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THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Epitope-Dependent Effect of Nef-Mediated HLA Class I Down-Regulation on Ability of HIV-1-Specific CTLs to Suppress HIV-1 Replication[1]

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It is believed that Nef-mediated HLA class I down-regulation is one of the mechanisms that allow HIV-1-infected cells to escape from being killed by HIV-1-specific human CTLs. In this study, we show that the effect of Nef-mediated HLA class I down-regulation on the ability of HIV-1-specific CTLs to suppress HIV-1 replication is epitope dependent. The CTLs specific for two Pol epitopes presented by HLA-B*5101, one of the HLA alleles associated with slow progression to AIDS, effectively killed HIV-1-infected CD4$^+$ T cells and suppressed HIV-1 replication. In contrast, those specific for the other four epitopes failed to kill HIV-1-infected CD4$^+$ T cells and partially or hardly suppressed HIV-1 replication. The difference of the ability between these two types of CTLs may result from the difference of the number of HLA class I epitope complex on the surface of NL-432-infected CD4$^+$ T cells. The Journal of Immunology, 2005, 174: 36–40.

Human immunodeficiency virus-1 escape from HIV-1-specific CD8$^+$ T cells occurs during acute and chronic phases of HIV-1 infections, although the mechanisms of the HIV-1 escape still remain unclear. Previously, various investigators proposed several hypotheses concerning mechanisms of HIV-1 escape from the host immune system such as mutations of immunodominant epitopes (1), reduction in the number of HIV-1-specific CTLs by apoptosis of CD8$^+$ T cells via Fas and TNF (2), skewed maturation of HIV-1-specific CD8$^+$ T cells (3), and impaired cytolytic activity of HIV-1-specific CTL toward HIV-1-infected CD4$^+$ T cells by Nef-mediated down-regulation of HLA class I molecules (4).

Nef down-regulates the surface expression of both HLA-A and -B molecules in HIV-1-infected cells because of internalization of these molecules from the cell surface by endocytosis in the presence of Nef (5). A previous study showed that the expression of HLA-A2 molecules on Nef-positive (Nef$^+$) HIV-1-infected primary CD4$^+$ T cells was 200- to 300-fold lower than that on Nef-defective (Nef$^-$) HIV-1-infected ones (6). These observations suggested that the Nef-mediated HLA class I down-regulation may decrease the recognition of HIV-infected cells by HIV-1-specific CTLs. In fact, it was shown that HLA-A*0201-restricted HIV-1-specific CTLs failed to kill Nef$^+$ HIV-1-infected CD4$^+$ T cells but effectively killed Nef$^-$ HIV-1-infected ones (4). This was further confirmed by a study using two HLA-B*3501-restricted, HIV-1-specific CTL clones (7). The ability of HIV-1-specific CTLs to suppress HIV-1 replication was also impaired by Nef-mediated HLA class I down-regulation (7, 8). These studies strongly suggest that Nef-mediated HLA class I down-regulation is one of the major mechanisms by which HIV-1 escapes from HIV-1-specific CTLs. However, because only a very restricted number of HIV-1-specific CTLs has been tested for their abilities to kill Nef$^+$ and Nef$^-$ HIV-1-infected CD4$^+$ T cells and to suppress the HIV-1 replication, it still remains uncertain whether Nef-mediated HLA class I down-regulation affects the killing ability of all HIV-1-specific CTLs.

HLA-B57, -B51, and -B27 alleles are associated with slow progression to AIDS (9). It has been speculated that long-term nonprogressors (LTNPs)[3] and slow progressors carry CTLs specific for conserved and dominant epitopes whose recognition is not affected by Nef-mediated HLA class I down-regulation. However, no study has yet investigated this hypothesis. To clarify the effect of Nef-mediated HLA class I down-regulation on CTLs specific for HIV-1 epitopes presented by HLA alleles that are associated with or not associated with slow progression to AIDS, we investigated the ability of both HLA-B*5101-restricted and HLA-A*3303-restricted HIV-1-specific CTLs to recognize HIV-1-infected CD4$^+$ T cells. In this study, we show that HIV-1-specific CD8$^+$ T cells have various ranges of ability to kill HIV-1-infected CD4$^+$ T cells and to suppress the replication of HIV-1.

Materials and Methods

HIV-1-specific CTL clones and lines

HIV-1-specific CTL clones and CTL lines were generated previously (10–12). All CTLs were cultured in R10 medium supplemented with 200 U/ml recombinant human IL-2 and stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

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Received for publication August 18, 2004. Accepted for publication October 27, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture; a Grant-in Aid for Scientific Research from the Ministry of Health, Labour and Welfare; the government of Japan; and a grant from Japan Health Science Foundation.

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[3] Abbreviation used in this paper: LTNP, long-term nonprogressor.

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0022-1767/05/$02.00

HIV-1 clones

An infectious proviral clone of HIV-1, pNL-432, and its mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously (13).

Infection of CD4$^+$ T cells with HIV-1

CD4$^+$ T cells were purified from PBMCs of HIV-1-seronegative individuals with HLA-B*5101 or HLA-A*3303 by means of anti-human CD4 mAb-coated magnetic beads (MACS beads; Miltenyi Biotec). The purified CD4$^+$ T cells were cultured and infected with HIV-1 clones as previously shown (7).

CTL assay

The cytotoxicity of CTL clones for cultured CD4$^+$ T cells infected with HIV-1 (>40% p24 Ag-positive cells) was determined by a standard ^{51}Cr release assay as shown previously (7).

Flow cytometric analysis

To assess HLA class I expression in HIV-1-infected CD4$^+$ T cells, the cells were stained with anti-B5 mAb 4D12 following staining with allophycocyanin-labeled anti-mouse Ig (BD Pharmingen), and thereafter were fixed and permeabilized for intracellular HIV-1 p24 staining with FITC-labeled anti-p24 mAb KC-57. The expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells was analyzed by using a FACSCalibur with CellQuest software (BD Biosciences). For detection of intracellular cytokines, HIV-1-specific CTL clones were cocultured with peptide-prepulsed CD4$^+$ T cells or HIV-1-infected CD4$^+$ T cells for 6 h at a CTL:CD4$^+$ T cell ratio of 1:2. CTLs cocultured with CD4$^+$ T cells were used as a negative control. After a 2-h incubation, brefeldin A was added to each well (10 μg/ml). The cells were then stained as previously described with a FITC-labeled anti-human CD8 mAb, PE-labeled anti-human IFN-γ mAb, and allophycocyanin-labeled anti-human TNF-α mAb.

Suppression of HIV-1 replication by HIV-1-specific CTLs

The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (7). After CD4$^+$ T cells had been incubated with the indicated HIV-1 clone following a 4-h incubation at 37°C with intermittent agitation, the cells were washed three times with R10 medium. HIV-1-infected CD4$^+$ T cells were cocultured with HIV-1-specific CTLs. From days 2 to 7 postinfection, 10 μl of culture supernatant was collected, and the concentration of p24 Ag in the supernatant was measured by enzyme immunoassay (HIV-1 p24 Ag ELISA kit; ZeptoMetrix). On days 3, 4, and 5 postinfection, cells were harvested and stained with a mixture of anti-CD4 and anti-CD8 mAbs and then with anti-p24 mAb. The percentage of intracellular p24 Ag-positive cells in the CD8$^-$ population was determined by flow cytometry.

Peptide binding assay

Binding of HIV-1 epitope peptides to HLA-B*5101 was examined by a peptide stabilization assay using RMA-S-B*5101 cells as previously described (10).

Results and Discussion

Ability of HIV-1-specific CTLs to suppress HIV-1 replication in HIV-1-infected CD4$^+$ T cells

To investigate the ability of HIV-1-specific CTLs to suppress HIV-1 replication, we selected the CTLs specific for four HLA-B*5101 epitopes and two HLA-A*3303 epitopes, whose sequences are found in the NL-432 clone. We measured the ability of seven CTL clones or lines specific for these epitopes to suppress HIV-1 replication in primary CD4$^+$ T cells infected with either HIV-1 clone NL-432 or its mutant NL-M20A, in which 1 aa of Nef has mutated and which has the ability to down-regulate cell surface expression of CD4 but not that of HLA class I molecules (13). The surface expression of HLA-B*5101 was indeed down-regulated in NL-432-infected CD4$^+$ T cells but not in NL-M20A-infected ones (Fig. 1*A*). CD4$^+$ T cells infected with the HIV-1 clones were cocultured with or without the HIV-1-specific CTLs. p24-positive CD4$^+$ T cells were not detected in the cultures of NL-M20A-infected CD4$^+$ T cells with the SF2-Pol283-8-specific CTL line, SF2-Pol743-9-51 CTL clone, or SF2-Gag327-9-249 CTL clone. They were also undetected in the cultures of NL-432-infected CD4$^+$ T cells with the SF2-Pol283-8-specific CTL line or SF2-Pol743-9-51 CTL clone, whereas the number of the p24-positive CD4$^+$ T cells was reduced by approximately one-half in the cultures with the SF2-Gag327-9-249 CTL clone. In contrast, the number of the p24-positive CD4$^+$ T cells was not reduced in the cultures of NL-432-infected and NL-M20A-infected CD4$^+$ T cells with HLA-mismatched HIV-1 Nef-specific CTL clones, SF2-6-218 and SF2-6-219 (Fig. 1*B*). These results suggest that SF2-Pol283-8-specific CTL line and SF2-Pol743-9-51 CTL clone completely suppressed Nef$^+$ HIV-1 replication and that SF2-Gag327-9-249 CTL clone only partially suppressed it. Two HLA-A*3303-restricted CTLs, the SF2-Gag144-152-10 clone and the SF2-Env697-706 line, gave the same results as the latter clones (data not shown).

