Large-restriction-fragment pattern comparison of *Mycobacterium avium* from 85 blood, stool, and respiratory specimens from 25 human immunodeficiency virus–infected San Francisco patients revealed 4 strains that infected multiple people (3 groups of 2 patients and 1 group of 3 patients). Most patients harbored a single *M. avium* strain, but 2 strains were recovered from 8 patients. The significance of recovering 2 strains is not clear, since the second strain was seldom recovered more than once. The strain recovered from blood was recovered from stool of 4 patients and respiratory secretions of 6 patients >4 weeks before detection of bacteremia, indicating that the intestinal and respiratory tracts are entry portals from which *M. avium* can disseminate. *M. avium* from 21 cities outside of California served as controls. Thus, a single *M. avium* strain can cause disseminated infection in multiple patients. This may represent infection from a common environmental source or person-to-person spread.

*Mycobacterium avium* is recognized as one of the most common opportunistic infections in patients with late-stage human immunodeficiency virus (HIV) infection [1]. Despite the prevalence of *M. avium* infections, little is known about the pathogenesis and epidemiology of the disease. The contribution of reactivation of latent infection (versus recent colonization) to the development of disseminated infections is unknown. The routes by which *M. avium* enters an HIV-infected person remain unconfirmed, but the respiratory and gastrointestinal tracts are implicated [2–4]. The source of *M. avium* responsible for most human infections has not been established, although potable water appears to be involved in at least some cases [5]. The pathogenic importance of *M. avium* cannot always be determined conclusively, since repeat cultures may be negative and colonization may not progress to disseminated infection [6–8].

The pathogenesis and epidemiology of disseminated *M. avium* infection can now be explored by use of restriction fragment length polymorphism (RFLP) analysis to identify and track specific *M. avium* strains. Digestion of *M. avium* DNA with an infrequent-cutting restriction endonuclease and separation of the resultant DNA fragments with pulsed-field gel electrophoresis (PFGE) produces strain-specific, large-restriction-fragment (LRF) patterns [9]. In addition, molecular techniques allow differentiation of *M. avium* from other species of the *M. avium* complex (MAC) [10, 11]. Early studies demonstrated that LRF patterns are strain-specific in that MAC isolates from different patients were distinct while isolates from the same patient had identical patterns [9]. The pathogenic significance of *M. avium* for these patients was demonstrated by repeatedly isolating organisms with the same LRF pattern. Others have compared MAC isolates with somewhat different results [5, 12–14]. Arbeit et al. [12] and Slutsky et al. [13] reported that some patients were infected by multiple strains of MAC when isolates with different LRF patterns were recovered from individual patients. Burki et al. [14] also noted different *M. avium* LRF patterns from individual patients but attributed the finding to culture contamination. They also noted the isolation of *M. avium* with identical LRF patterns from different patients. One strain was repeatedly recovered from respiratory secretions of HIV-infected patients. Von Reyn et al. [5] reported the recovery of *M. avium* with identical or similar PFGE patterns from multiple patients and from environmental sources, including potable water. Recovery of *M. avium* with the same LRF pattern from multiple patients suggests that infection was the result of recent colonization. Alternatively, the diversity of LRF pat-
tions may be limited such that epidemiologically unrelated strains give the same LRF pattern.

Guerrero et al. [15] described an alternative typing method after discovering the M. avium insertion element, IS1245. Discriminating patterns were generated when Southern blots of digested M. avium DNA were hybridized with IS1245 probes. Roiz et al. [16] used a similar typing method to study 75 M. avium isolates from 63 patients. Nineteen different patterns were noted when Southern blots were hybridized with DNA probes to the insertion sequence, IS1311. The M. avium isolates from 1 patient had different patterns, while isolates from multiple other patients gave identical patterns. The authors suggest that IS1311 has the discriminating power of PFGE, but a limited number of unrelated M. avium isolates has been examined.

