

Figure 2. Changes from baseline in total cholesterol, ALT and total bilirubin.

ALT and total cholesterol at week 96 were compared with the baseline values. Since participants who developed hyperlipidemia were treated with lipid-lowering agents during the study period, the highest levels registered in each participant during the follow-up were collected for analysis. There were no significant differences in total cholesterol and ALT between the two arms, while hyperbilirubinemia was significantly higher in the ATV/r arm. Modification of treatment due to hyperbilirubinemia was not required in any of the patients of the ATV/r arm. In these box-and-whisker plots, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

Considering many studies on HIV treatment held in western countries that enrolled few Asian HIV-1 patients, it is important to collect data from Asian population. The current United States Department of Health and Human Services guidelines recommend TDF/FTC as the first line regimen, while the European AIDS Clinical Society recommends 3TC and ABC addition to TDF and FTC alone (28, 29). TDF/FTC is a known potent antiretroviral agent, however, its long-term efficacy and safety remain unclear (11, 12). Considering that the combinations of NRTI are limited, the efficacy and safety of ABC in the low HLA-B*5701 population need to be evaluated for wider treatment options for HIV-1

patients (9, 10).

Conclusion

This study was designed as a selection study to compare the virologic efficacy and treatment safety of EFV and ATV/r, both with ABC/3TC, in Japanese patients. The results showed no significant differences in efficacy between the two regimens at week 48. The evaluation of safety was extended to 96 weeks, which also showed no significant difference in both arms. The results of the present study have already been applied as the basis of a follow-up study that is

currently being conducted in Japan to compare NRTI combinations of ABC/3TC and TDF/FTC with ATV/r as key drugs.

The authors state that they have no Conflict of Interest (COI).

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Original article

Effective recognition of HIV-1-infected cells by HIV-1 integrase-specific HLA-B*4002-restricted T cells

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Abstract

HLA-B*4002 is one of the common HLA-B alleles in the world. All 7 reported HLA-B*4002-restricted HIV epitopes are derived from Gag, Nef, and Vpr. In the present study we sought to identify novel HLA-B*4002-restricted HIV epitopes by using overlapping 11-mer peptides of HIV-1 Nef, Gag, and Pol, and found that 6 of these 11-mer Pol peptides included HLA-B*4002-restricted epitopes. Analysis using truncated peptides of these 6 peptides defined 4 optimal Pol (integrase) epitopes. All epitopes previously reported had Glu at position 2 (P2), suggesting that Glu at P2 is the anchor residue for HLA-B*4002; whereas only 2 of the integrase epitopes that we here identified had Glu at P2. CTL clones specific for the 2 epitopes effectively recognized HIV-1-infected cells whereas those for other 2 epitopes only weakly recognized them. The antigen sensitivity of the former clones for the epitope peptide was much higher than that of the latter clones, suggesting 2 possibilities: 1) the former T cells have high-affinity TCRs and/or 2) the epitope peptides recognized by the former T cells are highly presented by HLA-B*4002 in HIV-1-infected cells. These integrase-specific T cells with high antigen sensitivity may contribute to the suppression of HIV-1 replication in HIV-1-infected HLA-B*4002⁺ individuals.

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Keywords: HIV-1; Cytotoxic T lymphocytes; HLA-B*4002; Integrase

1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTL) play an important role in HIV-1 infections [1–4]. Previous studies demonstrated that HIV-1-specific CTL can inhibit viral replication in vitro [5–7] and that depletion of CD8⁺ T cells by treatment with an anti-CD8 mAb results in failure of the clearance of the virus in rhesus macaques infected with chimeric simian/human immunodeficiency virus [8]. These studies suggest that the CD8⁺ CTLs contribute to viral clearance and disease progression

in HIV-1-infected individuals. The study of CTL responses in an African cohort demonstrated that HLA-B-restricted T cell responses are associated with lower viral load than HLA-A-restricted or HLA-C-restricted ones [9], suggesting that HLA-B-restricted responses are important for the control of HIV-1. Therefore, the characterization of HIV-1 epitope-specific HLA-B-restricted CTLs is important for understanding the pathogenesis of HIV and developing an AIDS vaccine.

HLA-B*4001 and HLA-B*4002 are common HLA-B alleles in the world. These alleles are found in 10.8% and 16.6% of Japanese population, respectively, and the frequency of HLA-B*4002 is the third highest among HLA-B alleles [10]. Only residue 97 differs between these 2 alleles. So far 10 HLA-B*4001-restricted and 7 HLA-B*4002-restricted HIV epitopes have been reported in Caucasian cohorts [11–16]. These HLA-B*4002-restricted epitopes were derived from

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Gag, Nef, and Vpr; whereas the HLA-B*4001-restricted ones came from Gag, Nef, Pol, and Env.

In the present study, we sought to identify HLA-B*4001-restricted and HLA-B*4002-restricted HIV-1 epitopes in chronically HIV-1-infected Japanese cohorts by using 11-mer overlapping peptides derived from Pol, Gag, and Nef. We focused on these 3 proteins in the present study because these major proteins, which provide many CTL epitopes, are considered as vaccine targets. In addition, CD8⁺ T cell clones specific for these newly identified epitopes were generated and used to clarify their ability to recognize HIV-1-infected cells. In the present study, we found 4 novel integrase epitopes presented by HLA-B*4002 and further characterized the CD8⁺ T cells specific for these epitopes. Two of these epitopes were considered as immunodominant epitopes, because the specific T cells effectively recognized HIV-1-infected cells.

2. Materials and methods

2.1. Samples of HIV-1-infected individuals

This study was approved by the National Center for Global Health and Medicine and the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The HLA type of the patients was determined by standard sequence-based genotyping.

2.2. Synthetic peptides

We previously designed and generated overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences [17]. Each 11-mer peptide was overlapped by 9 amino acids. Truncated peptides of some 11-mer peptides were synthesized by utilizing an automated multiple peptide synthesizer and purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.3. Cells

The EBV-transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from PBMC of KI-400. C1R cells expressing HLA-A*0207 (C1R-A*0207) and those expressing HLA-B*4002 (C1R-B*4002) were generated by transfecting C1R cells with the HLA-A*0207 and HLA-B*4002 genes, respectively. C1R-A*3101 cells were previously generated [18]. 721.221-CD4 cells expressing HLA-B*4002 (.221-CD4-B*4002), HLA-Cw*0102 (.221-CD4-Cw*0102), and HLA-Cw*0304(.221-CD4-Cw*0304) were generated by transfecting 721.221-CD4 cells with the HLA-B*4002, HLA-Cw*0102, and HLA-Cw*0304 genes, respectively, and maintained in RPMI 1640 medium supplemented with 10% FCS and 2.0 mg/ml hygromycin B.

2.4. Intracellular cytokine production (ICC) assay

PBMCs from chronically HIV-1-infected patient KI-400 were stimulated with HIV-1-derived peptide (1 μ M) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN- γ production activity by using a FACSCalibur. Briefly, bulk cultures were stimulated with stimulator cells pulsed with HIV-1-derived peptide (1 μ M) for 2 h at 37 °C. Brefeldin A (10 μ g/ml) was then added, and the cultures were continued for an additional 4 h. Cells were collected and stained with phycoerythrin (PE)-labelled anti-CD8 monoclonal antibody (mAb; Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were made permeable by incubation in permeabilization buffer (0.1% saponin and 20% NCS in phosphate-buffered saline) at 4 °C for 10 min and then stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN- γ mAb (PharMingen, San Diego, CA). After a thorough washing with the permeabilization buffer, the cells were analyzed by using the FACSCalibur. Similarly IFN- γ production of established CTL clones was analyzed by use of this assay.

2.5. Generation of CTL clones

Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding 0.8 cells/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μ l of cloning mixture (RPMI 1640 medium containing 10% FCS, 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMCs from a healthy donor, and 1×10^5 irradiated C1R-B*4002 cells pulsed with a 1 μ M concentration of the appropriate HIV-1-derived peptides. Wells positive for growth after about 2 weeks were examined for CTL activity by performing the ICC assay. All CTL clones were cultured in RPMI 1640 containing 10% FCS and 200 U/ml recombinant human interleukin-2. CTL clones were stimulated biweekly with irradiated target cells pulsed with the corresponding peptides.

2.6. HIV-1 clones

NL-432, which is an infectious proviral clone of HIV-1, was previously reported [7,19].

2.7. HIV-1 infection of .221-CD4-B*4002 and .221-CD4 cells

.221-CD4-B*4002 and 721.221-CD4 cells were exposed to NL-432 for several days. These infected cells were used as stimulator cells for ICC assays when approximately 60% of cells had been infected, which was confirmed by intracellular staining for HIV-1 p24 antigen.

