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# Interleukin-4–Transgenic hu-PBL-SCID Mice: A Model for the Screening of Antiviral Drugs and Immunotherapeutic Agents against X4 HIV-1 Viruses

**Kazu Okuma,[1] Reiko Tanaka,[1] Tomoyuki Ogura,[2] Mamoru Ito,[2] Sei Kumakura,[3] Mikiro Yanaka,[3] Masako Nishizawa,[4] Wataru Sugiura,[4] Naoki Yamamoto,[4] and Yuetsu Tanaka[1]**

[1]Department of Immunology, Graduate School and Faculty of Medicine, University of the Ryukyus, Okinawa, [2]Central Institute for Experimental Animals, Kanagawa, and [3]Kureha Corporation and [4]National Institute of Infectious Diseases, Tokyo, Japan

**CXCR4-tropic (X4) human immunodeficiency virus type 1 (HIV-1) does not efficiently infect and replicate in severe combined immunodeficiency (SCID) mice reconstituted with human peripheral blood mononuclear cells, termed "hu-PBL-SCID mice," due to, at least in part, relatively low levels of expression of the CXCR4 coreceptor. To overcome this limitation, interleukin (IL)–4–transgenic hu-PBL-SCID mice were derived that spontaneously synthesized human IL-4, which has been shown to enhance CXCR4 expression and promote X4 virus infection in vitro. Experiments reported here show that (1) synthesis of human IL-4 in vivo augmented CXCR4 expression on human CD4$^+$ lymphocytes and importantly led to productive infection of not only X4 HIV-$1_{NL4-3}$ but also multidrug-resistant primary clinical isolates and that (2) the in vivo infection could be significantly blocked by the administration of a CXCR4 antagonist. Altogether, IL-4–transgenic hu-PBL-SCID mice provide a useful model for X4 HIV-1 study and testing/screening of anti-X4 viral drugs.**

HIV-1 isolates enter target cells primarily after binding to the CD4 receptor and via the CXCR4 and CCR5 coreceptors [1–5] and are classified into X4 and R5 strains, respectively [6]. The X4 isolates are frequently implicated in the decline of peripheral CD4$^+$ T cell counts characteristic of the late stage of HIV-1 infection proceeding to the development of AIDS [7].

hu-PBL-SCID mice have been extensively used as a small animal model to study HIV-1 pathogenesis [8–14]. Results from a previous study showed that, although infection of human peripheral blood mononuclear cell (PBMC)–reconstituted hu-PBL-SCID mice with a predominantly R5 HIV-1 caused intensive CD4$^+$ T cell depletion, infection of similarly reconstituted mice with the same infectious dose of an X4 HIV-1 resulted in little or no CD4$^+$ T cell depletion [11]. Thereafter, it was noted that this limitation of X4 HIV-1 infection was due, at least in part, to a decrease in the intensity of CXCR4 expression on CD4$^+$ T cells [13]. Thus, it was reasoned that the pathogenic effects of the X4 HIV-1 strains in the hu-PBL-SCID mice might be related to the relative levels of the expression of HIV-1 coreceptor (the state of activation/differentiation) on human CD4$^+$ T cells at the time of infection in these mice. This limitation has to date restricted our ability to use this mouse model for understanding the mechanisms of X4 HIV-1 pathogenesis and for the evaluation of candidate therapeutics against X4 viruses. These findings prompted us to seek alternative strategies for the development of an improved hu-PBL-SCID mouse system that is permissive for infection/replication of X4 isolates.

Human interleukin (IL)–4 has been shown to specifically enhance the cell-surface expression of CXCR4 on

Received 1 May 2007; accepted 11 July 2007; electronically published 4 December 2007.

Potential conflicts of interest: none reported.

Presented in part: First International Workshop on Humanized Mice, Tokyo, 11–12 October 2006 (abstract P-15); 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, 25–28 February 2007 (abstract 496).

Financial support: Health and Labor Science Research Grant (Research on Publicly Essential Drugs and Medical Devices) from the Ministry of Health, Labor, and Welfare of Japan (grant H16-soyaku-004).

Reprints or correspondence: Dr. Kazu Okuma, Dept. of Immunology, Graduate School and Faculty of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan (kokuma@med.u-ryukyu.ac.jp).

**The Journal of Infectious Diseases 2008; 197:134–41**

0022-1899/2008/19701-0021$15.00
DOI: 10.1086/524303

resting peripheral blood T cells [15]. Furthermore, it has been reported that human IL-4 plays an important role in rendering $CD4^+$ T cells susceptible to X4 HIV-1 infection via enhanced cell-surface expression of the CXCR4 coreceptor in vitro [15–17].

In efforts to overcome the limitation inherent with the use of hu-PBL-SCID mice for the study of X4 HIV-1 as described above, we developed an IL-4–transgenic immunodeficient mouse model that consistently secreted readily detectable serum levels of human IL-4. We show here that X4 isolates readily infect/replicate in this mouse model but not in wild-type (wt) non–IL-4–transgenic mice and that this model can now be exploited for the rapid evaluation of the therapeutic efficacy of new anti-X4 HIV-1 agents in vivo.

## METHODS

***IL-4–producing mice.*** Two strains of human IL-4–transgenic immunodeficient mice were bred on the C.B-17-*scid* [18] and BALB/cA-$Rag2^{-/-}\gamma_c^{-/-}$ (dKO) genetic background mice [19, 20] at the Central Institute for Experimental Animals (CIEA) as follows. PBMCs were isolated from a healthy human volunteer and activated in vitro with pokeweed mitogen. RNA was prepared from these PBMCs, and then cDNA was synthesized by reverse-transcriptase polymerase chain reaction (PCR). Human IL-4 cDNA was amplified from the cDNA using one set of primers: 5'-CCCGGGATCGTTAGCTTCTCCTGATAAAA-3' and 5'-GCGGCCGCTATTCAGCTCGAACACTTTGAAT-3'. The product was inserted into the PCR2.1 vector by use of the TA cloning kit (Invitrogen) and the insert sequenced. After confirmation of the sequence, IL-4 cDNA was inserted into pCMVb with a CMV promoter (Invitrogen). To produce transgenic mice, a DNA fragment containing the CMV promoter, IL-4 cDNA, and Poly(A) regions was excised with *Xho*I and *Hind*III sites of pCMVb and microinjected into the pronuclei of fertilized eggs from the 2 strains (C.B-17-*scid* and BALB/cA-dKO) of mice. These eggs were subsequently transplanted into oviducts of pseudopregnant foster recipient mice. The offspring mice were screened to confirm the insertion of the transgene into the genome by PCR, and serum from these mice was screened for levels of human IL-4 by ELISA with a commercial kit (BD). The IL-4 transgene–hemizygous mice were maintained by mating them with wt mice with the same genetic background in the specific-pathogen–free (SPF) facility of the CIEA. The mice were transferred to the SPF and biosafety level 3 facilities of the Institute for Animal Experiments, University of the Ryukyus, and were used for further experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee on the basis of the Regulation for Animal Experimentation of the CIEA and University of the Ryukyus before the initiation of the study.

***Viruses.*** X4 HIV-$1_{NL4\text{-}3}$ was obtained as described elsewhere [14]. Fourteen multidrug-resistant (MDR) HIV-1 clinical isolates were obtained from HIV-1–infected patients who had been treated with highly active antiretroviral therapy (HAART). The viruses were propagated in PBMCs stimulated with phytohemagglutinin (PHA; Sigma), IL-2 (National Institutes of Health AIDS Research and Reference Reagent Program), and IL-4 (Peprotec). Three isolates from these MDR isolates that efficiently grew in the activated cells were selected for further experiments. The titers of virus stocks were determined by end-point titration using a 2-fold limiting dilution of the stock and in vitro PHA-activated human PBMCs, and the infectious units (IU) were calculated.

***CXCR4 antagonist.*** The synthesis and purification of the CXCR4 antagonist KRH-1636 were performed at Kureha Corporation as described elsewhere [21]. As a control, the carrier tartrate was used in parallel.

***Transplantation and infection.*** The control (wt) and the IL-4–transgenic C.B-17-*scid* mice were depleted of NK cells by the intraperitoneal (ip) injection of 0.5–1.0 mg of anti–mouse IL-2R $\beta$ (TM$\beta$-1) [22] per animal. The IL-4–transgenic and the control BALB/cA-dKO mice do not require TM$\beta$-1 treatment because they lack NK cells [19, 20]. PBMCs were isolated from healthy human donors. Groups of 2–4-month-old IL-4–transgenic mice from each of the 2 background strains and their corresponding non–IL-4–transgenic wt mice were injected ip with PBMCs 3 days later. Groups of mice were challenged 24 h later ip with mock, HIV-$1_{NL4\text{-}3}$, or MDR isolates (2000 IU/500 $\mu$L/animal). For the experiments using the CXCR4 antagonist, groups of mice were administered 0.1 mL of 10 mmol/L KRH-1636, the tartrate carrier or saline ip at 1 h before and 1 day after virus infection. At 6–8 days after infection, the mice were killed, their blood was obtained by cardiocentesis, and human lymphocytes were collected from the peritoneal lavage fluids. The serum samples were assayed for levels of human IL-4 by use of an ELISA kit (R&D Systems). The human lymphocytes were analyzed using flow cytometry as described below. The remaining cells were cultured in RPMI 1640 medium (Sigma) supplemented with fetal calf serum and IL-2. The peritoneal lavage fluids, serum samples, and lymphocyte culture supernatants were examined for levels of p24 by use of an ELISA kit (Zepto Metrix).

