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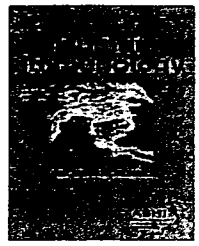

Requirements for the functional expression of OX40 ligand on human activated CD4+ and CD8+ T cells

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Summary Interaction between OX40 expressed on activated T cells and its ligand (OX40L) on antigen presenting cells (APC) provides a co-stimulatory signal for T cells to promote acquired immunity. In the present study, we have examined various culture conditions for optimum OX40L expression on T cells stimulated with immobilized anti-CD3/CD28 monoclonal antibodies (mAbs). Although the day 3 primed T cells expressed minimal OX40L, after repeated stimulations both the $CD4^+$ and $CD8^+$ T cells became OX40L positive as determined by flow cytometry. Interleukin (IL)-12 interfered with the OX40L expression. Among activated T cells, a higher frequency of $CD8^+$ T cells expressed OX40L than $CD4^+$ T cells. By blocking OX40L-OX40 interaction by an anti-OX40 mAb, the number of $OX40L^+$ T cells significantly increased. Screening of various cytokines showed that transforming growth factor (TGF)-β_1 was capable of induction of OX40L on the activated T cells within 3 days. The OX40L expressed on T cells was functional, as they bound soluble OX40 and stimulated human immunodeficiency virus-1 (HIV-1) production from cell lines chronically infected with HIV-1 and expressing OX40. Altogether the present study findings indicate that functional OX40L is inducible on human activated $CD4^+$ and $CD8^+$ T cells, and that the expression is enhanced by TGF-β_1.

Introduction

OX40/OX40L (CD134/CD252) represent a growing number of pairs of co-stimulatory molecules and ligands that have been reported to be critical for T-cell proliferation, survival, cytokine production, and the generation of memory T cells [1-5]. The OX40/OX40L are members of the tumor necrosis factor receptor (TNFR)-TNF super-family with OX40 being transiently expressed primarily by activated T cells after ligation of the T-cell receptor (TCR). Its cognate ligand OX40L (also termed gp34) [6,7] has a broad tissue distribution (including dendritic cells, Langerhans cells, B cells, natural killer cells, vascular endothelial cells, and mast cells) and is not normally detectable on resting cells but can be upregulated after activation [8-23]. The pathophysiologic

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ABBREVIATIONS

APCs	antigen-presenting cells
HIV	human immunodeficiency virus
mAb	monoclonal antibody
OX40L	OX40 ligand
PBMCs	peripheral blood mononuclear cells
TCR	T-cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
FCM	flow cytometry

relevance of such broad tissue distribution of OX40L requires further study. The interaction between OX40 and OX40L has been described as being highly plastic, a characteristic of the TNFR-TNF super-family that was recently confirmed by the delineation of the crystal structure of the OX40/OX40L complex [24].

Although initially the expression of OX40L was thought to be restricted to non-T cells, recent studies appear to question this view. Thus it has been shown that OX40L is in fact inducible on long-term cultured human $CD4^+$ and $CD8^+$ cytotoxic T-cell lines in addition to T-cell lines transformed by human T-cell leukemia virus type I (HTLV-I) [16,25,26]. In the murine system it has been reported that functional OX40L can be induced on TCR-transgenic T cells when they are stimulated *in vitro* [27,28]. More recently the data from Soroosh *et al.* [29] suggested that indeed OX40L expressed by murine T cells participates in an unique T-T-cell signaling system by interaction with OX40 and that such interactions are critical for long-term survival of such T cells. Although these studies have been performed using murine T cells, a role for such T-T-cell interaction, along with the optimum conditions and requirements for OX40L expression by human T cells, have yet to be defined. Thus the present studies were conducted to define first the culture conditions that promote OX40L expression by human T cells *in vitro*. We show here that activated human $CD4^+$ and $CD8^+$ T cells both can express functional OX40L *in vitro* specially after repeated stimulation, and that TGF-β1 is a strong inducer of OX40L. The role of OX40L expressed by T cells is discussed.

Subjects and methods

Reagents

Medium used was RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Sigma) (referred to as RPMI medium). No antibiotics were added. Recombinant human IL-2 (rIL-2) was provided courtesy of the US National Institutes of Health AIDS Research and Reference Reagent Program. The recombinant cytokines rIL-12, rIL-4, rIL-10, rIL-17, rIL-18 rIFN-γ, and rTGF-β_1 were purchased from Peprotec (London, UK). The fluorescent mAbs specific for human CD4 and CD8 (PE or PC5-labeled) and Streptavidin-PE were purchased from Beckman-Coulter (Fullerton, CA). The mAbs produced in our laboratory included mouse IgG1 anti-OX40L (clone 5A8) [30], rat IgG2b anti-HCV (clone Mo-8) [31], mouse IgG1 anti-human OX40 (clone B-7B5) [32], rat IgG2b anti-OX40 (clone W4-54 which was generated from a WKA rat and capable of blocking OX40/OX40L interaction), and control mouse IgG1 mAb TAXY-8 anti-HTLV-I tax antigen [33]. The mAbs from clones B-7B5 and W4-54 recognized different epitopes of OX40 and did not interfere with each other in terms of binding to the same molecule (unpublished data). These in-house mAbs were purified from SCID mouse ascites fluids by gel filtration using Superdex G-200 (Amersham Bioscience, Uppsala, Sweden). These mAbs were labeled using FITC- or Cy5-labeling kits (Dojin, Tokyo, Japan or Amersham) according to the manufacture's instructions. Human T-cell-negative isolation kit, magnetic beads conjugated with anti-human CD3 and anti-human CD28 mAbs, and magnetic beads conjugated with anti-human CD4 or CD8 were all purchased from Dynal Corporation (Oslo, Norway). Biotinylated recombinant soluble human OX40 (sOX40, CD134-muIgG2a/biotin fusion protein) and OX40L (sOX40L, CD134L-muCD8/biotin) were purchased from Ancell (Bayport, MN). A control for mouse IgG2a was purchased from Ancell. Neutralizing mAbs specific for human TNF-α and TNF-β were purchased from R&D (Minneapolis, MN). Anti-CD28 mAb with stimulating activity was purchased from R&D.

Cell lines

An ACH-2 cell line chronically infected with HIV-1 and expressing OX40 was derived by OX40-gene transfection as described previously [32]. Other OX40-, OX40L- and control-gene transfected cell lines used in the present study were generated from the human T-cell line Molt-4 expressing human CCR5 (kindly provided by Dr. M. Baba of Kagoshima University, Japan), and the human promonocytic cell line U1, which was derived from the HIV-1-chronically infected U937 cell line. These cell lines were transfected by electroporation using 10-15 μg of the individual plasmid, as described previously [32]. For the selection of transfectants, 1 μg/ml puromycin was added to the culture media. The expression of the OX40L or OX40 protein on the cell surface of the transfected cells was confirmed by an immuofluorescence followed by flow cytometry (FCM).

Stimulation of T cells *in vitro*

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized (5 U/ml) blood of normal healthy donors using standard density gradient centrifugation with the use of the human lymphocyte separation medium (Sigma). The cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. The PBMCs were resuspended at 1×10^6 cells/ml in RPMI medium supplemented with 20 U/ml IL-2. Then 1 ml of the cell suspension was dispensed into individual wells of 12-well plates (B-D, NJ, USA) and mixed with the anti-CD3/CD28 magnetic beads (anti-CD3/28 immunobeads) at a cell to bead ratio of 1:1, and cultured either in the presence or absence of exogenous IL-12, IL-4 or other recombinant cytokines at 20 ng/ml for 3 days at 37° C in a 5% CO_2-humidified incubator. In some

experiments, PBMCs were stimulated by plate bound OKT-3 (0.05, 0.5, or 5 μg/ml) and soluble anti-CD28 mAb (1 μg/ml). In addition, for some experiments, T cells were purified using a human $CD3^+$ T-cell-negative isolation kit. Viable cell numbers were assessed on an aliquot of such cells utilizing staining with 0.1% eosin-Y in PBS. After 3 days, activated cells were harvested, adjusted to 2×10^5 cells/ml and further stimulated using the same conditions every 3 days. The anti-OX40 blocking mAb (W4-54) or control mAb (Mo-8) was added to T-cell stimulation cultures at 5 μg/ml.

Flow cytometry

Cells to be analyzed were incubated in a FACS buffer (PBS containing 0.1% NaN_3 and 2% FCS) containing 2 mg/ml human IgG on ice for 15 minutes for the blocking of Fc receptors. Without washing, the cells were then subjected to staining with a set of dye-conjugated mAbs, 5A8-FITC and B-7B5-Cy5, with either anti-CD4-PE or anti-CD8-PE on ice for 30 minutes. Then after washing with FACS buffer, cells were fixed in 1% PFA-containing FACS buffer and analyzed using FACSCalibur, and data obtained were analyzed using Cell Quest software (BD, Bedford, MA). In order to determine whether activated T cells can bind OX40L and OX40, Fc-blocked cells were incubated with biotinylated sOX40 or sOX40L at 2.5 μg/ml together with either anti-CD4-PC5 or CD8-PC5 for 30 minutes on ice followed by staining with PE-labeled streptavidin (Beckman Coulter) for 30 minutes on ice. After washing with FACS buffer, cells were fixed in 1% PFA-containing FACS buffer and analyzed. In the experiments that were conducted to demonstrate the ability of W4-54 mAb to block the transfer of OX40 to OX40L and vice versa, aliquots of the Molt4/OX40L cells were mixed with Molt4/OX40 cells in the presence of 5 μg/ml W4-54 or control rat IgG for 30 minutes on ice. These cell aliquots were then incubated with predetermined optimum concentrations of the 5A8-FITC and B-7B5-Cy5 mAb and subjected to flow-cytometric analysis.

OX40 stimulation assay

Two HIV-1-chronically infected cell lines (ACH-2 and U1) that had been transfected with human OX40 gene (ACH-2/OX40 and U1/OX40) ([32] and Takahashi *et al.*, unpublished data) were suspended at 2×10^5 cells/ml and co-cultured with equal number of previously activated T cells in a volume of 0.5 ml in a 48-well plate (BD) in RPMI medium for 24-48 hours at 37° C in a 5% CO_2 humidified incubator. To neutralize endogenous TNF, anti-human TNF-α and TNF-β mAbs (at 5 μg/ml each) were included to block the potential effects of these cytokines synthesized endogenously. HIV-1 replication was determined by quantification of HIV-1 core p24 antigen in the supernatant fluid from such cultures utilizing commercial ELISA kits (Zeptometrix Corp., Buffalo, NY).

