Table 2 **Seroprevalence between high-risk and healthy controls in Mongolia**

	% Positive in each high-risk group					High risk (%)	Healthy controls (%)	OR (95% CI)	*P* value
	FSWs	MSM	Male STI clients	TB patients	Mobile men				
Anti-HIV-1/2	0	0	0	0	0	0	0	–	–
HBs Ag	11.5	18	15.4	16.4	20.7	15.5	14.7	1.1 (0.9–1.3)	0.570
HBs Ab	48.5	42	48.9	50.9	46.7	48.2	44.4	1.2 (1.0–1.4)	0.060
Anti-HCV	6	18	7.5	15.4	7	8	4.4	1.9 (1.3–2.7)	<0.001
Anti-TP	39.5	30	17.2	10.9	14.7	23.1	3.1	9.3 (6.4–13.4)	<0.0001

FSW = female commercial sex worker; MSM = men who have sex with men; TB= tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody; TP = *Treponema pallidum*; HCV = hepatitis C virus

were conducted using the *Stat View* software version 5.0 (SAS Institute, Cary, NC, USA). A P value of <0.05 was considered statistically significant.

RESULTS

The seroprevalences of anti-HIV-1/2, HBs Ab and Ag, anti-HCV and anti-TP of each group of the high-risk and healthy populations are presented in Table 2. None of the anti-HIV-1/2-positive samples was detected in this study. The prevalences of HBs Ag and HBs Ab in the high-risk population, including among each high-risk group, were not different compared with those in the healthy control. In contrast, the prevalences of anti-HCV (8%) and anti-TP (23.1%) in the high-risk population were significantly higher than those in the healthy control. The ORs of anti-HCV and anti-TP comparing between the high-risk population and the healthy control were 1.9 (95% CI: 1.3–2.7, $P < 0.001$) and 9.3 (95% CI: 6.4–13.4, $P < 0.0001$), respectively. The prevalences of anti-HCV in MSM and TB patients were higher than those of other risk groups. The prevalences of anti-TP in FSWs (39.5%) and MSM (30%) were surprisingly high.

Geographical differences of seroprevalence are shown in Table 3. Again, there were no significant differences of the prevalence of HBs Ab and Ag in different regions of specific high-risk groups. However, incidences of anti-HCV and anti-TP had some differences in different regions of the specific groups. A striking feature was that the prevalence of anti-TP in Ulaanbaatar FSWs was 54.7%.

The prevalence of HBs Ab was high. However, there were no differences in the prevalence between high-risk and healthy control populations over the country. One reason was that a hepatitis B virus (HBV) vaccination programme in childhood has been implemented 18 years ago. Therefore, we divided the subjects into two age-related groups (below 18 years and over 20 years) and analysed the seroprevalence of HBs Ab (Figure 2). There were no differences between the high-risk and healthy control populations in both age-related groups. However, in both high-risk and healthy control populations, higher age groups had significantly higher prevalence.

DISCUSSION

Since 1992 when the first case of HIV-1 infection was reported in Mongolia, the number of reported cases remained low until 2005. However, the number has been increasing sharply since 2005, and 36 cases have been reported as of February 2008 (Ministry of Health, Mongolia, unpublished data). By the estimated report of the Global Fund for AIDS, Tuberculoses and Malaria ('Impact of AIDS in Mongolia' 2004), without prevention measures, Mongolian HIV/AIDS prevalence will be doubled every two years and 2500 people will die of AIDS by 2014. Our result supports this estimation. A current prevalence of HIV-1 infection is still low but the risk status of HIV-1 infection must be high because of the very high prevalence of syphilis in FSWs (39.5%), especially in Ulaanbaatar (54.7%). Another report also presented similar prevalence among low-income FSWs in Mongolia (43%).[9] Schwebke *et al.* [7] reported the prevalence rate (8.6%) of syphilis among 137 male STI clients in

Table 3 **Seroprevalence of HBV, HCV and syphilis among a high-risk population by residence**

		% positive for				
	No.	Anti-HIV-1/2	HBs Ag	HBs Ab	Anti-HCV	Anti-TP
Ulaanbaatar						
FSWs	150	0	8.7	48.7	8.7	54.7
MSM	50	0	18	42	18	30
Male STI clients	200	0	10	51.5	5.5	16.5
TB patients	50	0	18	56	12	12
Mobile men	150	0	22	48	5.3	14.7
Subtotal	600	0	14	49.5	7.8	26.3
Darkhan – Uul						
FSWs	200	0	14.5	47	4	31.5
Male STI clients	100	0	28	44	7	26
TP patients	30	0	13.3	46.7	16.7	13.3
Subtotal	330	0	18.5	46	6	28.2
Dornogobi						
FSWs	20	0	5	55	5	30
Male STI clients	45	0	26.7	48.9	13.3	15.6
TB patients	10	0	20	70	10	10
Mobile men	150	0	19.3	45.3	8.7	14.7
Subtotal	225	0	19.6	48	9.3	16
Dornod						
FSWs	10	0	10	40	10	20
Male STI clients	100	0	15	46	5	17
TP patients	10	0	10	40	10	0
Subtotal	120	0	14.2	45	5.8	15.8
Huvsgul						
FSWs	30	0	10	56.7	6.7	30
Male STI clients	100	0	9	51	12	11
TP patients	10	0	20	30	40	10
Subtotal	140	0	10	50.7	12.9	15

HBV= hepatitis B virus; HCV = hepatitis C virus; TP= *Treponema pallidum*; FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody

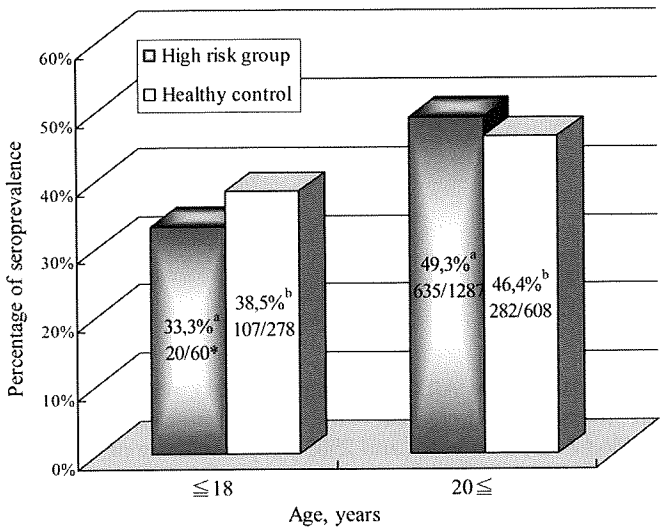

Figure 2 **Age-related seroprevalence of hepatitis B surface antibody (HBs Ab). *N-positive for HBs Ab/N-tested. [a]$P < 0.05$. [b]$P < 0.05$**

Ulaanbaatar in 1998. In our study conducted in 2007, this rate in Ulaanbaatar was 16.5%, suggesting that the prevalence of syphilis is increasing. It is true that these rates were anti-TP. Therefore, it did not mean active syphilis. However, these rates document that the exposure to syphilis is very high. A 100% condom programme is strongly recommended.

According to the unpublished data by NCCD, another risk factor for HIV-1 acquisition is that the predominant route of HIV-1 transmission in Mongolia is through sexual intercourse in MSM. The present study showed higher rates of anti-TP (30%) and anti-HCV (18%) in MSM than those in neighbouring countries: for example, 7% in Beijing (China) and 10% in St Petersburg (Russia) for syphilis and 0.8% or 5.2% in Beijing (China) for HCV.[14-16] These results indicate active high-risk sexual intercourse in Mongolian MSM. There is strong prejudice and discrimination against MSM in Mongolia. Hence, access to the MSM group was very difficult in this study. This barrier makes the delivery of information to MSM difficult. A quick countermeasure to MSM is crucial and a larger serological survey is necessary to grasp the actual prevalence of HIV-1 in Mongolian MSM.

Compared with other STIs, evaluation of hepatitis B was not simple because of the high-prevalence rate in the general population. A hepatitis B vaccination programme has been conducted 18 years ago. Around 35% of people below 18 years have HBs Ab. In contrast, those over 20 years had a significantly higher rate of HBs Ab in both high-risk and healthy control populations. Analysis of HBc-Ab could make it possible to discriminate between HBV-vaccinated and HBV-exposed individuals, which unfortunately we could not perform in this study. This result also suggests the frequent exposure to hepatitis B in Mongolians. Takahashi *et al.*[10] reported a comparable rate of HBs Ab prevalence, indicating a low selection bias of subjects in this study except for MSM and drug abusers.

The present study demonstrates that HIV prevalence is currently low. However, according to the high prevalence of syphilis and HCV in high-risk populations and the social stigma against MSM, the risk status for HIV-1 infection is estimated to be high. Close monitoring of the HIV epidemic is important in order to take quick measures for the high-risk populations and consequently keep the prevalence of HIV low in Mongolia.

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Strong Ability of Nef-Specific CD4$^+$ Cytotoxic T Cells To Suppress Human Immunodeficiency Virus Type 1 (HIV-1) Replication in HIV-1-Infected CD4$^+$ T Cells and Macrophages$^\nabla$

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A restricted number of studies have shown that human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic CD4$^+$ T cells are present in HIV-1-infected individuals. However, the roles of this type of CD4$^+$ T cell in the immune responses against an HIV-1 infection remain unclear. In this study, we identified novel Nef epitope-specific HLA-DRB1*0803-restricted cytotoxic CD4$^+$ T cells. The CD4$^+$ T-cell clones specific for Nef187-203 showed strong gamma interferon production after having been stimulated with autologous B-lymphoblastoid cells infected with recombinant vaccinia virus expressing Nef or pulsed with heat-inactivated virus particles, indicating the presentation of the epitope antigen through both exogenous and endogenous major histocompatibility complex class II processing pathways. Nef187-203-specific CD4$^+$ T-cell clones exhibited strong cytotoxic activity against both HIV-1-infected macrophages and CD4$^+$ T cells from an HLA-DRB1*0803$^+$ donor. In addition, these Nef-specific cytotoxic CD4$^+$ T-cell clones exhibited strong ability to suppress HIV-1 replication in both macrophages and CD4$^+$ T cells in vitro. Nef187-203-specific cytotoxic CD4$^+$ T cells were detected in cultures of peptide-stimulated peripheral blood mononuclear cells (PBMCs) and in ex vivo PBMCs from 40% and 20% of DRB1*0803$^+$ donors, respectively. These results suggest that HIV-1-specific CD4$^+$ T cells may directly control HIV-1 infection in vivo by suppressing virus replication in HIV-1 natural host cells.

Human immunodeficiency virus (HIV)-specific CD8$^+$ cytotoxic T cells (CTLs) play a central role in the control of HIV type 1 (HIV-1) during acute and chronic phases of an HIV-1 infection (5, 29, 34). However, HIV-1 escapes from the immune surveillance of CD8$^+$ CTLs by mechanisms such as mutations of immunodominant CTL epitopes and downregulation of major histocompatibility complex class I (MHC-I) molecules on the infected cells (9, 11, 12, 49). Therefore, most HIV-1-infected patients without highly active antiretroviral therapy (HAART) develop AIDS eventually.

HIV-1-specific CD4$^+$ T cells also play an important role in host immune responses against HIV-1 infections. An inverse association of CD4$^+$ T-cell responses with viral load in chronically HIV-1-infected patients was documented in a series of earlier studies (8, 36, 39, 41, 48), although the causal relationship between them still remains unclear (23). Classically, CD4$^+$ T cells help the expansion of CD8$^+$ CTLs by producing growth factors such as interleukin-2 (IL-2) or by their CD40 ligand interaction with antigen-processing cells and CD8$^+$ CTLs. In addition, CD4$^+$ T cells provide activation of macrophages, which can professionally maintain CD8$^+$ T-cell memory (17). On the other hand, the direct ability of virus-specific cytotoxic CD4$^+$ T cells (CD4$^+$ CTLs) to kill target cells has been widely observed in human virus infections such as those by human cytomegalovirus, Epstein-Barr virus (EBV), hepatitis B virus, Dengue virus, and HIV-1 (2, 4, 10, 19, 30, 31, 38, 50). Furthermore, one study showed that mouse CD4$^+$ T cells specific for lymphocytic choriomeningitis virus have cytotoxic activity in vivo (25). These results, taken together, indicate that a subset of effector CD4$^+$ T cells develops cytolytic activity in response to virus infections.

HIV-1-specific CD4$^+$ CTLs were found to be prevalent in HIV-1 infections, as Gag-specific cytotoxic CD4$^+$ T cells were detected directly ex vivo among peripheral blood mononuclear cells (PBMCs) from an HIV-1-infected long-term nonprogressor (31). Other studies showed that up to 50% of the CD4$^+$ T cells in some HIV-1-infected donors can exhibit a clear cytolytic potential, in contrast to the fact that healthy individuals display few of these cells (3, 4). These studies indicate the real existence of CD4$^+$ CTLs in HIV-1 infections.

