Serum (1→3) β-D-Glucan as a Noninvasive Adjunct Marker for the Diagnosis and Follow-Up of *Pneumocystis jiroveci* Pneumonia in Patients with HIV Infection

To THE EDITOR—We read with great interest the Brief Report by Watanabe et al [1] about the value of the (1→3) β-D-glucan (BG) assay as an adjunct for the diagnosis of *Pneumocystis jiroveci* pneumonia (PJP) in patients with AIDS. We congratulate the authors because their report has a large study population (111 patients with PJP and a control group with 425 patients). However, we would appreciate your taking into account the following observations.

First, we wonder whether the report is of a prospective study or a retrospective analysis of test performance in those patients with confirmed PJP.

Second, when using a control group, it is important to define accurately the risk factors of the matched control group, because the possible development of PJP is dependant on the host risk(s) for disease. From our point of view, in this setting, the control group should include human immunodeficiency virus (HIV)–positive patients with $CD4^+$ cell counts ≤200 cells/mm^3 or a $CD4^+$ cell percentage ≤14% with a clinical respiratory infection. These characteristics are not described by the authors in their control group.

Third, we would like to remark that the accuracy of a diagnostic test is defined by calculating the cutoff value, the sensitivity, the specificity, and the positive and negative predictive values. In their study, Watanabe et al [1] only report the sensitivity and specificity. In the clinical settings in which BG is used, negative predictive value is high, and it is consequently important to rule out the diagnosis of PJP and other invasive fungal diseases.

Fourth, bacterial pneumonia is a common respiratory infection in this subpopulation, with a 20% rate of positive blood culture results. Both gram-positive and gram-negative bacteremias have been reported to be the source of false-positive BG results [2]. The administration of some antibiotics may also be a cause of BG reactivity [3]. We miss these pertinent data in the cohort assessed by Watanabe et al [1], because both are possible confounding factors.

Fifth, Watanabe et al [1] state that serum BG levels are not suitable for monitoring the response to treatment and that they do not always return to normal levels during treatment. We agree that BG does not return to normal levels during the course of treatment, because 3 weeks is not enough time to achieve a serological cure, which usually requires several weeks after the end of treatment [4]. Our group [4] and others [5, 6] have reported that the kinetics of measured BG (Fungitell; Associates of Cape Cod) suggest that decreasing levels of BG correspond to a favorable response to treatment (Figure 1), whereas increasing levels are associated with treatment failure [4].

Therefore, we believe that prospective

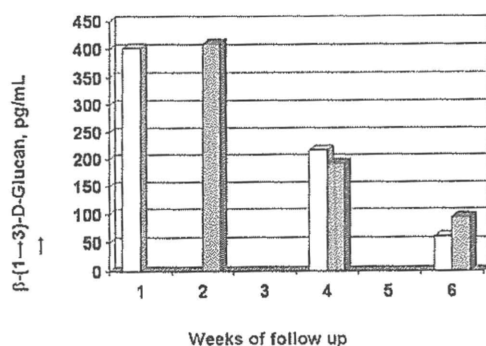

Figure 1. Kinetics of (1→3) β-D-glucan levels in a patient with *Pneumocystis jiroveci* pneumonia and human immunodeficiency virus infection *(white bars)* and in a renal transplant recipient with *P. jiroveci* pneumonia *(gray bars)*. Both patients responded to the treatment and survived the infection.

studies are needed to further evaluate the accuracy of serum BG assay for the diagnosis and follow-up of PJP in HIV-positive patients.

Acknowledgments

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Reply to del Palacio et al

To the Editor—We thank Palacio et al [1] for their interest in our study [2]. We would like to reply to their 5 comments.

First, our study [2] was performed retrospectively and analyzed >100 confirmed cases of *Pneumocystis* pneumonia (PCP).

Second, patients who had human immunodeficiency virus infection but did not have PCP were used as a control group, regardless of their CD4$^+$ cell counts in our original study. Among the 425 control patients, 273 had CD4$^+$ cell counts that were <200 cells/μL. If we analyzed data for these 273 patients, the median serum β-D-glucan level was 8.6 pg/mL (range, 1.0–283.0 pg/mL), which was almost same as the median value for all 425 control patients. This data indicates that serum β-D-glucan level is not influenced by CD4$^+$ cell counts.

Third, using the cutoff value of 23.2 pg/mL, positive predictive value and negative predictive value were 67.3% and 98.9%, respectively.

Fourth, the diagnosis of PCP was established by identification of *Pneumocystis jirovecii* in bronchoalveolar lavage (BAL) fluid with use of Diff-Quik (Dade Behring) staining method. We also examined the same sample by Gram stain and Ziehl-Neelsen stain for the detection of bacteria and mycobacteria, respectively, in BAL fluid. In addition, there were no patients who had comorbidity due to bacterial pneumonia or pulmonary tuberculosis. Therefore, it is unlikely that our data were biased by confounding factors, such as bacterial pneumonia or administration of antibiotics.

Fifth, as we mentioned in our report [2], β-D-glucan levels generally decrease soon after treatment in patients who experience a good clinical course, as Palacio et al [1] have presented, and levels are normalized several months or years after treatment in all patients. However, β-D-glucan levels are elevated in ~20% of patients during the early phase of treatment, and β-D-glucan levels seldom return to the normal level within a 21-day treatment period. In accordance with the Centers for Disease Control and Prevention guidelines [3], treatment of PCP is usually completed by 21 days in our hospital, regardless of the β-D-glucan levels, and there were no patients who experienced relapse caused by the cessation of treatment after 21 days. Therefore, it is apparent that increase of β-D-glucan levels soon after treatment does not always indicate treatment failure. Consequently, we concluded that serum β-D-glucan is a useful adjunct marker for diagnosis of PCP but is not suitable for monitoring of the disease.

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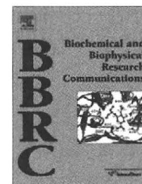

Naturally arising HIV-1 Nef variants conferring escape from cytotoxic T lymphocytes influence viral entry co-receptor expression and susceptibility to superinfection

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ABSTRACT

HIV-1 Nef is a key factor for pathogenesis and is known to down-regulate functionally important molecules, including viral entry co-receptor CCR5 and CXCR4, from the surface of HIV-infected cells. Some of these Nef activities are mediated by the well-conserved proline-rich region of Nef, and this region is highly targeted by cytotoxic T lymphocytes (CTLs). In the present study, we asked whether Nef variants selected under CTL-mediated selective pressure *in vivo* may constrain these important Nef activities. The analysis of autologous *nef* sequences isolated from a cohort of total 235 subjects in Japan revealed that the subjects showing amino acid variations, such as Arg75Thr and Tyr85Phe, located within the proline-rich region were significantly over-represented by those having *HLA-B*3501*. CTL assays corroborated that these mutations conferred escape from HLA-B*3501-restricted CTLs. The Arg75Thr variant Nef selectively impaired CCR5, but not CXCR4, down-regulation activity from the cell surface; whereas the Tyr85Phe variant Nef affected neither CCR5 nor CXCR4 down-regulation activity. Moreover, the cells expressing the Arg75Thr variant Nef significantly impaired protection from superinfection by CCR5-tropic, but not CXCR4-tropic, viruses. These results highlighted the importance of certain Nef-specific CTLs in modulation of viral co-receptor down-regulation activity and protection from HIV-1 superinfection, providing us with additional insight into vaccine design.

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1. Introduction

The HLA class I-restricted CD8$^+$ cytotoxic T lymphocyte (CTL) response is thought to play an important role in controlling HIV replication *in vivo* during an HIV infection [3,8,17]. Over the natural course of an infection, the CTL response acts as a major selective force driving HIV evolution, resulting in the selection of CTL escape mutants [7,14]. However, although CTL escape mutations enable the evasion of host immune responses, the requirements to preserve the basic structure and function of viral proteins may limit the accommodation of such sequence changes. Indeed, some CTL escape mutations modulate viral replication and may even revert upon transmission to an HLA-mismatched host [9,10]. It has also been reported that certain CTL escape mutations in well-conserved regions of Gag and Nef can impose functional constraints on these proteins and thus modulate viral replication [6,19,21].

