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Modified Dynabeads Method for Enumerating CD4+ T-Lymphocyte Count for Widespread Use in Resource-Limited Situations

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Summary: The Dynabeads method showed the potential for enumerating $CD4^+$ T lymphocytes (CD4 count) in HIV-1–infected individuals. The large volume of Dynabeads required for 1 sample and complex procedure made the method expensive and not easy for use, however. To decrease the cost and simplify the procedure, we reduced the volume of the Dynabeads, added wash times, and skipped over the staining step so as to count the CD4 cells directly under an optical microscope. The CD4 count of 246 blood samples using our modified Dynabeads method (DynabeadsCD4) showed a significant correlation with that obtained by flow cytometry (FlowcytoCD4) ($r = 0.91$ [$P < 0.0001$]; slope = 1.03, intercept = −16). The sensitivity and specificity for a CD4 count less than 200 cells/μL were 79% and 94%, and for a CD4 count less than 350 cells/μL, the sensitivity and specificity were 95% and 88%, respectively. The positive and negative predictive values for a CD4 count less than 350 cells/μL were 97% and 83%, respectively. The systematic error was 8 cells/μL (95% confidence interval [CI]: 0.4–16). The cost of Dynabeads for 1 sample was less than $1.00; thus, the estimated cost per DynabeadsCD4 test is less than $3.00, including the cost of other disposable materials. Our modified method is simple, economic, and accurate enough to monitor antiretroviral therapy in resource-limited situations.

Key Words: CD4, monitoring, Dynabeads, resource-limited situations

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The $CD4^+$ T-lymphocyte count (CD4 count) is an important surrogate marker for the clinical course of HIV infection, such as initiation of prophylactic treatment of opportunistic infections, initiation of antiretroviral therapy (ART), and monitoring the response to ART.[1–4] In developed countries, the CD4 count is usually measured by flow cytometry, which is considered to be the standard reference method.[3,4] In resource-limited areas, however, flow cytometry is available only in limited settings such as tertiary medical centers because it requires expensive reagents and well-trained technicians. Furthermore, equipment maintenance is another difficult issue, because a technical support system is needed in areas afflicted with frequent electrical power failures, which could potentially cause machine-related problems.

In recent years, lower cost and less technically demanding methods for enumerating CD4 cells have been tried but have not been used widely even in resource-limited settings for various reasons.[4,5] In the World Health Organization (WHO) guidelines for treatment of HIV-infected individuals in resource-limited environments, a total lymphocyte count (TLC) of 1200 cells/μL is recommended to represent a CD4 count threshold of 200 cells/μL in making a decision regarding therapy when the CD4 count is unavailable.[1] In addition, various research groups have recommended the use of a TLC,[5] absolute lymphocyte count or TLC,[6] and TLC combined with hemoglobin measurement[7] as surrogate markers for monitoring ART. These studies suggested that the lymphocyte count might have some value in monitoring ART. The lymphocyte count is readily available and inexpensive, but it is not sufficiently adequate to predict the absolute CD4 count in many settings.[4]

Among several low-cost and less technically demanding methods,[8–15] the Dynabeads assay, which uses magnetic particles coated with a monoclonal antibody to CD4 to capture $CD4^+$ cells, seems to be a good candidate as an alternative to flow cytometry based on its good correlation with the results of flow cytometry.[8,10,11,13] According to the protocol recommended by the manufacturer, however, CD4 and CD8 cells are enumerated at the same time using a large volume of Dynabeads. The large volume of Dynabeads used in each assay is also relatively expensive (approximately $5), particularly for poor settings. In addition, division of the samples into 2 aliquots during the procedure might jeopardize the accuracy of the results. Moreover, in this assay, the cells are lysed and nuclei are stained to count them, which makes the operation complex.

For monitoring ART in HIV infections, only the CD4 component is necessary and only the CD4 count (not CD8 count) is mentioned in ART guidelines.[1,2] For this reason,

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further modifications are needed for the expanded use of the CD4 count in resource-limited areas. In the present study, we modified the protocol to make it simple and inexpensive so that it could be applied widely in resource-limited facilities.

MATERIALS AND METHODS

Study Population and CD4 Enumeration

This study included 242 adult patients infected with HIV-1 who regularly consulted the AIDS Clinical Center of the International Medical Center of Japan between June and October 2003. The inclusion criteria were a CD4 count less than 1000 cells/μL and consent granted to participate in the study. Patient age ranged from 20 to 78 years (mean ± SD: 40 ± 11.5). A total of 315 blood samples were collected using EDTA-containing tubes and tested for CD4 count within 4 hours by flow cytometry (FlowcytoCD4; Coulter-EPICS XL-MCL, Beckman-Coulter, Fullerton, CA) with CD45–fluorescein isothiocyanate (FITC)/CD4-phycoerythrin (RD1)/CD8-pheycoerythrin-Texas Red (ECD)/CD3-pheycoerythrin-cyanin 5.1 (PC5) (Beckman-Coulter). The CD4 cell count in the rest of the blood sample was enumerated using Dynabeads (Dynabeads CD4; Dynal Biotech ASA, Oslo, Norway) within 24 hours. When different protocols such as 25-μL and 5-μL volumes of CD4 Dynabeads or 10 and 30 minutes of incubation time were compared, the same sample was used in each experiment.

