summarized in Table 1. For the second study, samples from an additional 38 patients were analyzed. The median numbers of HIV-1 RNA copies (32,289.3 copies/ml), CD4$^+$ T cells (449.4 copies/ml), and total white blood cells (5049.0 cells/ml) were determined in all 52 patients. Control healthy plasma samples were obtained from Teragenix Corporation (Kokusai Bio, Tokyo). A recombinant Vpr protein (rVpr) was first prepared as a fusion protein with glutathione *S*-transferase (GST) expressed by pGEX6-P-1, and purified according to the manufacturer's protocol (GE Healthcare Bio-Sciences, Piscataway, NJ). The purified rVpr appeared as a single band on Coomassie brilliant blue staining (supplementary information 1a: SI-1a). Two mouse monoclonal antibodies, 8D1 (IgG2a) and C217 (IgG2b), were generated by immunization with a full-length Vpr peptide, chemically synthesized based on the prototype NL4-3[10] (Osaka Peptide Institute, Osaka), and a synthetic 18-mer amino acid peptide encompassing its carboxy (C)-terminal region (Wako Pure Chemical Industries, Tokyo, Japan), respectively. An enzyme-linked immunosorbent assay (ELISA) was based on 8D1, as the primary antibody, and a purified rabbit IgG antibody, raised against the peptide of the C-terminal 18 amino acids of Vpr (IBL, Fujioka, Japan), as the second antibody. Although the Vpr-ELISA could clearly detect purified rVpr (SI-1b), we found that the system occasionally detected one or more cross-reacting peptide in healthy persons (data not shown). Therefore, we decided to carry out a semiquantitative analysis using immunoprecipitation-Western blotting (IP-WB) analysis, with rVpr quantified by ELISA as the standard. For the IP-WB analysis, 0.5 mg of C217 was bound to Protein G Sepharose (GE Healthcare Bio-Sciences). Each 200 μl of plasma was first treated with DNase I and RNase A for 5 min, and then incubated with 10 μl of C217-coupled beads for 2 h at 4°C. After being washed in buffer with 0.05% Tween-20, the immunoprecipitate was subjected to Western blot analysis. For standard samples, different amounts of purified rVpr were added to 200 μl of control plasma. No detergents were added when the samples were incubated with the primary antibody, so that the IP-WB would detect only soluble Vpr, and not Vpr in viral particles.[10] The detection limit of the system was about 1 ng/ml (0.07 nM) (SI-2a).

Representative results from the IP-WB analysis of 14 plasma samples are shown in Fig. 1a. A definite signal of the 14-kDa protein was observed in patients N-09, 11, and 13 (Fig. 1a). By contrast, no peptides around 14 kDa were detected in more than 60 specimens from healthy volunteers (Fig. 1b). Because the IP-WB could selectively detect the 14-kDa peptide in the culture supernatant of cells containing an expression plasmid encoding *vpr* (SI-2b), we concluded that the 14-kDa peptide detected by the IP–WB was Vper. A comparison of the signal intensities of the detected bands and standard rVpr (Fig. 1a; 5, 2.5, and 1.25 μg/ml signals, and N-11) indicated that the serum Vpr concentration was about 0.7 nM.

During the analysis, we did not detect the Vpr signal in one patient (N-10; Table 1) who had 11,000 copies/ml of HIV-1 RNA (Fig. 1a, lower panel). To evaluate whether our system failed to detect Vpr mutants differing from the prototype NL4-3 (GenBank accession number M19921), we amplified DNA fragments from peripheral blood mononuclear cells covering the entire *vpr* gene. Then we determined its nucleotide sequence (Fig. 2a, and primers in SI-3). The deduced amino acid sequences are also shown in Fig. 2b. Interestingly, the *vpr* gene from patient N-10 had a four-nucleotide (TTAA) insertion at

TABLE 1. CLINICAL DATA OF PATIENTS SUBJECTED TO ANALYSIS AND RESULTS OF THE IP-WB

						Clinical data				
						White blood cells (/mm^3)				
Case number	*Sex*	*Age*	*Causes of infection*	*Conditions*	*Treatment status*[a]	*Total number*	*Lymphocytes*	*CD4$^+$ T cells*	*HIV-1 RNA (copies/ml)*	*Vpr*[b]
N-01	M	39	HO[c]	AIDS[d]	2	8400	2612	771	<50	–
02	M	41	HO	AIDS	2	6800	2584	346	<50	–
03	M	59	HE[c]	AIDS	2	4700	2444	381	260	–
04	F	32	HE	AC[d]	3	6300	1890	302	4,400	–
05	M	38	HO	AIDS	2	8600	2417	585	<50	–
06	M	35	HO	AC	1	4900	1274	116	220,000	+++
07	M	45	BL[c]	AIDS	2	2600	546	38	73,000	++
08	M	58	HE	AIDS	2	6800	1632	366	<50	–
09	M	29	BL	AC	1	3000	1056	266	17,000	+
10	M	23	HO	AC	1	5200	1300	230	11,000	–
11	F	37	HE	AC	1	4600	1150	222	500,000	+++
12	F	40	HE	AC	1	6600	1584	598	98	–
13	M	42	BL	AIDS	2	3100	1054	110	70,000	++
14	M	23	HO	AC	1	5800	2656	553	71,000	++

[a]Group 1, no therapy; group 2, under medication; group 3, posttherapy.
[b]Based on results of the IP-WB, patients are divided into four groups; Vpr-negative (–) and Vpr-positive with less than 1 ng/ml (+), with 1–5 ng/ml (++), and with more than 5 ng/ml (+++).
[c]HO, homosexual; HE, heterosexual; BL, blood products.
[d]AIDS, acquired immunodeficiency syndrome; AC, asymptomatic carrier.

nucleotide 81, designated "clone 10," which generates a frameshift mutation within the inserted sequence (shown by the box in Fig. 2a). However, because this patient had no deletion in the 3′ region of the *vpr* gene, it was possible to clone the gene. Repeated sequence analyses of several clones of the amplified *vpr* DNA indicated that clone 10 was the major *vpr* in this patient (Table 2). The negative results of the IP-WB analysis for patient N-10 were therefore due to truncation of the C-terminal region.

Additional sequence analysis revealed that "clone N (Nara)," which differs by four amino acids from the prototype NL4-3 (Fig. 2b), was frequently observed in the analyzed patients (patients N-04, 05, 08, 09, 11, and 12). Interestingly, although patient N-09 had clone N as a major variant—all seven clones sequenced from the PCR products were identified as clone N (see Table 2)—the IP-WB analysis (Fig. 1a, lower panel) detected a positive Vpr signal in patient N-09. This suggests that C217 antibody, which was used as the first antibody in immunoprecipitation, reacts with the protein encoded by clone N, even though its C-terminal region differs from the prototype NL4-3 clone by two amino acids (Fig. 2b).

Next, we examined the possible correlation of Vpr and clinical manifestations. An analysis of 14 patients suggested a positive link between Vpr and viral titers (data not shown). To examine this possibility, we analyzed an additional 38 stocked samples using IP-WB. We detected Vpr in 14 samples. A representative result of the second analysis is shown in Fig. 1c. Positive Vpr signals were detected in patients T-166, 167, and 175. Then we examined the relationship between Vpr and RNA copy number in total 52 samples. As shown in Fig. 3a, we found a positive correlation between the detection of Vpr and RNA copy number ($p < 0.03$). In contrast, we did not detect a positive relationship between Vpr and the numbers of $CD4^+$ T cells or total white blood cells. The distribution of Vpr-positive patients based on the concentration of Vpr implied that the high amount of Vpr is observed in patients with high HIV-1 RNA copy numbers (Fig. 3b).