Results

Induction of OX40L on human T cells by repeated stimulation

Results from a series of preliminary studies carried out confirmed previous observations that the expression of both

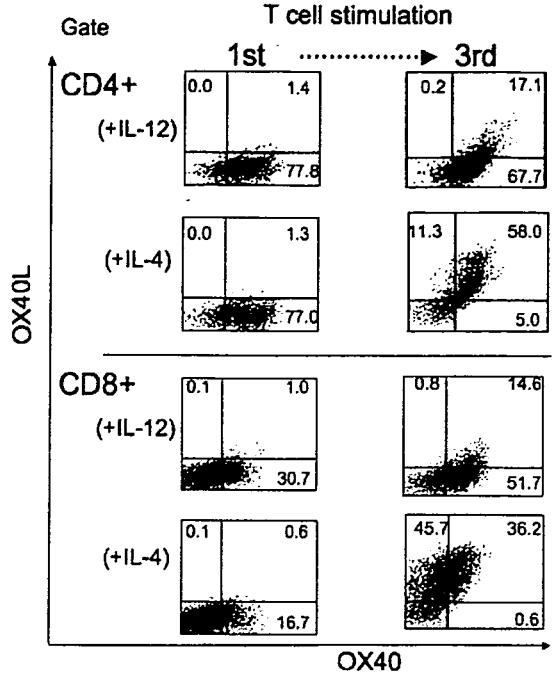

Figure 1. OX40L expression by human activated T cells. Peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3/CD28 immunobeads in the presence of interleukin (IL)-2 together with either IL-12 (+IL-12) or IL-4 (+IL-4) every 3 days. T cells 3 days after the first stimulation (1st) and third stimulation (3rd) were subjected to a multicolor staining using 5A8-FITC, B-7B5-Cy5 and either CD4-PE or CD8-PE. Flow-cytometric data on OX40L and OX40 expression by T cells of $CD4^+$ or $CD8^+$ gate were shown as dot blots. Numbers in the dot-plot graphs indicate percent positive cells. Data are representative of three similar experiments.

OX40L and OX40 were undetectable on resting T cells isolated from fresh human PBMCs from a number of normal donors as determined by standard FCM (data not shown). To examine the expression of OX40L and OX40 on activated human T cells, PBMCs were incubated *in vitro* with anti-CD3/28 immunobeads. Predetermined optimum concentrations of IL-2 and either IL-12 or IL-4 were added to the cultures to generate prototype Th1 or Th2-like populations of human $CD4^+$ and $CD8^+$ T cells to examine them for the expression of OX40L and OX40. After 3 days in such culture conditions, the cultures were harvested and the expression of OX40L and OX40 by the $CD4^+$ and $CD8^+$ T cells was examined by a triple-color staining method. As seen in Figure 1, although >75% of the $CD4^+$ T cells and 16-30% of the $CD8^+$ T cells expressed cell surface OX40, <1.5% of these cells expressed OX40L irrespective of the Th1 or Th2-inducing culture conditions. In efforts to determine if repeated stimulation would facilitate OX40L expression, similar cultures were set up except fresh anti-CD3/28 immunobeads and the same cytokine mixtures were added on days 3 and 6, and the cultures harvested on day 9. Such repeated stimulation had interesting results. Although IL-12 had a modest effect on OX40L expression by $CD4^+$ T cells (an increase from 1.4 to 17.3%) most of which co-expressed OX40, similar cultures incubated in IL-4 led to a dramatic increase (from 1.3% to

58.0%) in the frequency of $CD4^+$ T cells that co-expressed OX40 and OX40L. On the other hand, although similar repeated stimulation in the presence of IL-12 yielded similar effects on the co-expression of OX40L by $CD8^+OX40^+$ T cells (an increase from 1% to 14.6%) as $CD4^+$ T cells, stimulation in the presence of IL-4 not only led to an increase in $OX40^+OX40L^+$ (double positive (DP)) cells (from 0.6% to 36.2%) but also a discrete population of $CD8^+$ T cells that expressed only OX40L (from 0.1% to 45.7%). Repeated studies performed on different days using similar culture conditions showed some degree of variability in the levels of OX40L expression depending upon the donor of the PBMCs (data not shown). However, the basic trend was the same in that whereas the inclusion of IL-12 always led to a modest increase in the frequency of $OX40^+OX40L^+$(DP) cells within both the $CD4^+$ and the $CD8^+$ T-cell populations upon repeated stimulation, the inclusion of IL-4 consistently led to an increase exclusively in $OX40^+OX40L^+$(DP) cells within the $CD4^+$T-cell population and both $OX40^+OX40L^+$(DP) and single $OX40L^+$ (SP) cells in the $CD8^+$ T-cell population (Figure 1 and data not shown). Because previous reports using T cells from TCR transgenic mice suggested that weak TCR stimulation is more critical for OX40L expression [34], microtiter plates were coated with the OKT-3 mAb at 5, 0.5 and 0.05 μg/ml for 1 hour at 37° C, and then triplicate wells were cultured with human PBMCs for 3 days. Analyses of activated T cells from these cultures, however, failed to show any detectable changes in OX40L expression by T cells (data not shown), indicating that differences exist between murine TCR transgenic cells and human bulk T cells in terms of signals required for OX40L expression.

sOX40 binding by T cells

To examine whether the OX40L molecules expressed by these activated human T cells were functional, we examined the capacity of the repeatedly activated T cells to bind sOX40 by FCM. Figure 2 shows that although sOX40 binds preferentially to the restimulated $CD8^+$ T cells but not $CD4^+$ T cells, sOX40L binds preferentially to the restimulated $CD4^+$ T cells but not $CD8^+$ T cells. In addition, although a markedly higher frequency of the IL-4-treated $CD8^+$ T cells bound sOX40 than the IL-12-treated $CD8^+$ T cells, sOX40L bound preferentially to the restimulated $CD4^+$ T cells cultured in IL-12 as compared with IL-4. These data suggest that the induced OX40L molecules were functional in terms of their ability to bind OX40, and that the patterns of the expression of functional OX40L and OX40 by activated T cells differed depending on activation conditions and whether the T cells were of the $CD4^+$ or $CD8^+$ phenotypes.

Effect of anti-OX40 blocking mAb on OX40L expression

It has previously been shown in the murine system that OX40L expression on activated T cells is downregulated by cell-to-cell contact when co-cultured with OX40-expressing cells [29]. In addition, we have shown that human OX40L as well as OX40 molecules can be intercellularly transferred, indicating that co-expression of OX40L and OX40 by a single cell or co-cultures of cells individually expressing OX40L and

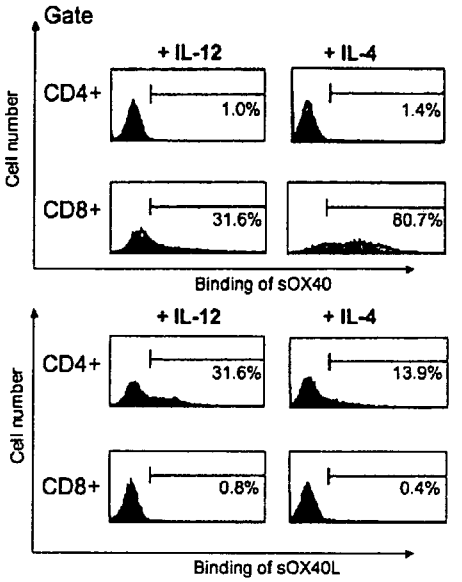

Figure 2. Expression of functional OX40L and OX40 on repeatedly activated T cells. Aliquots of cultures of peripheral blood mononuclear cells (PBMCs) restimulated three times every 3 days for a total of 9 days with anti-CD3/28 immunobeads in the presence of interleukin (IL)-2 together with either IL-12 (+IL-12) or IL-4 (+IL-4) were incubated with either biotinylated recombinant soluble forms of OX40 or OX40L along with either CD4-PC5 or CD8-PC5 mAb for gating. Binding of the soluble proteins was detected by using streptavidin-PE. The bars and the numbers displayed in the histogram graphs show positive area and percent positive cells. The control profile used to define the bar for the positive area was obtained on aliquots of the cultured cells untreated with the soluble proteins. Data are representative of three similar experiments.

OX40 in the same culture may interfere with OX40L expression and/or detection. To study OX40L expression without the involvement of interactions between these two molecules, a series of mAb specific for OX40 were first screened for their individual ability to block such interactions between OX40 and OX40L. A mAb clone W4-54 was thus identified that was capable of inhibiting the binding of sOX40L to membrane bound OX40. The effect of the addition of W4-54 on the binding of sOX40 to its cognate ligand OX40L was also studied. We initially used the OX40- and OX40L-transfected Molt4 cell lines for these studies in efforts to restrict the analysis to a study between these two molecules and to avoid the potential role of other cell surface molecules expressed by mixed cell populations in primary cultures. Figure 3 shows the data obtained. Thus, as seen in Figure 3a, the addition of the W4-54 mAb completely blocked the ability of sOX40L to bind to the Molt4 cell membrane-expressed OX40 (top panel). Similarly the addition of the same W4-54 mAb markedly decreased the binding of sOX40 to the Molt4 membrane bound OX40L (bottom panel). The partial blocking in this latter case may be caused by differences in the affinity of the mAb to bind to the sOX40 and the affinity of sOX40 to bind to the Molt4 membrane expressed OX40L. The ability of the W4-54 mAb to inhibit interaction between OX40 and OX40L is further exemplified by the data shown in Figure 3b.