The roles of CD4$^+$ CTLs in the control of an HIV-1 infection have not been widely explored. It is known that Gag-specific CD4$^+$ CTLs can suppress HIV-1 replication in a human T-cell leukemia virus type 1-immortalized CD4$^+$ T-cell line (31). However, the functions of CD4$^+$ T cells specific for other HIV-1 antigens remain unclear. On the other hand, the abilities of CD4$^+$ CTLs to suppress HIV-1 replication in infected macrophages and CD4$^+$ T cells may be different, as in the case of CD8$^+$ CTLs for HIV-1-infected macrophages (17). In this study, we identified Nef-specific CD4$^+$ T cells and investigated their ability to kill HIV-1 R5 virus-infected macrophages and HIV-1 X4 virus-infected CD4$^+$ T cells and to suppress HIV-1 replication in the infected macrophages and

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$CD4^+$ T cells. The results obtained in the present study show for the first time the ability of HIV-1-specific $CD4^+$ CTLs to suppress HIV-1 replication in natural host cells, i.e., macrophages and $CD4^+$ T cells.

MATERIALS AND METHODS

Patients. Informed consent was obtained from all subjects, in accordance with the Declaration of Helsinki. Plasma and PBMCs were separated from heparinized whole blood. The patients were sampled at the AIDS Clinical Center, International Medical Center of Japan, and the HLA types of the patients were determined by standard sequence-based genotyping. Patients with active opportunistic infections or psychological disorders and those treated with immunomodulatory agents were excluded.

Synthetic peptides. Peptides (17-mer) derived from the consensus sequence of the Nef protein of HIV-1 clade B were synthesized. These 17-mer peptides overlapped each other by 11 residues. For the feasibility of screening for T-cell epitopes, eight peptides were pooled in a cocktail. Peptides were prepared by using an automated multiple peptide synthesizer. The purity of the synthesized peptides was examined by mass spectrometry, and the peptides with >90% purity were used in the present study.

Cell surface and intracellular cytokine staining. For detection of intracellular cytokines of $CD4^+$ T cells, PBMCs or Nef-specific $CD4^+$ T-cell clones (effector cells) bulk cultured with peptides were stimulated with autologous EBV-transformed B-lymphoblastoid cells (B-LCLs) prepulsed with Nef-derived peptides or peptide cocktails (10^{-6} M) at an effector-to-stimulator (E/S) ratio of 1:4. The pulsed stimulator cells were washed twice in RPMI 1640–10% fetal calf serum (FCS) before use. The mixed cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A (Sigma-Aldrich) was added at a concentration of 10 μg/ml after the first 2 h of incubation to inhibit secretion of cytokines. In order to determine the MHC-II restriction of the $CD4^+$ T-cell epitopes, we also employed peptide-pulsed allogeneic B-LCLs with the HLA-DR allele partially matched or mismatched as stimulators in some assays. After a 6-hour incubation, the cells were stained with phycoerythrin (PE)-conjugated anti-human CD4 monoclonal antibody (MAb) (BD Biosciences, San Jose, CA). Then the cells were fixed, made permeable, stained with fluorescein isothiocyanate (FITC)-conjugated anti-human gamma interferon (IFN-γ) MAb (BD Biosciences, San Jose, CA), and analyzed by flow cytometry as previously described (16).

In order to determine the expression of cytotoxic effector molecules, we directly stained PBMCs or Nef-specific $CD4^+$ T-cell clones with allophycocyanin (APC)-conjugated anti-human CD4 or PE-conjugated anti-human CD4 MAb (BD Biosciences, San Jose, CA) without any stimulation of the cells. Then the cells were fixed, made permeable, stained with FITC-conjugated anti-human perforin, PE-conjugated anti-human granzyme A, or Alexa 647-conjugated anti-human granzyme B MAb (BD Biosciences, San Jose, CA), and analyzed by flow cytometry as previously described (44).

To detect the degranulation of Nef-specific $CD4^+$ T cells following antigen stimulation directly ex vivo, we incubated PBMCs with PE-conjugated anti-human CD107a MAb or PE-conjugated isotype control MAb in RPMI 1640–10% FCS containing the corresponding peptide (10^{-6} M), as previously described by Casazza et al. (10). Negative controls containing the PBMCs from the same individual but without peptides were also prepared. Cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A was added at a concentration of 10 μg/ml after the first 2 h of incubation. Then, the cells were stained with APC-conjugated anti-human CD4 MAb and FITC-conjugated anti-human IFN-γ MAb and analyzed as described above.

Generation of Nef-specific $CD4^+$ T-cell clones. Peptide-specific $CD4^+$ T-cell clones were generated from an established peptide-specific bulk $CD4^+$ T-cell culture by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium supplemented with 10% human serum from healthy donors and 200 U/ml recombinant human IL-2, 5×10^4 irradiated allogeneic PBMCs from a healthy donor as feeders, and 1×10^5 irradiated autologous EBV-transformed B-LCLs prepulsed with a 10^{-6} M concentration of the corresponding peptide). Wells positive for growth after 2 to 3 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for specific IFN-γ-producing ability by intracellular cytokine staining. All $CD4^+$ T-cell clones were cultured in RPMI 1640–10% human serum from healthy donors supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated autologous B-LCLs prepulsed with the appropriate epitope peptide.

Blocking of $CD4^+$ T-cell responses. To determine the MHC-II restriction of Nef-specific $CD4^+$ T-cell responses, we blocked the T-cell receptor–MHC-II interaction by using human MHC-II molecule-specific MAbs L243 (anti-HLA-DR), B7/21 (anti-HLA-DP), and Hu-11 and Hu-18 (anti-HLA-DQ4+5+6 and anti-HLA-DQ7+8+9, respectively), which were kindly donated by Y. Nishimura. Autologous B-LCLs prepulsed with the Nef epitope were incubated with the appropriate antibody (10 μg/ml) for 1 h on ice. Subsequently, the cells were washed in RPMI 1640–10% FCS and then incubated with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:2 for 6 h. Brefeldin A was added to the cultures (10 μg/ml) 4 h prior to termination of the cultures. To evaluate the ability of the effector cells to produce IFN-γ under blocking conditions, we stained the cells after stimulation with PE-conjugated anti-human CD4 MAb. Then the cells were fixed, made permeable, and stained with FITC-conjugated anti-human IFN-γ, as described above.

Intracellular cytokine production (ICC) assays for stimulator cells infected with recombinant vaccinia virus. Autologous B-LCLs were infected with 10 PFU per cell of recombinant vaccinia virus expressing HIV-1 Nef (rVac-Nef) or wild-type vaccinia virus (Vac-WT) and cultured for 16 h at 37°C in 5% CO_2. The infected cells were washed twice with RPMI 1640–10% FCS and then incubated with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:4 for 6 h. Brefeldin A was present in the cultures (10 μg/ml) for the last 4 h. To evaluate the ability of the effector cells to produce IFN-γ, we stained the cells with PE-conjugated anti-human CD4 MAb after stimulation. Then the cells were fixed, made permeable, and stained with FITC-conjugated anti-human IFN-γ, as described above.

ICC assays for stimulator cells pulsed with heat-inactivated HIV-1 particles. The virus particles of HIV-1 NL-432 and its Nef-defective mutant were generated by the HIV-1 clones and were heat inactivated at 56°C for 30 min. Autologous B-LCLs were incubated with the inactivated virus particles at 0.5 μg/ml (p24 antigen concentration) for 16 h at 37°C in 5% CO_2. The pulsed cells were washed twice with RPMI 1640–10% FCS and then incubated with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:4 for 6 h. Brefeldin A was present in the cultures (10 μg/ml) for the last 4 h. To evaluate the ability of effector cells to produce IFN-γ after stimulation, we sequentially stained the cells with PE-conjugated anti-human CD4 MAb, fixed them, made them permeable, and then stained them with FITC-conjugated anti-human IFN-γ MAb, as described above.

ICC assay for stimulator cells transfected with Nef-GFP fusion mRNA. For stimulator cells endogenously expressing Nef-green fluorescent protein (GFP) fusion proteins, m7GpppG-capped and poly(A)-tailed Nef-GFP fusion mRNA or GFP mRNA was delivered to autologous B-LCLs by electroporation, as previously described (46). Briefly, B-LCLs were suspended in a serum-free medium (Opti-MEM; Invitrogen Life Technologies) at the cell density of 2×10^6 cells/ml, mixed with 10 μg of mRNA, and electroporated by using a Gene Pulser device (Bio-Rad). The cells were immediately transferred to RPMI 1640–10% FCS, incubated at 37°C for 1.5 to 3 h, and then mixed with Nef-specific $CD4^+$ T-cell clones (effector cells) at an E/S ratio of 1:4. B-LCLs transfected with GFP mRNA were prepared as negative controls. Flow cytometry revealed that more than 60% of the viable B-LCLs expressed GFP. The cell mixtures were incubated for 6 h, and brefeldin A (10 μg/ml) was present for the last 4 h of the incubation. To evaluate the ability of the effector cells to produce IFN-γ after stimulation, we performed surface and intracellular cytokine staining to the cells, as described above.

Isolation and culture of macrophages and $CD4^+$ T cells. Monocytes and $CD4^+$ T cells were isolated from PBMCs of an HLA-DRB1*0803-positive or HLA-DRB1*0403-positive healthy donor by using anti-human CD14 MAb-coated and anti-human CD4 MAb-coated magnetic beads (magnetically activated cell sorting beads; Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. The isolated monocytes were cultured in complete medium containing macrophage colony-stimulating factor (50 ng/ml) for 1 week before use. The isolated $CD4^+$ T cells were cultured for 1 week in complete medium containing IL-2 (200 U/ml) and IL-4 (2.5 ng/ml) and stimulated with OKT3 anti-CD3 MAb (10 μg/ml) every 3 days during the culture period. These cultured macrophages and $CD4^+$ T cells were infected with HIV-1 as previously described (17, 45).

HIV-1 clones. Infectious proviral clones of an X4 HIV-1, pNL-432, and its Nef-defective mutant, pNL-Xh, which has a frameshift at a XhoI site (44th amino acid of the Nef protein), were kindly donated by Y. Koyanagi (Kyoto University, Kyoto, Japan). The infectious proviral clone of $pJRFL_{NL\text{-}432Nef}$ was previously constructed by exchanging the Nef region of R5 strain JRFL with that of NL-432 (17).

CTL assay. The cytotoxicity of Nef-specific $CD4^+$ T-cell clones against B-LCLs or HIV-1-infected target cells was measured by a standard ^{51}Cr release assay as previously described (17). Briefly, target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well)

were seeded in a 96-well round-bottom microtiter plate (Nunc). For the assays of B-LCLs, the desired amount of the corresponding peptide was coincubated with labeled target cells for 1 h. Then, effector cells were added at various E/T ratios, and the mixtures were incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the cpm in the supernatant in the wells containing only target cells (cpm spn). Maximum release was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was calculated by using the formula (cpm exp − cpm spn)/(cpm max − cpm spn) × 100 (%), where cpm exp is the counts per minute in the supernatant in the wells containing both target and effector cells.

Suppression of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1 Nef-specific CD4$^+$ CTLs to suppress HIV-1 replication was examined as previously described (45). Briefly, macrophages or CD4$^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C in 5% CO_2. After two washes with RPMI 1640–10% FCS, the cells were cocultured with the CD4$^+$ CTL clones. From days 3 to 9 after infection, 10 μl of culture supernatant was collected, and the concentration of p24 antigen was measured by use of an enzyme immunoassay (HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptMetrix, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 Ag in the supernatant of HIV-1-infected cells cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected cells culture without the CTLs) × 100.

RESULTS

Identification and characterization of two HIV-1 Nef-specific CD4$^+$ T-cell epitopes. PBMC from two HIV-1-seropositive individuals, KI-010 and KI-197, were cultured for 14 days after stimulation with either of four peptide cocktails comprising eight 17-mer overlapping Nef peptides. Specific IFN-γ production by each PBMC culture was tested by using intracellular IFN-γ staining after restimulating the cells with autologous EBV-transformed B-LCLs prepulsed with the corresponding peptide cocktail. Cocktail 1 and cocktail 4 induced specific IFN-γ-producing CD4$^+$ T cells among the PBMCs from KI-010 and KI-197, respectively (Fig. 1A). In order to determine which peptide was responsible for the specific CD4$^+$ T-cell responses in the peptide cocktails, we subsequently stimulated the responding PBMC cultures with autologous B-LCLs pulsed with each peptide included in the corresponding peptide cocktails. Nef17-7 and Nef17-8 peptides induced specific CD4$^+$ T-cell responses by the PBMCs cultured from KI-010, whereas Nef17-31 and Nef17-32 peptides induced specific ones by those from KI-197 (Fig. 1B). Considering that the flanking residues also contribute a small part to the overall binding energy of MHC-II-binding peptides, the core binding region is usually not the optimal ligand for MHC-II molecules. Therefore, we used the full-length 17-mer peptides Nef37-53 (Nef17-7) and Nef187-203 (Nef17-32) to generate CD4$^+$ T-cell clones for further studies. The clones specific for Nef37-53 and Nef187-203 epitopes were generated from KI-010 and KI-197, respectively.

