(Catalog No. PK-6102; Vector Laboratories; Burlingame, CA), slides were incubated for 20 min at room temperature in blocking solution (5% horse serum-PBS for uPAR, PAI-1, CAP18, IL-8 CD14, and cytokeratin; 5% goat serum for CD15) and washed thrice with PBS. Slides were then incubated in avidin blocking solution (Catalog No. SP-2001; Avidin/Biotin Blocking Kit; Vector Laboratories) for 15 min at room temperature, washed thrice with PBS for surface staining, and then washed in PBS-0.05% Tween 20–0.01% Thimerosal for cytoplasmic staining. Slides were then incubated with biotin blocking solution for 15 min at room temperature and washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal. Following this preparation, slides were incubated with primary monoclonal antibody (for uPAR, Catalog No. 3936; American Diagnostica; Greenwich, CT; for PAI-1, Catalog No. 3785; American Diagnostica; for CAP18, see primary antibody, ELISA methods above; for IL-8, Catalog No. 554717; BD Pharmingen mouse IgG2b clone G265–8; for CD14, Catalog No. 555396; BD Pharmingen; and for cytokeratin, Catalog No. 349205; BD Pharmingen) each diluted in 5% horse serum-1% BSA-0.05% Tween 20-PBS. For CD15, the antibody (Catalog No. 555400; BD Pharmingen) was diluted in 5% normal goat serum-1%BSA-0.05% Tween 20-PBS. Slides were incubated with primary antibody for 60 min at room temperature, and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. An isotype control antibody was run with each experiment (mouse IgG isotype control; Catalog No. I-2000; Vector Laboratories). For staining uPAR, PAI-1, CAP18, IL-8, CD14, and cytokeratin, reactions were developed with biotinylated horse anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% horse serum-1% BSA-0.05% Tween 20-PBS. For staining CD15, the reaction was developed with biotinylated goat anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% goat serum-1% BSA-0.05% Tween 20-PBS. Slides were incubated for 30 min at room temperature and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. For development, slides were incubated for 30 min at room temperature with Vector ABC reagent (Catalog No. PK-6102; Vector Laboratories) prepared in PBS 30 min before use and then washed thrice in PBS. Color was developed (3,3′-diaminobenzidine peroxidase substrate; Catalog No. SK-4100; or Vector VIP [peroxidase substrate, Catalog No. SK-4600; Vector Laboratories]) according to manufacturer's instruction. Color development was stopped by washing in distilled water, the slides were air dried, and the slides were viewed under microscope to assess the positive staining cells and determine cell type by comparing with Wright-Giemsa–stained slides (Hema 3 set, Catalog No. 122–911; Biochemical Science; Swedesboro, NJ).

Flow Cytometry

Five $\times$ 10^5 Sputolysin-treated (Calbiochem Corporation; San Diego, CA), PBS-washed sputum cells were added to each assay tube, incubated in 5% BSA-PBS for 10 min at room temperature to block nonspecific binding, washed once in PBS, fixed in 4% formaldehyde-PBS for 15 min at room temperature, and washed once with PBS. The cell samples were permeabilized in 0.5% saponin-PBS for 10 min at room temperature and washed once in 0.1% saponon-0.5% BSA-PBS. Monoclonal anti-human IL-8, uPAR, PAI-1, and CAP18 antibodies, and mouse IgG1, G2a, and G2b isotype control antibodies were labeled with fluorescein isothiocyanate (FITC) according to the zenon complex formation protocol (Zenon Alexa Fluor 488 mouse IgG1, IgG2a and IgG2b labeling kits, Catalog Nos. Z-25002, Z-25202, and Z-250102; Molecular Probes; Eugene, OR). Phycoerythrin (PE) labeled anti-human CD14, CD15, and cytokeratin were obtained from BD Pharmingen (Catalog Nos. 555398, 555402, and 347204, respectively). FITC- and PE-labeled anti-human antibodies (IL-8/FITC, uPAR/FITC, PAI-1/FITC, CAP18/FITC, CD15/PE, CD14/PE, and CK/PE) were added to respective tube with 1% mouse serum (Sigma Chemical), to reduce nonspecific staining. Mouse IgG1, IgG2a, IgG2b (Southern Biotech; Birmingham, AL), and IgM (Catalog No. 555584; BD Pharmingen) isotype-matched controls were included in each experiment. Tubes were incubated for 30 min at room temperature in the dark with slowing mixing followed by one wash with PBS and resuspension of the pellet in 300 μL of 1% formaldehyde. Samples were analyzed using a flow cytometer (FACSCalibur E3139; Becton Dickinson; Franklin Lakes, NJ).

Statistical Analysis

Data are expressed as mean ± SE unless otherwise indicated. Statistical comparisons were made using Student t test for unpaired two-group samples, analysis of variance for multiple group comparisons, and Pearson correlation coefficient. Two-tailed tests were performed, and a p value of < 0.05 was considered significant.

Results

Subject Groups

The CF patients (17 women and 11 men) were generally young adults (mean ± SD age, 23.7 ± 11.1 years) with well-preserved pulmonary function (mean ± SD FEV_1, 74.0 ± 17.4% predicted). The patients with COPD (24 women and 50 men) were, as expected, older (59.2 ± 9.9 years) with generally greater airflow obstruction (FEV_1, 54.6 ± 13.7% predicted). The patients with asthma (19 women and 15 men) were intermediate between these other groups in age (47.4 ± 13.9 years) and airflow obstruction (FEV_1, 65.9 ± 13.0% predicted). All but two patients with asthma were receiving regular bronchodilator medication (β_2-adrenergic aerosols and/or theophylline), while only seven patients were receiving regular inhaled corticosteroids.

CAP18 in Serum, BAL Fluid, and Sputum

To study the biology of CAP18 in CF, in a pilot study we first measured serum CAP18 levels by ELISA in patients with CF in stable condition (n = 15) and compared these to samples obtained from CF patients on admission to hospital for treatment of pulmonary exacerbation (n = 15) and 15 healthy adult control subjects. The ELISA showed linear parallelism in the range of 3 to 3,000 ng/mL with interassay coefficient of variation < 15%. Levels of CAP18 were similar in the three subject groups (stable CF, 966 ± 980 ng/mL; exacerbation CF, 1,137 ± 685 ng/mL; and control, 1,012 ± 648 ng/mL). We next compared CAP18 levels in BAL fluid of 23 patients with CF and 12 control subjects. CF patients had significantly higher levels of BAL fluid CAP18 than control subjects (189.7 ± 18.7 ng/mL vs

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120.7 ± 24.7 ng/mL, $p = 0.036$, by two-sided unpaired *t* test). As this suggested increased local production and/or reduced metabolism within the pulmonary compartment of inflamed CF airways, we proceeded to measure CAP18 levels in expectorated sputum of 30 patients with CF. Expectorated CF sputum levels (177.4 ± 14.7 ng/mL) were quite similar those in BAL, suggesting that sputum is an easily accessible and representative sample of airway secretions for measurement of CAP18 levels.

In order to obtain sputum samples from CF patients with mild disease severity and little or no sputum productivity (including nonexpectorators), we then proceeded to study CAP18 levels in induced-sputum samples using a validated standardized methodology. All nonexpectorators were able to produce adequate sputum for analysis after induction. CF patients were compared to patients with COPD, patients with asthma, and healthy control subjects. CF and COPD patients had comparable levels (79.6 ± 93 ng/mL vs 75.3 ± 38.9 ng/mL, respectively) and significantly elevated levels of sputum CAP18 when compared to control subjects (39.9 ± 24.2 ng/mL, $p < 0.009$ for either group vs control), while asthmatics had significantly reduced CAP18 levels (13.6 ± 9.8 ng/mL) compared to control subjects ($p < 0.0001$) or patients with CF or COPD ($p < 0.0001$) [Table 1].