It is well known that a number of viruses, including HIV-1 [13] as well as measles virus [18], influenza virus [12], hepatitis B virus [4], and retroviruses [16], evolved to gain ways to prevent a second infection or superinfection of cells in which viral replication has been already established. The protection against superinfection may be an important capacity of viruses for efficient replication and persistence in the host. Although different subsets of HIV-1 infect CD4-expressing cells either through CCR5 or CXCR4 as a co-receptor, the Nef protein of HIV-1 plays an important role in the down-regulation of all these viral receptors, e.g., CD4 [1], CCR5 [13], and CXCR4 [23], on the surface of virus-infected cells, and thus protects them from superinfection [13,23]. The well-conserved proline-rich region of Nef is important for the down-regulation of both chemokine receptors, CCR5 and CXCR4 [13,23], whereas a different motif of Nef is responsible for CD4 down-regulation [1]. We thus postulated that certain CTL responses toward this functional proline-rich region of Nef may constrain the down-regulation activity of chemokine receptors by Nef.

In the present study, to explore this hypothesis, we first analyzed the autologous *nef* sequences isolated from a total of 235 HIV-infected subjects. Indeed, two amino acid variations located in the proline-rich region of Nef appeared to be significantly over-represented in patients having HLA-B*3501. We further examined whether such naturally-arising Nef mutations influenced

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viral receptor down-regulation activity and the protection from superinfection.

2. Materials and methods

2.1. Genetic analysis of autologous nef genes

A total of 235 HIV-infected subjects followed at the AIDS Clinical Center, National Center for Global Health and Medicine of Japan, were enrolled in this study. Subjects were selected based on the availability of plasma and HLA class I alleles. HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 15 min) of patients' plasma, after which the viral RNA was extracted from them. DNA fragments encoding Nef proteins were amplified by a nested PCR, gel purified, and sequenced directly as previously described [20,21]. The study was conducted in accordance with the human experimentation guidelines of the International Medical Center of Japan and Kumamoto University.

2.2. Plasmid construction

DNA fragments encoding Nef (strain SF2) and GFP fusion proteins were cloned into plasmid pcDNA3.1 (Invitrogen Corp., Carlsbad, CA) as described previously [20]. The Nef variants created were as follow: Arg75Thr (75T), Tyr85Phe (85F), Arg75Thr plus Tyr85Phe (TF), Pro76Ala plus Pro79Ala (AxxA), and Glu174Ala plus Asp175Ala (EDAA).

2.3. Cytotoxic assays

CTL clones established previously [20,21] were maintained in RPMI1640 medium containing 10% FCS and 100 U/ml of recombinant IL-2 and stimulated with synthetic cognate peptides every 10–14 days. For target cells, mRNA encoding GFP alone or Nef-GFP fusion proteins having various mutations of interest were prepared by conducting *in vitro* transcription reactions, and then delivered to C1R cells expressing HLA-B*3501 (C1R-B3501) as described previously [20]. It should be noted that 15 ± 5% of the cells had died (positive for 7-amino actinomycin D [7-AAD; BD Biosciences, San Jose, CA] staining) by 16 h and that 85 ± 5% cells of the viable cells expressed GFP as revealed by flow cytometric analysis. At 16 h after transfection, CTL clones were then added; and their cytotoxic activity was determined by performing the standard ^{51}Cr-release assay described previously [22].

2.4. Receptor down-regulation analysis

TZM cells [24] were transfected for 48 h with plasmid DNAs encoding GFP alone or Nef-GFP fusion proteins having various mutations of interest. The resultant cells were stained with phycoerythrin (PE)-Cy7-conjugated anti-human CCR5 mAb (BD Biosciences, San Jose,CA), allophycocyanin-Cy7 anti-human CD4 mAb (Biolegend, San Diego, CA), allophycocyanin-conjugated anti-human CXCR4 mAb (R&D systems, Minneapolis, MN), and 7-AAD. The cells were then analyzed by flow cytometry (FACS Canto II, BD Biosciences, San Jose, CA).

2.5. Superinfection assay

For preparation of infectious HIV-1 particles, 293T cells were transfected with NL43 and JRFL proviral clones, after which the culture supernatant was collected 48 h later, as previously described [21]. The resultant virus stocks were quantified by ELISA for their p24 Gag concentration (ZeptoMetrix Corporation. Buffalo, NY). TZM cells [24], seeded at 5×10^5 cells/well in a 6-well plate, were first transfected for 24 h with plasmid DNAs encoding GFP alone or Nef-GFP fusion proteins having various mutations of interest, collected, and re-seeded at 2×10^5 cells/well in a 24-well plate. At 24 h after transfection, the resultant cells were then exposed to wild-type HIV-1, strain JRFL or NL43, at 100 ng/well of p24 Gag. At 48 h after infection, the resultant cells were stained with 7-AAD followed by intracellular staining with PE-labeled anti-p24 Gag mAb (KC-57; Beckman Coulter, CA), and analyzed by flow cytometry. The live GFP$^+$ subsets were gated and analyzed for their frequency of p24 Gag-expressing cells.

2.6. Statistical analysis

Statistical analysis and graphical presentations were done by using a computer program, SigmaPlot, with a statistical package (Hulinks, Inc., Tokyo, Japan). The results were given as the mean ± standard deviation. Statistical analysis of significance (p values) was based on the χ^2 test or one-way analysis of variance (ANOVA), and $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Mutational escape of HIV-Nef from CTLs

We first analyzed the autologous *nef* sequences isolated from plasma samples of 235 HIV-infected individuals in Japan and found that amino-acid variations at positions 75 and 85 (numbering based on SF2 strain) located in the proline-rich region in Nef were significantly over-represented in subjects having *HLA-B*3501*; whereas amino acid residues at other positions in this region were highly conserved (Fig. 1). More specifically, Arg75 to Thr (75T) and Tyr85 to Phe (85F) in Nef were more frequently observed in HIV-infected individuals with *HLA-B*3501* than in those negative for *HLA-B*3501* (Fig. 1).

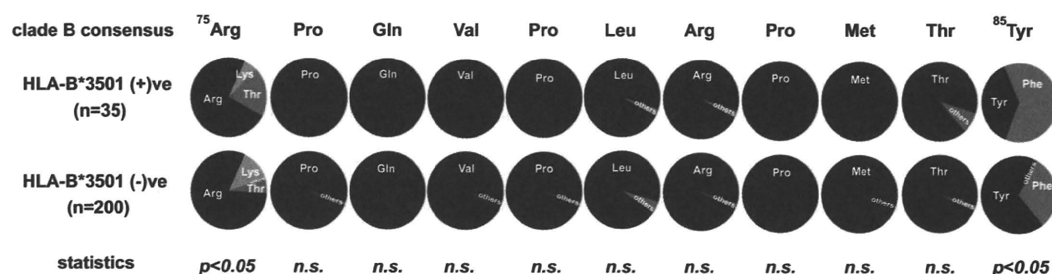

Fig. 1. Genetic analysis of autologous *nef* genes. A total of 235 HIV-infected subjects were divided into those negative or positive for *HLA-B*3501*. The amino acid variations at the proline-rich region of Nef (positions from 75 to 85) are shown. Amino acid sequence of the clade B consensus in this region is given at the top. The pie charts show the frequencies of individuals whose autologous Nef had the amino acid sequences indicated in the charts. Statistical analysis was performed by using the χ^2 test. *n.s.*, not significant.

