

The results in this retrospective study showed that anemia and adverse events occurred at comparable frequency in each group of patients. Consistent with previous reports (Hester and Peacock, 1998; Tseng et al., 1998) these adverse events occurred in less than a few months after starting each treatment. The frequency of anemia in the Combivir group was only 3.7% (2/54), and it was similar to that for ZDV 400 mg/day + 3TC group (5.0%) group. In other words there was no difference in these groups with respect to the frequency of anemia by the difference in the dose of ZDV. It is also of note that the efficacy of Combivir was comparable to that of 400 mg of ZDV of four times a day with a twice a day dosing of 3TC. However, we have to take into account the fact that Combivir was prescribed for heavy weight patients. And such may mask the occurrence of adverse events as well as the difference in efficacy.

We observed a certain degree of decrease in hemoglobin levels and neutrophil counts in the subgroups of patients in PCV (subgroups A and C, respectively). Interestingly, a gradual recovery of these hematological toxicities occurred despite the continuation of Combivir containing regimens. The mechanism whereby the risk of hematological toxicity associated with increasing ZDV dosages may be related to the intracellular accumulation of the toxic metabolite zidovudine monophosphate (AZTMP) (Tornevik et al., 1995). AZTMP interferes with both cellular DNA synthesis and exonuclease-catalyzed removal of ZDV from host cell DNA (Sommadossi et al., 1989; Harrington et al., 1993). In addition, at clinically

relevant concentrations, AZTMP acts as a potent inhibitor of the transport of pyrimidine nucleotide sugars into the Golgi complex, thereby inhibiting protein glycosylation and altering glycosphingolipid synthesis (Yan et al., 1995). Therefore, AZTMP may elicit cytotoxic effects on rapidly growing erythrocytes and neutrophil precursors, both by interfering with nuclear DNA replication and by compromising the function of membrane receptors involved in receiving of extracellular stimuli required for cell growth and differentiation. From these observations it seems reasonable to speculate that either decrease in the intracellular concentration of AZTMP or compensatory mechanisms that improve the signal transduction for erythropoiesis and myelopoiesis mediated by cytokines contributed the recovery from hematological toxicities.

Two mechanisms may be related to the decrease in the concentration of AZTMP: altered metabolism of nucleoside analogues due to impaired nucleoside phosphorylation and increased efflux of the compounds by membrane transport mechanisms (Schuetz et al., 1999; Wijnholds et al., 2000). These mechanisms have been considered to contribute to the cellular drug-resistance (Dianzani et al., 1994; Groschel et al., 1997; Fridland et al., 2000; Turriziani et al., 2000). However, there was no evidence of treatment failure for patients in our PCV group as we found an increase in $CD4^+$ cell counts and an undetectable HIV-RNA load. Furthermore, the MCV level which is associated with the intracellular increase of AZTMP was kept high. These observations suggest that decrease in the level of AZTMP in the course of long-term treatment is unlikely although we must determine longitudinal changes of intracellular AZTMP level in precursors of blood cells in patients on Combivir treatment. Other compensatory mechanisms against the hematological toxicity may occur. An increase in erythropoietin or granulocyte-colony stimulating factor (G-CSF) levels in compensation for chronic anemia or neutropenia is another notion.

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Short communication

Th1/Th2 balance and HTLV-I proviral load in HAM/TSP patients treated with interferon-α

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Abstract

We studied the immunological and virological effects of interferon-α (IFN-α) therapy in nine patients with HTLV-I-associated myelopathy (HAM/TSP). After therapy, the percentages of CCR5+ cells in CD4+ cells significantly decreased in the cerebrospinal fluid as well as blood. The therapy also significantly lowered the intracellular IFN-γ+/interleukin-4+ T-cell ratio in blood. Those helper T-cell type 1 (Th1)-related responses tended to be higher and reduce more evidently following therapy in three patients who clinically improved. Also, all the three patients had one or more HTLV-I copies in five blood mononuclear cells. These results suggest that IFN-α suppresses Th1 responses in HAM/TSP and that the patients with higher Th1 immunity and proviral loads may be responders of the therapy. Larger-scale studies are needed to confirm the findings.