In the current work, we successfully identified Vpr in 20 samples from 52 HIV-1-positive patients. A comparison of the signals obtained with standard rVpr revealed that the Vpr concentration was ~0.7 nM. Levy *et al.*[9] suggested that Vpr is present in patient plasma, with rVpr activating viral reproduction when added to the culture medium of latently infected cells. In addition, Muthumani *et al.* proposed that exogenous rVpr has various activities, such as inducing T cell apoptosis,[11] inhibiting macrophage function,[12] and suppressing NF-κB signaling.[13] However, these experiments did not consider the actual amount of Vpr present in the plasma samples. Our result is the first demonstration of Vpr in HIV-1-positive patients, and provides a rationale for the dose of rVpr suitable for *in vitro* experiments.

We observed a positive correlation between the detection of Vpr and HIV-1 RNA copy number ($p < 0.03$) (Fig. 3a). It has been reported that the exogenous Vpr induces viral production from latently infected cells, implying that Vpr is involved in viral reproduction *in vivo*. An important question still to be answered is how the Vpr titer changes in the context of viral replication during the clinical course of the disease. It is important to clarify whether Vpr functions as an initial trigger of viral expansion *in vivo*.

We did not detect a link between Vpr and the numbers of $CD4^+$ T cells. Recently, it was determined that WT-Vpr and its variant R77Q act differently in modifying the clinical features of HIV-1-positive patients. Based on several reports, it has been proposed that R77Q is a candidate marker for long-term nonprogression (LTNP),[14–16] although this is still controversial.[17,18] In this study, we observed that the main Vpr variants of patients N-04, 09, and 10 were R77Q or C-terminally truncated. However, we did not recognize these patients as candidates for LTNP (clinical observation by M. Konishi). The involvement of WT-Vpr and R77Q in patients is rationalized by *in vitro* experiments showing that rVpr induces the apoptosis of $CD4^+$ T cells,[11,12,14,15] whereas R77Q has less potent apoptosis activity than WT-Vpr.[15] It is important to note that the *in vitro* studies of the differential activities of exogenous WT-Vpr and R77Q used tremendous amounts of the proteins, and a difference in activity was observed only when 1.5–2.0 μM of the peptides was used.[15] As shown here, the concentration of Vpr in patient plasma was a maximum of 1.0 nM, and it is crucial to compare the functional difference of these molecules at a concentration comparable to that observed *in vivo*. Careful studies are required to address this matter.

SUPPLEMENTARY INFORMATION

SI-1. Purification of rVpr and measurement using ELISA. (**a**) Expression and purification profiles of rVpr. Vpr was expressed as a fusion protein with GST and purified in a glutathione column. Lane 1, marker; lane 2, initial lysate; lane 3, flow-through sample eluted from the glutathione column; lane 4, eluate from rVpr after treatment with precision protease; and lane 5, eluate from an affinity column containing a monoclonal antibody against Vpr (8D1). The arrowhead and arrow indicate the position of GST-Vpr and purified rVpr, respectively. Proteins were stained with Coomassie brilliant blue solution. (**b**) ELISA verion-1 for measuring rVpr. Synthesized full-length Vpr was used to make a standard curve. To the Vpr-ELISA were added 10 ng/ml each of GAPDH, HIV-1 integrase, and SARS-CoV Spike protein, which were expressed as a (His)-tagged protein, and purified using Ni-beads. Note that none of the samples gave cross-signals with Vpr. The amount of rVpr was assessed using the absorbance at OD450 nm, as shown with the dotted line.

SI-2. Detection of Vpr by the IP-WB. (**a**) Sensitivity of the system. The IP-WB analysis was conducted using C217 for IP and 8D1 for WB. To determine the sensitivity of the system, 10, 5, 2.5, and 1.25 ng of purified rVpr were added to 200 μl of plasma from a healthy human just before the IP-WB analysis. The signals obtained using IP-WB (upper panel) and the input rVpr (lower panel) detected by 8D1 are shown. (**b**) Detection of Vpr in a culture supernatant. Culture supernatants (sup.) of 293FS cells (Invitrogen) transfected with pcDNA3.1 (center lane, "Vec") or pcDNA3.1-*vpr* (right lane, "Vpr") were collected on day 6 after transfection, and the IP-WB analysis was carried out. The rVpr (400 pg/lane) was included in the same blot as a positive control of WB (left lane).

