Persistent Infection by a *Mycobacterium tuberculosis* Strain That Was Theorized To Have Advantageous Properties, as It Was Responsible for a Massive Outbreak

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The strains involved in tuberculosis outbreaks are considered highly virulent and transmissible. We analyzed the case of a patient in Madrid, Spain, who was persistently infected over an 8-year period by the same Beijing *Mycobacterium tuberculosis* strain. The strain was responsible for a severe outbreak on Gran Canaria Island. The case provides us with a unique opportunity to challenge our assumptions about *M. tuberculosis* Beijing strains. No clinical/radiological findings consistent with a virulent strain were documented, and the *in vitro* growth rate of the strain in macrophages was only moderate. No secondary cases stemming from this prolonged active case were detected in the host population. The strain did not acquire resistance mutations, despite constant treatment interruptions, and it remained extremely stable, as demonstrated by the lack of single-nucleotide-polymorphism (SNP)-based differences between the sequential isolates. Our data suggest that the general assumption about *M. tuberculosis* Beijing strains having advantageous properties (in terms of virulence, transmissibility, and the tendency to acquire mutations and resistance) is not always accurate.

Genotyping makes it possible to discriminate between different lineages of *Mycobacterium tuberculosis* and enables us to identify highly virulent strains with advantageous properties. The Beijing lineage (1–3) has generated the most attention for a number of reasons. First, it is highly transmissible and responsible for severe outbreaks (2). Second, studies based on cellular and animal models reveal higher virulence for most Beijing strains (4–6). Third, Beijing strains are thought to have a hypermutator phenotype (7), which could increase the tendency to acquire variability and, more specifically, resistance mutations (8).

In this study, we present a clinical case with persistent active infection by a Beijing *M. tuberculosis* strain over an 8-year period. This case provides us with a unique opportunity to analyze the behavior of a strain with advantageous properties. We examined virulence, transmissibility, variability, and acquisition of resistance. The added value of this case is that the Beijing strain involved is considered very highly transmissible: it caused a severe outbreak on Gran Canaria Island, where it spread after its introduction by a Liberian immigrant in 1993 (2), eventually accounting for one-third of all cases of tuberculosis on the island.

**MATERIALS AND METHODS**

**Immunological studies.** This research was approved by the research review board at our institution. The production of interleukin 12 p70 (IL-12p70) and tumor necrosis factor alpha (TNF-α) in response to recombinant human gamma interferon (rhIFN-γ) was assessed in whole blood cultures diluted 1:2 in RPMI 1640 (Lonza). Cultures were left unstimulated or were stimulated with 100 ng/ml lipopolysaccharide (LPS) from *Salmonella enterica* serovar Enteritidis (Sigma), either alone or in combination with various concentrations (10^4 to 10^7 IU/ml) of rhIFN-γ (R&D Systems). Supernatants were collected after 24 h of incubation at 37°C in a 5% CO₂ atmosphere (9). The production of IFN-γ was analyzed in whole-blood cultures diluted 1:1 in RPMI 1640. Cultures were left unstimulated or were stimulated for 48 h with phytohemagglutinin (PHA; 5 μg/ml; Roche) or live *Mycobacterium bovis* bacille Calmette-Guérin (BCG; 5 mg/ml; Sanofi Pasteur Limited), either alone or in combination with rhIL-12p70 (20 ng/ml; R&D Systems) (10). The response to TNF-α was analyzed by evaluating the production of IL-10 and IL-8 in whole-blood cultures diluted 1:1 in RPMI 1640. Cultures were left unstimulated or were stimulated for 48 h with 20 ng/ml of TNF-α.

Peripheral blood mononuclear cells (100,000/well) were resuspended in RPMI 1640 supplemented with 10% FCS (Biochrom). Cells were stimulated with 12.5 ng/ml soluble anti-CD3 (12.5 ng/ml; clone HT31a; Becton Dickinson) or PHA-L (10 μg/ml; Roche), both alone and in combination with IL-12 (20 ng/ml; R&D Systems), soluble anti-CD3 plus soluble anti-CD28 (250 ng/ml; Sanquin), staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB; 1 μg/ml; Sigma Chemical Co.), or 10 ng/ml of phorbol myristate acetate (PMA; Sigma Chemical Co.) plus 1 μg/ml of ionomycin (Sigma Chemical Co.). Cells were cultured in U-bottom, 96-well plates (0.2 ml/well) for 48 h.

