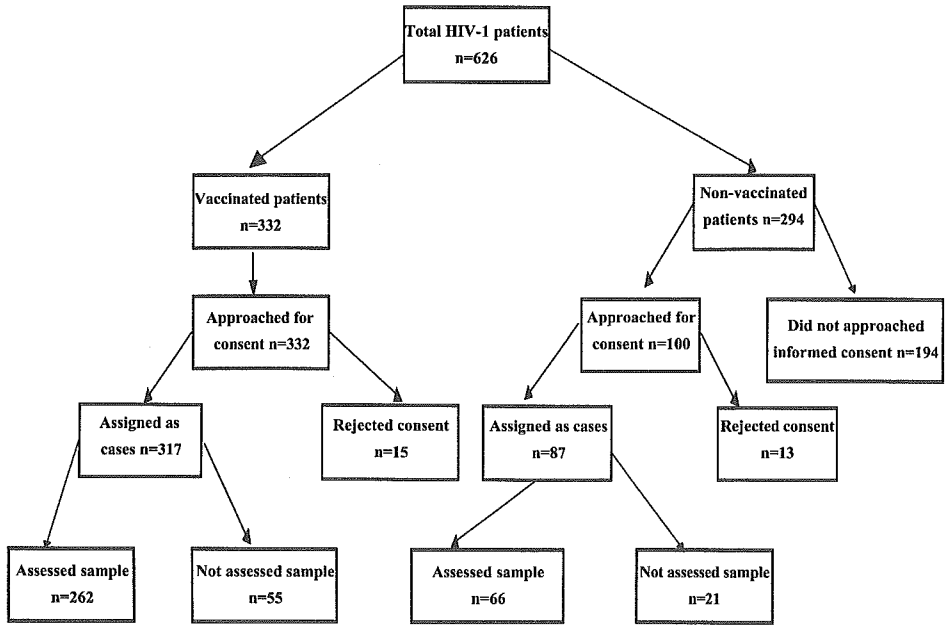

FIGURE 1. Profile of participants in this study.

4 with B strain); 3 by the symptoms, positive viral cultures, and antibody rise (2 with H1N1 strain and 1 with H3N2 strain); 5 by the symptoms, influenza test kit results, and antibody rise
(1 with H1N1 strain, 2 with H3N2 strain, and 2 with B strain); and 16 by the antibody rise between the symptoms (1 with H1N1 strain, 12 with H3N2 strain, and 3 with B strain). In total, 16 of 262 vaccinated patents had influenza illness (6.1%, confidence interval [CI]: 0.04–0.1) and 14 of 66 nonvaccinated patients had the illness (21.2%, CI: 0.13–0.35). The difference in the incidence between the 2 groups was significant ($P < 0.001$). The relative risk (RR) of influenza illness in vaccinated patients was 0.29 (CI: 0.14–0.55; $P < 0.001$) compared with nonvaccinated patients (Table 2). Eight patients who had a >4-fold rise in anti-H3 antibody titers between week 8 and week 16 without any clinical symptoms were not regarded as having influenza illness.

In patients with a CD4 count >200 cells/μL, the incidence of influenza illness in vaccinated patients (6.2%) was significantly lower than in nonvaccinated patients (21.0%) ($P < 0.001$). Conversely, in patients with a CD4 count <200 cells/μL, the same comparison showed no significant difference. Nevertheless, the incidences of influenza illness in vaccinated (5.9%) and nonvaccinated (22.2%) patients were the same as the incidence in patients with a CD4 count >200 cells/μL. Therefore, this analysis had lack of power because of the small number of nonvaccinated patients in this stratum. In vaccinated and nonvaccinated patients, the differences in the incidence were significant in patients with HAART ($P < 0.002$) and without HAART ($P < 0.05$) (see Table 2). When CD4 count was entered as a continuous variable, multivariate analysis using the logistic regression model identified vaccination ($P < 0.001$) and CD4 count ($P < 0.05$) but not HIV VL as independent predictors of influenza illness in HIV-1–infected patients.

In patients with influenza illness, 4 of 16 vaccinated patients and 4 of 14 nonvaccinated patients received an anti-influenza drug. None of the patients with influenza illness developed pneumonia that required treatment or hospitalization during the study period. Vaccination did not significantly change the HIV VL or CD4 count at weeks 8 and 16.

TABLE 1. Baseline Clinical and Immunologic Characteristics of Participants*

	Vaccinated	Nonvaccinated	*P*
No. participants (n)	262	66	—
Male/female ratio	7:1	15:1	n.s.
Median age, y (range)	41 (20–78)	40 (20–61)	n.s.
Received HAART (%)	75.2%	72.3%	n.s.
Median CD4 count at vaccination, μL (range)	380 (40–1137)	374 (66–1025)	n.s.
Median CD8 count at vaccination, μL (range)	778 (54–2649)	751 (163–1929)	n.s.
Median HIV VL at vaccination, $\log_{10}$/mL (range)	2.5 (1.5–6.2)	2.5 (1.5–6.4)	n.s.
Prior anti-H1 antibody-positive (%)	29.4%	26.4%	n.s.
Prior anti-H3 antibody-positive (%)	32.3%	30.3%	n.s.

*All participants were Japanese.
n.s. indicates not significant.

Anti-Hemagglutinin Antibody Responses Before and After Vaccination

HAI antibody titers against HA antigens (H1 and H3) were tested before and 8 and 16 weeks after vaccination (Table 3). To evaluate the effect of the single-shot influenza vaccine, subjects were divided into 2 groups based on the HAI titer before vaccination: the baseline HAI antibody-negative and antibody-positive groups. Furthermore, we excluded from this

TABLE 2. Incidence of Influenza Illness

	Vaccinated		Nonvaccinated		
	Illness/Patients	Rate (95% CI)	Illness/Patients	Rate (95% CI)	χ^2 Test
All patients	16/262	6.1% (0.04–0.1)	14/66	21.2% (0.13–0.35)	$P < 0.001$
CD4 count					
<200 cells/μL	3/51	5.9% (0.02–0.15)	2/9	22.2% (0.06–0.55)	n.s.
≥200 cells/μL	13/211	6.2% (0.03–0.1)	12/57	21.0% (0.12–0.33)	$P < 0.001$
HAART					
+	12/197	6.1% (0.04–0.1)	10/48	20.8% (0.11–0.34)	$P < 0.002$
−	4/65	6.2% (0.02–0.14)	4/18	22.2% (0.09–0.45)	$P < 0.05$

Incidence of influenza illness in healthy immunized controls was 3.8% (1 of 26, 95% CI: 0.01–0.19).
n.s. indicates not significant.

analysis the 13 patients who received the vaccination but had influenza illness (5 with H1N1 strain and 8 with H3N2 strain) during the study period so as to evaluate the antibody responses by the vaccination. The 8 patients who showed a >4-fold rise in anti-H3 antibody titers between week 8 and week 16 without any clinical symptoms were also excluded from this analysis, because the antibody rise in these cases was thought to be caused by influenza virus but not by vaccination. In the baseline HAI-negative group, the antibody responses to both antigens were significantly different compared with those in stratified HIV-1–infected patients by CD4 count (<200 cells/μL and ≥200 cells/μL; $P < 0.05$) at week 8 and week 16. These titers were low compared with those of the healthy immunized controls in both strata, however. In those with a CD4 count <200 cells/μL, 12 (27.9%) of 43 patients and 12 (32.4%) of 37 patients showed more than a 4-fold rise in the antibody responses against anti-H1 and anti-H3, respectively. In contrast, in those patients with a CD4 count >200 cells/μL, 62 (44.6%) of 139 patients and 61 (46.9%) of 130 patients showed a >4-fold rise in the antibody responses against anti-H1 and anti-H3, respectively. Although differences in the percentages of patients who showed both anti-H1 ($P = 0.05$) and anti-H3 ($P = 0.12$) antibody responses of the different CD4 strata were only marginal, there was a tendency for the single-shot vaccination to be more effective in terms of antibody responses in patients with a CD4 count >200 cells/μL. The antibody responses in both groups were not influenced by HIV VL (<100 copies/mL and ≥100 copies/mL; data not shown).

In the baseline HAI antibody-positive group, HAI titers to both antigens remained high and the sustainability of the antibody titers in HIV-1–infected patients was similar to those of the healthy controls, irrespective of CD4 counts (see Table 3). In terms of the antibody rise, in those with a CD4 count <200 cells/μL, 5 of 8 patients and 1 of 6 patients showed more than a 4-fold rise in the antibody response against anti-H1 and

TABLE 3. Anti-HA Antibody Responses After Vaccination in Baseline Anti-HA Antibody-Negative and Positive Individuals

	Anti-HA Antibody Responses* After Vaccination in HIV-1 Patients†						Healthy Immunized Controls	
	Stratum 1 (CD4 count <200 cells/μL)			Stratum 2 (CD4 count ≥200 cells/μL)				
	Week 0	Week 8	Week 16	Week 0	Week 8	Week 16	Week 0	Week 8
Baseline anti-H1 Ab-negative	n = 43			n = 139			n = 4	
Anti-H1 Ab responses	<10	26‡ (10–1280)	23‡ (10–1280)	<10	42 (10–1280)	36 (10–1280)	<10	135 (40–320)
Baseline anti-H3 Ab-negative	n = 37			n = 130			n = 4	
Anti-H3 Ab responses	<10	25‡ (10–640)	23‡ (10–1280)	<10	34 (10–1280)	32 (10–640)	<10	135 (40–320)
Baseline anti-H1 Ab-positive	n = 8			n = 67			n = 22	
Anti-H1 Ab responses	44 (20–320)	353 (40–1280)	208 (80–160)	54 (20–1280)	158 (20–1280)	143 (20–1280)	80 (20–640)	86 (20–640)
Baseline anti-H3 Ab-positive	n = 6			n = 73			n = 22	
Anti-H3 Ab responses	32 (20–80)	46 (20–160)	71 (20–640)	41 (20–1280)	105 (20–1280)	87 (10–1280)	59 (20–320)	66 (20–320)

*The data presented here are the geometric mean of anti-HA antibody titer. Range of the absolute titer is shown in parentheses.
†To analyze antibody responses to vaccination, patients with influenza infection were excluded from this analysis.
‡$P < 0.05$ compared with the respective value of stratum 2.
Ab indicates antibody. Change of the antibody titer from <10 to 40 U was considered a 4-fold rise.

anti-H3. Conversely, in those with a CD4 count >200 cells/μL, 16 of 67 patients and 19 of 73 patients showed more than a 4-fold rise.

Anti-H1 and Anti-H3 Antibody Responses in Patients With Influenza Illness Despite Vaccination

A total of 16 patients (5 with H1N1 strain, 8 with H3N2 strain, and 3 with B strain) had influenza illness among the vaccinated group during this study period. In the 5 patients with H1N1 illness, 3 were baseline anti-H1 antibody-negative and 2 had the antibody. Among the 3 baseline anti-H1 antibody-negative patients, 2 were infected before week 8 and 1 was infected after week 8. In the patient infected after week 8, no anti-H1 antibody was detected at week 8. In each of the 2 baseline anti-H1 antibody-positive patients, the titer was 20 U. Both patients were infected before week 8. In the 8 patients with H3N2 illness, 6 were baseline anti-H3 antibody-negative and 2 were positive for the antibody. In the 6 baseline anti-H3 antibody-negative patients, all were infected after week 8. Among these 6 patients, 4 were negative for anti-H3 antibody at week 8, whereas 2 had a 4-fold rise in the antibody before infection. In each of the 2 baseline anti-H3 antibody-positive patients, the titer was 20 U. Both patients were infected after week 8. Anti-H3 antibody at week 8 was increased to 40 U (a 2-fold rise) only in 1 patient. Overall, among the 9 infected patients (1 with H1N2 strain and 8 with H3N2 strain) in whom the antibody responses at week 8 could be evaluated, only 2 had a >4-fold rise of the antibody response before infection.