Our study compares the LRF patterns of M. avium isolates from different geographic locations, from different patients, from different types of specimens from the same patient, and from different colonies of the same specimen in an attempt to determine the genetic diversity of M. avium infecting patients with AIDS, to determine the frequency and significance of recovering genetically different M. avium from the same patient, to determine if colonization of the gastrointestinal or respiratory tract by a particular M. avium strain leads to bacteremia by that strain, and to determine the frequency and significance of recovering genetically identical M. avium from different patients.

Materials and Methods

Study population and control isolates. The study population comprised HIV-infected persons who had M. avium recovered from at least 2 different clinical specimens from different sites. These patients were selected from a larger group recruited for an epidemiologic study of MAC infection at San Francisco General Hospital [6, 17, 18]. Candidates for the larger study were patients who were HIV-seropositive, with CD4 cell counts ≤50/μL, and who had a blood specimen submitted for mycobacterial culture. A total of 325 patients were enrolled into the larger study between 1 November 1990 and 14 May 1992. Stool and respiratory tract specimens were obtained for mycobacterial culture at the time of enrollment. Blood, stool, and respiratory specimens were subsequently cultured for mycobacteria every 3 months and as clinically indicated. M. avium was recovered from at least 1 blood specimen from 111 patients by 14 May 1992. Seventy-five of these patients (68%) also had M. avium isolated from stool or respiratory tract specimens (or both). A convenient sample of 25 patients was selected from this group. We compared M. avium isolates from blood, stool, and respiratory secretions of these 25 patients as indicated in figure 1.

MAC isolates from HIV-infected persons who sought medical care in one of 21 different cities outside of California were used as geographically unrelated controls. These isolates were the first isolates received from each city. The isolates from Shreveport, Louisiana, and Wilmington, Maine, were Mycobacterium intracellulare; the other 19 isolates were M. avium.

Mycobacterial culture methods. Mycobacteria were isolated and identified by standard techniques. Respiratory tract specimens were decontaminated with sodium hydroxide—sodium citrate and the mucolytic agent N-acetyl-L-cysteine (NACL-NaOH). Stool specimens were decontaminated with either NACL-NaOH or 5% oxalic acid; both methods allow comparable recovery of mycobacteria from stool [6]. The respiratory tract and stool specimens were cultured on four types of media: Lowenstein-Jensen, selective Lowenstein-Jensen, Middlebrook 7H10, and selective 7H11 agar plates. Blood specimens, including bone marrow aspirates, were cultured for mycobacteria by two methods. Before January 1992, blood specimens were lysed and cultured on the same four types of media as the respiratory specimens. Beginning in January 1992, blood specimens were inoculated onto Middlebrook 7H11 agar plates and into BACTEC radiometric 13A broth (30 mL). All cultures were incubated for 8 weeks to detect growth of mycobacteria. Isolates were identified by standard biochemical methods, by DNA probes (AccuProbe [Gen-Probe, San Diego] or SNAP [Syngene, San Diego]), and/or by polymerase chain reaction—RFLP analysis [10].

Collection of isolates. M. avium was collected from blood, stool, or respiratory specimen cultures or subcultures by sweeping the media surface with a cotton applicator (sweep culture) and by picking 5 individual colonies. LRF patterns from all sweep cultures and at least 2 individual colony cultures (selected from a blood culture) were studied for each patient. Additional colony cultures were studied as indicated subsequently.