The levels of TNF-α, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, IL-2, and IL-17 in cultures were measured using a flow cytometry-based bead array system (BD Biosciences).
TABLE 1 Summary of the MTB isolates from the case

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Date</th>
<th>Specimen</th>
<th>Typing by*</th>
<th>Susceptibility to†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RFLP</td>
<td>MIRU-24</td>
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<tr>
<td>1</td>
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<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2</td>
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<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>Sep 2006</td>
<td>Sputum</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
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<td>Sputum</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
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<td>X</td>
</tr>
<tr>
<td>6</td>
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<td>X</td>
</tr>
<tr>
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</tr>
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<td>X</td>
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<td>X</td>
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<td>X</td>
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</tr>
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</tr>
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<td>X</td>
</tr>
<tr>
<td>17</td>
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<td>Urine</td>
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<td>X</td>
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<td>Sputum</td>
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</tr>
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</tr>
<tr>
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<td>X</td>
</tr>
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<td>Sputum</td>
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<td>X</td>
</tr>
<tr>
<td>23</td>
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<td>Sputum</td>
<td>X</td>
<td>X</td>
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</tbody>
</table>

* WGS, whole-genome sequencing; X, typing for the indicated isolate was performed.
† SM, streptomycin; EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; S, susceptible.

Microbiological methods. Clinical specimens were processed according to standard methods and inoculated on Lowenstein-Jensen slants and also in MGIT liquid medium (Becton Dickinson, Sparks, MD, USA). Testing for susceptibility to isoniazid, rifampin, streptomycin, and ethambutol was performed using MGIT SIRE (Becton Dickinson, Sparks, MD, USA). The M. tuberculosis cultures were stored at −70°C until analysis.

Genotyping methods. The fingerprinting methods applied were IS6110-based restriction fragment length polymorphism (RFLP) typing, which was performed as described in reference 11, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing with the 24-locus set (12, 13), and spoligotyping, which was performed following standard procedures (14) using a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands). We looked for identical patterns by comparing patterns from the case isolates and those in the population database. We also performed a second-line analysis in which subtle differences were allowed (1-band differences between the RFLP types or single locus variations [SLVs] between the MIRU-VNTR patterns).

High-resolution melting (HRM) analysis was performed as described elsewhere (15) to identify SNPs that are markers of Beijing strains.

Whole-genome sequencing. Four sequential isolates were used to identify SNPs as detailed elsewhere (16). We extracted the DNA from pooled colonies. We followed standard library preparation protocols based on the recommendations for Illumina TruSeq DNA sample preparation. A HiSeq 2000 device generating 101 bp-paired-end reads was used for sequencing. We mapped the reads for each strain using the Burrows-Wheeler Aligner to map the M. tuberculosis ancestral genome, as detailed in reference 16. SNP calls were made with SAMtools (coverage of at least 10×; mean SNP mapping quality of at least 10) later corroborated by VarScan (coverage of at least 10×, 95% of the reads to call a homozygous position, SNP found in both strands, and SNP quality of 20). Alternatively, VarScan also enabled us to analyze the strains using a multisample SNP calling approach. Multisample analysis is also implemented in VarScan and has the potential to exploit the joint evidence contributed by each strain to enhance SNP calling. The per-base depth of sequencing (715× to 1,252×) allowed us to examine low-frequency variants. We defined a low-frequency variant as any SNP call in which the alternative allele had a frequency of 10% to 90%. The threshold is based on a trade-off between detecting real variants and avoiding false-positive reporting.