In order to determine the HLA class II restriction molecules of these two CD4$^+$ T-cell epitopes, we employed HLA-DR-, -DP-, and -DQ-specific MAbs to block the T-cell receptor-HLA class II interaction between Nef-specific CD4$^+$ T cells and the stimulator cells. HLA-DR-specific MAb L243 blocked the recognition by both Nef37-53- and Nef187-203-specific CD4$^+$ T-cell clones after stimulation with the peptide-pulsed autologous B-LCLs, whereas HLA-DQ-specific MAb Hu11 or Hu18 and HLA-DP-specific MAb B7/21 failed to block it (Fig. 1C). These results indicate that these two epitope-specific T-cell responses were restricted by HLA-DR. To determine the

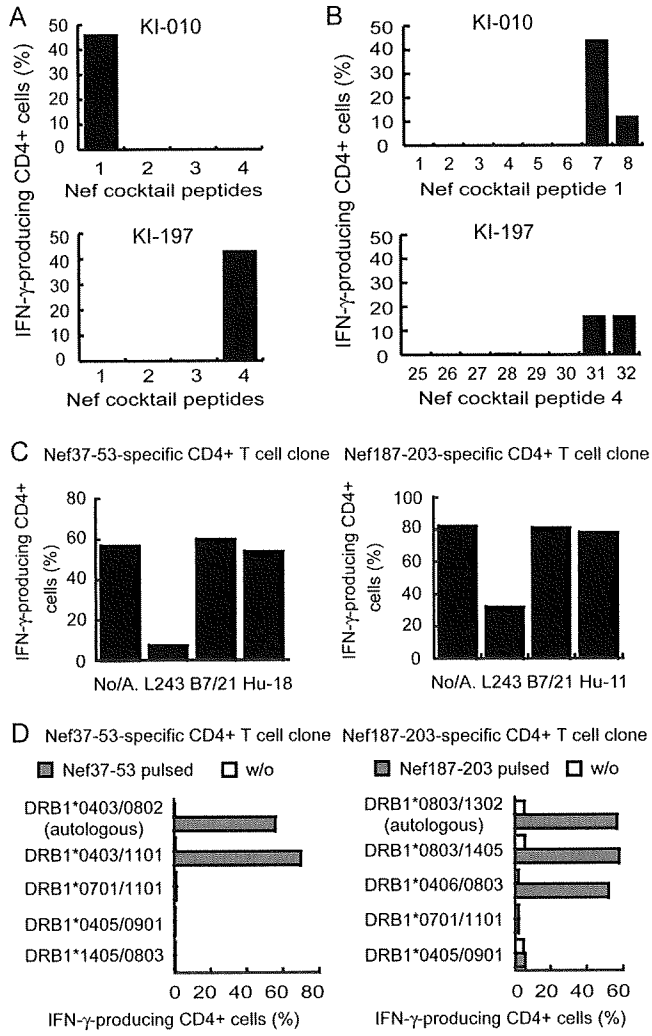

FIG. 1. Identification and characterization of two HIV-1 Nef-specific CD4$^+$ T-cell epitopes. (A) Induction of Nef-specific CD4$^+$ T cells from PBMCs of HIV-1-infected individuals. PBMCs from two HIV-1-seropositive donors (KI-010 and KI-197) were stimulated with cocktails comprising eight 17-mer overlapping Nef peptides and then were cultured for 2 weeks. IFN-γ-producing CD4$^+$ T cells (%) among these bulk-cultured PBMCs were detected by intracellular staining for IFN-γ after restimulation with autologous B-LCLs pulsed with the same cocktails. (B) IFN-γ-producing CD4$^+$ T cells induced by Nef single peptides. The PBMC bulk cultures that responded to the peptide cocktails were subsequently stimulated with B-LCLs pulsed with individual peptides included in those cocktails. IFN-γ-producing CD4$^+$ T cells (%) induced by single peptides were detected by intracellular staining for IFN-γ. (C) IFN-γ responses of Nef37-53-specific and Nef187-203-specific CD4$^+$ T-cell clones to the stimulation with peptide-pulsed B-LCLs were blocked by HLA-DR-specific antibody. Autologous B-LCLs prepulsed with epitope peptides were incubated with MHC-II-specific antibodies (No/A., no antibody; L243, anti-HLA-DR; B7/21, anti-HLA-DP; Hu11 and Hu18, anti-HLA-DQ) for 1 h. Then the two Nef epitope-specific CD4$^+$ T-cell clones were stimulated with the MHC-II-specific antibody-treated B-LCLs at an E/S ratio of 1:2. The percentage of IFN-γ-producing cells in the Nef-specific CD4$^+$ T-cell clones after stimulation was determined by intracellular staining for IFN-γ. (D) IFN-γ responses of Nef37-53-specific and Nef187-203-specific CD4$^+$ T-cell clones after stimulation with peptide-pulsed autologous B-LCL or peptide-pulsed allogeneic B-LCLs with partially matched and mismatched HLA-DR. The percentage of IFN-γ-producing cells among the Nef-specific CD4$^+$ T-cell clones after stimulation was determined by intracellular staining for IFN-γ.

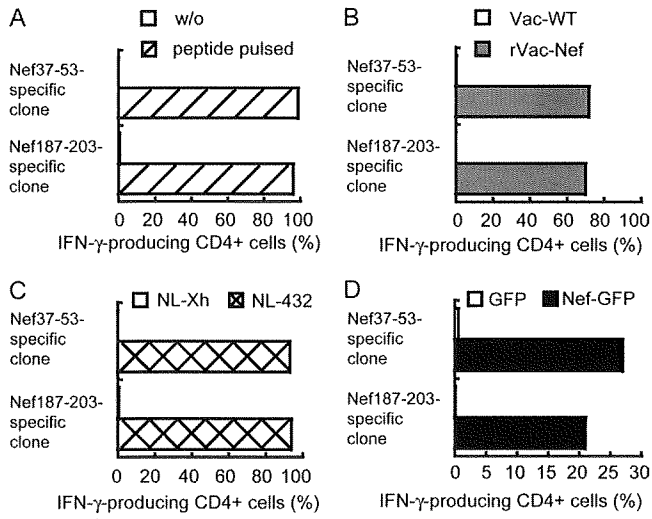

FIG. 2. Naturally occurring presentation of CD4+ T-cell epitopes. Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones were stimulated with peptide-pulsed, recombinant vaccinia virus-infected, HIV-1 particle protein-pulsed, or Nef-GFP fusion mRNA-transfected autologous B-LCLs. The percentage of IFN-γ-producing cells among the Nef-specific CD4+ T-cell clones after stimulation was determined by intracellular staining for IFN-γ. (A) Nef-specific CD4+ T-cell clones were tested for their IFN-γ production after stimulation with B-LCLs prepulsed with appropriate epitope peptides (peptide pulsed) or those without peptides (w/o). (B) Nef-specific CD4+ T-cell clones were tested for IFN-γ production after stimulation with B-LCLs infected with rVac-Nef or Vac-WT. (C) Nef-specific CD4+ T-cell clones were tested for their IFN-γ production after stimulation with B-LCLs prepulsed with heat-inactivated HIV-1 particles of X4 strain NL-432 (NL-432) or those of its Nef-defective mutant, NL-Xh (NL-Xh). (D) Nef-specific CD4+ T-cell clones were tested for their IFN-γ production after stimulation with Nef-GFP fusion mRNA-transfected B-LCLs (Nef-GFP) or GFP mRNA-transfected B-LCLs (GFP). Approximately 60% of the stimulator cells were Nef+ or GFP+ cells.

exact restriction alleles, we stimulated Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones with peptide-prepulsed B-LCLs from allodonors with partially matched or mismatched HLA-DR. The Nef37-53-specific CD4+ T-cell clone produced IFN-γ after stimulation with the corresponding peptide-pulsed B-LCLs from the donors sharing DRB1*0403, while the Nef187-203-specific clone produced IFN-γ after stimulation with the corresponding peptide-pulsed B-LCLs from the donors sharing DRB1*0803 (Fig. 1D). These results strongly suggest that the restriction alleles of CD4+ T-cell epitopes Nef37-53 and Nef187-203 were HLA-DRB1*0403 and HLA-DRB1*0803, respectively.

Naturally occurring presentation of CD4+ T-cell epitopes in rVac-Nef-infected or HIV-1 Nef protein-pulsed cells. To clarify the naturally occurring presentation of these two Nef epitopes, we investigated the ability Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones to produce IFN-γ after stimulation of them with autologous B-LCLs infected with rVac-Nef or those pulsed with heat-inactivated virus particles. The Nef37-53-specific and Nef187-203-specific clones used in this assay showed similar abilities to produce IFN-γ (>95%) after the stimulation with peptide-pulsed autologous B-LCLs (Fig. 2A). The B-LCLs infected with rVac-Nef induced about 70% of the two Nef-specific CD4+ T-cell clones to produce IFN-γ, whereas those cells infected with Vac-WT did not induce any IFN-γ production (Fig. 2B). In addition, the B-LCLs pulsed with NL-432 virus particles induced more than 90% of the CD4+ T cells from the Nef-specific clones to produce IFN-γ, whereas those cells pulsed with NL-Xh (Nef-depleted) virus particles failed to induce IFN-γ production (Fig. 2C). This result suggests that the Nef-specific CD4+ T cells also recognized the epitope antigen presented through endogenous MHC-II processing pathways. However, it still remains possible that Nef proteins from cells expressing Nef killed by vaccinia virus or HIV infection were presented by the exogenous HLA class II pathway. To exclude this possibility, we used stimulator cells transfected with Nef-GFP mRNA. Nef-GFP mRNA-transfected autologous B-LCLs induced IFN-γ production from both Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones, whereas GFP mRNA-transfected cells did not (Fig. 2D). In this assay, B-LCLs were used as stimulator cells within 3 h after the transfection. The frequency of dead cells among the Nef+ cells was approximately 0.6%. These results support the idea that endogenous HIV-1 Nef can be processed to MHC-II molecules in a manner similar to that of the previously observed endogenous presentation of HCMV CD4+ CTL epitopes (20). Thus, our results indicate that the Nef-specific CD4+ T cells recognized the epitope antigen presented through both exogenous and endogenous MHC-II processing pathways.

Cytotoxic activity and cytotoxic effector molecule expression of HIV-1 Nef-specific CD4+ T cells. Although antigen-specific CD4+ T cells are classically thought to function as helper T cells in antiviral immunity, HIV-1 Gag-specific cytotoxic CD4+ T cells were previously reported to exist (30–32, 50). In our study, Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones were tested for their ability to lyse autologous B-LCLs incubated with the epitope peptide (1,000 nM) at an E/T ratio of 5:1 (Fig. 3A). The Nef187-203-specific CD4+ T-cell clone showed a strong lytic activity against autologous B-LCLs incubated with the peptide, whereas the Nef37-53-specific CD4+ T-cell clone did not lyse autologous B-LCLs pulsed with the peptide. Furthermore, we stained for three cytotoxic effector molecules in these Nef-specific CD4+ T-cell clones and found that the expression levels of perforin and granzyme B were much higher in the Nef187-203-specific clone than in the Nef37-53-specific one, whereas the two clones showed similar levels of granzyme A expression (Fig. 3B). Considering that Th clones have been shown to develop cytotoxic activity after long-term culture in vitro (15), we sought to detect the cytotoxic activity of these two Nef epitope-specific CD4+ T cells ex vivo. We employed flow cytometric analysis to measure the cell surface mobilization of CD107a (6, 14), because only a very small number of these epitope-specific CD4+ T cells are suspected to exist among the PBMCs of these patients; thus, these cells would fail to kill the target cells in a chromium release assay. Epitope-specific CD4+ T cells among the PBMCs from two HIV-1-seropositive donors, KI-010 and KI-197, could be detected at very low frequency by revealing their specific IFN-γ responses following peptide stimulation for 6 h (Fig. 3C). We then gated the IFN-γ-producing CD4+ T cells and compared the levels of cell surface expression of CD107 for these two types of CD4+ T cells. The results showed that about 50% of Nef187-203-specific CD4+ T cells expressed CD107a on their