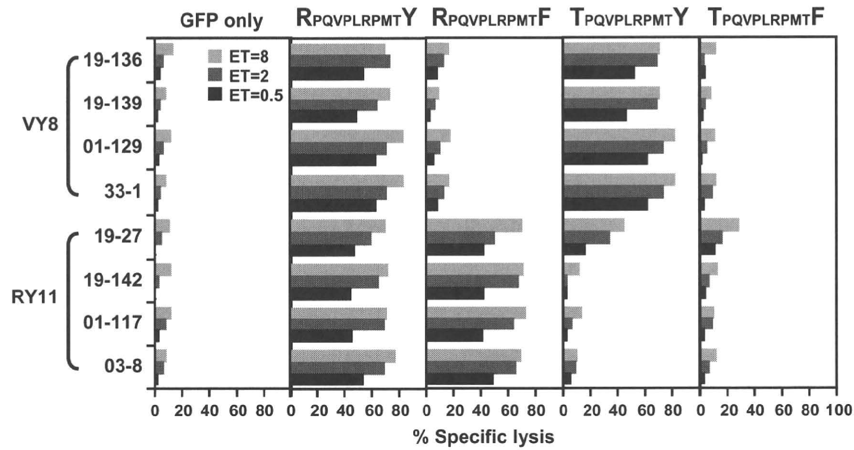

Fig. 2. Effects of the Nef variants on CTL recognition. The HLA-B*3501-restricted CTL clones specific for VY8 (VPLRPMTY) or RY11 (RPQVPLRPMTY) epitopes in Nef were analyzed for their cytolytic activity toward target cells at effector-to-target cell ratios (ET) of 0.5, 2, and 8, as indicated. The target cells were C1R-B3501 cells that had been transfected with DNAs encoding GFP alone or wild-type or various Nef variants. CTL clones were derived from four different donors: CTL19-136, 19-139, 19-27, and 19-142 were from patient No. 19; CTL01-117 and 01-129, from patient No. 1; and CTL 33-1 and 03-8, from patient Nos. 33 and 03, respectively. An additional experiment showed similar results.

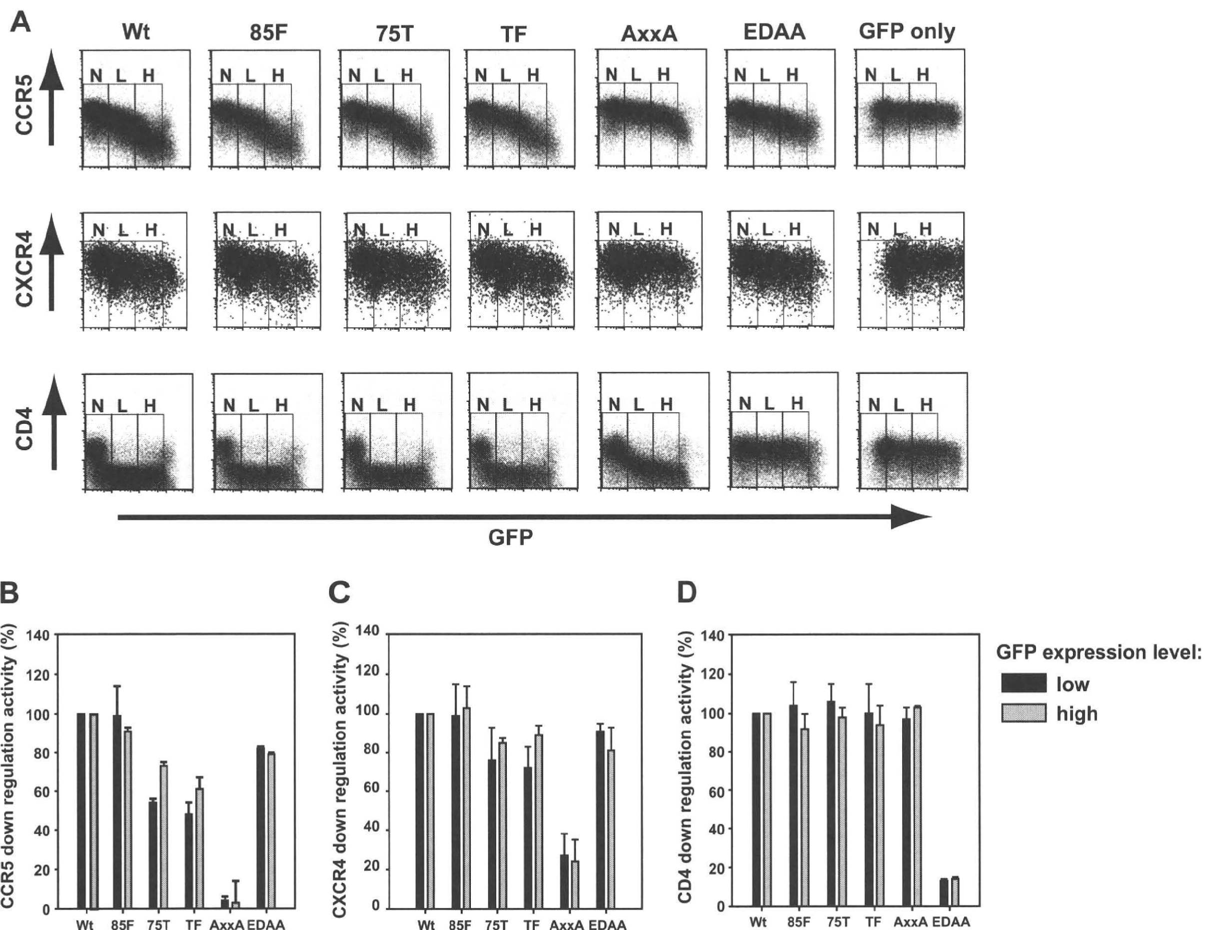

Fig. 3. Effects of the Nef variants on viral receptor down-regulation in TZM cells. (A) TZM cells were transfected with cDNA encoding GFP alone or Nef-GFP fusion proteins and analyzed for their expression levels of CCR5, CXCR4, and CD4 by flow cytometry. The Nef variants tested are indicated. N, L, and H indicate a negative, low, and high level of GFP expression, respectively. (B–D) The relative down-regulation activity of wild-type Nef and its various variants toward CCR5 (panel B), CXCR4 (panel C), and CD4 (panel D) is presented, with the wild-type Nef activity set to 100%. Data are presented as the mean ± SD of three independent experiments.

We then examined the HLA-B*3501-restricted CTLs for their recognition of these variants. Because we had previously identified two overlapping HLA-B*3501-restricted CTL epitopes, with the short epitope VY8 (Nef_{78-85}; VPLRPMTY) and the amino terminal-extended longer epitope RY11 (Nef_{75-85}; RPQVPLRPMTY) [15,21], we generated CTL clones specific for VY8 and RY11 from $CD8^+$ T cells isolated from four different HIV-infected patients and tested them for their cytotoxic activity toward target cells expressing wild-type or various variant Nef proteins (Fig. 2). Clearly, VY8-specific CTL clones failed to recognize the cells expressing the Nef variants having 85F or both 75T and 85F (TF) mutations (Fig. 2). Also, RY11-specific CTLs did not respond to cells expressing 75T or TF mutations (Fig. 2). These results indicate that the 75T and 85F were mutations that conferred escape from HLA-B*3501-restricted CTLs.

