Community-wide transmission of a strain of *Mycobacterium tuberculosis* that causes reduced lung pathology in mice

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Since 1992, *Mycobacterium tuberculosis* strain PG004 has been responsible for a large outbreak of tuberculosis in one northern Californian community. There are no epidemiological or host factors to explain this outbreak. PG004 was therefore analysed for biological characteristics that might explain its widespread distribution. BABL/c mice were infected intravenously with PG004, non-PG004 *M. tuberculosis* strains CCC20 and CCC23 isolated from patients in the same community, and the laboratory strain H37Rv. The susceptibility of PG004 to reactive nitrogen intermediates (RNIs) was compared with that of H37Rv. Because of the reported association of phenolic glycolipid production with mouse virulence, a junction sequence in the polyketide synthase gene cluster (*pkas15/1*) was compared among strains. It was found that the most virulent strain, based on mouse mortality, was not the outbreak strain PG004, but the non-outbreak strain CCC20. This strain had an intact *pkas15/1* sequence identical to that of another non-outbreak strain, CCC23, which caused death in only one out of ten mice in 300 days of follow-up. The outbreak strain PG004 had a frameshift mutation in the *pkas15/1* sequence identical to the sequence of H37Rv, and it was no more resistant to RNIs than H37Rv. The most distinguishing feature of PG004 was its failure to produce well-organized, coalescing granulomas in mouse lungs. The lack of organized granulomas and reduced pathology may prevent restriction of PG004 in the lungs and allow it to spread into alveolar air spaces and escape the host to transmit to others. Humans with reduced lung pathology may remain undiagnosed and untreated in the community longer than those with severe disease. The over-representation of an *M. tuberculosis* strain in a community, therefore, may be more associated with strains that cause reduced rather than severe lung pathology.

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INTRODUCTION

In 2000, a large outbreak of tuberculosis was described, caused by a strain of *Mycobacterium tuberculosis* referred to as PG004 due to its polymorphic GC-rich tandem repeat sequence (PGRS) genotype designation (Chin *et al.*, 2000). Several other genotyping methods, including IS6110-based RFLP analysis and spoligotyping, were used to confirm that strain PG004 had been circulating in Contra Costa County

in northern California, USA, since 1992. Between 1996 and 2000, 503 culture-confirmed tuberculosis patients were reported in the county. Of these, 117 (23%) were infected with PG004; 26 additional cases were found in surrounding San Francisco Bay Area counties, making the total size of the cluster of PG004 infections 143 cases. An epidemiological study of 73 patients in this cluster out of 221 tuberculosis patients from Contra Costa County in 1996–1997 found that factors significantly associated with the development of disease included the failure to identify contacts in a timely manner and diagnostic delays of source-case patients (Chin *et al.*, 2000). However, these factors did not explain the large outbreak caused by a single strain of *M. tuberculosis*. It was postulated that PG004

Abbreviations: ASN, acidified sodium nitrite; IL, interleukin; PGL, phenolic glycolipid; PGRS, polymorphic GC-rich tandem repeat sequence; p.i., post-infection; RNIs, reactive nitrogen intermediates; ROIs, reactive oxygen intermediates; Th, T helper.

strain might have distinct biological characteristics that caused it to be over-represented in this community.

Similar suggestions have been made recently with *M. tuberculosis* strains associated with other large tuberculosis outbreaks. Strain CDC1551, which caused a large outbreak of tuberculosis in rural counties in Tennessee and Kentucky, USA, in 1994–1996 (Valway *et al.*, 1998), was found to elicit a more vigorous cytokine response in mice compared with laboratory strains, as evidenced by a higher production of tumour necrosis factor alpha, interleukin 6 (IL-6) and IL-12 (Manca *et al.*, 1999). This response was attributed to apolar lipids produced by this strain (Manca *et al.*, 1999). Another strain (HN878), belonging to a widely distributed Beijing family clade, which caused a prison outbreak in Texas, USA (Sreevatsan *et al.*, 1997), was found to express a highly biologically active lipid product, phenolic glycolipid (PGL), not detected in *M. tuberculosis* strain CDC1551 or the laboratory strains H37Rv and Erdman (Reed *et al.*, 2004). This strain was 'hyperlethal' in mice (Reed *et al.*, 2004).

