Effect of serial subculturing on the genetic composition and cytotoxic activity of *Mycobacterium tuberculosis*

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Continuous subculture has been observed to produce changes in the virulence of micro-organisms, e.g. rabies virus, poliovirus and *Mycobacterium bovis* BCG. The latter has been used as a vaccine for tuberculosis for the last 100 years; however, in some instances its efficacy has been observed to be very low. In order to determine whether similar changes can be produced in *Mycobacterium tuberculosis*, we selected four isolates, *M. tuberculosis* H37Rv, a Beijing strain (DR-689), and two more isolates with deletion of the phospholipase C locus (*plcA-plcB-plcC*), and subjected them to serial culturing on Middlebrook 7H9 medium, with or without ox bile. After 100 passages, we performed RFLP-IS6110 analysis to determine whether genomic changes were produced. We also checked their genomic composition by microarray analysis. Changes in virulence were studied by measuring the cytotoxic effect of parental and subcultured isolates on a THP-1 macrophage monolayer. The most visible change was the change of position of an IS6110 band of ~1400 bp to ~1600 bp in the Beijing isolate subcultured in the ox bile medium. Analysis by microarray and PCR confirmation did not reveal any genomic changes. Cytotoxic activity was decreased in the isolates at levels close to that of BCG, and more consistently in those subcultured in the presence of ox bile.

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INTRODUCTION

According to estimates by the World Health Organization in 2007, an estimated 1.3 million deaths occurred among HIV-negative incident cases of tuberculosis (TB) and there were an additional 456 000 deaths among incident TB cases who were HIV-positive (WHO, 2009). The AIDS pandemic has complicated this problem even more. About 8% of AIDS patients develop TB (Small *et al.*, 1993; van Rie *et al.*, 1999). Although it is considered a treatable infection, isolates resistant to first-line anti-tuberculous drugs, and more recently to first- and second-line compounds (extensively drug-resistant TB), have been appearing (CDC, 2006). Therefore, it is important to create a better way to control this pandemic. Although many efforts have been made to obtain a vaccine based on genetically engineered bacteria, particularly BCG, pox virus or DNA encoding immunodominant antigens of *Mycobacterium tuberculosis*, to date there is nothing available for human use (Gupta *et al.*, 2007; Hoft, 2008; Ly & McMurray, 2008).

The only vaccine in use against TB was developed by Calmette and Guérin at the beginning of the 20th century by serial passaging a *Mycobacterium bovis* isolate on potato slices imbibed with glycerol and ox bile and monitoring its loss of virulence for several years (between 1908 and 1921) (Calmette & Guérin, 1909). The use of this as a vaccine strain started in 1948 in Europe, and afterwards it was expanded to other regions of the world (Martín *et al.*, 2007). After this, BCG strains underwent up to 1000 passages until the introduction of archival seed lots in the 1960s (Brosch *et al.*, 2007). The outcome has been many strains with phenotypic differences which possess a different ability to induce protection versus TB (Brosch *et al.*, 2007; Clemens *et al.*, 1983). In spite of this, the BCG vaccine is still the most used vaccine around the world.

At the time that BCG was prepared, the reason for the decrease in virulence was not known. Recently, complete sequencing of the genomes of *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur has allowed us to better understand their biological changes (Brosch *et al.*, 2007; Clemens *et al.*, 1983; Cole *et al.*, 1998; Kato-Maeda *et al.*, 2001). Genomic

Abbreviations: PDIM, phthiocerol dimycocerosate; TB, tuberculosis.

deletions and duplications are found in the *M. bovis* BCG chromosome. One of them, RD1, is considered to correlate with the loss of virulence. Its restitution re-establishes most of the virulent properties of *M. bovis* (Brosch *et al.*, 2007; Guinn *et al.*, 2004; Hsu *et al.*, 2003; Lewis *et al.*, 2003; Mahairas *et al.*, 1996; Stanley *et al.*, 2003).