In vitro infection. PMA-differentiated THP-1 cells were infected as described elsewhere (4, 17). Briefly, PMA-differentiated THP-1 cells were infected at a multiplicity of infection of 7 to 9 bacteria per cell and incubated for 3 h at 37°C in 5% CO2. To evaluate bacterial growth, supernatants were aspirated and monolayers were lysed at 3 h and days 1, 4, and 7 after infection. Serial 10-fold dilutions of cellular lysates were plated on Middlebrook 7H11 plates and incubated for 3 weeks at 37°C in 5% CO2, and colonies were counted. Intracellular growth was expressed as the growth rate, which is the slope of the function of log10 CFU values throughout the infection period (at 3 h and at days 1, 4, and 7). Three independent experiments were performed for each strain assayed.

RESULTS

The patient was a 45-year-old man who had been HIV positive since 1990 and was an intravenous-drug user (IVDU). He had not been vaccinated against BCG. He had spent 8 years in various prisons in the 1990s, after which time he was placed in an open prison regime. He registered with our institution and was diagnosed with Mycobacterium tuberculosis infection in September 2006. At the time, his CD4 count was below 100/μl, and his opportunistic infections were oropharyngeal candidiasis and mucocutaneous infection by herpes simplex virus. M. tuberculosis was subsequently cultured over 8 years (2006 to 2013) from 23 clinical specimens (Table 1). The Mantoux and Quantiferon tests were...
not performed because of their lack of clinical usefulness in this context.

Given the repeated isolation of *M. tuberculosis* over such a long period, we first investigated the involvement of host factors other than HIV-induced immunosuppression, namely, primary immunodeficiency, which predisposes to mycobacterial disease (OMIM 209950 [http://www.omim.org/entry/209950]), which can first appear during adulthood. We found a normal response to IL-12, which was measured as 2 ratios: the ratio of IFN-γ production in response to BCG plus IL-12p70 to IFN-γ production in response to BCG alone and the ratio of IFN-γ production in response to PHA plus IL-12p70 to IFN-γ production in response to PHA alone (Fig. 1). We also found that the levels of IL-12Rβ1 expressed by T cells activated with PHA plus IL-2 in cultures of cells from the patient were similar to those in cultures from healthy controls (data not shown). The patient’s cells also produced normal amounts of IL-12p70 and responded to IFN-γ. The ratios of IL-12p70 and TNF-α production in response to LPS, whether in the presence or absence of increasing concentrations of IFN-γ, were equivalent for the patient and for healthy controls (Fig. 1). Finally, production of IL-10 in response to TNF-α and production of IL-6 after activation with BCG were equivalent in both the patient and the healthy controls (data not shown).

Once the role of primary immunodeficiency had been ruled out, we evaluated the patient’s treatment history. During an in-depth interview, he admitted that he had taken treatment for only 1 to 2 months after every indication, enough until his symptoms improved. He repeatedly stopped taking his medication in order to remain ill, avoid prison, and gain access to social benefits. This new scenario was more consistent with a persistent untreated infection, which was confirmed by identical IS6110 RFLP and MIRU-VNTR patterns (Fig. 2a and b) for the isolates throughout the 8-year infection.

We extended the genotypic analysis to spoligotyping in order to assign the lineage to the *M. tuberculosis* strain involved in the persistent infection. The pattern revealed was characteristic of *M. tuberculosis* strains belonging to the Beijing lineage, that is, lack of spacers 1 to 34 (Fig. 2c). The Beijing lineage was confirmed using high-resolution melting analysis (Fig. 2d) and DNA sequencing to detect SNPs (in Rv2629 and Rv2952), which are markers for this family. Comparative analysis with MIRU-VNTR revealed that the Beijing strain persistently infecting our patient corresponded to the Beijing strain that was responsible for a severe outbreak on Gran Canaria Island (2). The patient was interviewed to establish epidemiological support for this finding and reported that he had spent a week on the island in the 1990s, which was before he was diagnosed with tuberculosis in our institution.

Given the fast and efficient transmission of this strain on Gran Canaria Island, the infectiveness of our patient (stain-positive in 2007 to 2012) and his transmission-associated epidemiological characteristics (IVDU, socioeconomic disadvantages, and prison stays), we looked for secondary cases infected by the same strain. Only 3 patients, all of whom had been on Gran Canaria Island before their stay in Madrid, were found to be infected by the same strain. In 2 of these patients, the diagnosis was made before that of the present case.