H1-Specific CD4 T-Cell Response Before and After Vaccination in Baseline Anti-H1 Antibody-Negative Subjects

H1-specific CD4 T-cell responses at week 8 were HIV VL dependent ($P < 0.005$) but not CD4 count dependent (Fig. 2A). Therefore, H1-specific CD4 T-cell responses were significantly increased by vaccination in HAART-treated patients ($P = 0.001$), because HIV VL was decreased by HAART (see Fig. 2B). In contrast, responses of HAI antibody titer were not different between HAART-treated and antiretroviral-naive patients (see Fig. 2C).

Comparison of Immune Responses to H1 Antigen at Week 8 Between Influenza A/H1N1–Infected and –Uninfected Patients

Five individuals were infected with influenza A/H1N1 during this season. HAI antibody titers at 8 weeks after the vaccination were not different between the infected and uninfected individuals. In contrast, H1-specific CD4 T-cell responses at week 8 were significantly low in the infected persons compared with those in the uninfected persons ($P < 0.05$; Fig. 3).

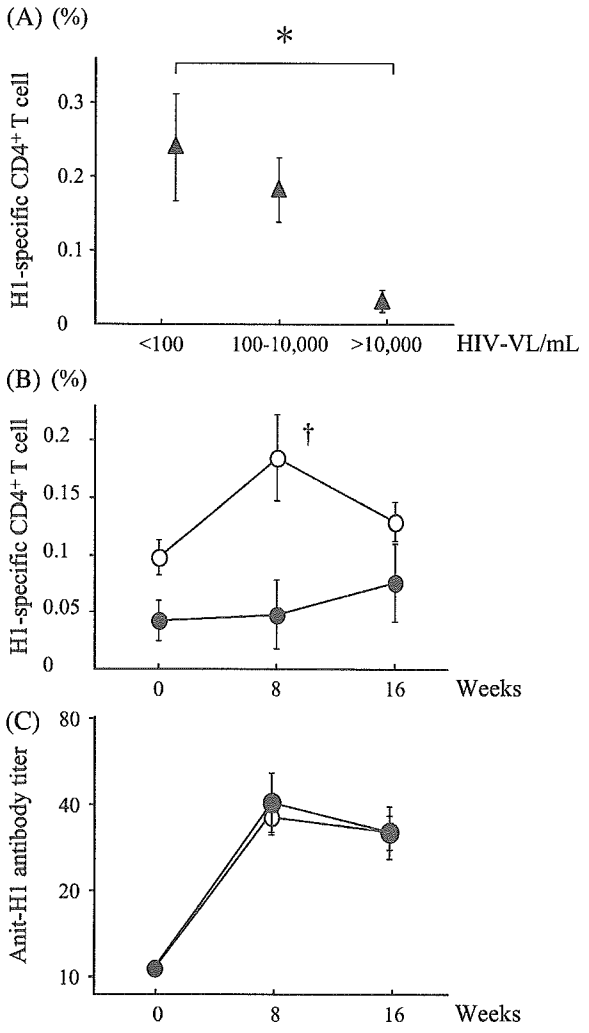

FIGURE 2. H1-specific CD4+ T-cell responses after influenza vaccine in baseline anti-H1 antibody–negative patients. A, Correlation of plasma HIV-1 viral load (HIV VL) and percentage of H1-specific CD4+ T cells. *H1-specific CD4+ T cells (▲) were significantly fewer in number in subjects with an HIV VL >10,000 copies/mL ($P < 0.005$). The number of samples with an HIV VL <100 copies/mL was 53, there were 19 samples with 100 to 10,000 copies/mL, and there were 11 samples with >10,000 copies/mL, because H1-specific CD4+ T cells were only examined in the first 10 samples per day as stated in the text. B, Changes in the percentage of H1-specific CD4+ Tcells in highly active antiretroviral therapy (HAART)–treated; (○; n = 63) and antiretroviral-naive patients (●; n = 12). †HAART-treated patients had significantly greater numbers of H1-specific CD4+ T cells at week 8 ($P < 0.01$) than antiretroviral-naive patients. C, Changes in anti-H1 antibody titer in HAART-treated (○; n = 131) and antiretroviral-naive patients (●; n = 35). Anti-H1 antibody responses were similar in both groups. Data are mean ± SEM.

DISCUSSION

Our prospective study confirmed many conclusions of previously reported small studies. First, we confirmed the protective effect of influenza vaccine in HIV-1–infected patients.[8–15] Second, anti-H1–specific and anti-H3–specific antibody responses were examined in HIV-1–infected patients after vaccination, and the responses were confirmed to be dependent on CD4 counts.[8–11]

To clarify the efficacy of a single-shot vaccination, we divided the participants by the positivity of anti-H1– and anti-H3–specific antibodies before vaccination and found that in

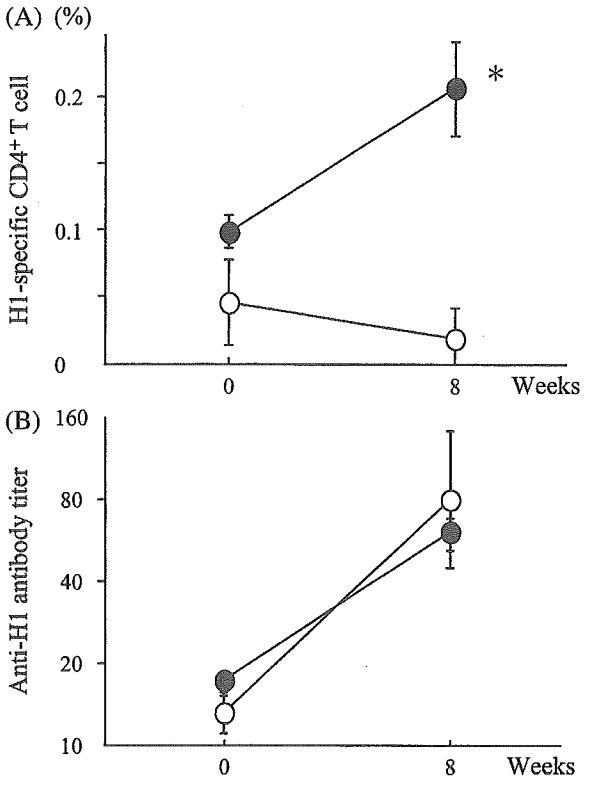

FIGURE 3. H1-specific CD4+ T cells and anti-H1 antibody responses at week 8 after vaccination in influenza A/H1N1–infected patients. Five vaccinated individuals were infected with influenza A/H1N1. A, Percentage of H1-specific CD4+ T-cell responses in infected (○; n = 4) and noninfected (●; n = 119) individuals. *H1-specific CD4+ T cells responded better to influenza A/H1N1 in noninfected patients than in infected patients ($P < 0.05$). One sample of 5 influenza A/H1N1–infected individuals was not examined because the sample was not among the first 10 samples per day as stated in the text. B, Anti-H1 antibody titers in infected (○; n = 5) and noninfected (●; n = 249) individuals. The anti-H1 antibody response at week 8 was similar in both groups. Data are mean ± SEM.

baseline antibody-negative HIV-1–infected patients, the antibody responses to the single-shot vaccination were less effective than those in healthy patients. In contrast, however, in baseline antibody-positive HIV-1–infected patients, the antibody responses were similar or more effective than those in the healthy controls and the titers exceeded >40 U in most cases, irrespective of CD4 count. Previous studies demonstrated that an antibody titer >40 U could be used as an index of vaccine protection.[12,25] In our study, the antibody titer was <40 U in most patients who became infected with influenza. Considered together, these results suggest that the antibody response may support the clinical efficacy of influenza vaccination. Kroon et al[8] reported that postvaccination antibody titers were higher in previously vaccinated HIV-1–infected patients than in nonvaccinated patients, although the difference was not significant. In the present study, the antibody titers showed a better response in individuals positive at baseline for anti-HA antibody than in those negative for the antibody. Furthermore, the response was well sustained, irrespective of CD4 count. Thus, it is conceivable that annual vaccination is specifically important for all HIV-1–infected patients. Sustainability of the antibody titer raised by the vaccination is to be followed in a future study.

In the immunologic part of our study, we examined antibody responses and specific CD4 T cells. The antibody response was almost the same as that reported previously[8,9]; the response correlated with the CD4 count. In contrast, specific CD4 T cells were much more influenced by HIV VL than by CD4 count.[1,8–15] Therefore, the specific CD4 T cells were higher in patients treated with HAART than in those untreated. This result indicates that HAART improves HA-specific CD4 T cells like in other infections,[21] or, in other words, the heightened cellular response to the influenza vaccine suggests functional reconstitution of the immune system after HAART.

Our data indicate that the specific CD4 T-cell responses may be related to HIV VL. The specific CD4 T-cell response needs antigen presentation by dendritic cells.[26] HIV-1 infection impairs the function of antigen presentation of dendritic cells.[27] Therefore, specific CD4 T-cell responses may be profoundly decreased in patients with a high HIV VL.

It is interesting to note that the percentage of H1-specific CD4 T cells at week 8 was significantly lower in influenza A/H1N1–infected patients. It is conceivable that the response of HA-specific CD4 T cells at week 8 can predict the efficacy of influenza vaccine. Influenza-specific CD4 T cells provide help (as Th cells) to B cells for the production of antibody to influenza HA and neuraminidase[28,29] and also promote the generation of virus-specific CD8+ cytotoxic T lymphocytes (CTLs).[26,30–33] Therefore, the specific CD4 T cell must have a protective role. This concept would be more reliable if we had analyzed H3-specific CD4 T cells rather than H1-specific CD4 T cells, because influenza A/H3N2 was the predominant subtype in this season. Further studies are necessary to elucidate this point.

Our study was designed as a prospective but nonrandomized study, because influenza vaccine has been already recommended for HIV-1–infected patients.[7] Practically, the number of nonvaccinated patients who did not participate in our study was higher than that of vaccinated patients (13% of nonvaccinated patients vs. 4.5% of vaccinated patients), and the violation rate of the study protocol was higher in nonvaccinated patients than in vaccinated patients (24.1% vs. 17.4%). Thus, 262 (78.9%) of 332 vaccinated patients and 66 (66%) of 100 nonvaccinated patients were analyzed in this study. Although a relatively high proportion of patients failed to complete the protocol, the main reason for the drop out may have been the lack of incentives and the need to visit our clinic on a fixed date for blood sampling. The vaccinated and nonvaccinated groups were well balanced in terms of baseline characteristics, however. Finally, we believe that the selection bias of participants, if any, is negligible.

In conclusion, our prospective study in a large population demonstrated that influenza vaccine provides protection of HIV-1–infected patients. In baseline antibody-negative patients, the 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. In contrast, in baseline antibody-positive patients, good antibody responses were observed, irrespective of CD4 counts. Annual vaccination of

HIV-1–infected patients is thus recommended. Therapy with HAART improves the cellular immune response to influenza HA.

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APPENDIX

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Patterns of Cytokine Production in Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Human CD8$^+$ T Cells after Stimulation with HIV-1-Infected CD4$^+$ T Cells

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Although human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells can produce various cytokines that suppress HIV-1 replication or modulate anti-HIV-1 immunity, the extent to which HIV-1-specific CD8$^+$ T cells produce cytokines when they recognize HIV-1-infected CD4$^+$ T cells in vivo still remains unclear. We first analyzed the abilities of 10 cytotoxic T-lymphocyte (CTL) clones specific for three HIV-1 epitopes to produce gamma interferon, macrophage inflammatory protein 1β, and tumor necrosis factor alpha after stimulation with epitope peptide-pulsed cells. These CTL clones produced these cytokines in various combinations within the same specificity and among the different specificities, suggesting a functional heterogeneity of HIV-1-specific effector CD8$^+$ T cells in cytokine production. In contrast, the HIV-1-specific CTL clones for the most part produced a single cytokine, without heterogeneity of cytokine production among the clones, after stimulation with HIV-1-infected CD4$^+$ T cells. The loss of heterogeneity in cytokine production may be explained by low surface expression of HLA class I–epitope peptide complexes. Freshly isolated HIV-1-specific CD8$^+$ T cells with an effector/memory or memory phenotype produced much more of the cytokines than the same epitope-specific CTL clones when stimulated with HIV-1-infected CD4$^+$ T cells. Cytokine production from HIV-1-specific memory/effector and memory CD8$^+$ T cells might be a critical event in the eradication of HIV-1 in HIV-1-infected individuals.