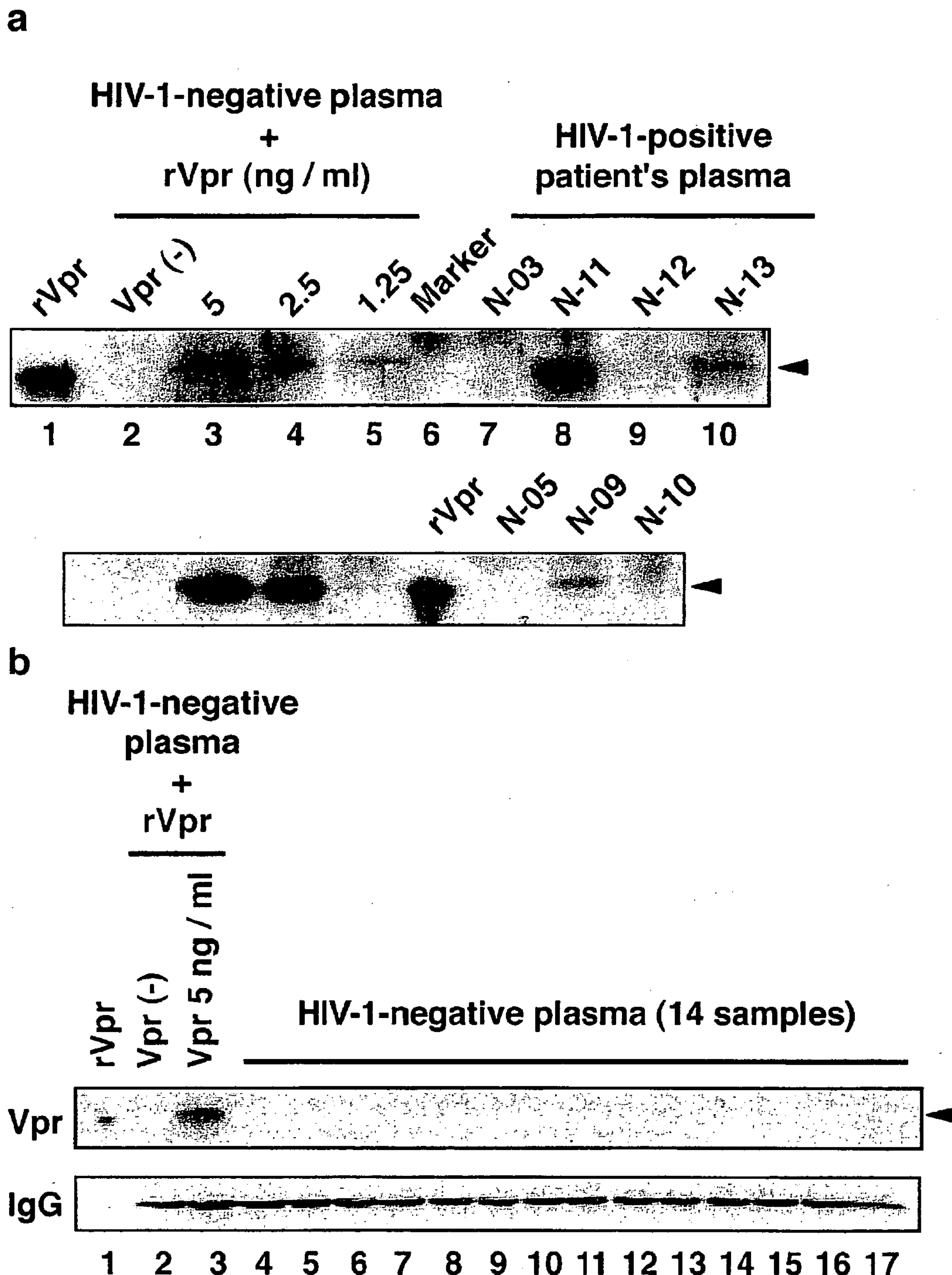

FIG. 1. Detection of Vpr in sera of HIV-1-positive patients. (**a**) Presence of the 14-kDa Vpr protein in HIV-1-positive patients. To semiquantify the Vpr concentration in patient samples, 5, 2.5, and 1.25 ng of standard rVpr (lanes 2–5), which had been measured using ELISA version-1 (see supplementary information 1b; SI-1b), were included. As a positive control for the WB analysis, 1 ng of rVpr (lane 1) was also included. Signals of HIV-1-positive plasma (lanes 7–10) and a molecular marker (lane 6) are shown. (**b**) Representative results of the IP-WB analysis of healthy volunteers. The IP-WB analysis was performed on more than 60 samples from healthy volunteers, and representative results from 14 cases (lanes 4–17) are shown. Note that no signals were detected around 14 kDa. The results for input rVpr (lane 1), no rVpr (lane 2), or 5 ng Vpr (lane 3) added to normal plasma are shown. IgG signals recovered after IP are also shown (lower panel). (**c**) Detection of the 14-kDa Vpr protein in HIV-1-positive patients in the second group. Also in this analysis, 5, 2.5, and 1.25 ng of standard rVpr (lanes 2–5) were included to assess the concentration of Vpr in patient plasma samples.

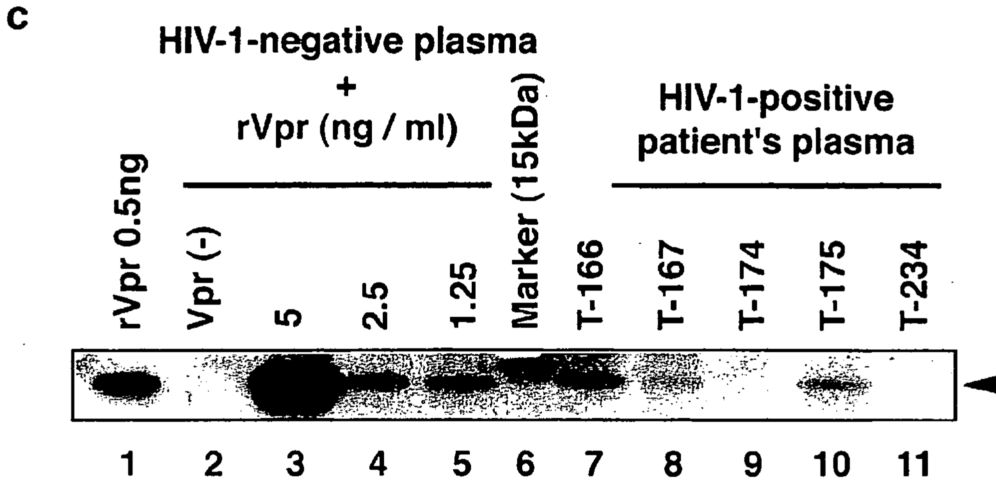

FIG. 1. (*Continued*).

SI-3. Cloning and sequence analysis of *vpr*. DNA covering *vpr* was amplified from the genomic DNA of peripheral blood cells using nested PCR. The primers used were Vpr1F (nt 4713–4733, 5′-GACCCTGACCTAGCAGACCA-3′) and Vpr1R (nt 5298–5318, 5′-CAAACTTGGCAATGAAAGCA-3′) for the first PCR. For the second PCR, Vpr2F (nt. 4854–4875, 5′-CAGTACTTGGCACTAGCAGCA-3′) and Vpr2R (nt 5243–5263, 5′-TAGGCTGACTTCCTGGATGC-3′) were used (GenBank accession number M19921). The first and second rounds of PCR were performed for 30 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min and for 95°C for 30 sec, 64°C for 30 sec, and 72°C for 45 sec, re-

a

```
NL4-3      1 ATGGAACAAG CCCCAGAAGA CCAAGGGCCA CAGAGGGAGC CATACAATGA ATGGACACTA
Clone-10   1 ---------- ---------- ---------- ---------- ---------- ----------

          61 GAGCTTTTAG AGGAACTTAA     GAGTGA AGCTGTTAGA CATTTTCCTA GGATATGGCT
          61 ---------- ---------- T[TAA]------ ---------- ---------- ----------

         117 CCATAACTTA GGACAACATA TCTATGAAAC TTACGGGGAT ACTTGGGCAG GAGTGGAAGC
         121 ---------- ---------- ---------- ---------- ---------- ----------

         177 CATAATAAGA ATTCTGCAAC AACTGCTGTT TATCCATTTC AGAATTGGGT GTCGACATAG
         181 ---------- ---------- ---------- ---------- ---------- ----------

         237 CAGAATAGGC GTTACTCGAC AGAGGAGAGC AAGAAATGGA GCCAGTAGAT CCTAG 291nt.
         241 ---------- ---------- ---------- ---------- ---------- ----- 295nt.
```

b

```
NL4-3     MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS
Clone-10  ••••••••••••••••••••••••••N*
Clone-N   ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••Q•••••II••••••••••••
```

FIG. 2. Sequence analysis of *vprs* and the deduced amino acids of Vpr variants in HIV-1-positive patients. The *vpr* gene was amplified and analyzed, as described in SI-3. (**a**) Nucleotide sequence of clone 10. The nucleotide sequence was compared with that of the prototype NL4-3. Clone 10 has a four-base insertion at nucleotide 81, generating a stop codon within the insert (indicated by the box). Nucleotides that are the same as those in NL4-3 are marked with small bars. (**b**) Amino acid sequences of Vpr variants found in the patients. The amino acids deduced from the obtained sequences and the NL4-3 clone are shown. As described in SI-3a, clone 10 was recognized as a major variant in patient N-10, while clone N was the major variant in patients N-04 and 09.