Modified Protocol (Original Protocol) of the Dynabeads Method

A well-mixed whole-blood sample (125 μL) was placed into a 1.5-mL microtube containing 375 μL (350 μL) of buffer (0.1% bovine serum albumin in phosphate-buffered saline [PBS]). CD14 Dynabeads were suspended with buffer (1:1 diluted buffer); 5 μL (25 μL) of CD14 Dynabeads was then added to the microtube containing the blood sample and the tube was inverted several times and then incubated in Dynal MX-1 for 10 minutes. The tube was spun down in a microcentrifuge and then placed in magnetic particle concentration for microcentrifuge tubes (Dynal MPC-S) (6 tubes per batch) for 2 minutes, followed by transfer of the entire volume (division into 2 200-μL aliquots) of monocyte-depleted blood into a new microtube. In the next step, 5 μL (25 μL) of CD4 Dynabeads was added to the tube and incubated in Dynal MX-1 for 30 minutes (10 minutes). The cells were washed with 500 μL of buffer, vortexed gently, and spun down; the tube was then placed in Dynal MPC-S for 2 minutes, the wash buffer was discarded, and the tube was removed from the Dynal MPC-S. The cells were washed 3 more times (once), resuspended by adding 125 μL of buffer, and kept at 4°C until counting (50 μL of lysing solution was added, followed by thorough vortexing for resuspension, and the cells were allowed to stand for 5 minutes, after which 50 μL of acridine orange staining solution was added and the sample was kept in darkness until counting). Finally, the sample was vortexed well, 10 μL of cells was applied to a hemocytometer, and the mononuclear cells with attached Dynabeads were counted as $CD4^+$ cells under a light microscope (the number of nuclei was determined under a fluorescence microscope). After reducing the volume of CD4 Dynabeads, it was not difficult to count the CD4 cells under an optical microscope, even without staining. All procedures were performed at room temperature at approximately 23°C. All Dynabeads-related equipments and reagents were products of Dynal Biotech ASA.

Statistical Analysis

All data are expressed as mean ± SD. StatView v5.0 software was used to analyze the correlation and single linear regression between DynabeadsCD4 and FlowcytoCD4. *P* values were calculated by 2-sided test and considered as significant if at a level less than 5%. All confidence intervals were 2-sided, with a significant level of 5%.

RESULTS

First, we examined the influence of a reduced volume of CD14 (from 12.5 μL to 5 μL) Dynabeads on monocyte depletion. The percentage of monocytes in 5 blood samples was analyzed by flow cytometry before and after treatment with 5 μL of CD14 Dynabeads. The result showed that 5 μL of CD14 Dynabeads deleted 92.4% to 97.5% (average = 95.6%) of monocytes from 125 μL of whole blood. The remaining experiments were performed using 5 μL of CD14 Dynabeads. Next, we examined the influence of a reduced volume of CD4 Dynabeads on the CD4 count in 23 samples. The volume of CD4 Dynabeads was reduced from 25 μL to 5 μL, but the incubation time was still 10 minutes (like that of original protocol), which we called modified protocol 1. CD4 counts by the original protocol and modified protocol 1 correlated significantly with those determined by flow cytometry: DynabeadsCD4 by the original protocol ($r = 0.90$ [$P < 0.0001$]; slope = 1.05, intercept = −32) and DynabeadsCD4 by modified protocol 1 ($r = 0.92$ [$P < 0.0001$]; slope = 1.05, intercept = 26). These results indicated that DynabeadsCD4 obtained by using the reduced volume of CD4 Dynabeads with a 10-minute CD4 separation correlated well with FlowcytoCD4. When the number of samples was increased to 56, however, the mean DynabeadsCD4 of 56 samples by modified protocol 1 was 269 ± 140 cells/μL compared with a mean FlowcytoCD4 of 336 ± 178 cells/μL (Table 1). The difference was −67 cells/μL ($P < 0.0001$). This result suggested that the 10-minute CD4 separation time was too short. We then examined the effect using a reduced volume of Dynabeads and a different incubation time.