***Flow cytometry analysis.*** Cell samples to be analyzed by flow cytometry were initially incubated with normal human IgG for blocking of the Fc receptors. For cell-surface staining, aliquots of cells were then stained with Cy5-labeled anti-CD4 (OKT4) and phycoerythrin-labeled anti-CXCR4 (12G5; Dako) or with Cy5-labeled anti-CD3 (OKT3). For intracellular staining, after CD3 staining the aliquots of cells were fixed, permeabilized, and incubated with fluorescein isothiocyanate–labeled anti–HIV-1 Gag p24 (2C2; Y.T. et al., unpublished data). Stained samples were analyzed on a FACSCalibur flow cytometer, using Cell Quest software (BD Pharmingen). Aliquots of cells stained

**Table 1. Expression of human CD4, CXCR4, and intracellular HIV-1 p24 in cells from X4 HIV-1–infected hu-PBL-SCID mice.**

| Category | X4 HIV-1 infection | Mice, no. | $CD4^+$ T cells, % | *P* | $CXCR4^+CD4^+$ T cells, % | *P* | $p24^+$ T cells, % | *P* |
|---|---|---|---|---|---|---|---|---|
| C.B-17-*scid* mice | | | | | | | | |
| Control | NL4-3 | 6 | 22.1 ± 8.3 | <.001 | 45.2 ± 4.7 | <.001 | 0.1 ± 0.1 | NS |
| IL-4 transgenic | NL4-3 | 6 | 66.3 ± 9.0 | | 65.5 ± 6.1 | | 0.1 ± 0.1 | |
| BALB/cA-dKO mice | | | | | | | | |
| Control | NL4-3 | 5 | 35.9 ± 5.1 | <.01 | 32.6 ± 1.4 | <.001 | 0.2 ± 0.1 | <.05 |
| IL-4 transgenic | NL4-3 | 3 | 57.4 ± 8.3 | | 68.2 ± 3.9 | | 3.2 ± 1.2 | |

**NOTE.** Cells in peritoneal lavage fluid from control and interleukin (IL)–4–transgenic hu-PBL-SCID mice on either the C.B-17-*scid* or BALB/cA-dKO background were labeled with appropriate monoclonal antibodies and analyzed by flow cytometry, as described in Methods. Analyzed data are shown as mean ± SD values. NS, not significant. The indicated *P* values for the comparison of control vs. transgenic mice for each category are based on Student's *t* test.

with or without each of the antibodies described above were used as controls for the purposes of establishing gates and for the determination of the frequency of positive cells.

***Statistical analysis.*** Data obtained by flow cytometry were analyzed by Student's *t* test with GraphPad Prism (version 4.0c for Mac OS X; GraphPad Software).

## RESULTS

***Production of human IL-4 in IL-4–transgenic mice.*** Efforts to construct the IL-4–transgenic mice constitutively synthesizing human IL-4 finally led to the establishment of mice on each of the 2 immunodeficient backgrounds expressing either high or low serum levels of human IL-4 (data not shown). On the basis of preliminary data obtained on the efficiency of virus replication, all subsequent experiments were done using only the 2 strains with high serum IL-4 expression levels. We assayed for levels of human IL-4 in the serum from the $IL\text{-}4^+$ hu-PBL-SCID mice and the wt hu-PBL-SCID mice on either the C.B-17-*scid* or BALB/cA-dKO background after infection with X4 HIV-$1_{NL4\text{-}3}$. Serum from each of the IL-4–transgenic mice on either background contained significant levels of human IL-4 (~800–1800 pg/mL), whereas serum from the control mice on the same background showed nondetectable levels of human IL-4. These data demonstrate that the human IL-4 synthesized by the IL-4–transgenic mice is generated from the transgene but not from the human PBMCs transplanted in these mice.

***Effect of human IL-4 on the levels of human CXCR4 and CD4 expression by cells transplanted into mice.*** Since human IL-4 has been previously documented to enhance the expression of CXCR4 in vitro, experiments were done to examine the expression of human CXCR4 on transplanted $CD4^+$ cells in the peritoneal lavage fluids from HIV-$1_{NL4\text{-}3}$–infected $IL\text{-}4^+$ hu-PBL-SCID mice and control hu-PBL-SCID mice on either the C.B-17-*scid* or BALB/cA-dKO background. There did not appear to be any detectable difference in the absolute amounts of cells recovered from the peritoneal lavage fluids from the IL-4–positive or IL-4–negative hu-PBL-SCID mice on either background (data not shown). Flow cytometry analysis demonstrated that the frequency of human $CD4^+$ cells from the IL-4–transgenic C.B-17-*scid* or BALB/cA-dKO mice was significantly higher than that from the control mice (table 1). As expected, there was a marked increase in the frequency of CXCR4-expressing $CD4^+$ cells from the IL-4–transgenic mice on either genetic background relative to that from the control mice (figure 1*A* and table 1). Thus, these data indicate that human IL-4 produced endogenously is functional in vivo in terms of its ability to enhance human CXCR4 expression on $CD4^+$ cells transplanted into the mice.

***Increased frequency of X4 HIV-1–infected cells from IL-4–transgenic hu-PBL-SCID mice.*** Since the constitutive synthesis of human IL-4 in IL-4–transgenic hu-PBL-SCID mice resulted in the enhanced expression of X4 HIV-1 receptors (human CXCR4/CD4) on the transplanted cells, we reasoned that such cells were likely to be more permissive to the infection and replication of X4 HIV-1. We thus challenged the IL-4–transgenic hu-PBL-SCID mice and control hu-PBL-SCID mice bred on the C.B-17-*scid* or BALB/cA-dKO mice with HIV-$1_{NL4\text{-}3}$. Cells obtained from the peritoneal lavage fluids were analyzed for cell-surface expression of human CD3 (since HIV-1 down-modulates CD4 expression) and the presence of intracellular p24. As seen in figure 1*B* and table 1, although very few if any $CD3^+$ cells from the control or IL-4–transgenic C.B-17-*scid* mice showed p24 expression, there was a >10-fold increase in the frequency of $CD3^+$ T cells that expressed p24 from the IL-4–transgenic BALB/cA-dKO mice relative to the control mice. These data suggest that, while transgene-induced human IL-4 increases the frequency of $CD4^+CXCR4^+$ T cells transplanted into both the C.B-17-*scid* and the BALB/cA-dKO mice, only the latter demonstrates increased sensitivity to X4 HIV-1 infection and replication, at least when this assay is used (see below).

***High production of X4 HIV-1 in the culture supernatants of cells from IL-4–transgenic hu-PBL-SCID mice.*** In an effort

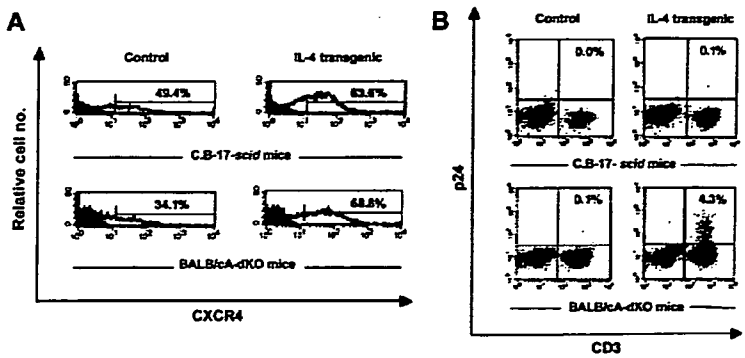


**Figure 1.** Enhancement of the expression of CXCR4 coreceptor and facilitation of X4 HIV-1 infection and replication in hu-PBL-SCID mice by in vivo production of human interleukin (IL)–4. Groups of hu-PBL-SCID mice, generated from IL-4–transgenic and nontransgenic (control) mice on either the C.B-17-*scid* or BALB/cA-dKO background, were injected intraperitoneally with HIV-$1_{NL4-3}$ at ~24 h after peripheral blood mononuclear cell (PBMC) reconstitution. Six to eight days later, peritoneal lavage fluids were harvested from mice in each group, and cells were collected from the fluids by density-gradient centrifugation. *A*, Cells analyzed for the frequency and mean density of human CXCR4 expression on $CD4^+$ cells by flow cytometry. Data for analyzed cells are depicted by a thick line, and the background control profile is depicted by a thin line and gray shading. The nos. above the bars represent the percentage of positive cells. Data shown are representative of mice in each group from 3 independent experiments. *B*, HIV-1 infectivity. Cells were subjected to flow cytometry after cell-surface CD3 and intracellular p24 staining. Analyzed data are depicted as dot plots. The nos. in the graphs indicate the percentage of $CD3^+p24^+$ cells. Data displayed are representative of mice in each group from 3 independent experiments.

to determine the reason for our failure to detect levels of intracellular p24 in the IL-4–transgenic mice on the C.B-17-*scid* background and to further support the above finding, peritoneal lavage fluids were collected from mock- or HIV-$1_{NL4-3}$–infected $IL\text{-}4^+$ hu-PBL-SCID mice and, for purposes of control, the HIV-$1_{NL4-3}$–infected non–IL-4–transgenic mice on the C.B-17-*scid* background. The cells were isolated from the peritoneal lavage fluids, and an aliquot was analyzed for the frequency and the relative density of human CXCR4/CD4; the remaining aliquot was cultured in vitro. In addition, the peritoneal lavage fluids and the culture supernatants of cells at days 1–3 after culture were assayed for levels of p24 production. As displayed in table 2, although the frequency of $CXCR4^+CD4^+$ cells in the IL-4–transgenic mice was significantly higher than that in the nontransgenic mice, the mean fluorescence intensity (MFI) of CXCR4 expressed by the $CD4^+$ T cells from these mice was not increased compared with the control (because of an increase in the frequency of $CXCR4^+CD4^+$ cells with relatively low MFI; see figure 1*A*). Analysis of levels of synthesized p24 demonstrated marked differences, as shown in figure 2. Thus, although the amounts of p24 produced were modest in the peritoneal lavage fluids and the cell-culture supernatants from HIV-1–infected control mice, the levels of p24 produced by those from HIV-1–infected IL-4–transgenic mice were strikingly higher (15,429, 11,844, 1696, and 53 pg/mL in the supernatants on day 3) (mean, 48.9 vs. 7255 pg/mL; >100-fold increase). Although the levels of p24 produced by one of the IL-4–transgenic mice (mouse 12) were similar to those in the control mice, this was likely due to the much lower relative level of human IL-4 (354 pg/mL in serum) produced by mouse 12 than those from the other 3 IL-4–transgenic mice (4227, 6313, and 2356 pg/mL in serum). The present data not only document the fact that the cells from these