Keywords: HAM/TSP; HTLV-I; Interferon-alpha therapy; Helper T cell; Chemokine receptor

1. Introduction

Human T-lymphotropic virus type I (HTLV-I) is associated with chronic inflammatory myelopathy, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986; Izumo et al., 2000). Previous studies demonstrated remarkable immune activation including helper T-cell type 1 (Th1)-associated responses (Kuroda and Matsui, 1993; Itoyama et al., 1996; Umehara et al., 1994; Jacobson et al., 1998) and high HTLV-I proviral loads (Nagai et al., 1998) in HAM/TSP. These immunological and virological changes are probably important in the pathogenesis of this myelopathy and effective immunotherapies for HAM/TSP need to suppress these abnormalities.

A randomized, double-blind study demonstrated that interferon-α (IFN-α) was clinically effective in HAM/TSP (Izumo et al., 1996). The immunological effects of the therapy had been unclear, but we recently found a significant reduction of CD4 cell subsets in the cerebrospinal fluid (CSF) of the patients receiving the therapy (Feng et al., 2003). Here, we report Th1 and Th2-associated chemokine receptor expression on T cells, intracellular cytokine levels in T cells and HTLV-I proviral loads in blood before and after the therapy in the same patients.

2. Materials and methods

2.1. Subjects

Nine patients (five women and four men) were enrolled in the present study as reported previously (Feng et al., 2003). Their ages ranged from 54 to 72 years old and the duration of disease was from 2 to 50 years. The clinical disability was graded according to the Osame's scale: motor

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disability: grade 0 (normal)–13 (bedridden), dysuria: grade 0 (normal)–3 (severe) (Izumo et al., 1996). The patients received intramuscular injections of IFN-α (3 million units) daily for 4 weeks (Izumo et al., 1996). None had received immunosuppressants for the last 3 months except for a single patient (HAM3) who was treated with a fixed dose of oral prednisolone (20 mg/day) throughout the therapy. Age- and sex-matched nine HTLV-I-seronegative control subjects were studied for peripheral blood mononuclear cells (PBMC). We obtained informed consents prior to the study and the present study conformed to the guidelines of Medical Ethics Committee of our medical school.

2.2. Mononuclear cell preparation

Heparinized venous blood and CSF were collected before the IFN-α therapy and the next day of the last IFN-α injection. PBMC were isolated by Ficoll-Paque and CSF cells were directly isolated by centrifugation.

2.3. Flow cytometric analysis

2.3.1. T-cell subset

We analyzed T-cell subsets using a standard direct immunofluorescent technique with monoclonal antibodies (MoAbs), a three-color flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA) and the CellQuest software.

The analyzed subsets were CCR5+ and CXCR3+ (helper T-cell type 1 [Th1]-associated chemokine receptor expressing cells) and CCR3+ (Th2-associated chemokine receptor expressing cells, especially in the early stage of Th2 response) among CD4+ and CD8+ cells. Peridinin chlorophyll protein-conjugated anti-CD4 and anti-CD8, fluorescein isothiocyanate-conjugated anti-CD8 MoAbs were provided by Becton Dickinson. Phycoerythrin-conjugated anti-CCR5 and anti-CCR3 and carboxy-fluorescein succinimidylester-conjugated anti-CXCR3 MoAbs were purchased from Dako (Tokyo, Japan).

The data were expressed as the percentages of T-cell subsets in CD4+ or CD8+ cells.

2.3.2. Intracellular Th1/Th2-associated cytokines

The ratio of IFN-γ producing cells to interleukin-4 (IL-4) producing cells in CD3+ cells was assayed with a FACSCalibur according to the previous report (Pala et al., 2000).