Cytokines and Plasmin Activation Component Levels in Induced Sputum of CF, COPD, and Asthma Patients, and Control Subjects

To see if this pattern was similar to other innate defense factors, we compared levels of IL-8, IL-10, INF-γ, TNF-α, uPA, uPAR, and PAI-1 in these subjects. As also shown in Table 1, levels of sputum IL-8 were significantly higher in patients with CF than in patients with COPD or asthma ($p < 0.001$ for each) or control subjects ($p < 0.0001$); COPD and asthma patients also had higher IL-8 levels than control subjects ($p < 0.05$). IL-10 levels were also higher in the CF patients than patients with COPD or asthma, or control subjects ($p < 0.05$), without differences noted between the latter three groups. TNF-α levels showed a similar pattern as IL-10 with elevation in patients with CF compared to patients with COPD or asthma, or control subjects ($p < 0.001$). INF-γ levels were similar among all four groups.

uPA levels were similar among the four groups. In contrast, uPAR levels were elevated and similar in patients with CF and COPD compared to patients with asthma and control subjects ($p < 0.001$ for each); uPAR levels were also higher in asthma than controls ($p < 0.05$). PAI-1 levels were comparably elevated in patients with CF, COPD, and asthma, compared to control subjects ($p < 0.05$; Table 1).

Neutrophil Origin of Sputum CAP18, IL-8, and PAI-1

In order to examine the cellular sources of these sputum substances in patients with CF, we examined cells obtained from induced-sputum samples by immunohistochemical staining and independently by flow cytometry. Most sputum cells were either intact or more commonly degenerated polymorphonuclear neutrophils with variable mucus component and occasional other cell types such as monocytes, macrophages, eosinophils, or squamous epithelial cells; ciliated epithelial cells were not seen (Fig 1, *top*). CAP18 heavily stained the cytoplasmic granules of these neutrophils (Fig 1, *center* and *bottom*), suggesting that a major source of sputum CAP18 was infiltrating neutrophils. CAP18 is known to be localized to secondary or specific neutrophil granules. Using flow cytometry, CAP18 expression was markedly increased in airway cells that coexpressed CD15 (3-fucosyl-N-acetyl-lactosamine), which is present in the intracellular secondary granules of neutrophils,

Table 1—***Levels of Innate Immune Factors in Sputum of Patients With CF, COPD, and Asthma, and Healthy Control Subjects****

Factors, pg/mL	CF (n = 28)	COPD (n = 74)	Asthma (n = 34)	Healthy (n = 44)
CAP18	79,623 ± 18,597†	75,262 ± 4,583†	13,646 ± 1,678†	39,936 ± 3,644
IL-8	29,880 ± 4,226†	11,852 ± 1,880†	11,683 ± 3,707†	1,713 ± 317
IL-10	386 ± 87†	53 ± 14	36 ± 4	72 ± 14
INF-γ	794 ± 137	392 ± 65	627 ± 225	684 ± 117
TNF-α	74 ± 31†	10 ± 4	8 ± 2	12 ± 6
uPA	59 ± 26	76 ± 24	64 ± 30	18 ± 6
uPAR	803 ± 112†	583 ± 87†	399 ± 103†	85 ± 11
PAI-1	3,812 ± 469†	6,410 ± 1039†	4,802 ± 1127†	592 ± 97

*Data are presented as mean ± SEM. pg/mL

†Significant at ≥ 95%. See text for p values.

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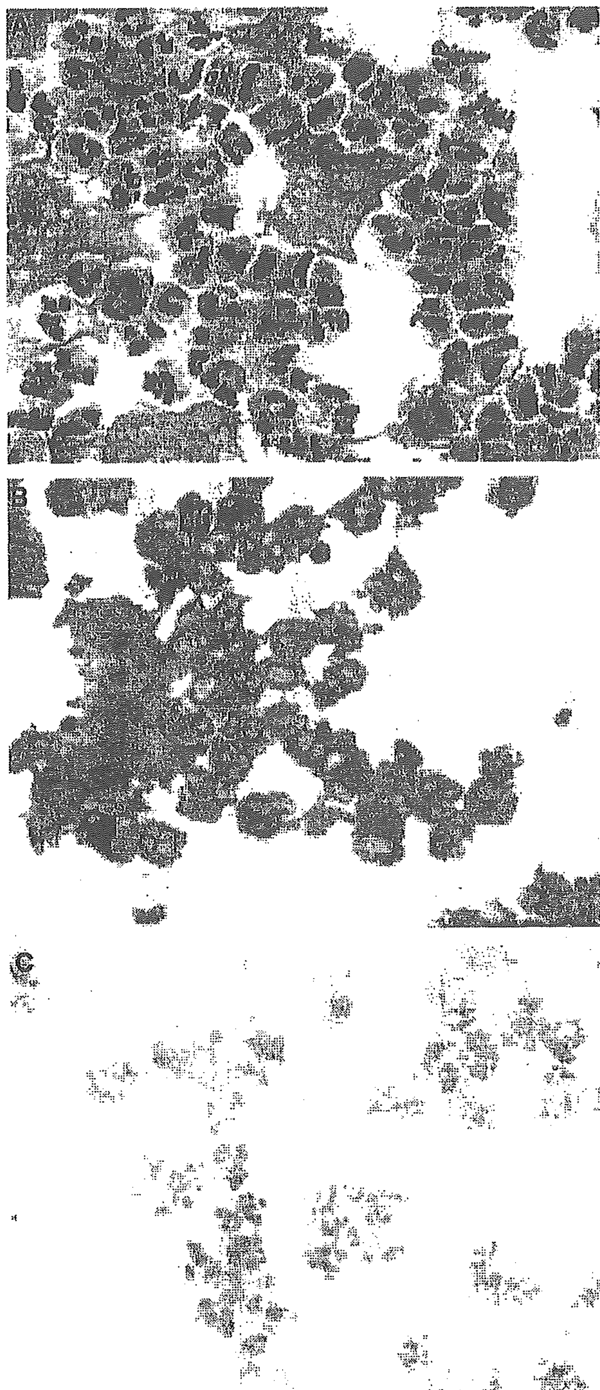

FIGURE 1. *Top*: Wright-Giemsa stain (original × 200) of induced-sputum sample from a CF patient showing neutrophil predominance. *Middle*: CF specimen stained for presence of CAP18 showing neutrophil granule localization. *Bottom*: isotype-negative control antibody result.

confirming the importance of neutrophil specific granules as a major source of airway CAP18 in CF (Fig 2).

Another neutrophil product important in CF pathobiology, IL-8, was also identified by flow cytometry in sputum cells that expressed CD15 (Fig 3). Similarly, PAI-1 was found, albeit at lower levels of expression, in CD15+ sputum cells (Fig 4).

Sputum levels of innate immune factors were correlated with each other in order to examine potential relationships in production or regulation. As shown in Table 2, levels of the three plasmin activation system components correlated with each other ($p < 0.0001$) but also with several cytokine responses (*eg*, uPAR and uPA with IL-8, $p < 0.0001$; uPAR and uPA with TNF-α, $p \leq 0.002$; and uPAR with IL-10, $p = 0.006$). In addition, uPAR was weakly correlated with CAP18 ($p = 0.01$). Among cytokines, IL-8 levels correlated with TNF-α and IL-10 ($p < 0.0001$), and IL-10 with IFN-γ ($p = 0.001$). CAP18 levels were correlated not only weakly with uPAR but also weakly with IL-8 ($p = 0.04$) and strongly with IL-10 ($p < 0.0001$).