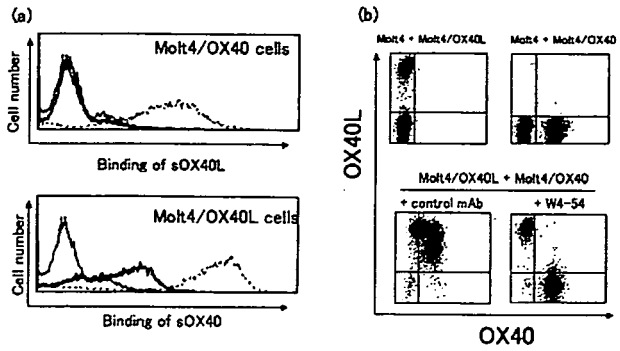

Figure 3. Anti-OX40 mAb (W4-54) blocks OX40-OX40L interaction. (a) Molt4 cells expressing OX40 (Molt4/OX40) or those expressing OX40L (Molt4/OX40L) were preincubated with 5 μg/ml W4-54 or negative control mAb (Mo-8) for 30 minutes on ice. These cells were then incubated with biotinylated sOX40L or sOX40 without washing for additional 30 minutes on ice followed by staining with streptavidin-PE. Top and bottom panels depict the sOX40L binding to Molt4/OX40 cells and sOX40 binding to Molt4/OX40L cells, respectively. Thin line represents background staining with streptavidin-PE; dotted line represents staining with sOX40L or sOX40 in the presence of the negative control mAb; thick dark line represents staining in the presence of the W4-54 blocking antibody. Data shown are representative of three similar experiments. (b) To test the ability of W4-54 to prevent OX40 and OX40L transfer by cell-to-cell contact, Molt4/OX40L cells and Molt4/OX40 cells were each mixed either with the mock-transfected Molt4 cells or mixed with each other in the presence of 5 μg/ml of W4-54 blocking antibody or, for purposes of control, the Mo-8 mAb on ice for 30 minutes. These mixed populations of cells were then stained with anti-OX40L-FITC (5A8) and a different clone of anti-OX40-Cy5 (B-7B5). Representative data from three experiments are shown. Note that when Molt4/OX40L cells and Molt4/OX40 cells were co-cultured in the control mAb, the Molt4/OX40 cells acquired OX40L, and at the same time, the Molt4/OX40L cells acquired OX40 as we showed previously [35].

Thus when the OX40 transfected Molt4 cells were co-cultured with the OX40L-transfected Molt4 cells and the mixed cells analyzed for OX40 and OX40L expression in the presence of the control antibody, there was significant interaction between these transfected cells (bottom left panel). However, the addition of the W4-54 mAb to the mixture of the transfected cells completely inhibited such interaction, as seen with the staining profile (bottom right panel). The top left and right panels depict the control profiles in which the OX40- and OX40L-transfected Molt4 cells were mixed with mock-transfected Molt4 cells. To determine the physiologic relevance of these findings, cultures of primary T cells were similarly examined for the expression of OX40L and OX40 after *in vitro* culture in media containing either IL-12 or IL-4 in the presence of either the same blocking W4-54 antibody or control Mo-8 mAb.

Addition of this blocking anti-OX40 mAb to the T-cell cultures stimulated by anti-CD3/28 (as described in Figure 1) did not show any significant increase in the OX40L expression after the first stimulation (Figure 4, top panel), indicating that the numbers of OX40L molecules on the T cells were few at early stage of stimulation. After the third stimulation, however, the antibody treatment showed a marked increase in the frequency of $OX40L^+$ cells in both $CD4^+$ and $CD8^+$ T-cell subpopulations treated with IL-12 (Figure 4, bottom panel), indicating that the OX40L molecules detected on the activated human T cells were endogenously synthesized but not transferred from other cells. In addition, these data highlight the problems associated with the analysis of OX40 and OX40L expression on such cultured cells if performed without preventing the interactions between these two molecules either by direct cell-to-cell interaction or by the transfer of soluble forms of such molecules.

Effect of TGF-β_1

We screened a number of recombinant human cytokines in combination with IL-2 that might influence the expression of OX40L by activated T cells. As shown in Figure 5, among the cytokines tested, only TGF-β_1 showed a low (by $CD4^+$ T cells) to modest (by $CD8^+$ T cells) enhancement in the induction of OX40L on the activated T cells after as little as 3 days in culture *in vitro*. The cytokines that failed to induce OX40L included IL-10, IL-13, IL-17, IL-18, IFN-γ, and IFN-β (Figure 5 and unpublished data), which was not secondary to the dose of the cytokine used. It is noteworthy that IL-12 and IL-13 also increased the frequency of $CD8^+$ T cells that express OX40. Studies carried out on primary cultures of PBMCs from another donor incubated *in vitro* in media containing TGF-β_1 for 3 days showed that TGF-β_1 influenced the expression of OX40L by a higher frequency of the $CD8^+$ T-cell population than the $CD4^+$ T-cell population (Figure 6a). In addition,

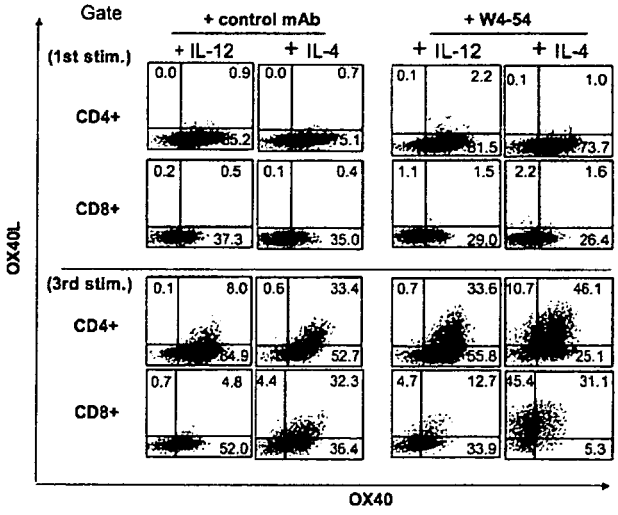

Figure 4. Effect of anti-OX40 blocking monoclonal antibody (mAb) on the expression of OX40L by T cells. Peripheral blood mononuclear cells were stimulated for 3 days (1st stim) or re-stimulated on days 3 and 6 and harvested on day 9 (3rd stim) as described in Figure 1 in the presence of either interleukin (IL)-12 or IL-4 and in the presence of either 5 μg/ml W4-54 or the control mAb (Mo-8). The cells were then analyzed for the expression of OX40L and OX40 using 5A8-FITC and B-7B5-Cy5, respectively. Data reflect the profile observed on gated populations of CD4-PE-positive or CD8-PE-positive cells; data are representative of three similar experiments.

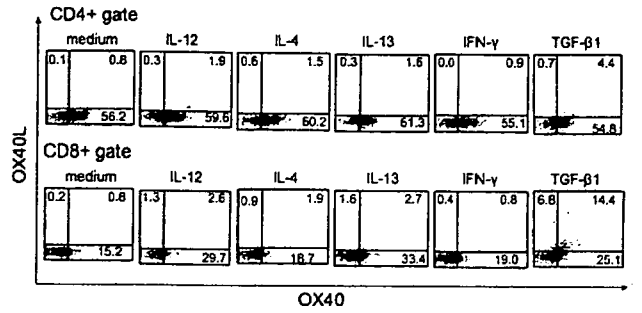

Figure 5. Effect of various cytokines on the induction of OX40L by primary activated T cells. Peripheral blood mononuclear cells were cultured *in vitro* for 3 days as described in Figure 1 in the presence of 20 U/ml interleukin (IL)-2 in combination with each of various cytokines at 20 ng/ml. Thereafter, the cells were stained with 5A8-FITC and B-7B5-Cy5 together with either CD4-PE or CD8-PE. The cells were then subjected to flow-cytometric analysis and the profile of OX40 or OX40L was determined on CD4- or CD8-gated populations of cells. Data are representative of three similar experiments.

there was a distinct population of $CD8^+$ T cells that only expressed OX40L but not OX40. To determine whether the TGF-β_1-induced OX40L was functional, aliquots of the TGF-β_1-induced PBMCs were incubated with biotinylated sOX40 or biotinylated sOX40L followed with streptavidin-PE. As seen in Figure 6b, OX40L expressed by the TGF-β_1-treated $CD4^+$ and $CD8^+$ T cells was quite functional (giving values of 24.3% and 61.7% positive, respectively). However, TGF-β_1 treatment rendered the OX40 molecules nonfunctional, as shown by a reduction of percentages of $CD4^+$ T cells bound by sOX40L from 33.2% to 3.4%. To exclude the possibility that the OX40L was passively acquired and synthesized by non-T cells in PBMCs, we stimulated purified $CD3^+$ T cells in the presence of both TGF-β_1 and anti-OX40 blocking mAb (W4-54), and confirmed that TGF-β_1 induced the endogenous expression of OX40L on both the $CD4^+$ and $CD8^+$ T cells as early as 12 hours (data not shown).

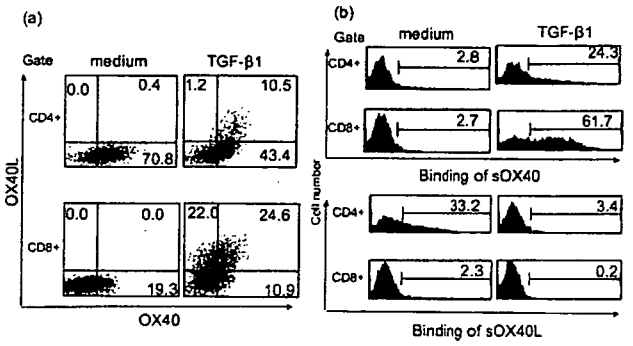

Figure 6. Transforming growth factor-β_1 (TGF-β_1) triggers functional OX40L expression on primary activated T cells. Peripheral blood mononuclear cells from another donor different from one examined in Figure 5 were activated (as described in Figure 1) in interleukin (IL)-2 medium either in the presence or absence of 20 ng/ml TGF-β_1 for 3 days. (a) Expression of OX40L and OX40 was determined by a multicolor staining with mAbs as outlined in Figure 1 legend. (b) Bindings of sOX40 and sOX40L proteins were examined as described in Figure 2 legend. Data are representative of three similar experiments.

Cell-stimulating function of OX40L expressed on T cells

Finally, to confirm that the OX40L induced on the activated human T cells are biologically functional (in addition to having the potential to bind their cognate ligand), we tested whether these $OX40L^+$ T cells could stimulate HIV-1 production from HIV-1-chronically infected T-cell (ACH-2) and the monocytic cell lines (U1) expressing OX40. Activation by the intracellular NF-κB pathway has been shown to be the pathway by which OX40 stimulation induces HIV-1 replication in such cells [32]. Figure 7 shows that human bulk T cells repeatedly stimulated in the presence of anti-CD3/CD28 immunobeads and IL-4 as well as those stimulated once in the presence of the immunobeads, and that TGF-β_1 could stimulate HIV-1 replication in the OX40-expressing T-cell and monocytic cell lines (Figures 7a and 7b, respectively). It is apparent that the cell activation which resulted in HIV-1 replication was mediated by OX40 stimulation by OX40L expressed on the T cells, because this induction of HIV-1 was significantly inhibited by the addition of anti-OX40L blocking mAb (clone 5A8).