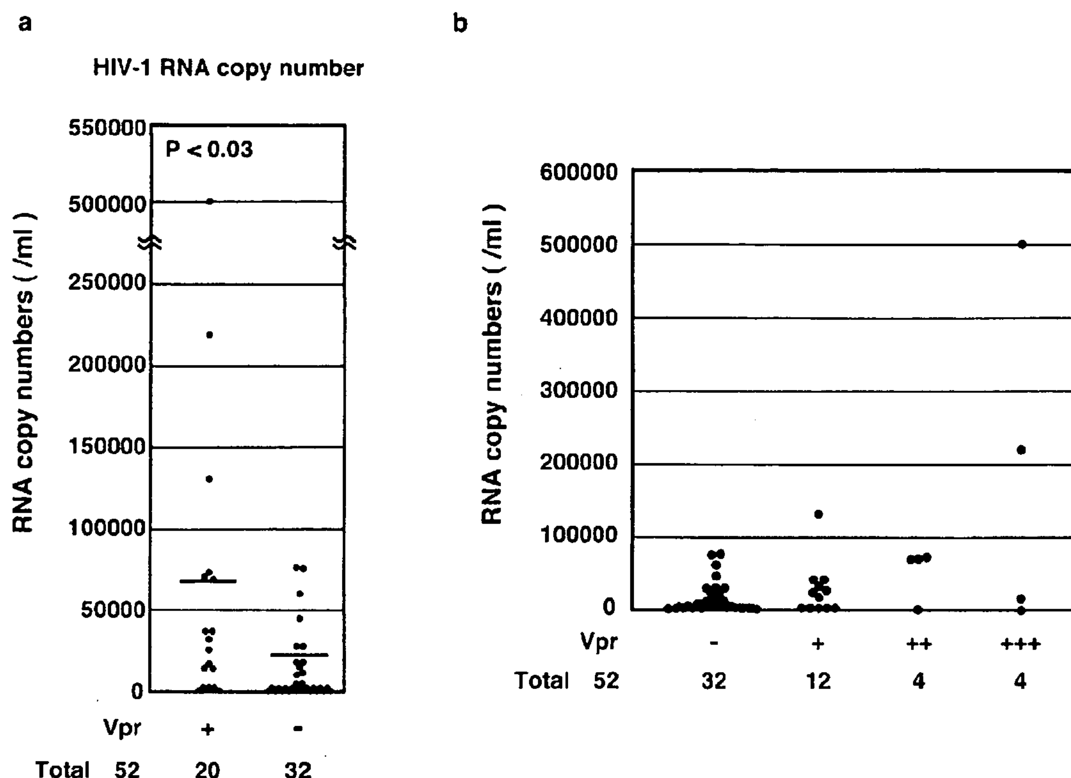

FIG. 3. Correlation between Vpr detection and clinical data. The analyzed cases were divided into Vpr-positive and Vpr-negative groups, and the statistical analysis was done using Student's t-test. (**a**). The relationships with the HIV-1 RNA copy number. The bars indicate the mean numbers in each group. The difference for HIV-RNA copy number with Vpr was statistically significant ($p < 0.03$). (**b**) Distribution of Vpr-positive patients according to the concentration of plasma Vpr. Based on the semi-quantitative analysis, patients were divided into four groups: Vpr-negative (−), Vpr-positive with less than 1 ng/ml (+), 1–5 ng/ml (++), and more than 5 ng/ml (+++). Each dot means a patient.

spectively. The PCR products were cloned into pZeroBlunt topo vector (Invitrogen, Carlsbad, CA). Several clones were sequenced for each PCR product.

SI-4. See Table 2.

TABLE 2. FREQUENCY OF *vpr* VARIANTS IN HIV-1 PATIENTS[a]

Cases	vpr *variants*		
	NL4-3	*Clone N*	*Clone 10*
N-03	7[b]	—	9
N-04	—	14	—
N-05	2	2	3
N-09	—	7	—
N-10	—	—	5
N-12	4	4	—

[a]PCR products amplified from patient genomic DNA were subcloned into the vector, and several clones were sequenced. The numbers in the table indicate the frequency of clones encountered in the sequence analyses. All 5 clones derived from patient N-10 were clone 10. In patients N-04 and 09, clone N was identified as the major variant; all 14 clones for patient N-04 and all 7 clones for patient N-09 were clone N. Patients N-03 and N-05 each had 2 *vpr* variants.

[b]Number of analyzed clones.

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The Polymorphisms in *DC-SIGNR* Affect Susceptibility to HIV Type 1 Infection

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ABSTRACT

Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (*DC-SIGN*) and its homologue *DC-SIGNR* (*DC-SIGN* related) have been thought to play an important role in establishing HIV infection by enhancing *trans*-infection of CD4$^+$ T cells in the regional lymph nodes. To identify polymorphisms associated with HIV-exposed seronegative (ESN) individuals in Thais, genomic DNA from 102 HIV-seronegative individuals of HIV-seropositive spouses, 305 HIV-seropositive individuals, and 290 HIV-seronegative blood donors was genotyped for two single nucleotide polymorphisms (SNPs) in *DC-SIGN* promoter (−139A/G and −336A/G), a repeat number of 69 bp in Exon 4 of *DC-SIGN* and *DC-SIGNR*, and one SNP in Exon 5 of *DC-SIGNR* (rs2277998A/G). We found that the proportion of individuals possessing a heterozygous 7/5 and 9/5 repeat and A allele at rs2277998 of *DC-SIGNR* in HIV-seronegative individuals of HIV-seropositive spouses was significantly higher than HIV-seropositive individuals [$p = 0.0373$, OR (95% CI) = 0.57 (0.32,1.01); $p = 0.0232$, OR (95% CI) = 0.38 (0.15,0.98); and $p = 0.0445$, OR (95% CI) = 0.61 (0.37,1.02), respectively]. Analysis after stratifying by gender showed that these associations were observed only in females but not in males. Moreover, HIV-seropositive females tend to have a homozygous 7/7 repeat more frequently than HIV-seronegative females with a marginal level of significance [$p = 0.0556$, OR (95% CI) = 1.79 (0.94,3.40)]. Haplotype analysis showed that the proportion of individuals possessing the 5A haplotype in HIV-seronegative females was significantly higher than HIV-seropositive females [$p = 0.0133$, OR = 0.50 (0.27,0.90)]. These associations suggest that *DC-SIGNR* may affect susceptibility to HIV infection by a mechanism that is different in females and males. Further studies are warranted to investigate the mechanisms of their function.

INTRODUCTION

SOME INDIVIDUALS, SUCH AS HIV-SERONEGATIVE COMMERCIAL SEX WORKERS and HIV-seronegative spouses of HIV-seropositive individuals, have been sexually exposed to HIV repeatedly, yet remain negative for anti-HIV antibody.[1,2] These individuals are recognized as HIV-exposed but seronegative persons (ESN). Our study in Thailand has identified a number of married couples in whom the serostatus of HIV infection was discordant between spouses and in the majority of cases, this discordance could not be explained by their sexual behavior, such as frequency of unprotected sexual contacts, or by viral load of their infected spouses (Rojanawiwat *et al.*, submitted). These observations made us speculate that these seronegative spouses were likely to have certain biological mechanisms that made them resistant to HIV infection.

A number of polymorphisms in host genes that encode products involved in HIV replication and/or immune regulation were reported to be associated with HIV infection and HIV disease progression.[3] Among these, only the homozygous *CCR5* 32-bp deletion (CCR5Δ32) was found to be consistently associated with resistance to HIV infection.[4–6] However, the CCR5Δ32 al-

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lele is very rare among Asians,[6–9] including Thais.[10] Thus other unidentified genes are likely to play a role in resistance to HIV infection among ESN in Thailand.

Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (*DC-SIGN*, also called CD209) is a receptor on dendritic cells (DCs) that binds to ICAM-3 expressed on T cells to facilitate the initial interaction between DCs and T cells. *DC-SIGN* has been considered important in HIV research because it acts as an intermediate receptor for binding to HIV-1 at mucosal sites and then enhancing *trans*-infection of $CD4^+$ T cells in regional lymph nodes.[11] A homologue of *DC-SIGN* called *DC-SIGNR* (*DC-SIGN* related or CD209L or L-SIGN; the official name is C-type lectin domain family 4, member M, CLEC4M) shares 77% amino acid identity and exhibits a similar capacity of binding to HIV-1.[12,13] *DC-SIGN* is expressed at high levels on DCs and some types of macrophages,[14,15] whereas *DC-SIGNR* is expressed on endothelial cells in liver and lymph nodes.[12,13,16] Recently, the presence of *DC-SIGNR* mRNA was demonstrated in the human vaginal and rectal mucosa by nested reverse transcriptase polymerase chain reaction (RT-PCR).[17] Because *DC-SIGN* and *DC-SIGNR* have an apparent role in DC–T cell interaction and HIV infection, the polymorphisms associated with these genes may have an impact on the transmission of HIV as shown in several studies.[18–23] The objective of this study is, therefore, to identify those reported polymorphisms in *DC-SIGN* and *DC-SIGNR* that are associated with ESN individuals in Thais. Having investigated polymorphisms of these two genes among Thai couples, we found that polymorphisms in the *DC-SIGNR* but not in the *DC-SIGN* were significantly associated with ESN females but not with ESN males.