Despite the treatment interruptions and lack of adherence, all the susceptibility tests performed revealed exclusively pansusceptible isolates. Whole-genome sequencing of 4 available isolates from the 5 isolates that were representative of the patient’s history and covered a 7-year period (2006, 2007, 2008, and 2012)
control isolates, the growth rate for this strain was average, and the phage infection model. Compared with the virulent Beijing phenotype for this strain was in line with the infective behavior (copies/ml).

Thanks to the high coverage achieved in the analysis (715× to 1,252×), we analyzed the possible presence of minority variants in greater detail. First, we analyzed variation in known drug resistance genes. No heterozygous sites were detected, suggesting that antibiotic pressure did not play a major role in the evolution of bacteria during treatment. In addition, we detected six positions with evidence of coexisting alleles. However, for three of them (2127067, 2295685, and 3119513), none of the sites had a frequency of the alternative allele below 90%, suggesting that they are true homozygous positions that are not at 100% because of background noise introduced by sequenc- ing or mapping errors. Another site (4124351) has a frequency of the alternative allele of 60% in two strains, while for the other two, there is a deletion. Manual inspection of the alignments revealed that the same deletion has likely created a misalignment around the region for the two strains exhibiting the heterozygous SNPs. Only 2 heterozygous positions with a minimum frequency difference between isolates of 20% (1986639 and 2128040) remained after all the filters. However, evidence of heterogeneity in both cases, although strong, has to be taken with caution, as the coverage of the site in both cases is far below the mean coverage for the strain. Again, this finding suggests that intrapatient microevolution did not play a major role in this patient.

We expected higher-than-average severity of *M. tuberculosis* infection owing to the present patient’s impaired T-cell function, which was probably secondary to HIV infection. Production of IL-2, IL-17, and IFN-γ in response to T-cell-receptor-mediated activation with SEB was considerably lower in cultures of the patient’s peripheral blood mononuclear cells than in those from healthy controls (Fig. 1D). In contrast, the response to PMA plus ionomycin, measured in terms of IFN-γ and IL-2 production, was not reduced; in fact, high production of IFN-γ was observed (Fig. 1E). Despite such a permissive environment, signs of severity such as miliary or marked cavitory disease were absent. From 2008 onward, sputum specimens were complemented by systematic blood and urine culture. However, the results were always negative except for a short period (August to September 2008) when urine was positive for *M. tuberculosi*, i.e., exactly when the CD4 counts were lowest (80/μl) and the HIV load was highest (>1,000,000 copies/ml).

The absence of clinical findings consistent with a virulent phenotype for this strain was in line with the infective behavior of the isolate when its growth rate was evaluated in a macrophage infection model. Compared with the virulent Beijing control isolates, the growth rate for this strain was average, and it was lower than that for the reference *M. tuberculosis* strain H37Rv (Fig. 3).

**DISCUSSION**

The case we present, that of continuous isolation of *M. tuberculosis* over an 8-year period, is exceptional. Primary immunodeficiency predisposing to mycobacterial disease was ruled out, and DNA fingerprinting combined with a detailed interview provided key information. All the sequential isolates corresponded to the same strain and were the result of conscious nonadherence, which enabled the patient to avoid incarceration and obtain social benefits.

The infection involved the Beijing strain responsible for one of the most extensive outbreaks of tuberculosis ever recorded (2, 3). It provided us with an extraordinary opportunity to review the validity of some of the assumptions generally accepted for this advantage-bearing *M. tuberculosis* lineage in terms of virulence, transmissibility, and ability to acquire variability.

Beijing strains are considered virulent (18). However, this strain showed an average ability to replicate in macrophages *in vitro* (4, 17). In addition, despite marked immunosuppression of the host and prolonged persistence and treatment interruptions, no radiological findings consistent with severity were found. Systematic culture of blood and urine specimens was performed, and all blood specimens were negative, whereas urine was positive only in the short period where CD4 counts were lowest.