2.4. HTLV-I proviral load in PBMC

DNA was extracted from PBMC. The HTLV-I proviral load was quantified using a real-time Taq-Man PCR method (PE Applied Biosystems, Foster City, CA). Standard curves of β-actin and HTLV-I tax genes were generated using DNA derived from an HTLV-I infected cell line, TloM1. TaqMan amplifications were carried out with the forward primers 5′-ACTCCTCAAGCGAGCTGCAT-3′, the reverse primer 5′-TTTTTCTTTGGGATCGGCG-3′ (Greiner Japan, Tokyo, Japan) and HTLV-I TaqMan Probe 5′-CCCAAGACCC-GTCGGAGGCC-3′ labeled with the 5′ FAM reporter dye and the 3′ TAMRA quencher dye molecules (Japan BioService, Asaka, Japan). The primers and probe for β-actin gene were obtained from PE Applied Biosystems. The thermal cycle conditions were 50 °C for 2 min followed by 95 °C for 10 min (hot start) and then 40 cycles were run by melting at 95 °C for 15 s and annealing/extension at 60 °C for 1 min in each cycle. Each sample was analyzed in triplicate. For each reaction, 100 ng of DNA, the equivalent of 2×10^4 cells, were subjected to the analysis. The amplifications were performed on an ABI PRISM 7700 sequence detector equipped with a 96-well thermal cycler. Copy numbers were reported as copy equivalents per 10^5 PBMC.

2.5. Statistical analysis

We used Mann–Whitney *U*-test to compare the unpaired values, Wilcoxon's signed rank test to compare the paired values. We also examined correlations in clinical disability, immunological and virological parameters (motor disability grade, percentages of CCR5+, CXCR3+ and CCR3+ in CD4+ cells, and CCR5+, CXCR3+ and CCR3+ in CD8+ cells in the CSF and blood, ratio of IFN-γ producing cells to IL-4 producing cells in blood CD3+ cells, and HTLV-I proviral load) of the HAM/TSP patients with Spearman rank correlation coefficient test. Correlations in both baseline values and the changes after the IFN-α therapy were analyzed. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Clinical effects

The motor disability grade improved in three patients after the IFN-α therapy (Patients HAM 3, grade 3 → 2; HAM 5, grade 8 → 6; HAM 6, grade 6 → 4) as we reported (Feng et al., 2003). The dysuria grade did not change.

3.2. T-cell subsets (Table 1)

3.2.1. CCR5

The percentage of CCR5+ cells in blood CD4+ cells was significantly higher before the IFN-α therapy in HAM/TSP than in control. In HAM/TSP, the mean percentages of all CCR5+ T-cell subsets in blood and CSF decreased after the therapy. Among them, the percentage of CCR5+ cells in blood CD4+ cells of HAM/TSP significantly decreased after the therapy, and they were no longer different between HSM/TSP and control. The percentage of CCR5+ cells in CSF CD4+ cells also significantly decreased after the therapy (Table 1).

Table 1
T cells expressing Th1/Th2-associated chemokine receptors in HAM/TSP patients treated with interferon-α and in control subjects

	CCR5+ in CD4+	CXCR3+ in CD4+	CCR3+ in CD4+
Control (n=9)	(%)	(%)	(%)
Blood	0.5 ± 0.1	27.3 ± 4.8	0.3 ± 0.1
HAM/TSP (n=9)			
Blood			
before IFN-α	1.5 ± 0.9 *	27.9 ± 12.3	0.3 ± 0.2
after IFN-α	0.9 ± 0.8 *	25.5 ± 7.6	0.4 ± 0.2
CSF			
before IFN-α	20.5 ± 8.8 *	88.5 ± 5.4	4.3 ± 4.3
after IFN-α	13.4 ± 5.3 *	81.3 ± 12.2	7.5 ± 3.1
	CCR5+ in CD8+	CXCR3+ in CD8+	CCR3+ in CD8+
Control (n=9)	(%)	(%)	(%)
Blood	0.9 ± 0.9	35.9 ± 8.1 *	0.5 ± 0.2
HAM/TSP (n=9)			
Blood			
before IFN-α	3.0 ± 4.5	55.5 ± 17.4 *	0.7 ± 0.4
after IFN-α	1.6 ± 1.0	36.4 ± 17.6 *	0.6 ± 0.2
CSF			
before IFN-α	27.1 ± 12.1	94.8 ± 4.4	4.3 ± 4.3
after IFN-α	17.9 ± 6.5	90.3 ± 9.3	7.5 ± 3.6

Data are mean percentages ± standard deviation.
*$P < 0.05$.