MATERIALS AND METHODS

Patients and samples

From 6 July 2000 to 15 October 2002 we conducted the Lampang HIV couple study at the HIV clinic in the Day Care Center of the Lampang Hospital, which is a referral hospital located about 600 km to the north of Bangkok (Rojanawiwat *et al.*, submitted). For this study of genetic polymorphisms, we recruited 188 couples, including 70 HIV-serodiscordant couples with 43 HIV-seronegative females and 27 HIV-seronegative males, and 118 HIV-seroconcordant couples. All 144 HIV-seropositive females and 155/161 (96.3%) HIV-seropositive males were heterosexually infected. There were two males with a history of injecting drug use, one male with blood transfusion, two males with more than one possible risk factor, and one male with no information. In terms of age, viral load, $CD4^+$ cell count, and clinical symptoms of HIV-infected spouses, there was no significant difference between discordant couples and concordant couples in both females and males, except that the proportion of HIV-infected males with HIV-seronegative wives was higher than that of HIV-infected males with HIV-infected wives (Table 1).

To increase the number of HIV seronegative spouses of HIV-infected individuals, we also included two HIV-seronegative individuals (one male, one female) who did not bring their HIV-seropositive spouses and 30 HIV-seronegative widows whose husband died of HIV/AIDS. These widows were included because they were significantly exposed to HIV: the median [interquartile range (IQR)] frequency of sexual contact was four (2, 6) times per month and 26/30 (86%) of them had never used a condom before they were aware of HIV. Furthermore, we believe that the level of HIV exposure was very high because their index case has already progressed and died: in 14/30 (46%) women, their husband died within 1 year of disclosure and in 21/30 women (70%) within 2 years of disclosure. There was one HIV-seropositive female who previously had an HIV-positive husband and remarried an HIV-negative husband after her first husband died. Therefore in total, 102 HIV-seronegative individuals (74 females and 28 males) at high risk for HIV infection and 305 HIV-seropositive individuals (144 females and 161 males) were enrolled.

The median age (IQR) of both HIV-seronegative and HIV-seropositive individuals was 32 (29, 36) years. Among 74 HIV-seronegative female spouses, one of them had known her HIV status before the marriage. In the other 73 females, the median (IQR) duration of marriage before they knew the HIV status of their husbands was estimated to be 5 (3, 8) years. The median (IQR) frequency of sexual contacts before the disclosure of the HIV status of their husband was five (3, 8) times per month. Of females, 64 (87.7%) reported that they had never used a condom during those sexual contacts and only four (5.5%) reported that they used a condom at every contact. Among 28 HIV-seronegative male spouses, nine of them had known the HIV status of their wives before the marriage. In the other 19 HIV-seronegative males, the median (IQR) duration of marriage before the disclosure of HIV status of their wife was estimated to be 1 (0, 2) year. The median (IQR) frequency of sexual contact before the disclosure of the HIV status of their wife was six (4, 24) times per month, and all of them reported that they had never used a condom during sexual contacts.

For a control HIV-seronegative group representing the Thai general population, 290 blood samples were collected from blood donors (171 males and 119 females) at the blood bank of the Lampang Hospital. All participants gave written informed consent. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

EDTA-treated blood samples were separated for plasma and buffy coat, then stored at −80°C until used. Genomic DNA was extracted and purified from the frozen buffy coat using a kit (QIAamp mini blood kit; QIAGEN GmbH, Hilden, Germany).

HIV-1 serology was screened by an ELISA kit (Enzynost anti-HIV-1/2 plus; Dade Behring Marburg GmbH, Marburg, Germany). The positive samples were then confirmed by another ELISA kit (Genscreen HIV 1/2 Diagnostic Pasture Ltd., France) and a gel particle agglutination test (Serodia HIV-1; Fujirebio Inc., Tokyo Japan).

Polymorphism genotyping

DC-SIGN promoter: −139A/G (rs2287886) and −336A/G (rs4804803): *DC-SIGN −139A/G* was genotyped by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Amplification of fragments containing this single nucleotide polymorphism (SNP) was performed using forward primer: 5′-GTCGGAATCCAAGGCCTCTC-3′ and

reverse primer: 5′-CAGGAAAGCCAGGAGGTCAC-3′. PCR was performed in a total of 25 μl containing 0.1 μM each primer, 100 μM dNTPs, 2.5 mM $MgCl_2$, and 0.5 unit of heat-activated DNA polymerase (IMMOLASE, Bioline USA Inc., MA) in 1× buffer supplied with the enzyme. The thermal profile was 7 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 20 sec at 58°C, and 1 min at 72°C. The 574-bp PCR products were incubated with *SpeI* restriction enzyme. The product, which contained the −139A allele, was cut into 318-bp and 256-bp fragments, and determined by electrophoresis in 2% agarose gel.

For *DC-SIGN −336A/G* genotyping, we used an allelic discrimination (AD) assay by 7500 real-time PCR with SDS analysis software (Applied Biosystems, CA). Two primers (forward primer: 5′-TGTGTTACACCCCCTCCACTAG-3′; reverse primer: 5′-GGACAGTGCTTCCAGGAACT-3′) were used to amplify 68-bp products containing the *DC-366A/G* SNP site, which was detected by two probes labeled with different fluorescent dyes: 5′-VIC-TACCTGCCTACCCTTG-MGB-3′ to detect the "A" allele and 5-FAM-CTGCCCACCCTTG-MGB to detect the "G" allele. Real-time PCR was performed in a 10-μl reaction mixture containing 5 μl of 2 × TaqMan universal master mix, 1× primers and probes mix, and 10 ng of DNA sample. Thermocycling consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C.

The 69-bp repeat number in Exon 4

The 69-bp repeat number was determined by the length of the PCR products. Primers and the thermal profile for *DC-SIGNR* 69-bp repeat number genotyping was performed as previously described.[13] For *DC-SIGN* 69-bp repeat number genotyping, forward primer: 5′-CCTTGGCTCTCACAAAT-GATGTCC-3′ and reverse primer: 5′-CACCCACTGCAG-CCTTCAGCTG-3′ were used in the PCR condition as described above. The thermal profile was 7 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 64°C, and 1 min at 72°C. The PCR products were analyzed by electrophoresis in 2% agarose gel.

DC-SIGNR Exon 5 (rs2277998)

A/G SNP of rs2277998 was genotyped by PCR-RFLP as previously described.[18]

Data management and sample analysis

We conducted this study in a blinded manner between field investigators and laboratory investigators. The proportions of individuals possessing a certain allele/genotype among different groups were compared by a chi-square test or Fisher's exact test. Odds ratio (OR) and 95% confidence interval (95% CI) were used to measure the strength of the genetic influence associated with susceptibility to HIV infection. All statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *DC-SIGNR* haplotypes were constructed and estimated for their frequencies by the expectation-maximization algorithm (Arlequin version 3.01, Genetica and Biometry Laboratory, Geneva, Switzerland).

RESULTS

Genotyping of polymorphisms in DC-SIGN and DC-SIGNR

We genotyped the five polymorphisms (three in *DC-SIGN* and two in *DC-SIGNR*) in 102 HIV-seronegative individuals, 305 HIV-seropositive individuals, and 290 blood donors (Table 2). We then compared the proportions of individuals possessing a specific genotype between HIV-seronegative and HIV-seropositive individuals (Table 3).

As for the two SNP sites in the *DC-SIGN* promoter, we did not find any significant differences in the proportion of individuals possessing the G allele (genotype G/G and A/G). The *DC-SIGN* 69-bp repeat number was highly conserved; the genotype 7/7 repeat was found almost exclusively at a frequency of 0.99. In contrast, we found some significant differences in genetic polymorphisms in *DC-SIGNR* associated with HIV infection.