**Table 2. Effect of the CXCR4 antagonist KRH-1636 on the expression of human CXCR4 by $CD4^+$ cells from X4 HIV-1–infected interleukin (IL)–4–transgenic hu-PBL-SCID mice.**

| C.B-17-*scid* mice | X4 HIV-1 Infection | CXCR4 antagonist | Mice, no. | $CXCR4^+CD4^+$ T cells, % | *P* | CXCR4 on $CD4^+$ T cells, MFI | *P* |
|---|---|---|---|---|---|---|---|
| Control | NL4-3 | Mock | 4 | 45.5 ± 9.3 | <.05[a] | 73.7 ± 36.0 | NS[a] |
| IL-4 transgenic | NL4-3 | Mock | 4 | 66.7 ± 7.4 | NS[b] | 73.1 ± 6.2 | <.05[b] |
| IL-4 transgenic | NL4-3 | KRH-1636 | 4 | 63.0 ± 4.2 | | 62.2 ± 4.8 | |

**NOTE.** Control or IL-4–transgenic hu-PBL-SCID mice on the C.B-17-*scid* background infected with X4 HIV-$1_{NL4-3}$ were administered mock KRH-1636 or real KRH-1636. Cells isolated from the peritoneal lavage fluid from the mice in each group were labeled with appropriate monoclonal antibodies and subjected to flow cytometry, as described in Methods. Data analyzed are displayed as mean ± SD values. MFI, mean fluorescence intensity; NS, not significant. The indicated *P* values are based on Student's *t* test.

[a] For the comparison between control mice and IL-4–transgenic mice that received a mock CXCR4 antagonist.

[b] For the comparison between IL-4–transgenic mice that received a mock CXCR4 antagonist and IL-4–transgenic mice that received KRH-1636.

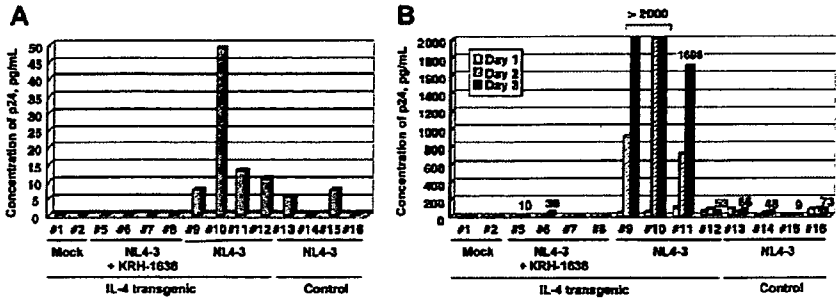


**Figure 2.** Efficient infection of interleukin (IL)–4–producing hu-PBL-SCID mice with X4 laboratory strain and an inhibitory effect of the CXCR4 antagonist KRH-1636 on infection. Twelve IL-4–transgenic and 4 nontransgenic (control) hu-PBL-SCID mice were generated on the C.B-17-*scid* background. Among them, 8 IL-4–transgenic and 4 control mice were infected intraperitoneally (ip) with the X4 laboratory strain (HIV-$1_{NL4-3}$) and 4 IL-4–transgenic mice were mock-infected at 1 day after engraftment. To evaluate the effect of KRH-1636, this drug was administered ip twice, at 1 h before and 1 day after infection of 4 IL-4–transgenic mice (NL4-3 + KRH-1636). At 8 days after infection, peritoneal lavage fluids were obtained from the mice in each group. Cells were isolated from the fluids and cultured in IL-2–containing medium. Levels of HIV-1 p24 in the peritoneal lavage fluids *(A)* and culture supernatants at days 1–3 after incubation *(B)* were quantitated for infectivity and replication efficiency by ELISA. With regard to the data on mock-infected mice, only 2 of 4 representative data are presented. The nos. listed above the bars in the graph indicate levels of HIV-1 p24 when it was detectable on day 3 (most of the values were <200 pg/mL, and select samples showed values of >2000 pg/mL). Pound signs (#) indicate mouse nos. Results shown are representative of 3 independent experiments.

IL-4–transgenic C.B-17-*scid* mice are susceptible but also demonstrate that the virus from such cells is replication competent. In addition, these findings suggest that the use of intracellular p24 levels is not a sensitive enough technique and that data using the intracellular p24 assay need to be carefully evaluated. These data also indicate that the IL-4–transgenic hu-PBL-SCID mice provide a powerful model for the study of X4 HIV-1 infection independently of the genetic background of the mice.

***Inhibitory effect of the CXCR4 antagonist on infection of IL-4–transgenic hu-PBL-SCID mice with the X4 laboratory strain.*** In an effort to further validate that the CXCR4 coreceptor was indeed used by the X4 HIV-1 virus in the IL-4–transgenic hu-PBL-SCID mice, we used the X4 virus-entry inhibitor, CXCR4 antagonist KRH-1636. Thus, the IL-4–transgenic hu-PBL-SCID mice on the C.B-17-*scid* background were infected with X4 laboratory strain HIV-$1_{NL4-3}$ and were either mock treated or treated with KRH-1636, and the peritoneal lavage fluids, cells in fluids, and cell-culture supernatants were examined as described above. As shown in table 2, the frequency of $CXCR4^+CD4^+$ cells in KRH-1636–treated IL-4–transgenic mice was marginally lower than that in mock-treated IL-4–transgenic mice. In addition, the MFI of CXCR4 expression by the $CD4^+$ T cells was clearly reduced by KRH-1636 administration. Importantly, treatment with KRH-1636 almost completely blocked X4 HIV-1 infection in these IL-4–transgenic mice (figure 2). These data indicate that X4 HIV-1 infection in transgenic mice is CXCR4 dependent and that our mouse model can be used to develop and test new anti-X4 HIV-1 drugs in vivo.

***Therapeutic effect of KRH-1636 on the infection of IL-4–transgenic hu-PBL-SCID mice with MDR clinical isolates.*** The appearance of MDR HIV-1 clinical isolates has been and continues to be one of the growing problems in a significant number of patients receiving HAART and seriously limits the use of the antiviral drugs that are currently available. Thus, the development of novel adjunct or alternative therapeutics is an urgent need. Since treated patients tend to harbor significantly higher levels of either dual/mixed or X4 viruses [23] and since MDR isolates are not usually refractory to new treatment with drugs from classes that have not been used previously in patients from which the viruses were derived, we finally wanted to examine the effect of KRH-1636 on MDR HIV-1 infection in IL-4–transgenic hu-PBL-SCID mice. For this experiment, we used the IL-4–transgenic BALB/cA-dKO mice instead of the IL-4–transgenic C.B-17-*scid* mice, because the former seems more permissive to X4 HIV-1 infection than the latter, as described above. Before the in vivo study, we confirmed that the in vitro infection by 3 MDR clinical isolates could be inhibited with KRH-1636 (more than ~90% inhibition at the 5-$\mu$mol/L level). Thus, groups of IL-4–transgenic hu-PBL-SCID mice were infected with a mixture of these selected MDR isolates containing equal IU of each virus and treated with KRH-1636 or the tartrate carrier control. Thereafter, the cells obtained from the peritoneal lavage fluids were analyzed for the expression of cell-surface human CD4, CD3, and intracellular p24. The serum, peritoneal lavage fluids, and supernatants following in vitro culture of the cells for 24 h were assayed for levels of p24 production. Flow cytometry analysis after CD4 staining demonstrated a significant decline in $CD4^+$ T cells in 2 (mouse 7 and mouse 8) of 4 control-treated mice (figure 3*A*; top profile shows data from 1 of these 2 mice), which was likely due to MDR HIV-1 pathogenesis. However, importantly, no detectable depletion of $CD4^+$ T cells was observed in any of 4 KRH-1636–treated mice (figure 3*A*; bottom profile). As summarized in table 3, the difference in the frequency of $CD4^+$ T cells between the control-treated mice and the