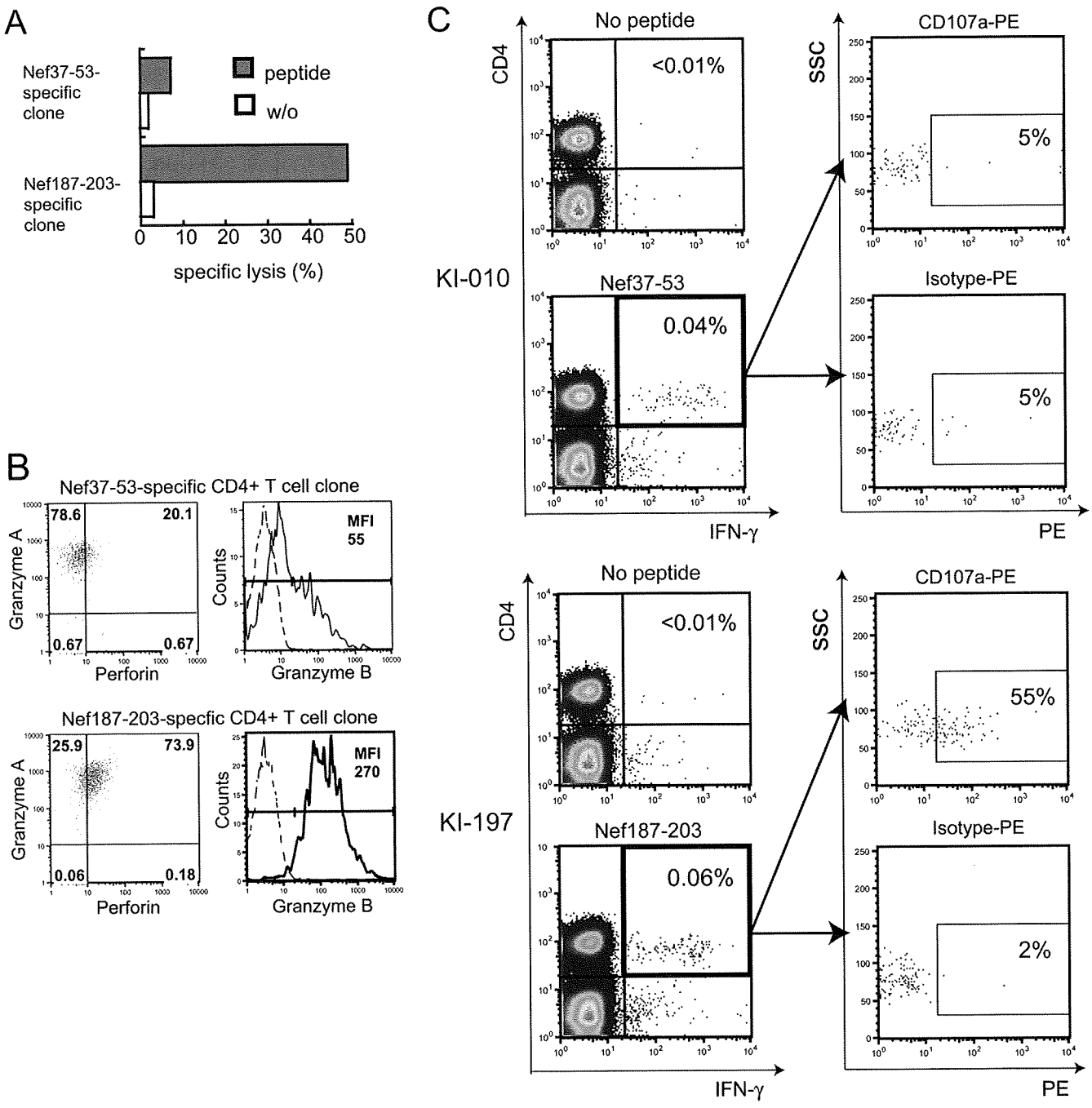

FIG. 3. Cytotoxic activity and cytotoxic effector molecule expression of HIV-1 Nef-specific $CD4^+$ T cells. (A) Cytotoxic activities of a KI-010-derived Nef37-53-specific $CD4^+$ T-cell clone and a KI-197-derived Nef187-203-specific $CD4^+$ T-cell clone against autologous B-LCLs incubated with the epitope peptides (1,000 nM) were measured by a standard ^{51}Cr release assay at an effector-to-target ratio of 5:1. Peptide, with peptide; w/o, without peptide. (B) Surface staining for CD4 and intracellular staining for perforin, granzyme A, and granzyme B were carried out on the Nef37-53-specific and Nef187-203-specific $CD4^+$ T-cell clones. The clones were stained without any stimulation. The stained clones were analyzed by flow cytometry, and the $CD4^+$ cells were gated. The expression levels of perforin and granzyme A are shown in dot plots. Values in dot plots show the frequencies (%) of the subsets among the $CD4^+$ T-cell clones. The expression levels of granzyme B are shown in histograms. Solid lines show the clones stained with anti-human granzyme B MAb; dashed lines show the same clones stained with isotype control antibody. Values in histograms show mean fluorescence intensities (MFI) of the solid lines. (C) Ex vivo analysis of CD107a surface expression on Nef37-53-specific and Nef187-203-specific $CD4^+$ T cells. PBMCs from two HIV-1-seropositive donors, KI-010 and KI-197, were incubated with or without their corresponding epitope peptide for 6 h. Then these PBMCs were stained with anti-CD4, anti-IFN-γ, and anti-CD107a or with mouse immunoglobulin G (IgG) MAb as an isotype control. Values in the IFN-γ/CD4 dot plots indicate the frequencies of IFN-γ-producing $CD4^+$ cells. The $CD4^+$ IFN-γ^+ cells in each PBMC population were gated, and then they were analyzed for the surface expression of CD107a. Values in the PE/side scatter (SSC) dot plots indicate the frequencies of the high-fluorescence subsets in the gated $CD4^+$ IFN-γ^+ population of the PBMCs stained with PE-conjugated anti-CD107a (CD107a-PE) and of the same PBMCs stained with PE-conjugated mouse IgG isotype MAb (Isotype-PE).

cell surfaces, whereas Nef37-53-specific CD4+ T cells did not, thus indicating that Nef187-203-specific CD4+ CTLs, but not Nef37-53-specific CD4+ T cells, have the ability to function as cytotoxic T cells.

Lysis of HIV-1-infected macrophages and CD4+ T cells by Nef187-203-specific cytotoxic CD4+ T cells. To investigate if the Nef-specific CD4+ T cells were able to kill HIV-1-infected target cells, we measured their cytotoxic activity against HIV-1-infected macrophages and CD4+ T cells. To exclude the possibility that different Nef sequences between two HIV-1 strains, NL-432 and JRFL, would affect the recognition of Nef-specific CD4+ CTLs, we used $JRFL_{NL\text{-}432Nef}$, a chimera R5 virus, with the Nef protein derived from the NL-432 strain in this study. Macrophages and CD4+ T cells from an HLA-DRB1*0803-positive healthy donor were infected with HIV-1 R5 strain $JRFL_{NL\text{-}432Nef}$ and X4 strain NL-432, respectively. Intracellular p24 staining of these cells showed that more than 80% of the cultured macrophages and CD4+ T cells were p24 antigen positive at day 3 postinfection, indicating the establishment of an HIV-1 infection in the cultured cells (Fig. 4A). Three Nef187-203-specific CD4+ CTL clones were used in our assays. They exhibited strong specific lysis of autologous B-LCLs incubated with 1,000 nM peptide; this lysis was dramatically decreased when the B-LCLs were incubated with 100 nM peptide (Fig. 4B), thus showing a lower sensitivity of peptide-pulsed target cells to Nef-specific CD4+ CTL clones than that of Nef-specific CD8+ CTL clones reported in our previous studies (18, 46). These Nef-specific CD4+ CTL clones killed both HIV-1-infected macrophages and CD4+ T cells, even at a decreased E/T ratio of 2:1 (Fig. 4C). The specific lysis of infected macrophages was higher than that of the infected CD4+ T cells. This difference may result from the intracellular p24 antigen expression levels of these two targets used in this assay (Fig. 4A).

Ability of HIV-1 Nef-specific cytotoxic CD4+ T cells to suppress HIV-1 replication in macrophages and CD4+ T cells. A previous study showed that Gag-specific CD4+ CTLs can suppress HIV-1 replication in human T-cell leukemia virus type 1-immortalized CD4+ T-cell line MT-2 (31). To clarify if CD4+ CTLs could also efficiently suppress HIV-1 replication in its natural host cells in vivo, we measured the ability of Nef-specific CD4+ CTLs to suppress the replication of HIV-1 in HIV-1-infected macrophages and CD4+ T cells in vitro. Macrophages and CD4+ T cells from an HLA-DRB1*0803-positive healthy donor were isolated, cultured, and then infected with HIV-1 $JRFL_{NL\text{-}432Nef}$ and NL-432 in vitro, respectively. To investigate the suppression ability of CD4+ CTLs by using an enzyme immunoassay, we measured p24 antigens in the supernatant of cultured HIV-1-infected target cells with or without a Nef187-203-specific CD4+ CTL clone at an E/T ratio of 0.1:1 (Fig. 5A). Two Nef187-203-specific clones revealed a strong ability to suppress HIV-1 replication in both HIV-1-infected macrophages and CD4+ T cells. The suppression ability of these T cell clones was E/T ratio dependent for both HIV-1-infected macrophages and CD4+ T cells (Fig. 5B), whereas the addition of an HLA class II-mismatched Nef37-53-specific CD4+ T-cell clone to HIV-1-infected macrophages or CD4+ T cells did not cause any suppression of p24 production (data not shown). Complete suppression of p24 production in both HIV-1-infected macrophages and CD4+ T cells

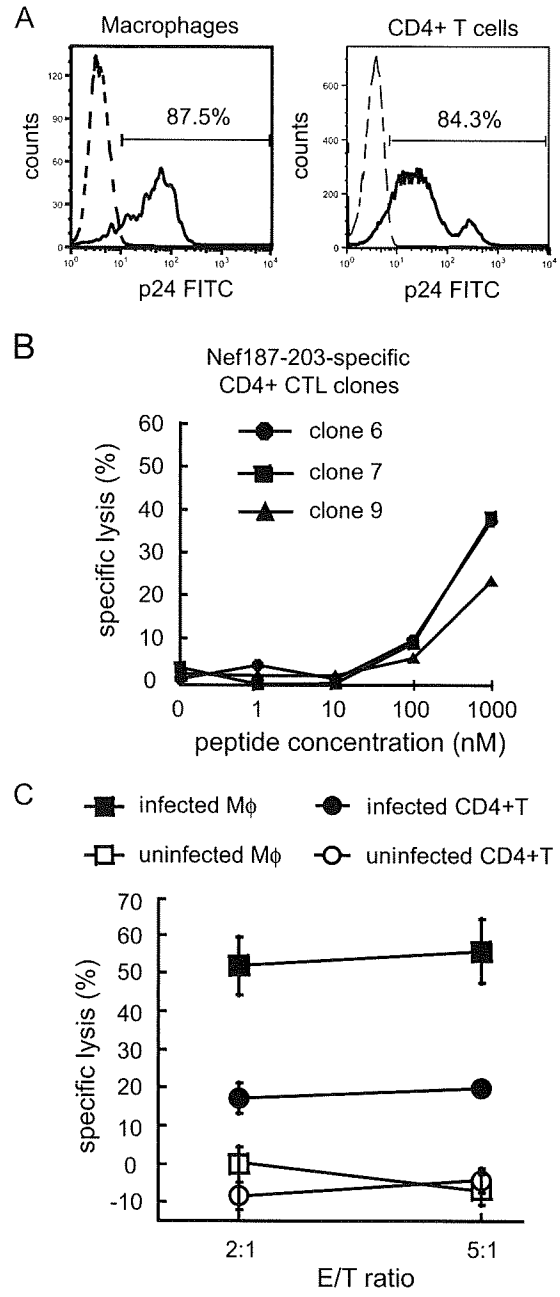

FIG. 4. Lysis of HIV-1-infected macrophages and CD4+ T cells by Nef187-203-specific cytotoxic CD4+ T cells. (A) Intracellular p24 antigen expression of macrophages and CD4+ T cells from an HLA-DRB1*0803-positive donor at day 3 postinfection. The dashed histogram represents uninfected cells, and the solid histogram represents HIV-1-infected cells. The values in each plot show the frequencies of p24 antigen-positive cells. The uninfected and HIV-1-infected macrophages and CD4+ T cells were then labeled with $Na_2{}^{51}CrO_4$ and incubated with Nef187-203-specific clones for CTL assays. (B) Cytotoxic activity of three Nef187-203-specific clones against autologous B-LCLs incubated with the peptide at the indicated concentrations. The cells were tested at an effector-to-target (E/T) ratio of 5:1. (C) Ability of Nef187-203-specific clones to lyse HIV-1-infected or uninfected macrophages (Mϕ) and CD4+ T cells. The cells were tested at the indicated E/T ratios by using the standard ^{51}Cr assay. Values represent averages ± standard deviations (error bars) of results from the assays of the three Nef187-203-specific clones.