Another widely disseminated strain, CB3.3, found in New York City in the 1990s, was found to be highly resistant to reactive nitrogen intermediates (RNIs) and hydrogen peroxide *in vitro* (Friedman *et al.*, 1997). Both CB3.3 and CDC1551 strains were found to be relatively more resistant to RNIs and reactive oxygen intermediates (ROIs) *in vitro* than the laboratory strains of *M. tuberculosis* and most other clinical isolates (Firmani & Riley, 2002a, b; Friedman *et al.*, 1997). More recently, a strain (CH) belonging to the East African–Indian lineage, which caused a large outbreak in Leicester, UK, was found to induce more anti-inflammatory IL-10 gene transcription in human monocyte-derived macrophages than strains CDC1551 and H37Rv (Newton *et al.*, 2006).

We studied PG004 to determine whether any of the biological characteristics previously found in other widespread *M. tuberculosis* strains were present in this Californian outbreak strain. Here, we report yet another phenotype associated with over-representation of a *M. tuberculosis* strain in the Californian community.

METHODS

Bacterial isolates. Two outbreak-associated *M. tuberculosis* strains with a PGRS designation of PG004 (IS6110 RFLP pattern designation 00708) and two non-outbreak-associated strains, CCC20 and CCC23, isolated from patients residing in the same community, were provided by the California State Department of Public Health and the Contra Costa County Health Department. Strains CCC20 and CCC23 had unique IS6110 RFLP patterns not represented by any other isolates in the collection of 2514 *M. tuberculosis* isolates. These strains were passaged no more than three times *in vitro* before use. Single-cell suspensions were prepared according to a published method (Grover *et al.*, 1967) and quantified by c.f.u. enumeration on Middlebrook 7H11 agar plates. *M. tuberculosis* H37Rv was used as a comparison strain. All of the strains were passaged once in mice before they were used for animal challenge experiments, as described below.

Mouse infection studies. Pathogen-free 8-week-old female BALB/c mice obtained from Charles River (Wilmington, MA, USA) were challenged via the tail vein with approximately 10^5 bacilli of each strain according to a previously reported procedure (Shimono *et al.*, 2003). Briefly, groups of five mice were infected with each mycobacterial strain for bacterial lung infection and histology studies, and groups of ten mice were infected with each mycobacterial strain for survival studies. The mice were monitored for signs of disease and then sacrificed. Differences in mortality over time were assessed using Kaplan–Meier survival analysis. Animal experimentation guidelines were followed in all murine experiments, as approved by the University of California, Berkeley, Institutional Animal Care and Use Committee.

Groups of five mice were euthanized at 1, 42 and 120 days post-infection (p.i.). The lungs and spleens were removed, homogenized, diluted and inoculated onto 7H11 agar plates for c.f.u. enumeration according to the procedure reported by Dunn & North (1995).

Histology slides were prepared commercially and examined by a veterinary pathologist specializing in mouse pathology who was blind to the information about the strains used to infect the mice. The left lung was removed from two mice at 42 and 120 days p.i., and fixed in 10 % buffered formalin, embedded in paraffin and processed. Sections were stained with haematoxylin and eosin for histological examination. At least two transverse sections of the upper third and lower third of the lung were examined for extent of damage, distribution of cell types, airway lesions and granuloma formation. Photomicrographs selected for the figures are representative of these sections obtained from two different animals.

RNIs susceptibility test. Several outbreak strains have been reported previously to be associated with increased resistance to RNIs (Firmani & Riley, 2002b; Friedman *et al.*, 1997). Therefore, one PG004 strain was compared against H37Rv for susceptibility to RNIs. Single-cell suspensions were prepared as described by Grover *et al.* (1967) and isolates were tested for resistance to RNIs in acidified sodium nitrite (ASN) as reported previously (Firmani & Riley, 2002b). The c.f.u. recovery from ASN-exposed strains was compared with that of strains exposed to ASN-free 7H9 broth. Comparison of the mean c.f.u. recovery of each strain, performed in triplicate, was done using Student's *t*-test, and a value of $P < 0.05$ was considered significant.