To examine the potential role of these factors in disease activity or progression, innate immune factors were correlated with level of pulmonary function as determined by spirometric assessment of airflow obstruction (Table 2). Among all study subjects, FEV_1 percentage of predicted showed a strong negative correlation with sputum CAP18 and PAI-1 levels ($p \leq 0.0009$) and a weak negative correlation with uPAR levels ($p = 0.01$; Table 2). The overall negative correlation between pulmonary function and CAP18 was due primarily to results in patients with CF ($r = -0.40$, $p = 0.06$) and to a slightly lesser extent COPD ($r = -0.19$, $p = 0.11$). A similar negative correlation trend was seen between FEV_1 percentage of predicted and IL-8 in patients with CF ($r = -0.39$, $p = 0.056$) and patients with COPD ($r = -0.21$, $p = 0.08$). However, when innate factors were correlated with each other in patients with CF, CAP18 and IL-8 did not correlate with each other; instead, CAP18 levels correlated strongly with IL-10 ($r = 0.78$, $p = 0.001$) and IFN-γ ($r = 0.59$, $p = 0.002$), suggesting that elevated CAP18 levels in CF may represent a counterinflammatory response along with IL-10 and IFN-γ (IL-10 correlated strongly with IFN-γ, $r = 0.58$, $p = 0.001$). In contrast, levels of IL-8, a known proinflammatory factor in CF and COPD that we confirmed correlated negatively with pulmonary function (see above), correlated strongly with levels of uPAR ($r = 0.59$, $p = 0.002$), PAI-1 ($r = 0.5$, $p = 0.009$), and TNF-α ($r = 0.5$, $p = 0.007$). TNF-α correlated strongly with uPA ($r = 0.65$, $p = 0.0004$).

Discussion

Research[1–2,30–31] has focused on the role of deranged aspects of innate immunity in the pathobiol-

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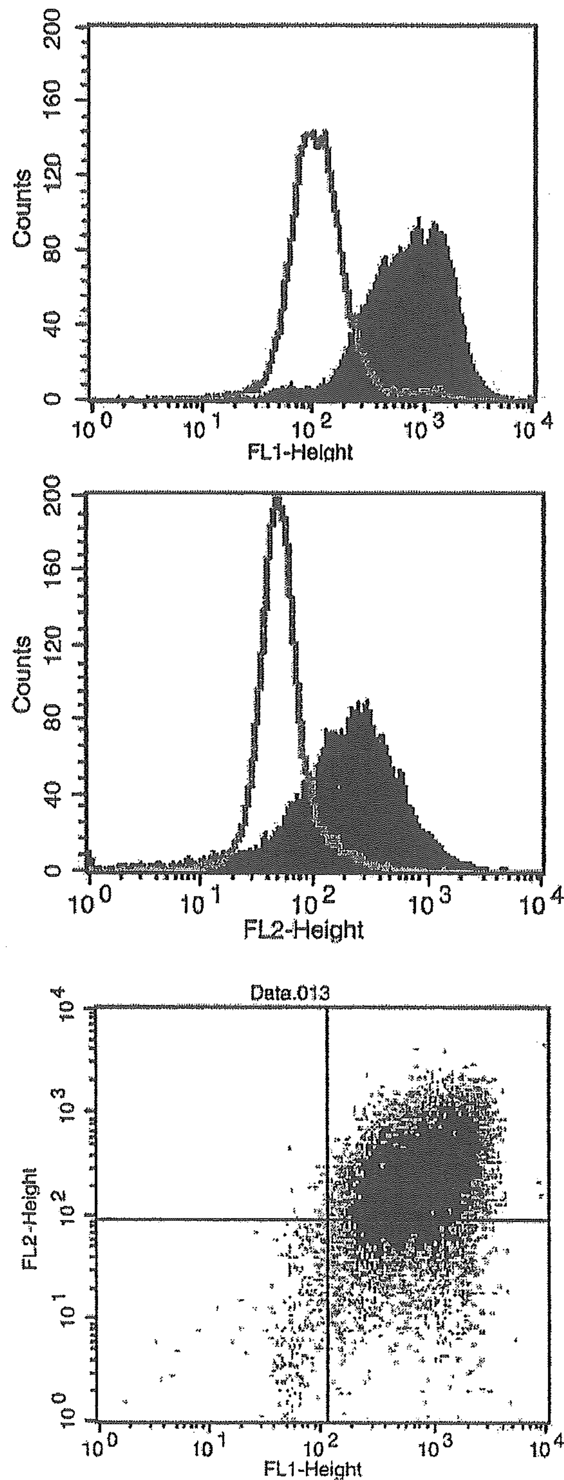

FIGURE 2. Flow cytometry for presence of CAP18 and CD15 in nonapoptotic (propidium iodide-negative) cells. *Top*: CF sample stained for CAP18 (purple) with isotype control antibody (open green) shown. *Middle*: same sample stained for CD15 with control. *Bottom*: two-color flow cytometry double-positive CAP18/CD15 cells are shown in upper right quadrant of gated cells (67.4% of total). Isotype control staining resulted in 1.0% double-positive cells (not shown). FL1 = fluorescence emissions wavelength 515 to 545 nm; FL2 = fluorescence emissions wavelength 564 to 606 nm.

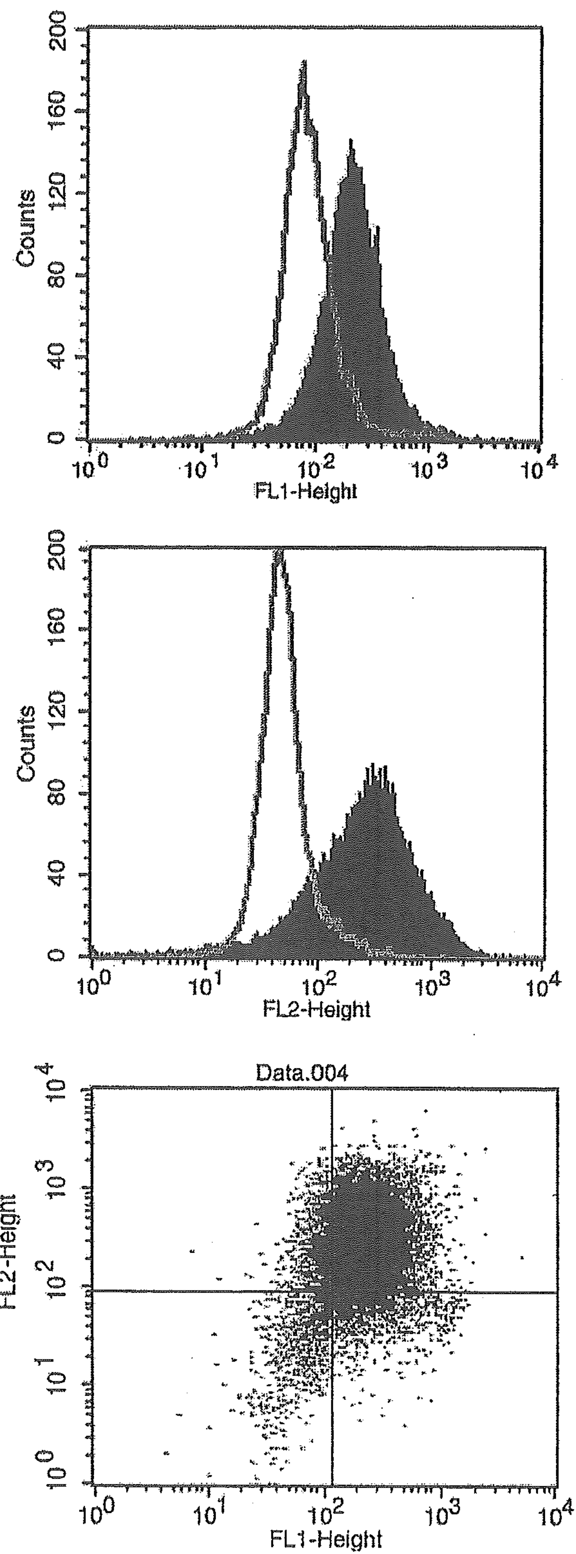

FIGURE 3. Flow cytometry for presence of IL-8 and CD15. *Top*: CF sample stained for IL-8 with isotype control shown as for Figure 2. *Middle*: same sample stained with CD15 and control. *Bottom*: double-positive IL-8/CD15 cells shown in upper right quadrant (64.4% of total). See Figure 2 legend for expansion of abbreviations.