3. Results

3.1. Identification of 11-mer peptides recognized by HLA-B*4001-restricted and HLA-B*4002-restricted HIV-1-specific CD8⁺ T cells

To identify novel HLA-B*4001-restricted CTL epitopes, we analyzed 5 HIV-seropositive HLA-B*4001⁺ Japanese individuals by Elispot assays with cocktails of overlapping 11-mer peptides spanning Gag (p17^{Gag}, p24^{Gag}, p2p7p1p6^{Gag}), Pol (Protease, RT, integrase), and Nef. The overlapping 11-mer peptide cocktails that gave more than 200 spots per 10⁶ cells were used to stimulate PBMC of each patient in order to identify the epitopes. After the PBMC had been cultured for 2 weeks, their IFN- γ production was analyzed by using the ICC assay. We found that 3 peptide cocktails induced IFN- γ production. Further analysis using 10 peptides in the peptide cocktails showed that three 11-mer peptides included HLA-B*4001-restricted epitopes but all of these peptides contained reported HLA-B*4001-restricted epitope sequences. Thus, we could not find any novel HLA-B*4001-restricted epitopes.

In order to identify CTL epitopes restricted by HLA-B*4002, we analyzed fresh CD8⁺ T cells from patient KI-400 (A*0207/A*3101, B*4002/B*4601, Cw*0102/Cw*0304) by performing Elispot assays with the cocktails of the overlapping 11-mer peptides. More than 200 spots per 10⁶ cells were observed with 7 out of 25 Gag cocktails, 11 out of 50 Pol cocktails, and 1 out of 10 Nef cocktails (data not shown). To find novel HLA-B*4002-restricted CTL epitopes, we focused on analyzing 5 peptide cocktails (Gag21-49, Pol781-809, Pol801-829, Pol901-929, and Pol921-949) that did not contain reported epitopes restricted by the 6 HLA-class I alleles this patient expressed. To determine which peptide in each cocktail induced the specific CD8⁺ T cells, we stimulated PBMCs from KI-400 with these peptide cocktails and then cultured the cells for 2 weeks. The responsiveness of the cultured CD8⁺ T cells toward ten 11-mer peptides in each peptide cocktail was measured by using the ICC assay. IFN- γ production was found in the bulk CD8⁺ T cells stimulated with autologous B-LCLs pre-pulsed with 2 Gag (Gag31-41 and Gag33-43) and 6 Pol peptides (Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929, and Pol921-931).

For determination of HLA restriction molecules of CD8⁺ T cells specific for these 11-mer peptides, the responsiveness of the bulk CD8⁺ T cells towards peptide-pulsed C1R cells expressing one of the HLA-A or -B alleles or .221 cells expressing one of the HLA-C alleles was measured by performing the ICC assay. HLA-B*4002-restricted responses were found in the bulk culture cells stimulated with the cells pre-pulsed with Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 or Pol921-931 (data not shown). These results indicate that these six 11-mer peptides included HLA-B*4002-restricted epitopes.

3.2. Identification of HLA-B*4002-restricted optimal epitope peptides

To determine the optimal epitopes for these 11-mer peptides, we stimulated bulk T cells with C1R-B*4002 cells

pre-pulsed with truncated peptide of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 or Pol921-931 at concentrations of 1000 nM and then measured the IFN- γ production of each bulk T cells was measured by conducting the ICC assay. Previous studies on HLA-B*4002-restricted epitopes suggested that Glu at position 2 is an anchor for HLA-B*4002 (11–16). Judging from the finding that Pol801-811 did not include HLA-B*4002-restricted epitopes, we speculated that 2E in Pol799-809 (IG11: IEAEVIPAETG) would be the anchor for HLA-B*4002 rather than 4E. We therefore generated 5 truncated peptides (IT10: IEAEVIPAET, IA8: IEAEVIPA, ET9: EAEVIPAET, AT8: AEVIPAET, and AG9: AEVIPAETG) of Pol799-809 and investigated whether CD8⁺ T cells induced by Pol799-809 would recognize these peptides. The T cells recognized only IG11 and IT10 at 1000 nM (Fig. 1A), whereas they showed higher sensitivity to IT10 than to IG11 (Fig. 1B). These findings indicate that Pol799-808 (IT10) was the optimal epitope.

For Pol807-817 (EL11: ETGQETAYFLL), we generated 4 truncated peptides (TL10: TGQETAYFLL, GL9: GQETAYFLL, GL8: GQETAYFL, and QL8: QETAYFLL). CD8⁺ T cells induced by the Pol807-817 peptide recognized EL11, TL10, GL9 and QL8, but not GL8 (Fig. 1A), indicating that L at position 11 was critical for the epitope. On the other hand, the T cells showed higher sensitivity to EL11 than to the other 3 peptides (Fig. 1C). These findings indicate that Pol807-817 (EL11) was the optimal epitope.

For Pol909-919 (YI11: YSAGERIVDII) and Pol911-921 (AT11: AGERIVDIIAT), we assumed 2 possibilities: 1) the two 11-mer peptides shared the same epitope, or 2) the two peptides included different epitopes. To clarify these possibilities, we analyzed Pol909-919 and Pol911-921 independently. For Pol909-919, we generated 5 truncated peptides (SI10: SAGERIVDII, SI9: SAGERIVDI, AI8: AGERIVDI, AI9: AGERIVDII, and GI8: GERIVDII). CD8⁺ T cells induced by Pol909-919 peptide recognized YI11, SI10, AI9, and GI8, but not SI9 and AI8 (Fig. 1A), indicating that I at position 11 was critical for this epitope. On the other hand, they showed higher sensitivity to GI8 than to the other 3 peptides (Fig. 1D). These findings indicate that Pol909-919 (GI8) was the optimal epitope. Regarding Pol911-921 (AT11), we generated 4 truncated peptides (AI9: AGERIVDII, AI8: AGERIVDI, GI8: GERIVDII, and GA9: GERIVDIIA). CD8⁺ T cells induced by Pol911-921 peptide recognized AT11, AI9, GI8 and GA9, but not AI8 (Fig. 1A), indicating that I at position 11 to be critical for this epitope. They also showed higher sensitivity to GI8 than to the other 3 peptides (Fig. 1E), indicating that GI8 (Pol912-919) was the optimal epitope. Thus, these results confirmed that Pol909-919 and Pol911-921 included the same epitope.

For Pol919-929 (IQ11: IATDIQTKELQ) and Pol921-931 (TQ11: TDIQTKELQKQ), we assumed that these two 11-mer peptides shared the same epitope. Therefore, we analyzed Pol919-929 and Pol921-931 independently. Regarding Pol919-929 (IQ11: IATDIQTKELQ) we speculated that 10L would be the C-terminus of the epitope because no hydrophilic residue is found in the C-terminus of HLA class I-binding peptides.