Comparison of the *pks15/1* sequence. The junction between the 3' end of *pks15* and the 5' end of *pks1* was amplified by PCR, and the product was sequenced, according to a published method (Camacho *et al.*, 2001). The sequences obtained from PG004, CCC20, CCC23 and H37Rv were aligned and compared visually.

RESULTS AND DISCUSSION

Mouse survival study

Both outbreak PG004 strains produced similar results in mice, and hence the results of only one strain are described below. Fig. 1(a) showed that the outbreak strain PG004, the non-outbreak strain CCC20 and the laboratory control strain H37Rv all grew at similar rates in the lungs of mice until day 42, at which point no further growth of strain CCC20 occurred. Strain CCC23, another non-outbreak strain, grew more slowly than the other strains throughout the experiment and did not achieve the same level of bacterial burden. There was no difference in bacterial growth in the spleen of mice infected with any of the strains (data not shown).

Fig. 1. (a) Growth of *M. tuberculosis* clinical strains in the lungs of BALB/c mice. Mice were infected intravenously via the tail vein with strain PG004 (■), CCC20 (▲), CCC23 (◆) or H37Rv (●). The bacterial load was calculated from c.f.u. enumeration on 7H11 agar plates of the culture of a lung or spleen homogenate after 3–5 weeks incubation at 37 °C. The infective dose was determined from c.f.u. recovery from organ homogenate 1 day after infection. Data are expressed as means ± SEM from four to five mice per time point. (b) Kaplan–Meier survival analysis of ten mice infected intravenously with 10⁵ c.f.u. PG004 (■), CCC20 (▼), CCC23 (◆) or H37Rv (●). This experiment was terminated before the mice infected with strain CCC23 had all died.

Infected mice were monitored for up to 300 days. The survival of mice infected with the outbreak isolate was not significantly different from that of mice infected with H37Rv ($P > 0.05$, Mantel–Haentzel test) (Fig. 1b). One non-outbreak strain (CCC23) caused death in only one out of ten mice during this period. This was the same strain that showed the lowest peak bacterial burden in lungs (Fig. 1a). Mice infected with this strain survived significantly longer than all other groups of mice ($P < 0.0001$). The other non-outbreak-associated strain, CCC20, caused accelerated mortality in mice. All of the mice infected with this strain were dead by 182 days compared with >250 days in the mice infected with the other strains ($P < 0.005$).

Histopathology

Lung pathology caused by each of the *M. tuberculosis* strains was examined histologically at 42 and 120 days p.i.

(Fig. 2). At day 42, the laboratory strain H37Rv produced a moderate to severe granulomatous pneumonia characterized by large multifocal to coalescing granulomas affecting 25–75 % of the lung parenchyma (Fig. 2; Table 1). The lungs of mice infected with the outbreak strain PG004 showed mild granulomatous pneumonia with scattered foci of small- to moderate-sized granulomas affecting less than 25 % of the lung parenchyma. Granulomas were composed of epithelioid and alveolar macrophages and a small number of lymphocytes. Many of the air spaces were preserved. At 42 days, lung sections of mice infected with the non-outbreak strain CCC20 had moderate granulomatous pneumonia affecting 25–75 % of the lung parenchyma. Granulomas were composed of alveolar macrophages and a few epithelioid macrophages and neutrophils. Much of the alveolar air space was filled with inflammatory cells. CCC23 invoked a granulomatous response similar to that of CCC20 at day 42.