Altogether the present data indicate that functional OX40L is inducible on both $CD4^+$ and $CD8^+$ *in vitro* activated human T cells, which is dependent not only on the degree of cell activation but also on the culture environment.

Discussion

The data shown here support the previous findings made by Takasawa *et al.* [25], who showed that long-term cultured cytotoxic $CD4^+$ and $CD8^+$ T-cell clones express OX40L after stimulation *in vitro*. The data reported here extend these previous findings and describe new culture conditions that lead to the induction of OX40L on human $CD4^+$ and $CD8^+$ T cells. Importantly, we demonstrated for the first time that the OX40L molecules induced on the T cells were functional, as they were capable not only of binding soluble recombinant OX40 but also of stimulating OX40-expressing cells (Figures 2 and 7). Importantly, the OX40L molecules detected on the activated T cells were endogenously synthesized, but not those transferred from other non-T cells [35], as demonstrated by using the anti-OX40 blocking mAb (W4-54) during *in vitro* activation in the cultures. Interestingly, the findings here also show for the first time that the immune-suppressive cytokine TGF-β_1 accelerated OX40L expression by primary cultures of activated human $CD4^+$ and $CD8^+$ T cells. The data presented here also show that the stimulated individual $CD4^+$ or $CD8^+$ T-cell could co-express OX40L along with OX40.

The reason why repeated activation was necessary for high levels of OX40L expression by the human T cells *in vitro* remains to be elucidated. In the murine system, it has been reported that bulk splenic T cells from normal adult mice do not express detectable levels of OX40L after stimulation with anti-CD3 with or without anti-CD28 stimulation either [13]. This is in contrast to the murine TCR-transgenic T cells that express OX40L readily after antigenic stimulation within

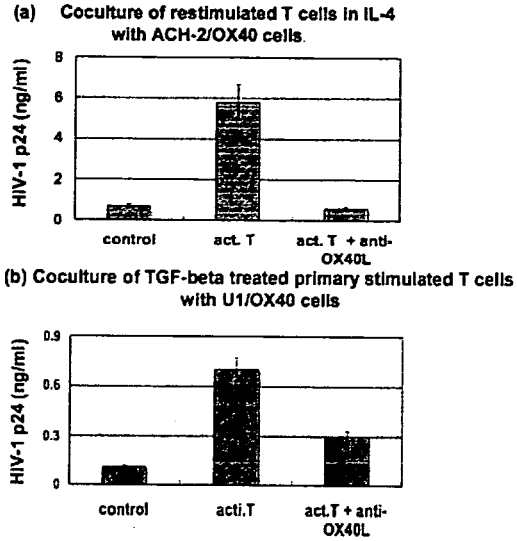

Figure 7. Effect of human T cells stimulated with anti-CD3/CD28 immunobeads either three times or once in the presence of interleukin (IL)-4 or transforming growth factor (TGF)-β_1, respectively, on HIV-1 production from HIV-chronically infected cell lines expressing OX40. (a) ACH-2 cells expressing OX40 (ACH-2/OX40) were co-cultured for 24 hours with T cells that had been previously stimulated three times on days 0, 3, and 6 with anti-CD3/CD28 immunobeads for a total of 9 days in the presence of IL-4 and IL-2. (b) U1 cells expressing OX40 (U1/OX40) were co-cultured for 2 days with peripheral blood mononuclear cells that had been stimulated with anti-CD3/CD28 immunobeads in the presence of TGF-β_1 and IL-2 for 3 days. Because endogenous tumor necrosis factor (TNF)-α and -β are capable of stimulating these two cell lines leading to HIV-1 production, 5 μg/ml anti-TNF-α and -β were included during the culture period. Anti-OX40L mAb (5A8) at 5 μg/ml was used to block OX40L-OX40 interactions. The levels of HIV-1 p24 produced in the culture supernatants were determined by enzyme-linked immunosorbent assay. Controls consisted of the ACH-2/OX40 and the U1/OX40 cells cultured alone (controls), and the ACH-2/OX40 and the U1/OX40 cells treated with anti-OX40L antibody before co-culture with the OX40L-expressing activated T cells (act.T + anti-OX40L). Each combination of culture was performed in triplicate; bars indicate standard errors. Data are representative of three similar experiments.

a few days [27,29]. Thus it is likely that the expression of OX40L by bulk T cells, but not single TCR-expressing T cells, is under tight control during the initial stage of T-cell activation in normal mice and human beings. Under physiologic conditions, OX40L expression by T cells might be programmed to occur at a later stage of immune responses in order to maintain induced effector memory helper T cells [29].

Our present data suggest that the cytokine environment in the cultures in which T-cell stimulation is carried out significantly affects OX40L expression. We found that IL-12 was inhibitory for the expression of OX40L by T cells (Figures 1 and 2). Because the percentages of $OX40^+$ T cells were higher in IL-12-treated T cells than those untreated or IL-4-treated, and anti-OX40 blocking mAb reversed the inhibitory effect of IL-12 on OX40L expression (Figure 4), it seems likely that IL-12 reduced the OX40L expression by T cells through enhanced production of functional OX40 molecules. Again, the effect of IL-12 on OX40L expression by human T cells is in contrast to the murine TCR-transgenic T cells. In this mouse system, the T cells preferentially express OX40L when they are stimulated in the presence of IL-12 via the activation of the STAT-4 pathway, and IL-4 severely downmodulates OX40L expression [27,28]. Also in contrast to human cases, these TCR-transgenic T cells express enhanced levels of OX40 after treatment with IL-4. These discrepancies in OX40L and OX40 induction may be ascribed to species-specific differences or simply because of a difference in the materials tested, *i.e.*, bulk T cells from normal adults versus T cells from the TCR-transgenic mice. Experiments using naive human T cells stimulated in the presence of anti-IL-4 (Th1-rich) or bulk T cells from normal mice may provide clues to such issues.

Even though there are some discrepancies between the human and murine systems, it is now apparent that endogenous OX40L molecules can be expressed on the activated T cells. Thus, functional OX40L molecules expressed at the cell surface may immediately interact with functional OX40 molecules expressed on the same T-cell or adjacent T cells. The fate of OX40L on the T cells is unknown, and thus studies are required to address this issue in more detail in the future. It is possible that some human OX40L may be downmodulated via a post-transcriptional and cell-to-cell contact-dependent mechanisms as the murine T-cell cases as shown by Soroosh *et al.* [29]. Our present data indicate that some OX40L and OX40 molecules remained on the T-cell surface without being bound by OX40 and OX40 molecules, respectively (Figure 2). In addition, some OX40L molecules on T cells may be transferred to adjacent cells via an OX40-dependent and/or independent manner [35].

The present study also revealed that the patterns of OX40L and OX40 expression were quite different between $CD4^+$ and $CD8^+$ T-cell subpopulations. Thus, whereas the $CD4^+$ T cells tend to preferentially express OX40, the $CD8^+$ T cells appear to preferentially express OX40L (Figures 1 and 4). This difference was not merely phenotypic but also functional, because functional OX40L molecules that could bind soluble OX40 were abundant in $CD8^+$ T cells and the OX40 molecules expressed by $CD4^+$ T cells could bind sOX40L (Figure 2). One possible mechanism for this may be that the molecular ratios of OX40L and OX40 synthesized in these subpopulations are different. This phenotypic difference indicates that a communication between $CD4^+$ and $CD8^+$ T cells via OX40L-OX40 interaction is also possible. Because OX40L-OX40 bindings have been shown to mediate cell to cell adhesion, it is highly possible that the OX40L-OX40 interaction may facilitate cross-talk between the two T-cell populations after activation. In the murine system, Soroosh *et al.* [29] hypothesized that OX40L expressed by $CD4^+$ T cells can provide autonomous OX40 signals through interactions between T cells, which contribute to the longevity of antigen-specific $CD4^+$ T cells and thus lead to the generation and survival of $CD4^+$ memory T cells. The other functions suggested in the murine sytem include deviation of a Th1 response to a Th2 response [28], and control of Th2 proliferation [27]. Another possible speculation is that in addition to the immune-stimulating roles, the OX40L/OX40 interaction

between activated T-T cells may serve as a feedback mechanism for the regulation of immune responses by rendering the $OX40^+$T cells unresponsive to further OX40L signals from APC after saturation or masking of functional OX40. In addition, interaction of OX40L/OX40 on T-T cells may result in apoptosis of the cells involved in select environments since OX40 signal alone or together with TNF has been shown to enhance apoptosis of OX40-transfectants ([36], our unpublished data). In addition to the signals generated by OX40 engagement, it is clear that engagement of OX40L also delivers a signal to the APC, which results in increased production of IL-12 by human DCs [10] leading to enhanced murine B-cell differentiation [8]. Thus, it is very interesting to examine whether cross-linking of OX40L generates such signals to the T cells. Soroosh *et al.* [29] mentioned that cross-linking OX40L on T cells with a recombinant soluble OX40 or anti-OX40L mAb did not promote T-cell proliferation, but rather suppressed it. Our preliminary experiments also failed to demonstrate any blocking or enhancing effects by anti-OX40L or anti-OX40 mAb on cell proliferation, cytokine production or T-cell survival *in vitro* (data not shown).

Finally, although some reagents that trigger human OX40L on a number of non-T-cell lineages such as the $CD11c^+$ DC [37], NK cells [21], and mast cells [22] have been reported, it should be emphasized that TGF-β_1 played a prominent role in inducing the expression of functional OX40L on both human $CD4^+$ and $CD8^+$ T cells within 3 days after activation (Figures 5-7). Because TGF-β_1 is known to induce IL-17 to generate Th17 cells [38], it is interesting to test whether the $OX40L^+$ cells are related to Th17 cells or not. Preliminary data obtained in our laboratory indicated that TGF-β_1 exerted its OX40L-inducing effect on T cells as early as 12 hours after T-cell activation even in cultures of T cells that had been previously treated with mitomycin-C (data not shown), suggesting that TGF-β_1 directly stimulates activated T cells to express OX40L without a requirement for either IL-17 or cell proliferation. The biologic function of this TGF-β_1-induced OX40L is not known at present. However, it can be speculated that the OX40L/OX40 interaction may be partly involved in multiple functions of TGF-β_1 [39] on immune responses including generation of regulatory T cells [40] and autonomous promoting survival of peripheral T cells [41]. It is interesting to examine whether such OX40L-expressing T cells express Foxp3. However, at present, we are unable to test this because our anti-OX40L mAbs used to stain the cells is not detectable on the cell surface after treatment of the OX40L-expressing cells with the permeabilization buffer provided with the human Foxp3 staining kit (eBioscience, Inc., San Diego, CA; data not shown). Further studies are in progress to elucidate the precise immunologic roles of OX40L expression by human T cells.