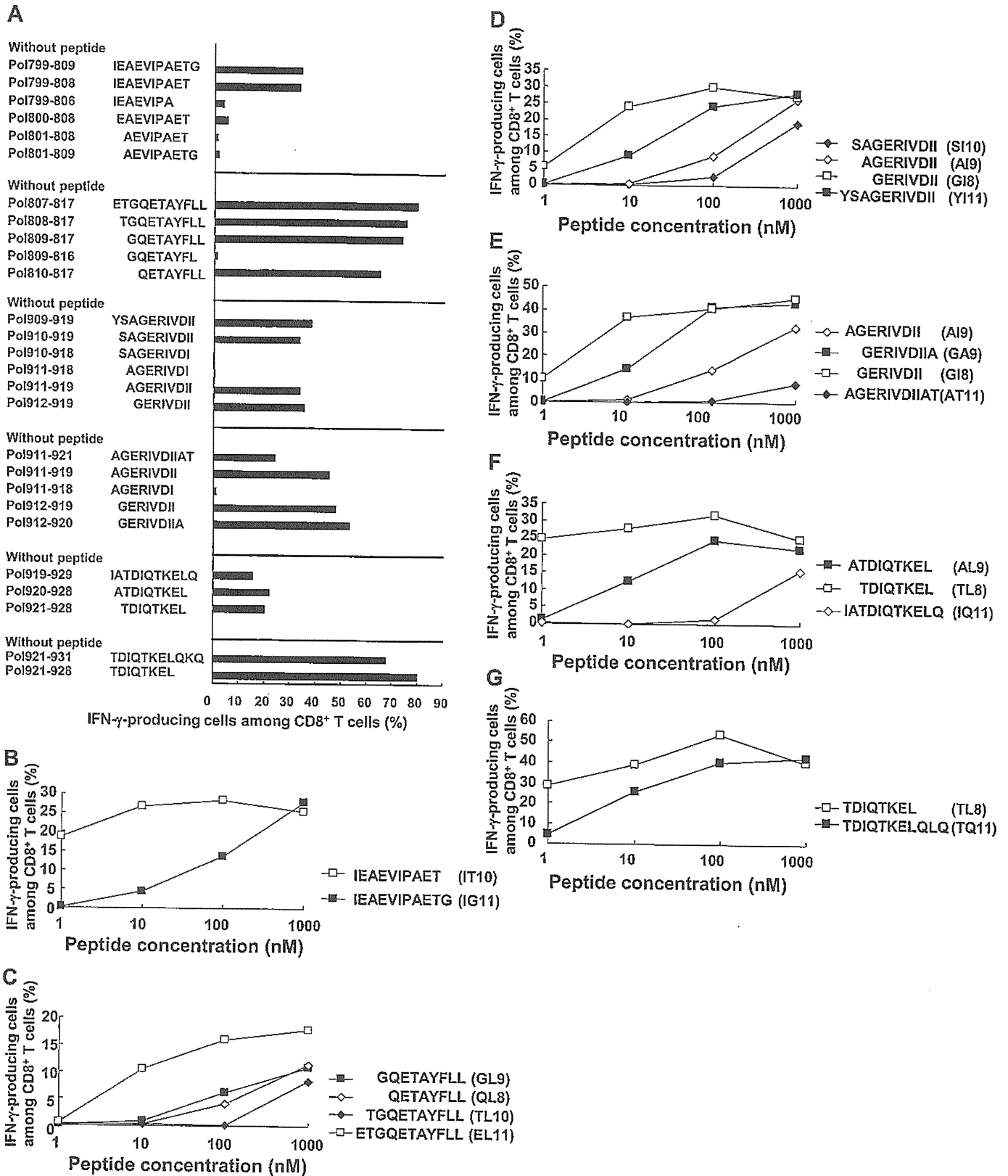


Fig. 1. Identification of HLA-B*4002-restricted HIV-1 CTL epitopes. A. For determination of the optimal epitopes of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 and Pol921-931, the recognition of the bulk T cells for the truncated peptides was examined by using C1R-B*4002 cells pre-pulsed with each truncated peptide at a concentration of 1000 nM. The responsiveness of the bulk CD8⁺ T cells toward each truncated peptide was measured by using the ICC assay. The percentages of IFN- γ -producing cells among the CD8⁺ T cells are shown in the figure. B–G. Optimal epitopes were not determined at concentrations of 1000 nM for Pol799-809 (B), Pol807-817 (C), Pol909-919 (D), Pol911-921 (E), Pol919-929 (F) or Pol921-931 (G). The responsiveness of the bulk CD8⁺ T cells was examined for C1R-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 1 to 1000 nM. The responsiveness of the bulk CD8⁺ T cells toward each truncated peptide was measured by performing the ICC assay. The percentages of IFN- γ -producing cells among CD8⁺ T cells are shown in the figure.

Therefore, we generated 2 truncated peptides (AL9: ATDIQTKEL and TL8: TDIQTKEL). Bulk CD8⁺ T cells induced by Pol919-929 peptide recognized all 3 peptides (Fig. 1A) and showed higher sensitivity to TL8 than to the other 2 peptides (Fig. 1F), indicating that Pol921-928 (TL8) was the optimal epitope. Similarly we speculated TL8 to be optimal epitope for Pol921-931 (TQ11: TDIQTKELQKQ), because no hydrophilic residue is found in the C-terminus of HLA-class I-restricted epitopes. Although bulk CD8⁺ T cells induced by Pol921-931 peptide recognized both TQ11 and TL8 peptides (Fig. 1A), they showed higher sensitivity to TL8 than to TQ11 (Fig. 1F). These findings indicate that Pol919-929 and Pol921-931 11-mer peptides included the same epitope, Pol921-928(TL8).

Thus, we identified 4 HLA-B*4002-restricted optimal peptides. Interestingly, these 4 Pol epitopes were all derived from integrase.

3.3. Generation and antigen sensitivity of HLA-B*4002-restricted Pol-specific CTL clones

To analyze the CD8⁺ T cells specific for these 4 integrase epitopes, IT10 (Pol799-808), EL11 (Pol807-817), GI8 (Pol912-919), and TL8 (Pol921-928), we established the specific CD8⁺ T cell clones and analyzed them for their antigen sensitivity by using the ICC assays. The result was shown in Fig. 2. The T cell clones and their EC₅₀ values were as follows: Pol799-808-specific T cells (27.7), Pol807-817-specific T cells (191.7), Pol912-919-specific T cells (443.1), and Pol921-928-specific T cells (7.6). These results indicate that Pol799-808-specific and Pol921-928-specific CD8⁺ T cell clones had higher antigen sensitivity than Pol807-817-specific and Pol912-919-specific ones.

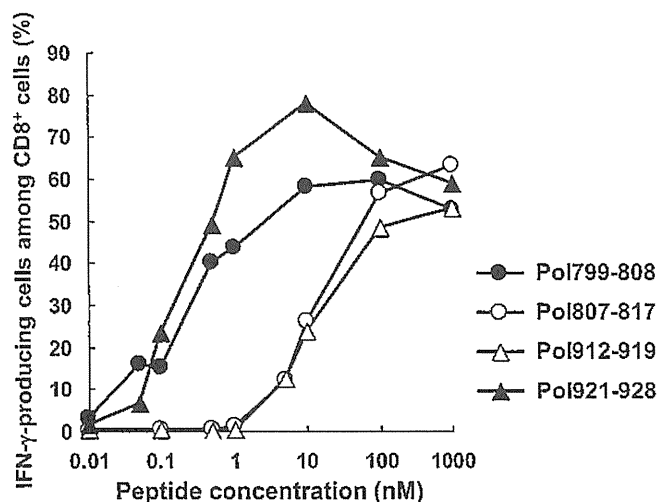


Fig. 2. Antigen Sensitivity of 4 HIV-1 integrase-specific CD8⁺ T cells. Antigen sensitivity of 4 HIV-1 integrase-specific CD8⁺ T cells was examined by using the ICC assay. The responsiveness of these CTL clones was examined for CIR-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 0.01 to 1000 nM.

3.4. Recognition of HIV-1-infected cells by specific T cells

To clarify whether Pol799-808, Pol807-817, Pol912-919, and Pol921-928 were naturally occurring peptides and whether CTLs specific for these epitopes had the ability to recognize HIV-1-infected cells, we investigated the response of these peptide-specific CD8⁺ T cell clones toward HIV-1 (NL-432)-infected .221-CD4 cell lines expressing HLA-B*4002. NL-432 includes wild-type sequences of these 4 epitopes. .221-CD4 cell lines and those expressing HLA-B*4002 were infected with NL-432, and then cultured for 4 days. The responses of the T cell clones toward these infected cells were measured by using the ICC assay. The percentage of the HIV-1-infected cells was determined by staining intracellular HIV-1 p24 (Fig. 3A). The Pol799-808-specific, Pol807-817-specific, Pol912-919-specific, and Pol921-928-specific CTL clones responded to .221-CD4-B*4002 cells infected with HIV-1 but not to uninfected .221-CD4-B*4002 cells or to HLA-B*4002-negative .221-CD4 cells infected with HIV-1. These results indicate that Pol799-808, Pol807-817, and Pol921-928 peptides were naturally processed and presented by HLA-B*4002 and that the T cells specific for these epitopes could recognize HIV-1-infected cells (Fig. 3B). On the other hand, the responses of Pol807-817-specific and Pol912-919-specific CTL clones was much weaker than those of the other CTL clones (Fig. 3B), indicating that the former CTLs only weakly recognized HIV-1-infected cells.

4. Discussion

There is only 1 amino acid substitution, at residue 97, on the peptide binding floor between HLA-B*4001 and HLA-B*4002. A previous study on the peptide motif of HLA-B*4001 showed that HLA-B*4001-binding peptide anchors are Glu at P2 (2E) and Leu at the C-terminus [20]. Indeed, 7 of 8 reported HLA-B*4001-restricted HIV-1-specific T cell epitopes have 2E and Leu at their C-terminus [11–13]. Although no HLA-B*4002-binding peptide motif had not yet been identified, we speculated that this motif would be similar to the HLA-B*4001-binding one. Indeed, all 7 HLA-B*4002-restricted epitopes previously reported have 2E (Table 1). However, 2 of the 4 epitopes identified in the present study did not have the 2E anchor. In addition, only 5 of 11 HLA-B*4002-restricted epitopes had Leu at their C-terminus. These findings suggest that the substitution from Ser to Arg at residue 97 may partially affect the structure of the F and B pockets. Pol807-817 (ETGQETAYFLL) does not have the 2E anchor. QL8 (QETAYFLL) is speculated to be an HLA-B*4002-restricted epitope because this peptide has 2E. However, the antigen sensitivity of the T cells specific for QL8 is much weaker than that for EL11. This result excludes the possibility that QL8 is the epitope peptide. Thr at position 2 of Pol807-817 may bind to the residues facing the B-pocket by hydrogen-bonding. Nine of the 11 HLA-B*4002-restricted epitopes have 2E, suggesting that the 2E is still anchor residue for HLA-B*4002.