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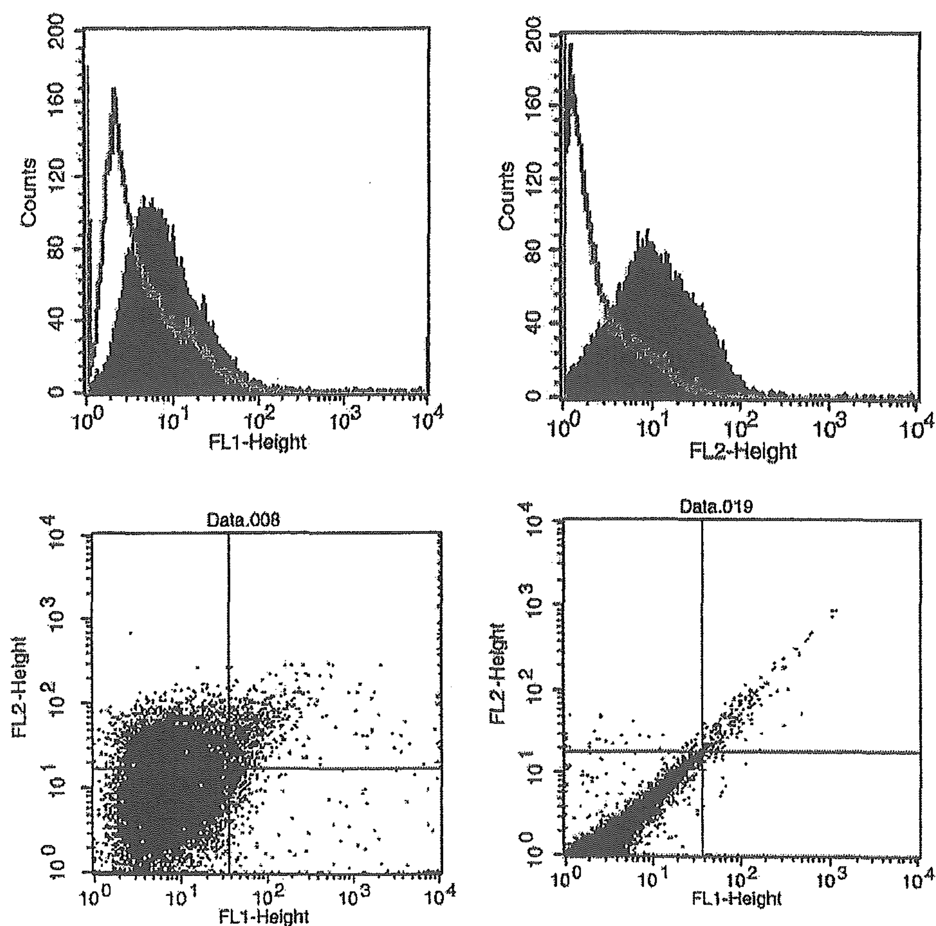

FIGURE 4. Flow cytometry for presence of PAI-1 and CD15. *Top left*: CF sample stained for PAI-1 with isotype control shown. *Top right*: same sample stained for CD15 and control. *Bottom left*: double-positive cells (upper right quadrant) representing 4.16% of total gated cells. *Bottom right*: double-positive cells in upper right quadrant (1% of total) using isotype control antibodies. See Figure 2 legend for expansion of abbreviations.

ogy of chronic inflammatory airways diseases such as CF, asthma, and COPD. In the present study, we were interested in aspects of the innate immune response in CF, in particular the following: (1) the role of the uPA system and the antimicrobial cathelicidin CAP18 that have not received much attention, and (2) how any abnormalities in CF compared to COPD and asthma as well as healthy persons. In order to study this local response noninvasively, we employed a standardized, validated sputum-induction protocol.[29]

The pulmonary plasminogen activator system involves multiple components including uPA, uPAR, and PAI-1. A saturable, specific binding of uPA to uPAR has been demonstrated in a number of cell types including monocytes, macrophages, mast cells, lymphocytes, fibroblasts, endothelial cells, and pulmonary airway epithelial cells.[32–33] Mature uPAR lacks the hydrophobic transmembrane and intracellular domains. Instead, a glycosylphosphatidylinositol moiety is added to the C-terminus providing the anchorage to the outer leaflet of the plasma membrane. Soluble uPAR has been isolated from normal and diseased plasma. By interacting with cell surface adapter molecules and extracellular proteins, uPAR acts beyond the traditional role of localizing and activating cell surface uPA and extends its effects to cellular adhesion and signal transduction.[32] The expression of uPAR is enhanced in various tumors and by a number of proinflammatory agents such as growth factors and cytokines.[32,34] Intraperitoneal injection of endotoxin to mice increased the expression of uPAR in many tissues.[35] Both membrane and soluble forms of uPAR were up-regulated in monocyte cultures by several bacterial surface proteins.[34] There is thus reason to suspect that chronic airways infection, such as seen in CF and COPD, may up-regulate uPAR, and indeed we found increased

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Table 2—*Significant Correlations of Induced Sputum Innate Immune Factors With Each Other and With Pulmonary Function Among All Study Subjects*

Factors	Correlates	r	p Value
CAP18	IL-10	0.53	0.0001
CAP18	IL-8	0.16	0.04
uPA	uPAR	0.61	0.0001
uPA	PAI-1	0.40	0.0001
uPA	IL-8	0.34	0.0001
uPA	TNF-α	0.24	0.001
uPAR	PAI-1	0.58	0.0001
uPAR	IL-8	0.47	0.0001
uPAR	TNF-α	0.22	0.002
uPAR	IL-10	0.20	0.006
uPAR	CAP18	0.19	0.01
PAI-1	IL-8	0.53	0.0001
IL-8	TNF-α	0.31	0.0001
IL-8	IL-10	0.30	0.0001
IL-10	IFN-γ	0.24	0.001
FEV_1	CAP18	− 0.31	0.0001
FEV_1	PAI-1	− 0.26	0.0009
FEV_1	uPAR	− 0.20	0.01

levels in sputum in CF and COPD, and to lesser degree in asthma, compared to normal. Interestingly, local uPA itself does not appear to be elevated in any of the disease groups, but its effects could be augmented by the up-regulated receptor levels seen.