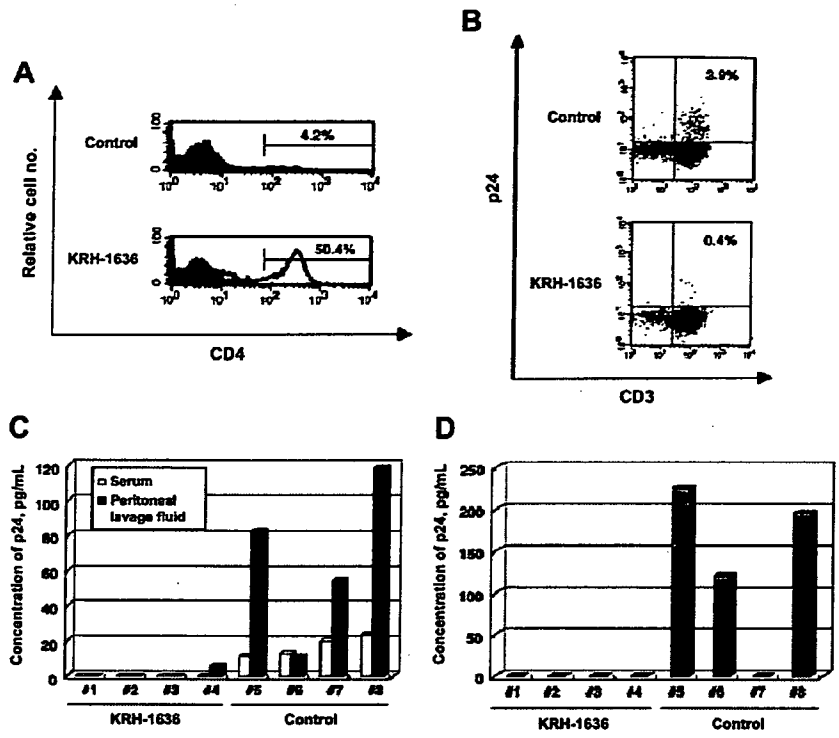


**Figure 3.** Prophylactic effect of KRH-1636 on infection and pathogenesis by multidrug-resistant (MDR) HIV-1 clinical isolates. Eight interleukin (IL)–4–transgenic hu-PBL-SCID mice (BALB/cA-dKO) were infected intraperitoneally (ip) with a mixture of MDR HIV-1 clinical isolates at 1 day after human peripheral blood mononuclear cell transfer. In an effort to assess the effect of KRH-1636 on HIV-1 infection, this agent or tartrate (control drug) was administered to 4 mice per group ip twice, at 1 h before infection and 1 day after infection. At 7 days after infection, serum and peritoneal lavage fluids were harvested from mice in each group, and cells were collected from the fluids. *A*, Cells examined for human CD4 expression by cell-surface staining and standard flow cytometry. Representative data from a single mouse from the control-treated or the KRH-1636–treated HIV-1–infected mice are shown. The frequency of $CD4^+$ T cells is depicted by a thick line, and the background control is depicted by a thin line with gray shading. The nos. above the bars indicate the percentage of positive cells. *B*, Aliquot of the peritoneal lavage cells analyzed by flow cytometry for the frequency of $CD3^+$ T cells that were positive for the intracellular presence of HIV-1 p24. Representative data of cells from the control-treated and the KRH-1636–treated HIV-1–infected mice are shown. The nos. in the graphs indicate the percentage of $CD3^+p24^+$ cells. *C*, Concentrations of p24 in serum and peritoneal lavage fluid. Concentrations were determined by ELISA to quantify MDR HIV-1 infection and replication efficiency. Pound signs (#) indicate mouse nos. *D*, Levels of in vitro p24 production. The remaining cells were cultured in a microtiter plate containing $IL\text{-}2^+$ medium for ~24 h, and the culture supernatants obtained were assayed for levels of in vitro p24 production by ELISA. Pound signs (#) indicate mouse nos. Results shown are representative of 3 similar independent experiments.

KRH-1636–treated mice was not significant. However, the MFI of CD4 expression was significantly decreased in the control-treated mice (229.3 vs. 296.3; $P < .05$). Results of CD3/p24 staining showed that the frequency of $CD3^+p24^+$ cells was markedly inhibited in the KRH-1636–treated mice, compared with that in the carrier-treated control mice (figure 3*B* and table 3). Furthermore, levels of HIV-1 p24 in the serum samples, peritoneal lavage fluids, and culture supernatants from the KRH-

**Table 3. Effect of KRH-1636 on infection and pathogenesis by multidrug-resistant (MDR) HIV-1 clinical isolates in interleukin (IL)–4–transgenic hu-PBL-SCID mice.**

| BALB/cA-dKO mice | X4 HIV-1 infection | CXCR4 antagonist | Mice, no. | $CD4^+$ T cells, % | *P* | $CD4^+$ T cells, MFI | *P* | $p24^+$ T cells, % | *P* |
|---|---|---|---|---|---|---|---|---|---|
| IL-4 transgenic | MDR | Control | 4 | 14.7 ± 11.9 | NS | 229.3 ± 33.0 | <.05 | 3.2 ± 0.8 | <.01 |
| IL-4 transgenic | MDR | KRH-1636 | 4 | 31.3 ± 15.7 | | 296.3 ± 25.2 | | 0.8 ± 0.7 | |

**NOTE.** IL-4–transgenic hu-PBL-SCID mice on the BALB/cA-dKO background were infected with MDR HIV-1 clinical isolates and administered tartrate (control) or KRH-1636. Cells in peritoneal lavage fluid from the mice in each group were stained with appropriate monoclonal antibodies and analyzed by flow cytometry, as described in Methods. Data shown here are mean ± SD values. MFI, mean fluorescence intensity; NS, not significant. The indicated *P* values for the comparison between control mice and mice that received KRH-1636 are based on Student's *t* test.

1636-treated HIV-1-infected mice were almost completely reduced relative to those in the control mice (figure 3*C* and 3*D*). Note that the failure to detect the in vitro production of p24 in mouse 7 might result from depletion of $CD4^+$ T cells (figure 3*D*). These data demonstrate that the CXCR4 antagonist KRH-1636 has a marked degree of prophylactic effect on infection with pathogenic MDR clinical isolates in vivo.

## DISCUSSION

Humanized mice that have served as valuable small animal models include the SCID-hu Thy/Liv mouse [24–28]. This mouse model, generated by implanting human hematopoietic tissues (human fetal thymus/liver) under the kidney capsule, has been used for the study of HIV-1 and is known for permissiveness to X4 HIV-1 infection [26–28]. However, the use of this model is limited by the fact that the implants are of human fetal organ origins that are not easily available. On the other hand, the hu-PBL-SCID mouse model provides another surrogate in vivo HIV-1 infection assay system. Although this model has led to a number of successful studies of HIV-1 [8–14], there was still a limitation in that it was difficult to demonstrate X4 HIV-1 infection and replication in such mice. Thus, to add extra value to the use of this mouse system for the study of HIV-1, in the present study we developed novel human IL-4–transgenic hu-PBL-SCID mice that enable CXCR4-using HIV-1 strains to efficiently infect and replicate in these mice.

Human IL-4 has low homology with murine IL-4 both at the gene and protein levels, accounting for the lack of cross-reactivity of this cytokine in the 2 species in vitro [29–32]. Results of the experiments reported here indicate that the high efficiency of X4 HIV-1 infection in the IL-4–transgenic hu-PBL-SCID mice was, at least in part, secondary to enhanced expression of viral receptors induced by human IL-4 synthesized endogenously. Interestingly, although there was no apparent increase in the number of cells recovered from the engrafted transgenic mice, there was a significant increase in the number of $CD4^+$ T cells recovered (1.5–3-fold). It is thus possible that the other cell lineages migrate from the peritoneal cavity to other tissues of the mice, resulting in enrichment of the $CD4^+$ T cell lineage. However, further studies of other tissues are needed to clarify this issue. Furthermore, our preliminary experiments indicate that the IL-4–transgenic hu-PBL-SCID mice remain permissive to R5 strain infection (data not shown).

In this report, we created 2 types of novel hu-PBL-SCID mice by transplanting human PBMCs into IL-4–transgenic C.B-17-*scid* and BALB/cA-dKO mice. The data obtained show that hu-PBL-SCID mice using the IL-4–producing BALB/cA-dKO mice appeared more permissive to X4 HIV-1 infection than did those using the IL-4–producing C.B-17-*scid* mice, at least as determined by the presence of intracellular p24. Although the reasons for this difference remain to be determined, it should be noted that, whereas the BALB/cA-dKO mice were derived by double mutation with defects in both the recombinase-activating gene 2 (Rag-2) and the gene encoding the $\gamma_c$ chain of select cytokine receptors [19, 20], the C.B-17-*scid* mice have only the Rag-2 mutation [18]. Thus, although the Rag-2 mutation prevents the normal maturation of T and B lymphocytes, the $\gamma_c$ chain mutation abrogates the expression of functional receptors for IL-2 and other cytokines, preventing the expansion of lymphocytes, including NK cells, which play an important role in the innate immune response such as nonspecific rejection of xenogeneic grafts. It is thus possible that the C.B-17-*scid* mice maintain a low but significant residual level of NK cell function, which may play a role in the difference noted above even though they were administered significant levels of anti–IL-2R$\beta$ antibody. Since the BALB/cA-dKO mice are completely deficient in NK cell lineage and function, they are more immunodeficient than the C.B-17-*scid* mice, suggesting that the level and type of immunodeficiency in the BALB/cA-dKO mice may facilitate better engraftment and more efficient viral infection and propagation within these mice. These select defects of the BALB/cA-dKO mice might render the IL-4–transgenic mouse model on this background more valuable and ideal for studies of X4 HIV-1.


### Acknowledgments

We thank the National Institutes of Health AIDS Research and Reference Reagent Program and M. Sasaki for supplying interleukin-2 and technical support, respectively. We are also grateful to Prof. Aftab Ansari for his critical reading of the manuscript and for his helpful discussion about and suggestions for the manuscript.