TABLE 1. CHARACTERISTICS OF HIV-SEROPOSITIVE INDIVIDUALS

	Male		*Female*	
	With HIV −ve spouse (n = 43)	*With HIV +ve spouse (n = 118)*	*With HIV −ve spouse (n = 27[a])*	*With HIV +ve spouse (n = 118)*
Median age; years (IQR)[b]	34 (31, 38)	33 (30, 37)	31 (28, 35)	30 (27, 35)
Median viral load,[c] $\log_{10}$ copies/ml (IQR)	5.257 (4.915, 5.757)	5.305 (4.773, 5.756)	4.939 (4.358, 5.579)	4.993 (4.214, 5.477)
Median CD4 count,[c] cells/mm^3 (IQR)	28 (13, 72)	38 (11, 246)	321 (157, 524)	277 (148, 427)
HIV-1-related symptoms,[c] % (*n*)	79.1 (34)	59.5 (69)	33.3 (9)	27.4 (32)
Diagnosis of AIDS,[c] % (*n*)	65.1 (28)	38.8 (45)	11.1 (3)	12.8 (15)

[a]This includes a female who was counted as a concordant couple with her previous HIV +ve husband.
[b]IQR, interquartile-range.
[c]Data not available on two concordant couples.

TABLE 2. DISTRIBUTION OF GENOTYPES [NO. (%)] OF POLYMORPHISMS IN DC-SIGN AND DC-SIGNR AMONG HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS

		HIV-seronegative			*HIV-seropositive*			*Blood donor*
		Male (n = *28*)	*Female* (n = *74*)	*Total* (n = *102*)	*Male* (n = *161*)	*Female* (n = *144*)	*Total* (n = *305*)	*Total* (n = *209*)
DC-SIGN								
Pomoter region								
−139 (rs2287886)	A/A	16 (57.14)	31 (41.89)	47 (46.08)	64 (39.75)	74 (51.39)	138 (45.25)	146 (50.34)
	A/G	9 (32.14)	35 (47.30)	44 (43.14)	86 (53.42)	56 (38.89)	142 (46.56)	120 (41.38)
	G/G	3 (10.71)	8 (10.81)	11 (10.78)	11 (6.83)	14 (9.72)	25 (8.20)	24 (8.28)
−336 (rs4804803)	A/A	22 (78.57)	59 (79.73)	81 (79.41)	119 (73.91)	121 (84.03)	240 (78.69)	237 (81.72)
	A/G	6 (21.43)	13 (17.57)	19 (18.63)	40 (24.84)	21 (14.58)	61 (20.00)	51 (17.59)
	G/G	0 (0.00)	2 (2.70)	2 (1.96)	2 (1.24)	2 (1.39)	4 (1.31)	2 (0.69)
Exon 4	7/5	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.62)	0 (0.00)	1 (0.33)	0 (0.00)
69-bp repeat numbers	7/7	28 (100.00)	73 (98.65)	101 (99.02)	160 (99.38)	144 (100.00)	304 (99.67)	288 (99.31)
	8/7	0 (0.00)	1 (1.35)	1 (0.98)	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.69)
DC-SIGNR								
Exon 5 (rs2277998)	G/G	19 (67.86)	46 (62.16)	65 (63.73)	111 (68.94)	115 (79.86)	226 (74.10)	215 (74.14)
	A/G	9 (32.14)	28 (37.84)	37 (36.27)	45 (27.95)	25 (17.36)	70 (22.95)	66 (22.76)
	A/A	0 (0.00)	0 (0.00)	0 (0.00)	5 (3.11)	4 (2.78)	9 (2.95)	9 (3.10)
Exon 4	5/5	0 (0.00)	0 (0.00)	0 (0.00)	4 (2.48)	4 (2.78)	8 (2.62)	11 (3.79)
69-bp repeat numbers	6/5	0 (0.00)	0 (0.00)	0 (0.00)	4 (2.48)	0 (0.00)	4 (1.31)	3 (1.03)
	6/6	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.34)
	7/5	6 (21.43)	21 (28.38)	27 (26.47)	33 (20.50)	19 (13.19)	52 (17.05)	47 (16.21)
	7/6	3 (10.71)	6 (8.11)	9 (8.82)	12 (7.45)	8 (5.56)	20 (6.56)	23 (7.93)
	7/7	9 (32.14)	22 (29.73)	31 (30.39)	52 (32.30)	62 (43.06)	114 (37.38)	133 (45.86)
	8/5	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.34)
	8/7	1 (3.57)	1 (1.35)	2 (1.96)	1 (0.62)	0 (0.00)	1 (0.33)	0 (0.00)
	9/5	3 (10.71)	7 (9.46)	10 (9.80)	8 (4.97)	4 (2.78)	12 (3.93)	8 (2.76)
	9/6	1 (3.57)	0 (0.00)	1 (0.98)	4 (2.48)	1 (0.69)	5 (1.64)	0 (0.00)
	9/7	5 (17.86)	14 (18.92)	19 (18.63)	39 (24.22)	40 (27.78)	79 (25.90)	56 (19.31)
	9/9	0 (0.00)	3 (4.05)	3 (2.94)	4 (2.48)	5 (3.47)	9 (2.95)	7 (2.41)
	10/7	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.69)	1 (0.33)	0 (0.00)

TABLE 3. COMPARISON OF PROPORTIONS [ODDS RATIO (95% CI) AND *p* VALUE] OF INDIVIDUALS POSSESSING CERTAIN GENOTYPE BETWEEN HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS

		Total		*Male*		*Female*	
Polymorphism	*Genotype*	*OR (95% CI)*	p	*OR (95% CI)*	p	*OR (95% CI)*	p
DC-SIGN							
−139 (rs2287886)	G/G and A/G	1.03 (0.64, 1.66)	0.8838	2.02 (0.84, 4.91)	0.0856	0.68 (0.37, 1.25)	0.1839
−336 (rs4804803)	G/G and A/G	1.04 (0.58, 1.89)	0.8769	1.29 (0.47, 4.17)	0.6012	0.75 (0.34, 1.64)	0.4283
69-bp repeat numbers	7/7	0.33 (0.00, 26.34)	0.4389	Undefined	1.0000	0.00 (0.00, 20.04)	0.3394
DC-SIGNR							
Exon 5 (rs2277998)	A/A and G/G	0.61 (0.37, 1.02)	0.0445	0.95 (0.37, 2.46)	0.9088	0.41 (0.21, 0.81)	0.0049
69-bp repeat numbers	5/5	Undefined	0.2097	Undefined	1.0000	Undefined	0.3022
	6/5	Undefined	0.5760	Undefined	1.0000	—	—
	6/6	—	—	—	—	—	—
	7/5	0.57 (0.32, 1.01)	0.0373	0.95 (0.34, 3.9)	0.9105	0.38 (0.18, 0.82)	0.0061
	7/6	0.73 (0.30, 1.79)	0.4412	0.67 (0.16, 3.97)	0.4699	0.67 (0.19, 2.44)	0.5613
	7/7	1.37 (0.82, 2.28)	0.2022	1.01 (0.40, 2.60)	0.9870	1.79 (0.94, 3.40)	0.0556
	8/5	—	—	—	—	—	—
	8/7	0.16 (0.00, 3.21)	0.1562	0.17 (0.00, 13.73)	0.2750	0.00 (0.00, 20.04)	0.3394
	9/5	0.38 (0.15, 0.98)	0.0232	0.44 (0.10, 2.73)	0.2112	0.27 (0.06, 1.13)	0.0477
	9/6	1.68 (0.19, 80.39)	1.0000	0.69 (0.06, 35.12)	0.5556	Undefined	1.0000
	9/7	1.53 (0.84, 2.78)	0.1369	1.47 (0.50, 5.28)	0.4619	1.65 (0.79, 3.47)	0.1513
	9/9	1.00 (0.24, 5.88)	1.0000	Undefined	1.0000	0.85 (0.16, 5.64)	1.0000
	10/7	Undefined	1.0000	—	—	Undefined	1.0000