Next, with 5 μL of CD4 Dynabeads, we lengthened the CD4 separation time from 10 minutes to 30 minutes in 34 samples. The correlations between DynabeadsCD4 and FlowcytoCD4 were $r = 0.91$ ($P < 0.0001$) and $r = 0.94$ ($P < 0.0001$), with slopes of 1.05 and 1.0 and intercepts of 22 and 8, for 10 and 30 minutes of incubation time, respectively. The mean difference with flowcytoCD4 was −32 cells/μL ($P = 0.008$) and −8 cells/μL ($P = 0.42$), respectively. According to these data, the 30-minute incubation time for CD4 separation yielded a better result than that of the 10-minute incubation time. We then fixed the protocol as 5 μL of CD14 Dynabeads with 10 minutes of incubation time and 5 μL of CD4

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TABLE 1. DynabeadsCD4 Determined by Different Protocols Compared with FlowcytoCD4

	Mean ± SD (Cells/μL)		Mean Difference		Regression Line		
Protocol	DynabeadsCD4	FlowcytoCD4	Cells/μL (95% CI)	P	Intercept	Slope	r^2
Original (n = 59)	364 ± 166	372 ± 193	−8 (−32 to 16)	0.521	−2	1.026	0.775
Modified 1 (n = 56)*	269 ± 140	336 ± 178	−67 (−93 to −41)	<0.0001	50	1.061	0.698
Modified 2 (n = 246)†	262 ± 136	254 ± 154	8 (0.4 to 16)	0.0396	−16	1.031	0.829

†Modified 2 (the final one): modified protocol with 30 minutes of CD4 separation.
*Modified 1: modified protocol with 10 minutes of CD4 separation.
$P > 0.05$ for all intercepts, $P < 0.0001$ for all slopes and r^2.

Dynabeads with 30 minutes incubation time (which we called modified protocol 2) and tested 246 samples. DynabeadsCD4 showed a significant correlation with FlowcytoCD4 ($r = 0.91$ [$P < 0.0001$]; slope = 1.03, intercept = −16; Fig. 1). At less than 200 cells/μL, the sensitivity and specificity of DynabeadsCD4 compared with FlowcytoCD4 were 79% and 94%, respectively, and at less than 350 cells/μL, the sensitivity and specificity were 95% and 88%, respectively. The mean DynabeadsCD4 was 262 ± 135 cells/μL and that of FlowcytoCD4 was 254 ± 154 cells/μL (see Table 1). The difference in the mean values was 8 cells/μL (95% confidence interval [CI]: 0.4–16; $P = 0.04$), with a random error of 64 cells/μL. The positive and negative predictive values of DynabeadsCD4 and FlowcytoCD4 for less than 200 cells/μL and less than 350 cells/μL were 90% and 87% and 97% and 83%, respectively. Other factors (eg, on therapy vs. off therapy, male vs. female) had no influence on DynabeadsCD4 (data not shown).

Table 2 shows the results of a comparison between the original protocol and our modified protocol. In our modified protocol, volumes of CD14 and CD4 Dynabeads were reduced from 12.5 μL and 25 μL, respectively, to 5 μL each against 125 μL of whole blood. Accordingly, the cost of the Dynabeads test decreased from $2.84 to $0.89. The incubation time for CD4 separation was prolonged to 30 minutes to obtain a better yield. In our protocol, after monocyte depletion, we transferred all treated blood to a new microtube for CD4 cell separation because we did not consider the CD8 count. We also skipped over lysis and nuclear staining steps so as to simplify the procedure.

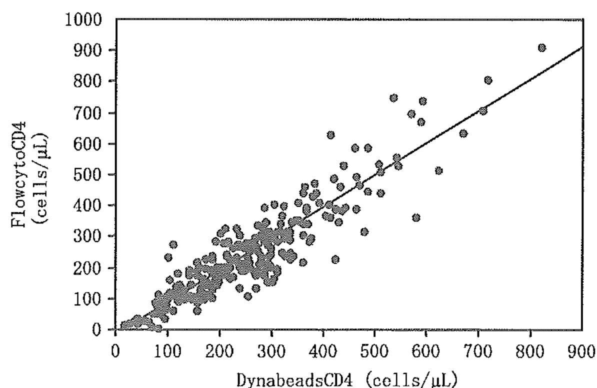

FIGURE 1. Correlation analysis of CD4+ T-lymphocyte count (CD4 count) using Dynabeads method and CD4 count using flow cytometry.

DISCUSSION

To attain the "3 by 5" goal of effective ART promoted by the WHO, precise monitoring of ART is indispensable. Low cost, in addition to good accuracy, is thus an important issue. In this regard, maintenance of a "high-tech" machine for long-term monitoring may be impossible. The Dynabeads method is currently used as an alternative method to flow cytometry for CD4 count in a number of countries. In this study, we successfully modified the protocol of the Dynabeads method to make it more suitable in resource-limited areas with 2 goals in mind: reasonable cost and sufficient accuracy.