3.2. Effects of the Nef mutations on down-regulation of viral receptors

We then examined the effects of wild-type and variant Nef proteins in TZM cells on their down-regulation activity of CD4, CCR5, and CXCR4 receptors. Transfection of these cells with the gene encoding wild-type Nef or GFP fusion proteins resulted in a remarkable reduction in the expression of CD4, CCR5, and CXCR4 receptors on the cell surface (Fig. 3A), confirming the observation previously reported [1,13,23]. Interestingly, the down-regulation of CCR5 and CXCR4 by the wild-type Nef appeared to be proportional to the level of Nef-GFP expression; whereas the down-regulation of CD4 was substantial even at a low level of Nef-GFP expression (Fig. 3A). The CCR5 (Fig. 3B) and CXCR4 (Fig. 3C) down-regulation activity of the AxxA variant was impaired by >90% and ~70%, respectively, compared with the wild-type activity; whereas the CD4 down-regulation activities of the wild type and the AxxA variant were not substantially different (Fig. 3D). In contrast, the CCR5 and CXCR4 down-regulation activity of the EDAA variant was impaired by only <20% (Fig. 3B and C), whereas its CD4 down-regulation activity was much impaired, by ~90% (Fig. 3D). These results confirmed the previous findings showing that genetically separable regions in Nef mediate down-regulation of CD4 and chemokine receptors [13,23].

In CTL escape-conferring mutations, we found that the 75T and TF variants were markedly impaired in their CCR5 down-regulation activity by ~40% (Fig. 3B); whereas the CXCR4 down-regulation activity was not much impaired by the variants (Fig. 3C). In contrast, they had no diminishment of their CD4 down-regulation activity (Fig. 3D). Also, there was no substantial difference either in CCR5, CXCR4 or CD4 down-regulation activity between the wild type and the RF variant (Fig. 3B–D). These data demonstrate that the 75T and TF variants selectively diminished the CCR5 down-regulation activity by Nef. Considering that the 75T mutation alone did not show substantial impairment in the down-regulation of HLA class I or enhancement of viral infectivity [21], it is possible that different molecular pathways could be involved in CCR5 down-regulation and the other Nef functions associated with the proline-rich motif.

3.3. Effects of the Nef mutations on susceptibility to HIV superinfection

We postulated that a functional consequence of the down-modulation of viral entry receptors by Nef would be that HIV-infected cells would be protected from a deleterious HIV superinfection, as

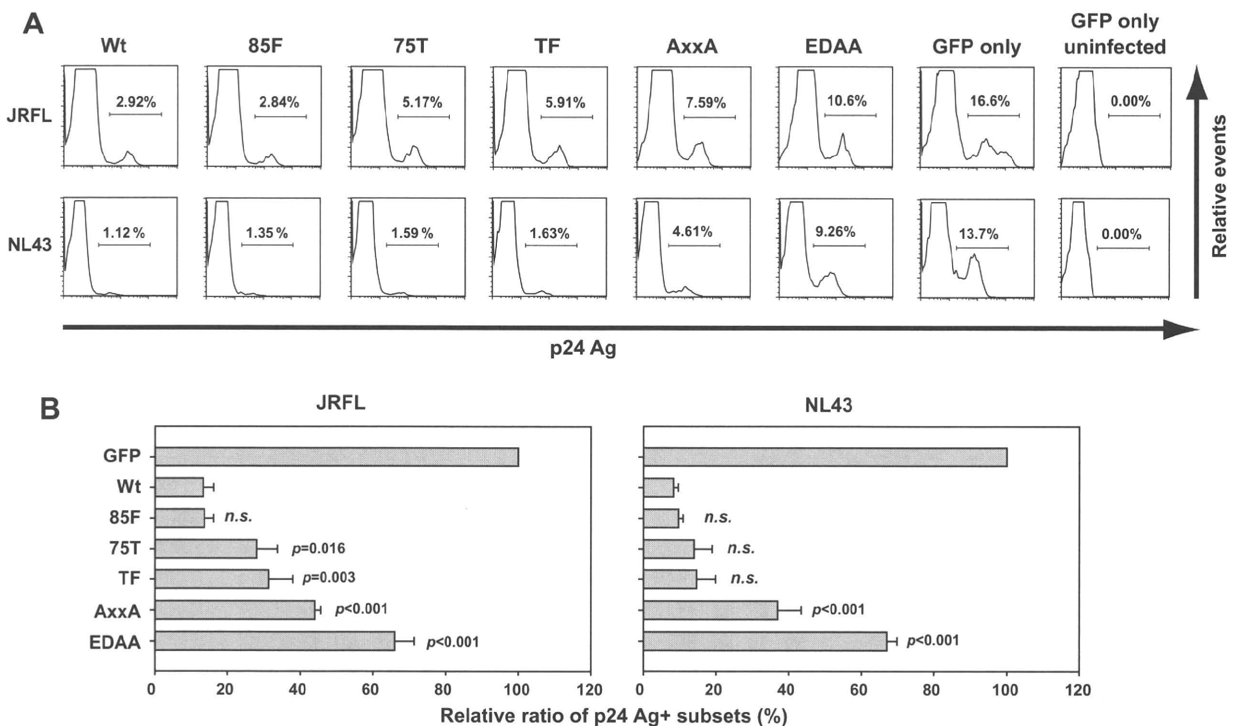

Fig. 4. Effects of the Nef variants on susceptibility to HIV-1 superinfection. (A) TZM cells that had been transfected with GFP alone or Nef-GFP constructs having various Nef mutations indicated in the figure were exposed to the wild-type HIV-1 (strain JRFL or NL43). Another fraction of GFP-expressing TZM cells remained uninfected as a negative control. The resultant cells were analyzed by flow cytometry. The live GFP^+ subset was gated and analyzed for its fluorescence intensity of p24 Gag. The frequency of p24 Gag^+ subsets within live GFP^+ subsets is indicated in the figure. (B) The relative ratio of p24 Gag^+ subsets is presented, with the value obtained for the TZM cells expressing GFP only set to 100%. Data are presented as the mean ± SD of at least three independent experiments, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

described before [2,11,13,16]. In addition, the selective impairment of chemokine receptor down-regulation by the Nef variants shown above may differently influence the superinfection protection against the CCR5- or CXCR4-tropic viruses. To test this possibility, we examined the susceptibility of TZM cells expressing wild-type or various Nef variants to infection by the JRFL (CCR5-tropic) and NL43 (CXCR4-tropic) viruses. We quantified HIV-infected cells by flow cytometry, because this method enabled us to directly look at HIV-infected cells in terms of intracellular expression of p24 Gag proteins. The susceptibility of the cells to infection by JRFL or NL43 was much decreased when the target cells had been expressing the wild-type Nef, by ~90% (Fig. 4A and B), whereas this protective capacity effected by Nef was impaired by either the AxxA or EDAA mutation (Fig. 4A and B), in good agreement with a previous report [13]. These results suggest that the down-regulation of viral entry receptors by Nef played a substantial role in protection of HIV-infected cells from HIV superinfection. However, the EDAA mutation showed the strongest impairment of protection against superinfection (Fig. 4A and B), indicating that the CD4 down-regulation activity by Nef was the most influential factor for protection against superinfection by both JRFL and NL43 viruses. Of the CTL-escape variants, both 75T and TF variants gave impaired protective capacity against superinfection by JRFL, whereas the 85F variant had no effect on this activity (Fig. 4A and B). In contrast, we observed no substantial effects on superinfection protection by the NL43 virus in all CTL-escape variants tested (Fig. 4A and B). In addition, the conventional single-round infectivity assay based on β-galactosidase activity in HIV-infected TZM cells gave similar results (data not shown). These data demonstrate that the 75T and TF variants selectively diminished the protection activity against superinfection by the CCR5-tropic viruses, suggesting the disadvantageous property of these Nef variants in virus spread and persistence during an HIV-1 infection. However, it is conceivable that the functional impairment in Nef induced by CTL-escape variants could be compensated later by mutations at secondary sites in Nef, as recently observed in the case of Gag proteins [5]. Further studies using a large number of subjects with various disease status are needed to extend this observation, such as how mutational escape from Nef-specific CTL responses, altered Nef functions, and clinical outcome of HIV-infected individuals are related to each other at the population level.