The percentage of CCR5+ cells in CSF CD4 cells was unequivocally higher in the three patients who clinically improved after the therapy (the lowest value was 26.8% in Patient HAM 6) than in the six patients without clinical effect (the highest value was 18.3% in Patient HAM 2).

3.2.2. CXCR3

In blood, the percentages of CXCR3+ cells in CD8+ cells were significantly higher in HAM/TSP before the therapy than in control. In HAM/TSP, the mean percentages of all CXCR3+ T-cell subsets in blood and CSF decreased after the therapy. Among them, the percentage of CXCR3+ cells in CD8+ cells significantly decreased after the therapy in HAM/TSP, and it was no longer different between HAM/TSP and control.

3.2.3. CCR3

No CCR3+ subset was significantly different between HAM/TSP and control or changed significantly after the therapy in HAM/TSP, although the mean percentages of CCR3+ cells in CSF CD4+ and CD8+ cells increased after the therapy.

3.3. Intracellular TH1/TH2-associated cytokines (Fig. 1)

The IFN-α therapy significantly decreased the ratio of intracellular IFN-γ- versus IL-4-producing T cells in blood (9.5 ± 7.6 before the therapy and 5.8 ± 4.9 after the therapy). The ratios in the three patients who clinically improved following the therapy (Patients HAM 3, 5 and 6) were over 5.0, while the therapy was not effective in any of the four patients with the ratios being less than 5.0 (Patients HAM 9, 4, 1 and 7).

3.4. HTLV-I proviral load (Fig. 2)

The HTLV-I proviral copy number before the IFN-α therapy in the nine patients was 13272 ± 9006 copies and

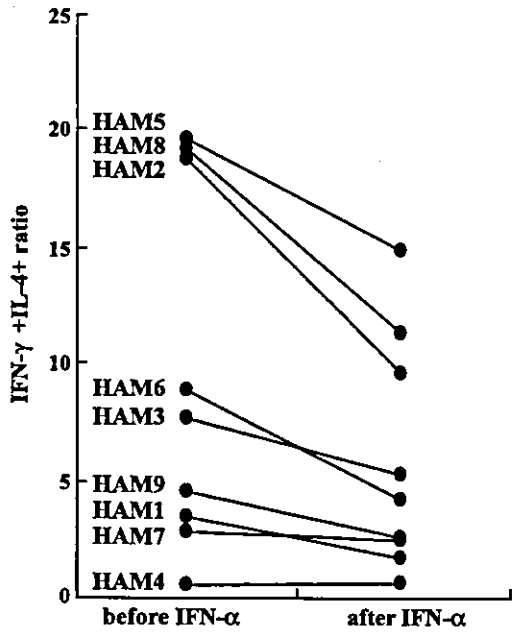

Fig. 1. Intracellular Th1/Th2 cytokine balance in T cells of the patients with HAM/TSP treated with IFN-α. The ratio of IFN-γ+ cells to IL-4+ cells among CD3+ T cells was significantly lower after the IFN-α therapy. Before-IFN-α, before IFN-α therapy; after-IFN-α, after IFN-α therapy.

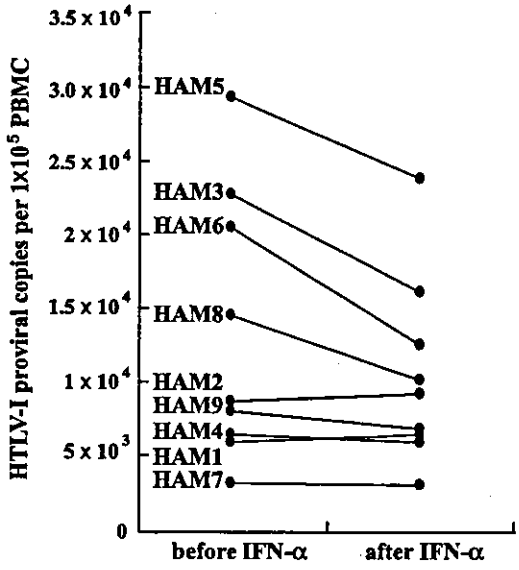

Fig. 2. HTLV-I proviral loads in the PBMC of the patients with HAM/TSP treated with IFN-α. After the IFN-α therapy, HTLV-I proviral loads apparently decreased in the patients with higher proviral loads, and clinical effect was seen in the three patients with 2×10^4 copies or more in 10^5 PBMC (HAM3, HAM5 and HAM6). Before-IFN-α, before IFN-α therapy; after-IFN-α, after IFN-α therapy.