Memory and effector CD8$^+$ T cells play an important role in viral eradication through their ability to produce cytokines involved in the suppression of viral replication (6, 10, 15, 26) as well as perforin and granzymes A and B, which are involved in the cytolysis of virus-infected cells (16, 24). The cytokines produced by these cells include gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and chemokines such as RANTES and macrophage inflammatory protein 1β (MIP-1β). IFN-γ increases the surface expression of HLA molecules and can activate macrophages that predominantly synthesize MIP-1β (8, 25). TNF-α induces apoptosis of human immunodeficiency virus type 1 (HIV-1)-infected cells (17). It has been shown that MIP-1β and RANTES can suppress HIV-1 replication in vitro by inhibiting the entry of HIV-1 via CCR5, while IFN-γ induces cellular proteins which suppress viral replication (2, 6).

A previous study showed that HLA-A2-restricted, HCMVpp65$_{495\text{-}503}$-specific CD8$^+$ T cells expressed various combinations of three cytokines, IFN-γ, TNF-α, and interleukin 2 (IL-2), after peripheral blood mononuclear cells (PBMC) containing these cells from three individuals had been stimulated with HCMVpp65$_{495\text{-}503}$ peptide, suggesting that HCMVpp65$_{495\text{-}503}$-specific CD8$^+$ T cells possess functionally heterogeneous cytokine production (18). This functional heterogeneity may be due to the heterogeneous populations in HCMVpp65$_{495\text{-}503}$-specific CD8$^+$ T cells. Indeed, these cells for the most part are CD8$^+$ T-cell populations with a memory/effector or effector phenotype (9, 23). Additional studies have revealed the heterogeneity of the production of cytokines and cytolytic effector molecules in human CD8$^+$ T cells (21, 23).

It is well known that virus-specific CD8$^+$ T cells can produce cytokines when they recognize virus-infected cells (4, 13, 14). However, virus-specific CD8$^+$ T cells stimulated with virus-infected cells may not produce cytokines more effectively than those stimulated with cells pulsed with an epitope peptide, because the expression of HLA class I–viral epitope peptide complexes is much lower on virus-infected cells than on peptide-pulsed cells. In particular, HIV-1-specific CD8$^+$ T cells may not produce cytokines effectively when they recognize HIV-1-infected cells, because HLA class I molecules have been actively down-regulated, mostly, although not exclusively, by the Nef protein produced by HIV-1-infected cells (4, 7, 20). An analysis of the cytokine production from HIV-1-specific CD8$^+$ T cells stimulated with HIV-1-infected CD4$^+$ T cells is needed to elucidate whether HIV-1-specific CD8$^+$ T cells can recognize HIV-1-infected CD4$^+$ T cells in vivo and to allow the formulation of testable hypotheses on the role of the cytokines from HIV-1-specific CD8$^+$ T cells in the suppression of HIV-1 replication in vivo.

In the present study, we investigated the production patterns of three cytokines, IFN-γ, TNF-α, and MIP-1β, in HIV-1-specific effector CD8$^+$ T-cell clones of the same or different specificities. In addition, we investigated the production of the three cytokines in these cytotoxic T-lymphocyte (CTL) clones and freshly isolated HIV-1-specific CD8$^+$ T cells exposed to

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HIV-1-infected $CD4^+$ T cells whose HLA class I molecules are down-regulated by HIV-1 Nef. The present study elucidates the cytokine production profile of HIV-1-specific $CD8^+$ T cells in response to HIV-1-infected $CD4^+$ T cells.

MATERIALS AND METHODS

CTL clones. Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cell/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human IL-2, 5×10^5 irradiated allogeneic PBMC from a healthy donor, and 1×10^5 irradiated C1R-A*2402, C1R-A*3303, or C1R-B*3501 cells prepulsed with 10^{-6} M of the corresponding peptide, Env77-85 [DPNPQEVVL] [19], Gag28-36 [KYKLKHIVW] [12], or Env830-837 [EVAQ RAYR] [11]). Wells positive for growth after about 2 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for CTL activity by a standard ^{51}Cr release assay. One Env830-837-specific, HLA-A*3303-restricted CTL clone (clone 1) had been generated previously (11). All CTL clones were cultured in RPMI 1640–10% FCS supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1 derived peptide.

Antibodies. The peridinin chlorophy protein-conjugated anti-human CD8 monoclonal antibody (MAb) and the ECD-conjugated anti-human CD45RA MAb were purchased from BD Biosciences (San Jose, CA) and the Immunotech Coulter Company (Marseille, France), respectively. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD27, phycoerythrin (PE)-conjugated anti-human IFN-γ, ECD-conjugated anti-human CD28, allophycocyanin (APC)-conjugated anti-human TNF-α, APC-conjugated anti-human IFN-γ, PE Cy7-conjugated anti-human TNF-α, and APC Cy7-conjugated anti-human CD27 MAbs were purchased from PharMingen (San Diego, CA). PE-conjugated anti-human CD28, APC-conjugated anti-human CD8, and FITC-conjugated anti-human MIP-1β MAbs were purchased from DAKO (Glostrup, Denmark). FITC-conjugated anti-HIV-1 p24 MAb KC-57 was purchased from Beckman Coulter (Miami, Fla.). A Cascade Blue-conjugated anti-human CD8 MAb was made by conjugating Cascade Blue (Molecular Probes, Eugene, OR) with the anti-CD8 MAb OKT8.

HLA-peptide tetrameric complexes. HLA class I–peptide tetrameric complexes were synthesized as previously described (3). Briefly, recombinant HLA class I proteins (HLA-A*2402, HLA-A*3303, and HLA-B*3501) and human β_2 microglobulin (β_2m) were produced in *Escherichia coli* cells transformed with the relevant expression plasmids. The heavy chain was modified by deletion of the transmembrane cytosolic tail and COOH-terminal addition of a sequence containing the BirA biotinylation site. Gag28-36, Env830-837, and Env77-85 peptides were used for refolding of the HLA-A*2402, HLA-A*3303, and HLA-B*3501 molecules, respectively. The HLA class I–peptide complexes were refolded in vitro. The 45-kDa complexes were isolated using gel filtration on a Superdex G75 column (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were biotinylated with the BirA enzyme (Avidity, Denver, CO). The biotinylated complexes were purified by using gel filtration first on a Superdex G75 column and then on a MonoQ column (Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England). HLA class I–peptide tetrameric complexes (tetramers) were mixed with PE-labeled streptavidin (extravidin-PE; Sigma-Aldrich, St. Louis, MO) at a molar ratio of 4:1.

Identification of HIV-1-specific CTLs by flow cytometry. A total of 0.2×10^6 to 1×10^6 cultured cells were mixed with the tetramers at concentrations of 0.02 to 0.04 mg/ml. After incubation at 37°C for 30 min, the cells were washed twice with RPMI 1640–10% FCS, and then an anti-CD8 MAb was added to the cell suspension. The cells were incubated at 4°C for 30 min, and then the cells were washed twice with phosphate-buffered saline (PBS)–10% FCS. The cells were analyzed using a FACSCalibur with CellQuest software (Becton Dickinson, San Jose, CA). The percentage of tetramer-positive cells was measured in CD8-positive cells.

Phenotype analysis of the CTL clones. Cells were stained for 30 min at 4°C using an FITC-conjugated anti-human CD27 MAb, a PE-conjugated anti-human CD28 MAb, an ECD-conjugated anti-human CD45RA MAb, and an APC-conjugated anti-human CD8 MAb; then they were washed twice in PBS supplemented with 10% NCS. The CD27 CD28 CD45RA phenotype of $CD8^+$ cells was analyzed using FACSCalibur.

Cell surface and intracellular cytokine staining. Specific CTL clones were stimulated with stimulator cells prepulsed with the appropriate HIV-1-derived peptide at each concentration at an effector-to-stimulator ratio of 1:1. Stimulator cells were washed in RPMI 1640–10% FCS before use. Cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A (Sigma-Aldrich) at a concentration of 10 μg/ml was added 2 h after stimulation. After a 6-h incubation, the cells were washed in PBS supplemented with 20% NCS. Cell surface staining was performed for 30 min at 4°C using a PerCP-conjugated anti-human CD8 MAb; then cells were washed twice in PBS supplemented with 10% NCS. Freshly isolated $CD8^+$ cells from HIV-1-infected individuals were stained with tetramers after a 6-h incubation, followed by staining with ECD-conjugated anti-human CD28, APC Cy7-conjugated anti-human CD27, and Cascade Blue-conjugated anti-human CD8 MAbs.

After a wash, the cells were fixed with 4% paraformaldehyde for 20 min at 4°C and permeabilized with PBS supplemented with 0.1% saponin containing 20% NCS for 10 min at 4°C. The cells were resuspended in permeabilizing buffer and then were stained with a PE-conjugated anti-human IFN-γ MAb or an APC-conjugated anti-human TNF-α MAb at room temperature for 20 min or with an FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C. When the cells were stained with all three MAbs, they were first stained with the PE-conjugated anti-human IFN-γ MAb and the APC-conjugated anti-human TNF-α MAb at room temperature for 20 min and then with the FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C. Freshly isolated $CD8^+$ cells were stained with an APC-conjugated anti-human IFN-γ MAb or a PE Cy7-conjugated anti-human TNF-α MAb at room temperature for 20 min or with an FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C.

Finally, the cells were washed three times with permeabilizing buffer and were resuspended in 2% paraformaldehyde. The percentages of intracellular IFN-γ-, MIP-1β-, and TNF-α-positive cells among tetramer-positive $CD8^+$ cells were analyzed using FACS Aria (Becton Dickinson, San Jose, CA).

Infection of $CD4^+$ T cells with HIV-1. Cultured $CD4^+$ T cells (purity, >98%) were incubated with an HIV-1 clone, NL-432 (1), or with the chimeric virus NL-432gag^{HXB2} for 4 h at 37°C with intermittent agitation. The cells were then washed once and cultured in RPMI 1640–10% FCS medium supplemented with recombinant human IL-2 (200 U/ml). On the following 2 to 7 days, the cells were harvested to determine the percentage of HIV-1-infected cells by measuring p24 antigen-positive cells using FACSCalibur. When HIV-1 p24-positive cells reached more than 40% of the cultured cells, they were used as stimulator cells.

CTL assay. Cytotoxicity was measured by a standard ^{51}Cr release assay as previously described (19). Target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added into a 96-well round-bottom microtiter plate (Nunc) with the indicated amount of the corresponding peptide. After a 1-h incubation, 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 was determined by measuring the cpm in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100. Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both the target and effector cells. The activities of the CTL clones on target cells pulsed with peptide were tested at an effector-to-target (E:T) ratio of 2:1.

CTL assay for target cells infected with recombinant vaccinia virus. Recombinant vaccinia virus containing the *env* or the *gag/pol* gene of HIV-1 SF2 was generated as described previously (19). Target cells (C1R-A*2402, C1R-A*3303, and C1R-B*3501 cells) were cultured with 10 PFU of recombinant or wild-type vaccinia virus per target cell overnight. These infected cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% NCS. Effector cells were added to the labeled target cells (5×10^3/well), and the mixtures were incubated for 4 h at 37°C. The activities of the CTL clones on target cells infected with recombinant vaccinia virus expressing *env* proteins were tested at an E:T ratio of 2:1.