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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 23, Number 12, 2007, pp. 1461–1467

DOI: 10.1089/aid.2007.0042

# An Efficient Tool for Surveying CRF01_AE HIV Type 1 Resistance in Thailand to Combined Stavudine–Lamivudine–Nevirapine Treatment: Mutagenically Separated PCR Targeting M184I/V

SIRIPHAN SAENG-AROON,[1] LAY MYINT YOSHIDA,[2,3] KOYA ARIYOSHI,[2] MASATAKA TAGUCHI,[2] PANITA PATHIPVANICH,[4] ARCHAWIN ROJANAWIWAT,[1] MASAKAZU MATSUDA,[3] MARI KANNAGI,[5] PATHOM SAWANPANYALERT,[1] WATARU SUGIURA,[3] and WATTANA AUWANIT[1]

## ABSTRACT

**Under programs organized by the government of Thailand, HIV-1-infected patients have been treated since 2002 with several regimens, including a tablet known as GPOvir, which contains lamivudine, stavudine, and nevirapine. The aim of this study was to establish an effective assay, based on mutagenically separated PCR (MS-PCR), with the goal of surveying GPOvir-resistant HIV-1 cases. To determine the target mutation point for the assay, we analyzed the patterns of acquired drug resistance in plasma samples from GPOvir-failed cases. Of 428 HIV-1-infected individuals treated with GPOvir at Lampang Hospital in northern Thailand from 2002 to 2004, 66 had detectable viral loads after 3 months of treatment. The HIV-1 sequences of these 66 GPOvir-failed cases and 55 pre-GPOvir baseline samples were analyzed. The most prevalent drug resistance mutation among the samples was the lamivudine resistance M184I/V mutation. Based on this finding, we developed a new MS-PCR assay to detect the M184I/V mutation, and evaluated the assay performance for detecting GPOvir-resistant CRF01_AE cases. Comparing the results of M184I/V MS-PCR and sequence analyses, we found a concordance rate of 95% and an overall sensitivity of the M184I/V MS-PCR for detecting GPOvir-resistant cases of 79%. Considering the relatively low price of the assay, approximately $12.50 per sample, M184I/V MS-PCR may be a candidate for monitoring a large number of GPOvir-treated patients, particularly in developing nations.**

## INTRODUCTION

AMONG THAILAND'S POPULATION of approximately 65 million people, 560,000 were estimated by the Joint United Nations Programme on HIV/AIDS (UNAIDS) to be living with HIV/AIDS at the end of 2005.[1] The most predominant HIV-1 subtype reported in Thailand is CRF01_AE, and the major route of infection is heterosexual contact.[2–4] Due to active and strategic HIV/AIDS prevention programs successfully promoted by the government of Thailand, the number of newly HIV-infected cases has decreased dramatically from an estimated 141,795 cases in 1991 to 15,166 cases in 2005.[5]

Nevertheless, for economic reasons only a small number of HIV/AIDS-infected patients in Thailand had access to adequate antiretroviral treatment (ART) until 2002, and the majority of these patients were either not treated or treated with suboptimal antiretroviral regimens, mostly dual therapy.[6] To overcome economic barriers and to open the door for more HIV/AIDS patients to be treated with antiretroviral drugs, the government of Thailand developed a new ART program that provides a locally produced generic drug, Government Pharmaceutical Organization-produced GPOvir. This drug, a combined tablet of stavudine (d4T), lamivudine (3TC), and nevirapine (NVP), has been used as the first-line treatment regimen. The program has been

[1]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.
[2]Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.
[3]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan.
[4]Lampang Hospital, Lampang, Thailand.
[5]Department of Immunotherapeutics, Tokyo Medical and Dental University, Tokyo, Japan.

running efficiently, and approximately 50,000 HIV/AIDS patients were receiving ART at the end of 2004.[7]

However, because of the unavoidable risk of emerging drug resistance mutations, an urgent need has developed for establishing a surveillance system to monitor drug resistance. This issue presents another challenge to the ART program in Thailand, as little standard drug resistance testing by direct sequencing is available due to the relatively high cost of its reagents and need for expensive equipment. To overcome this economic barrier, we propose in this paper a PCR-based point mutation assay, mutagenically separated PCR (MS-PCR), with high specificity and sensitivity,[8,9] as an excellent candidate to monitor and survey GPOvir-resistant HIV-1 cases.

## MATERIALS AND METHODS

### *Patient samples*

Plasma samples were collected from patients in Lampang Hospital, a government referral hospital in Lampang province in upper northern Thailand, approximately 600 km north of Bangkok. In total, 428 HIV-1-infected patients started GPOvir therapy at the hospital's Day Care Center clinic between 1 April 2002 and 31 January 2004.

Of these 428 cases, 55 were selected to serve as baseline cases before the initiation of GPOvir treatment and their plasma samples were analyzed for HIV-1 sequences. Among these cases, 28 were ART naive (Group A) and 27 had a history of ART exposure (Group C). These 27 cases had the following treatment histories: AZT monotherapy ($n$ = 3), AZT + ddC ($n$ = 13), AZT + ddI ($n$ = 6), AZT +ddI + ritonavir ($n$ = 1), AZT + 3TC + saquinavir + ritonavir ($n$ = 2), and two cases with no detailed information. Thus, two cases had 3TC exposure, but none was exposed to nonnucleoside reverse transcriptase inhibitors (NNRTIs).

Cases with a detectable viral load (>50 copies/ml) despite having received GPOvir therapy for at least 3 months were defined as treatment failures; this criterion was met by 66 cases whose plasma HIV-1 sequences were analyzed. Of these, 32 cases were ART naive (Group B) and the other 34 cases had previously been exposed to ART (Group D). Plasma samples were analyzed from these four groups of patients.

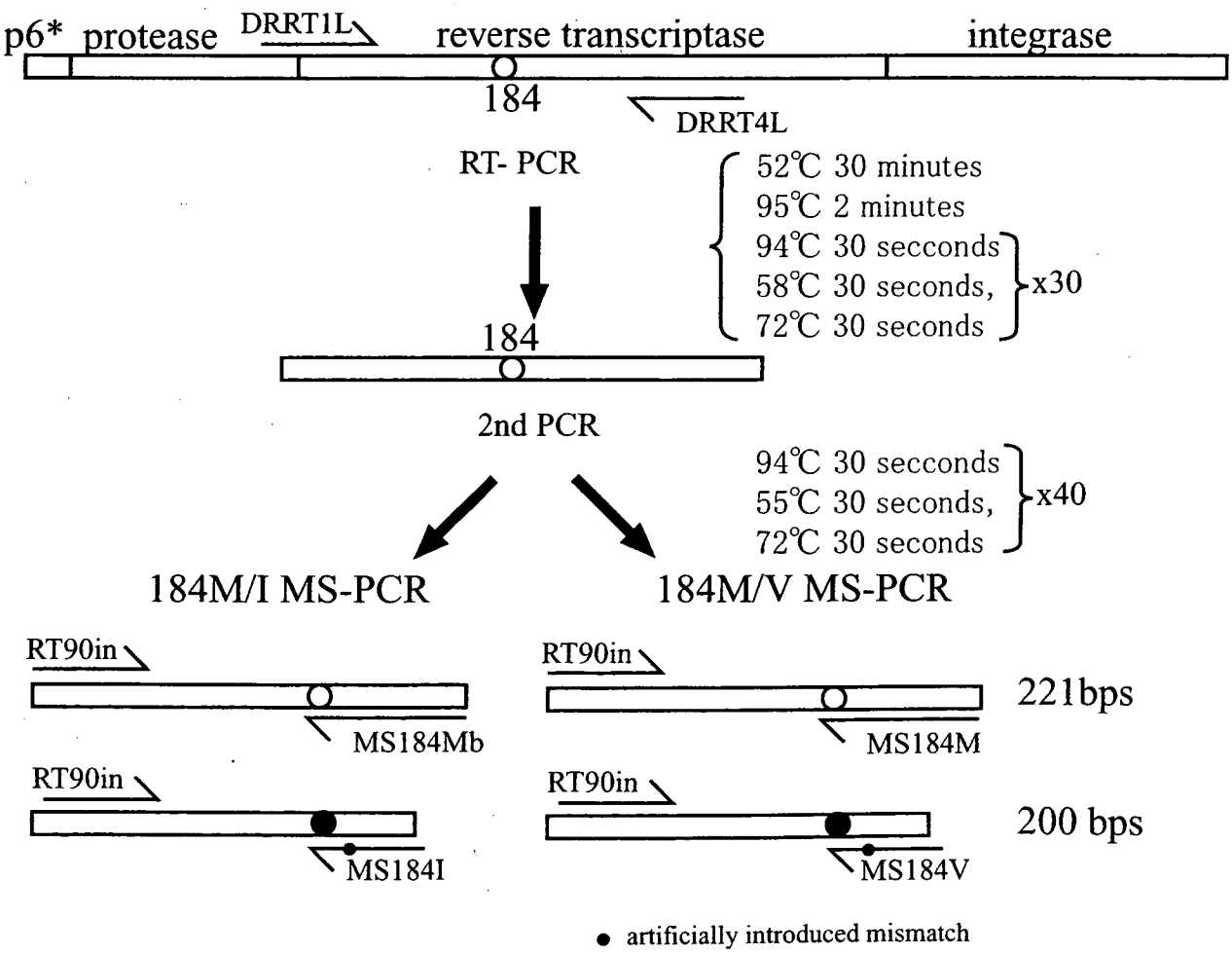


**FIG. 1.** Principle of M184I/V mutagenically separated PCR. The basic principle of mutagenically separated PCR and the primer regions of M184I and M184V determination are shown. Open and solid circles indicate wild-type (M) and mutant (I or V) 184 residues, respectively. Small solid circles indicate artificially introduced mismatch on reverse primers.

In addition to the samples from Lampang Hospital, 48 samples were analyzed from in-treatment cases collected at the National Institute of Infectious Disease (NIID) in Japan.