that after the therapy was 10472 ± 6323 copies ($P = 0.06$). Patients HAM 3, 5, 8 and 6, who had the highest proviral loads and who experienced the most obvious decline in proviral load as a result of therapy, were among the ones with the highest baseline ratios of intracellular IFN-γ- versus IL-4-producing blood T cells and with the most dramatic decline in those values following the therapy (Fig. 1). Furthermore, three of these four patients were the ones who clinically improved, and all the three patients had 2×10^4 HTLV-I copies or more in 10^5 PBMC. Meanwhile, clinical effect was not seen in any patient with lower HTLV-I proviral load and their proviral loads remained unchanged after the therapy (Fig. 2).

3.5. Correlation

None of the correlations in clinical disability, immunological and virological parameters in the HAM/TSP patients was statistically significant.

3.6. Comparison of immunological and virological findings in responders and non-responders to IFN-α therapy

We compared the immunological and virological findings in responders (Patients HAM 3, 5 and 6) and non-responders (Patients HAM 1, 2, 4, 7, 8 and 9) to the IFN-α therapy (Table 2). We could not analyze the data statistically due to the small sample size. However, among the significant parameters in the statistical analyses of HAM/TSP patients, there was a tendency for the percentages of CCR5+ cells in CD4+ cells in the CSF and blood, the ratio of intracellular IFN-γ- versus IL-4-producing T cells in blood, and the HTLV-I proviral load to be higher and reduce more evidently following the therapy in responders as compared with non-responders. In the other parameters, the percen-

Table 2
Immunological and virological data in "responders" and "non-responders" to the interferon-α therapy

	CCR5+ in CD4+ (%)	CXCR3+ in CD4+ (%)	CCR3+ in CD4+ (%)	CCR5+ in CD8+ (%)	CXCR3+ in CD8+ (%)	CCR3+ in CD8+ (%)	IFN-γ/ IL-4 ratio	HTLV-1 proviral load (copies/10^5 PBMC)
Blood								
Responders								
(A) Before therapy	2.0 ± 1.1	28.7 ± 7.8	0.4 ± 0.1	5.6 ± 7.6	49.1 ± 21.2	0.5 ± 0.1	11.8 ± 6.1	24156 ± 4666
(B) After therapy	1.1 ± 0.3	23.3 ± 4.8	0.4 ± 0.2	2.1 ± 1.0	37.2 ± 17.4	0.6 ± 0.4	6.4 ± 2.9	17506 ± 5694
(A)–(B)	0.9 ± 0.4	6.4 ± 7.6	0.0 ± 0.1	3.5 ± 6.6	11.8 ± 4.0	0.1 ± 0.2	5.4 ± 3.5	6650 ± 1107
Non-responders								
(A) Before therapy	1.2 ± 0.8	26.1 ± 10.9	0.4 ± 0.1	1.7 ± 1.7	51.2 ± 19.0	0.8 ± 0.5	8.3 ± 8.7	7830 ± 3802
(B) After therapy	1.0 ± 1.0	26.0 ± 8.8	0.4 ± 0.1	1.3 ± 1.0	37.4 ± 17.6	0.5 ± 0.1	5.6 ± 5.9	6954 ± 2538
(A)–(B)	0.4 ± 0.2	2.4 ± 3.2	0.1 ± 0.2	0.5 ± 0.0	13.8 ± 10.1	0.2 ± 0.5	2.7 ± 3.0	876 ± 1851
CSF								
Responders								
(A) Before therapy	33.5 ± 9.0	92.7 ± 0.8	8.8 ± 5.7	35.4 ± 17.1	97.8 ± 3.1	6.1 ± 2.8		
(B) After therapy	19.4 ± 3.0	89.5 ± 3.2	9.0 ± 4.6	25.4 ± 19.7	96.8 ± 0.5	7.5 ± 1.4		
(A)–(B)	14.1 ± 5.9	3.1 ± 4.0	−0.2 ± 1.1	9.9 ± 19.0	1.0 ± 2.6	−1.5 ± 1.3		
Non-responders								
(A) Before therapy	15.3 ± 2.0	86.8 ± 5.6	2.1 ± 0.6	23.9 ± 9.9	93.6 ± 4.5	3.1 ± 2.9		
(B) After therapy	10.9 ± 4.0	78.0 ± 13.2	6.1 ± 0.2	15.9 ± 5.3	87.9 ± 9.0	7.6 ± 5.0		
(A)–(B)	4.2 ± 2.4	8.8 ± 17.4	−4.5 ± 0.4	8.9 ± 12.2	5.8 ± 11.2	−4.8 ± 6.2		