RESULTS

Production of three cytokines in HIV-1-specific CTL clones. Three SF2-Env77-85-specific, HLA-B*3501-restricted CTL clones, three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones, and a further four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones were established from HIV-1-infected individuals. These CTL clones exhibited specific cytolytic activity in both target cells (C1R-A*2402, C1R-A*3303, or

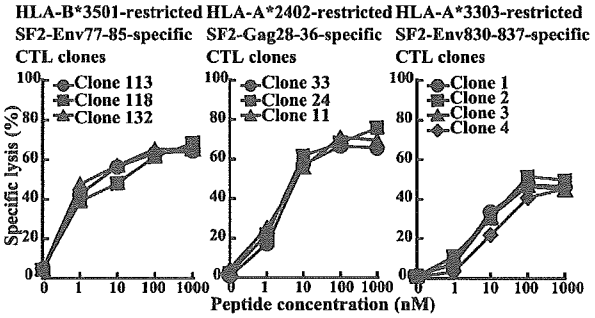

FIG. 1. Cytolytic activities of SF2-Env77-85-specific, SF2-Gag28-36-specific, and SF2-Env830-837-specific CTL clones. Cytolytic activities of three SF2-Env77-85-specific, HLA-B*3501-restricted CTL clones (clones 113, 118, and 132), three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones (clones 33, 24, and 11), and four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones (clones 1, 2, 3, and 4) were tested for C1R-B*3501, C1R-A*2402, and C1R-A*3303 cells pulsed with the corresponding peptides (1 to 1,000 nM), respectively. They were tested at an E:T ratio of 2:1.

C1R-B*3501 cells) prepulsed with a peptide epitope (Fig. 1) and target cells (C1R-A*2402, C1R-A*3303, or C1R-B*3501 cells) infected with HIV-1 recombinant vaccinia virus (data not shown). The results show no significant difference in cytotoxic activity between CTL clones specific for the same epitopes. We investigated the production of three cytokines, MIP-1β, IFN-γ, and TNF-α, from these HIV-1-specific CTL clones. Intracellular cytokine production by the clones was measured at 6 h after stimulation with the C1R transfectants prepulsed with the epitope peptide. Almost 100% of the cells produced at least one cytokine in all three of the SF2-Env77-85-specific CTL clones, all four of the SF2-Env830-837-specific CTL clones, and all three of the SF2-Gag28-36-specific CTL clones (Fig. 2). Different cytokine production patterns were found among three SF2-Env77-85-specific CTL clones though all of the clones produced IFN-γ more extensively than MIP-1β or TNF-α (Fig. 2A). Different cytokine production patterns were also found for the three SF2-Gag28-36-specific CTL clones (Fig. 2B) and the four SF2-Env830-837-specific CTL clones (Fig. 2C). Clone 11 exhibited much higher production of MIP-1β than of IFN-γ and TNF-α compared with clones 33 and 24. In addition, clones 2 and 3 exhibited much lower production of TNF-α than of IFN-γ and MIP-1β, while the difference in production between these cytokines was at a minimum for clones 1 and 4. These results were confirmed by performing the experiments twice. Thus, the results reveal heterogeneity in the production of these cytokines between CTL clones of the same as well as different specificities. To exclude contamination by T cells that are not epitope-specific CTLs, we stained the CTL clones with an epitope-specific HLA class I tetramer. More than 97% of the cells in nine CTL clones bound the epitope-specific tetramer, while only 92% of the cells bound to the specific tetramer in one (clone 33) of the SF2-Gag28-36-specific CTL clones (Table 1). However, this difference does not seem to be significant for the functions, since the three SF2-Gag28-36-specific CTL clones exhibited the same cytotoxic activity and IFN-γ production.

Since the T-cell clones used in the present study possess cytolytic activity (Fig. 1), they are thought to be mature effector

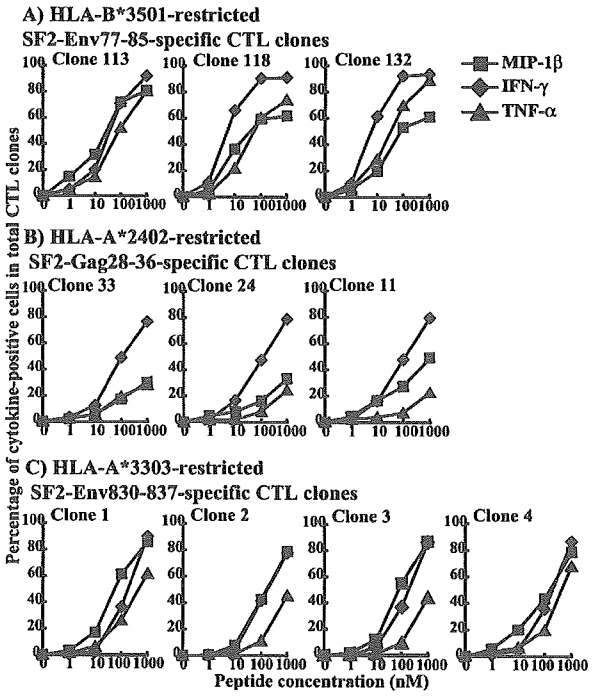

FIG. 2. Intracellular expression of cytokines in SF2-Env77-85-specific, SF2-Gag28-36-specific, and SF2-Env830-837-specific CTL clones responding to epitope peptide-pulsed cells. The 10 CTL clones were incubated for 6 h in the presence of C1R-B*3501, C1R-A*2402, or C1R-A*3303 cells pulsed with specific peptide. Brefeldin A was added 2 h after stimulation, and intracellular staining for IFN-γ, MIP-1β, and TNF-α was carried out using MAbs specific for each cytokine. Cytokine-producing cells were analyzed by flow cytometry. The frequency of each set of cytokine-producing $CD8^+$ cells was measured as the number of cytokine-producing $CD8^+$ cells per total $CD8^+$ cells.

$CD8^+$ T cells. To analyze the relation between the maturation stage of the CTL clone and cytokine production, we stained these CTL clones with anti-CD27, anti-CD28, and anti-CD45RA MAbs. Nine CTL clones were mostly of the $CD27^-$ $CD28^-$ $CD45RA^-$ type, while one (clone 2) of the SF2-Env830-837-specific CTL clones exhibited both $CD27^-$ $CD28^-$ $CD45RA^-$ and $CD27^+$ $CD28^-$ $CD45RA^-$ phenotypes (Table 1). Clone 2, stimulated with SF2-Env830-837 peptide, exhibited a much smaller number of TNF-α-producing cells than of IFN-γ-producing or MIP-1β-producing cells, but this is not a characteristic restricted to clone 2.

Coproduction of three cytokines by HIV-specific CTL clones stimulated with peptide-pulsed cells. The heterogeneity of the CTL clones shown in Fig. 2 suggests that the CTL clones are actually composed of cell populations which produce different combinations of the cytokines. To clarify this, we investigated the coproduction of these three cytokines in the CTL clones by simultaneously staining multiple intracellular cytokines with anti-IFN-γ, anti-MIP-1β, and anti-TNF-α MAbs. The results of the multiple staining of the three SF2-Env77-85-specific CTL clones are shown in Fig. 3A. The SF2-Env77-85-specific CTL clones stimulated with 1,000 nM of specific peptide revealed different patterns of cytokine production. Clone 113 included a high number of cells producing all three of the cytokines ($IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ $MIP\text{-}1\beta^+$) (72.0%). In contrast,

TABLE 1. CD27 and CD28 expression in HIV-1-specific CTL clones

CTL clone	% Tetramer-positive cells	% of total $CD8^+$ $CD45RA^-$ cells with the following phenotype:			
		$CD27^+$ $CD28^+$	$CD27^+$ $CD28^-$	$CD27^-$ $CD28^-$	$CD27^-$ $CD28^+$
Env77-85					
Clone 113	98.2	0.8	1.2	84.8	13.2
Clone 118	99.7	0.2	13.6	86.0	0.2
Clone 132	95.1	0.3	6.3	91.5	1.9
Gag28-36					
Clone 33	91.7	0.0	1.5	97.8	0.7
Clone 24	97.0	0.0	17.2	81.6	1.2
Clone 11	96.0	0.0	19.2	80.7	0.1
Env830-837					
Clone 1	99.5	0.0	13.0	86.0	1.0
Clone 2	99.7	0.0	46.1	53.9	0.0
Clone 3	99.7	0.1	8.9	89.8	1.2
Clone 4	99.2	0.0	0.2	99.8	0.0

clone 118 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (58.1%) and IFN-γ^+ TNF-α^+ cells (30.6%), while clone 132 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (54.7%), IFN-γ^+ TNF-α^+ cells (17.8%), and IFN-γ^+ cells (13.1%). This difference between these CTL clones became even more apparent when the CTL clones were stimulated with 10 nM of specific peptide. Clone 113 included MIP-1β^+ cells (16.1%) and IFN-γ^+ MIP-1β^+ cells (7.3%). In contrast, clone 118 included IFN-γ^+ cells (29.1%) and IFN-γ^+ TNF-α^+ cells (17.0%), while clone 132 included IFN-γ^+ cells (27.5%) and IFN-γ^+ MIP-1β^+ cells (20.6%). This difference was consistent over two different experiments performed on different days.

A similar analysis was performed on the four SF2-Env830-837-specific and the three SF2-Gag28-36-specific CTL clones. The seven CTL clones were stimulated with 1,000 nM of specific peptide (Fig. 3B and C). Among SF2-Gag28-36-specific CTL clones, clones 33 and 24 showed similar patterns of cytokine production. They included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (12 to 14%), IFN-γ^+ TNF-α^+ cells (11 to 13%), and IFN-γ^+ MIP-1β^+ cells (14 to 18%). In contrast, clone 11 included IFN-γ^+ TNF-α^+ cells (5%) and IFN-γ^+ MIP-1β^+ cells (34%). The difference became more complex when the clones were stimulated with a lower concentration (100 nM) of the peptide. The major populations of cytokine-producing cells were as follows: for clone 11, IFN-γ^+ cells (24.5%), IFN-γ^+ MIP-1β^+cells (15.4%), and MIP-1β^+cells (7.7%); for clone 33, IFN-γ^+ cells (28.0%), IFN-γ^+ MIP-1β^+cells (9.9%), and IFN-γ^+ TNF-α^+ cells (8.0%); and for clone 24, IFN-γ^+ cells (27.2%), IFN-γ^+ MIP-1β^+cells (9.1%), and IFN-γ^+ TNF-α^+ cells (7.6%). Among SF2-Env830-837-specific CTL clones, clones 1 and 4 showed similar patterns of cytokine production. These included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (50 to 60%), IFN-γ^+ TNF-α^+ cells (13 to 17%), and IFN-γ^+ MIP-1β^+ cells (12 to 14%). In contrast, clones 2 and 3 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (40 to 42%) and IFN-γ^+ MIP-1β^+ cells (35 to 37%). The difference became more complex when the clones were stimulated with a lower concentration (100 nM) of the peptide. At this concentration the major populations of cytokine-producing cells were as follows: for clone 1, MIP-1β^+ cells (17.6%) and IFN-γ^+ MIP-1β^+cells (10.9%); for clone 2, IFN-γ^+ MIP-1β^+ cells (19.1%), IFN-γ^+ cells (15.7%), and MIP-1β^+ cells (12.4%); for clone 3, MIP-1β^+ cells (20.8%), IFN-γ^+ MIP-1β^+ cells (20.6%), and IFN-γ^+ cells (10.9%); and for clone 4, IFN-γ^+ cells (15.1%), IFN-γ^+ MIP-1β^+ cells (12.0%), and IFN-γ^+ TNF-α^+ MIP-1β^+ cells (10.1%). These differences were also confirmed by two different experiments performed on different days.