The enzyme immunoassay analysis confirmed the results of the flow cytometric analysis (Fig. 1*C*). The SF2-Pol283-8 line and SF2-Pol743-9-51 clone completely suppressed replication of both NL-M20A and NL-432, whereas two CTL clones, SF2-Gag144-152-10 and SF2-Gag327-9-249, as well as one CTL line, SF2-Env697-706, partially suppressed NL-432 replication (21.7–44.0%) and effectively suppressed NL-M20A replication (82.4–89.9%). These results taken together suggest that the recognition by the latter CTLs was affected by Nef-mediated HLA class I down-regulation but that by the former ones was not.

To compare quantitatively the ability of these CTLs to suppress NL-432 replication, we tested the ability of the SF2-Pol283-8 or SF2-Pol743-9-51 at various E:T ratios to suppress NL-432 replication (Fig. 1*D*). Approximately 50% suppression of NL-432 replication was found when SF2-Gag144-152-10 and SF2-Gag327-9-249 CTL clones were tested at an E:T ratio of 1:1, whereas both SF2-Pol283-8 and SF2-Pol743-9-51 CTL clones showed ~50% suppression at an E:T ratio of 0.001:1, indicating that these CTLs have 1000-fold stronger ability to suppress NL-432 replication than SF2-Gag144-152-10 and SF2-Gag327-9-249 CTL clones.

The number of p24-positive CD4$^+$ T cells was not reduced in the culture of NL-432-infected CD4$^+$ T cells with the SF2-Rev71-11-55 clone, whereas it was partially reduced in that of NL-M20A-infected CD4$^+$ T cells with the same clone (data not shown). This clone also failed to suppress NL-432 replication but partially suppressed NL-M20A replication (Fig 1*C*). These results suggest that this CTL clone can weakly recognize NL-M20A-infected CD4$^+$ T cells but not NL-432-infected CD4$^+$ T cells.

Ability of HIV-1-specific CTLs to kill HIV-1-infected CD4$^+$ T cells and to produce cytokines by stimulation with HIV-1-infected CD4$^+$ T cells

To clarify the mechanism by which HIV-1-specific CTLs suppress HIV-1 replication, we investigated the activity of the HIV-1-specific CTL clones and lines to kill HIV-1-infected CD4$^+$ T cells and to produce cytokines when stimulated with HIV-1-infected CD4$^+$ T cells. SF2-Pol743-9-51 CTL clone and SF2-Pol283-8-specific CTL line, which showed strong suppression of NL-432 replication, effectively killed CD4$^+$ T cells infected with either NL-432 or NL-M20A. The result for the SF2-Pol743-9-51 clone was also confirmed by using the SF2-Pol743-9-specific CTL line (Fig. 2*A*). The cytolytic activity of these two CTLs for HLA-B*5101$^+$CD4$^+$ T cells infected with NL-432 was almost identical with that of those infected with NL-M20A at any E:T ratios (Fig. 2*B*). These results

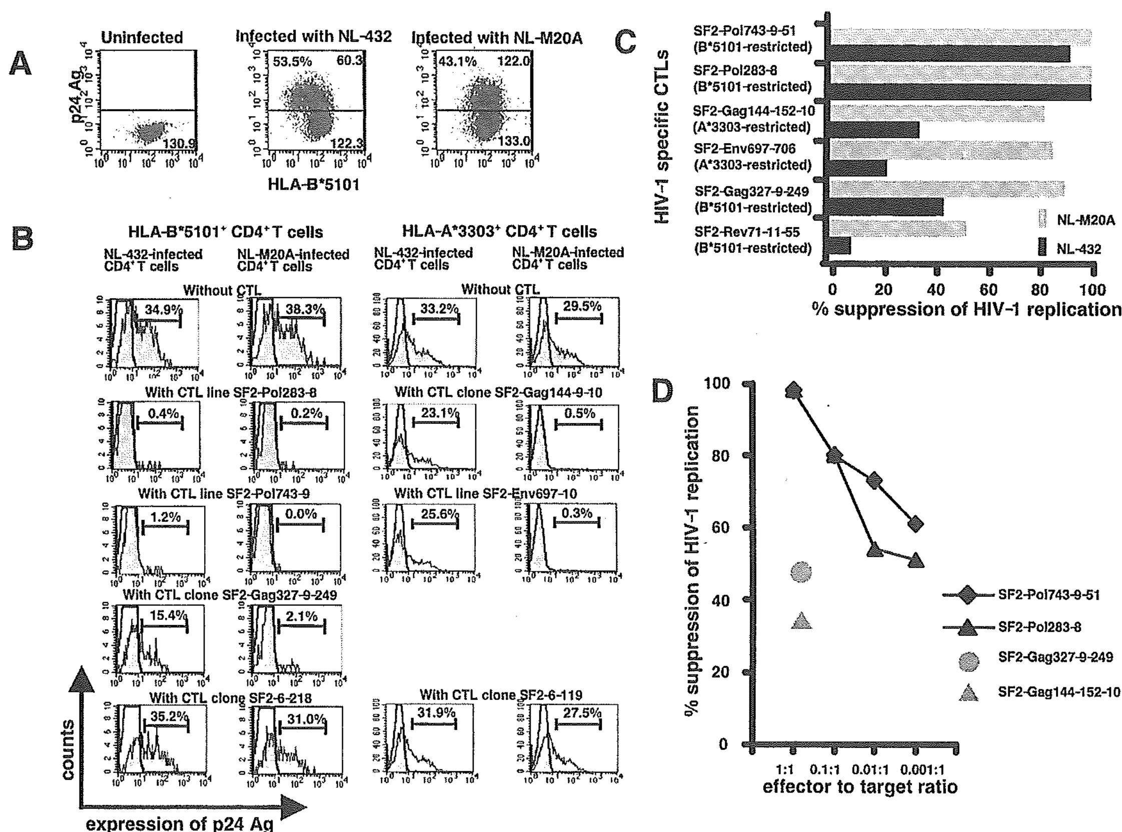

FIGURE 1. Suppression of HIV-1 replication in HIV-1-infected CD4$^+$ T cells by HIV-1-specific CTLs. *A*, Expression of HLA class I molecules on CD4$^+$ T cells infected with HIV-1 NL-432 or NL-M20A. CD4$^+$ T cells from donor U-13 with HLA-B*5101 were cultured and then infected with HIV-1 NL-432 or NL-M20A. On day 3 postinfection, the cells were stained with HLA-B5/B35-specific mAb 4D12 and anti-HIV-1 p24 Ag-specific mAb. The expression of HLA-B*5101 on p24-positive or p24-negative cells is shown as the mean fluorescence intensity (MFI) in each figure. *B*, Number of HIV-1-infected CD4$^+$ T cells in the cultures of HIV-1-infected CD4$^+$ T cells with HIV-1-specific CTLs. CD4$^+$ T cells isolated from donor U-13 were infected with NL-432 or NL-M20A and cocultured with HIV-1-specific CTLs at an E:T ratio of 1:1. The number of HIV-1 p24 Ag$^+$CD8$^-$ cells in the culture was measured by flow cytometry at the peak of HIV-1 infection. Uninfected and HIV-1-infected CD4$^+$ T cells stained with anti-p24 mAb are shown as bold line and filled histogram, respectively. Percentages of p24 Ag$^+$ cells are shown in each figure. *C*, Amount of HIV-1 p24 Ags in the cultures of HIV-1-infected CD4$^+$ T cells with HIV-1-specific CTLs. Cultured CD4$^+$ T cells from donors U-13 and U-27(A*3303$^+$) were infected with NL-432 or NL-M20A and then cocultured with HIV-1-specific CTLs at an E:T ratio of 1:1. HIV-1 p24 Ags in the supernatant were measured on days 2–7 postinfection by enzyme immunoassay. The percentage of suppression of HIV-1 replication was calculated. *D*, Comparison of the ability of HIV-1-specific CTLs to strongly suppress Nef$^+$ HIV-1 replication. CD4$^+$ T cells isolated from donor U-13 were infected with NL-432 and then cocultured with the HIV-1-specific CTLs at various E:T ratios. HIV-1 p24 Ag in the supernatant at the peak of infection was measured by enzyme immunoassay, and the percent suppression of NL-432 replication was calculated.

indicate that Nef-mediated HLA class I down-regulation does not affect the ability of these CTLs to kill HIV-1-infected CD4$^+$ T cells. In contrast, the three HIV-1-specific CTLs (SF2-Gag144-152-10 and SF2-Gag327-9-249 CTL clones, and SF2-Env697-706 CTL line) killed NL-M20A-infected CD4$^+$ T cells but failed to kill NL-432-infected CD4$^+$ T cells (Fig. 2*A*), suggesting that Nef-mediated HLA class I down-regulation affected the ability of these CTLs to kill HIV-1-infected CD4$^+$ T cells. These results are consistent with those of a previous study showing that 2 HLA-B*3501-restricted CTL clones killed NL-M20A-infected CD4$^+$ T cells but failed to kill NL-432-infected CD4$^+$ T cells (7).