Acknowledgments

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MAJOR ARTICLE

Novel Mutation of Human DNA Polymerase γ Associated with Mitochondrial Toxicity Induced by Anti-HIV Treatment

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(See the editorial commentary by Lewis, on pages 1399–401.)

Mitochondrial toxicity is a major adverse effect of the nucleoside reverse-transcriptase inhibitors (NRTIs) used for treatment of human immunodeficiency virus type 1 (HIV-1) infection and can result in life-threatening lactic acidosis. The toxicity is due to inhibition of polymerase γ (Pol γ), which is required for replication of mitochondrial DNA (mtDNA). Genetic factors could be involved in this process, given that not all NRTI-treated patients experience the toxicity. In 1 patient with lactic acidosis, a novel homozygous Pol γ mutation (arginine to cysteine at codon 964 [R964C]) was identified at a site close to polymerase motif B, which is highly conserved among family A polymerases. Recombinant R964C Pol γ showed only 14% activity, compared with that of wild-type Pol γ. Culture with stavudine significantly reduced mtDNA levels in patient-derived lymphoblastoid cell lines (LCLs) harboring R964C Pol γ, compared with those in LCLs harboring wild-type Pol γ. The novel Pol γ mutation could be associated with the severe lactic acidosis induced by long-term NRTI use.

Today's antiretroviral regimens are highly effective at suppressing HIV-1 replication and restoring immune function. However, long-term use of some antiretroviral agents is often associated with a variety of toxicities that can decrease quality of life or jeopardize the patient's health [1, 2]. The nucleoside reverse-transcriptase inhibitors (NRTIs) that represent the backbone of current anti–HIV-1 regimens are associated with a variety of long-term adverse effects, most of which are attributed to mitochondrial toxicity, possibly due to inhibition of mitochondrial DNA (mtDNA) replication. mtDNA replicates by a multienzyme complex, the main component of which is the nuclear-encoded DNA polymerase γ (Pol γ) [3]. NRTIs are thought to induce mitochondrial toxicity by inhibiting Pol γ, which results in the depletion of mtDNA, damage of the respiratory chain, elevation of serum lactate levels, and life-threatening lactic acidosis [3–6].

The antiretroviral agent stavudine (d4T) was once used but later dropped from the preferred first-line combination regimens because of its high mitochondrial toxicity [1, 2]. In vitro studies showed that d4T causes the greatest inhibition of Pol γ activity, and clinical studies showed that d4T use is most significantly associated with the elevation of serum lactate levels among clinically used NRTIs [4, 5, 7, 8]. However, d4T is a component of GPO-VIR and Triomune, which are widely used generic drugs in resource-limited situations, and it is still commonly prescribed, especially in developing countries [9, 10]. Furthermore, not only

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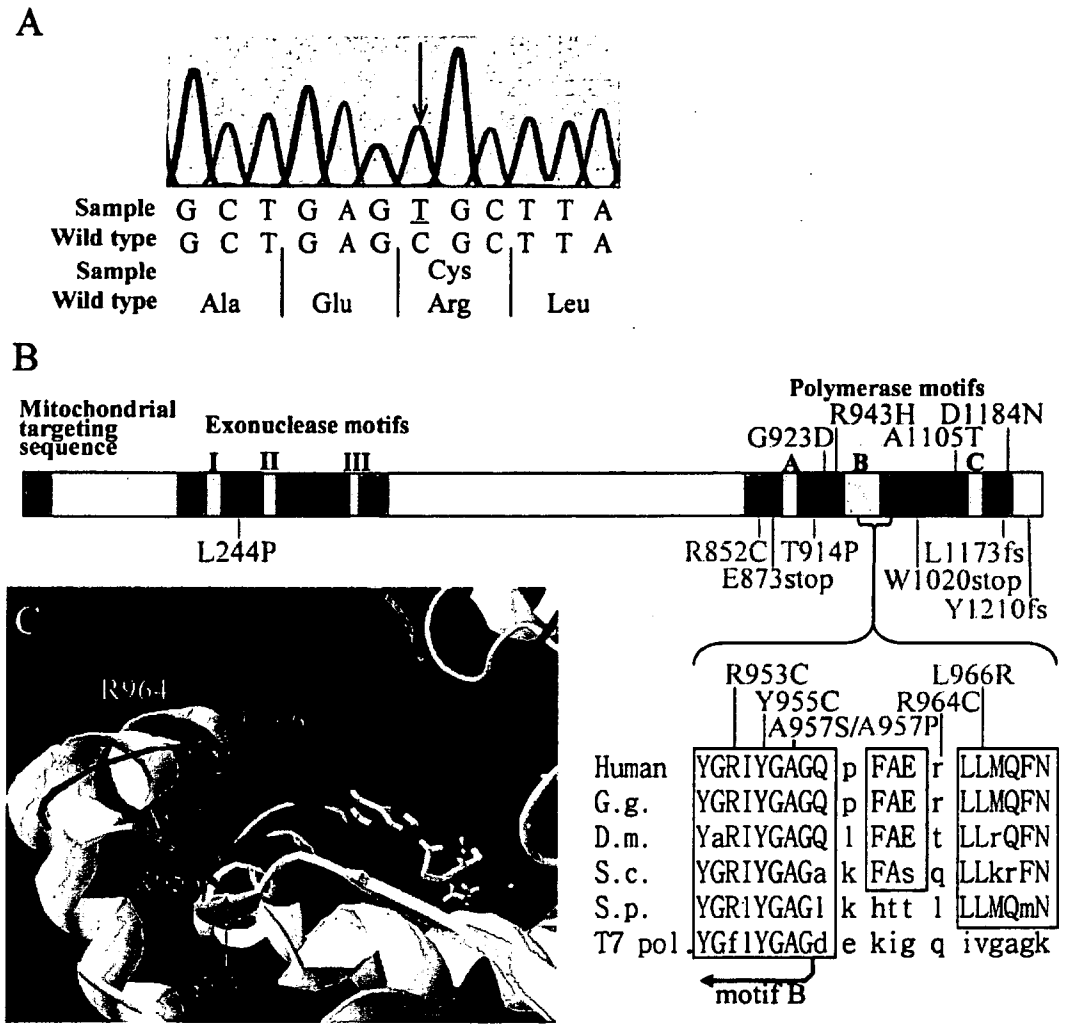

Figure 1. A novel polymerase γ (Pol γ) mutation, R964C, close to polymerase motif B. *A*, Direct sense-strand sequence around the R964C mutation in Pol γ. Ala, alanine; Arg, arginine; Cys, cysteine; Glu, glutamic acid; Leu, leucine. *B*, Novel and reported mutations in Pol γ. Active sites of exonuclease and polymerase are shown in green boxes. Mutations shown in green are associated with Alpers syndrome, and mutations shown in blue are associated with autosomal dominant progressive external ophthalmoplegia (PEO). G.g., *Gallus gallus;* D.m., *Drosophilia melanogaster;* S.c., *Saccharomyces cerevisiae;* S.p., *Schizosaccharomyces pombe*. *C*, Homologous-structure modeling of the Pol γ active site from the T7 polymerase complex structure with incoming ddATP. Motif B, O helix, is shown in pink, and motif A is shown in blue. The position of autosomal dominant PEO and Alpers syndrome mutations are shown in green. Position 964 is shown in yellow. Primer and template DNA strands are shown in red and green, respectively.

d4T but didanosine and zidovudine (AZT), both of which are often used in salvage therapy after virological treatment failure, also cause significant mitochondrial toxicity [4, 5, 7]. Therefore, mitochondrial toxicity is still a major critical problem in the management of patients treated with antiretroviral regimens [1, 2].

Because not all patients receiving long-term NRTI treatment experience mitochondrial toxicity, genetic factors as well as other environmental conditions could be involved. Human DNA Pol γ is composed of a 140-kDa catalytic subunit and a 55-kDa accessory subunit. Mutations in the gene for the catalytic subunit *(POLG)* have been shown to be a frequent cause of mitochondrial disorders, including progressive external ophthalmoplegia (PEO), which is often associated with multisystemic disorders (such as deafness, cataracts, depression, dysphagia, hypogonadism, neuropathy, and sensory ataxia) and Alpers syndrome (a fatal childhood disease caused by brain and liver failure often associated with refractory seizures, episodic psychomotor regression, cortical blindness, and liver disease with micronodular cirrhosis) [11–15]. The main hypothesis of the present study was that genetic variations in *POLG* promote sensitivity to NRTI treatment. To test our hypothesis, we se-

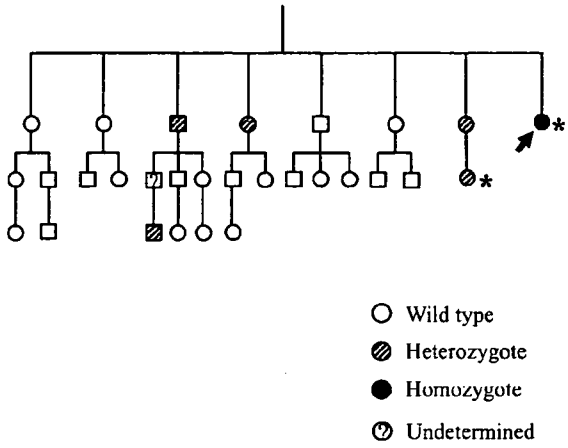

Figure 2. Pedigree of a family with the polymerase γ R964C mutation. Asterisks indicate individuals whose lymphoblastoid cells were established and analyzed; circles indicate females; and squares indicate males.

quenced all 22 coding exons of *POLG* in 11 patients with a history of hyperlactatemia induced by d4T, as well as in 5 patients receiving long-term treatment with d4T who had normal serum lactate levels.