The effect of serial passage *in vitro* of *M. tuberculosis* has been reported; van Soolingen *et al.* (1991) investigated the stability of the IS6110 insertion element. In their study, a passage *in vitro* of six *M. tuberculosis* strains and two *M. bovis* BCG strains was done every week for 6 months in a liquid culture medium. Furthermore, they investigated whether serial passage in increasing concentrations of antimicrobial drugs would select drug-resistant mutants with modified fingerprints. No change was observed in the banding patterns of the IS6110 elements in any of the strains passaged on drug-free or drug-containing media (van Soolingen *et al.*, 1991).

Aranaz *et al.* (2004) reported a change in the hybridization profiles (spoligotyping) of isogenic strains (a clinical isolate and the derivative strain cultured several times), caused by a deletion in the direct variable repeat. In this study, the serial passage had been done only eight times.

Recently, it has been reported that the *in vitro* culture passaging of the laboratory strain H37Rv can reduce its ability to synthesize the cell wall lipid phthiocerol dimycocerosate (PDIM), an important *M. tuberculosis* virulence factor (Domenech & Reed, 2009; Andreu & Gibert, 2008). This loss is irreversible and related to genetic changes, particularly the deletion of the *pps* and *drr* operons (Domenech & Reed, 2009).

Based on these observations, we consider that extended subculturing of *M. tuberculosis* may produce mutations affecting its genetic and/or virulence characteristics. In order to study this hypothesis, we subcultured 100 times several *M. tuberculosis* strains, including *M. tuberculosis* H37Rv and three more strains lacking the *plcA-plcB-plcC* locus, including one isolate belonging to the Beijing group. Gross genetic changes of their IS6110 elements were measured by RFLP analysis and by spoligotyping. Minor changes were studied by microarray assays. Changes in their cytotoxic activity on a macrophage monolayer were determined as a measure of their virulence, and neutral red staining was used to detect changes in the cell wall PDIMs.

METHODS

Mycobacterial strains. For this study, three *M. tuberculosis* strains [donated by Kristin Kremer from the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands] were used. These isolates have the common characteristic that they are $\Delta plcA-plcB-plcC$; their genetic characteristics were described by Vera-Cabrera *et al.* (2007). Strain DR-689 belongs to the 'Beijing' cluster. All strains were grown on Löwenstein–Jensen agar and the stocks were prepared by growing on Middlebrook 7H9 medium with Tween 80 supplemented with oleic acid, bovine albumin, dextrose and catalase (OADC). The cultures were grown to mid-exponential

phase, and stored at -70°C until needed. Cell density for each stock culture was determined in triplicate using Middlebrook 7H10 agar. *M. bovis* BCG Pasteur was used as a control of attenuated mycobacteria.

DNA extraction. DNA was extracted from the colonies on Löwenstein–Jensen agar using the CTAB–NaCl technique with some modifications (Wilson, 1987).

Determination of optimum ox bile concentration for mycobacterial growth. Calmette & Guérin (1909) originally used the addition of ox bile (Difco) to emulsify the bacterial cultures. Since bile salts have also been observed to induce genetic changes, we decided to utilize ox bile in our experiments. In order to determine the concentration of ox bile in the cultures, a series of culture media were prepared using Middlebrook 7H9–Tween 80–OADC with ox bile at 0.5, 1, 2.5, 5, 7.5, 10 and 25% (w/v); we also tested potato dextrose broth with ox bile at the same concentrations. The tubes with the media were inoculated with a suspension of a young culture of *M. tuberculosis* H37Rv and incubated at 37°C ; turbidity was observed and registered at 7, 14, 21 and 28 days.

Fitness curve of the *M. tuberculosis* strains in medium with and without ox bile. Bacterial suspensions from liquid cultures were prepared and their OD₆₀₀ was adjusted to between 0.5 and 0.7 (equivalent to 1×10^7 c.f.u.). Ten microlitres of each of these suspensions was inoculated into 50 ml Erlenmeyer flasks containing 10 ml M7H9. The cultures were done in triplicate, and the content of the flasks was incubated at 37°C with constant agitation. Five hundred microlitre aliquots were taken and the OD₆₀₀ was measured using 1 cm cuvettes with a MBA 2000 spectrophotometer (Perkin-Elmer). The readings were taken for 18 days, and the optical density was plotted against incubation time.