Cells expressing all three cytokines were most frequently found in the CTL clones stimulated with the stimulator cells prepulsed with 1,000 nM peptide, while cells expressing either two or all three of the cytokines were not found in the CTL clones stimulated with the stimulator cells prepulsed with 1 nM peptide (Fig. 4). The CTL clones stimulated with stimulator cells prepulsed with 1 nM peptide predominantly produced a single cytokine. These results indicate that CTL clones produce multiple cytokines when they are stimulated with cells prepulsed with a high concentration of an HLA–epitope peptide complex but produce a single cytokine when they are stimulated with cells prepulsed with a low concentration. This implies that HIV-1-specific CTL clones produce a single cytokine when they are stimulated with HIV-1-infected cells because a small number of HIV-1 CTL epitope peptides is presented in HIV-1-infected cells.

Coproduction of three cytokines by HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells. Nef-mediated down-regulation of HLA class I critically affects the ability of HIV-1-specific CTLs to kill HIV-1-infected cells; this could be the result of a lowered frequency of cytokine-producing cells after stimulation with HIV-1-infected $CD4^+$ T cells. Indeed, our previous study showed that the lower frequency of HIV-1-specific CTL clones produced fewer cytokines by stimulation with Nef^+ HIV-1-infected $CD4^+$ T cells than by stimulation with Nef^- HIV-1-infected $CD4^+$ T cells (22). We investigated the coproduction of the three cytokines by HIV-1-specific CTL clones after stimulation with Nef^+ HIV-1-infected $CD4^+$T cells. Approximately 7 to 9% of the cells in five clones produced cytokines after stimulation with Nef^+ HIV-1-infected $CD4^+$ T cells (Fig. 5). Most of these cytokine-secreting cells produced only a single cytokine but were able to produce any one of the three. Thus, the results indicate that after stimulation with HIV-1-infected $CD4^+$T cells, HIV-1-specific CTL clones can produce various kinds of

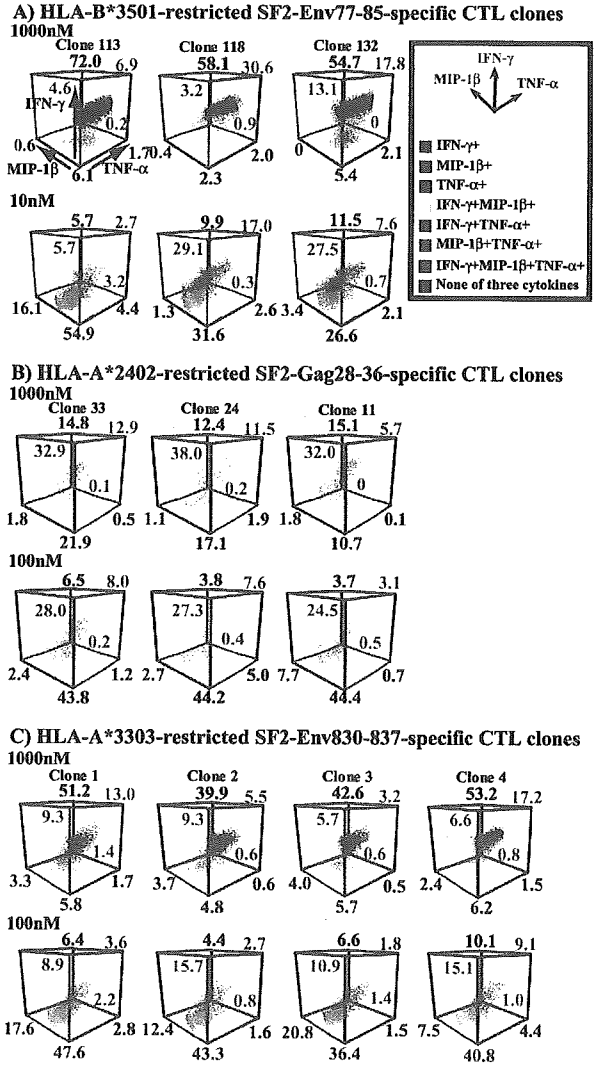

FIG. 3. Coexpression of three cytokines in HIV-1-specific CTL clones responding to epitope peptide-pulsed cells. (A) Env77-85-specific CTL clones. Intracellular cytokine production of three SF2-Env77-85-specific CTL clones was measured 6 h after stimulation with C1R-B*3501 cells prepulsed with the corresponding peptides (1,000 nM and 10 nM). Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. (B) Gag28-36-specific CTL clones. Intracellular cytokine production of three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones (clones 33, 24, and 11) was tested 6 h after stimulation with C1R-A*2402 cells prepulsed with the corresponding peptides (1,000 nM and 100 nM). (C) Env830-837-specific CTL clones. Intracellular cytokine production of four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones (clones 1, 2, 3, and 4) was tested 6 h after stimulation with C1R-A*3303 cells prepulsed with the corresponding peptides (1,000 nM and 100 nM). Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. Fluorescence-activated cell sorter data were analyzed with Paint-A-Gate^{PRO} (BD Biosciences). The frequencies of cells expressing these cytokines are shown as percentages of the total number of cells. Cells expressing these cytokines are shown in a 3-dimensional presentation as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

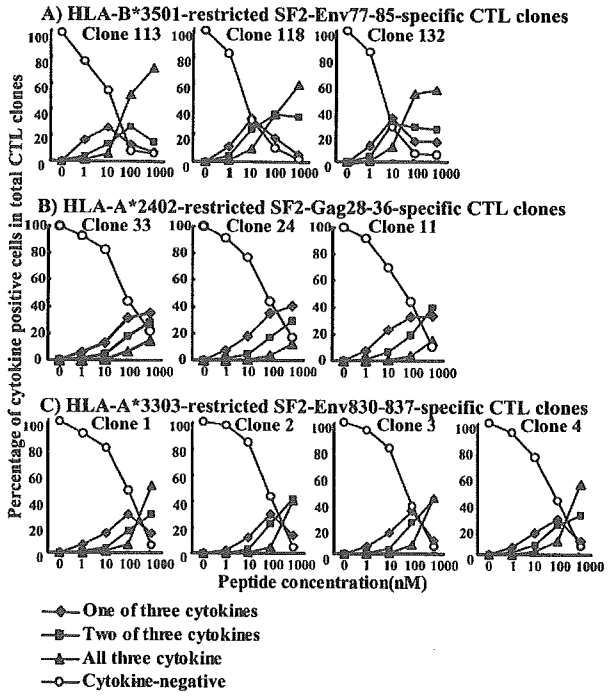

FIG. 4. Kinetics of multiple cytokine expression in the HIV-1-specific CTL clones corresponding to epitope peptide stimulation. The frequency of CTL clones expressing a single cytokine, two cytokines, or all three cytokines was measured 6 h after stimulation with cells prepulsed with the corresponding peptide at different concentrations as follows: the frequency of cells expressing a single cytokine was the sum of the frequency of cells expressing IFN-γ only, MIP-1β only, and TNF-α only in the total CD8$^+$ cells, while the frequency of cells expressing two cytokines was the sum of cells expressing IFN-γ and MIP-1β, IFN-γ and TNF-α, and MIP-1β and TNF-α in total CD8$^+$ cells.

cytokines but that each CTL clone is able to produce only a single cytokine. Heterogeneity in the production of these cytokines among CTL clones of the same specificity is barely noticeable.

Cytokine production of freshly isolated HIV-1-specific CD8$^+$ T cells stimulated with HIV-1-infected CD4$^+$ T cells. The cytokine production results for HIV-1-specific CTL clones stimulated with HIV-1-infected CD4$^+$ T cells suggest the possibility that when HIV-1-specific CD8$^+$ T cells recognize HIV-1-infected CD4$^+$ T cells in HIV-1-infected individuals, they produce various combinations of cytokines but each of them produces only a single cytokine. To clarify the accuracy of this hypothetical picture, we performed ex vivo analysis of the cytokine production of HIV-1 Gag28-36-specific CD8$^+$ T cells after stimulation with Nef$^+$ HIV-1-infected CD4$^+$ T cells (Fig. 6). We isolated CD8$^+$ T cells from fresh PBMC of two HIV-1-infected individuals (KI-144 and KI-158). Analysis using a Gag28-36-specific HLA-A*2402 tetramer as well as anti-CD27 and anti-CD28 MAbs showed that approximately 0.2 to 0.3% of CD8$^+$ T cells were Gag28-36-specific CD8$^+$ T cells and that the Gag28-36-specific CD8$^+$ T cells predominantly have either a CD27$^+$ CD28$^-$ memory/effector phenotype or a CD27$^+$ CD28$^+$ memory phenotype (Fig. 6). We then stimulated the CD8$^+$ T cells with NL-432-infected CD4$^+$ T cells. Approximately

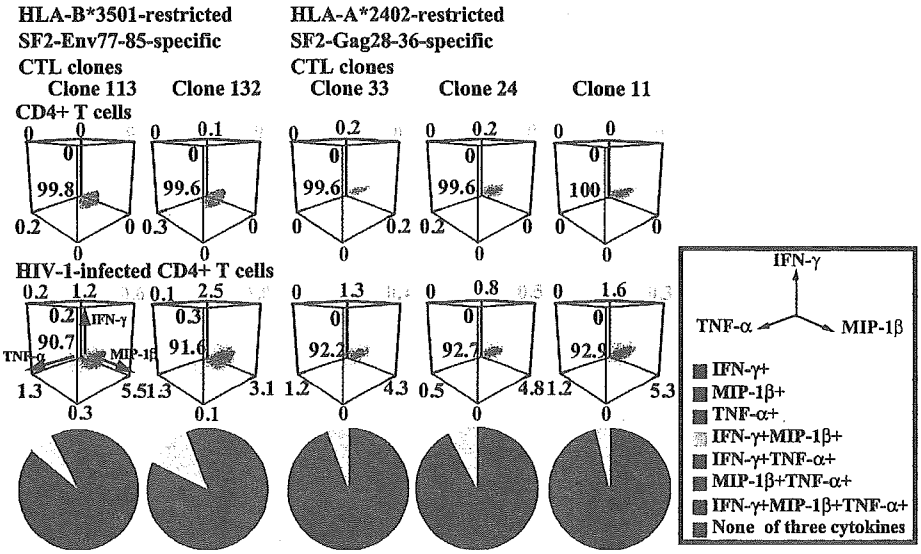

FIG. 5. Coexpression of all three cytokines in HIV-1-specific CTL clones responding to HIV-1-infected $CD4^+$ T cells. Intracellular cytokine production of two SF2-Env77-85-specific CTL clones and three Gag28-36-specific CTL clones was measured 6 h after stimulation with $CD4^+$ T cells infected with the Nef^+ HIV-1 clone NL-432 or NL-432gag^{HXB2}. Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. The frequency of cells expressing these cytokines is shown as a percentage of the total number of cells in a 3-dimensional presentation. The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown as a pie chart. Cells expressing these cytokines are shown as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

30 and 10% of Gag28-36-specific $CD8^+$ T cells produced at least one cytokine in PBMC from two different HIV-1-infected individuals (Fig. 6). The frequency of cytokine-producing cells was much higher in freshly isolated Gag28-36-specific $CD8^+$ T cells than in Gag28-36-specific CTL clones. KI-144 cells predominantly produced only MIP-1β or both MIP-1β and IFN-γ, while KI-158 cells predominantly produced only MIP-1β or both MIP-1β and TNF-α. This is in contrast to the finding that Gag28-36-specific CTL clones produced a single cytokine: either MIP-1β, IFN-γ, or TNF-α.

