Molecular Evidence of Endogenous Reactivation of \textit{Mycobacterium tuberculosis} after 33 Years of Latent Infection

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Since Robert Koch described the cause of tuberculosis in 1882, the natural history of the disease after primary infection has been subject to debate. Only \textasciitilde10\% of infected individuals develop active disease, which may appear years to decades after infection. Late onset has been attributed to the endogenous reactivation of dormant bacteria. However, this has not been documented by molecular means for latencies of more than a few years. In Denmark, we have recently recultured 205 freeze-dried \textit{Mycobacterium tuberculosis} strains obtained from 1961 through 1967. These “historical” strains are analyzed by DNA restriction fragment–length polymorphism testing, and their DNA patterns are compared with those of 4008 recently obtained clinical specimens. This has, surprisingly, yielded molecular evidence of \textit{M. tuberculosis} reactivation after 33 years of latent infection. A father and son who developed tuberculosis in 1961 and in 1994, respectively, were the only patients infected with strains that share an identical DNA pattern.

Primary infection with \textit{Mycobacterium tuberculosis} leads to clinical disease in only \textasciitilde10\% of cases [1]. Most infected individuals are able to mount an effective immune response, which limits proliferation of the bacilli and produces a long-lasting partial immunity, both to new infections (often referred to as “exogenous reinfections”) and to the reactivation of latent bacilli (often referred to as “endogenous reactivation”) [2, 3]. Approximately one-half of infected individuals who develop clinical disease experience “early” progressive disease that occurs within 5 years of infection, and the rest have “late” disease, which is caused by reactivation as long as several decades after infection [2, 4]. The relative contributions of reactivation and reinfection, and even whether each occurs, have been the subject of considerable controversy [2, 3, 5–7]. The introduction of molecular subtyping methods has permitted characterization of specific strains of \textit{M. tuberculosis} and determination of whether isolates from different patients might have a common origin [8]. Therefore, the potential exists for determining whether disease in a particular patient is caused by a new infection with a strain in current circulation or by reactivation of infection with a previously prevalent strain. Using molecular subtyping, both reinfection and reactivation have been convincingly demonstrated over a span of \textasciitilde5 years [6, 9]. Although the possibility of reactivation after longer periods of time is a generally accepted dogma, so far it has not been demonstrated. Here we present evidence of reactivation of \textit{M. tuberculosis} infection after 33 years.

Methods

Data collection. In Denmark, all microbiological tests for mycobacteria have been carried out at the International Reference Laboratory for Mycobacteriology (IRLM) at Statens Serum Institut (SSI) in Copenhagen since 1922. This is the only laboratory that performs culture-based tuberculosis (TB) diagnosis for Denmark, Greenland, and the Faroe Islands. It also serves as an international reference laboratory for Iceland and Lithuania. Because all \textit{M. tuberculosis} specimens from Denmark, Greenland, and the Faroe Islands are processed in a single laboratory and because of the long-standing mandatory centralized TB notification system in Denmark, we believe that our data are nearly complete and highly representative of culture-positive TB from the areas we cover. This is of major importance in interpreting DNA band clustering [8].

From 1961 through 1967, 205 strains of \textit{M. tuberculosis} were collected from samples obtained from small groups of epidemiologically related patients. The strains were divided into 2 groups by means of the bacteriophage BK1, and the results were compared with the epidemiologic linkage information [10]. The strains were
then stored as freeze-dried samples for 33–39 years, until they were recultured in 2001. The DNA patterns of these strains are now being analyzed, and, at present, DNA patterns from 130 of the historical strains have been compared with those from *M. tuberculosis* strains collected in the 1990s. In 1992, DNA analysis of *M. tuberculosis* strains was implemented in Denmark on a nationwide basis, using the internationally standardized restriction fragment–length polymorphism (RFLP) method [11]. Since then, the RFLP patterns of 4008 strains collected from 3781 patients with TB have been analyzed, representing 97% of all culture-positive patients with TB in Denmark, as well as the Faroe Islands and Greenland. Table 1 gives basic epidemiologic data for both the historical and the recent strains.