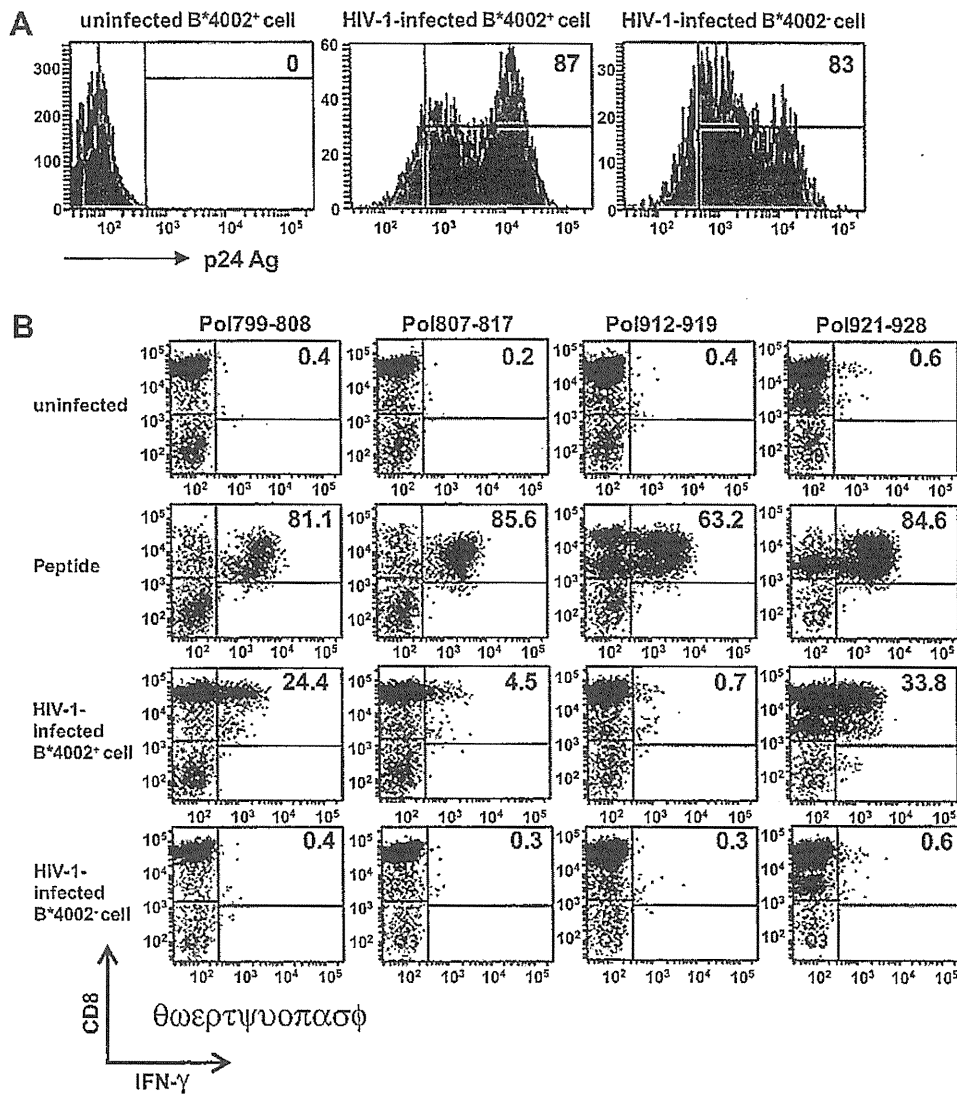


Fig. 3. Ability of 4 HIV-1 integrase-specific CD8⁺ T cells to recognize HIV-1-infected cells. A. The .221-CD4 and B*4002⁺.221-CD4 cell lines were infected with HIV-1 (NL-432) and cultured for 4 days. The frequency of HIV-1-infected cells was detected by using staining of intracellular p24 with anti-p24 mAb. The percentage of HIV-1-infected cells is shown in each figure. B. Recognition of HIV-1-infected cells by the Pol799-808-, Pol807-817-, Pol912-919- or Pol921-928-specific CD8⁺ T cell clones. The activities of these peptide-specific CD8⁺ T cell clones to recognize B*4002⁺.221-CD4 cell lines infected with HIV-1 or those pre-pulsed with the corresponding peptide (1000 nM) were measured by use of the ICC assay. The percentages of IFN- γ -producing cells among CD8⁺ T cells are shown in each figure.

Although the 7 HLA-B*4002-restricted epitopes previously reported do not include Pol-derived ones, we identified novel 4 HLA-B*4002-restricted Pol-specific T cell epitopes in the present study. Interestingly, all of these Pol epitopes were derived from integrase. Though 29 integrase epitopes were reported as 20 different HLA class I-restricted epitopes (Los Alamos HIV Molecular Immunology Data), integrase epitopes were not found among HLA-B*4001-restricted Pol epitopes. Regarding the integrase epitopes, HLA-B*4201 and HLA-B*1503 present 3 different epitopes, whereas the other 18 alleles present 1 or 2 epitopes. Thus, HLA-B*4002 is so far the only HLA-class I allele that can present more than 3 integrase epitopes.

Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, whereas Pol807-817-specific and Pol912-919-specific ones weakly recognized these cells. Antigen sensitivity of the former T cells was much

higher than that of the latter ones. Thus, the ability to recognize HIV-1-infected cells was associated with the antigen sensitivity. However, it is difficult to clarify why the 2 T cells weakly recognize HIV-1-infected cells because we did not measure the bindings of these epitope peptides to HLA-B*4002 molecules and of the specific tetramers to the specific T cells. We can suggest 2 possibilities from the data shown in Fig. 2 and Fig. 3: 1) The former T cells may have higher affinity TCR and/or 2) these former epitope peptides are more highly presented than the latter by HLA-B*4002 in HIV-1-infected cells. Since Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, we proposed that they would effectively recognize and kill HIV-1-infected cells *in vivo*.

HLA-B*4001 and HLA-B*4002 are found in 10.8% and 16.6% of the Japanese population, respectively. Since both

Table 1

A list of HLA-B*4002-restricted epitopes identified previously and in this study.

Sequence	Protein	Reference
GELDRWEKI	Gag (p17)	*15
KETINEEAA	Gag (p24)	*15
AEWDRVHPV	Gag (p24)	*15
AEAMSQVTNS	Gag (p2p7p1p6)	*16
TERQANFL	Gag (p2p7p1p6)	*15
REPHNEWTL	Vpr	*14
KEKGGLEGL	Nef	*15
IEAEVIPAET	Pol (Integrase)	This study (Pol799-808)
ETGQETAYFL	Pol (Integrase)	This study (Pol807-817)
GERIVDII	Pol (Integrase)	This study (Pol912-919)
TDIQTKEK	Pol (Integrase)	This study (Pol921-928)

HLA-class I alleles are detected in approximately 25% of Japanese individuals, T cell epitopes presented by these alleles are useful for studies on HIV-1 immunopathogenesis and the development of AIDS vaccines.

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Case of relapsed AIDS-related plasmablastic lymphoma treated with autologous stem cell transplantation and highly active antiretroviral therapy

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Abstract

Plasmablastic lymphoma is a rare and aggressive malignancy strongly associated with HIV infection. The refractory/relapsed disease rate is high, and the survival rate is characteristically poor. There are no satisfactory salvage regimens for relapsed cases. We successfully performed autologous stem cell transplantation using a regimen consisting of MCNU (ranimustine), etoposide, cytarabine, and melphalan in a Japanese patient with relapsed AIDS-related plasmablastic lymphoma of the oral cavity. Highly active antiretroviral therapy continued during the therapy. Therapy-related toxicity was tolerable, and a total of 40 Gy of irradiation was administered after autologous stem cell transplantation. The patient has remained in complete remission for 16 months since transplantation.

Introduction

Plasmablastic lymphoma (PBL) was proposed in 1997 as a new distinct subtype of diffuse large B-cell lymphoma (DLBCL).¹ PBL has a strong predilection for the oral cavity of HIV-positive patients, predominantly in males. The response duration is generally short, and the refractory/relapsed disease rate is high.^{2,3} Previously, we reported that a MEAM [MCNU (ranimustine), etoposide, cytarabine, melphalan] regimen with autologous stem cell trans-

plantation (ASCT) was effective and well tolerated in 3 patients with refractory or relapsed AIDS-related lymphoma, even though the patients were infected with HIV.⁴ Here, we report a case of relapsed PBL treated with high-dose chemotherapy using a MEAM regimen followed by ASCT.

Case Report

A 51-year-old Japanese man was diagnosed with HIV infection 8 years previously presented with night sweats, fever, weight loss, and a gingival ulcer. Gingival biopsy showed PBL [LCA⁺, CD20⁻, CD79a⁻, CD38⁺, CD138⁺, MUM-1⁺, CD30 (partially⁺), CD10⁻, CD3⁻, CD5⁻, CD56⁻, IgG⁺, IgA⁻, IgM⁻, lambda⁺, kappa⁻, EBER-ISH⁺, LMP-1⁻, and HHV-8 LANA-1 (latency-associated nuclear antigen-1)⁻], Mib-1 index 40-90% (Figure 1), and computed tomography (CT) confirmed stage IBE PBL. The patient's performance status was 1, and his serum LDH level was above normal. No bone marrow involvement was detected in the biopsy sample. His age-adjusted IPI (International Prognostic Index) was a low intermediate. The patient's HIV viral load was less than 50 copies/mL, and his CD4 count was 520 cells/ μ L. The patient was treated with 3 courses of CHOP (cyclophosphamide, adriamycin, vincristine, and prednisolone every 21 days, but not given G-CSF routinely), 36 Gy of involved-field radiation, and highly active antiretroviral

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Key words: autologous stem cell transplantation, plasmablastic lymphoma, AIDS.