As for the *DC-SIGNR* 69-bp repeat number, we found a variation from 5 to10 repeats with 13 genotypes. The most common genotype in our study population was the 7/7 repeat, followed by the 9/7 and 7/5 repeats. We found that HIV-seronegative individuals had a significantly higher frequency of possessing the heterozygous 7/5 or 9/5 repeat than HIV-seropositive individuals ($p = 0.037$ and 0.023, respectively). Interestingly, we did not find any individuals with three or four repeats, which were found occasionally in whites. Instead we found one individual with 10 repeats that had not been reported elsewhere. For the SNP in *DC-SIGNR* Exon 5 (rs2277998), the proportion of individuals possessing the A allele (genotype A/A and A/G) was significantly higher in HIV-seronegative than that in HIV-seropositive individuals ($p = 0.0445$).

We further analyzed these associations after stratifying the study population into male and female groups (Table 3). Interestingly, we found that these associations remained significant in females ($p = 0.0061$ for the 7/5 repeat, $p = 0.0477$ for the 9/5 repeat, and $p = 0.0049$ for the A allele at the SNP rs2277998A) but not in males ($p = 0.9105$ for the 7/5 repeat, $p = 0{,}2112$ for the 9/5 repeat, and $p = 0.9088$ for the A allele at the SNP rs2277998A). Moreover, we found that HIV-seropositive females tend to have the homozygous 7/7 repeat more frequently than HIV-seronegative females with a marginal level of significance ($p = 0.0556$). Again, this association was not found in males.

TABLE 4. DISTRIBUTION OF DC-SIGNR HAPLOTYPE [NO. (%)] AMONG HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS AND BLOOD DONORS

	HIV-seronegative			*HIV-seropositive*			*Blood donor*
DC-SIGNR haplotype[a]	*Male (n = 56)*	*Female (n = 148)*	*Total (n = 204)*	*Male (n = 322)*	*Female (n = 288)*	*Total (n = 610)*	*(n = 580)*
5A	9 (16.07)	28 (18.92)[b]	37 (18.14)	51 (15.84)	30 (10.42)[b]	81 (13.28)	77 (13.28)
6A	0 (0.00)	0 (0.00)	0 (0.00)	3 (0.93)	3 (1.04)	6 (0.98)	1 (0.17)
7A	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.31)	1 (0.35)	1 (0.16)	2 (0.34)
5G	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.62)	1 (0.35)	3 (0.49)	5 (0.86)
6G	4 (7.14)	6 (4.05)	10 (4.90)	17 (5.28)	6 (2.08)	23 (3.77)	26 (4.48)
7G	33 (58.93)	86 (58.11)	119 (58.33)	188 (58.39)	190 (65.97)	379 (62.13)	390 (67.24)
8G	1 (1.79)	1 (0.68)	2 (0.98)	1 (0.31)	1 (0.35)	2 (0.33)	1 (0.17)
9G	9 (16.07)	27 (18.24)	36 (17.65)	59 (18.32)	55 (19.10)	114 (18.69)	78 (13.45)
10G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.35)	1 (0.16)	0 (0.00)

[a]Haplotypes were constructed from repeat number of 69-bp (Exon 4) and A/G allele at rs227799 (Exon 5).
[b]HIV-seronegative female versus HIV-seropositive female; $p = 0.0133$, OR (95% CI) = 0.50 (0.27, 0.90).

We also analyzed frequencies of the *DC-SIGNR* haplotypes constructed from a 69-bp repeat number in Exon 4 and an allele at rs227799 in Exon 5 in relation to HIV infection as summarized in Table 4. We found nine haplotypes in our study population. The 7G was the most common haplotype in all groups. The 5A was the second most common haplotype in the HIV-seronegative group whereas the 9G was the second most common haplotype in the HIV-seropositive and blood donor groups. We found that the proportion of individuals possessing the 5A haplotype was significantly higher in HIV-seronegative females than HIV-seropositive females [$p = 0.0133$, OR (95% CI) = 0.50 (0.27,0.90)].

DISCUSSION

We identified the polymorphisms at the two loci in *DC-SIGNR* that showed a statistically significant association with Thai HIV-seronegative individuals of HIV-seropositive spouses, especially among females. Our data on their marital history and sexual behavior indicated that most of these seronegative females were exposed to HIV repeatedly. There were four HIV-seronegative females reporting condom use at every contact. However, three of the four women had had a child with their HIV-infected index case, indicating that there was a possibility of HIV exposure. Thus we included these women in our analysis. We have also done the analysis excluding this group, but the conclusions remain the same. Therefore we think that *DC-SIGNR* may play an important role in conferring resistance to HIV infection.

The previous study from the United States showed that the heterozygous 7/5 repeat in *DC-SIGNR* was associated with resistance to HIV infection, whereas the homozygous 7/7 repeat was associated with susceptibility to HIV infection.[19] Our study is the first conducted in Asia showing consistent results with the American study, although in our study the significance of the association between the homozygous 7/7 repeat and HIV infection was marginal and was found only in females. In another published study, however, such associations of repeated number with susceptibility to HIV infection were not shown.[20] We think this is because the comparison was made only with the HIV-negative general population but not with ESN. In our study population as well, we did not find a significance difference between HIV-seropositive individuals and HIV-negative blood donors. Gramberg *et al.*[21] investigated the effect of polymorphisms in the *DC-SIGNR* neck domain on the interaction with HIV the envelope protein in *in vitro* experiments, but they found that coexpression of seven repeats with five repeats did not decrease the interaction with HIV compared with seven repeats only. However, there remains a possibility that they did not show the inhibitory effect because their experiments were conducted under high level expression of the gene.

Our study is the first showing associations of the other two polymorphisms that are heterozygous 9/5 repeat and A allele at the SNP site rs2288997 in Exon 5 of *DC-SIGNR* with HIV-seronegative individuals. The A-to-G change at this SNP site is particularly interesting as it causes an aspatate-to-asparagine substitution in the carbohydrate recognition domain (CRD). This amino acid change may affect the binding affinity of CRDs to HIV-1 gp120 and/or ICAM-3. But the relevance of this genetic polymorphism to HIV infection has not yet been investigated in *in vitro* experiments. Since we found a significant linkage between the A allele in Exon 5 and five repeats in Exon 4, the association of the 7/5 and 9/5 repeat with HIV-seronegative individuals may merely be due to a confounding effect by the A allele in Exon 5 and it may be the polymorphism in the CRD coding region that truly affects susceptibility to HIV infection. It is also possible that these two polymorphisms reported here are in linkage disequilibrium with another variant elsewhere in this region that is actually responsible for the observed protective effect.

In our study population, we found that these associations with polymorphisms in *DC-SIGNR* were not observed when only males were included in the analysis. Instead, the stronger associations were observed when only females were analyzed than when males were combined. We have two possible reasons for this difference. First, there might be a different mechanism of HIV infection between female and male, and *DC-SIGNR* plays a role only in female HIV infection. Second, according to our information on marital history, the duration of marriage before the disclosure of HIV status was much longer in females than in males and one-third of HIV-negative males had known the HIV status of their wives before marriage. Therefore, a considerable proportion of HIV-seronegative males was unlikely to have been highly exposed to HIV; thus they may still be susceptible to HIV infection. In fact, our follow-up data of their serostatus showed a three times higher seroconversion rate in male seronegative individuals than female seronegative individuals (data not shown).