Next, we investigated the ability of these CTLs to produce IFN-γ and TNF-α after having been stimulated with HIV-1-infected CD4$^+$ T cells (Fig. 2*C*). The total percentages of IFN-γ- and TNF-α-producing cells were ~2–5% and 4–9% in the HIV-1 Pol-specific CTLs stimulated with NL-432-infected and NL-M20A-infected ones, respectively. In contrast, the total percentages of IFN-γ- and TNF-α-producing cells were ~4 and 4–6% in the HIV-1 Gag- and Env-specific CTLs stimulated with CD4$^+$ T cells infected with NL-432 and NL-M20A, respectively. Thus, there was no difference in the number of cytokine-producing cells between these two groups of HIV-1-specific CTLs. These results suggest that the difference in the ability to suppress HIV-1 replication between the two groups results from that in cytolytic activity between them, and that cytokines secreted from the CTLs are partially involved in the suppression of HIV-1 replication.

The SF2-Rev71-11-55 clone failed to produce cytokines after stimulation with either CD4$^+$ T cells infected with NL-432 or those infected with NL-M20A (Fig. 2*C*). This result together with that of suppression of HIV-1 replication indicate that the SF2-Rev71-11 epitope is very weakly presented by HLA-B*5101 in HIV-1-infected CD4$^+$ T cells.

*Ability of four HLA-B*5101-restricted CTLs to recognize HIV-1 epitopes*

It is thought that the ability of CTLs to kill HIV-1-infected cells and to suppress HIV-1 replication is determined by the ability

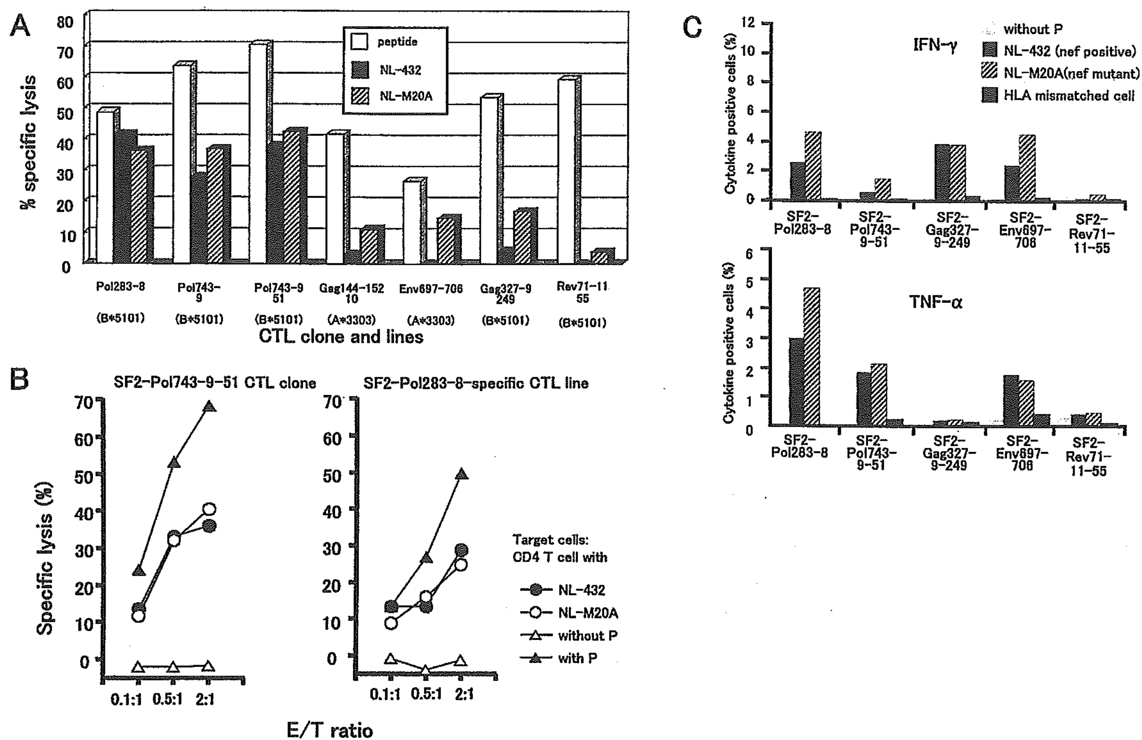

FIGURE 2. Activity of HIV-1-specific CTLs to kill HIV-1-infected CD4$^+$ T cells and to produce cytokines after stimulation with HIV-1-infected CD4$^+$ T cells. *A* and *B*, CD4$^+$ T cells from donors U-13 and U-27 were infected with NL-432 or NL-M20A. On day 3 postinfection, the cells were harvested and used as target cells in the standard ^{51}Cr release assay. Cytotoxic activity of two CTL lines and five CTL clones was examined for CD4$^+$ T cells prepulsed with each epitope peptide (□) and those infected with NL-432 (■) or with NL-M20A (▨) at an E:T ratio of 2:1 (*A*). Cytotoxic activity of SF2-Pol283-8 and SF2-Pol743-9-51 for NL-432-infected CD4$^+$ T cells or NL-M20A-infected CD4$^+$ T cells was examined at E:T ratios of 2:1, 0.5:1, and 0.1:1 (*B*). The specific lysis of the CTLs for CD4$^+$ T cells pulsed with epitope peptide (1 μM) or without peptide was examined by the same assay. Percentages of p24-positive cells in HLA-B*5101-positive CD4$^+$T cells infected with NL-432 and with NL-M20A were 42.0 and 37.0%, respectively. *C*, CD4$^+$ T cells purified from the same donors were infected with NL-432 or NL-M20A. On day 3 postinfection, uninfected CD4$^+$ T cells and NL-432- or NL-M20A-infected CD4$^+$ T cells were mixed with HIV-1-specific CTLs, at an effector-to-stimulator ratio of 1:2 and then incubated for 6 h. Intracellular staining of IFN-γ or TNF-α was performed by using PE-labeled anti-IFN-γ and anti-TNF-α mAbs. The percentages of p24 Ag-positive cells among U-13 CD4$^+$ T cells infected with NL-432 or NL-M20A were 36.6 and 32.1%, respectively, and those among U-27 CD4$^+$ T cells infected with NL-432 or NL-M20A were 33.6 or 31.0%, respectively. The percentages of IFN-γ- and TNF-α-producing cells in the CTLs stimulated with peptide-pulsed CD4$^+$ T cells were as follows: Pol283-8, IFN-γ, 46.3%, TNF-α, 22.0%; Pol745-9-51, IFN-γ, 49.7%, TNF-α, 18.5%; Gag327-9-249, IFN-γ, 17.4%, TNF-α, 0.4%; Env697-706, IFN-γ 33.0%, TNF-α, 31.5%; and Rev71-11-55, IFN-γ, 40.0%, TNF-α, 21.8%.

of TCR to recognize the epitope and by the amount of the epitope presented on the surface of HIV-1-infected cells. We investigated the ability of TCR to recognize the epitope among four HLA-B*5101-retricted CTLs. We measured the ability of the peptides to bind to HLA-B*5101 molecules (BL_{50}) by an HLA-B*5101 stabilization assay, and also measured the ability of CTLs to kill epitope peptide-pulsed cells (LL_{50}, peptide concentration providing a half of maximum percent specific lysis; Table I). A high BL_{50}/LL_{50} ratio indicates a high ability of TCR to recognize the epitope. BL_{50} values of Pol743-9 and Pol283-8 peptides were 10- and 100-fold lower than those of Rev71-11 and Gag327-9, respectively, indicating that the former peptides had higher ability to bind to HLA-B*5101 than the latter ones. In contrast, LL_{50} values of Pol743-9-51 and SF2-Rev71-11-55 CTLs were 6- and 20-fold lower than those of SF2-Pol283-8 and SF2-Gag327-9-249 CTLs, respectively. Thus, the Pol743-9-51and Pol283-8-specific CTLs showed lower BL_{50}/LL_{50} ratio than SF2-Gag327-9-249 and SF2-Rev71-11-55 CTLs (Table I). These results indicate that the ability of TCR of the former CTLs to recognize the epitope was much lower than that of the latter ones. Both Pol743-9-51 and Pol283-8-specific CTLs effectively killed NL-432-infected CD4$^+$ T cells, whereas SF2-Gag327-9-249 and SF2-Rev71-11-55 CTLs failed to kill them. These findings together suggest that the