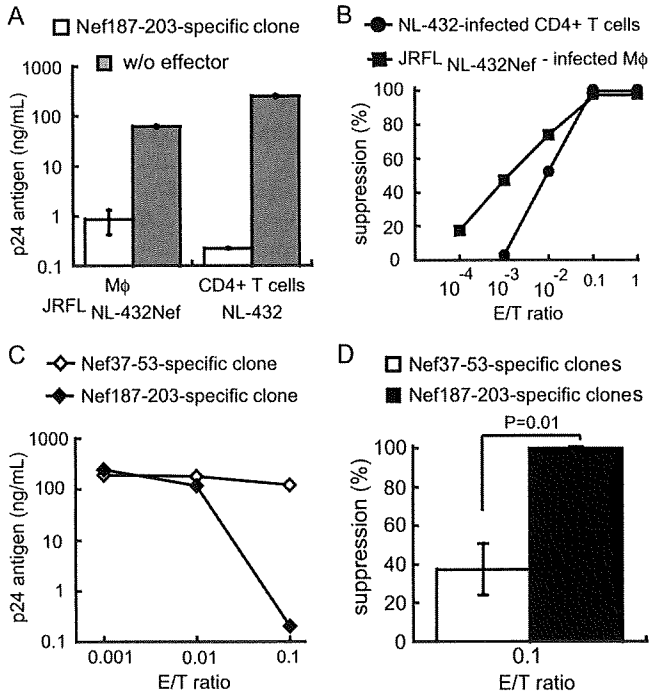

FIG. 5. Ability of HIV-1 cytotoxic CD4$^+$ T cells to suppress HIV-1 replication in vitro. (A) Ability of Nef187-203-specific CD4$^+$ CTL clones to suppress $JRFL_{NL\text{-}432Nef}$ virus and NL-432 virus replication in macrophages and CD4$^+$ T cells, respectively. Macrophages and CD4$^+$ T cells from an HLA-DR-compatible healthy donor were infected with HIV-1 and subsequently cocultured or not with Nef-187-203-specific CD4$^+$ CTL clones at an effector-to-target (E/T) ratio of 0.1:1. The concentration of p24 antigen in the supernatant on day 6 postinfection was measured by using an enzyme immunoassay. Values are presented as the averages ± standard deviations of results from the assays of two Nef187-203-specific clones. (B) The ability of Nef187-203-specific CD4$^+$ CTL clone to suppress HIV-1 infection in target cells was E/T ratio dependent. $JRFL_{NL\text{-}432Nef}$-infected macrophages or NL-432-infected CD4$^+$ T cells were subsequently cocultured with a Nef187-203-specific clone at the indicated E/T ratios. The concentration of p24 antigen in the supernatant on day 6 postinfection was measured as described above. (C) Ability of a Nef37-53-specific CD4$^+$ T-cell clone with no CTL activity to suppress HIV-1 replication in HIV-1-infected CD4$^+$ T cells. CD4$^+$ T cells from two healthy donors expressing the corresponding HLA-DR alleles were infected with HIV-1 and were subsequently cocultured with a Nef37-53-specific or Nef187-203-specific clone at the indicated E/T ratios. The concentration of p24 antigen in the supernatant on day 6 postinfection was measured as described above. (D) The ability of Nef37-53-specific CD4$^+$ T-cell clones to suppress HIV-1 replication in HIV-1 infected CD4$^+$ T cells was less than that of Nef187-203-specific CD4$^+$ CTL clones. Values are presented as averages ± standard deviations (error bars) of results from the assays of three Nef37-53-specific or Nef187-203-specific clones. Statistical differences were determined with Student's t test, and the double-sided P value is shown.

was detected at a low E/T ratio of 0.1:1, indicating that these Nef-specific CD4$^+$ CTLs had a very strong ability to suppress HIV-1 replication. To investigate if this strong suppressor ability could be attributed to the cytolytic activity of CD4$^+$ T cells, we compared the suppressor ability of Nef37-53-specific CD4$^+$ T cells, which did not show significant CTL activity, with that of the Nef187-203-specific CTL clones. A Nef37-53-specific clone with no CTL activity revealed weak suppression activity at an E/T ratio of 0.1:1 against the HIV-1-infected CD4$^+$ T cells from an HLA-compatible healthy donor (Fig. 5C), with this ability being significantly lower than that of the Nef187-203-specific CD4$^+$ CTL clone (Fig. 5D). This result indicates that the Nef-specific cytotoxic CD4$^+$ T cells have strong ability to suppress HIV-1 replication and that noncytotoxic Nef-specific CD4$^+$ T cells may have weak ability to suppress HIV replication via cytokines or by some other mechanism(s).

Detection of Nef187-203-specific CD4$^+$ T cells in chronically HIV-1-infected individuals. To investigate if CD4$^+$ T cells specific for Nef187-203 could be frequently found in HLA-DRB1*0803-positive HIV-1-infected individuals, we expanded our investigation to include nine more chronically HIV-1-infected patients carrying the HLA-DRB1*0803 allele. PBMCs from these patients and KI-197 were stimulated with Nef187-203 peptide and cultured for 2 weeks to expand the population of epitope-specific CD4$^+$ T cells. IFN-γ-producing cells were determined by intracellular staining after restimulation of the bulk cultures with HLA-DRB1*0803-positive B-LCLs prepulsed with the peptide. We observed Nef187-203-specific CD4$^+$ T cells in the bulk cultures from three of these nine donors, i.e., KI-105, KI-121, and KI-154. Taken together, our data indicate that Nef187-203-specific CD4$^+$ T cells were detected among cultured PBMCs from 4 of 10 HLA-DRB1*0803-positive HIV-1-infected individuals (Table 1).

Among the PBMCs from donors KI-154 and KI-197, who showed strong CD4 responses tested by the assay using in vitro-cultured PBMCs, we also detected Nef187-203-specific CD4$^+$ T cells directly ex vivo (Table 1). Furthermore, more than 50% of the Nef187-203-specific CD4$^+$ T cells from both KI-154 and KI-197 mobilized CD107a after stimulation with Nef187-203 peptide (Table 1), demonstrating the existence of cytotoxicity-associated degranulation of Nef187-203-specific CD4$^+$ T cells in these two HIV-1-infected patients.

DISCUSSION

Previous studies showed that Gag and Nef are immunodominant proteins of HIV-1-specific CD4$^+$ T-cell responses in patients at various stages of an HIV-1 infection. Such studies also

TABLE 1. Detection of Nef187-203-specific CD4$^+$ T cells in chronically HIV-1-infected individuals

Subject	HAART	CD4 count (cells/ml)	Viral load (RNA copies/ml)	Frequency (%) of:		
				CD4$^+$ IFN-γ^{+a} cells in:		CD4$^+$ IFN-γ$^+$ CD107a$^+$ cells in ex vivo PBMCs^b
				Cultured PBMCs	Ex vivo PBMCs	
KI-097	+	322	14,000	0	NT^c	NT
KI-105	+	485	<50	2.2	0	0
KI-121	−	265	24,000	18.4	0	0
KI-139	+	505	110,000	0	NT	NT
KI-144	+	496	17,000	0	NT	NT
KI-152	+	303	<50	0	NT	NT
KI-154	+	481	7,700	70.3	0.01	70.0
KI-163	+	419	26,000	0	NT	NT
KI-185	+	331	<50	0	NT	NT
KI-197	+	350	<50	60.7	0.06	55.0

[a] IFN-γ$^+$, IFN-γ-producing.
[b] Frequency among Nef187-203-specific CD4$^+$ IFN-γ$^+$ cells.
[c] NT, not tested.

revealed that only a limited number of peptides may induce CD4 T-cell responses in a genetically diverse population (1, 27). In the present study, we found two Nef CD4+ T-cell epitopes, Nef37-53 and Nef187-203, from two HIV-1-seropositive donors. A previous study showed that a group of subjects with CD4 T-cell responses targeted the peptide Nef187-203; however, the MHC-II restriction of it was not reported (27). Here we characterized both Nef epitopes as HLA-DR restricted in our subjects. Classically, HLA class II-restricted epitopes are processed through the exogenous pathway. However, for CD4+ T-cell recognition of virus-infected cells, the endogenous pathway for HLA class II presentation was also identified in some virus infections (20, 33, 35). In our present study, Nef-specific CD4+ T-cell clones recognized the epitope presented in recombinant vaccinia virus-infected or Nef-GFP fusion mRNA-transfected B-LCLs through the endogenous pathway as well as through the classical exogenous pathway in the antigen protein-pulsed B-LCLs. Furthermore, Nef187-203-specific CD4+ CTLs recognized HIV-1-infected macrophages and CD4+ T cells, suggesting that these HIV-1 host cells could present Nef protein to MHC-II molecules through the endogenous pathway during an HIV-1 infection. Thus, we demonstrated for the first time both endogenous and exogenous presentation of an HIV-1 CD4 epitope by HLA class II molecules.

Since previous studies showed that Gag-specific CD4+ T cells exhibit cytotoxic activity (4, 30, 31), here also we investigated if the same mechanism exists for another immunodominant HIV-1 antigen, Nef. Strong cytotoxic activity was found in the Nef187-203-specific clones in our present study. Compared with the noncytotoxic Nef37-53-specific clone, the cytotoxic Nef187-203-specific clone showed higher perforin and granzyme B expression levels. Although Th clones can acquire cytotoxic behavior during in vitro culture (15), ex vivo studies have directly indicated the persistence of HIV-1-specific cytotoxic CD4+ T cells (31). In addition, a significantly higher perforin expression in a CD4+ subset of PBMCs from HIV-1-infected patients was also observed earlier, suggesting a high prevalence of cytotoxic CD4+ T cells during an HIV-1 infection (4). In our present study, it is unlikely that the observed Nef-specific cytolysis was an artifact of prolonged culture, because ex vivo analysis showed that Nef187-203-specific CD4+ T cells from two donors mobilized CD107a after stimulation with Nef187-203 peptide. Our observations on the cytotoxic effector molecule expression of Nef-specific CD4+ CTL clones suggest that these CTLs kill their target cells by a perforin-dependent pathway, just as in the case of the Gag-specific CD4+ CTLs reported previously (31). The perforin expression in HIV-1-specific CD4+ T cells may be controlled by the CD8 responses during an infection, producing cross-regulation between HIV-1-specific CD4+ and CD8+ T-cell responses (47).

Although Gag-specific CD4+ CTLs were demonstrated to be able to suppress HIV-1 replication in a CD4+ T-cell line, MT-2 (31), the ability of HIV-1-specific CD4+ CTLs to kill infected natural target cells and to suppress HIV-1 replication in these cells has not been explored. CD4+ T cells under normal conditions do not express any HLA class II molecules. Naturally, HIV-1 can replicate only in activated CD4+ T cells, which express MHC-II and are susceptible to CD4+ CTL killing (22). However, the question as to whether the levels of HLA class II expression on HIV-1-infected activated T cells are high enough for efficient recognition by CD4+ CTLs remains unresolved. In addition, previous studies revealed differential susceptibility to CD8+ CTL killing between HIV-1-infected macrophages and CD4+ T cells, showing the complexity of CTL killing of natural target cells during an HIV-1 infection (12, 17, 40). Here we demonstrated higher specific lysis of infected macrophages by Nef-specific CD4+ CTLs than of infected CD4+ T cells by these cells. This result implies that HIV-1-infected macrophages can present virus antigen to HLA class II molecules more effectively than HIV-1-infected CD4+ T cells. On the other hand, naturally higher HLA class II expression on macrophages may also contribute to more-efficient killing of them by CD4+ CTLs. We observed significant HLA class II downregulation on HIV-1-infected CD4+ T cells but not on the infected macrophages (data not shown), in line with a previous report indicating that HIV-1 proteins impair HLA class II expression on infected CD4+ T cells (26). These findings, taken together, may explain why Nef-specific CD4+ CTLs killed HIV-1-infected macrophages more efficiently than HIV-1-infected CD4+ T cells.

Although a difference between cytotoxic activity against HIV-1-infected macrophages and that against CD4+ T cells was observed, Nef-specific CD4+ CTL clones exhibited complete suppression of HIV-1 replication in both kinds of host cells, even at an initial E/T ratio of 0.1 in the assay. The Nef187-203-specific CD4+ CTL clones exhibited a more than 10-fold-stronger ability to suppress HIV-1 replication in macrophages or CD4+ T cells than Nef- or Gag-specific CD8+ CTL clones investigated in our previous studies (17, 18), which employed the same assays, suggesting that Nef187-203-specific CD4+ T cells may be capable of suppressing HIV-1 replication in vivo. In principle, HIV-1-specific T-cell clones can suppress virus replication in two ways: by suppressing cytotoxic activity and cytokine production. A recent study showed that in vitro-cultured noncytotoxic CD4+ T cells produced CCR5 chemokines to suppress HIV-1 replication in those cells themselves (28). In our study, the high level of Mip-1β production by Nef187-203-specific CD4+ CTL clones (data not shown) might also have partly contributed to the suppression of virus replication.