Given the high number of secondary cases caused by the same strain on Gran Canaria Island, one would also expect a high number of secondary cases in Madrid, especially considering that the index patient was smear positive, had spent periods of time in prison, and was an IVDU. However, against all expectations, only 3 additional cases (2 of which had been diagnosed before the present case) were caused by this strain. This value is far from the 28% of the total number of cases of tuberculosis on Gran Canaria Island. In addition, since the 3 patients had previously been on Gran Canaria Island, they were more likely infected there.

Additional cases in Madrid that were caused by the same strain may have gone undetected owing to insufficient fingerprinting coverage. The database used in the survey included the genotypes of the *M. tuberculosis* isolates from 2,669 cases. These were obtained from a multicenter molecular epidemiology study performed over 9 years in Madrid (2001 to 2009) and involving 8 hospitals (19–21). The sample corresponded to 79% and 32% of all culture-positive cases in immigrants and autochthonous patients, respectively. While it is true that only the analysis of the whole population can ensure the complete lack of additional cases infected by the strain under study, the population covered in our sample is reasonably representative.

In addition, our findings for cases infected by the Madrid strain are far from the 28% obtained on Gran Canaria Island, thus showing that transmission is clearly different in each of these 2 settings. Apart from the secondary cases that might be expected from our persistent case, 2 of the other patients in Madrid infected by the same strain had pulmonary tuberculosis (the remaining one was articulare) and were diagnosed before the present case (in 2002 and 2005). These cases could also have generated secondary cases owing to the patients’ characteristics and history (prison stays, IVDU, alcoholism, and homelessness), but secondary cases were not detected during the 8-year molecular epidemiology survey. These data suggest that the successful transmission of this strain on Gran Canaria Island is more probably a consequence of epidemiological factors and/or social networks than of bacterial factors. The index case on Gran Canaria Island was that of a nonadherent patient with
laryngeal tuberculosis \( (2) \), which could be the main reason for the outbreak, thus minimizing the role of bacterial factors.

The Beijing lineage is thought to be more prone to resistance than other strains \( (8) \). In the present case, the strain always remained pansusceptible despite a long history of treatment interruptions and poor adherence. Acquisition of variability in \( M. \) \textit{tuberculosis} can also prove to be advantageous in contexts other than resistance \( (16, 22) \), since it can aid in escape from the immune system and in the acquisition of more infective phenotypes \( (23) \) and can lead to the emergence of more infective variants. The finding of a potential hypermutator phenotype for Beijing strains \( (7) \) could point to a high tendency to accumulate diversity. In the present case, the lack of an appropriate control precludes a more rigorous analysis of whether the Beijing strain studied has a lower underlying mutation rate, leading to lower variability. However, our findings seem to challenge the assumption that Beijing strains are more prone to acquire variability because, despite the persistent and prolonged active infection, the strain was observed to be extremely stable, even when whole-genome sequencing was applied. Given the high coverage achieved in the whole-genome sequencing analysis \( (715 \times \text{to } 1,252 \times) \), the power to identify minority variants was very high. Consequently, we were able to rule these variants out with sufficient confidence and no further need to analyze single colonies. A more in-depth analysis of the readings including heterozygous calls and based on 2 independent algorithms enabled us to rule out minority variants in all cases. We found that the present patient had a previous \( M. \) \textit{tuberculosis} isolation in 1990 in Barcelona; however, the isolate was unavailable, with the result that it was not possible to expand the genomic analysis backwards.

In summary, we performed an in-depth analysis of a patient with an 8-year history of persistent infection by a Beijing \( M. \) \textit{tuberculosis} strain that had previously caused an extensive and prolonged outbreak. None of the advantages generally assumed for such an epidemiologically successful strain were found in our study. In the case we present, the strain did not generate secondary cases, was not virulent, and remained susceptible and extremely stable from a genomic point of view. Our findings suggest that the general assumption of an advantageous microbiological phenotype for specific strains involved in severe outbreaks might not always be correct.
REFERENCES