METHODS

Sequence analysis of* POLG *in patients and healthy volunteers. All 22 coding exons of *POLG* were sequenced in 11 patients with a history of hyperlactatemia induced by d4T treatment and in 5 patients who were receiving long-term d4T treatment but had normal serum lactate levels. To analyze the prevalence of the identified mutation, the region encompassing *POLG* exons 17 and 18 was sequenced in 26 additional HIV-1–infected individuals, 110 healthy volunteers, and 27 relatives of the identified mutation carriers. All analyzed patients were treated or followed-up at the AIDS Clinical Center, International Medical Center of Japan (IMCJ), and the healthy volunteers were recruited at IMCJ and Srinagarind Hospital, Khon Kaen University, Thailand. The institutional review boards of IMCJ (H13-20) and Khon Kaen University (HE460318) approved this study, and informed consent was obtained from all participants. Genomic DNA was extracted from whole blood by use of the QIAamp Blood Mini Kit (Qiagen), followed by polymerase chain reaction (PCR) with One Shot LA PCR Mix (Takara Shuzo) using primers that have been described elsewhere [16]. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems) and an automated DNA sequencer (model 3730; Applied Biosystems). The amino acid sequences were deduced using the Genetyx-Win program (version 6.1; Software Development).

Production and purification of recombinant human Pol γ. The human Pol γ catalytic subunit cDNA was constructed from RNA derived from a wild-type Pol γ carrier and a mutant Pol γ carrier. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) by use of the Catrimox-14 RNA Isolation Kit (version 2.11; Takara Shuzo). Reverse-transcriptase PCR was performed, followed by nested PCR. The outer primer pairs were A1F (nt 87–108; NM 002693 as referential sequence) and A1R (nt 2037–2016) for fragment A, B1F (nt 1063–1084) and B1R (nt 3296–3275) for fragment B, and CDF (nt 2081–2101) and CDR (nt 4425–4404) for fragments C and D. The inner primer pairs were A2F (5′-AGATCTGGTCTCCAGCTCCGTC [*Bgl*II restriction site plus nt 357–377]) and A2R (nt 1646–1625) for fragment A, B2F (nt 1362–1382) and B2R (nt 2667–2646) for fragment B, CDF and C2R (nt 3296–3275) for fragment C, and D2F (nt 2485–2506) and D2R (nt 4054–4037) for fragment D. The obtained PCR product fragments (A, *Bgl*II restriction site plus nt 357–1646; B, nt 1362–2667; C, nt 2081–3296; D, nt 2485–4054) were cloned by using Original TA Cloning Kit (Invitrogen). Unintended mutations were corrected by the oligonucleotide-based mutagenesis method. Fragments A and B were combined by the PCR-mediated recombination method [17]. The *Bgl*II–*Nde*I portion of fragment A+B, the *Nde*I–*Stu*I portion of fragment C, and the *Stu*I–*Eco*RI portion of fragment D were inserted into a histidine-tagged transfer vector (pYNGHis; Katakura Industries) [18]. Thusly obtained transfer vector and baculovirus (CPd strain) genomic DNA were cotransfected into BmN cells [19]. Then, the recombinant baculovirus was screened by Western blot analysis with anti–human Pol γ serum (Lab Vision). Successful recombinant viruses were used to inoculate silkworm *(Bombyx mori)* larvae, and the infected larvae were reared until pupal state [19]. Pupae were mushed with mushing buffer (50 mmol/L Tris [pH 8.0], 10% glycerol, 0.3 mol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1% 2 mercaptoethanol [2-ME], 1 mg/mL leupeptin, 1 mmol/L EDTA, and 0.5% Triton X-100) and centrifuged. The supernatant was loaded onto a Ni-chelate–affinity resin column, and the column was eluted with 50 mmol/L Tris (pH 8.0) and 250 mmol/L imidazole. The eluted solution was loaded onto an ion-exchange column, and the column was eluted with 50 mmol/L Tris (pH 8.0), 1 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L 2-ME, and 0.05% nonidet P-40. Protein concentration was measured by use of Coomassie Protein Assay Reagent (Pierce). Purified protein was stocked with 50% glycerol at 4°C until use. All purification procedures were done at 4°C.

Polymerase assay. The polymerase activity of recombinant Pol γ was determined using a chemiluminescent reverse-transcriptase assay kit (Asahi Kasei) according to the protocol provided by the manufacturer [20]. Recombinant Pol γ (1.2 μg) was incubated with 10 μg of polyA-oligo$(dT)_{27}$, 30 μmol/L dTTP, and 4.2 μmol/L biotin-16–dUTP at 37°C for 15 m for

the standard reaction. Inhibition of Pol γ was measured in this standard reaction in the presence of ddTTP (Takara Shuzo), AZT triphosphate (AZT-TP; Moravek Biochemicals), and d4T triphosphate (d4T-TP; Moravek Biochemicals). Steady-state kinetic analysis determined $K_{m(dTTP)}$, V_{max}, and K_i values from initial linear steady-state velocities with Lineweaver-Burk plot analysis by use of Graph Pad PRISM (version 4; GraphPad Software), and k_{cat} values were calculated by dividing the V_{max} value by active enzyme concentrations [21].

Measurement of mtDNA/nuclear DNA (nDNA) in lymphoblastoid cell lines (LCLs). The d4T-induced depletion of mtDNA in patient-derived LCLs was assessed as described elsewhere [22]. LCLs were established by Epstein-Barr virus transformation from the PBMCs of wild-type Pol γ carriers and heterozygous and homozygous mutant Pol γ carriers. The established LCLs (5×10^5) were cultured in triplicate for 1 week in the presence or absence of d4T (1 or 10 μmol/L), and the culture experiment was repeated 3 times for each LCL. Total DNA was extracted from LCLs before and after the culture, and the change in the mtDNA/nDNA ratio during the culture was measured by real-time PCR using the ABI PRISM 7700 sequence detection system (Applied Biosystems) [22, 23]. TaqMan β-actin control reagents (Applied Biosystems) were used for nDNA measurement. Specific primers and probe for the mitochondrial NADH dehydrogenase subunit 1 gene were used for mtDNA measurement [24].

RESULTS

Novel* POLG *mutation in a patient with hyperlactatemia. All 22 coding exons of *POLG* were sequenced in 11 patients with a history of d4T-induced hyperlactatemia and in 5 patients with normal serum lactate levels despite long-term d4T use. There is a known variation in the number of CAG repeats in the second exon, and a correlation between male infertility and the absence of the common 10-CAG repeat has been reported [25]. Analysis of the second exon in our patients showed that all 5 with normal lactate levels and 9 of 11 patients with d4T-induced hyperlactatemia were homozygous for a 10-CAG repeat allele, whereas 2 patients with hyperlactatemia were heterozygous for *POLG* allele with 7/10-CAG and 11/13-CAG repeats. Sequencing of the other exons identified 2 synonymous mutations (both of which were previously reported single-nucleotide polymorphisms) heterozygous with wild-type nucleotides in exons 12 and 18 in 2 different patients with hyperlactatemia, although these mutations were not found in the other patients. In addition to these 2 mutations, a novel homozygous mutation in which arginine is replaced with cysteine at position 964 (R964C) was identified in *POLG* exon 18 in 1 patient with hyperlactatemia (figure 1*A*). Sequence analysis of peripheral blood samples obtained on other days and subclonal analysis of PCR products containing the region of exons 17 and 18 confirmed that this mutation was not an artefact from the PCR procedure and that the patient had the R964C mutation homozygously. Interestingly, position 964 is located close to polymerase motif B, which is highly conserved among family A DNA polymerases, and many mutations associated with PEO

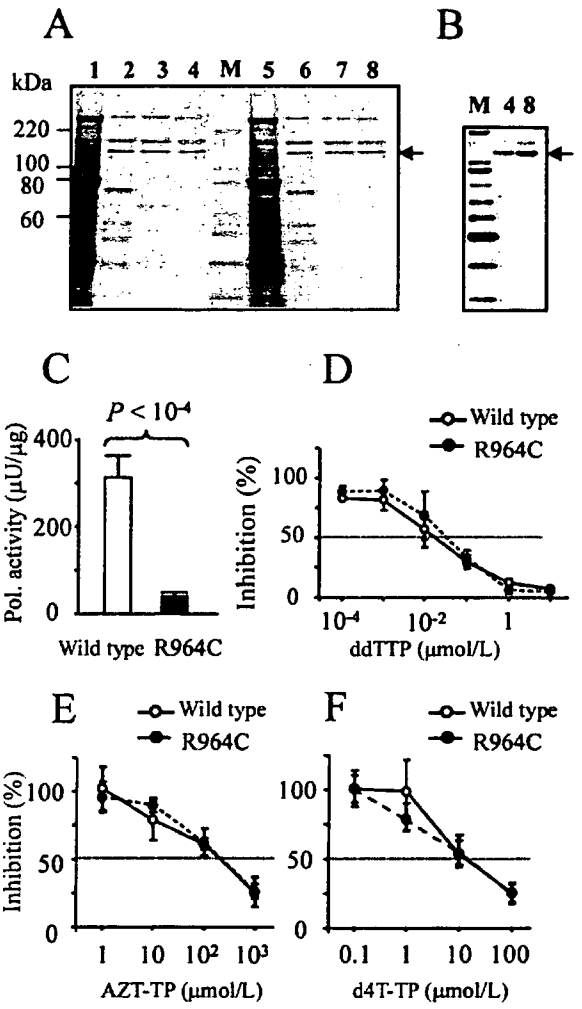

Figure 3. Purification and analysis of recombinant polymerase γ (Pol γ). *A*, Purity of wild-type *(lanes 1–4)* and mutant *(lanes 5–8)* recombinant Pol γ, shown in silver staining. *Lanes 1 and 5,* mushing buffer; *lanes 2 and 6,* Ni-chelate–affinity resin column elutant; *lanes 3 and 7,* ion-exchange column elutant; *lanes 4 and 8,* 50% ion-exchange column elutant plus 50% glycerol; *lane M,* molecular-weight marker. *B,* Western blot of final purified products for the wild type *(lane 4)* and mutant *(lane 8).* A molecular-weight marker is also shown *(lane M). C,* Polymerase activity of wild-type and mutant recombinant Pol γ. One unit is defined as the enzyme activity that incorporates 1 nmol of dTTP in 1 min at 37°C. The indicated *P* value is based on Student's *t* test. *D–F,* Inhibition of Pol γ by nucleoside analogue triphosphates (*D,* ddTTP; *E,* zidovudine triphosphate [AZT-TP]; *F,* stavudine triphosphate [d4T-TP]). Data are mean ± SD values from 3 independent experiments. Each experiment was performed in triplicate.

and Alpers syndrome are located around this site, indicating that the region is critical for normal function of Pol γ (figure 1*B*) [15, 26]. Furthermore, in homologous-structure modeling for the polymerase domain of the human Pol γ catalytic subunit (defined as residues 871–1145) developed from ternary T7 polymerase complex structure (Protein Data Bank entry 1SKR), position 964 in human Pol γ is analogous to position 534 in T7 polymerase, which is located in the O1 helix next to the O helix corresponding to polymerase motif B, a motif that is involved in the binding of incoming dNTPs; this suggests that R964C might change the interaction between Pol γ and incoming dNTPs (figure 1*C*) [26–28].