*Viral load and drug resistance genotyping*

Patient plasma viral loads were measured by the Amplicor HIV-1 Monitor Test, version 1.5 (Roche Diagnostics Gmbh, Mannheim, Germany) kit, following the ultrasensitive protocol included in the kit. Drug resistance genotyping was performed as described.[9] In brief, viral RNA was extracted from 200 $\mu$l patient plasma, and 1030 bps DNA fragments, including full protease and the N-terminal half of reverse transcriptase, were amplified by RT-PCR. The nucleotide sequence of the amplicon was determined by a cycle sequencing reaction, analyzed by the autosequencer ABI-3100 (Applied Biosystems, Foster City, CA), and edited with ABI PRISM SeqScape Software, version 2.0 (Applied Biosystems). Based on the International AIDS Society-USA Drug Resistance Mutations Chart,[10] GPOvir-resistant strains were defined as viruses with at least one mutation at the d4T, 3TC, and NVP resistance-associated mutation sites: M41L, K65R, D67N, K70R, M184I/V, L210W, T215Y/F, K219Q/E, L100I, K103N, V106A/M, V108I, Y181C/I, Y188C/L/H, and G190A.

*Development of M184I/V MS-PCR*

Mutagenically separated-PCR (MS-PCR) is a PCR-based point-mutation detection assay with high sensitivity and specificity, whose principles have been reported in detail.[8,9] As summarized in Fig. 1, the key principle of this method is in its unique primer design with artificially introduced mismatches. The lengths of wild-type and mutant primers are designed with about 20 bases difference so that their PCR products can be easily differentiated by gel electrophoresis. Using the principles of MS-PCR, we designed new primer sets and established 184 MS-PCR by using control templates.

In the first round, one-step RT-PCR was identical for sequencing and MS-PCR. Thus, the same RT-PCR product was used as a template for both the sequencing and MS-PCR assays. The codon for residue 184 could be methionine (M: ATG, wild type), isoleucine (I: ATA, mutant type), or valine (V: GTG, mutant type),[11-13] and the positions of the nucleotide mutations that induce 184I and 184V are different, so we set up two 184 MS-PCR reactions: one to differentiate 184M from 184V and another to differentiate 184M from 184I. Thus, each reaction tube contained one common forward primer and two competitive detection reverse primers. The common primer was RT90in: 5′-GGAAGTTCAATTAGGAATACCGCATCCAGCAGG. The reverse primers were MS184M: 5′-GTGCTGCCCTATTTCTAAATGTGATCCTACATACAAGTCATCGAT, MS184V: 5′-CAGATCCTACATACAAGTCATGCAC), MS184Mb: 5′-GTGCTGCCCTATTTCTAAATGTGATCCTACATACAAGTCTTCC), and MS184I (5′-CAGATCCTACATACAAGTCAACT). The introduced mismatches are underlined. The detailed PCR cycles used in the assay are shown in Fig. 1. The sizes of the wild-type specific primers, MS184M and MS184Mb, were designed about 20 nucleotide bases longer than the mutant type-specific primers so that the wild-type PCR product could be differentiated from the mutant-type PCR product by 3% agarose gel electrophoresis (Fig. 2). Clinical samples, whose drug-resistant genotypes were confirmed by the sequencing method, were used as positive controls and included in every experiment.

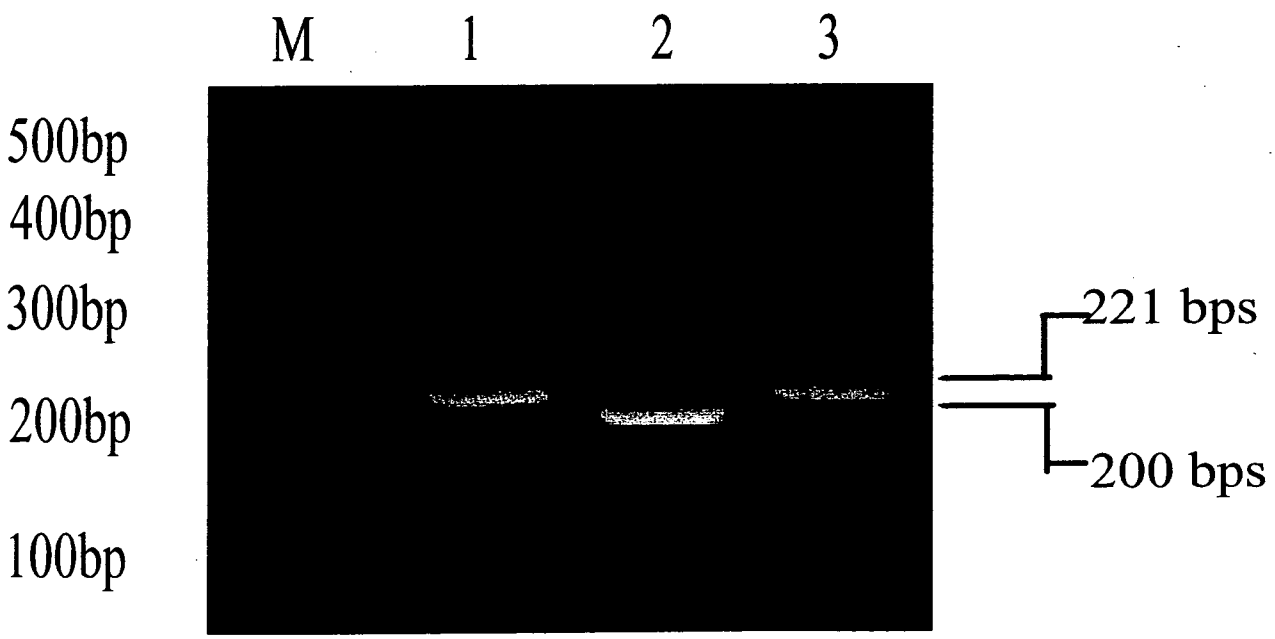


**FIG. 2.** Agarose gel electrophoresis results of M184I/V MS-PCR. M: 100 bp ladder marker. Lane 1: M184 wild-type sample with 221 bps amplicon. Lane 2: M184V mutant sample with 200 bps amplicon. Lane 3: Sample with a mixture of M184 wild-type and M184V mutant. Amplicons were observed at both 221 and 200 bps.

TABLE 1. PREVALENCE OF RESISTANCE MUTATIONS IN BASELINE AND GPOVIR-FAILED CASES

| | *Naive* | | | | *Previously exposed to ART* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Group A Baseline (n = 28)* | | *Group B Failed (n = 32)* | | *Group C Baseline (n = 27)* | | *Group D Failed (n = 34)* | |
| *Mutation* | n | % | n | % | n | % | n | % |
| NRTI resistance mutation | | | | | | | | |
| M41L | 0 | 0 | 0 | 0 | 2 | 7.4 | 12 | 35.3 |
| K654 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2.9 |
| D67N | 0 | 0 | 5 | 15.6 | 4 | 14.8 | 18 | 52.9 |
| K70R | 0 | 0 | 0 | 0 | 2 | 7.4 | 8 | 23.5 |
| Q151M | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2.9 |
| M184V/I | 0 | 0 | 15 | 46.9 | 0 | 0 | 23 | 67.6 |
| L210W | 0 | 0 | 0 | 0 | 3 | 11.1 | 8 | 23.5 |
| L215F/Y | 0 | 0 | 0 | 0 | 3 | 11.1 | 16 | 47.1 |
| K219Q/E | 0 | 0 | 0 | 0 | 3 | 11.1 | 8 | 23.5 |
| NNRTI resistance mutations | | | | | | | | |
| L100I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| K103N | 0 | 0 | 5 | 15.6 | 1 | 3.7 | 3 | 8.8 |
| V106M/A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| V108I/V | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 5.9 |
| Y181C/I | 0 | 0 | 8 | 25.0 | 1 | 3.7 | 17 | 50.0 |
| Y188L/C | 0 | 0 | 1 | 3.1 | 0 | 0 | 1 | 2.9 |
| G190A | 0 | 0 | 5 | 15.6 | 1 | 3.7 | 12 | 35.3 |

## RESULTS

### *Sequence analyses of baseline and GPOvir-failed samples*

To identify mutations associated with GPOvir treatment, reverse transcriptase regions from patient samples were sequenced. As shown in Table 1, no resistance mutations were found in sequences from baseline, ART-naive samples (Group A). In 32 ART-naive, GPOvir-failed cases (Group B), we most frequently found M184V (15 cases, 46.9%). We also found D67N NRTI-resistant mutations and K103N, Y181C, Y188L, and G190A NNRTI-resistance mutations.

In contrast to Group A, the baseline sequences of ART-experienced cases (Group C) demonstrated different mutation patterns, and all thymidine analogue mutations such as M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E were observed in high frequencies. These drug resistance mutations were found among patients suboptimally treated with AZT and ddI or ddC[14] and their patterns matched those of antiretrovirals commonly used in Thailand before GPOvir was introduced. However, no M184V resistance mutation was found among the samples in Group C. In addition, K103N, Y181C/I, and G190A NNRTI mutations were found in Group C. These mutations have been associated with nevirapine resistance. In 34 ART-experienced, GPOvir-failed cases (Group D) as well, M184V/I was most frequently observed (23 cases, 67.6%). In this group, we also found many other mutations, e.g., M41L, K65R, D67N, K70R, Q151M, L210W, T215Y/F, and K219Q/E NRTI, as well as K103N, V108I/V, Y181C/I, Y188C, and G190A NNRTI.

The drug resistance mutation patterns of the four groups indicated that M184I/V was by far the most prevalent drug resistance mutation in both ART-naive and ART-experienced GPOvir-failed patients. Furthermore, M184I/V was not found in any baseline sample. These findings indicate that the M184I/V mutation would be a feasible marker for monitoring GPOvir drug-resistant cases.