Serial passaging of the strains. In order to obtain a homogeneous population, we first 'cloned' the isolates by separating individual colonies on Middlebrook 7H10 agar. Each individual colony was then separated three times; after that, we started the serial passaging. From the cloned strains one colony was taken and inoculated into 25 ml Erlenmeyer flasks containing 7.5 ml of either M7H9–Tween 80–OADC, or the same medium plus 0.5% (w/v) ox bile. Cultures were incubated at 37°C with constant agitation at 150 r.p.m. for 5 days. The passage was done using a sterile pasteur pipette, using a drop from the culture in exponential phase to inoculate a flask containing fresh medium, until completing 100 passages. Every 10 passages, an aliquot was taken and stored in M7H9 with 5% (v/v) glycerol at -70°C .

Once we reached 100 serial passages, genomic DNA was extracted, and bacterial suspensions were prepared from each isolate.

RFLP analysis for IS6110. Southern blotting with labelled IS6110 DNA was performed as previously described using an international protocol (van Embden *et al.*, 1993). Briefly, *M. tuberculosis* DNA was digested with *Pvu*II, electrophoresed, and hybridized with a labelled 245 bp PCR-generated probe as previously described.

Spoligotyping. This assay was carried out with the same objective as RFLP. The spoligotyping method described by Kamerbeek *et al.* (1997) was used. PCR amplification of the direct repeat locus was performed on heat-treated cell suspensions. Biotin-labelled PCR products were detected by hybridization onto a spoligotyping membrane (Isogen Bioscience).

Microarray analysis. The customized arrays were produced to our specifications by Oxford Gene Technology using Agilent technology carrying 21 725 non-redundant oligonucleotides (60-mers) essentially

covering all parts of the genomes of *M. tuberculosis* strains H37Rv and CDC1551, and *M. bovis* strains AF2122/97 and BCG Pasteur. On average, there was an oligonucleotide probe every 203 bases.

PCR for deletions reported in microarray analysis. In order to verify any deletions observed in the microarray analysis, a set of primers was designed using Primer Select (Lasergene DNASTAR) software for each of the absent genes according to the microarray analysis. PCRs were carried out using 25 µl reaction mix, which contained 50 nM of each primer, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl₂, 200 µM nucleotide mix, 1 U *Taq* polymerase and 50 ng of the DNA template; the volume was adjusted with sterile water. As a positive control, we used *M. tuberculosis* H37Rv DNA. The PCRs were done in an MJ Research thermal cycler. Amplicons were visualized by electrophoresis on a 1.2 % (w/v) agarose gel and amplicon sizes were compared using a 100 bp marker (Promega).

Monocyte cell cultures. Human monocyte cell line THP-1 was maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10 % (v/v) fetal bovine serum (Gibco-BRL) and 1 mM sodium pyruvate (Sigma). In order to transform the cells into macrophages, the cells were subcultured four times without sodium pyruvate and then seeded into 24-well microplates (Costar Corning) at a concentration of 4×10^5 cells per well in complete RPMI 1640 supplemented with phorbol 12-myristate 13-acetate (Calbiochem Biosciences) at a concentration of 6.25 ng ml⁻¹. Cell cultures were washed twice with RPMI 1640 every 48 h for no longer than 4 days.