Contributions: HG, chemotherapy and stem cell transplantation performing and manuscript preparation; SH, therapeutic strategy planning and manuscript preparation and editing; RH, chemotherapy and stem cell transplantation preparation and performing; TM, stem cell transplantation preparation and performing; HH, treatment for HIV infection planning; AT, TI, PBL diagnosis contribution; MM, figures preparation; KT, YK, SO, treatment for HIV infection supervision; AM, chemotherapy and stem cell transplantation supervision and PBL diagnosis contribution.

Conflict of interest: the authors report no conflicts of interest.

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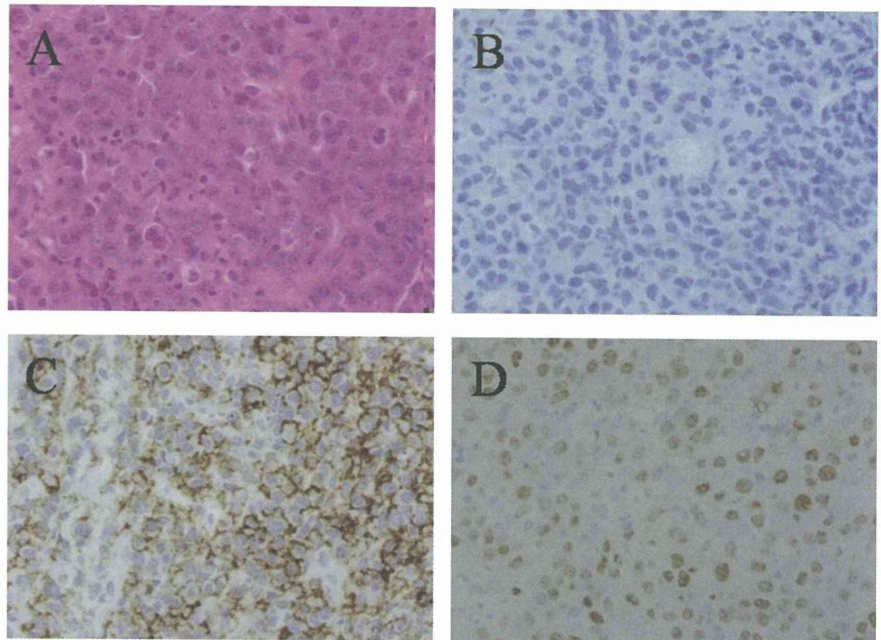


Figure 1. Pathologic findings in the oral cavity ($\times 400$). (A) Hematoxylin and eosin staining showing large cells with plasmablastic morphology. (B) The cells were negative for CD20. (C) The cells expressed CD138, which is a plasma cell-related antigen. (D) EBV-encoded RNA *in situ* hybridization (EBER-ISH) was positive.

therapy (HAART) with abacavir/lamivudine/nelfinavir concurrently. He achieved a complete remission (CR) confirmed by a CT scan and was followed up at our outpatient clinic for 1 year after the completion of treatment. However, the patient subsequently discontinued regular visits and HAART. Two years later, he developed an oral cavity tumor (Figure 2) and was readmitted to our hospital. A CT scan showed a gingival tumor with destruction of the maxillary bone. A fluorodeoxyglucose positron emission tomography (FDG-PET) scan (Figure 3a) showed increased uptake of FDG in the tumor lesion. The initial diagnosis was confirmed by a biopsy. The patient's HIV viral load was 1.2×10^5 copies/mL and his CD4 count was 90 cells/ μ L at the time of relapse. First, the patient was administered modified-ESHAP (etoposide, methylprednisolone, cytarabine, and carboplatin) as salvage chemotherapy, but there was no significant change in the tumor lesion after one course of modified-ESHAP. Next, he was treated with two courses of ICE (ifosfamide, carboplatin, and etoposide), and a reduction in tumor was achieved. We performed intrathecal administration of 15 mg methotrexate and 20 mg prednisolone on the day preceding each course. HAART with abacavir/lamivudine/raltegravir was administered concurrently with the chemotherapy. He was also treated with prophylactic agents such as sulbactam/ampicillin for aerobic and anaerobic bacteria in his oral cavity, acyclovir for herpes simplex and zoster, fluconazole for fungal infection, sulfamethoxazole/trimethoprim for *Pneumocystis jiroveci* (discontinued on day 1 of ASCT and restarted when the engraftment was confirmed), and azithromycin for *Mycobacterium avium-intracellulare* complex (MAC). At the time of hematological recovery from modified-ESHAP, a CD34-positive cell count of 28.3×10^6 cells/kg was obtained by G-CSF (2.5 μ g/kg lenograstim subcutaneously every 12 hours for 6 days). The patient achieved a partial response (PR) after completion of salvage therapy, and he was subsequently treated with MEAM followed by infusion of CD34+ cells at 4.7×10^6 cells/kg (Table 1). Nine days after transplantation, he achieved complete hematological recovery, and the regimen-related toxicity was mild showing grade 1 nausea, grade 2 diarrhea, and grade 3 febrile neutropenia. HIV control was optimal throughout transplantation, and the CD4 count rapidly increased after stem cell transplantation. At the time of relapse, the CD4 count was less than 100 cells/ μ L; however, 1 year after transplantation it increased to more than 400 cells/ μ L (Figure 4). The patient achieved CR after ASCT (Figure 3b). Subsequently, 40 Gy of irradiation was administered to the oral cavity and the left maxillary sinus. The patient has remained in complete remission for 16 months after transplantation.

Discussion

Plasmablastic lymphoma is a distinctive B-cell neoplasm that manifests diffuse proliferation of large atypical lymphoid cells, most of which resemble B-immunoblasts and have the immunophenotype of plasma cells.⁵ PBL is uncommon and accounts for 2.6% of all AIDS-related lymphomas.⁶ The clinical course is very aggressive. A retrospective analysis of 112 published cases showed that the refractory/relapsed disease rate was 54%; mortality rate was 53%, and the median overall survival rate was 15 months.³ Initially, our patient achieved a CR by chemoradiotherapy combined with HAART. However, the lymphoma relapsed 20 months after discontinuation of HAART. A meta-analysis showed that the prognosis of PBL was statistically better in patients who received HAART in addition to chemotherapy and/or radiotherapy than in patients who received chemotherapy and/or radiotherapy alone.⁷ Franciscini *et al.* reported a case of PBL that recurred after interruption of HAART thus substantiating the findings.⁸ Immunologic and virologic control with HAART may be beneficial for treating PBL and may possibly maintain continued CR.⁹ In our case, HAART was initiated with chemotherapy concurrently. We avoided the use of zidovudine and stavudine because of its associated bone marrow toxicity and neurotoxicity. The use of protease inhibitors was also avoided because of their inhibitory effect on CYP3A4. No serious side effects associated with HAART were noted in this patient.

Epstein-Barr virus (EBV) is identified in the neoplastic cells of approximately 40% of HIV-related lymphoma cases, and human herpesvirus (HHV)-8 is specifically associated with primary effusion lymphoma and Castleman disease.¹⁰ We detected the EBV genome in atypical cells by *in situ* hybridization, and it is considered to play a vital role in the pathogenesis of PBL.¹ Our case was negative for HHV-8 LANA-1. Previous studies have shown the absence of HHV-8 in oral PBL cells, but Cioc *et al.* reported the presence of HHV-8 in oral PBL cells using reverse transcriptase *in situ* PCR method.¹¹ It is possible that some cases of PBL are associated with HHV-8.

Recently, high-dose chemotherapy and ASCT as a salvage therapy for relapsed or

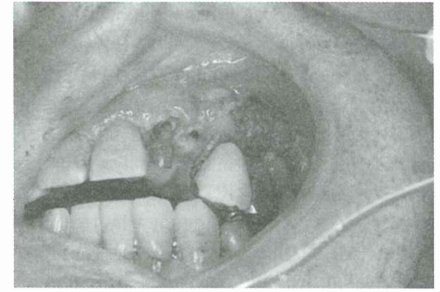


Figure 2. Clinical appearance of the lesion at relapse.

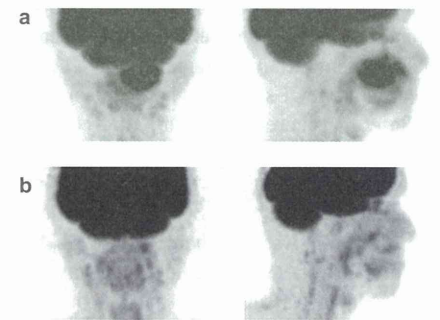


Figure 3. (a) Fluorodeoxyglucose positron emission tomography scan of the oral cavity prior to salvage therapy. FDG uptake was shown in the tumor. (b) Fluorodeoxyglucose positron emission tomography scan of the oral cavity after autologous stem cell transplantation. No abnormal uptake was present in this region.

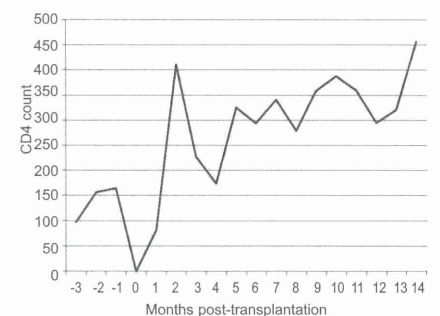


Figure 4. Transition of CD4+ cell counts (/μL) before and after autologous stem cell transplantation.