LRF patterns. Cells from the sweep cultures and individual colonies were grown in 20 mL of 7H9 broth, supplemented with 10% OAD (0.6% saponified oleic acid, 5% bovine albumin fraction V, and 2% dextrose) and 0.4% Tween 80. A portion of the cell pellet from these cultures was resuspended in 1% low-melting-point agarose, and high-molecular-weight DNA was isolated as described previously [9] with the following modifications. The steps involving antibiotics and phenylmethylsulfonyl fluoride were deleted. After protease K digestion, the pluses were washed six times with 10 mM TRIS and 1 mM EDTA. The embedded DNA was digested overnight with 20 U of XbaI, Asnl, or DraI in the buffer provided by the manufacturer. Plugs containing digested DNA were loaded into a 1% agarose gel in 0.025 M TRIS, 0.5 mM EDTA, and 0.025 M boric acid. PFGE was carried out (CHEF-DR II system [Bio-Rad, Richmond, CA] or CHEF-Mapper) at 14°C for 20–24 h at 200 V; ramped pulse times varied according to the enzyme used. Molecular weight standards were included with each electrophoresis: polymerized bacteriophage λ DNA (Bio-Rad) or Saccharomyces cerevisiae (yeast) chromosomal DNA (Bio-Rad). Gels were stained with ethidium bromide and photographed with UV illumination.

LRF pattern comparisons and strain identification. We compared the LRF patterns prepared with the same enzyme by visual inspection. LRF patterns were classified as identical if there were no apparent differences in any of their LRF bands, similar if the patterns differed by the presence or absence of no more than 2 LRF bands, and distinctly different if the patterns differed by the presence or absence of ≥3 LRF bands. We assumed that isolates with identical or similar LRF patterns were the same strain and isolates with distinctly different LRF patterns were unique strains. Others have made similar assumptions [12, 19]. Isolates with identical or similar LRF patterns were assigned the same strain designation regardless of their origin.

When LRF patterns of isolates from different patients appeared identical or similar following Asnl and XbaI digestion, the relationship was confirmed or refuted by a third enzymatic digestion with DraI. If the LRF pattern of a blood sweep differed from either of its 2 individual colonies, LRF patterns of additional colonies (up
to 5) were studied to determine if the discrepancy was due to the presence of DNA from multiple strains. If the LRF patterns of a patient’s blood, respiratory, or stool isolate differed, we analyzed additional LRF patterns from individual colonies to see if the discrepancy was due to the presence of multiple strains. If the intensity of multiple bands of an LRF pattern differed markedly, LRF patterns from individual colonies were compared to see if the discrepancy was due to the presence of multiple strains.

**Results**

LRF patterns were generated for 219 MAC isolates. These isolates were from 106 clinical specimens obtained from 46 persons, 25 of whom resided in the San Francisco area. MAC from the remaining patients were recovered from persons in 21 different cities outside of California. In total, the isolates gave 57 AsnI LRF patterns and 54 XbaI LRF patterns. All strains with distinctly different AsnI LRF patterns had distinctly different XbaI patterns. One pair of strains with similar AsnI LRF patterns had distinctly different XbaI and DraI LRF patterns. On the basis of the appearance of their LRF patterns and the criteria described above, the 219 isolates represented 48 different strains. The strains recovered from San Francisco patients are listed in figure 1. The collection date of the first M. avium-positive blood culture is listed and the interval between blood cultures is shown. The LRF patterns of the isolates are shown as dots connected by lines.
Comparison of M. avium LRF patterns of individual colonies of specimens from San Francisco. We compared the LRF patterns of individual colonies from 27 blood, 8 respiratory, and 6 stool specimens. For most of these clinical specimens (25/27 blood specimens, 4/8 respiratory specimens, and 6/6 stool specimens), the LRF patterns of the individual M. avium colonies were identical to the others from the specimen and to the LRF pattern obtained from a sweep of the original culture. For example, figure 2A shows the identical Asn I LRF pattern of a sweep culture (lane 2) and individual colony cultures (lanes 3–5) from a blood specimen from patient 15.

Subtle variations occurred in the LRF patterns of individual colonies from 1 clinical specimen, a respiratory specimen from patient 15 (figure 2A, lanes 6 and 7). These variations consisted of the presence or absence of two large restriction fragments following Asn I digestion. Subtle variations were also seen following XhoI and DraI digestion. This degree of variation did not warrant classification of the colonies as different strains as defined earlier.