Table I. *Ability of HLA-B*5101-restricted CTLs to recognize the epitopes*

CTLs	Epitope Peptide	Sequence	(A) Binding Ability of Peptide (BL_{50})	(B) Cytolytic Activity for Peptide-Pulsed Cells (LL_{50})[a]	(A)/(B) Ability of TCR to Recognize the Epitope	Cytolytic Activity for NL-432-Infected Cells (% specific lysis)
Pol743–9-51	Pol743–9	LPPVVAKEI	6.1×10^{-6} M	5.0×10^{-9} M	1,220	36.0
Pol283–8	Pol283–8	TAFTIPSI	6.8×10^{-6} M	3.0×10^{-8} M	227	28.7
Gag327–9-249	Gag327–9	NANPDCKTI	4.0×10^{-4} M	1.0×10^{-7} M	4,000	8.0
Rev71–11-55	Rev71–11	VPLQLPPLERL	5.0×10^{-5} M	5.0×10^{-9} M	10,000	0

[a] LL50, Peptide concentration providing a half of maximum percent specific lysis.

difference in the ability between these CTLs to kill NL432-infected $CD4^+$ T cells is due to that in the number of epitopes presented by HLA-B*5101 on the surface of NL-432-infected $CD4^+$ T cells rather than that in the ability of TCR to recognize the epitope. A recent study also showed that the abilities of HIV-1-specific CTLs to kill cell lines infected with Nef-defective HIV-1 IIIB clone and to suppress replication of this clone were associated with specificity of the CTLs but not with functional avidity of the CTLs (14). Thus, the number of HLA-epitope complex presented on HIV-1-infected $CD4^+$ T cells may be critical for recognition of HIV-1-specific CTLs.

HLA-B57 and -B27 alleles are well-known factors associated with slow progression to AIDS (10). A recent study revealed that HIV-1-specific $CD8^+$ T cells have a high proliferation capacity that is coupled to perforin expression in HLA-B*5701$^+$ LTNPs but not in HLA-B*5701$^+$ or HLA-B*5701$^-$ progressors (15), suggesting that HIV-1-specific $CD8^+$ T cells, which have a high proliferation capacity and effector function, control HIV-1 replication in HLA-B*5701$^+$ LTNPs. However, the mechanism of the association of these HLA alleles with slow progression to AIDS still remains unclear. The present study revealed that the CTLs specific for the two Pol epitopes presented by one of the HLA class I molecules associated with slow progression to AIDS, HLA-B*5101, completely suppressed HIV-1 replication and killed HIV-1-infected $CD4^+$ T cells, implying that these cells effectively control HIV-1 replication in vivo. Because we investigated a limited number of CTLs restricted by HLA alleles that are not associated with slow progression of AIDS in the present and previous studies (8), it still remains unclear that the existence of these CTLs is associated with slow progression of AIDS. Further analysis of HIV-1-specific CTLs restricted by various HLA alleles will clarify the mechanism of the association of these HLA alleles with slow progression to AIDS.

In the present study, we showed that the effect of Nef-mediated HLA class I down-regulation on recognition by HIV-1-specific $CD8^+$ T cells of HIV-1-infected $CD4^+$ T cells vary in epitopes, and particularly demonstrated the existence of HIV-1-specific CTLs that could completely suppress Nef^+ HIV-1 replication and effectively kill primary $CD4^+$ T cells infected with Nef^+ HIV-1. These CTLs are expected to suppress HIV-1 replication in vivo.

Acknowledgments

We thank Dr. Adachi for the generous gift of the NL-M20A clone; Sachi Doki for technical assistance; and Sachiko Sakai for secretarial assistance.

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Identification and characterization of HIV-1-specific CD8$^+$ T cell epitopes presented by HLA-A*2601

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Received 3 August 2004; received in revised form 8 February 2005; accepted 17 February 2005
Available online 17 March 2005

Abstract

Since HLA-A*26 is one of the most common alleles in Asia, where approximately 20% of people have this allele, identification of HIV-1-specific epitopes presented by HLA-A*26 is necessary for studies on the immunopathogenesis of AIDS and vaccine development in Asia. As presented herein, we used the reverse immunogenetics approach to identify HIV-1 epitopes presented by HLA-A*2601, one of the major HLA-A*26 subtypes. We selected 24 HLA-A*2601-binding peptides out of 110 HIV-1 peptides by using a HLA-A*2601 stabilization assay. The ability of these HLA-A*2601-binding peptides to induce peptide-specific CD8$^+$ T cells was tested by stimulating PBMCs from HIV-1-infected individuals having HLA-A*2601 with these peptides. Four HLA-A*2601-binding peptides induced peptide-specific CD8 T cells. Analysis using HIV-1 recombinant vaccinia-infected C1R-A*2601 cells indicated that these four peptides were HIV-1 epitopes endogenously presented by HLA-A*2601. Two epitope-specific CD8$^+$ T cells were predominantly detected in HIV-1 infected individuals, suggesting that these epitopes may be useful for vaccine development.
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Keywords: HIV-1; CTL; HLA-A*2601

1. Introduction

In acute and chronic phases of human immunodeficiency virus type-1 (HIV-1) infection, an HIV-1-specific cytotoxic T lymphocyte (CTL) response is effectively induced [1–3]. Several studies have provided direct evidence for high levels of HIV-1-specific CTLs in patients in whom HIV-1 replication is controlled [4,5], suggesting that CTLs may control HIV-1 replication. Therefore, HIV-1 vaccine development and therapy to induce HIV-1-specific CTL might be expected to prevent HIV-1 infection and the development of AIDS.

On the other hand, it is believed that HIV-1 escapes from the host immune system. There are several proposed mechanisms that would allow HIV-1-infected cells to escape from being killed by HIV-1-specific CD8$^+$ T cells [6–11]. A mutation within the viral epitopes recognized by CTL is one of these mechanisms [7]. Therefore, identification and characterization of such epitopes are necessary for studies on vaccine development and immunopathogenesis of AIDS. We previously showed a strategy to determine HIV-1 epitopes by testing whether HIV-1-specific CTLs are induced in PBMCs from HIV-1-seropositive individuals by stimulating the cells with HLA class I-binding HIV-1 peptides [12,13]. Subsequent studies employing this strategy, which is called reverse immunogenetics, identified a large number of HIV-1 epitopes presented by HLA-A*1101, HLA-A*2402, HLA-A*3303 and HLA-B*5101 [14–19].

HLA-A*26 is one of the most common alleles in Asian countries, where approximately 20% of the people have this allele. Although 20 HLA-A*26 subtypes from A*2601 to

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0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.02.022

A*2620 have been reported, A*2601, A*2602 and A*2603, are predominantly found in Asian countries including Japan [20,21]. Therefore, identification of HIV-1 epitopes presented by these alleles is required for studies on AIDS pathogenesis and vaccine development in Asia. Since HLA-A*2601 is the most frequently found HLA-A*26 subtype [20,21], we first focused on identification of HIV-1 epitopes presented by this subtype. The strategy of reverse immunogenetics was used to identify HLA-A*2601-restricted epitopes. Peptide-specific $CD8^+$ T cells were measured by counting IFN-γ-producing $CD8^+$ T cells after stimulating PBMCs from HIV-1-infected HLA-$A*2601^+$ individuals with HLA-A*2601-binding HIV-1 peptides. $CD8^+$ T cell epitopes were finally identified by testing whether peptide-specific $CD8^+$ T cells produced IFN-γ after stimulation with HIV-1 recombinant (r-HIV-1) vaccinia-infected HLA-$A*2601^+$ cells. We herein describe 4 HLA-A*2601-restricted HIV-1 epitopes identified by using this reverse immunogenetics technique.

2. Materials and methods

2.1. Cells

Cells of C1R and TAP-defective mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). C1R cells expressing HLA-A*2601 (C1R-A*2601) were generated by transfecting the C1R cells with the HLA-A*2601 gene. RMA-S transfectants expressing HLA-A*2601 (RMA-S-A*2601) were previously generated [22]. C1R-A*2601 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin; and RMA-S-A* 2601, in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

2.2. Synthetic peptides

Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2601 binding motifs. Peptides were prepared by utilizing an automated multiple peptide synthesizer, with the Fmoc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% of purity were used in the present study.

2.3. HLA-stabilization assay

RMA-S-A*2601 cells express empty HLA-A*2601 on their cell surface when they are cultured at 26 °C. The surface expression of empty HLA-A*2601 rapidly decreases after RMA-S-A*2601 cells are incubated at 37 °C, whereas HLA-A*2601 molecules are stably expressed on the surface of the cells at 37 °C if they bind peptides. Binding of HIV-1 derived peptides to HLA-A*2601 was measured as previously described by using RMA-S-A*2601 cells [22]. Briefly, RMA-S-A*2601 cells were cultured at 26 °C for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class I α_3 domain-specific mAb TP25.99 [23] and the FITC-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACS Calibur (BD Bioscience, San Jose, California, USA). HLA-A*2601-binding peptides were defined as those which at a concentration of 10^{-3} M caused >25% increase in MFI compared with the MFI of control RMA-S-A*2601 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL50 value. Binding peptides were classified into three categories according to their BL_{50}: high binding ($BL_{50} < 10^{-5}$), medium binding ($10^{-5} \leq BL_{50} < 10^{-4}$), and low binding ($BL_{50} \geq 10^{-4}$). High-, medium-, low- and non-binding peptides were ranked as 3, 2, 1 and 0, respectively. The mean binding rank (MBR) of a group of peptides was then calculated. For example, if in a group of 10 peptides, three were high binding, one was medium binding, one was low binding and five non-binding, then the MBR is 12/10 = 1.20. The MBR of each amino acid group and each peptide length were analyzed by the Mann-Whitney *U*-test (Stat View 4.02; Abacus Concepts, Berkeley, CA, USA).