Table 1. MEAM regimen.

	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day 0
MCNU 300 mg/m ²		○					
Etoposide 200 mg/m ²		○	○	○			
Cytarabine 200 mg/m ²		○	○	○			
L-PAM 140 mg/m ²					○		SCT↓

refractory AIDS-related lymphoma was shown to be feasible and effective.^{12,13} However, its role in relapsed AIDS-related PBL was unclear. We found that at least 7 cases of AIDS-related PBL were treated with ASCT,^{3,12,14-17} but the efficacy of ASCT has not yet been analyzed. In this case, we performed ASCT using a MEAM regimen based on a BEAM regimen by replacing BCNU (carmustine) with MCNU. The regimen-related toxicity was mild. The patient developed febrile neutropenia but it was not caused by *Pneumocystis pneumonia*, MAC infection, or any other serious infection. He had an ulcer with necrotic tissue in his oral cavity which seemed to be related to a severe infection. The use of sulbactam/ampicillin as prophylaxis was effective. High-dose chemotherapy using a MEAM regimen with HAART followed by ASCT seemed to be well tolerated.

Re *et al.* showed that a low CD4 count, a poor performance status, and bone marrow involvement were negative prognostic factors in patients treated with ASCT.¹⁶ In our patient, the CD4 count was less than 100 cells/ μ L, the performance status was 1, and no bone marrow involvement was detected at the time of relapse. The patient is currently alive with no relapse or major opportunistic infection. The CD4 count increased rapidly after transplantation. Recently, some studies showed that immune recovery after ASCT for HIV-related lymphoma was similar to that observed in HIV-negative patients.^{18,19} High-dose chemotherapy followed by ASCT for relapsed AIDS-related PBL seems to be feasible and effective.

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SHORT REPORT

Paediatric HIV and elimination of mother-to-child transmission of HIV in the ASEAN region: a call to action

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Recent achievements in scaling up paediatric antiretroviral therapy (ART) have changed the life of children living with HIV, who now stay healthy and live longer lives. However, as it becomes more of a chronic infection, a range of new problems have begun to arise. These include the disclosure of HIV serostatus to children, adherence to ART, long-term toxicities of antiretroviral drugs and their sexual and reproductive health, which are posing significant challenges to the existing health systems caring for children with HIV with limited resources, experiences and capacities. While intensified efforts and actions to improve care and treatment for these children are needed, it is crucial to accelerate the prevention of mother-to-child transmission (PMTCT) of HIV, which is the main cause of paediatric HIV in the ASEAN region so as to eliminate the fundamental cause of the problem. This report argues that given over 70% of women have access to at least one antenatal care visit in the region and acceptance of HIV testing after receiving counselling on PMTCT could be as high as 90%, there is an opportunity to strengthen PMTCT services and eventually eliminate new paediatric HIV infections in the ASEAN countries.

Keywords: paediatric HIV; antiretroviral therapy; disclosure; prevention of mother-to-child transmission; elimination of paediatric HIV

Introduction

Of the two million children under 15 estimated to be living with HIV globally, 160,000 of them live in Asia and the Pacific. Among them 30,000 children were receiving antiretroviral therapy (ART) in 2008 with a proportion of them on treatment for several years (UNICEF, UNAIDS, WHO, & UNFPA, 2009). Recent achievements in scaling up services for the provision of paediatric ART have changed the life of children living with HIV; they can stay healthy, live longer lives and also attend school. However, as HIV infection becomes more of a chronic condition, a range of new problems and challenges have begun to arise.

The ASEAN and Japan HIV/AIDS workshop (ASEAN AIDS workshop) which aims at sharing the experiences and exploring possible solutions for common challenges among ASEAN member countries, namely Brunei Darussalam, Cambodia, Indonesia, the Lao People's Democratic Republic, Malaysia, Myanmar, the Philippines, Singapore, Thailand and Viet Nam, has been organised annually

since the year 2003 hosted by the Ministry of Health, Labour and Welfare Japan. The sixth ASEAN AIDS workshop was held in Tokyo in February 2009 with participants from ASEAN member countries, World Health Organization Regional Office for the Western Pacific, the National Center for Global Health and Medicine and the Ministry of Health, Labour and Welfare Japan. Paediatric HIV and prevention of mother-to-child transmission (PMTCT) of HIV was the one of the main issues discussed during the workshop.

Currently, relatively few initiatives have drawn attention on the issues of paediatric HIV and PMTCT of HIV in the region despite serious challenges they face and their negative consequences on children. The aim of this report is to highlight these issues by drawing the discussions and conclusions reached in the ASEAN AIDS workshop and the review of the existing evidences, and to contribute to the ongoing efforts to eliminate mother-to-child transmission of HIV, thus virtually eliminating paediatric HIV in the region.

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Challenges in paediatric HIV care, support and treatment

Four major challenges on paediatric HIV care, support and treatment were raised and discussed during the workshop. The first issue concerned with the disclosure of HIV serostatus to children, a highly complex issue as also reported by several authors (Lesch et al., 2007; Wiener, Mellins, Marhefka, & Battles, 2007). The participants reported that the challenges often combine the reluctance of families to disclose HIV infection to their children together with a limited experience of health care providers in managing this critical step. Although most countries represented in the workshop have reportedly set 10 years of age as the time for disclosure, doubts about its appropriateness were voiced by participants, along with concerns related to a lack of experience and confidence about the process of disclosure. The workshop agreed that the most appropriate time would be when children start asking questions regarding their condition and reasons for the treatment, and that disclosure should be done gradually and through age-sensitive approaches and contents.

The second issue referred to adherence to anti-retroviral (ARV) treatment. It was stated that relatively high rates of adherence are currently reported among young children, whose compliance almost entirely relies on their caregivers. However, it appears that adherence gradually decreases as these children become adolescents, and accordingly assume more responsibility for their medication-taking. Adolescents often discontinue medication due to medication fatigue (Saitoh et al., 2008), which was also reported from the workshop participants. It is also known that adolescents sometimes use non-adherence to medication as a way to express their autonomy (Simoni et al., 2007). Moreover, appearance change due to lipoatrophy or even the perception of this potential risk could lead adolescents to discontinue medication (DeLaMora, Aledort, & Stavola, 2006). Keeping adherence rates among paediatric patients, especially adolescents, continues to pose a major challenge.

Thirdly, as children living with HIV grow into adolescents, sexual health becomes a significant issue of concern. They start to develop intimate relationships, which bring new complex issues, such as disclosure of their HIV status to their boyfriend/girlfriend, fear of stigmatisation and discrimination, and risk of transmission of HIV infection (Thorne et al., 2002). Furthermore, it was reported that in some western countries their perspective marriage, pregnancy and parenting were raising additional challenges and concerns.

Fourth, long-term toxicities of ARV drugs, such as mitochondrial toxicity and metabolic abnormality, are of significant relevance for paediatric cases, as length of treatment may be expected to span several decades. A WHO survey found that in Cambodia, among 3236 children who were on the first line regimens in 2009, 3058 (94.5%) of them were on regimens including d4T and in Viet Nam, 1324 children among 1817 (72.9%) were on regimens including d4T (WHO, 2010). Since the majority of children are younger than 10 years of age, it is expected that most of them may experience d4T-related toxicities such as lipoatrophy as they become teenagers. As mentioned above, lipoatrophy is reported as one of the main obstacles to drug adherence among adolescents in developed countries (DeLaMora et al., 2006). As the new WHO guidelines recommend phasing out the use of d4T (WHO, 2009a), awareness of this potential risk should be raised and strategies for more appropriate regimen choices should be discussed.

As argued above, paediatric HIV is already confronted with several challenges; it is expected that these problems could become more complex in the future as children grow into adolescents and young adults. Continuous, dedicated efforts and infinite patience from children themselves as well as support from family and health care providers are necessary to keep and achieve high adherence to ART. Drug toxicities are unavoidable unless new less toxic ARVs are produced and made available at affordable cost for resource-limited settings. Psychosocial impact related to HIV infection in children is immense and immeasurable, which may affect them not only psychologically but also socially, for example, by affecting their relationships with others and their future opportunities. This workshop agreed that all these challenges require immediate attentions and dedicated efforts are urgently needed to overcome these seemingly inextricable difficulties.

Strengthening prevention of mother-to-child transmission (PMTCT) of HIV

While intensified efforts and actions to improve care, support and treatment for children living with HIV are needed urgently, it is fundamental and crucial to strengthen HIV prevention and stop new infection from occurring among children. Since over 90% of new HIV infections among infants and young children occur through mother-to-child transmission of HIV (WHO & UNICEF, 2007), it is obvious that prevention remains the top priority. It is well-documented that focused and well-established interventions for

PMTCT have virtually eliminated paediatric HIV in high-income countries, with antenatal care (ANC) playing an important role as a platform for HIV testing and provision of prevention services (WHO & UNICEF, 2007).

