Polyclonal and Compartmentalized Infection by *Mycobacterium tuberculosis* in Patients with Both Respiratory and Extrapulmonary Involvement

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Infection by *Mycobacterium tuberculosis* (MTB) is assumed to be caused by a single strain, and several MTB strains within the same patient are rarely considered. The present study analyzes the phenomenon of mixed infections by MTB in a group of 50 patients with both respiratory and extrarespiratory tuberculosis. First, the proportion of patients with infection by >1 strain was defined, and second, the clonal composition of the MTB populations at different infected sites was studied. In 3 (6%) of 50 patients, >1 strain was cultured, which indicates that mixed infections are not anecdotal. The coinfected strains were not equally distributed at the respiratory and extrarespiratory site, which reflects a compartmentalization of the infection. In 1 patient, although 2 strains were found at the respiratory site, only 1 of these strains was involved in the extrarespiratory infection, which suggests that clonal selection can occur in the dissemination of the infection.

An episode of tuberculosis (TB) is traditionally thought to be caused by a single strain of *Mycobacterium tuberculosis* (MTB), and recurrences are usually considered to be due to reactivation by the strain that caused the first episode. Equally, extrarespiratory TB is generally assumed to be caused by the same strain that infects the respiratory site.

However, some reports have described different MTB strains within a single host in different circumstances. In this sense, exogenous reinfection with a strain different from the one involved in the primary episode has been reported in several contexts [1–7]. Mixed infection with >1 mycobacterial strain has been reported only anecdotally [6, 8–10], and some of these studies are old and are, therefore, limited by the methodology available at the time. Dual infection by MTB is especially relevant in patients with TB who are infected at different anatomical sites, although it has received little attention in the literature. Our study aimed to ascertain the proportion of mixed infection by different MTB strains in a group of patients with disseminated TB and to characterize the clonal distribution of strains between the different infected sites.

### Patients, Materials, and Methods

Of the 123 patients with disseminated TB (culture positive for MTB from 1 respiratory and 1 extrapulmonary sample obtained <30 days apart) over the past 5 years in our institution, we were able to analyze 107 isolates (median time between isolates from the same patient, 4.5 days) from 50 patients (42 infected with human immunodeficiency virus [HIV]). Sample distribution was sputum (50), urine (28), blood (8), lymph nodes (7), cerebrospinal fluid (CSF; 5), peritoneal fluid (3), bone marrow (1), pleural fluid (1), biopsy (1 from the lung and 1 from an abdominal adenopathy), and abscess (2).

Clinical specimens were processed according to standard methods. Susceptibility testing for isoniazid, rifampin, streptomycin, and ethambutol was performed for all the strains, using the MB/BacT system (Organon Teknika). Three different molecular methods were used to type the MTB isolates: spoligotyping, double repetitive elements polymerase chain reaction (DRE-PCR), and IS6110 restriction fragment–length polymorphism (RFLP) analysis. The analysis was performed by typing the whole bacterial population cultured from the clinical samples, except when it was necessary to analyze single colonies (for patients with mixed infection). In these patients, the positive cultures were plated onto Middlebrook 7H11 agar plates to obtain single colonies. Ten colonies from each plate were picked and subcultured in mycobacterial growth indicator tube (MGIT) medium for molecular analysis. All cultures grown from these single colonies were typed by spoligotyping, DRE-PCR, and RFLP, to define the clonal composition of the MTB populations. The spoligotyping assay was performed as de-
Results. In our institution, over the past 5 years, 123
(13.8%) of 892 patients with TB had an episode of disseminated
TB, defined by isolation of MTB from at least 1 respiratory
and 1 extrarrespiratory sample. In 47 of 50 patients available
for study, the molecular characterization of MTB strains by
spoligotyping indicated the existence of identical typing pat-
tterns for the strains isolated from the respiratory and extrarrespiratory
sites. For the remaining 3 (6%) patients, different spo-
ligotypes were obtained for the respiratory and extrarrespiratory
(uran, lymph node, and peritoneal fluid) sites (figure 1). Lab-
oratory cross-contamination was ruled out in all cases after
typing isolates processed on the same day and determining that
no other isolate shared the typing pattern of the study isolates.

To confirm the genotypic data obtained by spoligotyping,
the isolates were typed by 2 additional molecular methods, and,
again, different DRE-PCR types and IS6110 RFLP patterns were obtained for isolates from the 3 patients whose strains differed by spoligotyping (figure 1), and, for the remaining 47 patients, identical DRE-PCR types were obtained (data not shown).

Of the 3 patients with dual infection, all were HIV infected and were injection drug users (IDUs); 2 had spent some time in prison, and 1 was homeless. All strains, respiratory and extrarespiratory, were susceptible to antituberculous drugs. For the 47 patients with identical strains from both the respiratory and extrarespiratory site, 39 were HIV infected, among whom 22 were IDUs, 3 were homeless, 4 were alcoholics, and 5 had been in prison. Of the 8 HIV-uninfected patients, 4 were alcoholics.

Typing analysis is generally performed with the whole bacterial population of MTB cultured from each clinical sample. Therefore, it does not allow us to precisely know the distribution of strains (clonal composition) at each of the anatomical sites for patients with mixed infection. To increase the resolution of our analysis for the 3 patients with dual infection, we performed typing assays with a selection of multiple single colonies picked from the MTB cultures obtained from each of the infected sites.