At 120 days, the laboratory strain H37Rv produced severe granulomatous pneumonia characterized by large coalescing granulomas affecting more than 75 % of the lung (Fig. 2). Compared with day 42, the granulomas were composed of a denser cellular infiltrate of alveolar macrophages with few neutrophils scattered throughout the granulomas. The lymphocytic infiltrates around and within the granulomas were also denser than those at day 42. The outbreak strain PG004 produced moderate, rarely coalescing granulomatous pneumonia affecting 25–75 % of the lung. Granulomas were composed of epithelioid macrophages with infiltrates of lymphocytes and neutrophils. There were foci of necrosis within the granulomas and cholesterol cleft formation that were not seen at day 42. Mice infected with strain CCC20 had severe granulomatous pneumonia affecting nearly 100 % of the lung parenchyma. Granulomas were coalescing and composed of epithelioid macrophages with cellular necrosis and cholesterol cleft formation. At day 120, lungs infected with strain CCC23 had moderate multifocal granulomatous pneumonia affecting less than 25 % of the lung parenchyma. Granulomas were similar to those found at day 42; however, the lymphocytic infiltrates adjacent to and within granulomas were denser and cholesterol cleft formation was present.

RNI susceptibility

After exposure to 3 mM ASN, PG004 showed 56 % survival (as determined by comparison of c.f.u. recovery) and H37Rv showed 76 % survival compared with the respective ASN-unexposed control strains ($P > 0.1$); exposure to a 6 mM concentration of ASN produced 3 % survival in the PG004 strain and 11 % survival in H37Rv ($P > 0.2$).

pks15/1 gene sequence

The junction sequence of the polyketide synthase gene cluster *pks15/1* was aligned for strains PG004, CCC20,

Fig. 2. Lung histology at 42 and 120 days p.i. for mice infected with strain PG004, CCC20, CCC23 or H37Rv. Lungs were stained with haematoxylin and eosin. Magnification $\times 12.5$ (first and second columns) and $\times 100$ (third and fourth columns).

CCC23 and H37Rv (Fig. 3). Their sequences were compared with the published corresponding sequence of HN878, a strain reported to express PGL. The outbreak strain PG004 had the same sequence found in the laboratory strain H37Rv, whilst the two non-outbreak strains from the same community in California shared the same sequence found in HN878, with a 7 bp insertion.

Molecular epidemiology studies have shown that certain *M. tuberculosis* strains are over-represented in some communities. Whilst some of this over-representation can be explained by epidemiological or host-related factors, often there is no obvious explanation, and hence biological reasons for their enhanced transmissibility are sought. By definition, all human clinical isolates of *M. tuberculosis* are virulent; thus, the ability of a strain to cause active disease in a human host per se is not an explanation for its transmissibility.

Strain CDC1551, which caused an outbreak of tuberculosis and tuberculin skin test conversions in Tennessee and

Kentucky, was initially found to be more virulent in mice than laboratory strains (Valway *et al.*, 1998). However, subsequent studies have shown that, rather than being more virulent than H37Rv or Erdman, it induced a more vigorous pro-inflammatory cytokine response (Manca *et al.*, 1999). Apolar lipid fractions from CDC1551 induced higher levels of tumour necrosis factor alpha, IL-6 and IL12 by monocytes than those from the other strains.

Another outbreak strain (HN878) reported from a prison outbreak in Texas was found to express PGL, which had an inhibitory effect on the release of pro-inflammatory cytokines by macrophages (Reed *et al.*, 2004). The strain that expressed this lipid product belonged to a subset of the internationally widespread Beijing family. All strains belonging to this family share an identical *pk15/1* gene sequence, which is expressed as a single open reading frame (Camacho *et al.*, 2001; Reed *et al.*, 2004). Strains H37Rv, Erdman and CDC1551 have a natural frameshift mutation at this locus and they do not produce PGL. An extra 7 bp