DISCUSSION

In the present study, we employed three kinds of HIV-1-specific CTL clones of the same specificity from the same individual. These CTL clones mostly exhibited an effector phenotype ($CD27^-$ $CD28^-$ $CD45RA^-$) and strong cytolytic activity. The HIV-1-specific CTL clones exhibited functional heterogeneity in the production of three cytokines in clones of the same specificity as well as of different specificities, indicating that HIV-1-specific effector $CD8^+$ T cells, even when of the same specificity, display functional heterogeneity in cytokine production. The mechanism for this heterogeneity of function, however, is still unclear. One possibility is that these CTL clones carry different T-cell receptors (TCR), with the difference in receptor signaling triggering the functional heterogeneity, although it is not understood how different activation of TCR influences cytokine production. The fact that cells expressing different cytokine production patterns do in any event exist in each clone tends to exclude this hypothesis but does support the idea that the cytokine production pattern is determined by various combinations of interacting factors, such as certain specific characteristics of the T cells, including the usage and expression level of TCR, the expression level of accessory molecules, and the relative activation status of the T cells.

Cytokine production by HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells reflects the response of HIV-1-specific CTLs in vivo much better than cytokine production by such clones stimulated with peptide-pulsed cells. The analysis of HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells revealed that only 7 to 9% of cells in each CTL clone produce one of the cytokines IFN-γ, TNF-α, and MIP-1β, suggesting limited cytokine production by HIV-1-specific effector $CD8^+$ T cells carrying the $CD27^-$ $CD28^-$ phenotype in vivo. This limited cytokine production may be explained by either or both of two factors: (i) the amount of HLA class I–virus peptide complexes on virus-infected cells is much smaller than that on peptide-pulsed cells; (ii) HLA class I–HIV-1 peptide complexes are down-regulated on the surfaces of HIV-1-infected cells. Previous studies have demonstrated that HIV-1-specific CTL clones may partially suppress HIV-1 replication, although these cells hardly kill HIV-1-infected $CD4^+$ T cells because of the Nef-mediated down-regulation of the HLA-A and -B molecules (20). The partial suppression may be explained by the limited cytokine production of HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells. Thus, it is hypothesized that cytokines produced by HIV-1-specific $CD8^+$ T cells play an important role in the suppression of HIV-1-replication.

Heterogeneity in cytokine production between CTL clones of the same specificity as well as of different specificities was not found when the CTL clones were stimulated with HIV-1-infected $CD4^+$ T cells. This may be explained by the fact that

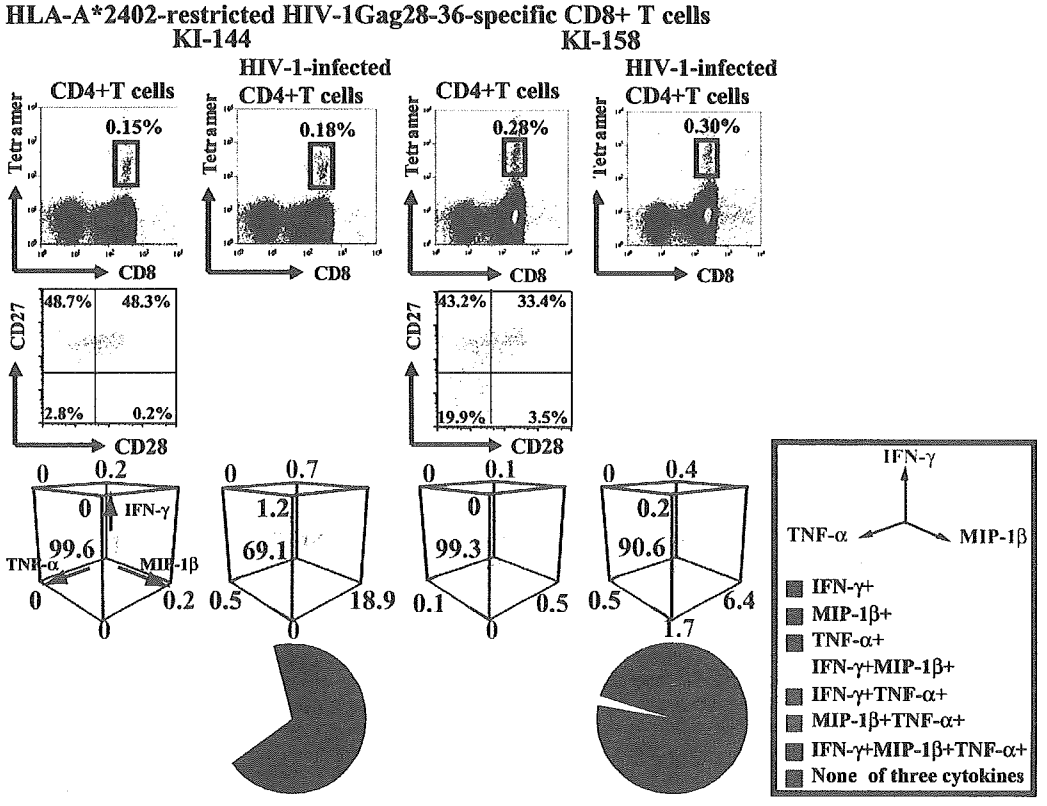

FIG. 6. Coexpression of three cytokines in freshly isolated $HIV_{Gag28-36}$-specific $CD8^+$ T cells responding to HIV-1-infected $CD4^+$ T cells. Intracellular cytokine production of Gag28-36-specific $CD8^+$ T cells was measured 6 h after stimulation with $CD4^+$ T cells infected with the Nef^+ HIV-1 clone NL-432gag^{HXB2}. Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. The $CD8^+$ tetramer-positive cells are gated to determine both the frequency of cells expressing these cytokines and the CD27 and CD28 expression. The frequency is shown as a percentage of the total number of cells in a 3-dimensional presentation. The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown in a pie chart. Cells expressing these cytokines are shown as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

heterogeneity in cytokine production between the CTL clones was not found when they were stimulated with cells pulsed with lower concentrations of HIV-1 peptides. Therefore, it is still unclear whether the heterogeneity in cytokine production between CTL clones of the same specificity as well as of different specificities influences disease progression.

A previous study revealed that HLA-A2-restricted, $HCMVpp65_{495-503}$-specific $CD8^+$ T cells are able to produce various combinations of IFN-γ, TNF-α, and IL-2 after PBMC from healthy individuals are stimulated with $HCMVpp65_{495-503}$ peptide, suggesting that the virus-specific $CD8^+$ T cells possess a functional heterogeneity of cytokine production in vivo (18). Since the HCMV-specific $CD8^+$ T cells are heterogeneous with regard to the surface markers CD45RO, CD45RA, CD27, CD28, CD57, and CD62L (18, 21), it is thought that they include various population types, ranging from memory to effector T cells. Therefore, the heterogeneity at maturation of $CD8^+$ T cells may reflect the capacity for functional heterogeneity in cytokine production. Our recent study revealed that $CD8^+$ T cells with effector phenotypes ($CD27^-$ $CD28^-$ $CD45RA^{+/-}$) or memory/effector phenotypes ($CD27^{low}$ $CD28^-$ $CD45RA^{+/-}$) can produce IFN-γ after stimulation of $CD8^+$ T cells with an anti-CD3 MAb (23), indicating that effector and memory/effector $CD8^+$ T cells have a more pronounced ability to produce IFN-γ than memory $CD8^+$ T cells. Thus, the heterogeneity in maturation or differentiation of $CD8^+$ T cells reflects the functional heterogeneity in cytokine production capacity seen in vivo.

Freshly isolated Gag28-36-specific $CD8^+$ T cells produced more cytokines than CTL clones with the same specificity. Since these Gag28-36-specific $CD8^+$ T cells carried either a $CD27^+$ $CD28^-$ memory/effector or a $CD27^+$ $CD28^+$ memory phenotype, it is likely that they are less mature than the CTL clones. These results suggest that HIV-1-specific $CD8^+$ T cells with a $CD27^+$ $CD28^-$ or $CD27^+$ $CD28^+$ phenotype can produce more cytokines than those with an effector phenotype. Approximately 20 to 30% of cytokine-producing $CD8^+$ T cells produced two or three cytokines, whereas most CTL clones of the same specificity produced a single cytokine. This indicates the ability of HIV-1-specific memory and memory/effector $CD8^+$ T cells to produce multiple cytokines when they recognize HIV-1-infected cells. Various differentiation ranges of HIV-1-specific $CD8^+$ T cells, from memory to effector, are found in PBMC from HIV-1-infected individuals (5, 22). HIV-

1-specific $CD8^+$ T cells with a $CD27^+$ $CD28^-$ or $CD27^+$ $CD28^+$ phenotype may play both a direct and an indirect role in the suppression of HIV-1 replication in vivo via cytokines secreted from these $CD8^+$ T cells. Approximately 30 and 10% of Gag28-36-specific $CD8^+$ T cells produced cytokines in KI-144 and KI-158, respectively. This difference might be explained by the fact that approximately 20% of Gag28-36-specific $CD8^+$ T cells from KI-158 carried effector phenotype $CD27^-$ $CD28^-$, whereas no Gag28-36-specific $CD8^+$ T cells from KI-144 carried it.

In summary, HIV-1-specific CTL clones for the most part produced a single cytokine and did not exhibit heterogeneity of cytokine production among clones after stimulation with HIV-1-infected $CD4^+$ T cells, although they exhibited multiple cytokine production and functional heterogeneity of cytokine production between clones after stimulation with HIV-1 peptide-pulsed cells. Freshly isolated HIV-1-specific $CD8^+$ T cells with an effector/memory or memory phenotype produced much greater amounts of the cytokines than CTL clones with the same epitope specificity after stimulation with HIV-1-infected $CD4^+$ T cells. HIV-1-specific $CD8^+$ T cells with an effector/memory or memory phenotype might directly or indirectly play a crucial role in the eradication of HIV-1 via the cytokines secreted from these T cells in HIV-1-infected individuals.

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Survey of human immunodeficiency virus (HIV)-seropositive patients with mycobacterial infection in Japan

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KEYWORDS
Mycobacterial infection;
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Abstract *Objective.* To assess DNA polymorphisms in mycobacterial isolates obtained from human immunodeficiency virus (HIV)-seropositive patients with tuberculosis in Japan from 1996 to 2003.

Methods. Restriction fragment length polymorphisms (RFLP) from *Mycobacterium tuberculosis* and *Mycobacterium avium* isolates obtained from individual seropositive patients with tuberculosis ($n=78$) were analysed with the use of IS*6110* and $(CGG)_5$ or IS*1245* and IS*1311*, respectively, as markers. As a control, the same procedures were applied to isolates from HIV-seronegative tuberculosis patients ($n=87$).

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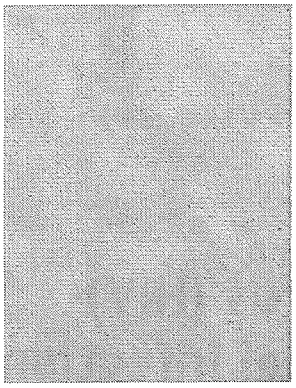

Results. Of 86 mycobacterial strains, *M. tuberculosis*, *M. avium* and *Mycobacterium chelonae* were identified in 48 (55.8%), 36 (41.9%) and 2 (2.3%) isolates, respectively. The obtained RFLP patterns of *M. tuberculosis* isolates from both the HIV-seropositive and -seronegative groups were variable, suggesting no obvious clustering among the isolates. Similar results were obtained in isolates of *M. avium*.