TABLE 2. Comparison Between the Original Protocol and Modified Protocol 2 for Enumeration of CD4 Count

Step	Original Protocol	Modified Protocol 2
Buffer (μL)	350	375
Blood (μL)	125	125
CD14 Dynabeads (μL)	25 (1:1 dilution)	5
Incubation temperature (°C)	RT	RT
Incubation duration (min)	10	10
Monocyte-depleted supernatant (μL)	200*	505†
CD4 Dynabeads (μL)	25	5
Incubation temperature (°C)	RT	RT
Incubation duration (min)	10	30
Repeat of washing (total min)	2 (10 min)	4 (20 min)
Staining time (min)	5 min	—
Resuspension volume (μL)	—	125
Time of total experiment per sample (min)	50	75
Samples comfortably analyzed per operator	12–18	12–18
Cost of CD14 Dynabeads ($)	1.63	0.65
Cost of CD4 Dynabeads ($)	1.21	0.24
Total cost of Dynabeads ($)	2.84	0.89

*Transferring 200 μL to a new tube.
†Transferring the entire volume to a new tube.
Values are for 1 test.
RT indicates room temperature (approximately 23°C).

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In the present study, DynabeadsCD4 obtained by using the original protocol also showed a good result (see Table 1). During the operation, we found 2 problems with the original protocol, however. One was the transfer of 200 μL of blood from 500 μL of blood to a new tube after monocyte depletion. This step might lead to inaccurate results because we could not mix the blood well while the tube was on the Dynal MPC-S. The other was that too many free Dynabeads (which did not attach to CD4 cells) and red blood cells were identified when the cells were counted under a light microscope. This might be the reason for recommending lysis of the cells, staining the nuclei, and using a fluorescent microscope in the last step of the original protocol. In our modified protocol, the entire sample was transferred to a new tube after monocyte depletion. The number of free Dynabeads decreased after the volume of CD4 Dynabeads was reduced. Furthermore, we washed the sample 4 times after CD4 cell separation in spite of the original protocol recommending washing only twice. The red blood cells could be almost completely removed by 4 washes, especially when the washing buffer had been discarded completely at each wash. These modifications made a direct count under a light microscope possible.

After reduction of the volume of Dynabeads used in the assay, the cost of reagents used for analysis of 1 sample decreased to less than $1.00. Thus, the total cost of 1 CD4 count, including other disposable materials such as syringes, tubes, and tips, could be less than $3.00.

In conclusion, the present study demonstrated that our final modified protocol of Dynabeads assay could be used as a good alternative to flow cytometry with sufficient accuracy, reliability, and simplicity at a reasonable cost. Therefore, the assay could be suitable for monitoring ART in resource-limited settings.

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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

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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.

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

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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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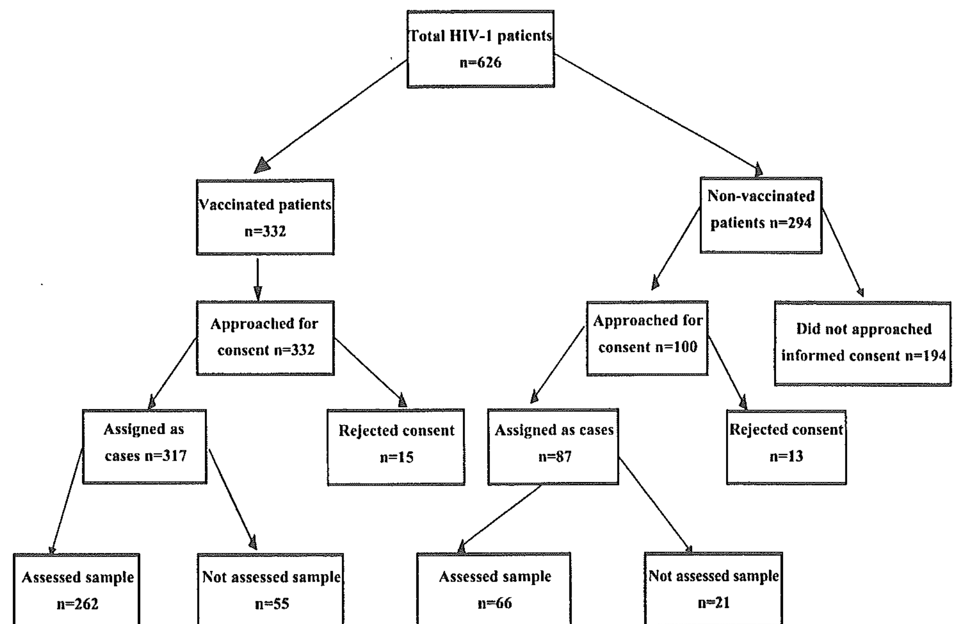

FIGURE 1. Profile of participants in this study.

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.

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.

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.

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.

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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).