Classically, virus-specific CD4+ T cells play a key role in the maintenance of CD8+ CTL memory (24, 42). In the present study, we sought to demonstrate roles of Nef-specific CD4+ CTLs beyond such helper functions. Notably, we found the suppression of HIV-1 replication in host macrophages and CD4+ T cells by Nef-specific CD4+ CTL clones. Previous investigations showed macrophages to be major reservoirs for HIV-1 in an early infection and in patients with an undetectable viral load on HAART (13). Furthermore, HIV-1-infected macrophages mediate infection of nonlymphoid tissues such as lung or brain (43). Therefore, the strong ability of Nef-specific CD4+ CTLs to suppress HIV-1 replication in macrophages might help to control HIV-1 rebound in structured treatment interruption patients and to relieve the neuropathology associated with AIDS. In addition, Nef-specific CD4+ CTLs may target HIV-infected host cells that resist CD8+ CTL recognition due to an impaired HLA class I antigen-processing pathway. Studies on EBV-specific CD4+ CTLs indicated that they killed EBV-positive Burkitt's lymphoma cells, which are resistant to CD8+ CTL killing, through impaired MHC-I antigen

presentation (2, 37). Thus, particularly in the tissues that can express HLA class II molecules, such as dendritic cells, macrophages, and activated T cells, HIV-1-specific $CD4^+$ CTLs may take the position left vacant due to escape from $CD8^+$ CTL surveillance. However, $CD4^+$ CTLs can also target the antigen-presenting cells and bystander $CD4^+$ T cells, which present epitope peptides through the exogenous pathway. As mentioned by Norris et al. (31), this effect may result in depletion of healthy immune cells during an HIV infection. These results, taken together, indicate that the influence of $CD4^+$ CTLs in vivo on the disease development of AIDS requires more consideration. The frequency of HLA-DR0803-positive patients that responded to the Nef187-203 epitope assessed in our study was 40%, although this value probably was underestimated because previous reports showed that some patients might lose CD4 responses specific for HIV-1 antigens due to vigorous HIV-1 reproduction (7). Exact assessment of the frequency of HIV-1-specific $CD4^+$ T cells would require the use of more-sensitive and cytokine/cytotoxicity response-independent techniques, such as those involving MHC-II tetramers (21).

Overall, our results demonstrated that Nef-specific cytotoxic $CD4^+$ T cells killed HIV-1-infected $CD4^+$ T cells and macrophages in a perforin-mediated manner and that the cytotoxic $CD4^+$ T cells exhibited strong ability to suppress HIV-1 replication in the natural host cells. In addition, our ex vivo analysis revealed that these cytotoxic $CD4^+$ T cells could be detected in 20% of the chronically HIV-infected patients tested. These results, taken together, suggest the importance of Nef-specific $CD4^+$ T cells in the control of HIV-1 infections in vivo.

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Outbreak of *Pneumocystis jiroveci* Pneumonia in Renal Transplant Recipients: *P. jiroveci* Is Contagious to the Susceptible Host

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Background. Prophylaxis against *Pneumocystis jiroveci* pneumonia (PCP) is only recommended during some periods after renal transplantation. Recent advances in immunosuppressive therapy have considerably reduced acute rejection. However, the reported PCP outbreaks are increasing in renal transplant recipients.

Methods. Only three sporadic PCP cases had occurred since 1976 in our Renal Transplant Unit until the index case in July 2004. A PCP outbreak of 27 cases occurred mainly in the outpatient clinic within 1 year, followed by six additional cases during the next 3 years. Molecular analysis of *P. jiroveci* and surveys of reservoir were performed.

Results. Molecular analysis documented that all cases were caused by the same strain. Among 27 cases of the outbreak, human-to-human transmissions were traceable in 22 cases based on dates of outpatient clinic visits and in four cases during hospitalization. Based on the confirmed cases, airborne transmission was suspected with an estimated median PCP incubation period of 53 days (range 7–188 days). Surveys for reservoir of *P. jiroveci* identified asymptomatic carriers and environmental contamination. Some sporadic cases might be caused by reservoirs. Among the 33 cases, none had received PCP prophylaxis, 22 cases had PCP over 12 months, and six cases over 10 years after renal transplantation.

Conclusion. On documentation of a PCP case, we recommend PCP prophylaxis for a maximum period of 6 months (upper limit of incubation period) in all renal transplant recipients including those on regular maintenance immunosuppressive therapy.

Keywords: *Pneumocystis jiroveci* pneumonia, Renal transplant recipients, Outbreak, Prophylaxis, Immunosuppressive therapy.

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Pneumocystis jiroveci is an opportunistic pathogen that causes fatal pneumonia in immunocompromised persons such as organ transplant recipients, patients on immunosuppressants, and HIV-infected individuals. The lack of a culture method for *P. jiroveci* makes it difficult to prove the mode of spread and source of infection. The transmission modes include direct transmission from the infected individuals and the environment. For example, cancer patients treated at hospitals admitting AIDS patients were reported to have a higher incidence of *P. jiroveci* pneumonia (PCP) (*1*), and the increased incidence of PCP in renal transplant units is considered to be due to admission of AIDS patients (*2*). In addition, there is strong evidence for person-to-person transmission of *P. jiroveci* as confirmed by molecular typing (*3*). Rabodonirina et al. (*3*) identified PCP in 10 renal transplant recipients (RTRs) and using single-strand conformation polymorphism molecular typing confirmed that it was transmitted from a known AIDS patient with PCP. Single-strand conformation polymorphism was also used to confirm interhuman PCP infection in a pediatric transplant unit (*4*). Furthermore, *P. jiroveci* DNA was detected in air samples, suggesting environmental risk to susceptible persons (*5*). Based on these evidences, PCP must occur by de novo infection from exogenous sources.

Mycophenolate mofetil (MMF) was introduced in Japan in 2000, and its use has markedly reduced the incidence of graft rejection. Currently, the combination of MMF, a calcineurin inhibitor (CNI), and prednisolone is the main immunosuppressive therapy used in the maintenance phase of postrenal transplantation. In contrast, PCP prophylaxis has been abandoned because of the nephrotoxicity of trimethoprim-sulfamethoxazole (TMP-SMX), especially during the maintenance phase. The European Renal Trans-

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plant Guidelines recommends PCP prophylaxis for at least 4 months after the transplantation, (*6*) whereas another report recommended the use of PCP prophylaxis for 6 to 12 months (*7*). However, in this decade, large outbreaks of PCP after an index case have been reported in RTRs in 2004 in France, 2005 and 2008 in Germany, and 2007 in The Netherlands (*3, 4, 7, 8, 9*). Here, we report another large outbreak of PCP in 33 RTRs in whom human-to-human transmission was suggested in 26 cases.

MATERIALS AND METHODS

Diagnosis of PCP and Transmission Map

An outbreak of PCP in 27 cases occurred from July 2004 through April 2005, and six additional cases were diagnosed during the next 3 years in the Department of Transplant and Endocrine Surgery, Nagoya Daini Red Cross Hospital. The diagnosis of PCP was based on clinical symptoms such as fever and dyspnea, interstitial shadow on the chest X-ray, and detection of *P. jiroveci* by Grocott stain of bronchoalveolar lavage fluid (BALF) samples. The demographics of all 33 patients are listed in Table 1. All dates of outpatient clinic visits and hospitalization were confirmed by the medical charts for the transmission map. This hospital has 812 beds distributed in three buildings. The outpatient clinic for renal transplant patients is located in the second floor of building 1, and the renal transplant patients are hospitalized in the third floor of building 3. Therefore, the outpatients have no direct contact with inpatients.

Immunosuppressive Therapy

All patients except one received MMF combined with prednisolone, and three patients did not receive CNI. Thus, a

TABLE 1. Patient demographics in this study

Case number	Sex	Age (yr)	Date of PCP	Postrenal transplantation (mo)	Incubation period (d)	Immunosuppressive therapy	Contact with active PCP
R1	M	61	July 14, 2004	2	—	PSL+CNI+MMF	—
R2	M	59	August 30, 2004	2	49	PSL+MMF	Yes
R3	M	64	October 3, 2004	92	93	PSL+CNI+MMF	Yes
R4	F	42	October 12, 2004	14	102	PSL+CNI+MMF	Yes
R5	F	56	November 22, 2004	4	7	PSL+CNI+MMF	Yes
R6	M	20	November 22, 2004	13	143	PSL+CNI+MMF	Yes
R7	M	24	November 27, 2004	15	58	PSL+CNI+MMF	Yes
R8	M	50	November 29, 2004	3	91	PSL+CNI+MMF	Yes
R9	M	39	November 30, 2004	41	26	PSL+CNI+MMF	Yes
R10	F	44	December 6, 2004	3	21	PSL+CNI+MMF	Yes
R11	F	41	December 10, 2004	3	15	PSL+CNI+MMF	Yes
R12	F	27	December 13, 2004	45	126	PSL+CNI+MMF	Yes
R13	M	39	December 17, 2004	3	22	PSL+CNI+MMF	Yes
R14	M	47	December 24, 2004	87	39	PSL+CNI+MMF	Yes
R15	M	51	December 25, 2004	47	40	PSL+CNI+MMF	Yes
R16	F	21	January 4, 2005	2	63	PSL+CNI+MMF	Yes
R17	M	55	January 6, 2005	118	45	PSL+CNI+MMF	Yes
R18	M	29	January 7, 2005	3	87	PSL+CNI+MMF	Yes
R19	M	48	January 7, 2005	135	50	PSL+CNI+MMF	Yes
R20	M	50	January 13, 2005	173	101	PSL+CNI+MMF	Yes
R21	M	24	February 5, 2005	34	89	PSL+CNI+MMF	Yes
R22	M	57	February 5, 2005	135	40	PSL+CNI+MMF	Yes
R23	M	62	February 21, 2005	11	56	PSL+CNI+FTY	Yes
R24	F	38	February 22, 2005	93	22	PSL+MMF	Yes
R25	M	46	March 2, 2005	48	13	PSL+CNI+MMF	Yes
R26	F	39	May 11, 2005	106	83	PSL+MMF	Yes
R27	F	21	May 13, 2005	38	Unknown	PSL+CNI+MMF	No
R28	F	43	November 17, 2005	176	188	PSL+CNI+MMF	Yes
R29	M	56	March 24, 2006	2	Unknown	PSL+CNI+MMF	No
R30	M	55	June 5, 2006	235	42	PSL+CNI+MMF	Yes
R31	F	35	November 2, 2006	139	Unknown	PSL+CNI+MMF	No
R32	F	34	January 26, 2008	115	Unknown	PSL+CNI+MMF	No
R33	F	26	January 31, 2008	73	128	PSL+CNI+MMF	Yes

PCP, *Pneumocystis jiroveci* pneumonia; PSL, prednisolone; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; FTY, sphingosine 1–phosphate receptor agonist.

combination immunosuppressive therapy with MMF, CNI, and prednisolone was used in 29 of 33 patients (Table 1).

Biological Specimens

Thirteen BALF samples and one sputum sample obtained from RTRs with PCP had been stored at −20°C since January 2005 (from cases 16–33; Table 1). In January 2005, one BALF sample was obtained from a patient with malignant lymphoma and PCP hospitalized in another ward of the same hospital. In addition to these clinical samples, environmental and mouthwash surveys were conducted to determine the reservoir of *P. jiroveci*. Environmental surveys were conducted three times: in June 2005, May 2006, and the last one was in July 2006 just after a thorough sterilization of the outpatient clinic. A total of 88 environmental swabs were obtained from the outpatient clinic, waiting rooms, hospital rooms, nurses' stations, daycare room, laboratory, hospital corridors, escalator, elevators, in the first, second, and third surveys with 40, 30, and 18 swabs, respectively. Mouthwash was obtained by using 10 mL of saline for 10 sec gargle. We obtained 327 mouthwash samples from 323 RTRs (who gave informed consent among 500 RTRs regularly consulting this clinic), three physicians involved in the management of these patients, and one nurse in charge of the outpatient clinic in June and July 2006 after documentation of the environmental contamination at the second environmental survey (see below). Furthermore, we also arbitrarily used 11 BALF samples obtained from AIDS patients with PCP in the AIDS Clinical Center, International Medical Center of Japan located in Tokyo, for comparison of *P. jiroveci* genotype.

P. jiroveci Genotyping

Total DNA was extracted from specimens by use of QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the instructions provided by the manufacturer. *P. jiroveci* genotyping was performed based on sequence analysis of the internal transcribed spacer (ITS) 1- and ITS 2-containing regions of the nuclear operon. The ITS 1+5.8S+ITS 2 sequences were amplified by nested polymerase chain reaction (PCR). Two rounds of PCR were performed under the same conditions. In the first PCR, we used the primer pair of N18FS (5′-GGT CTT CGG ACT GGC AGC-3′) and N26SRX (5′-TTA CTA AGG GAA TCC TTG TTA-3′), described previously by Tsolaki et al. (*10*), for 45 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min. The second PCR was performed with the *P. jiroveci*-specific primer pair of ITSF3 (5′-CTG CGG AAG GAT CAT TAG AAA-3′) and ITS2R3 (5′-GAT TTG AGA TTA AAA TTC TTG-3′) (*11*) for 45 cycles. The PCR products from the first and second rounds were applied for electrophoresis on a 1.5% agarose gel containing ethidium bromide to visualize the expected bands (band sizes, 580 and 530 bps). To avoid contamination, each step was performed in different areas of the hospital with different sets of micropipettes. Reagents used in the PCR mixtures were prepared in a laminar-flow cabinet. To monitor possible contamination, negative controls were included in each PCR round. Second round PCR products were purified by the Montage PCR Centrifuge Filter Devices (Millipore, Billerica, MA), and direct sequencing was performed by dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA) using model 3700 automated DNA Sequencer (Applied Biosystems). Some first PCR products were cloned into plasmid vector of pT7 Blue T-Vector (Novagen, Madison, WI), transformed into competent *Escherichia coli*, and cultured on Luria-Bertani medium (LB) medium overnight. White clones were subjected to nested PCR and sequenced. The fragment consisting of the ITS 1+5.8S+ITS 2 regions was analyzed by using the Genetix-Win program version 6.1 (Software Development, Tokyo). The ITS 1 and ITS 2 alleles were typed subsequently by using the scores described by Lee et al. (*12*) The *P. jiroveci* ITS type was defined by the combination of ITS 1 and ITS 2 genotypes.