Three respiratory specimens (1 from patient 20 and 2 from patient 24) and 2 blood specimens (1 from patient 22 and 1 from a control patient) contained 2 different M. avium strains with distinctly different LRF patterns. Dashed lines in figure 1 connect different strains that were recovered from the same clinical specimen. A mixture of strains was indicated by marked variation in band intensity within the LRF pattern and by differences in the LRF patterns of individual colonies picked from the culture. Examples are shown in figure 2B, in which different Asn I LRF patterns for individual colonies (lanes 3 and 4) are compared with the sweep culture (lane 2) of a respiratory specimen from patient 24. The sweep’s pattern is a combination of the patterns derived from the individual colonies. Comparison of sweep patterns with individual colony patterns revealed differences for each of the 5 mixed cultures.

In prior unpublished studies, such differences were apparent when the sweep culture contained as many as three genetic entities, with any one representing as little as 10% of the microorganisms.

Comparison of individual colonies from at least 1 specimen from each patient and comparison of multiple specimens from the same patient demonstrated that most of the clinical specimens studied (101/106) contained a single M. avium strain.

Comparison of M. avium recovered from patients in different cities. Twenty-one distinctly different Asn I LRF patterns (figure 3) were derived from MAC isolates recovered in 21 different US cities from blood of HIV-infected patients. The XhoI and DraI LRF patterns were also different for these isolates. Two of the isolates were M. intracellulare (figure 3A, lanes 2 and 4) and 19 were M. avium. The digestion patterns from these control isolates differed from each other by >5 LRF bands regardless of which enzyme (Asn I, XhoI, or DraI) was used to digest the DNA. While the Asn I LRF pattern of the Memphis isolate (figure 3B, lane 6) resembles the pattern from the Dallas isolate (figure 3B, lane 9), these patterns differed by >11 bands and there was no resemblance between their XhoI or DraI LRF patterns. For 1 specimen from Charleston, South Carolina, the presence of both heavy and light LRF bands suggested that the specimen contained 2 strains. Asn I digestion of DNA from individual colonies revealed two distinct LRF patterns (figure 3B, lanes 4 and 5) and confirmed that this specimen contained 2 different strains of M. avium.

One of the Asn I LRF patterns (figure 3B, lane 5) was identical to the pattern from the Memphis isolate (figure 3B, lane 6). Digestion with XhoI and DraI confirmed the similarity. Cross-contamination was suspected, since processing of these 2 samples was initiated in our laboratory on the same day. We could not confirm our suspicion of contamination because only 1 slant was available from each patient.

Comparison of M. avium from different body sites of the same patient. For the most part, each patient from the San Francisco
Francisco area harbored a single *M. avium* strain, and isolates from different body sites had identical or similar LRF patterns. Two different strains were recovered from 8 of the 25 patients, and none of the patients harbored >2 strains.

*M. avium* was recovered from at least 1 blood, 1 respiratory, and 1 stool specimen in 16 patients. In 9 of these patients, all isolates from the respiratory tract, stool, and blood had identical LRF patterns. One patient (patient 20) produced respiratory isolates whose LRF patterns differed distinctly from those derived from his blood, stool, and other respiratory isolates. Three patients (patients 11, 19, and 21) yielded blood isolates that had LRF patterns similar, but not identical, to those of respiratory or stool isolates. Some, but not all, of the isolates with subtle *Asn*I LRF pattern differences had subtle differences following *Xba*I or *Dra*I digestion. Three patients (patients 2, 3, and 15) had isolates from blood and respiratory specimens with the same pattern, but isolates from their stool specimens were distinctly different.

For 8 patients, *M. avium* was recovered from respiratory and blood specimens but not from any stool specimens. Five of these patients had blood and respiratory isolates with identical LRF patterns. One (patient 24) had respiratory isolates with distinctly different LRF patterns, one of which was identical to the LRF pattern of blood isolates. Two isolates with distinctly different LRF patterns (as previously described) were obtained from a single blood specimen from 1 patient (patient 22). One of the patterns was identical to the pattern obtained from his respiratory isolates. The remaining patient in this group provided 1 *M. avium* blood isolate and a single respiratory isolate with distinctly different LRF patterns (patient 13).