As shown in Table 1, although most countries report high-ANC coverage ranging from 70% to over 90% (UNICEF et al., 2009), coverage of PMTCT service in this region remains low with estimated coverage of 25% in average (WHO, UNAIDS, & UNICEF, 2009). This indicates that weak PMTCT services and low coverage rates are leaving mother-to-child transmission of HIV largely unabated and resulted in high number of new paediatric infections, which in turn aggravate the complex problems related to the management of paediatric HIV, especially in settings where available expertise for such services is quite limited. In the absence of stronger and more effective PMTCT services, it is expected that the number of children with HIV will continue to increase and pose additional challenges to health systems.

One of the reasons suggested to explain the low coverage of PMTCT services is the dominance of treatment and care over prevention in HIV control efforts (Horton & Das, 2008). Other reasons include limited financial and human resources (Paintsil & Andiman, 2009); fragile maternal, newborn, and child health (MNCH) services; low acceptance of HIV testing due to the poor quality of services; limited information; lack of access to ART (Dahl, Mellhammar, Bajunirwe, & Bjorkman, 2008); and problems related to the involvement of male partners (Kakimoto et al., 2007). Moreover, weak coordination within ministries of health and among public

health programmes, often caused by incoherent policies, financing and institutional mechanisms, is often recognised as a significant factor (Druce & Nolan, 2007).

However, there is now an effort to strengthen PMTCT services in the region by linking HIV and MNCH services by applying the Asia-Pacific operational framework for linking HIV/STI services with reproductive, adolescent, MNCH services, which is known as Guilin Framework (WHO, UNICEF, UNFPA, & UNAIDS, 2008) with some promising success in some countries. The Asia-Pacific United Nations task force for the prevention of parent-to-child transmission of HIV has also strongly committed to pursue the virtual elimination of paediatric HIV in the region.

Call to action

Given that over 70% of women have access to at least one ANC visit in the region, and acceptance of HIV testing after receiving counselling for PMTCT could be as high as 90% (Kakimoto et al., 2007; Pai et al., 2008), there is an opportunity to strengthen PMTCT services, all the more so in the ASEAN region with low HIV prevalence and a relatively well-established health infrastructure compared to other resource-limited countries. Drawing from successful experiences of countries like Thailand, where rates of new HIV infections through mother-to-child transmission have been dramatically reduced from 6.4% in 2001 to 1.3% in 2006 (National AIDS prevention and alleviation committee, 2008), the workshop recognised political commitment and full integration of PMTCT

Table 1. Prevention of mother-to-child transmission of HIV in the ASEAN region.

	Estimated adult HIV prevalence rate (%), 15–49 years), 2007 ^a	ANC coverage (%), 2003–2008 ^a	Percentage of pregnant women with HIV who received ARVs for PMTCT: low–high estimates (%), 2008 ^b	Children (0–14 years) living with HIV, 2007 ^a
Brunei Darussalam	–	–	–	–
Cambodia	0.8	69	35– > 95	4400
Indonesia	0.2	93	4–15	4360 ^c
Lao PDR	0.2	35	8–28	–
Malaysia	0.5	79	10–39	–
Myanmar	0.7	–	14–65	–
Philippines	–	91	<1–1	50 ^c
Singapore	0.2	–	–	–
Thailand	1.4	98	33– > 95	14,000
Viet Nam	0.5	91	27–87	3055 ^c

^aSee UNICEF et al. (2009).

^bSee WHO et al. (2009).

^cThe sixth ASEAN and Japan HIV/AIDS Workshop 2009 country report.

services into well-organised MNCH care systems as a key to success.

With the new WHO rapid advice on use of ARV drugs for treating pregnant women and preventing HIV infection in infants recommending the use of more efficacious ARV regimens (WHO, 2009b), it is believed that through close linkage and collaboration between HIV and MNCH programmes sustained by strong political commitment and joint effort from all the stakeholders, elimination of paediatric HIV in the ASEAN region is an achievable goal.

Acknowledgements

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Identification of a Current Hot Spot of HIV Type 1 Transmission in Mongolia by Molecular Epidemiological Analysis

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Abstract

We investigated the current molecular epidemiological status of HIV-1 in Mongolia, a country with very low incidence of HIV-1 though with rapid expansion in recent years. HIV-1 *pol* (1065 nt) and *env* (447 nt) genes were sequenced to construct phylogenetic trees. The evolutionary rates, molecular clock phylogenies, and other evolutionary parameters were estimated from heterochronous genomic sequences of HIV-1 subtype B by the Bayesian Markov chain Monte Carlo method. We obtained 41 sera from 56 reported HIV-1-positive cases as of May 2009. The main route of infection was men who have sex with men (MSM). Dominant subtypes were subtype B in 32 cases (78%) followed by subtype CRF02_AG (9.8%). The phylogenetic analysis of the *pol* gene identified two clusters in subtype B sequences. Cluster 1 consisted of 21 cases including MSM and other routes of infection, and cluster 2 consisted of eight MSM cases. The tree analyses demonstrated very short branch lengths in cluster 1, suggesting a surprisingly active expansion of HIV-1 transmission during a short period with the same ancestor virus. Evolutionary analysis indicated that the outbreak started around the early 2000s. This study identified a current hot spot of HIV-1 transmission and potential seed of the epidemic in Mongolia. Comprehensive preventive measures targeting this group are urgently needed.

Introduction

MONGOLIA HAS A LOW PREVALENCE of HIV with estimated infected individuals comprising less than 0.01% of the general population. However, this number is increasing rapidly and recent statistical data estimated a 10-fold increase in HIV/AIDS incidence during the past 5 years. Since 1992, when data on HIV/AIDS began to be compiled in Mongolia, there had been only five cases reported as of December 2004. In 2005, the number of infected cases increased sharply and 11 cases were registered in that year. According to an unpublished report from the Ministry of Health Mongolia, the total number of HIV infected cases was 56 as of May 2009. The infected individuals were men who have sex with men (MSM) 64.3%, heterosexual males (HSM) 14.3%, and females (HSF) 21.4%, of whom 50% were female sex workers (FSW).

The Second Generation HIV/Sexually Transmitted Infections (STI) surveillance program (SGS) for HIV/STI serolog-

ical studies in various behaviors was initiated in Mongolia in 2002. According to the latest results of the SGS conducted in 2007, the HIV prevalence among blood donors and pregnant women was zero and among 1350 tuberculosis patients was 0.15% (95% CI 0.00–0.35).¹ The results of our 2007 prevalence survey of 2465 individuals (1415 high-risk and 1050 healthy control populations) in Mongolia demonstrated that the current HIV prevalence is low, but according to the high prevalence of syphilis (anti-TP 23.1%) and HCV (anti-HCV 8%) in high-risk populations, the risk status for HIV-1 infection is estimated to be high.² The high-risk populations included FSW, MSM, mobile men, tuberculosis (TB) patients, and male STI clinic clients; healthy control populations were youth and blood donors.

Knowledge about current patterns and trends of HIV infections is essential for planning and evaluating prevention programs and for resource allocation. In the past, epidemiological data on newly diagnosed HIV/AIDS and results of a

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surveillance study of HIV/STI have been used for planning and targeting HIV prevention programs in Mongolia. However, these data were not sufficient to implement a comprehensive, effective, targeted strategy for prevention of HIV infection in Mongolia. To gain a better understanding of the current HIV status, a more detailed molecular epidemiological study using phylogenetic analyses is needed. Molecular epidemiological analyses are useful tools to gain information about the origin of HIV epidemics and transmission patterns.^{3,4} Moreover, information regarding the genetic diversity of HIV strains, and the geographic prevalence of genotypes, is important for the evaluation of diagnostic tests and vaccines and for their effective application.^{5,6}

In the present study, we used phylogenetic analyses to determine the HIV-1 subtypes circulating in Mongolia, especially the dominant subtypes responsible for the outbreak in the infected population. Then, we performed a Bayesian coalescent-based framework to investigate the origin and estimate the onset year of the dominant HIV-1 subtypes in Mongolia.

Materials and Methods

Study population

After obtaining informed consent, blood samples were collected anonymously from 39 Mongolian HIV-1-positive patients attending the National Center for Communicable Diseases of Mongolia (NCCD) in November 2007 and May 2009. For all of them, HIV infection was diagnosed from 1997 to 2009, and all participants were infected through sexual contact. In this study, we also examined two stored serum samples at the NCCD obtained from a Russian married couple who were diagnosed with HIV-1 in Mongolia in 2007. Therefore, a total of 39 (69.6%) samples out of all 56 reported cases in Mongolia as of May 2009 was examined in this study. The study subjects were 32 men [mean age (\pm SD): 31.3 \pm 7.8 years, range: 17–52 years] and 9 females (25.9 \pm 5.1, 18–33 years) and 63.4% of them had a high level (college/university) of education (Table 1). The *StatView* software version 5.0 (SAS Institute, Cary, NC) was used for statistical analyses. The collected serum samples were sent to the AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, Japan, for further analysis. The study protocol was approved by the ethics committees of NCGM (H19-448 and H20-545) and the Ministry of Health, Mongolia (2007-#7 and 2008-#3).