Phylogenetic Tree Analysis

Comparison and alignment of ITS 1+5.8S+ITS 2 sequences was conducted by using the Clustal-W program. Results of the alignment were then analyzed by the neighbor-joining method, and the distance matrixes were generated by the Maximum Composite Likelihood method (*13*). Bootstrap resampling (500 data sets) of the multiple alignments was performed to test the statistical robustness of the tree. Phylogenetic analyses were conducted in MEGA4 (*14*).

Nucleotide Sequence Accession Numbers

The accession numbers of the identified ITS 1+5.8S+ITS 2 alleles registered in GenBank are as follows: patient A1, A4, A5, AB481404; patient A2, A7, AB481405; patient A3, AB481406; patient A6, AB481407; patient A8, AB481408; patient A9, AB481409; patient A10, AB469817; patient A11, AB481410; patient R16, R18, R19, R20, R21, R22, R24, R25, R26, R29, R30, R31, R32, R33, RM2, RE2, AB481411; patient ML, AB481412; patient RM1, AB481413; patient RE1, AB481414.

RESULTS

Outbreak of PCP

In our hospital, more than one thousand renal transplantations have been performed since 1976. Until the first index case occurred in July 2004, there were only three sporadic PCP cases in our renal transplantation unit. Among 27 cases of the outbreak, human-to-human transmission was suggested in 22 cases from the hospital visits' dates at outpatient clinic and four cases during hospitalization (Fig. 1). In this outpatient compartment, patients shared the same waiting room until they were called for examination. Specifically, three patients (cases 3, 4, and 6) were at the outpatient clinic on the same date (July 2) when the index case (case 1) was also in the clinic. Case 1 was hospitalized on July 12 for 3 days in the room next to that of case 2. Case 2 was diagnosed with PCP on August 30 when case 8 was hospitalized for 3 days in the room next to that of case 2. Therefore, transmission routes from cases 1 to 2 and from cases 2 to 8 were suspected to be airborne. Cases 6 and 12 were in the outpatient clinic on August 9 when case 6 was still asymptomatic. Case 12 required immediate hospitalization due to PCP on her next visit 4 months later. This case suggests that the asymptomatic reservoir can be infectious. In this outbreak, only case 27 was untraceable. Based on the traceable cases, our estimated median incubation period of PCP was 53 days (range 7–188

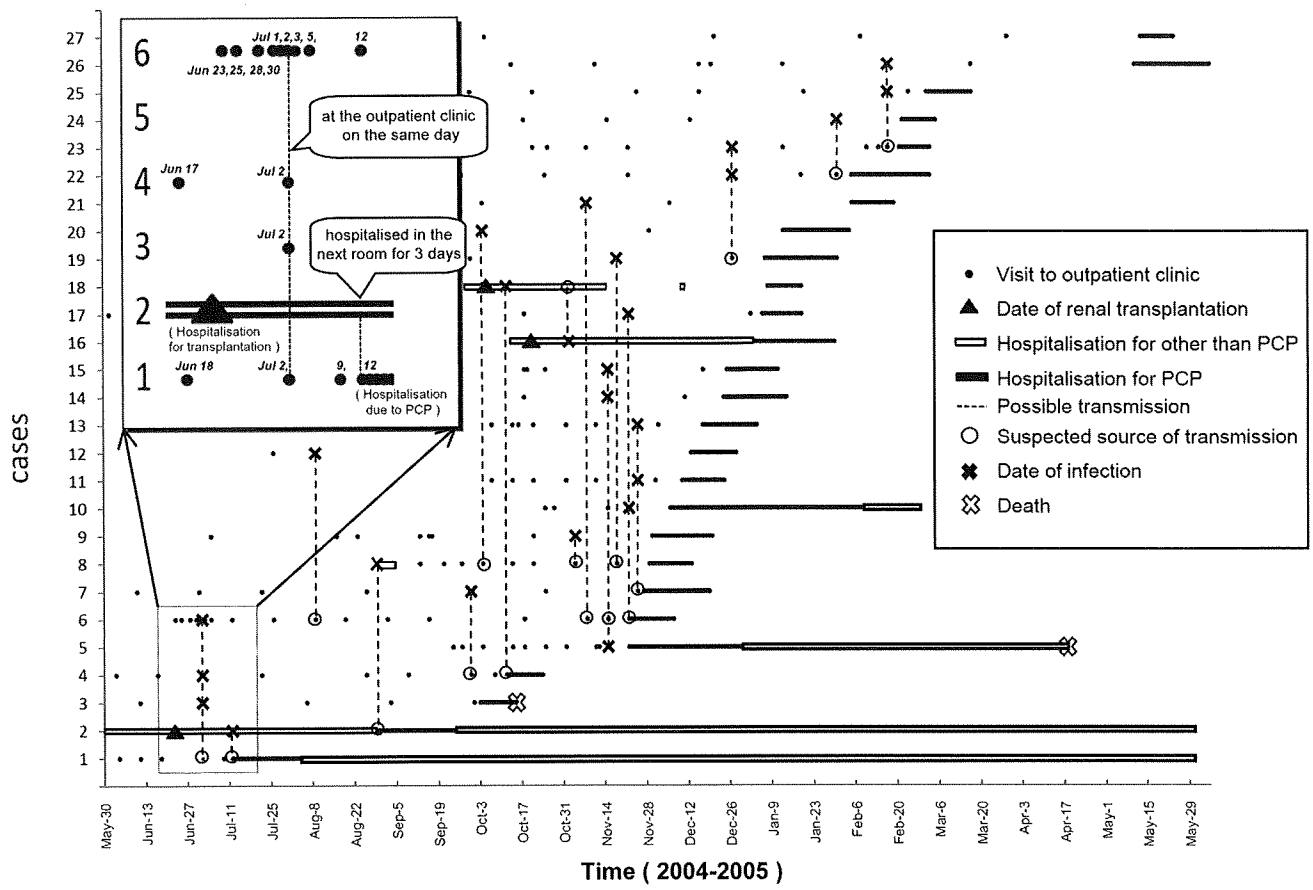

FIGURE 1. Transmission map of *Pneumocystis jiroveci* pneumonia (PCP) in renal transplant recipients. In this outpatient department, outpatients shared the same waiting room until their examination. Among 27 cases with PCP, possible human-to-human transmission was suggested in 22 cases from the visiting dates of the outpatient clinic. Case 1 was hospitalized on July 12 for PCP (see the *left upper* magnified corner). On July 2 (10 days before the onset of PCP in Case 1), case 1 had direct contact with cases 3, 4, and 6 in the same waiting room of the outpatient clinic. None of these cases had direct contact with each other thereafter before the onset of their PCP caused by the same strain. In contrast, 4 cases were suspected to be infected during hospitalization. Only case 27 was untraceable. The abscissa denotes calendar date (month/day).

days). This outbreak was concluded with case 27 by April 2005.

PCP prophylaxis with a single strength tablet (80 mg TMP/400 mg SMZ) 3 times per week had been provided to all RTRs who received renal transplantation within 1 year. However, apprehensive about possible nephrotoxicity of TMP-SMX, some RTRs refused taking TMP-SMX. Six additional PCP cases were diagnosed in the following 3 years. None of these 6 cases had taken TMP-SMX. In cases 27, 29, 31, and 33, we were unable to identify the contact date with the active PCP case. The infection route could not be explained except from an asymptomatic carrier(s) or the environment.

Molecular Analysis of *P. jiroveci*

From January 2005 to April 2005, nine BALF and one sputum samples were available for sequencing. Eight BALF and one sputum samples were sequenced successfully, and all sequences were identical as ITS type Bi (AB481411). Despite the lack of molecular analysis of the first 15 cases, this result strongly indicates that the outbreak must have been caused by the same organism. During the same period, a pathologically confirmed PCP case who suffered from malignant lymphoma was hospitalized in another ward. The genotype of this case was the ITS Ep. These findings indicate that the *P. jiroveci* strains of PCP in the renal transplant patients and the malignant lymphoma patient were different.

Surveys for *P. jiroveci* Reservoir

We obtained 40 swabs in the first environmental survey conducted in June 2005. *P. jiroveci* DNA was detected in one swab from the floor of an inpatient room where no PCP patient had been hospitalized. The genotype was the ITS type Ip. Thus, we were unsuccessful in detecting the environmental reservoir in the first survey. The second environmental survey with 30 swabs obtained from the outpatient consulting rooms was conducted in May 2006 when case 29 hospitalized due to PCP. We detected *P. jiroveci* DNA from one swab, and the genotype was the ITS type Bi (AB481411). Therefore, we identified the possible environmental reservoir in the second environmental survey. In June 10 (after case 30), the outpatient consulting room was cleaned thoroughly with 70% alcohol to remove contaminating organisms. Then, we again

conducted the third environmental survey with 18 swabs of the outpatient consulting rooms soon after the cleaning. The third survey did not detect *P. jiroveci* DNA. However, cases 31 to 33 were diagnosed after June 10, indicating that the environmental cleaning did not prevent the occurrence of PCP.

The mouthwash survey, in which 327 samples were collected from June to July 2006 when environmental contamination was documented, detected *P. jiroveci* DNA in two samples obtained from asymptomatic RTRs. The genotypes in these samples were ITS Bi (AB481411) and ITS In. Both patients did not receive TMP-SMX prophylaxis, and none has developed PCP so far. It is possible that the patient harboring ITS Bi (AB481411) is the asymptomatic reservoir of *P. jiroveci* of this outbreak.

Comparison of Genotype of *P. jiroveci* in AIDS Patients and Phylogenetic Analysis

To compare *P. jiroveci* genotype, we sequenced 11 BALF samples obtained from AIDS patients with confirmed diagnosis of PCP. The results showed eight sequences among 11 patients. Two samples from these patients were classified as ITS Bi (AB481405). However, the *P. jiroveci* DNA sequences of ITB Bi obtained from AIDS patients (AB481405) was clearly different from that of RTRs (AB481411) (Fig. 2).

DISCUSSION

We reported a large outbreak of PCP in RTRs by the same organism. Phylogenetic analysis clearly indicated this fact. Given the historical data of only three PCP cases in the same renal transplant unit over nearly 30 years and the potential nephrotoxicity of TMP-SMX, PCP prophylaxis had not been prescribed to RTRs in this unit. Both the medical staff and RTRs had considered that PCP prophylaxis was not necessary for RTR, especially for patients at a stable state. However, we were alarmed of the large PCP outbreaks in RTRs as reported in the literature (*3*, *4*, *7*, *8*). In this regard, PCP prophylaxis is recommended strictly for HIV-infected patients with CD4 counts less than 200/μL (*15*). Therefore, in HIV-infected patients who regularly consult their physicians, PCP is less likely to happen in both outpatients and hospitalized patients even though rates of colonization may be as high as 69% in HIV-infected patients (*16*). In RTRs, PCP prophylaxis is only recommended during the early posttransplantation period (*6*, *7*). However, in our outbreaks presented here, 22 of 33 cases had PCP over 12 months and six cases over 10 years after the transplantation, suggesting the need for prophylaxis when a definite PCP case is documented (i.e., a case-guided prophylaxis) not only for renal transplantation inpatients in the early postoperative period but also for all RTRs. Introduction of