Conclusions. This is the first report on the molecular epidemiology of *Mycobacterium* spp. isolated from HIV-seropositive patients in Japan. The results indicate that no particular clones of *M. tuberculosis* or *M. avium* prevail in HIV-seropositive patients in Japan. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

Introduction

In recent years, a decline in the number of new patients with acquired immunodeficiency syndrome (AIDS) has been observed in several industrialized countries, including the United States, Western European countries, Australia and New Zealand.[1-6] However, no decline in patients with human immunodeficiency virus (HIV) has been observed in Japan.[7] Mycobacterial infections, such as those of *Mycobacterium tuberculosis* and *Mycobacterium avium*, are important opportunistic infections in HIV-seropositive patients. With respect to tuberculosis (TB), several studies based on clinical observations[8-12] and on epidemiologic surveys[13-17] have provided evidence that HIV infection is a risk factor for the development of active and often lethal TB. Outbreaks of TB among communities of HIV patients have been reported in the United States, but multidrug resistant (MDR) *M. tuberculosis* strains were rarely isolated from these patients.[12,18] In sub-Saharan Africa, TB associated with HIV has played an important role in increasing TB transmission throughout the population.[17,19]

Non-tuberculous mycobacterial infection can be difficult to treat because of primary resistance against most of the commonly used anti-tubercular drugs, such as isoniazid, rifampin, streptomycin, ethambutol, pyrazinamide and kanamycin.[20] A relatively high prevalence of non-tuberculous mycobacterial infections has been observed in HIV/AIDS patients, and 25-50% of patients with AIDS in the United States and Europe are infected with this group of bacteria, primarily with *M. avium*, which mainly causes disseminated mycobacteraemia in AIDS patients.[21]

Japan is considered to have a low prevalence of HIV/AIDS, with a cumulative number of 2556 AIDS cases and 5140 HIV cases reported by the end of 2002.[7] However, the recent trend of HIV cases shows a substantial increase, particularly among men who have sex with men and youth/young adults. A considerable number of HIV patients in Japan have experienced discrimination or breach of confidentiality and they feel insufficiency of social and economical supports.[22] Patients with mycobacterial infection used to be discriminated, but the prejudice toward the patients declines. The medical, social and economic backgrounds of HIV patients in Japan differ considerably from those in regions such as North America, Europe and Africa. The correlation between HIV and mycobacterial infections in Japan may also differ from that in countries where research on AIDS-related diseases is well developed. Survey of the occurrence and clinical profiles of these infections is important for the development of countermeasures against mycobacteria and HIV coinfection. In this study, we analysed the current prevalence, clinical features and epidemiologic findings of mycobacterial infection associated with HIV infection in Japan.

Materials and methods

Bacterial isolates and clinical data

From 1996 to 2003, 86 clinical mycobacterial isolates were obtained from eight hospitals in Japan: the International Medical Centre of Japan (IMCJ) (Tokyo); Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (JATA) (Tokyo); National Tokyo Hospital (Tokyo); Tokyo Metropolitan Komagome Hospital (Tokyo); Social Insurance Central General Hospital (Tokyo); National Nishi-Kofu Hospital (Yamanashi); National Osaka National Hospital (Osaka) and National Kyushu Medical Centre (Fukuoka). Clinical information on individual patients was obtained by the physicians in charge with questionnaire on mycobacterial isolation date, history of previous mycobacterial infection, microscopic observation of

Table 1 Nationality and sex of HIV-positive patients with mycobacterial infection in Japan

Nationality	No. of patients	Male:female	Mycobacteria species
Japanese	33	31:2	*M. tuberculosis*: 21 *M. avium*: 11 *M. chelonae*: 1
Non-Japanese	16	9:7	*M. tuberculosis*: 9 *M. avium*: 6 *M. chelonae*: 1
Unknown[a]	37	33:2, unknown[a]:2	*M. tuberculosis*: 18 *M. avium*: 19 *M. chelonae*: 0
Total	86	74:10, unknown[a]:2	*M. tuberculosis*: 48 *M. avium*: 36 *M. chelonae*: 2

[a] Nationality or sex of these patients was not disclosed due to the ethics code of the corresponding hospital.

sputa, sites of infection (pulmonary or extrapulmonary), peripheral blood $CD4^+$ lymphocyte number, chemotherapeutic regimens and standard demographic data. The Ethics Committees in each hospital approved this study (IMCJ-H13-54) and all patients gave a written informed consent.

As a control for *M. tuberculosis* genotyping, 87 clinical isolates from adult HIV-seronegative tuberculosis patients without any serious complication at IMCJ were used. Since other hospitals, except JATA, have no ward for TB patients and the RFLP patterns of *M. tuberculosis* isolates from JATA and IMCJ were variable, and showed no obvious clustering among the isolates.

Mycobacterial culture and identification of strains

Bacteria were grown on egg-based Ogawa medium (Kyokuto Pharmaceutical Co., Ltd, Tokyo, Japan) for 3-5 weeks. Cultured organisms were applied to a polymerase chain reaction (PCR) kit for *M. tuberculosis* diagnosis (Amplicor *Mycobacterium tuberculosis* Test, Roche Diagnostic Systems, Inc., Branchburg, NJ), and PCR-negative organisms were further applied to an identification kit for mycobacterial species that uses DNA-DNA hybridization (DDH Mycobacteria, Kyokuto Pharmaceutical Co., Ltd).

Drug sensitivity testing

Drug sensitivity of *M. tuberculosis* strains was tested by two agar proportion methods, one with Middlebrook 7H10 agar medium, as recommended by the U.S. Public Health Service,[23] and the other with egg-based Ogawa medium, as recommended by the Japanese Society for Tuberculosis (Vit Spectrum-SR™, Kyokuto Pharmaceutical Co., Ltd).

DNA fingerprinting

Chromosomal DNA from mycobacterial isolates was prepared as described previously[24,25] but with slight modification. The DNA was precipitated in isopropanol, and the precipitates were redissolved in 20 μl 0.1X TE buffer.

For IS6110- and $(CGG)_5$-restriction fragment length polymorphisms (RFLP)[26] of *M. tuberculosis*, DNA was digested overnight with restriction enzymes *Pvu*II and *Alu*I (Takara Bio, Inc., Shiga, Japan), respectively. The digested fragments were separated by electrophoresis on 1% agarose gels. A 1-kb DNA ladder (Promega Corp., Madison, WI) was used as a marker. The agarose gels were stained with ethidium bromide, and the results were recorded photographically. DNA fragments were transferred onto N^+ Hybond membrane (Amersham Biosciences, Little Chalfont, UK), and the DNA was fixed to the membrane by UV illumination. The *IS6110* probe was a 245-bp DNA fragment amplified by PCR as described previously.[25] The 15-mer oligonucleotide $(CGG)_5$ was synthesized by Nippn Techno Cluster, Inc., Tokyo, Japan. The probes were labelled with horseradish peroxidase by the ECL Direct™ System (Amersham Biosciences). Hybridization was conducted with the ECL Direct™ System, according to the recommendations of the manufacturer. Autoradiographs were obtained by exposing the membranes to X-ray film.

For IS*1245*-[27] and IS*1311*-RFLP[28] of *M. avium*, DNA was digested overnight with *Pvu*II. The IS*1245* and IS*1311* probes were 427 and 200-bp DNA fragments, respectively, and were amplified by

PCR as described previously.[27,28] Briefly, the oligonucleotides for IS*1245*, 5′-GCCGCCGAAACGATCTAC-3′ and 5′-AGGTGGCGTCGAGGAAGAC-3′,[27] and for IS*1311*, 5′-GTCGGGTTGGGCGAAGAT-3′ and 5′-GTGCAGCTGGTGATCTCTGA-3′,[28] were used to amplify the fragments prepared from purified chromosomal DNA from *M. avium* ATCC 25291 by PCR.

Analysis

Fingerprinting patterns of *M. tuberculosis* or *M. avium* were analysed with Molecular Analyst Fingerprinting Plus Software, version 1.6 (Bio-Rad Laboratories, Inc., Hercules, CA). To facilitate comparison of the fingerprinting patterns, normalization was performed relative to the molecular-weight markers. Each dendrogram was calculated according to the unweighted-pair group method with average linkage according to the supplier's instructions.

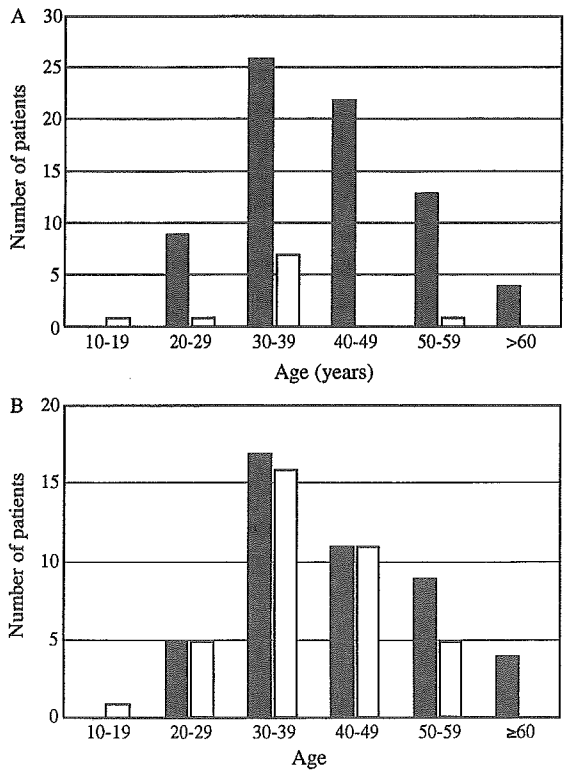

Figure 1 Distribution of 84 mycobacterial infections in HIV-seropositive patients. Panel A: age (years) and sex distribution. Filled bars, male; open bars, female. Panel B: age (years) and pathogenic agent distribution. Filled bars, tuberculosis patients; open bars, non-tuberculous mycobacterial-infected patients.

Results

Mycobacterial infection in HIV-seropositive patients

From 86 HIV-seropositive patients, 48 (55.8%) *M. tuberculosis*, 36 (41.9%) *M. avium*, and 2 (2.3%) *Mycobacterium chelonae* isolates were identified (Table 1).

Nationality and sex are also listed in Table 1. Mean age was 40.5±12.2 years, ranging from 11 to 68 years. Most mycobacteria and HIV coinfected patients were aged 30-39 years (Fig. 1). The most frequent route of HIV infection was sexual transmission (90%); other routes were infection by blood products (5%), drug abuse (5%), mother-to-child infection (1%) and unknown (1%). With respect to mycobacterial infection, 48 and three individuals had primary and recurrent infection, respectively. There was no corresponding record for the remaining patients.

Profile of HIV-seropositive patients with *M. tuberculosis*

In 46 of the 48 tubeculosis patients, the ratio of males/females was 43/3 (Table 1). Mean age was 42.7±11.9 years, ranging from 22 to 68 years. Twenty-five patients had combined pulmonary and extra-pulmonary infection, mainly due to miliary tuberculosis. A total of 56.3% of the 48 patients had pulmonary tuberculosis, as evidenced by positive microscopy smears. Peripheral blood $CD4^+$ cell counts at the time of TB diagnosis ranged from 6 to 331/mm^3, and the median was 62/mm^3.

According to drug sensitivity testing, 43 isolates (89.6%) were sensitive to anti-tubercular drugs, 3 (6.3%) were resistant to a single drug, and 2 (4.2%) were resistant to 2 and 5 drugs, respectively.

In 87 HIV-seronegative TB patients with tuberculosis, 82 were Japanese and five were non-Japanese. The ratio of males/females was 56/31. Mean age was 53.3±20.5 years (56.1±19.0 years for males and 48.6±22.0 years for females), ranging from 18 to 95 years (18-90 for males and 18-95 for females) and patients over 40 years of age accounted for 66.7% of the total. According to drug sensitivity testing, 75 isolates (86.2%) were sensitive to anti-tubercular drugs, 6 (6.9%) were resistant to a single drug, and 6 (6.9%) were resistant to 2 and 6 drugs, respectively.

RFLP analysis of *M. tuberculosis*

To determine whether specific strain(s) of tubercular bacilli prevail among HIV-seropositive patients in Japan, we analysed DNA fingerprints of the isolates by RFLP analysis. Thirty-three of the 48 *M. tuberculosis* clinical isolates were analysed by RFLP, and the patterns are shown in Fig. 2.