PAI-1, a serine protease inhibitor belonging to the serpin family, is a single-chain 45- to 50-kd glycoprotein secreted by many cell types.[36–37] It binds to uPA to modulate the activity of uPA beyond plasmin activation to involve cell adhesion and tissue remodeling. PAI-1 may be inactivated by binding to uPA or forming complexes with uPA and uPAR that are internalized and digested in lysosomes. PAI-1 is secreted by many cell lines, and its expression can be regulated by hormones, growth factors, cytokines, and endotoxin in cell cultures.[37] Depending on the presence of specific regulatory agents, the expression of PAI-1 can either be enhanced or reduced. We found comparably increased levels of PAI-1 in all three chronic inflammatory airways diseases studied (Table 3). Marshall and Shute[38] reported quantitatively similar elevations of PAI-1 in CF sputum (mean, 5.7 ng/mL vs 1.0 ng/mL in control subjects, as compared with our mean of 3.8 ng/mL vs 0.6 ng/mL). During the inflammatory processes, autocrine and paracrine chemokine and cytokine secretion by neutrophils, monocytes, lymphocytes, and airway epithelial cells is enhanced. This may result in increased PAI-1 in sputum by different paths in these diseases.

Table 3—*Schematic Representation of Disease-Specific Patterns of Innate Immune Factors in Sputum as Compared to Healthy Control Subjects**

Factors	CF	COPD	Asthma
CAP18	↑	↑	↓
IL-8	↑↑	↑	↑
IL-10	↑	⟷	⟷
INF-γ	⟷	⟷	⟷
TNF-α	↑	⟷	⟷
uPA	⟷	⟷	⟷
uPAR	↑↑	↑↑	↑
PAI-1	↑	↑	↑

*↑ = elevated; ↓ = depressed; ↔ = equivalent.

Increases of the neutrophil chemotactic cytokine IL-8 level in airways of CF and COPD patients have been previously reported and related to pathogenesis.[4,28,39] IL-8 is produced by a variety of cells including neutrophils, monocytes, T-cells, and endothelial and airway epithelial cells.[39–42] IL-8 and uPAR both are chemotactic with monocyte and airway epithelial cell expression induced by bacterial products.[34,43] The interaction between leukocytes and endothelial cells is regulated by IL-8 via changes in integrin expression that also involve uPAR.[44–47] Therefore, integrins are potential mediators connecting the functions of IL-8 and uPAR (sputum levels of which we found to be highly correlated). Marshall et al[48] have also related IL-8 activity to PAI-1 by demonstrating that PAI-1 enhances IL-8 activity via inhibition of shedding IL-8/heparan sulfate/syndacan-1 complexes from endothelium. Indeed, IL-8 levels were highly correlated in our study not only with uPAR but also with PAI-1, suggesting a coordinate response in these diseases. The plasminogen activator system may play a prominent role in sustaining airway inflammation by increasing β_2-integrin–mediated leukocyte adhesion and also leukocyte adhesion to extracellular matrix vitronectin.[49–51]

With regard to cathelicidin CAP18, we found comparably elevated levels in CF and COPD patients and subnormal levels in asthmatics. These differences were due to local production, as systemic CAP18 levels in CF patients and control subjects were similar to each other and previously reported plasma levels (approximately 1 μg/mL).[52] CAP18 levels inversely correlated with pulmonary function, a relationship seen most strongly in CF patients but also in COPD. These changes have not been previously reported, although recently Chen et al[53] found increased CAP18 in BAL fluid from patients with CF; CAP18 levels correlated with neutrophilia and decreased lung function. Under normal conditions, CAP18 is secreted by airway epithelial cells and alveolar macrophages, but in conditions of neutrophilia this cell type would be expected to be the dominant source, as we found. The antimicrobial characteristics of CAP18 are related to its α-helical

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structure; positive ions, pH, and its own concentration affect the structure and change its antimicrobial activity.[54] It is unknown how changes in pH, ion concentration, sputum viscosity, and other factors in patients with chronic airways disease may affect the bioactivity of CAP18. We found that increases in CAP18 correlated with IL-10 and IFN-γ rather than IL-8 or TNF-α levels in CF, suggesting a possibly compensatory antiinflammatory rather than proinflammatory role. However, high concentrations of CAP18 may be cytotoxic to eukaryotic cells, and a deleterious effect certainly cannot be excluded.[54]

Sputum levels of PAI-1 also correlated negatively with pulmonary function. This suggests a relationship between PAI-1 and the degree of inflammation and tissue remodeling. PAI-1 binds uPA/uPAR, forming uPA/uPAR/PAI-1 complexes that are internalized across the cell membrane together with low-density lipoprotein receptor-related protein and degraded in the lysosome. uPAR is recycled to the cell membrane. PAI-1 thus not only controls the proteolytic activity of uPA but also modulates the number of uPAR on the cell surface.[15,55] The parallel increase of PAI-1 with uPAR suggests a finely tuned proteolytic balance is critical in the airway plasminogen activator system.[56] The dissolution and remodeling of extracellular matrix depends on a tightly controlled dynamic that maintains a proper balance between uPA/uPAR and PAI-1.[57] The increase in the PAI-1 may be homeostatic for the proproteolytic activity of increased uPAR.

In conclusion, comparison of CF to COPD and asthma as well as normal control subjects revealed interesting differences in innate immune factor levels. CAP18 is elevated in mild CF as well as COPD and inversely related to lung function, but correlation to IL-10 and IFN-γ suggests it may homeostatic rather than proinflammatory. The low levels of CAP18 in asthma are unexpected, perhaps reflecting the eosinophilic character of asthmatic inflammation or metabolic differences. Elevations of IL-8 in CF and COPD confirm prior studies, while levels of IL-10 have been variously reported to be low, normal, or elevated. TNF-α is only modestly increased in CF sputum, and IFN-γ levels are normal. CF and COPD, as noted above, share a common elevation of uPAR that is greater than that seen in asthma, while all three diseases show comparable increases in PAI-1. Neutrophils seem to be a prominent source of CAP18, IL-8, and PAI-1 in these diseases, These differing patterns allow may allow disease differentiation from a pathobiologic perspective and offer avenues for further research on pathogenetic pathways. For example, the striking differences observed in sputum CAP18 (elevated in CF and COPD, depressed in asthma) suggest further studies on the role of cathelicidins in specific forms of airway inflammation. The practical implications of such studies will likely emerge only after considerably more investigation.

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Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation

Wei Xiao, Yao-Pi Hsu, Akitoshi Ishizaka, Teruo Kirikae and Richard B. Moss
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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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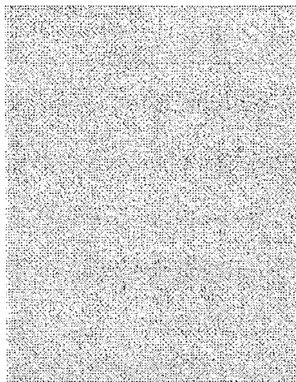

Results. Of 86 mycobacterial strains, *M. tuberculosis*, *M. avium* and *Mycobacterium chelonae* were identified in 48 (55.8%), 36 (41.9%) and 2 (2.3%) isolates, respectively. The obtained RFLP patterns of *M. tuberculosis* isolates from both the HIV-seropositive and -seronegative groups were variable,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

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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.