**Specimen processing.** Freeze-dried *M. tuberculosis* strains were resuspended in distilled water, and each was transferred directly into 1 tube of Dubos culture medium containing Tween 80 (SSI Diagnostika) and 2 tubes of Löwenstein-Jensen medium (SSI Diagnostika). Bacteria were harvested after 3–4 weeks of incubation. The recent strains were cultured in the Bactec culture system (Becton Dickinson), and, when species identification by AccuProbe (Gene-Probe) revealed the presence of *M. tuberculosis* complex, the isolates were subcultured for 3–4 weeks in Dubos medium containing Tween 80. Harvested bacteria were heat killed (90°C for 30 min), and RFLP testing was done by the internationally standardized method [11].

**Results**

**DNA analysis.** Two of the *M. tuberculosis* strains that we examined exhibited identical 13-band DNA patterns (figure 1). The strains were isolated, at an interval of 33 years, from a father (in 1961) and son (in 1994). This particular DNA pattern was not found in any other strain, either among the 130 strains that were analyzed from the group of 205 historical strains or among the 4008 recent strains (table 1).

**Index case: father.** The index case patient was born in 1926. According to both hospital and IRLM records, he was given a diagnosis of tuberculous infection of a knee joint in 1946 and of pulmonary TB in 1961. The patient presented in 1961 with a 1-year history of fever, weakness, and productive cough. Chest radiography showed several small nodular pulmonary opacities and a large (4 × 5 cm) cavity in the apex of the right lung. The diagnosis was confirmed by microscopy of a sputum smear and by culture. The patient was treated for 1 year with streptomycin, isoniazid, and para-aminosalicylic acid (PAS) and was discharged from the hospital without symptoms. During the next 20 years, the patient was hospitalized several times, mainly for problems related to chronic alcohol abuse, but never again received a diagnosis of TB. The last hospital records for this patient are from 1982, when he died of metastatic lung cancer.

**Secondary case: son.** The second case patient was born in 1954. According to both hospital and IRLM records, the patient was given a diagnosis of pulmonary TB in 1994. He presented with a 2-month history of night sweats, weight loss of 5 kg,
fever, and nausea. Chest radiography showed a left pleural effusion and both dense and diffuse opacities in the apex of the right lung. The diagnosis of TB was confirmed by culture. The patient underwent treatment for 6 months and then was discharged without symptoms. Recently, the patient was interviewed and could verify and supplement the information in the hospital records. He had no history of travel outside Scandinavia, episodes of earlier disease compatible with TB, or contact with persons who had TB, other than his father and mother. He was vaccinated with bacille Calmette-Guérin before incurring the presumed initial infection and could not recollect any tuberculin skin test evaluation during the contact investigation in 1961. He left home at age 18 years.

**Other family members.** The family was from a small municipality in a rural district of Denmark. The mother died from surgical complications in October 1966, when she underwent resection of the sixth segment of the right lung to remove a large tuberculous cavity. The *M. tuberculosis* isolate from this patient shows 2 clearly visible extra bands in comparison with the DNA pattern obtained from the isolates from the father and son. TB was diagnosed in the mother’s niece in 1961, but this niece had no contact with the son. The DNA pattern for the niece’s isolate differs by 1 clearly visible extra band from that of the isolate identified in samples from the father and son and by another clearly visible extra band from the isolate from the mother. The son shared the living room of a small 2-room apartment with his sister, who never had any disease compatible with TB, according to the interview with the son and the medical records. All the DNA patterns of isolates obtained from this family were located in a separate branch of a dendrogram and showed <60% similarity to any other historical or recent strains.