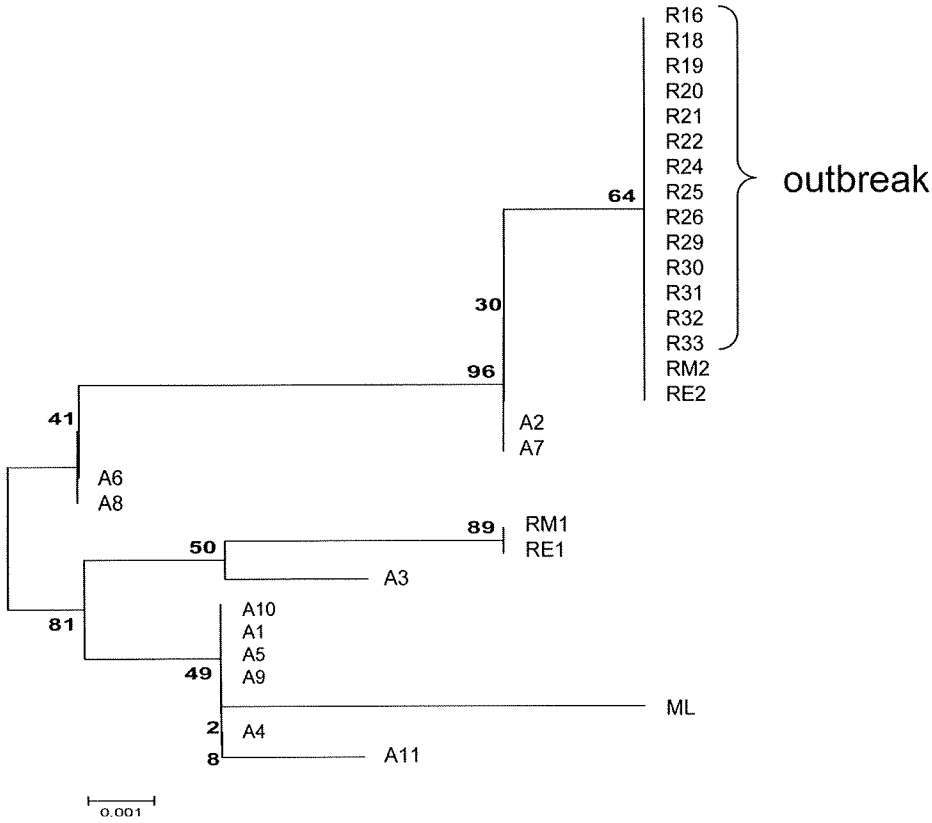

FIGURE 2. Phylogenetic tree analysis of *Pneumocystis jiroveci* DNA. Sequences of 530 bp of the internal transcribed spacer (ITS) 1+5.8S+ITS 2 gene regions were analyzed. R16 to R33 are *P. jiroveci* DNA sequences identified in renal transplant recipients, A1 to A11 in AIDS patients, ML in a patient with malignant lymphoma, RM 1 and 2 in mouthwash samples, and RE1 and 2 in environmental swabs. Numbers on branches are bootstrap values with 500 replicates of neighbor-joining analysis. Scale bar=0.001 substitutions.

MMF has dramatically reduced the rate of graft rejection. It is possible that newer and more potent immunosuppressants may increase the risk of PCP and render RTRs more susceptible to PCP.

Another new finding in this study is that *P. jiroveci* can be transmitted at the outpatient clinic. Some patients might be infected through airborne transmission. Detection of *P. jiroveci* DNA in air samples (*5*) suggested this infectious route. Based on the opportunistic nature of this strain, not all exposed patients develop PCP. Thus, some of the exposed patients could become asymptomatic reservoirs. Other investigators also reported human reservoirs of *P. jiroveci* (*17, 18*). We do not know at this stage whether *P. jiroveci* is persistently present in the asymptomatic reservoir. Until proven otherwise, it is possible that *P. jiroveci* transiently colonizes both the asymptomatic carrier and environment simultaneously and exists for long periods without a PCP patient. In this regard, the incubation period of PCP in our cases ranged from 7 days to almost 6 months. During this period, patients with PCP were confirmed to be infectious based on analysis of the outbreak in our hospital. Therefore, further unexpected sporadic cases can be registered now and in the future. These results add further support to the importance of continuous PCP prophylaxis in RTRs. Based on the longest incubation period in our study, further work is needed to decide on the importance of PCP prophylaxis for 6 months.

Our mouthwash and environmental surveys detected four *P. jiroveci* DNAs. Two of them were the ITS type Bi (AB481411) but the other two were the ITS types Ep (AB481412) and Ip (AB481415). Whether or not the ITS type Bi was the highly pathogenic is not clear at this stage. In 11 AIDS patients, various ITS types were noted. Interestingly, another reported large PCP outbreak in RTRs was caused by other ITS types (*8*). Future studies should identify the pathogenic genotype of *P. jiroveci*.

In conclusion, to prevent further outbreak of PCP, a case-guided 6-month prophylaxis seems warranted for all RTRs even those under regular maintenance immunosuppressive therapy including CNI, steroid, and anti-metabolite.

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BRIEF REPORT

Serum (1→3) β-D-Glucan as a Noninvasive Adjunct Marker for the Diagnosis of *Pneumocystis* Pneumonia in Patients with AIDS

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High serum (1→3) β-D-glucan levels are described in patients with *Pneumocystis* pneumonia (PCP). We evaluated the diagnostic value of β-D-glucan in 111 patients with AIDS who had PCP and confirmed its usefulness. However, it does not correlate with disease severity and is not suitable for monitoring response to treatment.

Pneumocystis pneumonia (PCP) is associated with significant morbidity and mortality in patients with human immnuodeficiency virus type 1 (HIV-1) infection [1, 2]. PCP is usually diagnosed microscopically by identifying *Pneumocystis jirovecii* in bronchoalveolar lavage fluid (BALF) or bronchoscopically obtained lung tissue [3]. Bronchoscopy, however, is invasive, especially in patients with hypoxemia associated with PCP. Therefore, a minimally invasive method is desirable for diagnosis.

Serum (1→3) β-D-glucan (hereafter, β-D-glucan) is a common component of the cell wall of most fungi and is the major component of the cyst of *P. jirovecii*. Therefore, it is measured in patients who are suspected to have PCP, as well as in those with deep-seated mycotic infections [4]. Although β-D-glucan has been used as an adjunct test for the diagnosis of PCP [5], only a few reports have evaluated its level [5–7] and its correlation with other parameters (such as lactate dehydrogenase [LDH] level) in mixed populations that included a small number of HIV-infected patients [6]. For this purpose, we analyzed the correlation between β-D-glucan levels and other parameters among patients with AIDS who have PCP.

Methods. We evaluated data from 111 consecutive HIV-1–infected patients with PCP at the International Medical Center of Japan, an 885-bed tertiary care hospital in Tokyo, from April 1997 through July 2007. This study was approved by the Ethics Review Committee of the hospital (IMCJ-H20-569). Patients who did not undergo diagnostic bronchoscopy were excluded from the study.

Medical records were reviewed, and the following data were collected: age; sex; mode of infection; $CD4^+$ cell count; serum levels of LDH, β-D-glucan, and C-reactive protein (CRP); and alveolar-arterial oxygen tension gradient ($AaDO_2$). Serum β-D-glucan levels were measured using the Fungitec G MK test (Seikagaku). Manipulation was performed described elsewhere [4, 5], in accordance with the manufacturer's instructions. Serum β-D-glucan levels in HIV-1–infected patients without PCP determined during the same period were used as a control. If serum β-D-glucan levels had been determined several times for the same patient, only the first measurement was included. Although oral and esophageal candidiasis are superficial infections, they were included as an independent factor and analyzed. In this report, the term *candidiasis* refers to oral and/or esophageal candidiasis.

The diagnosis of PCP was established by identification of *P. jirovecii* in BALF. Each BALF specimen (100 μL) was centrifuged at 900 *g* for 2 min by means of a Shandon Cytospin III device, and a monolayer of deposited cells were stained using Diff-Quik (Dade Behring) and examined microscopically for the presence of *P. jirovecii*.

Data were expressed as means ± standard deviations (SDs) or as medians. Differences in categorical variables between patients with PCP and control patients were assessed using the Mann-Whitney *U* test. The Mann-Whitney *U* test (for comparison of 2 groups) and the Kruskal-Wallis test (for comparison of 3 groups) were used for analysis of differences in serum β-D-glucan levels. A receiver-operating-characteristic (ROC) curve was constructed to illustrate the cutoff value for β-D-glucan. The relationships were analyzed by linear regression analysis. Differences were considered significant at $P < .05$. Statistical analyses were performed using SPSS, version 17.0 (SPSS).

Results. A total of 111 patients had a definite diagnosis of PCP, and serum β-D-glucan level was measured in each. Of

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these patients, 67 also had candidiasis at admission. Of the control group (425 patients who did not have PCP), 28 had candidiasis, 3 had cryptococcal infection, and 394 had neither.

The patients with PCP were older than the control patients (mean ± SD, 42.3 ± 11.9 vs 38.7 ± 11.7 years; $P < .01$), and $CD4^+$ cell counts were significantly higher in the control patients than in the patients with PCP (mean ± SD, 178.6 ± 155.6 vs 49.1 ± 63.1 cells/μL; $P < .001$). Sex and mode of transmission of HIV were similar in both groups ($P = .81$ and $P = .53$, respectively). All patients with PCP received treatment, and 6 patients died of PCP.

Of the patients with PCP, 67 had candidiasis and 44 did not; of the control patients, 28 had candidiasis, 3 had cryptococcal infection, and 394 did not have any fungal infection. The median (range) serum β-D-glucan level in each group was 171.2 (14.9–2966), 209.6 (2.4–2469), 7.40 (1.0–73.0), 22.7 (9.3–69.7), and 8.25 (1.0–310) pg/mL, respectively (Figure 1). The median serum level of β-D-glucan among all patients with PCP (174.8 [2.4–2966] pg/mL) was significantly higher than that among the control patients (8.2 [1.0–310.1] pg/mL) ($P < .001$). The presence of candidiasis in both the PCP group and the control group and of cryptococcal infection in the control group did not significantly influence serum levels of β-D-glucan ($P = .53$, $P = .83$, and $P = .08$, respectively).

With respect to the diagnostic value of β-D-glucan, the area under the ROC curve for β-D-glucan level was 0.964 (95% confidence interval, 0.945–0.984) (Figure 2). A β-D-glucan cutoff value of 23.2 pg/mL (which represented the technique's threshold of detection) had a sensitivity of 96.4% and a specificity of 87.8%.

There was no correlation between serum levels of β-D-glucan and $AaDO_2$ at room air ($r = 0.125$; $P = .30$), LDH ($r = .030$; $P = .76$), or CRP ($r = .002$; $P = .62$). In 42 instances, serum β-D-glucan levels were measured before and after treatment. On the basis of a cutoff value of 23.2 pg/mL, normalization of serum β-D-glucan levels was noted in 7 patients. In contrast, serum β-D-glucan levels slightly increased in 9 patients despite clinical improvement being noted at week 3. This finding indicates that β-D-glucan levels reflected the clinical course in only 16.7% of patients (7 of 42) within 3 weeks of treatment.

Discussion. The present study has reported 3 major findings. The first major finding is the usefulness of quantitative measurement of serum β-D-glucan levels for the diagnosis of PCP. With a cutoff value of 23.2 pg/mL, β-D-glucan level had

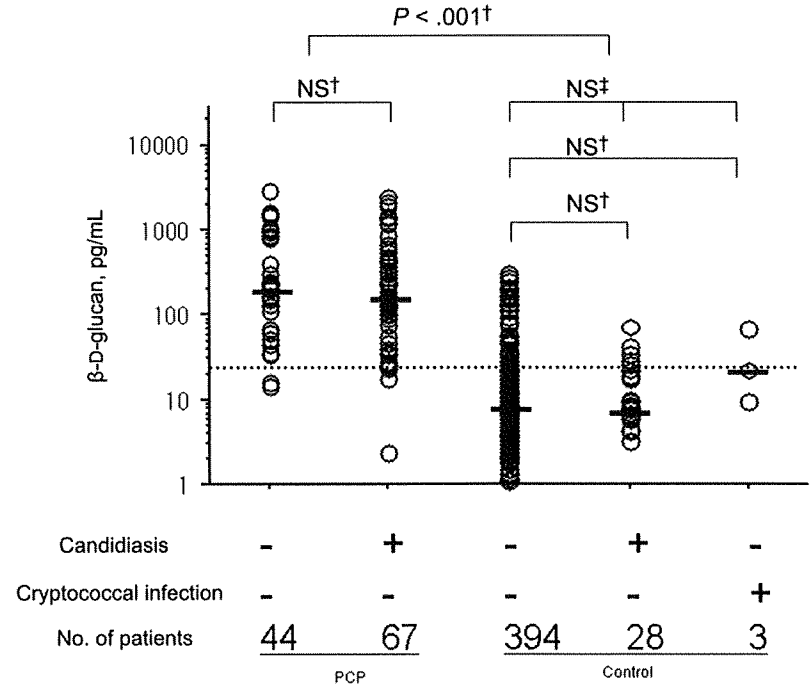

Figure 1. Serum levels of (1→3) β-D-glucan. Levels of β-D-glucan in serum were examined before treatment of *Pneumocystis* pneumonia (PCP), candidiasis, and cryptococcal infection. The Mann-Whitney *U* test (†) and the Kruskal-Wallis test (‡) were used for comparison of serum β-D-glucan levels. Individual values are plotted, and horizontal bars represents medians. The presence of candidiasis in both the PCP group and the control group and of cryptococcal infection in the control group did not significantly influence serum β-D-glucan levels ($P = .53$, $P = .83$, and $P = .08$, respectively). Serum β-D-glucan levels were significantly higher in patients with PCP than in those without PCP, despite the presence of candidiasis and cryptococcal infection ($P < .001$). NS, not significant.