2.4. Patients

Blood samples were collected with informed consent from seven HIV-1 infected patients with HLA-A*2601 (KI-098 with acute HIV-1 clade B infection, and KI-003, KI-134, KI-034, KI-060, KI-123 and KI-125 with chronic HIV-1 clade B infection).

2.5. Detection of IFN-γ-producing $CD8^+$ T cells after stimulation of PBMCs with peptide-pulsed C1R-A*2601 cells

After C1R-A*2601 cells had been incubated for 60 min with each peptide (1 μM) or each peptide cocktail (1 μM concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed C1R cells (8×10^4 per well) and cultured PBMC cells (2×10^4 per well) were added to a 96-well round-bottomed plate, which was incubated for 2 h. Subsequently, Brefeldin A (10 μg/ml) was then added and incubation was continued for an additional 4 h. Next the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4 °C for 20 min, and then permeabilized with PBS containing 20% newborn calf serum (Summit Biotechnology, Greely, Co.) and 0.1% saponin (permeabilizing buffer) at 4 °C for 10 min. Thereafter the cells were resuspended in permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in PBS containing

2% paraformaldehyde and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by flow cytometry.

2.6. Detection of IFN-γ-producing $CD8^+$ T cells after stimulation with C1R-A*2601 cells infected with recombinant HIV-1 vaccinia

C1R-A*2601 cells were infected with 10 plaque-forming units of recombinant vaccinia virus expressing a given protein (Gag and Pol, Nef, or Env) or WT vaccinia virus per target cells at 37 °C for 1 h, and then cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells in a 96-well round-bottomed plate at 37 °C for 2 h. The activities of the effector cells to produce IFN-γ were tested at an E:T ratio of 1:4. Brefeldin A (10 μg/ml) was added, and then incubation was continued for an additional 4 h. The cells were thereafter stained with anti-CD8 mAb, fixed with 4% paraformaldehyde at 4 °C for 20 min, and incubated at 4 °C for 10 min in the permeabilizing buffer. They were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb. The cells were finally resuspended in PBS containing 2% paraformaldehyde and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by flow cytometry.

2.7. Generation of cytotoxic T lymphocyte (CTL) clones

Gag169–177-specific, Pol604–612-specific, Pol647–656-specific, and Env464–473-specific CTL clones were generated from HIV-1-specific bulk cultured T cells by limiting dilution in U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-A*2601 cells pre-pulsed with the corresponding peptide at 1 μM) in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human recombinant IL-2(rIL-2)(Ajinomoto, Tokyo, Japan).

2.8. CTL assay

Cytotoxicity was measured by use of the standard ^{51}Cr release assay. Target cells (5×10^5) were incubated for 60 min with 150 μCi $Na_2{}^{51}CrO_4$ in saline, and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (5×10^3/well) were added to each well of a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) with the desired amount of the corresponding peptide and were incubated for 1 h at 37 °C. Effector cells were added and the mixtures were incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release (cpm spn) was determined by measuring the cpm in the supernatant in the wells containing only target cells. The maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% TritonX-100. Percent specific lysis was calculated as follows: percentage specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the cpm in the supernatant from wells containing both target and effector cells.

3. Results

3.1. Identification of HLA-A*2601-binding peptides from HIV-1 peptides carrying HLA-A*2601-binding motif

A previous study revealed that HLA-A*2601-binding peptides have two anchor residues, one at position 2 and the other at the C-terminus [24]. Five (Val, Thr, Ile, Leu and Phe) and 2 (Tyr and Phe) amino acids prevail at position 2 and the C terminus, respectively. Our recent study using an HLA-A*2601 stabilization assay demonstrated that acidic amino acids, Asp and Glu, and a broad range of amino acids with the exception of positively charged ones function as anchors at position 1 and the C-terminus, respectively [22]. Therefore, we chose the sequences of 8-mer to 11-mer containing these anchor residues at position 1, position 2 and the C-terminus from the sequences of Gag, Pol, Nef and Env proteins in the HIV-1 SF2 strain. One hundred-ten peptides matched to these sequences were synthesized. The binding of these synthetic peptides to HLA-A*2601 was then tested by the HLA-stabilization assay using RMA-S-A*2601 cells. A representative result for peptides with high ($BL50 < 10^{-5}$), medium ($10^{-5} \leq BL50 < 10^{-4}$), and low affinity ($BL50 \geq 10^{-4}$) is shown in Fig. 1. Twenty-

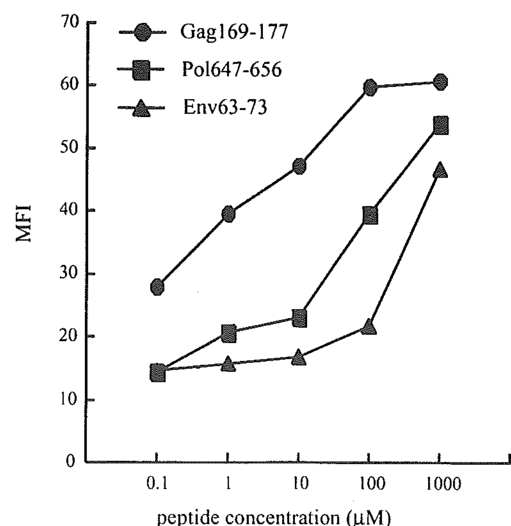

Fig. 1. Binding to HLA-A*2601 of HIV-1 peptides carrying HLA-A*2601 motif. Binding of the peptides carrying A*2601 anchors was measured by a stabilization assay using RMA-S-A*2601 cells. Representative results of binding peptides with high-(Gag169–177), medium-(Pol647–656), and low-(Env63–73) affinity are shown.

Table 1
HLA-A*2601-binding HIV-1 peptides

Sequence	Position	BL50 (M)
EVFRPGGGDM	Env464–473	1.5×10^{-6}
EVIPMFSAL	Gag169–177	2.9×10^{-6}
ETKLGKAGY	Pol604–612	4.1×10^{-6}
ETWEAWWMEY	Pol551-560	6.4×10^{-6}
EVHNVWATHA	Env63–72	1.1×10^{-5}
ELKKIIGQV	Pol872–880	1.9×10^{-5}
ETPGIRYQY	Pol293–301	2.7×10^{-5}
EVNIVTDSQY	Pol647–656	5.2×10^{-5}
ETINEEAAEW	Gag205–214	1.4×10^{-4}
EIYKRWIIL	Gag262–270	1.6×10^{-4}
EILGHRGWEA	Env782–791	2.6×10^{-4}
ETKLGKAGYV	Pol604–613	3.3×10^{-4}
ELYPLTSLRSL	Gag484–494	3.3×10^{-4}
EVVIRSDNF	Env272–280	3.3×10^{-4}
EVYYDPSKDLV	Pol471–481	3.9×10^{-4}
DTTNQKTEL	Pol626–634	4.0×10^{-4}
DVKNWMTETLL	Gag314–324	4.0×10^{-4}
EVNIVTDSQYA	Pol647–657	5.9×10^{-4}
ETGQETAYF	Pol807–815	6.0×10^{-4}
EVHNVWATHAC	Env63–73	$>1.0 \times 10^{-3}$
EICGHKAIGTV	Pol121–131	$>1.0 \times 10^{-3}$
DIISLWDQS	Env106–114	$>1.0 \times 10^{-3}$
EVIPLTEEA	Pol446–454	$>1.0 \times 10^{-3}$
DIVIYQYMDDL	Pol332–342	$>1.0 \times 10^{-3}$

four peptides bound to HLA-A*2601. They included four high-, four medium- and 16 low-affinity peptides (Table 1).

Twenty of seventy-two peptides (27.8%) carrying Glu at P1 bound to HLA-A*2601, whereas only 4 of 38 peptides (10.5%) carrying Asp at P1 bound to this allele (Table 2). This supports a previous study using only 38 peptides, which revealed higher binding ability of peptides carrying Glu at P1 than those carrying Asp at the same position [22]. In addition, peptides carrying Val and Thr at P2 exhibited higher affinity than those carrying Leu and Ile at the same position (Table 2), supporting also the results in a previous study using mutated peptides at position 2 [22].