Mycobacterial cytotoxicity assay. The cytotoxicity assays were done according to the technique previously reported by Castro-Garza *et al.* (2007). Before the beginning of the experiment, bacteria were thawed at 37 °C, mixed vigorously using a vortex, and diluted in tissue-culture medium to obtain the desired density to infect the cells with an m.o.i. of 10:1 (cells:bacilli). Macrophage (THP-1 cell line) monolayers were infected in triplicate with the *M. tuberculosis* strains tested. At 24 h post-infection, the wells were washed twice with pre-warmed media at 37 °C. Later on days 1, 4 and 7, the culture medium from three wells from each strain and controls was discarded and the cells were fixed in 10 % (v/v) buffered formalin for at least 24 h at 4 °C. A different microplate was used for each time point to avoid the effect of formaldehyde vapour on the rest of the cell culture. Subsequently, cytotoxicity was quantified by crystal violet assay (Castro-Garza *et al.*, 2007). The quantitative analysis of cytotoxicity on the stained cultures infected with *M. tuberculosis* was based on the percentage of dead macrophages, employing the following equation: percentage of dead macrophages = $[(\text{control OD}_{600} - \text{sample OD}_{600}) / \text{control OD}_{600}] \times 100$. All experiments were repeated in triplicate. Statistically significant differences among the groups were assessed by the variance ANOVA test using multiple comparisons. We compared the cytotoxicity value at days 1, 4 and 7 of parental against subcultured strains in both media, against *M. bovis* BCG, and the passage number 100 in ox bile against passage number 100 without ox bile and against *M. bovis* BCG.

Neutral red staining. In order to detect changes in the cell wall PDIMs, neutral red staining was performed as previously described (Soto *et al.*, 2002) but adapted to microcentrifuge tubes (Andreu & Gibert, 2008). *M. tuberculosis* H37Rv was stained as a positive control. Bacterial growth on Middlebrook 7H10 was washed twice with 1 ml 50 % methanol for 1 h at 37 °C with regular shaking. Then, 1 ml 0.002 % neutral red in barbital buffer (5 % NaCl in 1 % sodium barbital, pH 9.8) was added to the cells, which were incubated at 37 °C with occasional agitation. Results were evaluated after 1 h and 24 h.

RESULTS

Determination of optimum ox bile concentration for mycobacterial growth

After inoculating M7H9/OADC media containing several concentrations of ox bile, the turbidity was registered visually. The best growth was observed in M7H9/OADC with 0.5 % (w/v) ox bile; this bile concentration was chosen for the next subcultures. The *M. tuberculosis* isolates did not show any growth in the potato-dextrose broth, therefore this medium was discarded for future experiments.

Fitness growth curve of *M. tuberculosis* strains in medium with and without ox bile

All *M. tuberculosis* strains had grown by 18 days in Middlebrook 7H9/OADC and Middlebrook 7H9/OADC + 0.5 % (w/v) ox bile. The results are shown in Fig. 1. Isolates grown in medium without bile developed remarkably faster than those cultured in medium plus bile. In order to do the serial passaging, we selected 5 days, when most of the isolates had reached the exponential phase.

RFLP

In Fig. 2, we show the IS6110 RFLP patterns of the parental and subcultured strains, which were identical except for that of strain DR-689 grown on medium with bile added, which showed movement of one band from ~1400 bp to 1600 bp.

Spoligotyping

None of the strains subjected to serial passage showed any change using this genotyping method (not shown), demonstrating the stability of the chromosomal DR region in the strains analysed here.

Microarray analysis

Microarray analysis for these specific *M. tuberculosis* isolates has been reported before (Vera-Cabrera *et al.*, 2007). In the new analysis, after 100 passages some genes were observed to be absent; however, in the analysis done by PCR, they were confirmed to be present and constituted false-negatives.

Mycobacterial cytotoxicity assay

The *M. tuberculosis* isolates were incubated on a THP-1 macrophage monolayer in order to determine whether changes in cytotoxicity were produced. In Fig. 3, we show the effect of the original isolates and the isolates subcultured 100 times with and without ox bile on the macrophage monolayer. Parental strains H37Rv, DR-689, RIVM 97-0448 and RIVM 97-1177 produced a greater than

Fig. 1. Growth curve of the mycobacterial isolates in Middlebrook 7H9 with (left) and without (right) ox bile. The OD₆₀₀ of the cultures was plotted against time of incubation. For this experiment, *M. tuberculosis* H37Rv (Δ), *M. tuberculosis* DR-689 (▼), *M. tuberculosis* RIVM 97-0448 (●) and *M. tuberculosis* RIVM 97-1177 (○) were used.