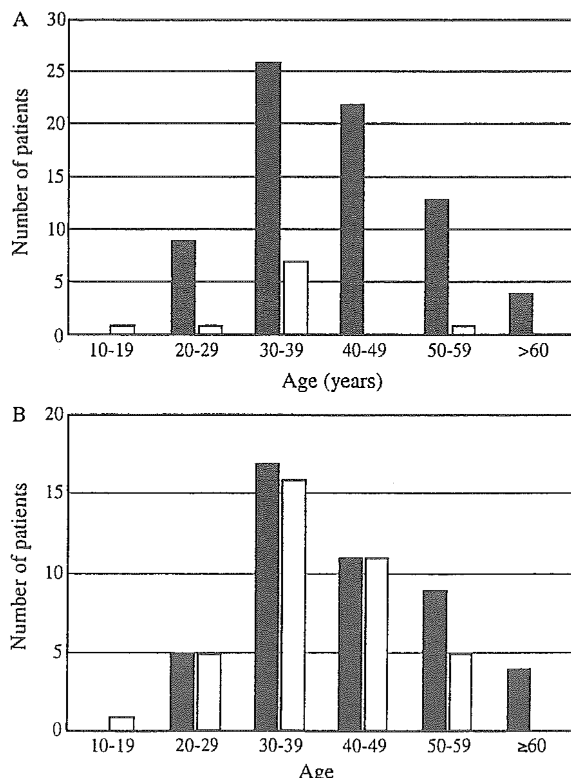


Figure 1 Distribution of 84 mycobacterial infections in HIV-seropositive patients. Panel A: age (years) and sex distribution. Filled bars, male; open bars, female. Panel B: age (years) and pathogenic agent distribution. Filled bars, tuberculosis patients; open bars, non-tuberculous mycobacterial-infected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

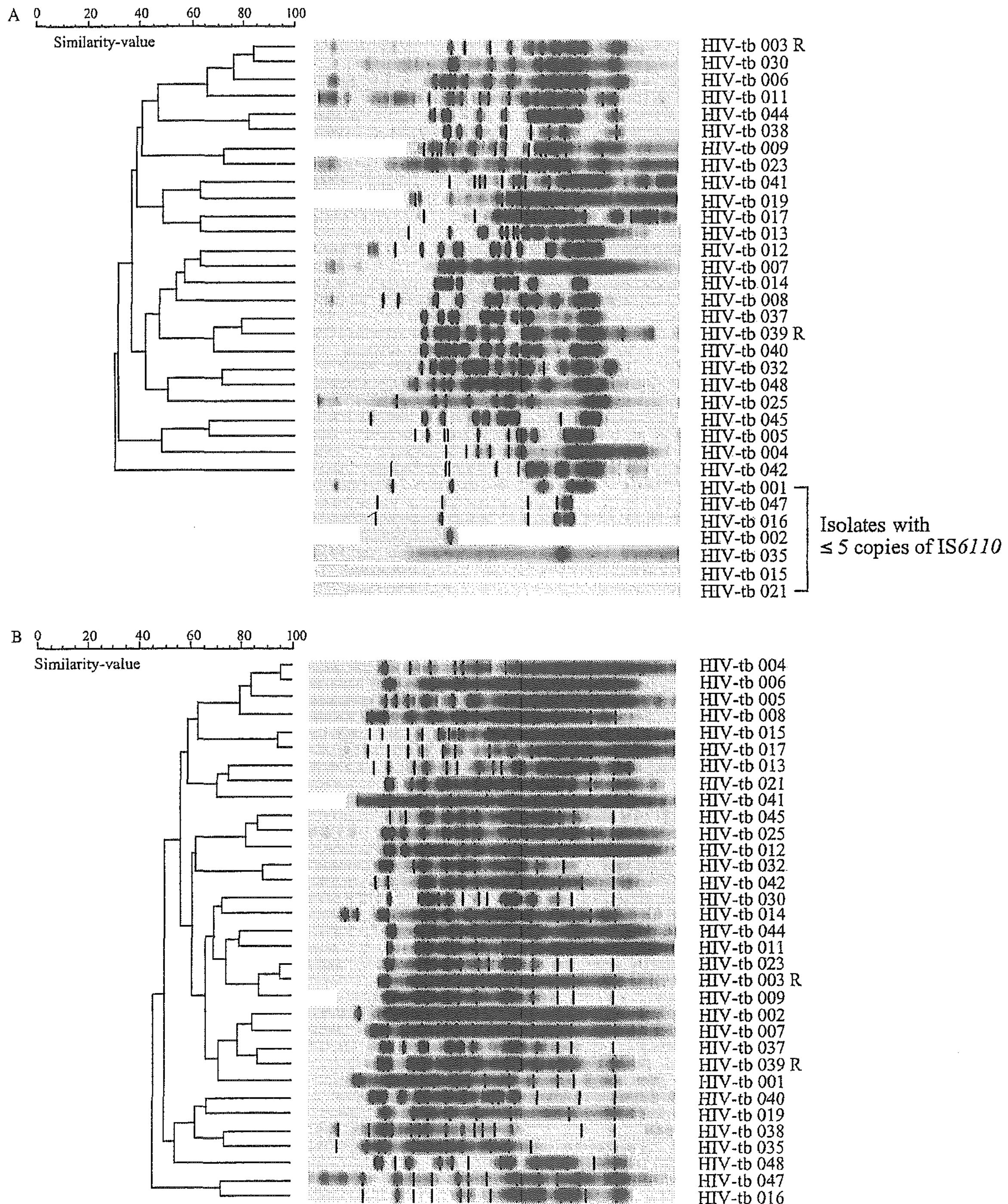


Figure 2 IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each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