Table 1. Histopathological examination of infected mouse lung sections

<i>M. tuberculosis</i> strain	Lung parenchyma lesion (%)	Granuloma distribution	Cell types (macrophages/neutrophils/lymphocyte distribution)	Necrosis/cholesterol cleft	Alveolar air spaces
42 days p.i.					
PG004	<25	Scattered foci	Alveolar and epithelioid macrophages, few lymphocytes	None	Mostly preserved
CCC20	25–75	Multifocal, nodular	Alveolar and a few epithelioid macrophages, neutrophils present, few lymphocytes	None	Filled with inflammatory cells
CCC23	<25	Small multifocal	Alveolar and epithelioid macrophages, few lymphocytes	None	Preserved
H37Rv	25–75	Multifocal, coalescing	Alveolar and epithelioid macrophages, dense lymphocytes	None	Filled with inflammatory cells
120 days p.i.					
PG004	25–75	Moderate, rarely coalescing	Epithelioid macrophages, neutrophils and lymphocytes present	Present, cholesterol cleft seen	Some spaces filled with inflammatory cells
CCC20	Nearly 100, severe granulomatous pneumonia	Large, coalescing	Epithelioid macrophages, neutrophils present, dense lymphocytes	Present, cholesterol cleft seen	Mostly filled with inflammatory cells
CCC23	<25	Moderate, multifocal	Dense epithelioid macrophages and lymphocytes	Absent, cholesterol cleft seen	Largely preserved
H37Rv	>75, severe granulomatous pneumonia	Large, coalescing	Dense, mostly alveolar macrophages, neutrophils scattered, dense lymphocytes	Present, cholesterol cleft seen	Mostly filled with inflammatory cells

sequence in *pks15/1* found in HN878 and some other clinical strains corrects the frameshift mutation, which generates a single *pks15/1* open reading frame and restores PGL production (Camacho *et al.*, 2001). It should be noted that an intact *pks15/1* sequence does not necessarily correlate with PGL production (Camacho *et al.*, 2001).

More recently, Newton *et al.* (2006) described yet another phenotype associated with a strain (CH) of *M. tuberculosis* that caused a large outbreak among school children in Leicester, UK, in 2001. Strain CH belongs to the so-called East African–Indian lineage of *M. tuberculosis* and was

shown to induce more IL-10 gene transcription than strain H37Rv or CDC1551 in human monocyte-derived macrophages. It was also more susceptible to hydrogen peroxide but not to nitric oxide than the other strains. It has a deletion affecting *Rv1519*, the function of which is unknown. The authors suggested that its anti-inflammatory effect on macrophages contributes to its persistence in the community and its potential for causing outbreaks (Newton *et al.*, 2006).

Resistance to RNIs and ROIs has been shown to be associated with the outbreak strain CB3.3 from New York

Fig. 3. Sequence alignment of the *pks15/1* junction locus. Strain CCC20, which was the most virulent in mice, had the same sequence as strain CCC23, which caused the least number of murine deaths; both were non-outbreak strains. This sequence was identical to that of the Beijing clade HN878, which caused an outbreak in Texas (Sreevatsan *et al.*, 1997). The Contra Costa County outbreak strain, PG004, had a *pks15/1* sequence identical to that of H37Rv.

City (Friedman *et al.*, 1997). However, strain RJ2E, which is most commonly distributed in Rio de Janeiro, Brazil, was no more resistant to RNIs and ROIs than the laboratory strains (Firmani & Riley, 2002b). In this study, strain PG004 from California did not exhibit resistance to RNIs when compared with H37Rv.

Here, we examined three clinical *M. tuberculosis* isolates from patients residing in the same community. The three strains examined (PG004, CCC20 and CCC23) were distinct by IS6110 RFLP pattern, PGRS and spoligotyping. Interestingly, all of the clinical isolates examined showed a distinct pattern of infection in mice. Strain CCC23, which was isolated only once in northern California among more than 2000 culture-confirmed cases, caused only one death among ten mice infected after 300 days, whilst another non-outbreak strain, CCC20, also seen only once in the same community, caused death in all mice after only 182 days. Although the latter did not attain a high bacterial burden compared with the other isolates, it caused more extensive lung damage and a faster time to death in mice.

Interestingly, in our study, the isolates associated with the outbreak (PG004) did not grow any faster or attain a higher bacterial burden than isolates seen only once during the study period or than strain H37Rv. These observations suggest that the ability of *M. tuberculosis* to cause outbreaks is not necessarily correlated with its rate of growth in a host or the ability to cause severe disease or death in the mouse model.