**Discussion**

Molecular methods are proving to be useful in epidemiology in distinguishing recent from previous infection. The identification of certain insertion sequences occurring in the genome of *M. tuberculosis* in a variable number of copies and at variable insertion sites allows a high degree of discrimination between strains of the bacillus. RFLP typing, using the insertion sequence IS6110 as a genetic marker, has been the method of choice and increasingly is applied in epidemiologic research, both during outbreaks of TB and in large population-based studies [8]. An important conclusion of many studies has been the unexpectedly high contribution of recent transmission to the incidence of TB [6, 12, 13], which has distracted attention from endogenous reactivation to such an extent that some investigators have even doubted its existence [5]. Today, the relative frequency of exogenous reinfection versus that of endogenous reactivation is still undetermined, and the existence of reactivation in humans after a latency of more than a few years and its contribution to the total incidence of TB have never been demonstrated directly [3]. Indirect evidence derives from models based on trends in historical epidemiologic data, often tuberculin surveys, which have sometimes been analyzed in combination with more recent data [2, 3, 5, 7].

During analysis of freeze-dried *M. tuberculosis* strains obtained in the 1960s, we detected a 13-band DNA pattern in a specimen collected in 1961 that was identical to the DNA pattern of a specimen from 1994. These 2 specimens differed from all other Danish specimens obtained in the 1960s or the 1990s. They proved to be from a father (1961) and son (1994) (figure 1). The father had highly infectious pulmonary TB with cavitation for 1 year before he received the diagnosis in 1961. During this period, we assume that the father infected his son, who was living in the same household, and that the son subsequently developed TB in 1994 as a result of reactivation of the dormant bacilli. This theory is supported by the fact that the DNA pattern of the isolates from the father and son was not found in any of the 4138 other strains that were examined, which were obtained on a national scale in the 1960s and 1990s. This eliminated a large number of potential sources (table 1). We are aware that the RFLP method cannot directly reveal the occurrence of a transmission event and that other scenarios are also possible [8]. For instance, the son could have been infected by another source within or outside his family at some point between the 1960s and the implementation in 1992 of nationwide DNA analysis of *M. tuberculosis* isolates. However, we cannot imagine more convincing direct molecular epidemiologic evidence of *M. tuberculosis* reactivation after several decades, and we believe this to be the first instance of molecular evidence of endogenous reactivation of *M. tuberculosis* in humans, in this case after 33 years of latent infection.

TB due to reactivation of latent bacilli is presumed to result from a failure of immune surveillance, for instance because of immunosuppressive therapy or infection with human immunodeficiency virus. However, in the vast majority of cases, the specific cause of reactivation is unknown [5]. The hospital case records for the son report evidence of heavy alcohol abuse at approximately the time of reactivation of the disease. Alcohol abuse is a known risk factor for progression to active TB, because the poor eating habits and inadequate nutrition associated with alcohol abuse can lead to immunologic impairment.

The lifetime risk of developing TB after primary infection is often said to be ~10%, and it is stated that approximately one-half of those who develop the disease will do so within the first 5 years after infection [1]. Recent studies suggest that both the lifetime risk of developing disease and the distribution of morbidity after infection are strongly dependent on the age of the individual at the time of the primary infection [14]. Infection acquired at an early age is associated with appreciably longer incubation periods than infection acquired in adulthood. However, little is known about the generation time of TB, which has never been measured directly [14]. In this report, the son was only 7 years old at the time of the presumed initial infection, and the incubation period may have been as long as 33 years.
Recently, an enzyme in a metabolic pathway critical for lipid metabolism in *M. tuberculosis* was identified as important to the capacity of the bacteria to stay dormant for years before it causes active infection [15]. It has also been demonstrated that the bacteria may persist intracellularly, not only in macrophages but also in nonphagocytic cells in lung tissue, in the absence of histologic evidence of tuberculous lesions [4]. *M. tuberculosis* is a resilient organism that can adapt to a wide variety of environmental conditions, making it a successful human pathogen. Better understanding of the molecular pathogenesis of latency, which will also point to methods for preventing reactivation, is a prerequisite for eliminating the disease. Primary infection with *M. tuberculosis* is associated with a substantial early risk of developing TB [2], but even if the primary infection does not lead to progressive disease within the first few years after transmission, we can now confirm, by molecular means, that the *M. tuberculosis* bacilli may remain dormant for decades and thereby constitute a persistent, although probably small, risk of reactivation.

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**References**