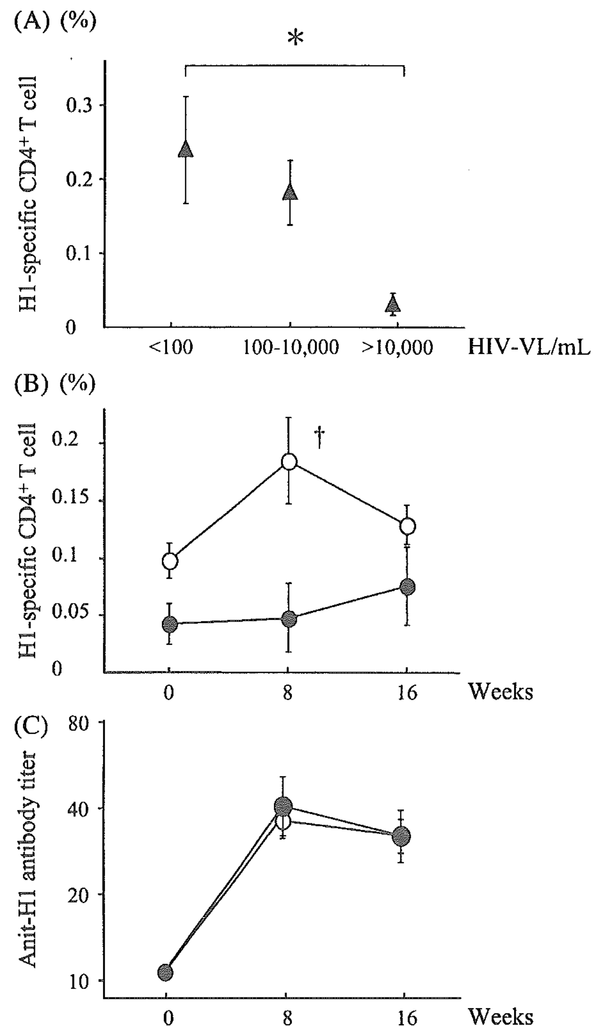

FIGURE 2. H1-specific CD4+ T-cell responses after influenza vaccine in baseline anti-H1 antibody–negative patients. A, Correlation of plasma HIV-1 viral load (HIV VL) and percentage of H1-specific CD4+ T cells. *H1-specific CD4+ T cells (▲) were significantly fewer in number in subjects with an HIV VL >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

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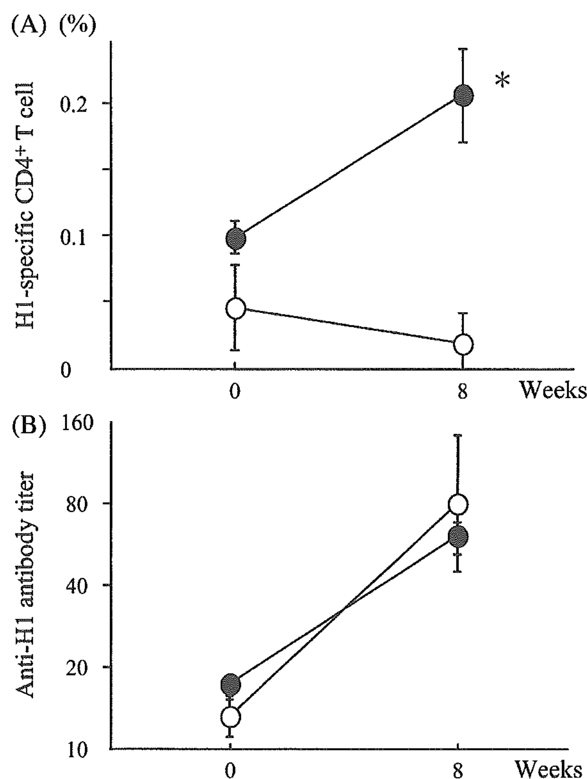

FIGURE 3. H1-specific $CD4^+$ T cells and anti-H1 antibody responses at week 8 after vaccination in influenza A/H1N1–infected patients. Five vaccinated individuals were infected with influenza A/H1N1. A, Percentage of H1-specific $CD4^+$ T-cell responses in infected (○; n = 4) and noninfected (●; n = 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

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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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Short Communication

Trial Surveillance of Cases with Acute Respiratory Symptoms at IMCJ Hospital

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SUMMARY: We have developed a surveillance system that can detect a severe acute respiratory syndrome (SARS) outbreak in a hospital as quickly as possible using the "SARS alert" strategy proposed by the World Health Organization (WHO). Our research examined hospital staff and in-patients during the winter of 2003/2004. We defined patients with a fever of over 38°C and respiratory symptoms as "cases with acute respiratory symptoms." During the study period, 215 such cases (78% in-patients; 22% hospital staff members) were reported. A rapid diagnostic test for influenza was performed on 131 individuals, with 52 having positive results. There were no cases fulfilling the definition of SARS provided by the WHO in their SARS alert. The present surveillance system will be of use in the early detection of a SARS epidemic in a hospital as well as in early detection of similar illnesses accompanied by acute respiratory symptoms, such as influenza.