4. Conclusions

We showed that some naturally arising amino acid variations in the well-conserved proline-rich region of Nef are associated with escape from HLA-B*3501-restricted CTLs. One of these mutations, Arg75Thr selectively impaired CCR5, but not CXCR4, down-regulation activity by Nef and decreased protection capacity against superinfection by CCR5-tropic HIV-1. Thus, certain Nef-specific CTL responses can constrain HIV-1 spread and persistence in the HIV-infected host, providing us with additional insight into the designing of vaccines against HIV-1.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Clinical Symptoms and Courses of Primary HIV-1 Infection in Recent Years in Japan

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Abstract

Background The natural course of HIV-1 infection includes 10 years of an asymptomatic period before the development of AIDS. However, in Japan, the disease progression process seems faster in recent years.
Methods The study subjects were 108 new patients with primary HIV-1 infection during the period from 1997 through 2007. We evaluated their clinical symptoms and laboratory data, and then analyzed disease progression in 82 eligible patients. Disease progression was defined as a fall in CD4 count below 350/μL and/or initiation of antiretroviral therapy.
Results Ninety percent of the patients were infected via homosexual intercourse. All patients had at least one clinical symptom (mean; 4.75±1.99) related to primary HIV-1 infection, with a mean duration of 23.2 days (±14.8) and 53.3% of them had to be hospitalized due to severe symptoms. The mean CD4 count and viral load at first visit were 390/μL (±220.1) and 4.81 log10/mL (±0.78), respectively. None developed AIDS during the study period. Estimates of risk of disease progression were 61.0% at 48 weeks and 82.2% at 144 weeks. In patients who required antiretroviral therapy, the median CD4 count was 215/μL (range, 52-858) at initiation of such therapy. Among the patients with a CD4 count of <350/μL at first visit, 53% never showed recovery of CD4 count (>350/μL) without antiretroviral therapy.
Conclusion Despite possible bias in patient population, disease progression seemed faster in symptomatic Japanese patients with recently acquired primary HIV-1 infection than the previously defined natural course of the disease.

Key words: HIV-1, primary infection, disease progression

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Introduction

The natural course of HIV-1 infection has been well described in large cohorts from the United States and Europe before the introduction of highly active antiretroviral therapy (HAART); primary HIV-1 infection (PHI) is followed by a clinical latency, usually lasting around 10 years, which precedes the eventual collapse of the immune system (1, 2). However, there is a common feeling among clinicians at present that the natural disease progression of recently infected patients is faster than in previous years (3, 4). Disease progression depends on various factors such as HLA type (5), concomitant infections (6, 7), and available medical resources (8). In addition to these factors, events occurring during PHI could also determine the natural course of the disease. Initial studies suggested that patients with more symptoms related to primary PHI and longer duration of illness exhibit faster rates of progression to AIDS (9-13). Plasma viral load at a set point is also an independent predictor of disease progression (14, 15). However, to determine the viral set point is sometimes difficult. Therefore, for clinicians, the severity of clinical symptoms is the only predictor of subsequent disease progression. The latency be-

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tween the development of PHI and commencement of HAART is also important in the present HAART era.

The main aim of this study was to evaluate the natural disease progression of recently infected Japanese patients. To determine whether or not the disease progression of recently infected patients is accelerated, their CD4 decline was compared with that of hemophiliacs infected before 1985 as the first HIV-1 infection in Japanese.

Furthermore, we also evaluated the correlation between initial CD4 count, viral load, and clinical events and subsequent changes in CD4 and/or time to start HAART in symptomatic Japanese patients with PHI.

Patients and Methods

Study site and patients with PHI

This study was conducted at the AIDS Clinical Center (ACC), National Center for Global Health and Medicine (NCGM: formerly International Medical Center of Japan). The NCGM (925 beds) is a tertiary general hospital located in central Tokyo and the ACC is the main referral clinic for treatment of HIV infected patients in Japan. As part of the follow-up service, HIV-1 infected patients usually visit the ACC on a monthly basis and CD4 count and viral load are measured at each visit. In the present retrospective study, we reviewed the medical records of 108 patients with PHI who were newly diagnosed with PHI between 1997 through 2007 at the ACC. We had conducted a clinical trial of structured treatment interruptions in patients with PHI from November 2000 through December 2002 and 26 patients were enrolled in that trial (16, 17). In terms of the data of these 26 patients, only the initial clinical and laboratory data were included in the present analysis, while all other data, such as time to events, were excluded from this study. To compare the natural CD4 decline of previously and recently infected patients, CD4 counts of 42 Japanese hemophiliacs recorded in the database in 1988 were analyzed as a previous control. Japanese hemophiliacs were infected with HIV-1 through contaminated blood products before 1985 (the estimated mean year of infection was 1983). Therefore, CD4 counts at the end of 1988 were the data at least 3 years after infection. In this comparison, the number of eligible recently infected patients was 59 patients; untreated and CD4 count at 3 years after infection was available.

Definition of PHI

PHI was diagnosed based on the presence of the following three criteria: 1) negative or incomplete western blot finding at the first visit with subsequent change to positive, 2) negative or weakly reactive enzyme-linked immunosorbent assay (ELISA) result for plasma HIV-1 RNA, and 3) confirmed HIV-1 infection on the first visit with documentation of negative ELISA result within 6 months. Symptomatic PHI was defined as PHI accompanied by at least one symptom related to acute retroviral syndrome, such as fever, lymphadenopathy, or skin rash.

Definition of disease progression

Disease progression was defined as fall in CD4 count below 350/μL and/or initiation of antiretroviral therapy. Specifically, patients with an AIDS-defined illness [listed under Centers for Disease Control and Prevention (CDC) category C], patients with AIDS requiring initiation of HAART, and those with severe symptomatic PHI on HAART were defined to have disease progression. The selection of a cutoff value of 350/μL for CD4 count was based on the fact that treatment is generally indicated during the chronic phase of infection when CD4 count falls below 350/μL (18). Patients were considered to be in immunologic progression at the first visit when the initial CD4 count was <350/μL and never subsequently reached 350/μL. For patients who showed a spontaneous increase in subsequent CD4 counts to ≥ 350/μL (such recovery occurred within 3 months from the first visit in all such patients), disease progression was set to have started at the time when such change in CD4 count occurred.

Statistical analysis

Continuous variables are presented as mean value ± SD. Categorical variables were presented as absolute numbers and proportions. Time to events was analyzed by the Kaplan-Meier survival curves, and compared using log-rank test. For patients who did not experience the events described above, data were censored at their last visit. To evaluate the differences between patients groups, the Student t test and χ^2 test were used when appropriate. The relationships between variables were analyzed by the Spearman rank-over correlation test. Statistical significance was defined as $p<0.05$. Data were analyzed using SPSS for Windows (version 15, SPSS, Inc., Chicago, IL).

Results

Table 1 lists the demographics of the enrolled patients with PHI. All patients had at least one documented symptom consistent with PHI (median 5; range 1-11). Fever, cervical lymphadenopathy, pharyngitis, and rash were found in more than 50% of patients (Table 2). The mean duration of symptoms was 23.2 days (SD ±14.8). Fifty-eight (53.7%) patients had to be hospitalized due to severe clinical symptoms. The initial viral loads in hospitalized patients were significantly higher than those of non-hospitalized patients. A longer duration of symptoms was associated with higher initial viral load (R=0.31, p=0.002) (Fig. 1A), and lower CD 4 count (R=-0.22, p=0.03) (Fig. 1B). Consequently, a higher viral load slightly was correlated with a lower CD4 count at the first visit (R=-0.22, p=0.033) (Fig. 1C).