Data are shown as mean ± standard deviation.
IFN-γ, interferon-gamma; IL-4, interleukin-4; IFN-γ/IL-4, ratio of IFN-γ producing cells to IL-4 producing cells in CD3+ cells; PBMC, peripheral blood mononuclear cells; CSF, cerebrospinal fluid.

tages of CCR3+ cells in CSF CD4+ and CD8+ cells tended to be lower and increase more after the therapy in non-responders than in responders.

4. Discussion

Our previous analysis revealed a significant reduction of CD4+ cells in CSF of HAM/TSP after IFN-α therapy (Feng et al., 2003). In the present study, we focused on the Th1/Th2 balance and showed that IFN-α therapy significantly reduced CCR5+CD4+ cells, a Th1 subset, in the patients' CSF as well as blood. The CCR5+CD4+ cell subset in CSF reflects the disease activity in multiple sclerosis (Misu et al., 2001). This subset is increased in the synovium of active rheumatoid arthritis (Mack et al., 1999), and the inhibition of CCR5 successfully treated adjuvant arthritis in rats, an animal model of rheumatoid arthritis (Barnes et al., 1998). In HAM/TSP, elevated levels of CCR5 on memory CD4+ cells in PBMC (Wu et al., 2000) and macrophage inflammatory protein-1α, a CCR5 ligand, in CSF (Miyagishi et al., 1995) were reported. These findings suggest a pathogenic role of CCR5+CD4+ cells in HAM/TSP and other immunologic diseases, and a suppression of the subset by IFN-α might relate to the alleviation of myelitis in HAM/TSP.

There was also a tendency for the CXCR3+CD4 cell, another Th1 subset, to be decreased and CCR3+CD4+ cells, a Th2 subset, to be increased in CSF of the treated patients. A significant decrease in blood CD8+ cell number in the treated patients (Feng et al., 2003) may be attributable to the reduction in CXCR3+CD8+ cells. Moreover, intracellular Th1/Th2-associated cytokine ratio in T cells, which was analyzed only in blood because of the limited volumes of CSF, reduced significantly following the therapy. Taken together, our data suggests that the IFN-α therapy suppressed Th1-related responses in HAM/TSP, although our small-scale study did not address whether the immunological changes were directly associated with the clinical efficacy of IFN-α in HAMTSP.

The present study suggested some interesting differences in baseline immunological and virological findings between responders and non-responders to the IFN-α therapy, that is, responders showed higher Th1 responses and more viral replication than non-responders, and the therapeutic suppression to those parameters was more evident in responders. Our small-scale study could not confirm the associations statistically, but those analyses will be critically important to reliably predict therapeutic efficacy of intramuscular injections of IFN-α beforehand. Whether such laboratory data as (1) baseline percentage of CCR5+ cells in CSF CD4+ cells >20%, (2) baseline ratio of intracellular IFN-γ- versus IL-4-producing blood T cells >5.0 and (3) baseline HTLV-I proviral load more than one copy in five PBMC are really linked to clinical efficacy of IFN-α therapy in HAM/TSP need to be examined in a larger cohort of patients by statistical analyses.

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