### *Comparison of M184I/V MS-PCR and sequencing results*

We conducted M184I and M184V MS-PCR for all 121 samples and successfully obtained results for 120 samples. To evaluate the performance of MS-PCR, we compared the results of M184I/V MS-PCR and sequence analysis. As shown in Table 2, the concordance rate between M184I/V MS-PCR and se-

TABLE 2. COMPARISON BETWEEN M184I/V MS-PCR AND DIRECT SEQUENCE ANALYSES

| | *Sequence results* | | |
|---|---|---|---|
| *MS-PCR results* | *Wild type* | *Mutant* | *Total* |
| Wild type | 76 | 1 | 77 |
| Mutant | 5[a] | 38 | 43 |
| Total | 81 | 39 | 120 |

[a]Four cases had previously been exposed to antiretrovirals.

TABLE 3. SENSITIVITY OF M184I/V MS-PCR TO DETECT GPOVIR-RESISTANT HIV-1

| | *Total* | *Wild type* | *Mutant* | *Sensitivity (%)* |
|---|---|---|---|---|
| All samples ($n$ = 120) | | | | |
| No mutation | 62 | 60 | 3 | — |
| With mutations | | | | |
| Overall | 56 | 17 | 39 | 70 |
| 1 | 10 | 10 | 0 | 0 |
| 2 | 19 | 2 | 17 | 90 |
| >3 | 27 | 5 | 22 | 82 |
| ART-naive cases before GPOvir treatment ($n$ = 59) | | | | |
| No mutation | 40 | 40 | 0 | — |
| With mutations | | | | |
| Overall | 19 | 4 | 15 | 79 |
| 1 | 4 | 4 | 0 | 0 |
| 2 | 12 | 0 | 12 | 100 |
| >3 | 3 | 0 | 3 | 100 |

quencing results was high, 95% (114/120). Only six cases showed discordant results. In five cases, samples were determined as mutant types by MS-PCR, but as wild types by the sequencing method. One sample was diagnosed as M184I by sequencing, but as wild type by MS-PCR.

The 120 samples were analyzed by direct sequencing, and 56 samples were found to have at least one GPOvir-resistance mutation. In comparison or on the other hand, M184I/V MS-PCR detected resistance mutations in 39 of 56 resistance cases (Table 3). Thus, the overall sensitivity of MS-PCR for detecting GPOvir-resistant viruses was 70% (39/56). Among the 10 cases with one resistance mutation, the detection rate was low. Single mutations observed in these 10 cases were D67N ($n$ = 1), T215S ($n$ = 4), K103N ($n$ = 3), and Y181C ($n$ = 2). On the other hand, among the cases with more than one drug resistance mutation, the sensitivity of MS-PCR was considerably higher. Of the 46 samples with more than one GPOvir resistance mutation, 39 (85%) were diagnosed as having resistant viruses by MS-PCR. This percentage includes ART-experienced cases, which developed drug resistance mutations because of ART exposure. If we excluded such cases from the analyses and reevaluated the sensitivity of M184I/V MS-PCR for ART-naive cases (Table 3), the overall sensitivity for detecting GPOvir-resistant cases with at least one mutation became 79%. However, the sensitivity for detecting cases with one resistance mutation remained low.

Although we designed M184I/V MS-PCR for CRF01_AE samples to increase the likelihood of using the assay in other developing nations, we also evaluated the assay performance against 48 non-AE samples randomly collected at the NIID. These 48 cases included subtypes B (30 cases), C (10), D (1), F (4), and G.[15] The overall concordance rate between M184I/V MS-PCR and sequencing in the 48 non-AE samples was 92% (Table 4).

## DISCUSSION

The M184I/V mutation, which demonstrates high-level resistance to lamivudine,[16–21] has been reported to be the first mutation selected by lamivudine-containing regimens.[15] Since the GPOvir tablet contains a combination of lamivudine, stavudine, and nevirapine, we reasoned that M184I/V might be the most frequently observed drug resistance mutation in cases of GPOvir treatment failure. Therefore, we expected that M184I/V could be an efficient marker for surveying GPOvir-resistant cases. Indeed, the M184I/V mutation was the most common mutation found; it was detected by direct sequencing in 38 of 66 treatment failure cases. To create a less expensive and simpler altenative to direct sequencing for monitoring treatment and surveying for drug resistance, we developed a new diagnostic PCR assay to detect M184I/V mutations. The performance of this assay, which employs a MS-PCR method, was evaluated by comparing it to the results of direct sequencing. As described in the results, our newly designed assay demonstrated a high concordance rate (95%) with sequence analysis, suggesting that M184I/V MS-PCR could be a useful tool to survey for drug resistance in GPOvir-treated populations.

However, analysis of the MS-PCR and sequencing results showed six discordant cases. Typical discordant cases were defined as mutants by MS-PCR but as wild type by direct sequencing. This discordance pattern between MS-PCR and sequencing has also been observed in our previous reports.[9,14] We explained this discordance as due to the higher sensitivity of MS-PCR in detecting minor drug resistance populations, which cannot be detected by direct sequencing. Thus, this type of discordance may not be a drawback, but an advantage of the assay, since it detects resistance earlier than sequence analysis.

TABLE 4. COMPARISON BETWEEN M184I/V MS-PCR AND DIRECT SEQUENCE ANALYSES IN NON-AE SAMPLES ($n$ = 48)

| | *Sequence results* | | |
|---|---|---|---|
| *MS-PCR results* | *Wild type* | *Mutant* | *Total* |
| Wild type | 17 | 0 | 17 |
| Mutant | 4 | 27 | 31 |
| Total | 21 | 27 | 48 |

On the other hand, one discordant case was found with the opposite pattern; wild type was detected by MS-PCR and mutant by sequencing. This case indicated a limitation of MS-PCR reliability. Detailed analysis of this sample clarified that the base sequence of codon 184I was "ATC," not "ATA" as we had designed for the primer. Of the 18 cases of M184I in our samples, 17 had isoleucine coded at codon 184 as ATA, and ATC was identified only in this one discordant case. We also searched a large database (the Los Alamos drug resistance database), but could not find any reported case of ATC coded as isoleucine at codon 184, suggesting that the case we observed was exceptional and the prevalence of ATC coding for isoleucine is likely to be small. Nevertheless, this discordant case indicates an intrinsic limitation of MS-PCR, i.e., that polymorphism in primer binding sites is critical for assay performance and accuracy. Therefore, when constructing an MS-PCR assay, it is important to find a target point within a relatively conserved region of the HIV-1 genome. Though we faced a minority polymorphism in M184I coding, downstream of 184 where we designed the detection primer is the active site of HIV-1 reverse transcriptase with D185 and D186, and one of the most conserved regions within the HIV-1 genome. In fact, we confirmed the sequences of all 120 CRF01_AE samples in our study to search for polymorphisms in the 3′-end of the detection primer, and found no coding polymorphisms at D185 and D186. Although it may be not practical to cover all possible polymorphisms within the primer binding region, it is still important to accumulate additional information on the M184I/V mutation in CRF01_AE and to improve the assay reliability as much as possible.

In non-AE samples, we found four discordant cases, i.e., mutant by MS-PCR and wild type by sequencing. These four cases were also checked for sequence polymorphisms in the primer binding region, and no polymorphisms were found. This analysis suggests that 3′-end polymorphisms were not the cause of the discordance.

The sensitivity of M184I/V MS-PCR in detecting GPOvir-resistant viruses was 85% for viruses with two or more resistance mutations (Table 3). However, we had difficulty detecting resistance cases with only one mutation. To improve the detection rate of drug resistance cases, we searched for additional drug-resistance mutations, which salvaged M184V/I-negative resistance cases and improved the detection rate. The results in Table 1 show that it was difficult to detect cases with a single mutation, which is very common among these cases. However, adding D67N and Y181C/I as targets of MS-PCR may decrease false-negative results.

Although M184V/I MS-PCR failed to detect all drug resistance cases (14% misdiagnosed), we still propose MS-PCR as a promising candidate assay for drug resistance surveillance. In most developing nations, where even routine CD4 counts and measures of viral load have not been achieved, routine diagnosis of drug resistance by direct sequencing is too expensive, $200 to $250. On the other hand, MS-PCR screening costs only $12.50 per sample, a more affordable price than sequencing, and more patients can access MS-PCR. The drawbacks of MS-PCR observed in our study can be overcome by collecting more sequence information and designing multitarget MS-PCR.

Thus, we believe that MS-PCR can be used to achieve maximum benefits for the HIV/AIDS population in Thailand and probably in other developing countries, where the most commonly delivered antiretroviral combination includes d4T, 3TC, and NVP. We have also confirmed that our M184I/V MS-PCR assay can be applied to non-AE samples such as subtypes B, C, D, F, and G. Therefore, our MS-PCR-based drug resistance surveillance sysytem can be readily useful in developing countries worldwide.

## ACKNOWLEDGMENTS

We are grateful to all the participants of the Lampang cohort. We appreciate the technical assistance of Dr. Yoshinao Kubo, Ms. Suthira Kasemsuk, Ms. Sriprai Seneewong-na-ayuthaya, Ms. Anong-nard Suyasarojna, Ms. Nutira Boonna, and Mr. Prapan Wongnamnong. We also thank Ms. Claire Baldwin for her help in preparing the manuscript. This study was supported by The Ministry of Public Health Thailand, Ministry of Health, Social Welfare and Labour of Japan, and the Japan International Cooperation Agency.