Disease progression was analyzed in 82 patients. None of the patients had AIDS-defining events. Estimates of the risk of disease progression were 50.6% at 24 weeks, 61.0% at 48 weeks, 67.0% at 96 weeks, and 82.2% at 144 weeks

Table 1. **Baseline Characteristics of 108 Patients with Primary HIV-1 Infection in this Study**

Characteristics	Total number or mean (±SD) or %	Hospitalized patients (n = 58)	Non-hospitalized patients (n = 50)	p
Age (year)	31.8±8.48	32±9.07	31±7.82	NS
Sex				
Male	102	56	46	NS
Female	6	2	4	NS
Predisposing factor				
MSM	97	53	44	NS
Heterosexual	8	3	5	NS
IDU	1	0	1	NS
Unknown	2	2	0	NS
PMH of STD	75 (69.7)	44 (40.4)	31 (29.3)	NS
Syphilis	49 (45.5)	27 (25.3)	21 (20.2)	NS
Acute hepatitis A	11 (10.1)	6 (6.1)	5 (4.0)	NS
Acute hepatitis B	36 (33.3)	22 (20.2)	14 (13.1)	NS
Amebiasis	10 (9.1)	9 (8.0)	1 (1.1)	0.035
Others	7 (6.1)	2 (2.0)	5 (4.1)	NS
No. of symptoms	4.75±1.99	4.98±1.94	4.48±2.04	NS
Duration of symptoms (days)	23.2±14.8	27.8±13.1	18.0±15.1	0.001
Laboratory findings				
CD4 count/μL	390.0±220.1	356.1±204.1	443.7±236.0	0.06
HIV RNA $\log_{10}$/mL	4.81±0.78	5.03±0.68	4.48±0.81	0.001
STI trial*	26	12	14	NS

*Patients enrolled in a clinical trial of structured treatment interruptions in recently HIV-1-infected patients. Abbreviations; MSM: men who have sex with men, PMH of STD: past medical history of sexual transmitted diseases, STI: structured treatment interpretations, IDU: intravenous drug user, Others: genital herpes infection, chlamydial urethral infection condyloma acuminata, NS: not significant

Data are presented as mean ± SD or percentage (%) unless otherwise indicated

Table 2. **Symptoms and Physical Findings Observed in the Patients with >10% Frequencies (n=108)**

Symptoms and physical findings	frequency (%)
Fever	91
Lymphadenopathy	63
Pharyngitis	53
Rash	50
Diarrhea	37
Fatigue	32
Headache	26
Myalgia	20
Weight loss	19
Nausea	16
Appetite loss	14
Neurological sign	13
Hepatomegaly	13
Thrush	12

(Fig. 2). Eighteen of 34 (53.3%) patients with an initial CD 4 cell count below 350 cells/μL had immunologic progression at the first visit. Their CD4 counts never increased above 350/μL until initiation of HAART. Forty-eight (58.5%) required initiation of HAART in this study. The reasons for the initiation of HAART were severe clinical symptoms related to PHI in 16 patients and immunologic progression in 32 patients. The median CD4 count of those patients at initiation of HAART was 215/μL (range, 52-858).

We analyzed the clinical course in 66 patients (excluding 26 patients who enrolled in a clinical trial of structured treatment interruptions in PHI and 16 patients who received HAART for PHI) to determine the factors associated with disease progression. Half of these patients (33 patients) required hospitalization. As shown in Fig. 3A, the mean time to disease progression of the hospitalized patients [57.4 weeks, 95% confidence interval (95%CI); 34.9-79.8 weeks] was shorter than that of the non-hospitalized (33 patients, 94.4 weeks, 95%CI; 71-117 weeks, p=0.002). Among the 32 patients with CD4 count >350/μL at first visit, 24% had documented disease progression within 1 year, whereas among 34 patients with CD4 count <350/μL at first visit, 76.4% showed disease progression (Fig. 3B). The mean times to disease progression for the two groups were 111.9 weeks (95%CI; 92.8-131) and 39.5 weeks (95%CI; 18.6-60.5), respectively (p<0.001). Disease progression in 39 patients with high viral load (≥5.0 $\log_{10}$/mL) was not significantly different (p=0.41) from that in 27 patients with low viral load (<5.0 $\log_{10}$/mL) (Fig. 3C). The number of symptoms was not significantly different in each group (Fig. 3D). The mean time to disease progression was 69.8 weeks (95% CI; 47.2-92.5) in patients with a high viral load and 80.4 weeks (95%CI; 54.9-105.8) in those with a low viral load.

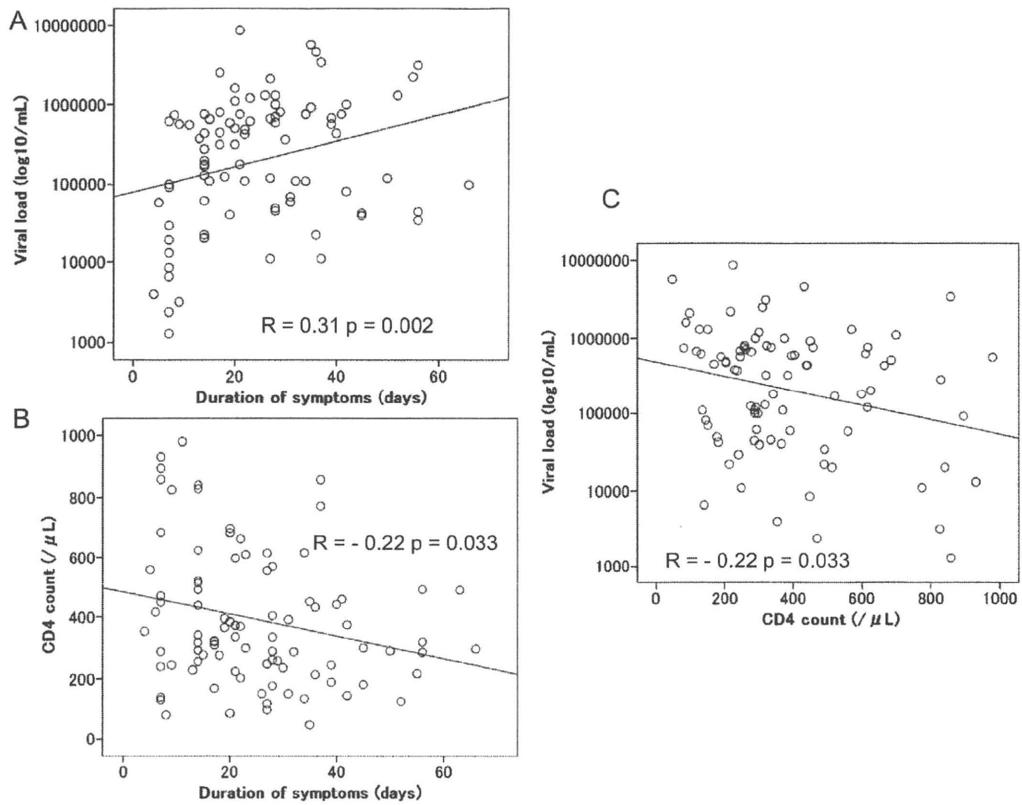

Figure 1. Correlations among plasma viral load, CD4 count, and clinical symptoms. A; Plasma viral load correlated with duration of symptoms (R=0.31, p=0.002). B; CD4 count correlated inversely with duration of symptoms (R=-0.22, p=0.033). C; plasma viral load correlated inversely with CD4 count (R=-0.22, p=0.033).