70 % cytotoxic effect on the macrophage monolayer at 7 days post-infection; however, strains subjected to serial

Fig. 2. RFLP analysis by Southern blotting for IS6110. Lanes: 1, *M. tuberculosis* DR-689 without subculturing; 2, *M. tuberculosis* DR-689 subcultured 100 times in Middlebrook 7H9; 3, *M. tuberculosis* DR-689 subcultured 100 times in Middlebrook 7H9 plus ox bile; 4, *M. tuberculosis* H37Rv without subculturing; 5, *M. tuberculosis* H37Rv subcultured 100 times in Middlebrook 7H9; 6, *M. tuberculosis* H37Rv subcultured 100 times in Middlebrook 7H9 plus ox bile. The arrow shows the change of the position of the IS6110 element.

passage produced a much lower cytotoxicity. The highest value was produced by RIVM 97-0448 subcultured 100 times without ox bile (50 % cytotoxicity). All the other subcultured isolates produced less than 20 % cytotoxicity. *M. bovis* BCG Pasteur showed cytotoxicity close to 0 % at 7 days of incubation. At 7 days, statistically significant differences were found in the analysis of the cytotoxic activity of the noncultured isolates against the subcultured isolates tested ($P < 0.05$). In the case of RIVM 97-0448 and RIVM 97-1177, there was no statistically significant difference in the cytotoxic activity of the bacterial cells subcultured in the presence of bile and of *M. bovis* BCG.

When observed under the microscope, the non-subcultured isolates presented extensive destruction of the monolayer, and abundant acid-fast bacilli were observed; outside the phagocytes they formed cumuli and cords of mycobacteria (Fig. 4). The mycobacteria subcultured 100 times presented much less destruction of the monolayer, bacilli were scarce and most were present inside the macrophages, in a similar way to that observed with BCG Pasteur, which was used as a control of attenuated bacteria.

Neutral red staining

We analysed the change in the neutral red phenotype of *M. tuberculosis* parental and subcultured strain colonies growing on M7H10 agar. All of the strains subjected to serial passage kept a similar colour to that of the parental strains.

DISCUSSION

It has been observed that *in vitro* serial passage of micro-organisms may produce important changes in their phenotypic characteristics (Basombrio *et al.*, 2000;

Fig. 3. Cytotoxic activity of *M. tuberculosis* isolates on the THP-1 macrophage monolayer. Culture plate wells were seeded with 4×10^5 macrophages and infected with the bacilli at an m.o.i. of 1:10. For this assay we utilized the non-subcultured isolates (●), the isolates subcultured 100 times in Middlebrook 7H9 (○) and the isolates subcultured 100 times in Middlebrook 7H9 plus bile (▼). *M. bovis* BCG Pasteur (△) was utilized as a control of attenuation.

González-Garza *et al.*, 2000; Jennings & Potter, 1999); attenuated micro-organisms obtained by this means include rabies virus, poliovirus, measles virus and *M. bovis* BCG, which have been very important in the fight against infectious disease over the last 110 years. We studied the effect on genomic composition and virulence of *M. tuberculosis* after 100 serial subcultures. The culturing was carried out in liquid culture medium (M7H9). We also used M7H9+ox bile to emulate the composition of the media used by Calmette and Guérin. The bile is composed of salts that act as detergents emulsifying fatty acids, and it has been observed that this can cause DNA damage in *Salmonella enterica* and induce precise rearrangements and mutations (Prieto *et al.*, 2004). Ox bile can have similar effects on the DNA of *Escherichia coli*, as observed by Bernstein *et al.* (1999), where exposure to bile salts induced oxidative stress chain genes producing damage to DNA. Evidence that bile salts cause base oxidative damage has also been obtained in eukaryotic cells (Bernstein *et al.*, 2005). Several lines of evidence indicate that bile salts may cause oxidative damage: (i) exposure to bile induces GC/AT transitions; (ii) sodium deoxycholate activates tran-

scription of genes belonging to the oxidative-damage-responsive OxyR and SoxSR regulons. We observed changes in the RFLP for IS6110 in one of the isolates utilized (DR-689), but only in the isolate subcultured with ox bile. It seems that this substance may be an important inducer of genetic changes. Although we have not determined the changes in the transposition site of this IS6110 element, it has been observed that these elements can modify the expression or transcription of neighbouring genes, which can give rise to important biological bacterial changes (McEvoy *et al.*, 2007; Wall *et al.*, 1999).