*3.2. Induction of HIV-1 peptide-specific CD8$^+$ T cells from PBMCs of HIV-1-infected individuals with HLA-A*2601*

PBMCs from three HIV-1-infected individuals with HLA-A*2601 (KI-003, KI-098 and KI-134) were cultured for 10–14 days after they had been stimulated with cocktails of HLA-A*2601-binding peptides. The cultured cells were then tested for IFN-γ production by CD8$^+$ T cells after stimulation with C1R-A*2601 cells pre-pulsed with the peptide cocktails (Table 3). Cocktail 1 induced a high number of the specific CD8$^+$ T cells in PBMCs from KI-098 and KI-134 and a low number of them in PBMC from KI-003. Cocktail 2 induced a high number of the specific CD8$^+$ T cells in PBMCs from KI-003 and KI-134, whereas cocktails 3, 4 and 5 induced a low number of the specific CD8$^+$ T cells in PBMCs from KI-098, KI-003 and KI-134, respectively. To determine which peptides in the cocktails induced the specific CD8$^+$ T cells, we stimulated the cultured cells with C1R-A*2601 cells pre-pulsed with each peptide contained in the cocktails. Env464–473, Pol604–612, Pol647–656 and Gag169–177 peptides induced the specific CD8$^+$ T cells in 1 (KI-003), 2 (KI-003 and KI-134), 1 (KI-003) and 2 (KI-098 and KI-134) individuals, respectively (Fig. 2).

Table 2
Effect of residues at P1 and P2 on the binding of peptides to HLA-A*2601

Amino acid	NBP[a]/NTP[b]	MBR[c]
Position 1		
E	20/72 (27.78%)	0.44
D	4/38 (10.53%)	0.11
Position 2		
V	10/24 (41.67%)	0.67
T	6/22 (27.28%)	0.55
L	2/37 (5.41%)	0.01
I	5/27 (18.52%)	0.19

[a] Number of binding peptides.
[b] Number of total peptides tested.
[c] Mean binding rank.

*3.3. Identification of HIV-1-specific CD8$^+$ T cell epitopes endogenously presented by HLA-A*2601*

To clarify whether these peptides are endogenously presented in HIV-1-infected cells, we investigated the ability of these peptide-specific CD8$^+$ T cells to produce IFN-γ after stimulation of these cells with C1R-A*2601 cells infected with r-HIV-1 vaccinia. The cultures containing the four peptide-specific CD8$^+$ T cells significantly produced IFN-γ after stimulation with r-HIV-1 vaccinia-infected cells as

Table 3
Induction of peptide-cocktail-specific CD8$^+$ T cells in cultured cells stimulated with the peptide cocktail

Peptide cocktail	Percentage of IFN-γ-producing cells in CD8$^+$ T cells		
	KI-003	KI-098	KI-134
Cocktail 1 (Gag169–177, Pol551–560, Env464–473, Env63–72)	0.9	55.4	4.7
Cocktail 2 (Pol872–880, Pol293–301, Pol647–656, Pol604–612)	24.5	0	10.3
Cocktail 3 (Gag205–214, Gag262–270, Env782–791)	0	0.8	0.1
Cocktail 4 (Pol604–613, Gag484–494, Env272–280, Pol471–481)	2.5	0	0
Cocktail 5 (Pol626–634, Gag314–324, Pol647–657, Pol807–815)	0	0	0.8
Cocktail 6 (Env63–73, Pol121–131, Env106–114, Pol446–454, Pol332–342)	0	0	0

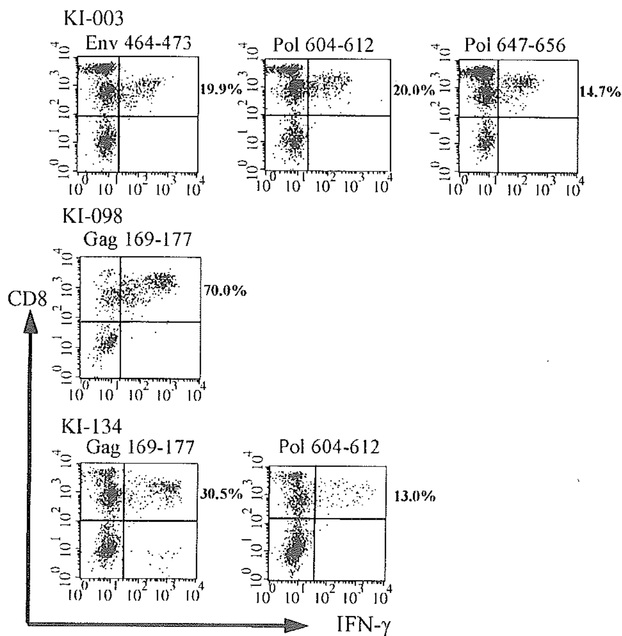

Fig. 2. Induction of HIV-1-specific CD8 T cells from PBMCs of HIV-1-infected individuals with HLA-A*2601. PBMCs from three HIV-1-infected individuals (KI-003, KI-098 and KI-134) were stimulated with cocktails of HLA-A*2601 binding peptides (Table 3), and were then cultured for 10–14 days. The cultured cells were stimulated with C1R-A*2601 cells pre-pulsed for 6 h with the cocktail of HLA-A*2601 binding peptides. IFN-γ-producing CD8⁺ T cells were measured by using flow cytometry. Cultured cells containing IFN-γ-producing CD8⁺ T cells from three HIV-1-infected individuals were stimulated with C1R-A*2601 cells pre-pulsed with individual peptides included in the cocktails shown in Table 3. IFN-γ-producing CD8⁺ T cells were measured by using flow cytometry. Percentage of IFN-γ-producing CD8⁺ T cells is presented in each figure.

compared with those stimulated with WT vaccinia-infected cells (Fig. 3). These results indicate that Gag169–177, Pol604–612, Pol647–656 and Env464–473 are endogenously presented in HIV-1-infected cells and recognized as CD8⁺ T cell epitopes.

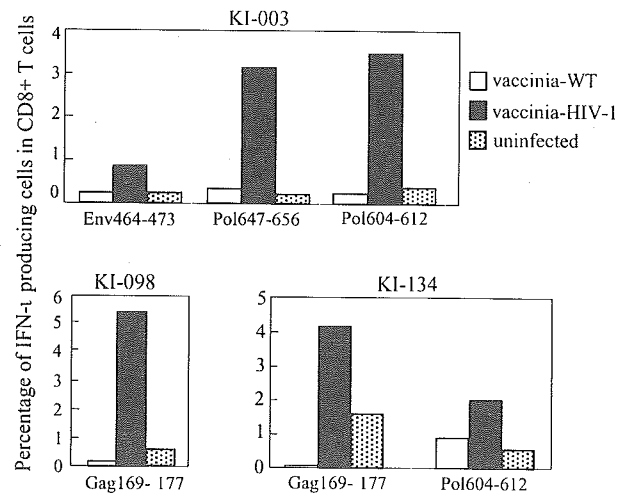

Fig. 3. Recognition of HLA-A*2601-restricted HIV-1 epitopes presented on r-HIV-1 vaccinia-infected cells. Cultured cells containing peptide-specific CD8⁺ T cells shown, were examined for IFN-γ production after they had been stimulated with C1R-A*2601 cells infected with wild-type vaccinia (vaccinia-WT), those with r-HIV-1-vaccinia-infected C1R-A*2601(vaccinia-HIV-1), or uninfected C1R-A*2601 cells (uninfected).

To confirm that these CD8⁺ T cell epitopes are recognized by specific CTLs, we established CTL clones specific for these epitopes. Pol647–656-specific and Env464–473-specific CTL clones were established from patient KI-003, whereas Gag169–177-specific and Pol604–612-specific CTL ones were established from patients KI-098 and KI-125, respectively. These CTL clones effectively killed not only epitope–peptide-pulsed C1R-A*2601 cells but also C1R-A*2601 cells infected with recombinant HIV-1(r-HIV-1)-vaccinia (Fig. 4). These results show that the peptides were epitopes presented by the HLA-A*2601 and indicated that they were recognized as CTL epitopes by the specific CTLs.

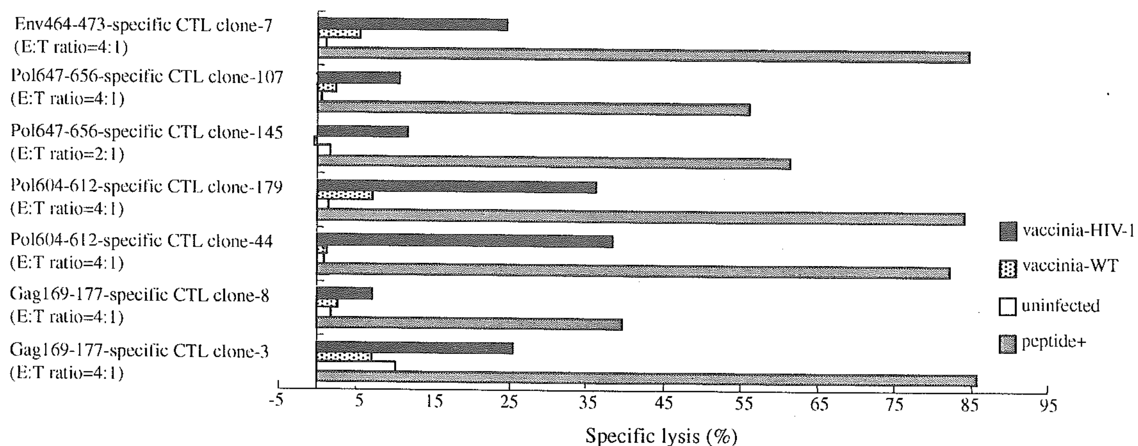

Fig. 4. Cytolytic activity of the HLA-A*2601-restricted CTL clones toward peptide-pulsed or r-HIV-1 vaccinia-infected cells. The activities of HLA-A*2601-restricted CTL clones toward C1R-A*2601 cells pre-pulsed with 1 μM epitope peptides (peptide+) or infected with recombinant vaccinia virus expressing the corresponding proteins, Gag and Pol, or Env (vaccinia-HIV-1), or wild-type vaccinia virus (vaccinia-WT) were tested at an effector-to-target (E:T) ratio of 2:1 or 4:1.