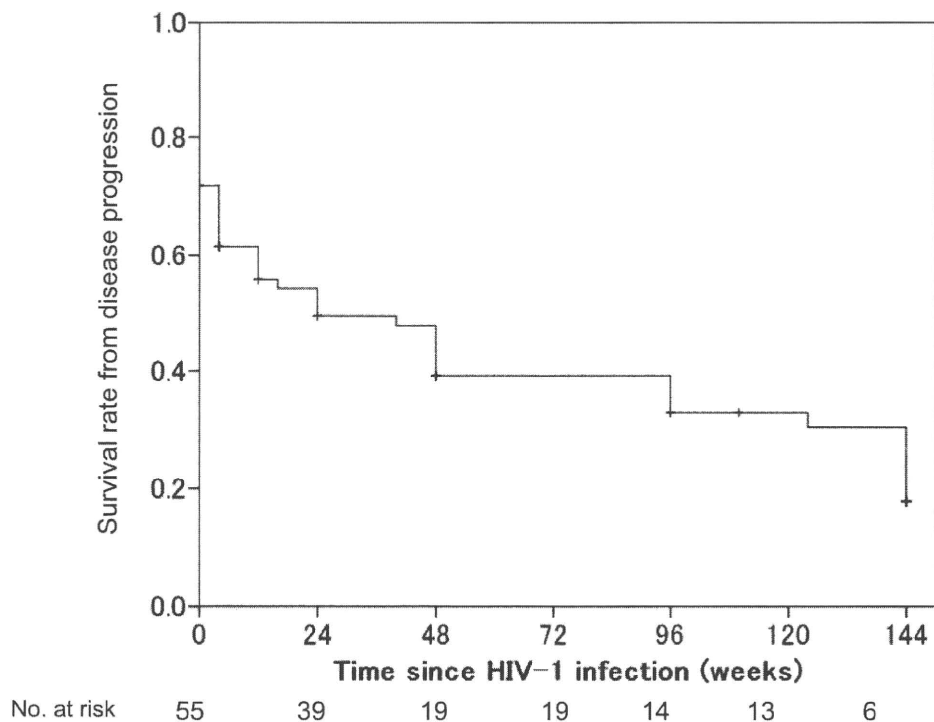

Figure 2. Progression-free survival in 82 patients. Progression was defined as CD4 count <350/μL or initiation of HAART. No. at risk: the number of CD4 count >350/μL or HAART naïve patients

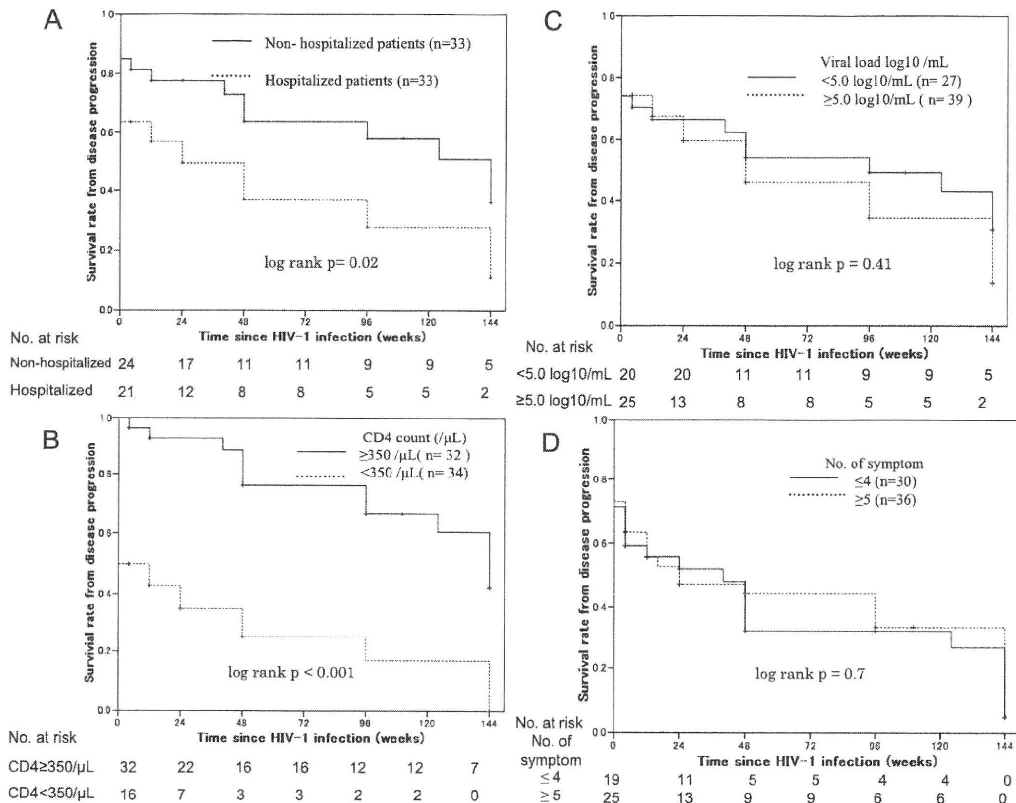

Figure 3. **Progression-free survival among 66 patients according to rate of hospitalization, baseline CD4 count, and viral load. No. at risk: the number of CD4 count >350/μL or HAART naïve patients. A; Solid line: patients who required hospitalization due to PHI, dashed line: patients who did not require hospitalization (p=0.02, by log-rank test). B; Solid line: patients with CD4 count >350/μL at first visit, dashed line: patients with CD4 count <350/μL (p<0.001). C; Solid line: patients with viral load <5.0 log_{10}/mL, dashed line: patients with viral load ≥5.0 log_{10}/mL (p=0.41). Disease progression was defined as CD4 count <350/μL or initiation of HAART. D; Solid line: patients with the number of PHI symptoms ≤4, dashed line: patients with the number of PHI symptoms ≥5 (p=0.7, by log-rank test).**

Comparison of percentage of recently infected patients with CD4 counts >350/μL at 3 years after infection and that of hemophiliacs as the first HIV-1 infected population in Japanese is shown in Fig. 4. The percentage (13.5%) of recently infected patients was significantly lower than that (47.6%) of Japanese hemophiliacs (p<0.001), clearly indicating the rapid decline of CD4 count in recently infected patients.

Discussion

In this study, we demonstrated rapid disease progression of symptomatic PHI Japanese patients in this decade. However, when we divided our study subjects into two groups according to the first half (1997-2002) and the latter half (2003-2007), disease progression of each group was not different (data not shown). In contrast, disease progression surrogated with natural CD4 decline of recently infected patients was significantly accelerated compared with Japanese hemophiliacs infected with HIV-1 before 1985. However, there are two quite different backgrounds; one is the route of infection and the other is the year of infection. Almost all hemophiliac patients are also co-infected with hepatitis C but do not have other sexually transmitted diseases (STDs). In contrast, most patients in the present study were infected via homosexual intercourse with many other STDs that may facilitate acceleration of the disease progression (7). In the present study, 69.7% patients had a past medical history of STDs, and the mean number of STDs was 1.08/patient (0: 31.3%, 1: 37.4%, 2: 23.2%, 3: 8.1%). In this regard, most published data on disease progression were obtained from men who have sex with men (MSM) cohorts (1, 2). Therefore, it is unlikely that the recent rapid disease progression is due to Japanese MSM. Whether or not the rapid disease progression in the recently HIV-1-infected Japanese can be generalized is to be elucidated in future studies.