The identified R964C carrier was a 34-year-old HIV-1–infected Thai woman who had been asymptomatic until the development of *Pneumocystis jiroveci* pneumonia. She had suffered from severe lactic acidosis after 1 year of use of d4T and lamivudine (3TC). Her peak lactate level was 67 mg/dL, and paresthesia was still present in both legs after >5 years of cessation of d4T treatment. To our knowledge, the R964C mutation in Pol γ had not been reported previously. To analyze the prevalence of the R964C mutation, the region including *POLG* exons 17 and 18 was sequenced in 26 additional Thai patients and 110 healthy volunteers (including 100 Thais), but the mutation was not detected in any of these individuals. However, 5 of the patient's 27 relatives had the mutation heterozygously (figure 2). One of the 5 heterozygous mutation carriers was the father of 3 children, and the index case patient's father, who was considered to be a heterozygous carrier, had 8 children, suggesting that heterozygous R964C mutation is not associated with male infertility.

Low polymerase activity of mutant Pol γ. To characterize the biochemical effect of the R964C mutation, wild-type and mutant recombinant protein of the Pol γ catalytic subunit were constructed and purified from baculovirus-infected silkworm pupae through Ni-chelate–affinity resin and ion-exchange columns [18, 19]. The purity of wild-type and mutant Pol γ was almost the same at each purification step (figure 3*A*), and no degradation was observed in the final products (figure 3*B*). Surprisingly, analysis of polymerase activity showed that the mutant Pol γ had only 14% activity, compared with that of wild-type Pol γ (figure 3*C*). Steady-state kinetic analysis showed that the R964C mutation did not significantly alter $K_{m(dTTP)}$ but decreased k_{cat} to <10%, resulting in a decrease in the $k_{cat}/K_{m(dTTP)}$ ratio to 11% (table 1) and suggesting that the binding affinity to dTTP was not altered by the R964C mutation, although catalytic efficiency was reduced significantly. Inhibition analysis showed that 0.02 μmol/L ddTTP, 200 μmol/L AZT-TP, and 15 μmol/L d4T-TP inhibited 50% of wild-type Pol γ activity, respectively. Furthermore, the analysis also showed that the R964C mutation did not alter the susceptibility of Pol γ to these nucleoside triphosphates (figure 3*D*–3*F*) and that K_i values for these 3 nucleoside-analogue phosphates were not altered by the R964C mutation (data not shown). The above experiments were repeated 3 times from baculovirus inoculation of silkworm larvae through enzymatic analyzes of recombinant Pol γ, and the results were found to be reproducible.

Decrease in mtDNA level in LCLs with mutant Pol γ caused by d4T. Previous studies have demonstrated that ratios of mtDNA level to nDNA level in PBMCs from patients with hyperlactatemia are markedly low and that mtDNA/nDNA ratios decrease in various cell lines and PBMCs after an 8-day incubation with NRTI [22, 23]. These findings prompted us to analyze the biological effects of the R964C mutation in a cell culture system. LCLs were established from the PBMCs of wild-type Pol γ carriers, including 2 patients with normal lactate levels despite long-term d4T use (wild-type 1, a 58-year-old HIV-1–infected Japanese man with a history of 5 years of d4T and 3TC use, and wild-type 2, a 45-year-old HIV-1–infected Japanese man with a history of 7 years of d4T and 3TC use), 1 patient with a history of hyperlactatemia induced by d4T use (wild-type 3, a 58-year-old Japanese man with a history of severe lactic acidosis [peak lactate level of 69.3 mg/dL] accompanied by peripheral neuroparalysis induced by 1 year of d4T and 3TC use), 1 heterozygous mutant Pol γ carrier (an HIV-1–uninfected niece of the index case patient), and the homozygous mutant Pol γ carrier (the index case patient) (figure 2). DNA sequencing of all 22 coding exons of *POLG* confirmed that these individuals did not have any other mutation apart from R964C. An LCL derived from each individual was cultured in the absence or presence of d4T (1 or 10 μmol/L) for 1 week, and the change in the mtDNA/nDNA ratio was assessed by real-time PCR [24]. Because the mtDNA/nDNA ratio differed widely among individual LCLs, the ratio from before the culture was used as the baseline reference for the relative comparison in each LCL. One-week culture in the absence or presence of 1 μmol/L d4T did not significantly change the mtDNA/nDNA ratio in each type of LCL (figure 4). However, when the d4T concentration was increased to 10 μmol/L, which is equivalent to the peak plasma concentration at the standard dosage [29], the mtDNA/nDNA ratio decreased in LCLs derived from 1 wild-type Pol γ carrier with a history of hyperlactatemia (wild-type 3, 0.71-fold) and the heterozygous (0.53-fold) and homozygous (0.50-fold) mutant Pol γ carriers. However, the ratio

Table 1. Effect of the R964C mutation on the polymerase kinetics of polymerase γ (Pol γ).

Pol γ	$K_{m(dTTP)}$, μmol/L	k_{cat}, s^{-1}	$k_{cat}/K_{m\ (dTTP)}$
Wild type	10.9	0.1	0.009
R964C	8.3	0.008	0.001

NOTE. $K_{m(dTTP)}$ and k_{cat} kinetic values for recombinant Pol γ proteins were determined with polyA-oligo$(dT)_{27}$ as the substrate, as described in Methods. s, seconds.

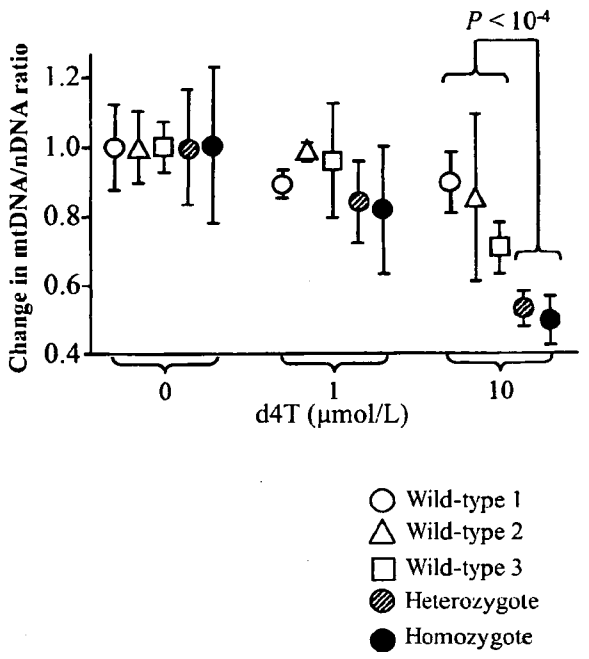

Figure 4. Changes in mitochondrial DNA (mtDNA)/nuclear DNA (nDNA) ratios in lymphoblastoid cell lines (LCLs) cultured with and without d4T. For each LCL established from the peripheral blood mononuclear cells from 4 patients and from a niece of the index case patient (see Results), the mtDNA/nDNA ratios after culture at the indicated stavudine (d4T) concentration are presented relative to the baseline ratios (before culture). Data are mean ± SD values from 3 independent experiments. Each experiment was performed in triplicate. The indicated *P* value is based on Student's *t* test.

did not change significantly in LCLs derived from the other wild-type Pol γ carriers with normal lactate levels (wild-type 1, 0.90-fold; wild-type 2, 0.85-fold). Both LCLs harboring mutant Pol γ heterozygously and homozygously contained significantly reduced mtDNA levels after 1 week of culture in the presence of 10 μmol/L d4T, compared with those in LCLs harboring wild-type Pol γ, indicating that the R964C mutation–induced phenotype was dominantly expressed in heterozygous LCLs. There were other heterozygous mutant Pol γ carriers in the family of the homozygous mutant carrier (figure 2), but unfortunately we could not obtain from them viable PBMCs to prepare LCLs.

DISCUSSION

A novel mutation of Pol γ, R964C, was identified homozygously in 1 Thai patient with a history of severe lactic acidosis induced by 1 year of treatment with d4T, the prevalence of which seemed rare even in the Thai population, although at least 2 familial lineages were considered to harbor the mutation because her father and mother were not close relatives. She had been healthy until the symptomatic development of AIDS, and no member of her family suffered from mitochondrial disorders, indicating that the R964C mutation does not necessarily induce symptomatic disease under normal conditions. However, enzymatic analysis of recombinant Pol γ showed that the R964C mutation decreased Pol γ activity to one-tenth of normal, although it did not alter the percent susceptibility to nucleoside analogue triphosphates, including d4T-TP. Furthermore, LCLs derived from the homozygous and the 1 heterozygous mutant Pol γ carriers contained significantly low mtDNA levels after 1 week of culture with 10 μmol/L d4T, compared with those in LCLs derived from wild-type Pol γ carriers. Considered together, the results suggest that the R964C mutation compromised the patient's mitochondrial level to just above the clinical threshold and that further inhibition of mitochondrial DNA replication by d4T therapy caused the level to fall below this energetic threshold and, thus, the patient to present with acute mitochondrial failure and lactic acidosis.

The 964th position of the Pol γ catalytic subunit seems to be critical to its enzymatic function, around which there are many mutations reported to be associated with genetic mitochondrial diseases [15]. Some of them are inherited in autosomal dominant fashion, indicating that mutant Pol γ could suppress the normal function of wild-type Pol γ [15, 30]. In the present study, mtDNA levels were significantly decreased by culture with d4T in the LCLs holding the R964C mutation heterozygously, suggesting that the phenotype caused by the R964C mutation could also be expressed in a dominant negative fashion.