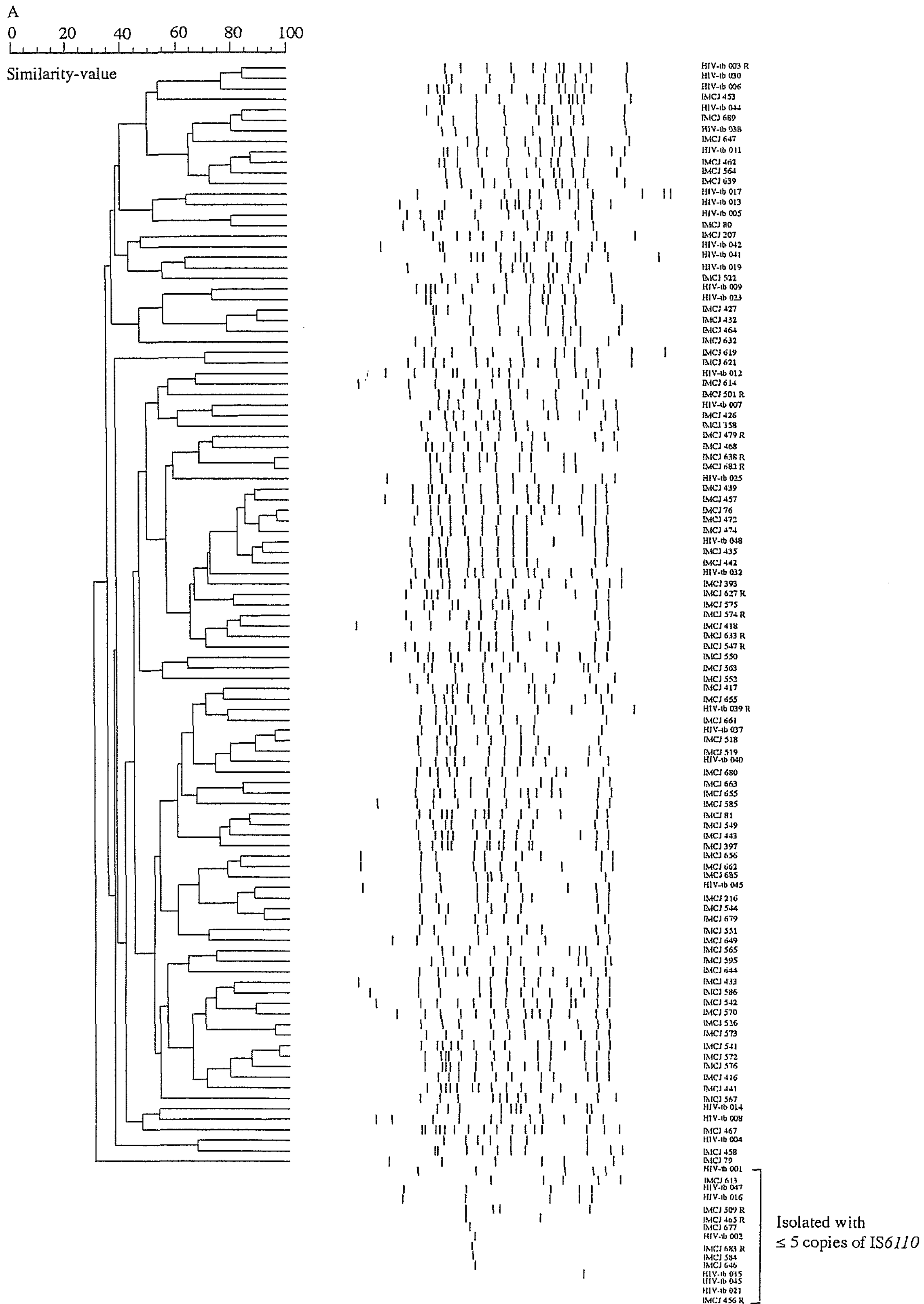
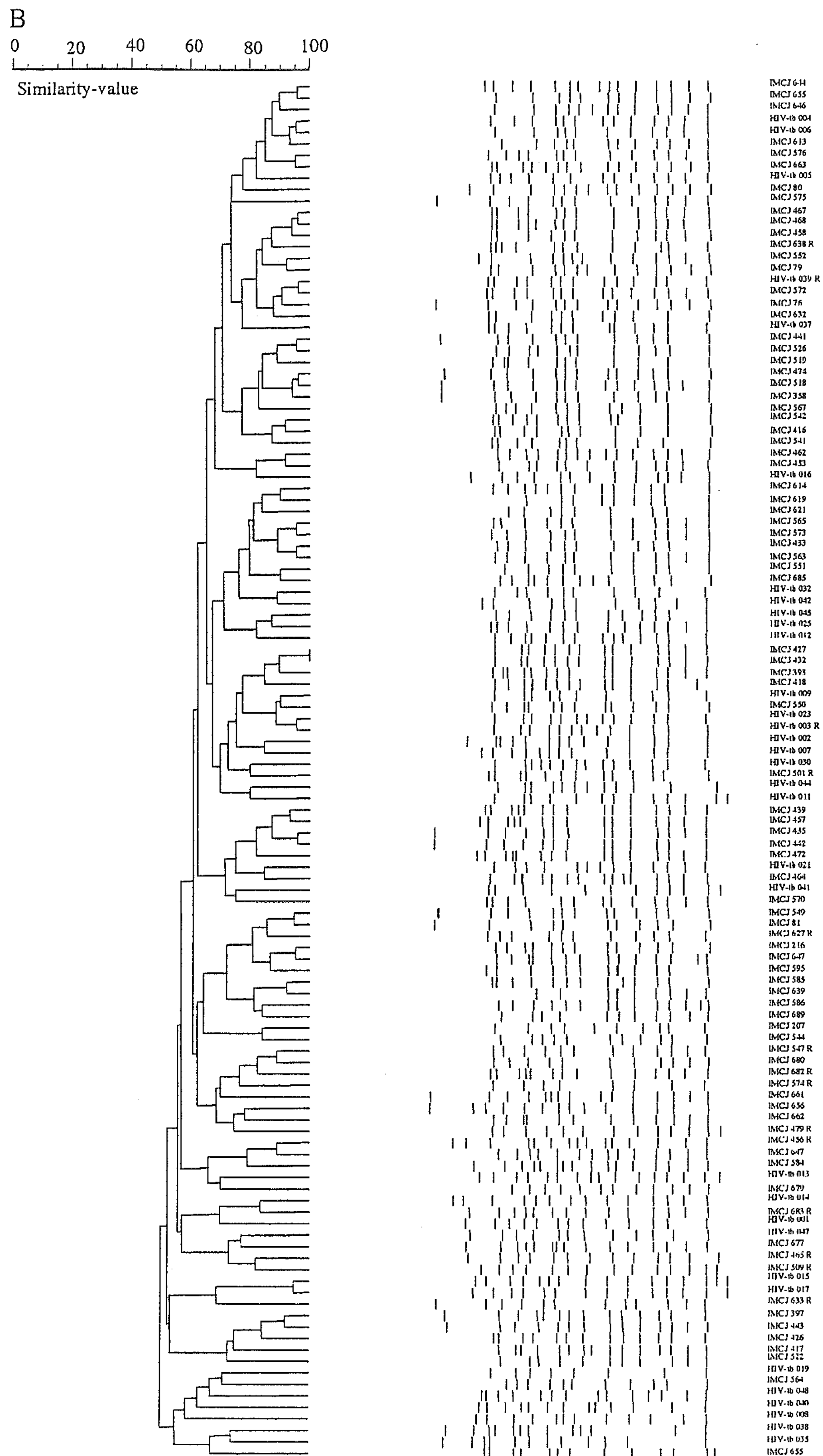


Figure 3. IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive and HIV-seronegative patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. 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.



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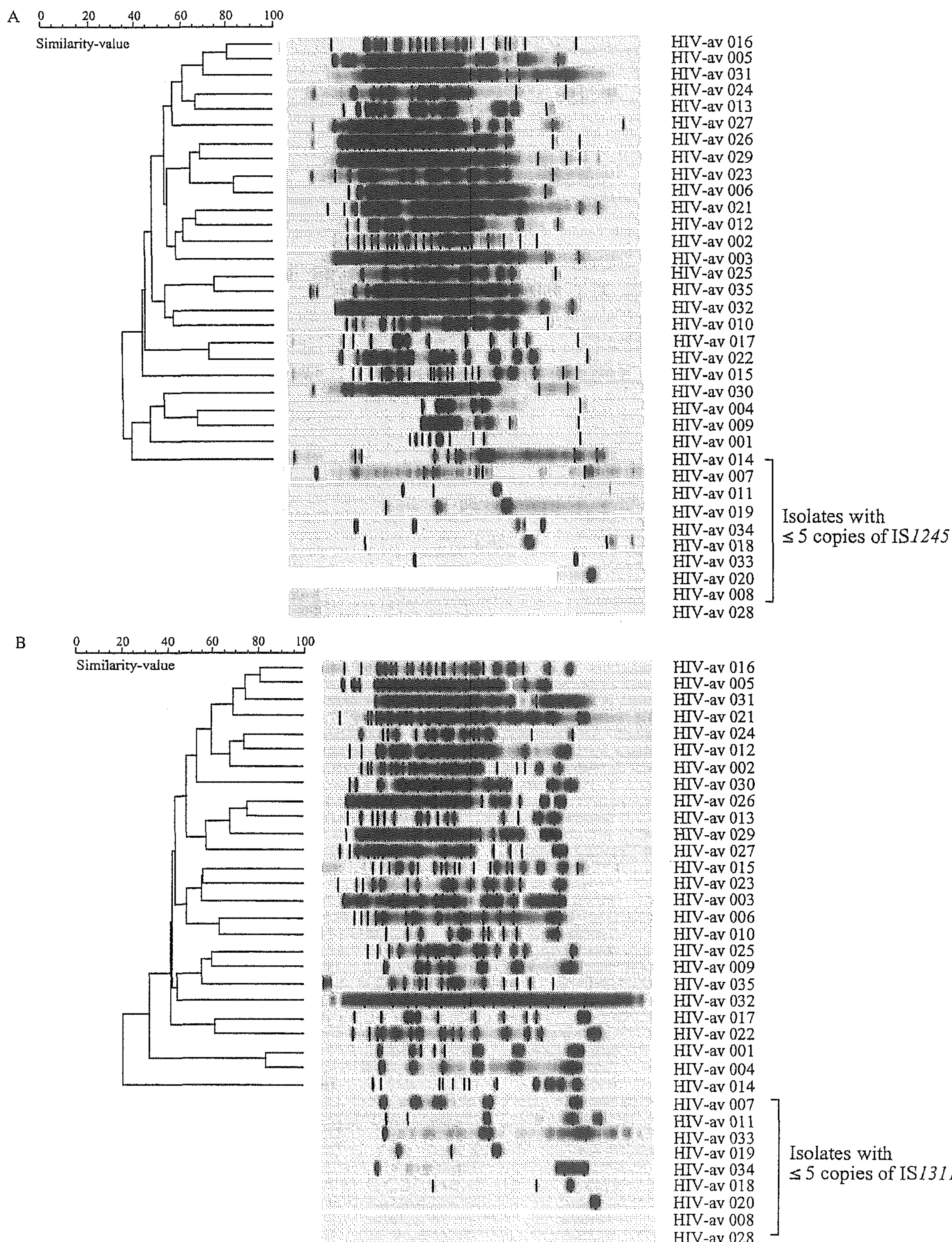