Some HLA types are protective against disease progression such as HLA-B57 (19) and HLA-B51 (20) because HLA-restricted cytotoxic T lymphocytes (CTLs) play an important role on viral control. On the other hand, virus can easily escape from CTLs (17, 21). In some prevalent HLA types, escape virus can transmit and accumulate in the population (21). In this situation, some HLA types are no more

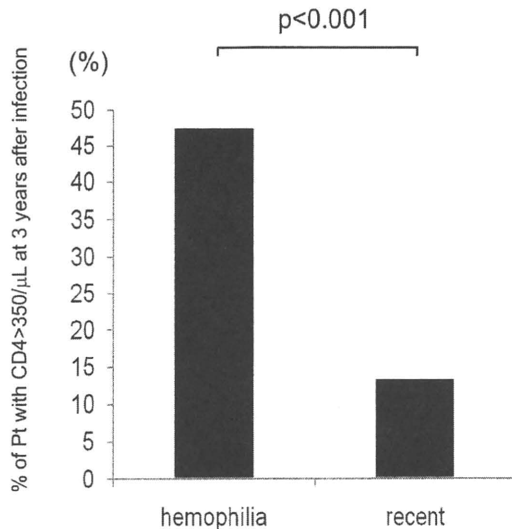

Figure 4. **Comparison of percentage of previously and recently infected patients with CD4 counts >350/μL at 3 years after infection. In this analysis, Japanese hemophiliacs (designated "hemophilia" in the figure) were regarded as a previously infected patient, because they were infected with HIV-1 before 1985. The number of hemophiliacs was 42 patients. The eligible number of recently infected patients (designated "recent" in the figure) was 59 patients; infected with HIV-1 after 1997, untreated, and CD4 count at 3 years after infection.**

protective. The HLA distribution is different in Americans compared to Japanese. Another possible hypothesis for the different disease progression is that Japanese hemophiliacs were exposed to HIV-1 through contaminated blood products imported from US as the first Japanese population infected with the virus around 1983. However, in recent years, most HIV-1 infection in Japanese is transmitted from Japanese patients. It can be postulated that current HIV-1 in Japan has adapted to the Japanese population, indicating acquisition and accumulation of escape virus from immune pressure of the otherwise protective HLA in Japanese population (21). From a negative point of view, the situation is similar to the epidemic of drug-resistance virus in treatment of naïve patients (22). The clinical relevance of the prevalence of immune escape virus in Japanese is a potentially serious matter in terms of the natural course of HIV-1 infection.

In the present study, all patients have had at least one symptom associated with PHI. During the follow-up period, no patient developed AIDS, whereas around 70% of the patients experienced immunologic progression as defined by a CD4 count <350/μL. It is noteworthy that the majority of these patients exhibited immunologic progression within 3 years and, surprisingly, >60% of them were documented within the first year. HAART was initiated in nearly 60% of patients during this period, including initiation for PHI-related severe symptoms in 20% of these patients. Previous studies on PHI have suggested that the number, duration, and/or severity of symptoms can predict faster disease progression to AIDS (23, 24). Our findings are compatible with these previous studies. Considered together, these results suggest that the duration of illness rather than the number of symptoms is more likely to be a major determinant of immunological progression. The estimated risks of disease progression were more than 50% by week 24 and 80% by week 144. Comparison with those observed elsewhere during the natural course of HIV-1 infection (24), these disease progression rates are surprisingly high. Among the patients with CD4 counts >350/μL at first visit, a quarter of them showed disease progression within 1 year. In contrast, in patients with CD4 count <350/μL, three quarters of them showed disease progression within the same period. Goujard et al (25) suggested possible recovery of CD4 count after the primary infection phase even in patients with very low count because it fluctuates during that period. In contrast, our results suggest that patients with a CD4 count of <350/μL during primary infection should be monitored carefully because spontaneous recovery of CD4 cell count during primary infection was rare. This cautionary remark could also apply to patients with a CD4 count of >350/μL because they exhibited nearly 60% risk of disease progression within 3 years. These observations may allow more targeted clinical monitoring and timely initiation of HAART. The impact of a short-term HAART during symptomatic primary infection on the subsequent disease progression needs to be elucidated in future study.

Although we included all recent seroconverters during the study period, it could be argued that this study carries some institution bias (i.e., a high proportion of cases with severe disease). However, the present finding of a surprisingly rapid disease progression in our patient population is new. Whether or not the natural course of disease progression has recently become accelerated in other countries or other cohorts is a matter of great interest.

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 μM) 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 μM) for 2 h at 37 °C. Brefeldin A (10 μg/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 μl 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 μM 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 10^6 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 10^6 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 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.

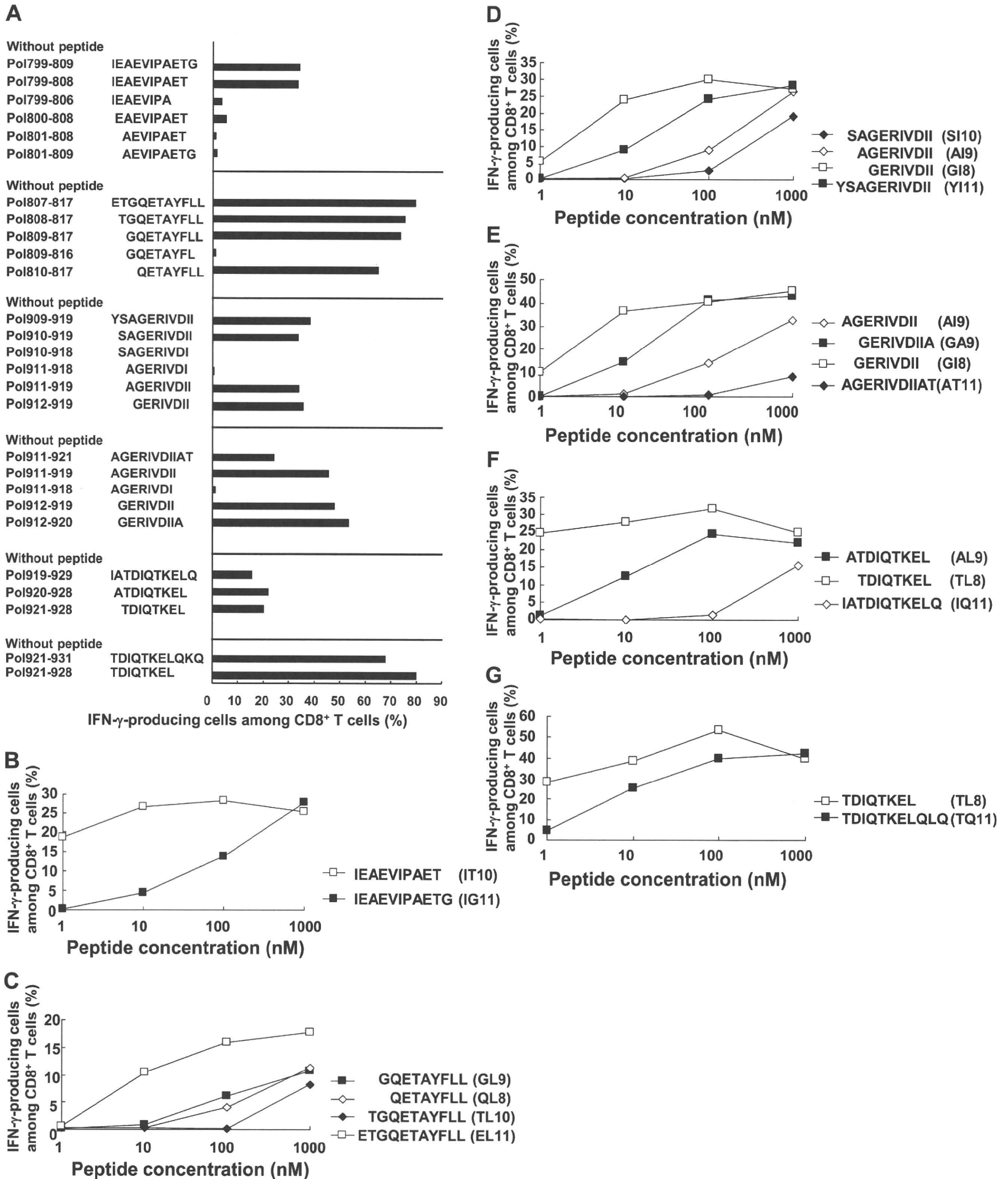

Fig. 1. Identification of HLA-B*4002-restricted HIV-1 CTL epitopes. A. For determination of the optimal epitopes of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 and Pol921-931, the recognition of the bulk T cells for the truncated peptides was examined by using C1R-B*4002 cells pre-pulsed 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.