We found that the repeat number of *DC-SIGN* in Thais was highly conserved in the homozygous 7/7 repeat and was not associated with susceptibility to HIV infection as showed in the previous study.[22] We did not find any association between polymorphisms in the *DC-SIGN* promoter (−139A/G and −336A/G) and susceptibility to HIV infection in our study group, whose risk for acquiring HIV infection was heterosexual contact. This finding confirmed the previously reports, which showed an association of −336G with risk for parenteral risk, but not mucosal risk for HIV infection.[23]

Although *DC-SIGN* and *DC-SIGNR* are quite similar in amino acid sequences and both have a binding ability to carbohydrate ligands, there are differences in their characteristics, including expression distribution,[16,24] carbohydrate binding profiles,[25–29] alternatively splicing,[17,30] and level of polymorphism in repeat numbers.[13,19,21,29] Thus it is plausible that they may play a different role in HIV infection. *DC-SIGNR* expression at mucosal sites (vaginal and rectal) has been found to have an alternative splicing that produces predicted soluble isoforms of *DC-SIGNR* molecules.[17] This soluble isoform may modulate the efficiency of viral transmission and dissemination.[17] Our experiment in monocyte-derived DCs cultured *in vitro* revealed the expression of *DC-SIGNR* by nested RT-PCR (data not shown). The 375-bp nested PCR product had 100% identity to the *DC-SIGNR* mRNA isoform I [variant 1 (NM_04257) and variant 2 (NM_214675)] and isoform II [variant 3 (NM_214676)]. Further *in vivo* and *in vitro* studies are warranted to investigate the mechanisms of their functions.

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MAJOR ARTICLE

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

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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

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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_{NL4-3} 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 β (TMβ-1) [22] per animal. The IL-4–transgenic and the control BALB/cA-dKO mice do not require TMβ-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_{NL4-3}, or MDR isolates (2000 IU/500 μL/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 $IL-4^+$ 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 $HIV-1_{NL4-3}$. 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 $HIV-1_{NL4-3}$–infected $IL-4^+$ 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 $HIV-1_{NL4-3}$. Cells obtained from the peritoneal lavage fluids were analyzed for cell-surface expression of human CD3 (since HIV-1 downmodulates 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

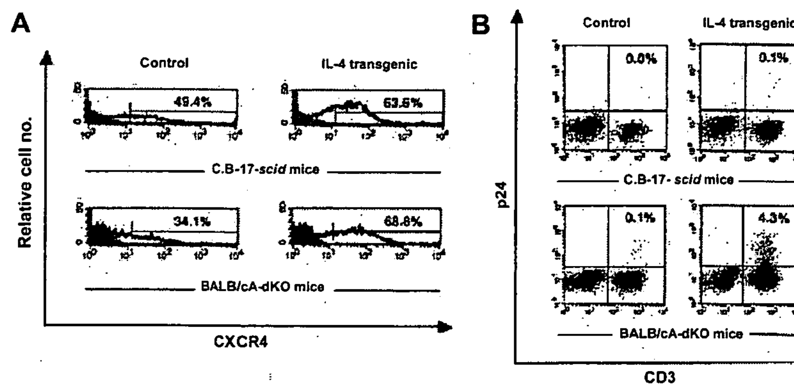

Figure 1. Enhancement of the expression of CXCR4 coreceptor and facilitation of X4 HIV-1 infection and replication in hu-PBL-SCID mice by in vivo production of human interleukin (IL)–4. Groups of hu-PBL-SCID mice, generated from IL-4–transgenic and nontransgenic (control) mice on either the C.B-17-*scid* 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-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.

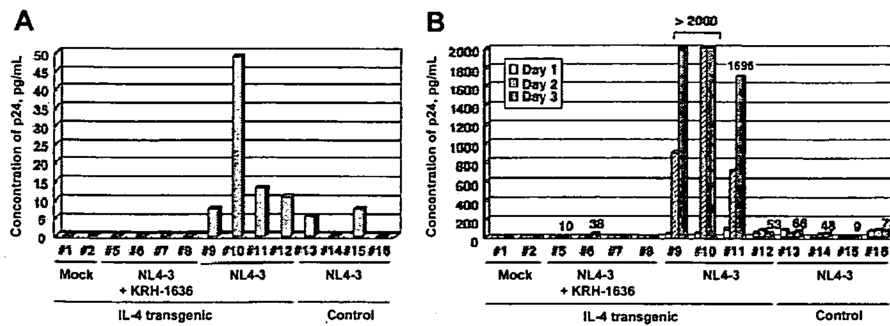

Figure 2. Efficient infection of interleukin (IL)–4–producing hu-PBL-SCID mice with X4 laboratory strain and an inhibitory effect of the CXCR4 antagonist KRH-1636 on infection. Twelve IL-4–transgenic and 4 nontransgenic (control) hu-PBL-SCID mice were generated on the C.B-17-*scid* background. Among 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-μmol/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

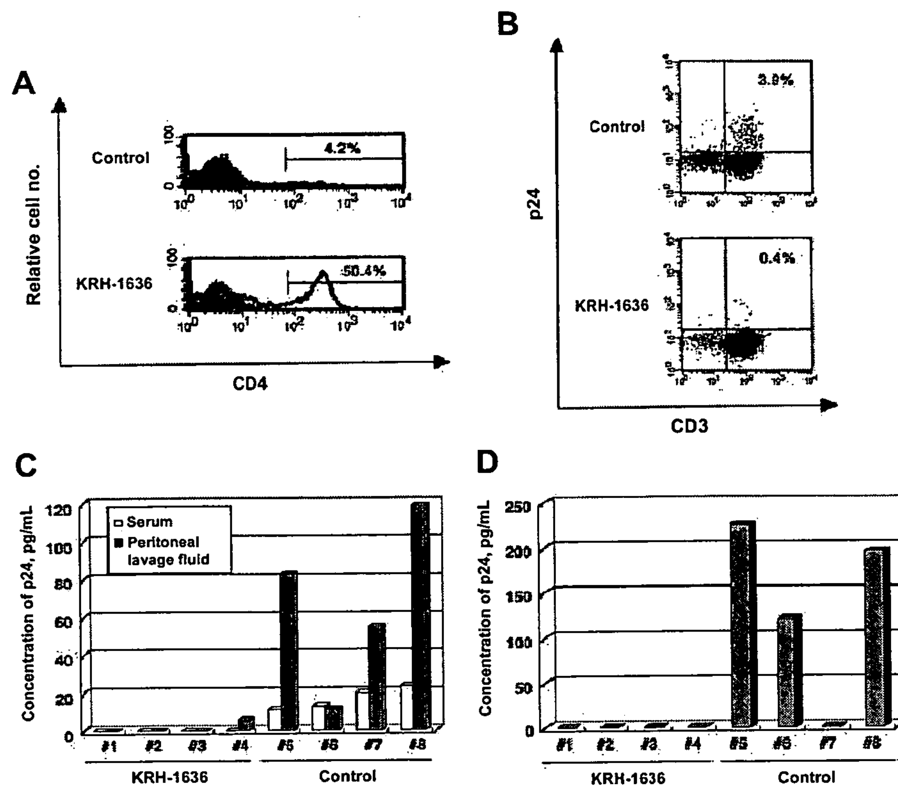

Figure 3. Prophylactic effect of KRH-1636 on infection and pathogenesis by multidrug-resistant (MDR) HIV-1 clinical isolates. Eight interleukin (IL)–4–transgenic hu-PBL-SCID mice (BALB/cA-dKO) were infected intraperitoneally (ip) with a mixture of MDR HIV-1 clinical isolates at 1 day after human peripheral 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 $IL-2^+$ 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 γ_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 γ_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-2Rβ 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.

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