Severe acute respiratory syndrome (SARS) haunted the world from November 2002 to July 2003. According to the World Health Organization (WHO), over 8,000 infected patients and nearly 800 deaths were reported in 26 regions during this period. An extremely large problem in the case of SARS is the number of health care workers (HCWs) infected; at 1,706 persons, the figure accounted for 21% of all reported cases (1; http://www.who.int/csr/sars/country/table2004_04_21/en/). Because of this problem, the WHO has proposed a new surveillance strategy known as the "SARS alert" (2; http://www.who.int/csr/sars/postoutbreak/en/). If a SARS alert occurs, the WHO recommends that strict infection control procedures be adopted immediately. However, the introduction of this policy requires daily surveillance in accordance with the definition of a SARS alert. Additionally, this surveillance targets not only in-patients but also hospital personnel. To date, the WHO has not yet indicated any specific methods for the application of SARS alert surveillance to hospital personnel.

Therefore, we attempted to create a new surveillance system to detect clinical SARS cases as defined by the SARS alert in both patients and HCWs. To facilitate the detection of SARS as well as other respiratory infectious diseases such as influenza, the present surveillance focused on cases with "acute respiratory symptoms".

These definitions used for this surveillance were a fever of over 38°C and one or more symptoms of respiratory tract illness (RTI), including both upper RTI (rhinorrhea or sore throat) and lower RTI (coughing, sputum, shortness of breath, decreased SpO_2, or radiographic evidence of lung infiltrates consistent with pneumonia or respiratory distress syndrome [RDS]).

The subjects were all in-patients, nurses, doctors, technicians, pharmacists or other medical staff at the International Medical Center of Japan (IMCJ) hospital, Tokyo, Japan. The study period was from December 2003 to March 2004. If a patient or HCW with acute respiratory symptoms was identified, the head of each section filled in a surveillance report and submitted it to an infection control team (ICT). The results of the surveillance were analyzed and released weekly to hospital staff by hospital intranet.

During the study period, 215 cases with acute respiratory symptoms were reported. Their median age was 39.0 years of age (range: 5 mos - 99 years of age), and the male:female ratio was 1:1.05. Wards in which numerous cases were reported were the pediatric ward (36 cases), the respiratory ward (20 cases) and the private room ward (18 cases). The identified cases included 168 in-patients (78%), 26 nurses (12%), 15 doctors (7%), 4 technicians (2%) and 2 pharmacists (1%). A rapid test for influenza (Espline®; Fujirebio, Inc., Tokyo, Japan) (3) was performed in 131 cases (61%), and 40% of tested individuals were found to be positive. Trends in the reported cases are shown in Figure 1. There was a peak in the number of reported cases from the 3rd week of January to the 2nd week of February, coinciding with a peak in influenza cases at the IMCJ hospital. Additionally, these peaks coincided with a peak in the nation wide spread of influenza in Japan (4; http://idsc.nih.go.jp/idwr/kanja/weeklygraph/01flu-e.html).

During the surveillance period, one cluster of cases with acute respiratory symptoms was found in our hospital. The episode was observed in the respiratory ward and included 11 cases with acute respiratory symptoms; 4 of which tested positive on the rapid diagnostic test for influenza. This finding caused the ICT to quickly introduce appropriate infection control measures such as cohort isolation, prophylactic use of oseltamivir, and limitations on the admission of new patients. With this intervention, the cluster was quickly controlled.

During the study period, no actual SARS alert cases that met the WHO definition were observed.

SARS is characterized by its high transmissibility to HCWs and becomes widespread via nosocomial infection (5,6). Therefore, both in-patients and HCWs with symptoms must be constantly monitored in order to detect a SARS outbreak

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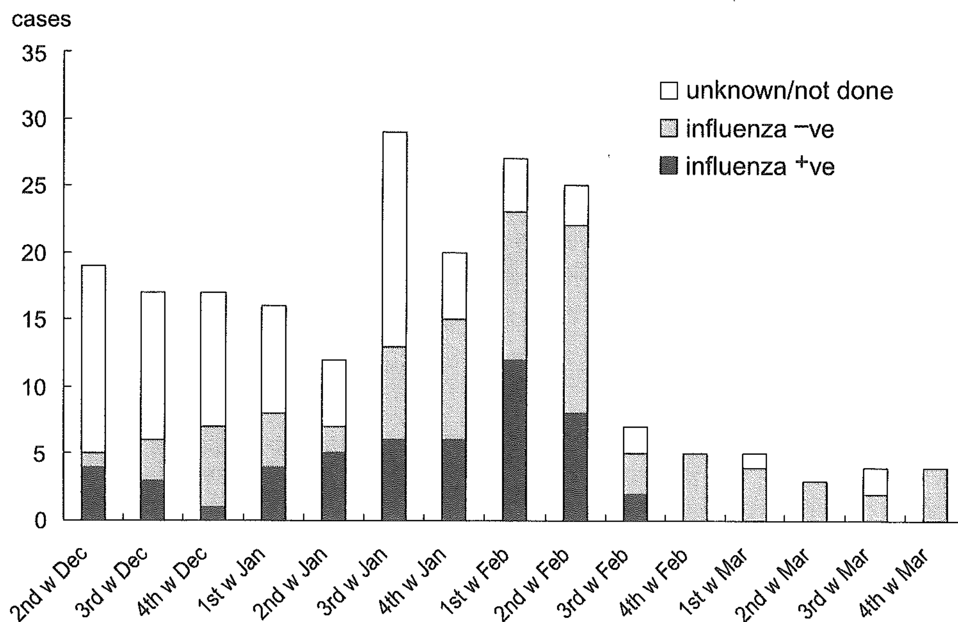