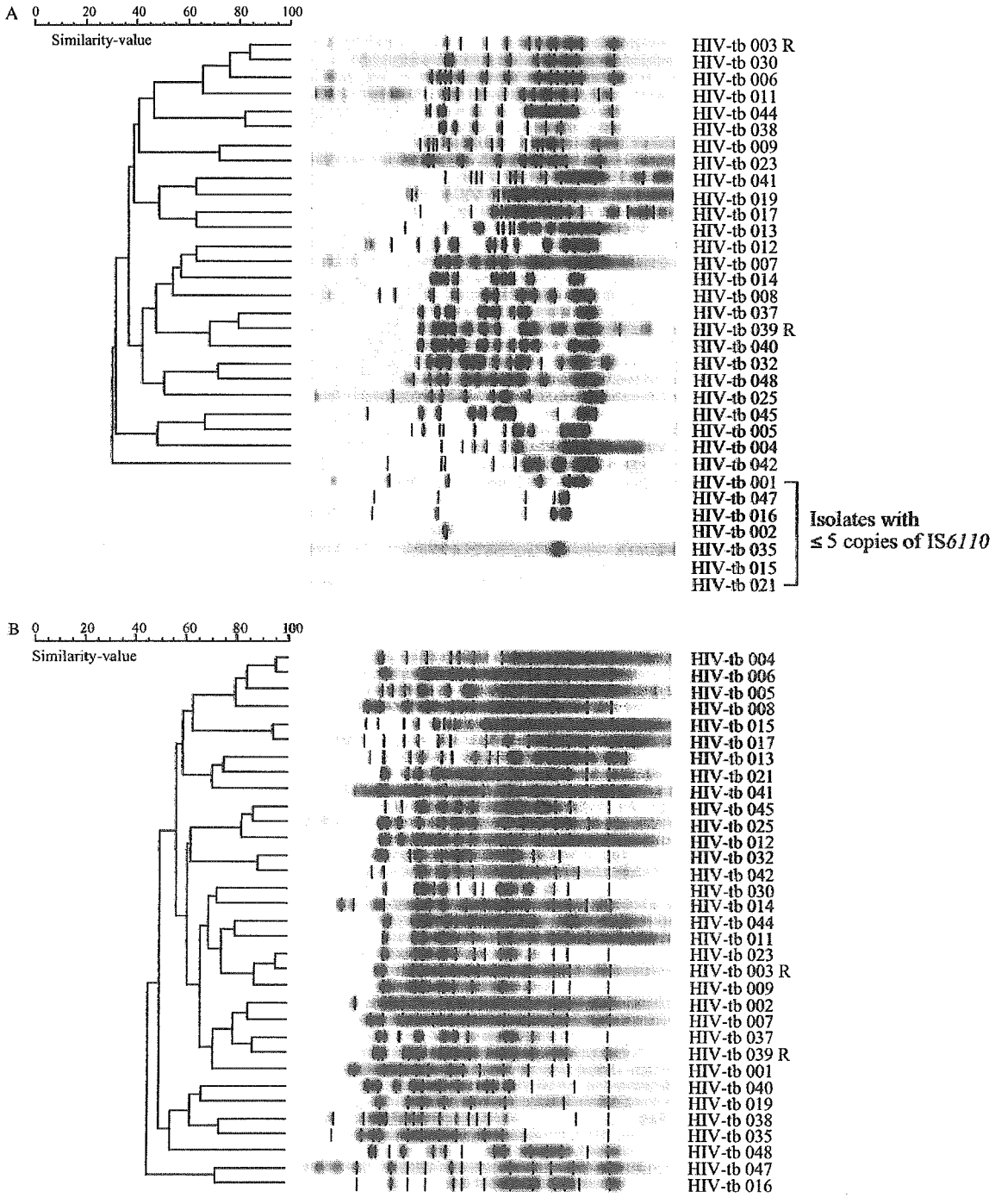

Figure 2 IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. In the IS*6110*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, HIV-tb 003 R is an HIV-seropositive patient-derived isolate.

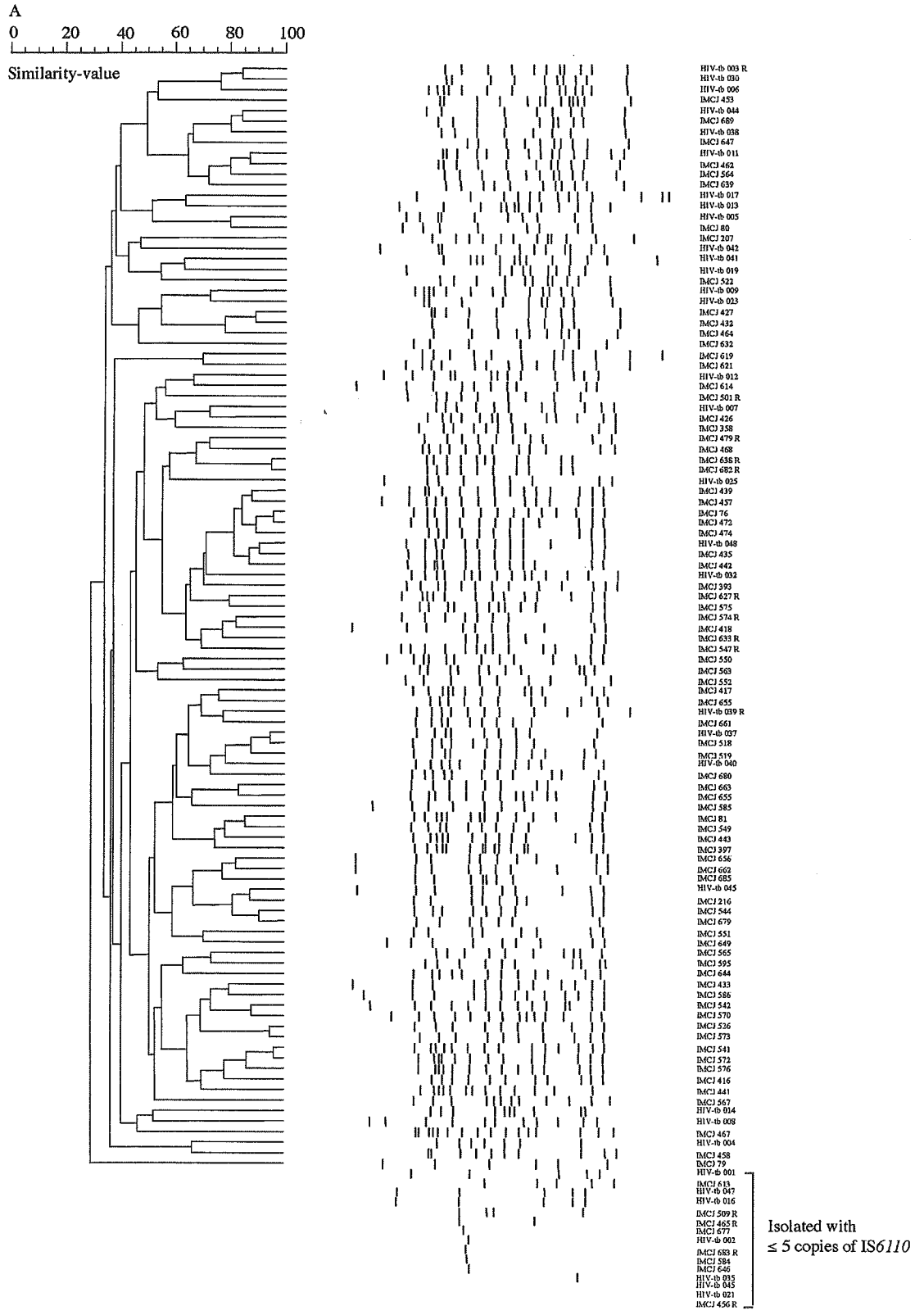

Figure 3. IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive and HIV-seronegative patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. In the IS*6110*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, a prefix of 'IMCJ' indicates an HIV-seronegative patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, IMCJ 627 R is an HIV-seronegative patient-derived isolate.

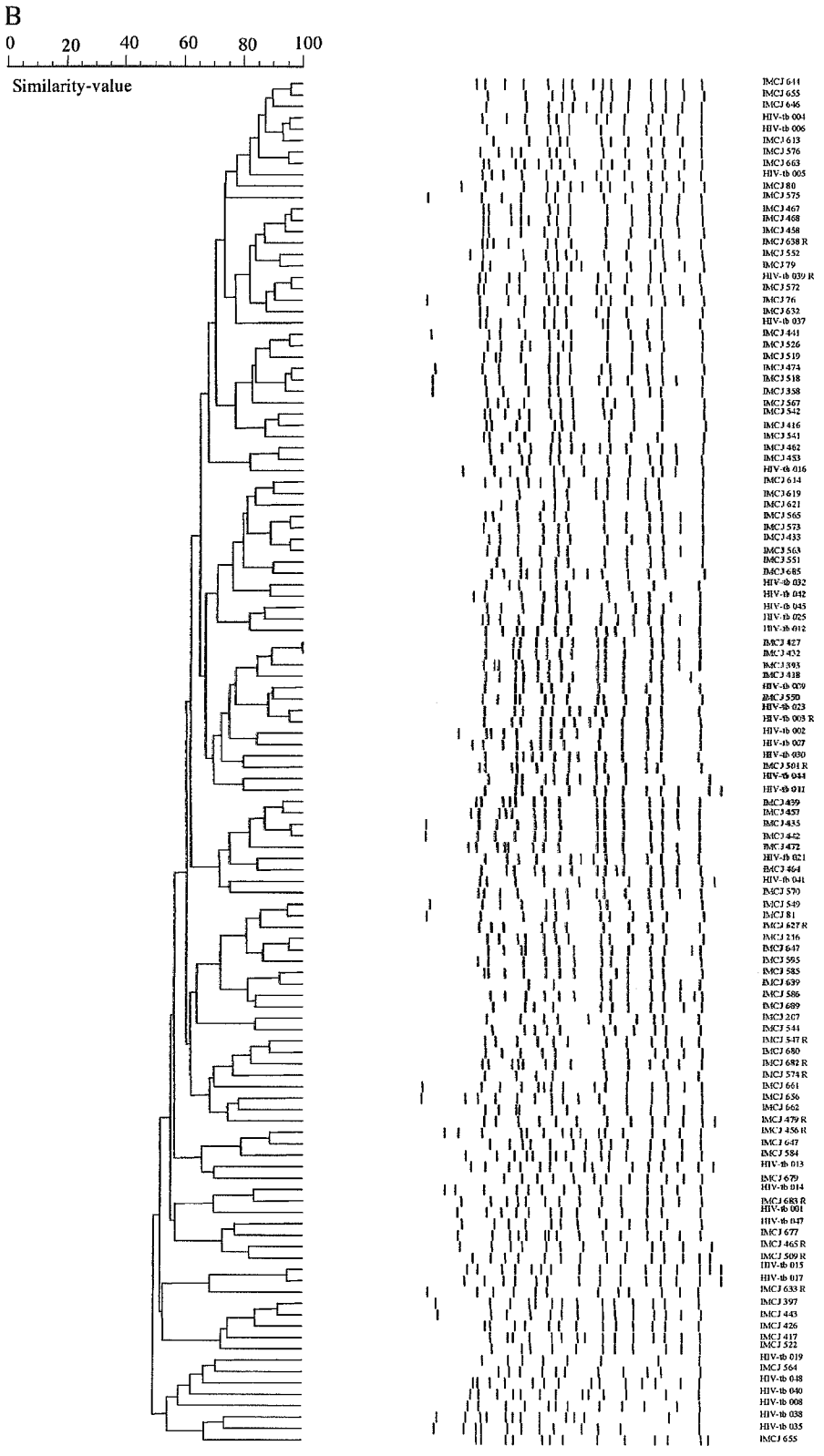

Figure 3 (*continued*)