Amplification and sequencing of HIV-1

Total RNA was extracted from 140 μ l of serum, using the QIAamp RNA Mini Kit (Qiagen, Valencia, CA) according to the instructions supplied by the manufacturer. HIV-1 cDNA was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) using the TaKaRa One Step RNA PCR kit (AMV, TaKaRa Bio Inc., Japan) and then DNA fragments were amplified using the TaKaRa Ex Taq Hot Start Version (TaKaRa Bio Inc, Japan) with the following primer sets. A total of 1065 bp of the polymerase (*pol*) fragment (HXB2: 2243–3308) containing the regions encoding the full protease and first 560 nucleotides of RT was amplified by RT-PCR with primers of F-1849 (5'-GAT GAC AGC ATG TCA GGG AG-3') and R-3500 (5'-CTA TTA AGT CTT TTG ATG GGT CAT AA-3'). DRPRO5 (5'-AGA CAG GYT AAT TTT TTA GGG A-3'),

TABLE 1. EPIDEMIOLOGICAL CHARACTERISTICS OF THE 41 HIV-1 INFECTED PATIENTS

Characteristics	Male (%)	Female (%)
Gender	32 (78)	9 (22)
Age groups (based on age at first diagnosis)		
<20	1 (2.4)	1 (2.4)
20–29	15 (36.6)	5 (12.2)
30–39	10 (24.4)	2 (4.9)
40–49	4 (9.8)	0 (0)
>50	1 (2.4)	0 (0)
Unknown	1 (2.4)	1 (2.4)
Ethnicity		
Mongolian	31 (75.6)	8 (19.5)
Russian	1 (2.4)	1 (2.4)
Transmission category		
Homosexual	27 (65.8)	0 (0)
Heterosexual	4 (9.8)	9 (22) ^a
Unknown	1 (2.4)	0 (0)
Education		
Uneducated	1 (2.4)	0 (0)
Primary	0 (0)	0 (0)
Incomplete secondary	2 (4.9)	3 (7.3)
Complete secondary	5 (12.2)	2 (4.9)
College/university	23 (56.1)	3 (7.3)
Unknown	1 (2.4)	1 (2.4)
HIV-1 subtype		
B	30 (73.1)	2 (4.9)
C	0 (0)	2 (4.9)
G	2 (4.9)	0 (0)
CRF01_AE	0 (0)	1 (2.4)
CRF02_AG	0 (0)	4 (9.8)

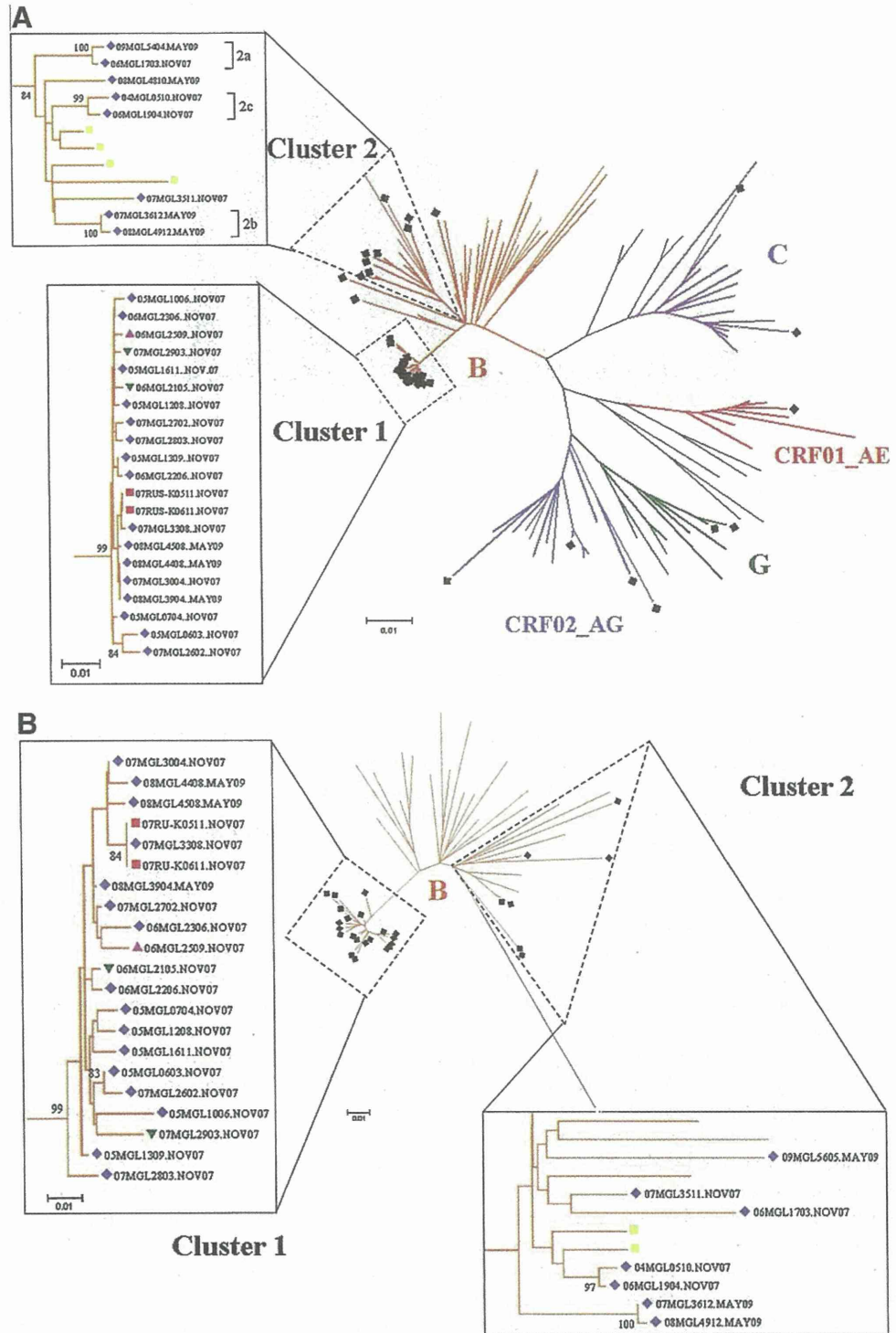
^aFive (55.6%) of the female cases were female sex workers.

DRPRO2L (5'-TAT GGA TTT TCA GGC CCA ATT TTT GA-3'), DRRT1L (5'-ATG ATA GGG GGA ATT GGA GGT TT-3'), and DRRT4L (5'-TAC TTC TGT TAG TGC TTT GGT TCC-3') primer sets were used for nested PCR. A total of 447 bp of the envelope (*env*) fragment (HXB2: 6834–7281) containing the region C2V3 was amplified by RT-PCR with primers of V1V2-1 (5'-TGT GTA CCC ACA GAC CCC AAC CC-3') and IC462M (5'-GCC CAT AGT GCT TCC TGC TGC T-3'). ENV02 (5'-ATG GTA GAA CAG ATG CAT GA-3') and E115 (5'-AGA AAA ATT CCC CTC CAC AAT TAA-3') primer sets were used for nested PCR. The amplified DNA was purified using the QIAquick PCR purification kit (Qiagen Inc.) according to the protocol provided by the manufacturer. Purified DNA was sequenced by using the ABI BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and processed with an automated ABI 3730 DNA Analyzer (Applied Biosystems).

HIV-1 subtype determination and distance-based phylogenetic inference

Analyses of HIV-1 subtype and circulating recombinant form were performed using the REGA HIV-1 Subtyping Tool.⁷ The basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) was used to search and select the HIV-1 reference sequences with the highest similarity score (>95%) to the Mongolian strains. Additional reference

FIG. 1. Distance-based phylogenetic tree. Unrooted radial phylogeny of (A) 41 tested sequences and 67 reference HIV-1 *pol* (PR to RT; 1065 nt) sequences with different subtypes; (B) 28 tested sequences and 21 reference HIV-1 subtype B *env* (C2V3; 447 nt) sequences. The color of the branches represents the different subtypes and recombinant forms. *Solid diamonds*: sequences from Mongolians. The clustered sequences are framed and subtrees of the clusters are represented in the rectangle panels on the left. Numbers on the branch in the subtrees represent bootstrap probabilities. The different symbols and different colors in the subtrees indicate risk factors and/or countries of origin of sequences [*blue diamonds*: MSM (men who have sex with men); *green triangles*: HSM (heterosexual male); *pink triangle*: HSF (heterosexual female) for Mongolians only; *red squares*: sequences isolated from Russian patients; *yellow squares*: reference sequences of Korean origin].



sequences of different subtypes and recombinant forms were retrieved from the Los Alamos National Laboratory (www.hiv.lanl.gov). Sequences were aligned using the CLUSTAL-W software in the MEGA (molecular evolutionary genetics analysis) version 4.1.⁸ Phylogenetic trees were constructed by the neighbor-joining (NJ) method based on the Maximum Composite Likelihood distance matrix listed in the MEGA software. The reliabilities of the branching patterns were tested by bootstrap analysis with 1000 replicates.

Bayesian coalescent inference using the subtype B sequences

Evolutionary rates, molecular clock phylogenies, and other evolutionary parameters were estimated from heterochronous genomic sequences of subtype B in Mongolian, non-Mongolian, and some reference sequences by using the Bayesian Markov chain Monte Carlo (MCMC) method. The reference sequences were obtained from the HIV sequence