Fig. 1. Trend graph of reported case with acute respiratory symptoms between the 2nd week of December 2003 and the 4th week of March 2004.

in a hospital in the early stages. The SARS alert strategy proposed by the WHO is an operational definition used to ensure that appropriate infection control and public health measures are implemented until SARS has been ruled out as a cause of pneumonia or RDS.

This policy defines SARS cases clinically as cases with a fever of over 38°C, with one or more symptoms of lower RTI (coughing, difficulty breathing, or shortness of breath), with radiographic evidence of lung infiltrates consistent with pneumonia or RDS, and with no alternative diagnosis that can fully explain the illness. SARS alert situation is defined as one or both of the following:

i) two or more HCWs in the same health care unit fulfilling the clinical case definition of SARS and whose onset of illness occurs within the same 10-day period; and

ii) hospital-acquired illness in three or more persons (HCWs and/or other hospital staff and/or patients and/or visitors) in the same health care unit fulfilling the clinical case definition of SARS and whose onset of illness occurs within the same 10-day period.

Because the threat of infection involves not only SARS but also other emerging respiratory virus infections (i.e., new types of influenza), we attempted to create a system that can also detect acute respiratory infections such as influenza in a hospital. Because the early clinical features of SARS and influenza are quite similar, some confusion in clinical settings is expected. Hence, a "syndromic surveillance" system, that is, a system that detects acute respiratory symptoms without regard to the pathogenic virus, must be developed. Therefore, we partially modified the WHO's SARS alert strategy and introduced a new method of surveillance for the early detection of SARS and influenza.

Our criteria for the definition of disease differed from that of the WHO in that it included upper RTI and (ii) it did not require pneumonia findings in chest X-rays. We felt that adding these changes would allow the detection of influenza outbreaks in a hospital as well.

An epidemic of cases with acute respiratory symptoms during the aforementioned period was effectively monitored during surveillance at IMCJ hospital. An outbreak of influenza at the hospital was also detected by the present surveillance system. Information provided by surveillance was effectively used for infection control. Fortunately, there were no cases that met the definition of SARS provided by the WHO in their SARS alert. Hospital staff should be informed as soon as possible about the spread of infectious diseases in the hospital. We used hospital intranet for this purpose, and information was quickly conveyed to the appropriate divisions of the hospital.

The present surveillance strategy will be of use in the early detection of a SARS epidemic in a hospital as well as in the early detection of similar illnesses accompanied by acute respiratory symptoms such as human influenza and new types of influenza. Further study is needed to improve the sensitivity and specificity of this surveillance.

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

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KEYWORDS
Mycobacterial infection;
HIV-seropositive patients;
RFLP

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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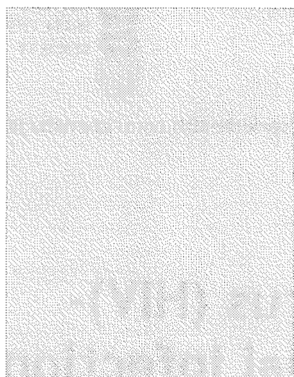

Results. Of 86 mycobacterial strains, *M. tuberculosis*, *M. avium* and *Mycobacterium chelonae* were identified in 48 (55.8%), 36 (41.9%) and 2 (2.3%) isolates, respectively. The obtained RFLP patterns of *M. tuberculosis* isolates from both the HIV-seropositive and -seronegative groups were variable, 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.

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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 extra-pulmonary), 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 IS*6110* 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.