In the other patients with a history of d4T-induced hyperlactatemia, no amino acid–altering mutations were found in *POLG* exons, suggesting that other factors could be involved in the development of NRTI-induced mitochondrial toxicity. Given that decreased mtDNA levels in PBMCs have been reported in treatment-naive HIV-1–infected patients compared with non–HIV-1–infected subjects [23, 31], HIV-1 infection itself has mitochondrial toxicity and predisposes infected individuals to NRTI toxicity. Patients infected with some specific HIV-1 subtype or strain might be more sensitive to NRTI-induced toxicity than other infected patients. Furthermore, the intracellular and intramitochondrial phosphorylation of NRTIs by cellular kinases and the intramitochondrial transport of NRTIs or their phosphorylated prodrugs by transport proteins are also pathophysiologically important and might be genetically or environmentally different among individuals [32].

We identified a nonsynonymous *POLG* mutation in only 1 patient with a history of severe lactic acidosis. Nonetheless, the present study represents the first identification of a mutation in *POLG* that predisposes patients to mitochondrial toxicity induced by antiretroviral treatment, which strongly supports the current understanding that inhibition of Pol γ by NRTIs

leads to mtDNA depletion and thereby causes mitochondrial dysfunction.

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glomerular filtration rate 15 ml/min per 1.73 m^2) avoiding the need for renal replacement therapy. In addition, there was a dramatic decline in proteinuria from an estimated 5 g to 1 g per 24 h.

Epidemiological studies have shown a correlation between increasing HIV viral load, decreasing CD4 cell count and the occurrence of proteinuria and renal failure [1], as well as a reduction in the incidence of HIVAN in patients treated with HAART [2]. Previous case reports have demonstrated a reversal of acute renal failure and histological changes on biopsy after short courses of antiretroviral therapy [3]. Several observational cohort studies have demonstrated a decreased need for renal replacement therapy for patients treated with antiretroviral therapy compared with those not so treated [4–6]. This case supports the hypothesis that HIVAN can be treated by the suppression of viral replication with HAART, and when initiated early may remove the need for renal replacement therapy and provide long-term stabilization of disease. Unlike the previous reported cases, this case suggests a risk of relapse of disease after discontinuing HAART. This suggests that a history of HIVAN alone may be an indication for indefinite HAART, even with an adequate CD4 cell count and despite mild to moderate side effects.

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HLA-Cw8 primarily associated with hypersensitivity to nevirapine

We read with interest the report by Littera *et al.* [1] about human leukocyte antigen (HLA)-dependent hypersensitivity to nevirapine in Sardinian HIV patients. The authors state that high levels of genetic homogeneity and linkage disequilibrium make the Sardinian population particularly suitable for genetic association studies, and they observed a statistically significant association between a nevirapine-hypersensitivity reaction and the HLA-Cw*0802-B*1402 haplotype. In the Sardinian population, however, HLA-Cw*0802 and B*1402 are in such strong linkage disequilibrium that they could not establish which one of these two alleles is primarily associated with the hypersensitivity reaction to nevirapine. Considering that HLA-B14(65) can not be found in the Japanese population, it might be helpful to analyse the patients in our clinic for a determination of the primarily associated HLA allele [2–5].

In our outpatient clinic, a total of 326 HIV-1-infected individuals (309 were Japanese) had given written informed consent for HLA analysis and the study of its association with HIV-1 disease progression and drug-induced adverse events. High resolution typing of the alleles at the HLA-A, HLA-B, HLA-Cw, HLA-DRB1, and HLA-DQB1 loci had been performed by polymerase chain reaction amplification using sequence-specific primers in all of them. The allele frequency of HLA-Cw8 and HLA-B14 was 13 and 0%, respectively, which is compatible with previous reports of HLA frequency in the Japanese population [2–5]. Forty-three of the analysed patients were on nevirapine treatment or had a history of nevirapine treatment. One of them died of malignant lymphoma 4 weeks after the introduction of nevirapine-containing treatment. In another patient, nevirapine-containing treatment was terminated 17 days after initiation because of granulocytopenia probably induced by co-administered zidovudine. These two patients were excluded from further analysis and the remaining 41 patients were divided into two groups; a nevirapine-hypersensitive group and a nevirapine-tolerant group (Table 1). The nevirapine-hypersensitive group included 11 patients who experienced extensive skin rash (accompanied by fever > 38°C in three) and one patient with chronic hepatitis C who developed nevirapine-induced hepatotoxicity with aspartate aminotransferase/alanine aminotransferase values three times above the baseline. The nevirapine-tolerant group included 29 others who had been treated with nevirapine for a period of more than 6 months and did not develop any hypersensitive reaction [1]. There were no significant

Table 1. Demographics and immunological variables in the nevirapine-hypersensitive group and nevirapine-tolerant group.

Variable	Nevirapine hypersensitive ($n=12$)	Nevirapine tolerant ($n=29$)	P value
Mean age, years (SD)	33	40	0.07
Sex, *n* (%)			
Male	11 (92%)	26 (90%)	>0.99
Female	1 (8%)	3 (10%)	
Ethnicity, *n* (%)			
Japanese	11 (92%)	28 (97%)	0.50
Mean weight, kg (SD)	62 (13)	61 (8)	0.88
Plasma HIV-1 RNA, *n* (%)			
>400 copies/ml	9 (75%)	14 (48%)	0.17
Immunological status, cells/μl (SD)			
CD4	306 (186)	291 (184)	0.81
CD8	587 (246)	765 (416)	0.17
HLA, *n* (%)			
Cw8	5 (42%)	3 (10%)	0.03

differences in age, sex, ethnicity, weight, HIV-1 viral load, CD4 and CD8 cell counts between the two groups (Fisher's exact test for dichotomous variables, Student's *t*-test for continuous variables). The frequency of HLA-Cw8-positive patients in the nevirapine-hypersensitive group was 42%, which was significantly higher than those of the nevirapine-tolerant group (10%) and the general Japanese population (9–14%) [2–5]. In the nevirapine-hypersensitive group, four patients including one who developed hepatotoxicity had HLA-Cw*0801 and one had HLA-Cw*0803. In the nevirapine-tolerant group, three patients had HLA-Cw*0801. HLA-Cw*0802 was not identified in the patients we analysed. There was no significant difference in the frequency of the other HLA alleles between the two groups.

Considering our data together with that of Littera *et al.* [1], HLA-Cw8 antigen rather than specific alleles of other genes linked with HLA-Cw*0801 or HLA-Cw*0802 may be primarily associated with a nevirapine-hypersensitivity reaction. Nevirapine or nevirapine metabolite coupled with HLA-Cw8 antigen may be expressed on the cell surface and may induce hypersensitive reactions including skin rash and hepatotoxicities. We totally agree with Littera *et al.* [1] that a careful choice of drugs in susceptible patients identified by HLA typing would considerably reduce the risk of severe and sometimes life-threatening hypersensitive reactions.

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K65R development among subtype C HIV-1-infected patients in tenofovir DF clinical trials

A recent study demonstrated a greater propensity for HIV-1 subtype C to develop a K65R mutation under in-vitro selection with tenofovir compared with subtype B or other non-B subtypes [1]. The mechanistic basis for this in-vitro observation was not clear because the single nucleotide change is identical for the K65R substitution in either subtype B or subtype C. For subtype B, a switch from AAA to AGA occurs. For subtype C, a switch from AAG to AGG occurs. The third codon position, however, is different for subtype C, reflecting redundancy in the genetic code for lysine and a natural variation among different HIV-1 subtypes. The authors speculate that this third codon position difference or other genetic changes may influence the propensity for the development of K65R in subtype C HIV-1.

We evaluated patients with subtype C, subtype B, and other non-B HIV-1 subtypes for virological failure and the development of resistance in two phase III clinical trials of tenofovir disoproxil fumarate (DF). These studies enrolled patients primarily from the United States, Europe and South America. Within these studies, approximately 7% of the 1200 patients enrolled were infected with non-B HIV-1 subtypes. Study 903 assessed the combination of tenofovir DF with lamivudine and efavirenz, and study 934 assessed the combination of tenofovir DF with emtricitabine and efavirenz. Although neither study was statistically powered to address efficacy in patients with non-B subtypes, the virological failure rates were similar between patients with subtype B or non-B HIV-1 subtypes (15.5 versus 19%, respectively,

ABSTRACT 2

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Effects of protease and reverse transcriptase inhibitor-resistance mutations on integrase polymorphism in multidrug resistance cases

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BACKGROUND: HIV protease (Pr), reverse transcriptase (RT) and integrase (IN) are encoded in tandem in the *pol* gene and are translated as one precursor protein, which is processed by Pr into the final enzymes. RT and IN also form a complex during the early stage of the viral life cycle. Since these three enzymes interact, acquisition of mutations in one protein may affect selection of mutations in others. Thus, the aim of this study was to clarify any interference between IN, Pr and RT, and effects of acquiring PI and RTI resistance mutations on integrase polymorphisms.

METHODS: Multidrug resistance (MDR) cases were randomly selected from patient samples sent to the NIID between 2002 and 2006 for routine drug-resistance genotyping. Newly diagnosed treatment-naive (naive) cases were collected between January 2003 and December 2006. Viral RNA extracted from 200 µl plasma was reverse transcribed, and 1.3 Kbps protease–RT and 0.9 Kbps integrase regions were amplified by nested PCR. PCR products were analysed by auto-sequencer. Drug-resistant mutations were defined by the IAS-USA chart of drug-resistance definitions.

RESULTS: Fifty-three MDR cases (subtype B: 47; CRF01_AE: 5, C: 1), and 51 naive cases (B: 41; CRF01_AE: 8, AG: 2) were enrolled. Integrase mutations observed in MDR and newly diagnosed cases were compared for subtypes B and CRF01_AE. For subtype B, we found the following mutations: D10E (naive: 97%, MDR: 87%), K14R (3%, 9%), S17N (29%, 41%), V31I (11%, 28%), V72I (68%, 63%), L101I (41%, 50%), T112V (11%, 13%), S119T (16%, 37%), A124T (89%, 51%), T125A (16%, 27%), K156N (35%, 22%), V201I (38%, 49%), S230N (3%, 16%), N232D (100%, 89%), L234I (10%, 2%) and S283G (5%, 9%). No diketo acid-related resistance mutation was observed in either the MDR or naive group. However, S119T and S230N were observed in higher frequencies in the MDR group ($P<0.05$). For CRF01_AE cases, no diketo acid resistance or significant differences were observed between MDR and naive cases

CONCLUSION: Accumulation of PI and RTI resistance mutations apparently affected integrase polymorphism to some extent. However, the data for MDR patients were encouraging in that no diketo-acid-related resistance mutation was observed in either the MDR or naive group.