PG004 was found to have the same frameshift mutation in the *pks15/1* locus as that found in strains H37Rv, Erdman and CDC1551 (Fig. 3). Interestingly, the most virulent (CCC20) and least virulent (CCC23) strain in mice each shared the same *pks15/1* sequence found in HN878 (also known as strain 210) (Fig. 3). Hence, in this study, *pks15/1* locus sequence differences did not correlate with mouse lung lesions and cannot explain the outbreak caused by PG004 in Contra Costa County. This finding is consistent with a recent report from Thailand that showed that an intact *pks15/1* sequence is found among many non-Beijing family clinical *M. tuberculosis* strains isolated in similar proportions (>80%) from patients with severe manifestations of tuberculosis such as tuberculosis meningitis, as well as from those with lung disease (Chaiprasert *et al.*, 2006).

The most striking feature of PG004 was the histopathological changes it produced in the mouse lung: the granulomas were composed of pro-inflammatory cells that rarely coalesced. Alveolar air spaces in mice infected with the outbreak strain were visible even after 120 days of infection, and yet mice infected with CCC20, the non-outbreak strain, showed no visible air spaces, despite having the same bacterial burden (Fig. 2). Mice infected with the other non-outbreak strain (CCC23) had preserved air spaces, but the granulomas were densely packed with lymphocytes and had a lower bacterial burden after the same duration of infection (Fig. 1a). The dense infiltration of inflammatory cells and coalesced granulomas may restrict tissue spread of the tubercle bacilli and thus

prevent their entry into the alveolar air space. Reduced lymphocyte infiltration observed in mice infected with the outbreak strain may preclude effective containment of the viable bacilli inside granulomas, allowing the bacilli to enter the alveolar air space to escape the host and infect others. Whilst acknowledging that this observation made in a mouse model may not apply to the human disease, this differential host response elicited by PG004 may explain its over-representation in the Californian community.

It is often noted that patients with a cavitary lung lesion or granulomas that erode into bronchial trees transmit *M. tuberculosis* at a higher frequency than those without these lesions. This may be true, but such patients, due to the severity of their disease, are also more likely to be recognized, diagnosed and initiated on treatment sooner than those with less severe disease. Those with indolent, less severe disease are likely to remain undetected in the community for longer and have a greater opportunity to transmit their infection to their close contacts. This could potentially explain the over-representation of PG004 in one Californian community.

The above observation for PG004 could also explain the spread of strains such as HN878. This strain, although hypervirulent in mice, was reduced in its ability to elicit a strong T helper (Th)1-type immune response in mice (Reed *et al.*, 2004). Although not tested in mice, another large outbreak strain, CH, from the UK was found to induce an enhanced anti-inflammatory cytokine response in macrophages (Newton *et al.*, 2006). A reduced Th1-type immune response would affect the cell-mediated immune response and might preclude bacterial clearance and restriction of bacterial spread, which could lead to the strain's enhanced transmission to other hosts. A reduced Th1 response has also been shown to be associated with a hypervirulent phenotype in a mutant *M. tuberculosis* strain disrupted in its *mce1* operon (Shimono *et al.*, 2003). The *mce1* mutant was found to be unable to establish a tightly organized granuloma in mouse lungs and was reduced in its ability to elicit pro-inflammatory cytokines in murine macrophages infected *ex vivo* (Shimono *et al.*, 2003).

Thus, a *M. tuberculosis* strain associated with one of the largest tuberculosis outbreaks in the USA was found to elicit a distinct lung pathology in mice. Whether this phenotype observed in mice is related to its ability to cause an outbreak in a human community is not clearly established, but this phenotype appears to be a distinct biological property of a clinical *M. tuberculosis* isolate that to the best of our knowledge has not been described previously. Bacterial factors that elicit this pathology are not known, but the identification of such factors may lead to a better understanding of tuberculosis transmission in general.

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