Molecular typing analysis with single colonies revealed compartmentalization of the infection in these 3 patients, because the clonal composition of the respiratory and extrarespiratory populations was different. In 2 of 3 patients (patients 13 and 32), all colonies from the respiratory site shared a unique typing pattern that was different from the pattern shared by all the extrarespiratory colonies, which indicates that each site had an homogeneous MTB population that was different from that of the other infected site. For the third patient (patient 29), the analysis of single colonies by spoligotyping (figure 2) showed the coexistence of 2 different strains at the respiratory site (proportion, 4:6), and only 1 of these strains was detected after analysis of the multiple colonies in the extrarespiratory sample. Additional typing by DRE-PCR and IS6110 RFLP analysis confirmed these results (figure 2). Single-colony analysis revealed the real differences in the RFLP patterns of the coinfesting strains that had not been detected previously in the mixed population analysis (figure 1).

Discussion. In our institution, 14% of patients with TB simultaneously had respiratory and extrarespiratory infection by MTB. In our study, we found that mixed infections by >1 MTB strain are not anecdotal in this group of patients. The possibility that laboratory cross-infection played a role in the misassignment of a strain was ruled out.

Mixed infection by several MTB strains can be explained by (1) the appearance of a new strain that diverged from a preexisting persistent MTB clone, (2) simultaneous coinfection by 2 recently acquired strains, or (3) superinfection of an uncured MTB infection. With regard to the first supposition, microevolution phenomena in persistent infections by MTB have been reported to lead to the appearance of strain variants that diverge during the development of the infection. Usually, these strains are highly similar with respect to the parental strain [6, 14], although this was not the case in our study, in which RFLP analysis showed clear differences between the strains. With regard to the second possibility, it is difficult to assume a simultaneous coinfection with >1 strain, and this has only been found for an extremely high risk of exposure [15]. It is more reasonable to consider that the coexistence of 2 strains within the same host is due to the overlapping of 2 independent infections, a preexisting infection and superinfection by a new strain [9]. This has been found in circumstances of high reexposure to TB [6]. In our study, the 3 patients with dual infection were HIV infected and had similar sociological/epidemiological circumstances (e.g., injection drug use, prison/homelessness, and alcoholism) that are consistent with a high risk of overexposure to TB.

The most relevant aspect of our study, apart from the proportion of mixed infections, is the fact that the 2 strains in our patients were not equally distributed at the respiratory and extrarespiratory sites. In all 3 patients, differences in the clonal composition of the cultured bacterial populations were defined for both body sites. This indicates the compartmentalization of TB. In 2 patients, after analysis of multiple independent colonies, the molecular patterns were found to be the same for all colonies within each infected site but different for respiratory and extrarespiratory sites. For the third patient, 2 different strains were detected simultaneously at the respiratory site, but only 1 of them was found at the extrarespiratory site. This last observation suggests that certain strains may have a greater ability to disseminate, and this fact could be responsible for the compartmentalization found in our patients. One hypothesis would be that different strains were involved initially in the respiratory infection (the 3 patients were candidates for reexposure to TB), but, after the course of the infection, one strain is more adapted to disseminate, whereas the other remains at the respiratory site, thus leading to compartmentalization of the infection. Some authors go further and stress the possibility that compartmentalization could occur within the lung, with different MTB strains infecting different sites [15]. Another hypothesis is that compartmentalization could stem from the differences in susceptibility to the innate antimycobacterial effects of extrapulmonary organs.

We cannot rule out the possibility that compartmentalization could be due to the overlapping of a reactivation of a previous, unresolved infection at the extrarespiratory site with a recent infection. An exhaustive revision of the medical charts of coinfected patients showed no clinical/radiological features that suggested active TB or TB treatment in the past, although we could not assign a purified protein derivative–negative status because of the low CD4 cell counts in the 3 cases.

In any case, the compartmentalization of MTB infection was
clear in our study and could be due either to (1) mixed infection followed by a differential selection of clones in the respiratory/extrarespiratory compartments or (2) overlapping of a reactivated extrarespiratory strain with another recently acquired one. The definition of compartmentalization in TB is especially relevant in therapy if the susceptibility patterns of the coinfecting strains differ. In our study, all patients shared a susceptible pattern, although our findings are still highly relevant in epidemiological/pathogenic terms. From the epidemiological point of view, compartmentalization stresses the need to consider in the laboratory the clonal composition of the cultures from patients with respiratory and extrarespiratory involvement, to guarantee precision in molecular epidemiological studies. Deficiencies in the tracking of transmission chains could be caused by the assumption that respiratory and extrarespiratory strains within the same patient are always the same. Moreover, if mixed infections are not considered for the first episode of patients with recurrences, this could lead us to wrongly classify as exogenous reinfections cases of recurrence that are really due to clonal selection from a polyclonal first episode [8].

Our study population consisted mainly of HIV-infected patients. This is the unavoidable consequence of having studied mixed infection specifically in a population with disseminated TB. In our geographic setting, almost all cases of disseminated TB are found in HIV-infected patients. Nevertheless, our observations should draw attention to the possibility that compartmentalization may be present not only in HIV-infected patients but also whenever infection by >1 MTB strain occurs as result of overexposure or superinfection.
From a pathogenic point of view, our study also could help to detect strains with a higher tropism for respiratory or extrarespiratory sites and to select them for additional analysis to understand the mechanisms involved in the dissemination of TB. We are running experiments in a model of infection to assess whether the strains that showed high efficiency to disseminate have a greater ability to infect macrophages.

Finally, compartmentalization of MTB infection, as described in the present study, is a relevant issue that is worthy of a more in-depth analysis. This would help us understand the mechanisms involved in extrarespiratory infection by this microorganism.

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References