Figure 4 IS*1245*- and IS*1311*-probed DNA fingerprinting patterns of *M. avium* clinical isolates from HIV-seropositive patients in Japan and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*1245* (A) or IS*1311* (B) band is normalized so that the patterns for all strains are comparable. In both the IS*1245*- and IS*1311*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated. The isolates are named as follows: a prefix of 'HIV-av' indicates an HIV-seropositive patient-derived isolate.

The number of IS*6110* bands in the isolates ranged from 0 to 15 (Fig. 2A). Thirty-one different IS*6110* fingerprinting patterns were observed in the isolates. Seven isolates (21.2%) showed 0-5 IS*6110* bands, these isolates were insufficient in band number for cluster analysis. Identical patterns were not found among the isolates (Fig. 2A).

The number of $(CGG)_5$ bands of the copy isolates ranged from 8 to 16 (Fig. 2B). Thirty-three different $(CGG)_5$ fingerprinting patterns were observed in the isolates. Identical patterns were not found among the isolates (Fig. 2B). Three pairs of isolates (HIV-tb 004 and HIV-tb 006, HIV-tb 015 and HIV-tb 017 and HIV-tb 03 and HIV-tb 023) were closely related, with 90% or more similarity. However, the corresponding patients did not show any linkage such as hospital or date of sample isolation.

Comparison of RFLP patterns between HIV-seropositive and -seronegative TB patients

To assess whether the same kinds of mycobacteria prevail in HIV-seropositive and -seronegative patients, RFLP patterns of *M. tuberculosis* clinical isolates were investigated in both groups. In IS*6110*- or $(CGG)_5$-patterns from both groups, the patterns from both HIV-seropositive and -seronegative patients did not consist of apparent clusters and appeared to segregate randomly in the dendrograms (Fig. 3).

Profile of HIV-seropositive patients with *M. avium* infection

The number of HIV-seropositive patients with *M. avium* infection was 36 (Table 1). Mean age was 37.4 ± 9.9 years, ranging from 11 to 56 years. Most of the *M. avium*-infected patients (58.3%) suffered from disseminated infection, and the sputa of 88.9% patients were culture-positive but smear-negative upon preliminary mycobacterial examination. Almost all the *M. avium* isolates were resistant to all anti-TB drugs. Peripheral blood $CD4^+$ cell counts of 34 patients (unknown: 2) at the time of *M. avium* diagnosis ranged from 0 to $202/mm^3$, and the mean $CD4^+$ cell count was $38.6 \pm 60.4/mm^3$. In 26 of 34 patients (76.4%), the $CD4^+$ cell counts were less than $50/mm^3$.

RFLP analysis of *M. avium*

The RFLP patterns of 35 of 36 *M. avium* isolates were investigated (Fig. 4). The number of IS*1245*- and IS*1311*-bands ranged from 0 to 25 and from 0 to 23, respectively, and analysis showed 33 different patterns of each. Nine isolates (25.7%) showed 0-5 bands; these isolates were insufficient for cluster analysis because of few numbers of IS*1245* or IS*1311* bands. Among the isolates, identical patterns were not found. Cluster analysis revealed no clusters. These results indicate that no particular strain of *M. avium* prevailed among HIV-seropositive patients.

Discussion

We analysed mycobacterial isolates obtained from HIV-seropositive patients and found that *M. tuberculosis* and *M. avium* accounted for a large proportion of HIV-associated mycobacterial infection in Japan. Although *Mycobacterium kansasii* is also known to be associated with AIDS,[29,30] it was not isolated in this study. Two isolates of *M. chelonae* were obtained from stool specimens of patients.

It has been suggested that recurrent TB is responsible for most cases of HIV-associated TB, particularly in countries with high-level of transmission.[31] Kanazawa et al.[32] reported that the majority of HIV-positive Japanese patients with TB (83%) were more than 40 years of age and had recurrent TB. In the present study, the age of HIV-seropositive patients shifted to the 30s, suggesting that TB incidence among HIV-positive patients in Japan is transforming from recurrence in older persons to primary infection in younger persons.

With respect to drug resistance, 10.4% of the strains obtained from HIV-seropositive patients showed resistance to one or more anti-TB drugs. Abe et al.[33] reported that 10.3% of *M. tuberculosis* isolates from patients in Japan were resistant to one or more of the four first-line anti-TB drugs: isoniazid, rifampin, streptomycin and ethambutol. A 1996 report noted that the drug resistance rate in New York City was 33%.[34]

We found that both the IS*6110* and $(CGG)_5$ fingerprinting patterns of *M. tuberculosis* isolates from HIV-seropositive patients in Japan differed from those of a TB outbreak in New York City[12,18] and of isolates from the patients in Lima, Peru.[16] Comparing RFLP patterns of *M. tuberculosis* isolates from HIV-seropositive patients with those from HIV-seronegative patients, we found that the DNA fingerprints did not distinguish between these two TB patient groups. These data indicate that TB transmission in Japan occurs via HIV-seronegative TB patients rather than via HIV-seropositive TB patients. The epidemiological studies in Botswana[17] and Tanzania[19] showed no clustering any particular

pattern of DNA fingerprints. These findings are consistent with our present results.

Patients infected with *M. avium* suffer from chronic lung disease. In patients with HIV-associated *M. avium* infection, it is thought that pulmonary symptoms will develop when $CD4^+$ lymphocyte counts fall below $100/mm^3$. The median $CD4^+$ lymphocyte count at *M. avium* diagnosis was $10/mm^3$, and at that time the majority of patients showed disseminated *M. avium* infection. Almost all *M. avium*-infected patients in the present study were in advanced stages of AIDS. *M. avium* organisms can be isolated from environmental sources such as water or soil.[35-37] Because they are capable of causing infection in animals, e.g. birds and pigs, it has been postulated that the source of human infection is either the environment or from animals. Ichiyama et al.[38] searched sources of soil, water and dust in Japan and found *M. avium* isolates in 68.0% of dust samples tested. It is believed that the most frequent mode of *M. avium* infection in humans occurs by inhalation or by deglutition of the agent from environmental sources.[37,39,40] To prevent infection with this agent in HIV-seropositive patients, further studies are needed to identify original sources and to further elucidate infectious routes.

In conclusion, the number of HIV patients in Japan is increasing; according to the latest report,[7] the number is over 10 000. The number of TB patients in Japan remains higher than in other developed countries.[41] However, the number of HIV-infected patients with mycobacterial infection in Japan is limited. With respect to TB, no outbreak among HIV-seropositive patients was found. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

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