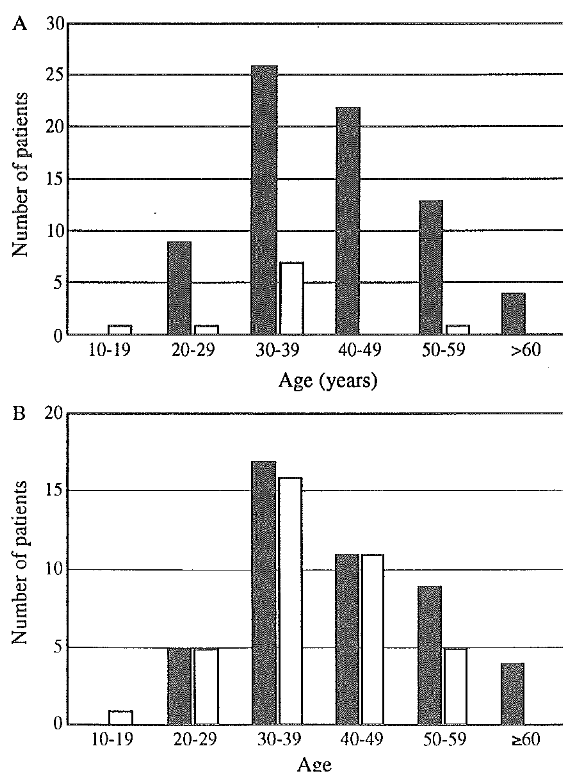

Figure 1 Distribution of 84 mycobacterial infections in HIV-seropositive patients. Panel A: age (years) and sex distribution. Filled bars, male; open bars, female. Panel B: age (years) and pathogenic agent distribution. Filled bars, tuberculosis patients; open bars, non-tuberculous mycobacterial-infected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

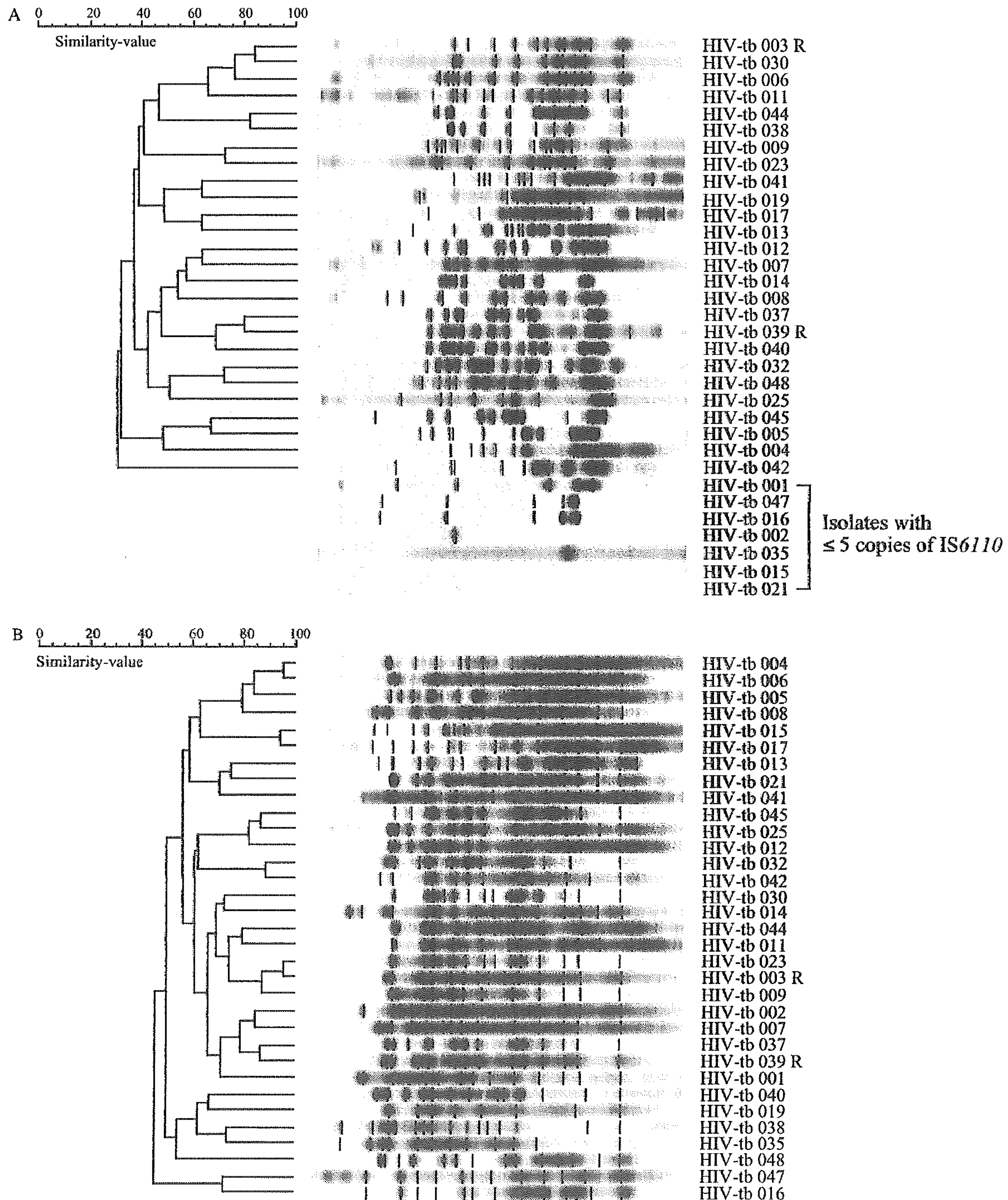

Figure 2 IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each 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.