For 1 patient (patient 17), *M. avium* was recovered from stool and blood specimens but not respiratory tract specimens. *M. avium* isolates from 3 of this patient’s stool specimens had 2 distinctly different LRF patterns. The isolates from 2 stool specimens were identical to those derived from blood, but the LRF pattern from a third stool specimen was distinctly different from blood or other stool isolates.

**Comparison of *M. avium* recovered in San Francisco from different patients.** Isolates from 9 of 25 San Francisco area patients had LRF patterns that were identical or similar to the patterns of at least 1 other patient’s isolate. There were three instances of 2 patients (patients 1 and 2, 6 and 7, 8 and 9) whose isolates had identical LRF patterns and one cluster of 3 patients (patients 3, 4, and 5) whose isolates had similar LRF patterns. Examination of 2–6 isolates collected on different days from different sites of each patient confirmed this finding. In figure 4A, the identical *Asn*I LRF pattern of multiple isolates from patients 1 and 2 can be seen in lanes 2–5 and those from patients 6 and 7 in lanes 6–9; those from patients 8 and 9 can be seen in figure 4B (lanes 2–5). Similarity in *Asn*I LRF patterns of isolates from patients 3, 4, and 5 can be seen in figure 4B (lanes 7–12). Differences in the isolates from the latter 3 patients consisted of the presence of 1 additional *Asn*I LRF band, a shift in size of 1 *Xba*I LRF band, and the shift in size of 2 *Dra*I LRF bands.

**Discussion**

We used LRF patterns as epidemiologic markers to identify and track specific *M. avium* strains infecting patients with AIDS. Initial studies demonstrated that LRF patterns of *M. avium* isolates from different patients were distinctly different, but subsequent reports indicated that different patients, with no obvious epidemiologic link, had identical restriction patterns [9, 13, 14]. The patients with identical patterns were from the same geographic area, and their cultures were processed in the same facilities. Our comparison of blood isolates from different cities demonstrates the diversity of *M. avium* LRF patterns.
These isolates, which were assumed to represent unique strains, had distinctly different LRF patterns with one exception. The exception appears to have resulted from contamination in our laboratory. Contamination involving conventional and radiometric mycobacterial culture methods has been reported [14, 15, 20–26] and these reports probably underestimate the actual frequency of the problem.

Disseminated *M. avium* in patients with AIDS is frequently associated with gastrointestinal and respiratory tract invasion or colonization [2–4]. Furthermore, *M. avium* is frequently recovered from stool or respiratory secretions before development of bacteremia, and its recovery from either of these sites is associated with an increased risk of *M. avium* bacteremia [6]. LRF pattern analysis demonstrated that the *M. avium* strain recovered from an individual’s respiratory tract and gastrointestinal tract is not always the same as the one recovered from blood. However, for 40% of our patients, the *M. avium* strain recovered from blood was also recovered from respiratory secretions or stool >4 weeks prior to development of bacteremia. More frequent culture screening may have demonstrated that colonization preceded bacteremia in other patients as well. Thus, our findings provide strong evidence that *M. avium* does disseminate from the gastrointestinal and respiratory tracts.

Isolates from 36% of the San Francisco area patients had LRF patterns that were identical or similar to the patterns of at least 1 other patient’s isolate. This is in contrast to the marked differences between unrelated controls. Clusters infected by the same strain included three clusters of 2 patients and a cluster of 3 patients. The pathogenic significance of the shared strains was confirmed by repeatedly demonstrating the same pattern from multiple isolates, from multiple specimens and sites, for each patient. The finding that multiple patients were infected by the same strain suggests that active disease in some patients is the result of recent rather than latent infection. This raises the question of person-to-person transmission of *M. avium*, which heretofore has not been shown to occur. The clustering of patients with the same *M. avium* strain could represent a recent common environmental exposure. von Reyn et al. [5] reported the recovery of *M. avium* with identical or similar PFGE patterns from multiple patients and from environmental sources. This is less plausible in our patients, because attempts to recover similar *M. avium* strains from the same patient at San Francisco General Hospital, but environmental cultures of San Francisco General Hospital, including the hospital water system, failed to yield *M. avium*. More frequent culture screening may have demonstrated that colonization preceded bacteremia in other patients as well. Thus, our findings provide strong evidence that *M. avium* does disseminate from the gastrointestinal and respiratory tracts.