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Address reprint requests to:
*Wataru Sugiura*
*AIDS Research Center*
*National Institute of Infectious Diseases*
*4-7-1 Gakuenn Musashimurayama*
*2080011 Tokyo Japan*

*E-mail:* wsugiura@nih.go.jp

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Transplant Immunology
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# Sensitization to enhanced green fluorescence protein minor histocompatibility antigen by gene transduction into dendritic cells and peritoneal exudate macrophages ☆

Eigo Satoh [a,b], Xiao-Kang Li [a], Yuhko Hara [a], Keiichi Ogata [a], Lei Guo [a], Yuhsuke Kitazawa [a], Naoko Funeshima-Fuji [a], Takashi Satoh [a], Tohko Miyagi [a], Wataru Sugiura [c], Naoki Yamamoto [c], Kenichi Teramoto [b], Shigeki Arii [b], Hiromitsu Kimura [a,*]

[a] *Department of Research Surgery, National Research Center for Child Health and Development, Japan*
[b] *Department of Surgery, Tokyo Medical and Dental University, Japan*
[c] *Aids Research Center, National Institute of Infectious Disease, Japan*

Received 9 November 2006; received in revised form 28 April 2007; accepted 30 April 2007

**Abstract**

Enhanced green fluorescence protein (EGFP) has been widely applied to gene transduction in cellular and molecular biology as a reporter element. When applied to cell transplantation, it raises fundamental issues concerning cell-associated antigens, in particular, a model of minor histocompatibility antigen(s). Although it is well known that immunological behavior of minor histocompatibility antigens mimic tumor associated antigens (TAA), identified genes coding minor histocompatibility antigens are few and far between. Inasmuch as immunity and tolerance to TAA are provided by immunological behavior of minor histocompatibility antigen such as histocompatibility antigen of the Y chromosome, H–Y, it occurs to us that transgenic as well as transduced EGFP provides a useful model system to be applied to tumor immunology. In this respect, genetic modification of specialized antigen-presenting cells (APC), i.e., dendritic cells (DC), such as gene transduction of EGFP into DC, would provide one of the most important strategies in transplantation as well as tumor immunology inasmuch as DC play a key role in initiating primary immune responses, As far as gene transduction into DC is concerned, others have reported that successful gene transduction occurs in DC by adenoviral vector systems. However, our previous studies concerning EGFP transduction into DC suggested that this view should be carefully examined and interpreted.

Employing adenoviral and lentiviral vector systems as well as specialized APC of rat DC and peritoneal exudate macrophages (PEM), EGFP-transduced APC were examined to determine whether and to what extent the EGFP-transduced APC were able to sensitize non-transgenic littermates against transgenic EGFP as antigen(s). Thus EGFP-transgenic cardiac isografts were transplanted to non-transgenic littermates and examined to determine if sensitization of non-transgenic littermate recipients with the EGFP-transduced APC was able to reject the test grafts in an accelerated manner.

In this study, we examined this and provide further evidence that widely used viral vector systems are unable to transfer the reporter gene EGFP into *mature* rat DC generated from bone marrow cells (BMC), driven by Flt3/Flk2 ligand and IL-6. Nevertheless, successful gene transduction was obtained by either applying a lentiviral vector system to the *developing DC progenitor cells* during a long-term culture of rat BMC or by applying an adenoviral vector system to PEM. Thus, successful gene transduction into specialized APC was verified by in vivo priming of non-transgenic littermates with the EGFP-transduced APC, followed by accelerated rejection of EGFP-transgenic cardiac isografts.


*Keywords:* CD161a (NKR-P1A); Dendritic cells (DC); Enhanced green fluorescence protein (EGFP); Minor histocompatibility antigen(s); Peritoneal exudate macrophages (PEM); Viral vector

☆ This study was supported by a Research Grant from the Ministry of Health and Labor Welfare of Japan, a Grant-in-Aid from the Ministry of Education, Culture, Sports and Technology of Japan, and the Human Science Foundation of Japan.

* Corresponding author. Department of Research Surgery, National Research Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo, 157-8535, Japan. Tel.: +81 3 3417 2594; fax: +81 3 3417 2864.
*E-mail addresses:* chimera@nch.go.jp, rschimera@yahoo.co.jp (H. Kimura).

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doi:10.1016/j.trim.2007.04.003

## 1. Introduction

For the last decade, enhanced green fluorescence protein (EGFP) has been widely used as a reporter gene to visualize a target molecule in cellular and molecular biology [1]. However, when applied to cell transplantation study in vivo, it becomes an apparent culprit that induces immune responses, inasmuch as transduced-EGFP is a foreign protein. Indeed, cytosolic localization of virally transduced proteins including EGFP in general becomes a target of cell-mediated immunity regardless of their cytotoxicity or helper function, i.e., CD8+ T lymphocytes recognize target cells expressing eight or nine peptide sequences derived from viral components in association with class I molecule [2,3]. In this regard, it occurs to us that transgenic and/or transduced EGFP provides a quite unique model of a minor histocompatibility antigen. Only a few classical minor histocompatibility antigens as well as their coding genes have been identified [4,5]. In contrast to any other unknown minor histocompatibility antigens, EGFP is well defined. Thus the EGFP-introduced cells also provide an ideal model for analyzing immunological behavior of minor histocompatibility antigens such as H–Y [6,7] and tumor-associated antigens (TAA).

As far as genetic modification of dendritic cells (DC) is concerned, gene transduction into DC would provide one of the most important strategies in transplantation as well as tumor immunology inasmuch as DC play a key role in initiating primary immune responses as specialized antigen-presenting cells (APC) [8]. Indeed, DC gene-modification has been shown to be a powerful tool in immunotherapy for both experimental and clinical settings [9–12]. Nevertheless, despite numerous studies concerning with gene transfer into DC where several vector systems including adenoviral vectors [13,14], retroviral vectors [12,15], HVJ-related vectors [16,17] and electroporation [18,19] are applied to gene transduction studies, our previous studies have demonstrated that this is not the case for highly purified rat DC [20].

The cause of the apparent resistance of gene transduction into *mature DC* is currently not known. The efficiency of gene transduction appears be affected by at least two major factors.

One concerns the target cell population for the gene to be transduced. In mouse DC systems, and specifically in gene-transfer studies *in vitro*, DC cell lines [21] or DC-like cells (as APC) are often used instead of primary bone marrow cells (BMC)-derived DC. In general, it is difficult to obtain a large number of homogeneous primary DC. When primary DC separated *in vivo* are employed, the DC population is usually very small and heterogeneous. Likewise, in human gene-transfer studies [14,22], CD 14+ monocyte-derived DC have been isolated from peripheral blood, and these populations are also usually small in number and differentiate into different type of cells. Moreover, most of the studies by others concerned with gene transfer into DC have employed heterogeneous cell populations, making it unclear whether and to what extent the gene is indeed transduced in *bona fide DC* or a DC subset such as *myeloid DC* or *plasmacytoid DC* [23].

However, it is not the case where rat DC systems are concerned. With recent advances in techniques for rat DC culturing [24] and cell separation [25], attempts have become feasible to test the gene transduction into a subset of primary rat DC generated from BMC culture.

The second major aspect of gene transduction studies concerns the vector system. Recently, a lentiviral vector system has been developed as a new vehicle for the retroviral vector. Unlike the precedent retroviral system [9–12], this system transduces genes into non-dividing cells [26]. This lentiviral vector system was developed using the Human Immunodeficiency Virus-1 (HIV-1). A third generation of lentiviral vectors has been improved as a self-inactivated and VSV-G pseudotyped vector [27].

Our previous studies and current study employ this third generation of lentiviral vector system instead of the more widely used retroviral vectors [9–12], because we attempt to transduce genes into non-dividing *fully mature rat DC*. The EGFP gene was employed as a reporter gene as well as an indicator of minor histocompatibility antigen(s) that provide a prototype of tumor immunology. To monitor the function of EGFP-modified APC, both adenoviral and lentiviral vectors were employed and examined.

This study attempted to further verify and extend our findings in previous studies [20] that neither vector system is able to transduce genes into fully mature rat DC generated from a BMC culture. Hence, it failed to prime normal rats toward EGFP-expressing cardiac isografts as a minor histocompatibility antigen. However, when developing DC progenitors are subjected to gene transduction, the lentiviral vector is able to do so. Furthermore, like the developing DC progenitors subjected to the lentiviral vector, peritoneal exudate macrophages (PEM) transduced by either lentiviral or adenoviral EGFP vector are likewise able to sensitize normal rats to EGFP antigen.

Our studies strongly indicate that the currently used viral vector systems are unable to transduce functional genes into *fully mature* DC. Studies by others regarding gene transduction into DC should be carefully interpreted based on a small population of fully functional macrophages in DC preparation.

## 2. Objective

Genetically modified and/or foreign proteins, including EGFP, introduced into the target cells provide a prototype of cell- or tissue-specific antigens such as TAA as well as minor histocompatibility antigens. Inasmuch as these cytosolic antigens are subjects of cell-mediated immunity not by humoral immunity, we address a fundamental question regarding whether and to what extent non-transgenic littermates are sensitized to EGFP minor histocompatibility antigens by EGFP-modified DC and/or PEM. Employing two representative viral vector systems, i.e., adenoviral and lentiviral vectors, we thus examined whether accelerated graft rejection of EGFP-transgenic cardiac isografts occurred in non-transgenic littermates following the EGFP-modified DC and/or PEM immunization.

## 3. Materials and methods

### 3.1. Animals

An inbred strain of DA (MHC: RT1avl) rats was obtained from Harlan Olac Ltd. (Blackthorn, Bicester, England). An outbred strain of male SD TgN (act-