Table 4
Induction of epitope-specific CD8$^+$ T cells in PBMCs from HIV-1-infected individuals

Patients[a]	Viral load[b]	CD4[c]	CD8[c]	Percentage of IFN-γ-producing cells in CD8$^+$ T cells			
				Gag169–177	Pol604–612	Pol647–656	Env464–473
KI-003	3.1×10^3	262	3469	0.1	3.5	3.2	1.1
KI-098	2.2×10^2	981	740	5.3	0.1	0.1	0.2
KI-134	3.7×10^5	422	1545	4.1	0.4	0.7	0.4
KI-123	6.6×10^4	406	1328	2.7	1.2	0.2	0.5
KI-060	8.4×10^3	542	1085	0.5	ND	0.5	0.5
KI-125	2.6×10^4	258	115	4.3	10.6	0.7	0.3
KI-034	2.2×10^4	242	997	4.1	2.9	0.2	0.3

[a] HIV-1-infected individuals with HLA-A*2601.
[b] Copy/ml.
[c] Cell/μl.

*3.4. Gag169–177- and Pol604–612-specific CD8$^+$ T cells are predominantly found in HIV-1-infected individuals with HLA-A*2601*

To clarify whether CD8$^+$ T cells specific for these epitopes were predominantly induced in HIV-1-infected individuals bearing HLA-A*2601, we investigated the induction of the specific CD8$^+$ T cells in PBMCs from 7 HIV-1-infected individuals by stimulating them with these epitope peptides. Gag169–177- and Pol604–612-specific CD8$^+$ T cells (more than 1% of total CD8$^+$ T cells) were found in five and four, respectively, of the seven HIV-1-infected individuals (Table 4). In contrast, Env464–473- and Pol647–656-specific CD8$^+$ T cells were induced in only one of these seven individuals. These results suggest that Gag169–177 and Pol604–612 are predominantly recognized in most HIV-1-infected individuals with HLA-A*2601.

4. Discussion

A previous study that analyzed the sequences of self-peptides eluted from HLA-A*2601 molecules identified the motifs of HLA-A*2601-binding peptides (P2: Val, Thr, Ile, Phe, and Leu, C terminus: Phe and Tyr, Ref. [24]). A subsequent study using an HLA-A*2601 stabilization assay confirmed the anchor residues at position 2 and the C-terminus by using mutated peptides at position 2 and the C-terminus, and further revealed by using 38 peptides that Glu/Asp and non-polar amino acids are preferred at position 1 and the C-terminus, respectively [22]. In the present study using 110 eight- to eleven-mer peptides, we confirmed these anchor residues at positions 1, 2 and the C-terminus. These anchor residues are useful for identification of HLA-A*2601-restricted epitopes including those of viral antigens, tumor antigens, and self-antigens by using reverse immunogenetics.

Although we employed a ^{51}Cr-release cytotoxic assay to identify peptide-specific CD8$^+$ T cells in previous studies employing reverse immunogenetics [15–19], we used the IFN-γ-production assay for the present study. Since the peptide-stimulated, cultured PBMCs contain NK cells, they often show non-specific killing activity toward target cells such as C1R cells, which are sensitive to NK cells. On the other hand, the effect of NK cells is excluded in the IFN-γ-production assay, since peptide-specific CD8$^+$ T cells can be specifically identified by using flow cytometry with anti-CD8 and anti-IFN-γ mAbs. Therefore, this assay is useful for identification of epitope-specific responses by HIV-1-specific CD8$^+$ T cells. On the other hand, it is impossible to show whether these specific CD8$^+$ T cells include cytotoxic T cells by the IFN-γ-production assay. We therefore generated epitope-specific T cell clones and tested whether these CTL clones could kill the target cells. The results showed that these epitopes were indeed recognized by specific CTLs.

The induction of the four epitope-specific CD8$^+$ T cells varied among seven HIV-1-infected individuals carrying HLA-A*2601 (Table 4). Gag169-specific and Pol604-specific CD8$^+$ T cells were found in five of seven and in four of six HIV-1-infected individuals, respectively. In contrast, Pol647-specific and Env464-specific CD8$^+$ T cells were detected only in KI-003. These results suggest that Gag169-specific and Pol604-specific CD8$^+$ T cells were predominantly induced in HIV-1-infected individuals bearing HLA-A*2601. The induction of these HIV-1-specific CD8$^+$ T cells was not correlated with viral load or the number of CD4$^+$ or CD8$^+$ T cells. Epitope mutation may be one factor for the failure to induce some specific CTLs in HIV-1-infected individuals. However, it is difficult to conclude that this would account for all cases where specific CTLs are not induced in HIV-1-infected individuals, because no mutation was detected in some cases [25].

We searched reported HIV-1 sequences of HIV-1 clade A–E (HIV sequence database, Los Alamos, New Mexico, USA) for major variants of these epitopes. The sequences of Gag169–177 (EVIPMFSAL) and Pol604–612 (ETKLGKAGY) were found in 35 of 36 HIV-1 clade B isolates and in 25 of 33 HIV-1 clade B isolates, respectively (Table 5), indicating that the sequences are relatively conserved in clade B. Since CTLs specific for these epitopes were frequently induced in HIV-1-infected individuals with HLA-A*2601, they may be useful for making a vaccine to induce specific CTLs. These sequences were also conserved in clades A, D and E (Table 5), implying that these sequences are epitopes

Table 5
Variation of HLA-A*2601-restricted epitopes in clades A–E

Epitope	Sequence	Clade A	Clade B	Clade C	Clade D	Clade E
Gag 169-177	EVIPMFSAL	8/11	35/36	1/28	5/5	8/9
	------T--	2/11	1/36	25/28		
	----V----	1/11				
	-I----T--			1/28		
	----I-T--			1/28		
	------P--					1/9
Pol 604-612	ETKLGKAGY	11/11	25/33	7/26	4/5	9/9
	---I-----		1/33	8/26	1/5	
	D--I-----			2/26		
	---VK----		1/33			
	--R------		2/33			
	---K-----		1/33			
	D--S-----		1/33			
	D--------		1/33			
	-----R---		1/33			
	---M-----			2/26		
	-I-M-----			1/26		
	--------C			1/26		
	---K-----			1/26		
	---V-----			1/26		
	-S-I-----			1/26		
	--RI-----			1/26		
	D--K-----			1/26		

in these clades as well. Indeed, our previous study revealed that clade B CTL epitopes, whose sequences were conserved between clades B and E, were recognized as epitopes by CTLs in clade E-infected individuals [26]. That study also revealed that mutants of clade B epitopes, which were predominantly found in clade E, were recognized as CTL epitopes in clade E-infected individuals. The Gag169–177 mutant carrying Thr at position 7 was the consensus sequence in clade C, whereas the Pol604–612 mutant carrying Ile at position 4 was predominantly found in this clade (Table 5). Therefore, these mutants might be recognized as T cell epitopes in clade C-infected individuals. In Asian countries where clades C and E HIV-1 are prevalent in addition to clade B, HLA-A*2601 is one of the commonly found alleles. Identification of HLA-A*2601-restricted HIV-1 epitopes in clades C and E would also be useful for HIV-1 vaccine development in Asia.

In conclusion, we identified 4 HLA-A*2601-resticted $CD8^+$ T cells epitopes by using reverse immunogenetics in the present study. These four epitopes will be useful for studies on the immunopathogenesis of AIDS in HIV-1 clade B-infected individuals. Two epitopes in particular, Gag169–177 and Pol604–612, are promising for the development of an HIV-1 vaccine, since $CD8^+$ T cells specific for these epitopes were predominantly induced in HIV-1-infected individuals bearing HLA-A*2601.

Acknowledgements

The authors thank Dr. S. Ferrone for the gift of mAB TP25.99 and Sachiko Sakai for secretarial assistance. This research was supported by a grant-in aid for scientific research from the Ministry of Health, Labour and Welfare, the government of Japan, by a grant from the Japan Health Science Foundation, and by a grant from the Organization for Pharmaceutical Safety and Research.