Comparison of LRF patterns of multiple isolates from individual patients revealed that different *M. avium* strains were occasionally recovered from the same patient. Although most patients harbored a single strain, 2 different *M. avium* strains were occasionally recovered from the same patient. However, both strains were rarely recovered from >1 specimen. Plausible explanations for recovering 2 different strains from the same patient include infection or colonization with different *M. avium* strains and erroneous culture results.
Polyclonal infections due to MAC have been reported [12, 13]. Slutsky et al. [13] recovered 2 MAC strains from 4 of 12 patients with multiple MAC-positive cultures >1 week apart. This is similar to 32% in the present study. In both studies, isolation of both strains from >1 specimen was possible for only 8% of patients. Arbeit et al. [12] used PFGE to examine multiple colonies of *M. avium* recovered from blood, sputum, and/or stool of 14 HIV-infected patients. Two strains were detected in 2 (15%) of 13 blood cultures and 1 (17%) of 6 stool cultures. In contrast, examination of individual colonies from 6 stool specimens, 26 blood specimens, and 8 respiratory specimens from San Francisco demonstrated that none of the stool samples, 4% of the blood samples, and 25% of the respiratory samples contained multiple *M. avium* strains. The differences in the study results may be due to subculture technique or selection bias in that our patients were early in the course of their *M. avium* infection.

The clinical significance of recovering multiple MAC strains from an individual has not been determined. Our results, and those of Slutsky and colleagues, suggest that the second strain is present transiently in the majority (75%) of these patients. When recovered more than once, the second strain is typically cultured from nonsterile sites, as was noted for 2 of our patients. Transient colonization of the respiratory and gastrointestinal tract has been reported and may clear without antimycobacterial therapy [6, 7]. In addition, blood from some patients is transiently positive by culture for *M. avium*. Of 221 patients whose blood cultures grew *M. avium* in a local laboratory, 34 (15%) had negative cultures when subsequently screened to participate in an *M. avium* treatment trial [8]. None of these patients had received therapy for *M. avium*. Another possibility is that the strains recovered only once are false-positive culture results. Recognition of erroneous mycobacterial culture results has increased since the advent of RFLP analysis and strain-specific identification of mycobacteria [14, 15, 27–30]. Burki et al. [14] examined LRF patterns of multiple *M. avium* isolates from 11 patients. From 4 patients, isolates with two different patterns were identified; 1 isolate from each patient was a contaminant.

Small variations in the LRF patterns of *M. avium* isolates from the same patient were encountered. For 5 patients (of 25 providing multiple specimens), at least 1 isolate gave an LRF pattern that differed by the presence or absence of 1 or 2 bands. These subtle differences were seen between colonies of the same specimen and between colonies from different specimens of the same patient. These LRF patterns were classified as similar, and the isolates were considered closely related for five reasons: minimal changes in the organism’s DNA sequence can produce noticeable differences in its LRF pattern [19]; epidemiologic investigations of other bacteria, including mycobacteria, have demonstrated small variation in LRF patterns of some related isolates [12, 31]; small differences in the RFLP patterns of serially passaged bacteria, including mycobacteria, have been observed [32, 33]; such differences may occur in the LRF patterns produced with one or more enzymes but not with others [30, 32]; and the differences in the LRF patterns of control strains from different geographic areas were obvious, not subtle.

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