When we looked for other genetic changes, either in the DR locus or in the rest of the genome, we could not detect any. *M. bovis* BCG was subcultured about 230 times, which resulted in severe changes in its genomic composition, including two large duplications and multiple deletions around the bacterial chromosome. However, it is not mandatory to have enormous changes in the DNA to have important biological differences, as has been observed in *M. tuberculosis* H37Ra, where point mutations in the transcriptional regulator *phoP* gene contribute importantly

Fig. 4. Microscopic features of THP-1 monolayers after 7 days of infection with *M. tuberculosis* RIVM 97-1177. (a, b) Monolayer infected with the isolate without subculturing at $\times 10$ and $\times 100$, respectively. (c, d) Monolayer infected with the isolate subcultured in Middlebrook 7H9 plus bile at $\times 10$ and $\times 100$, respectively.

to its avirulence (Chesne-Seck *et al.*, 2008; Zheng *et al.*, 2008).

M. tuberculosis virulence has been determined in animal and cell culture models. The ability of the bacilli to produce granulomas, disseminate in the lung tissue and produce lethality in mice has been used as a marker of virulence (Abdul-Majid *et al.*, 2008; Lewis *et al.*, 2003; Shimono *et al.*, 2003). Cell culture models using pneumocytes or macrophages are adequate for measuring cytotoxicity, which reflects the virulence of the different strains studied (Castro-Garza *et al.*, 2002; Danelishvili *et al.*, 2003; Dobos *et al.*, 2000; McDonough & Kress, 1995). In order to analyse the virulence of the different strains used in our study, as well as the strains obtained after subculturing, we used the crystal violet assay in macrophages derived from the THP-1 cell line (Castro-Garza *et al.*, 2007). In our case, we observed a dramatic decrease in the ability to destroy the macrophage monolayer to levels close to those produced by BCG. Other authors have obtained avirulent or low virulence derived strains. The best known is the case of *M. tuberculosis* H37Ra obtained by leaving the original

culture for months and selecting a smooth colony derived from the original (Steenken, 1950). *M. tuberculosis* H37Ra avirulence has been explained by a decrease in the synthesis of the superficial lipids, which seem to be essential for *M. tuberculosis* virulence (Chesne-Seck *et al.*, 2008; Zheng *et al.*, 2008). This hypothesis has been confirmed by recent findings in the *M. tuberculosis* H37Ra genome (Zheng *et al.*, 2008). Other authors have subcultured the H37Rv strain for a period of 2 years and not observed changes in virulence (van Soolingen *et al.*, 1991). However, they did not use ox bile and performed the subcultures every 4–6 weeks, reaching a total of 20 passages. In our experiments, we subcultured every 5 days, 100 times, and we observed a decrease in cytotoxicity for the macrophage monolayer, most evidently in the bacteria subcultured in 7H10 plus ox bile. Cardona *et al.* (2006) subcultured for 1 year a clinical isolate that was already deficient in the production of cell wall sulfolipids (*M. tuberculosis* MT103), which showed a decrease in lethality for SCID mice. This attenuation was linked to a negative neutral red reaction and to a deficiency in the synthesis of PDIMs. More recently, Domenech & Reed (2009) observed that after 20 subcultures, *M.*

tuberculosis H37Rv loses the ability to produce the cell wall lipid PDIMs. By DNA microarray analysis they observed the deletion of several genes involved in mycobacterial lipid metabolism, including both the *pps* and *drr* operons.

Based on these studies, we performed neutral red staining of our subcultured isolates and did not observe changes in the phenotype. It is possible that after this length of time the isolates have not changed their cell-wall composition. We intend to continue the subculturing for 150 more passages to determine whether more genetic and biological changes are produced as with *M. bovis* BCG, which required 230 passages to be completely avirulent for immunocompetent animals. Neutral red staining will be a quick method to determine changes associated with virulence, although it will be necessary to perform animal experiments and determine the complete genome sequences of the serially passaged strains.

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