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Efficacy and Immunologic Responses to Influenza Vaccine in HIV-1–Infected Patients

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Summary: Influenza vaccine is recommended for HIV-1–infected patients. The present prospective study was conducted to evaluate the clinical efficacy and immunologic responses to the vaccine. From November 1 to December 27, 2002, 262 HIV-1–infected patients received a trivalent influenza subunit vaccine, whereas 66 did not. Influenza illness occurred in 16 vaccinated and 14 nonvaccinated patients (incidence = 6.1% [95% confidence interval (CI): 4%–10%] in vaccinated vs. 21.2% [CI: 13%–35%] in nonvaccinated persons, $P < 0.001$; relative risk = 0.29 [CI: 0.14–0.55]). Influenza vaccine provided clinically effective protection against influenza illness in HIV-1–infected patients. In baseline antibody-negative patients, anti-H1 and anti-H3 antibody responses to the vaccination were significant in those patients with a CD4 count >200 cells/μL compared with those with a CD4 count <200 cells/μL ($P < 0.05$). In contrast, in baseline antibody-positive patients, good antibody responses were observed irrespective of CD4 counts, like the healthy controls. Based on these results, annual vaccination is recommended. Specific CD4 responses correlated with HIV-1 viral load (VL), especially in patients treated with highly active antiretroviral therapy (HAART) compared with those without HAART ($P < 0.01$), although the clinical efficacy did not correlate with HIV-1 VL. HAART may enhance the immunologic efficacy of influenza vaccine.

Key Words: HIV-1, influenza, vaccination, antibody response, specific CD4

(*J Acquir Immune Defic Syndr* 2005;39:167–173)

After the recent approval of various anti-influenza drugs and rapid diagnosis kits for influenza infection by the Ministry of Health, Labor, and Welfare of Japan, it has become easier to diagnose this infection. Along with the developments in diagnostic methods and treatment of the infection, influenza vaccination programs have been actively applied in HIV-1–infected individuals. Influenza virus infection may be more prolonged in individuals with immunodeficiency[1] and can cause a transient increase in plasma HIV-1 viral load (VL)[2] that might become relevant to the clinical course of HIV-1 infection.[2,3] Therefore, influenza vaccine has been generally recommended for HIV-1–infected patients,[4–6] as is already stated in the guidelines of the Advisory Committee on Immunization Practices.[7] Few studies have reported the protective effect of such vaccination in patients with HIV-1 infection, however. Previous studies demonstrated that the number of CD4 T cells (CD4 count) could predict the efficacy of and/or antibody response to the vaccine but did not clearly demonstrate the correlation between the vaccine efficacy and HIV VL.[1,8–15]

Activated memory $CD4^+$ T cells are the predominant target of HIV-1,[16] and the antibody response to hemagglutinin (HA) is T-cell dependent.[17–19] Therefore, highly active antiretroviral therapy (HAART) may reconstitute the immune function of not only the antibody responses but T helper (Th)–cell responses. In this large prospective clinical study, we investigated the clinical efficacy of influenza vaccine in HIV-1–infected patients and correlated it with the immune response to the vaccine as determined by increased antibody titer and/or HA-specific CD4 T cells.

Received for publication July 7, 2004; accepted March 16, 2005.
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Supported in part by a grant for AIDS Research from the Ministry of Health, Labor, and Welfare of Japan (H15-AIDS-001) and by the Japanese Foundation for AIDS Prevention (H.Yamanaka).
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MATERIALS AND METHODS

Study Design and Participants

A 0.5-mL dose of single-shot trivalent influenza subunit vaccine, which contains 15 μg of influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Shanton/7/87, was prepared for adults in the 2002 through 2003 winter season in Japan. All HIV-1–infected patients who consulted the outpatient clinic of the AIDS Clinical Center at the International Medical Center of Japan from November 1 to December 27, 2002 were advised to receive the vaccine, although the final decision was left to the individual. In previous seasons, nearly half of HIV-1–infected patients received influenza vaccine in our clinic. This study was designed to be prospective in nature but nonrandomized. Only individuals, vaccinated and nonvaccinated, who understood the purpose of the study were enrolled, without any incentives. To keep selective bias to a minimum, all vaccinated and consecutive first-come 100 nonvaccinated patients were asked to participate in this study. All study participants gave

informed consent, and the institutional ethical committee approved this study (protocol IMCJ-141). Twenty-six hospital staff members who were vaccinated with the same vaccine batch were enrolled as healthy immunized controls after consenting to participate in this study. Among them, 4 had no anti-influenza antibodies before vaccination. All participants were asked to visit to our clinic at least at week 0, 8, and/or 16 after enrollment to allow the withdrawal of 17 mL of blood at each visit for analysis of immunologic responses and routine examinations, including CD4 count and HIV VL.

Definition and Diagnosis of Influenza Virus Infection

In this study, influenza infection (illness) was defined if the patient had flulike symptoms associated with at least 1 adjunct diagnosis such as a serologic or virologic diagnosis. Flulike symptoms were defined as a fever of ≥38.0°C combined with 2 of the following 5 clinical symptoms: cough, rhinitis, myalgia, sore throat, and headache. All participants were asked to visit the clinic if they developed flulike symptoms. To avoid a bias in the clinical diagnosis, a history of influenza vaccination was written out on a separate colored sheet, which was removed from medical records before the outpatient clinic physician attended and examined the patient. The serologic diagnosis was defined as a >4-fold rise in anti-influenza antibody titer compared with before and 4 weeks after the symptoms. In addition, a change of the antibody titer from <10 to 40 U was defined as a 4-fold rise. Patients who had only the antibody rise but no flulike symptoms were not considered to have influenza-related illness. The virologic diagnosis was made by means of viral culture and/or a Rapidvue influenza test kit (Quidel, San Diego, CA) using a nasal or throat swab.

Laboratory Investigations

At each visit, CD4 T cells were enumerated by standard flow cytometry and HIV VL was measured using the Roche Amplicor assay kit, version 1.5 (Roche Diagnostic Systems, Branchburg, NJ). Antibody responses to each of the 3 individual vaccine components were examined by the standard hemagglutinin inhibition (HAI) assay.[20] Titers ≥40 U were defined as protective, and a >4-fold rise in the antibody titer was considered an adequate response in previously antibody-negative patients.

For assessment of HA-specific CD4 T-cell responses, intracellular γ-interferon (IFN) production was examined by flow cytometry using the method described previously.[21,22] Because of the limited availability of peripheral blood mononuclear cells (PBMCs), we analyzed the H1-specific CD4 T cells only. Because fresh PBMCs must be used for this assay, as a result of a labor limitation, only the first 10 participants per day were examined on any particular day. Briefly, HA was purified from influenza virus strain, A/New Caledonia/20/99 (H1N1), as described previously.[23] PBMCs were isolated from the fresh heparinized blood and cultured (2×10^6 cells/mL) with diluted H1 plus anti-CD28 antibody (1 μg/mL) or medium alone for 16 hours at 37°C. Brefeldin A (10 μg/mL) was added to each sample in the final 5 hours of incubation. After 16 hours of stimulation, the cells were collected and stained with anti-CD4 allophycocyanin antibody (Beckman Coulter, Fullerton, CA) and anti-CD69–fluorescent isothiocyanate antibody (Becton Dickinson). Subsequently, the cells were fixed and permeabilized to examine for the intracellular production of γ-IFN as described previously.[21,22] The flow cytometry analysis was performed by means of the FACSCalibur fluorescence-activated cell sorter with CellQuest software (BD Biosciences, San Jose, CA), and 10,000 CD4 T cells were collected for each analysis.

Statistical Analysis

The data on HA-specific CD4 T cells are presented as the arithmetic mean ± SEM. The data on anti-HA antibody titer are presented as the geometric mean. Statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA). Differences in the proportion of influenza virus infection between vaccinated and nonvaccinated groups were analyzed by the χ^2 test. Multiple logistic regression analysis was used to identify factors that contributed to protection against influenza illness. For the analyses of immune responses, participants were stratified by their CD4 count or HIV VL. Changes in antibody titer and HA-specific CD4 T cells were analyzed using the Kruskal-Wallis test or the Mann-Whitney U test. In all tests, a P value <0.05 was considered significant.

RESULTS

Subjects

During the period of vaccination, 626 HIV-1–infected patients visited our clinic, and 332 of these received the vaccine, whereas 294 did not. Among them, 317 of those vaccinated and 87 of 100 approached to participate as nonvaccinated patients agreed to participate in the present study. Consequently, 76 patients dropped out of the study (55 of 317 vaccinated patients and 21 of 87 nonvaccinated patients). There were no characteristic differences at baseline between the analyzed and drop-out patients (data not shown). None of the patients dropped out from the study because of HIV-1 disease progression, and none received anticancer or immunosuppressive agents during this study. The final composition of the study group based on compliance with the study protocol, including visits on the fixed dates, was 262 vaccinated (82.6%) and 66 nonvaccinated (75.9%) patients (Fig. 1). Table 1 summarizes the baseline characteristics of the participants.

Efficacy of Influenza Vaccine

The peak of the influenza epidemic of the 2002 through 2003 winter season in Japan was documented during the fourth week of January 2003 and was predominantly caused by influenza A/H3N2. The prevalence of influenza infection in this season was the third highest in the last decade.[24] In this study, 30 participants were diagnosed as having definitive influenza illness (5 patients with A/H1N1 strain, 16 with A/H3N2 strain, and 9 with B strain). Six patients were confirmed to have an influenza illness by flulike symptoms, positive viral cultures, positive influenza test kit results, and a >4-fold rise in antibody titer (1 with H1N1 strain, 1 with H3N2 strain, and

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