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Original article

Neutral-red reaction is related to virulence and cell wall methyl-branched lipids in *Mycobacterium tuberculosis*

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Abstract

Searching for virulence marking tests for *Mycobacterium tuberculosis*, Dubos and Middlebrook reported in 1948 that in an alkaline aqueous solution of neutral-red, the cells of the virulent H37Rv *M. tuberculosis* strain fixed the dye and became red in color, whereas the cells of the avirulent H37Ra *M. tuberculosis* strain remained unstained. In the 1950 and 1960s, fresh isolates of *M. tuberculosis* were tested for this neutral-red cytochemical reaction and it was reported that they were neutral-red positive, whereas other mycobacteria of diverse environmental origins that were non-pathogenic for guinea pigs were neutral-red negative. However, neutral-red has not really been proven to be a virulence marker. To test if virulence is in fact correlated to neutral-red, we studied a clinical isolate of *M. tuberculosis* that was originally neutral-red positive but, after more than 1 year passing through culture mediums, turned neutral-red negative. We found that, in comparison to the original neutral-red positive strain, this neutral-red negative variant was attenuated in two murine models of experimental tuberculosis. Lipid analysis showed that this neutral-red negative natural mutant lost the capacity to synthesize pthiocerol dimycocerosates, a cell wall methyl-branched lipid that has been related to virulence in *M. tuberculosis*. We also studied the neutral-red of different gene-targeted *M. tuberculosis* mutants unable to produce pthiocerol dimycocerosates or other cell wall methyl-branched lipids such as sulfolipids, and polyacyltrehaloses. We found a negative neutral-red reaction in mutants that were deficient in more than one type of methyl-branched lipids. We conclude that neutral-red is indeed a marker of virulence and it indicates important perturbations in the external surface of *M. tuberculosis* cells.

Keywords: Mycobacterium tuberculosis; Virulence; Neutral-red

1. Introduction

It is estimated that the etiologic agent of human tuberculosis, *Mycobacterium tuberculosis*, infects one-third of the world's population and kills 2 million people each year [1]. These data clearly indicate that new vaccines and drugs are needed to control tuberculosis. To develop these new antituberculosis agents, it is crucial to understand *M. tuberculosis–host* interaction. After about 100 years of research, knowledge of the cellular components responsible for *M. tuberculosis* pathogenicity is still limited. Throughout this time, the virulence of *M. tuberculosis* strains has been measured using experimental animal models, ascertaining which bacterial deficiencies or modifications affect the progression of the disease. The successful development of methods for creating mutations in specific genes combined with studying these mutants in animal models has enabled several viru-

Abbreviations: BAL, broncoalveolar liquid; DIM, pthiocerol dimycocerosates; HPRT, hypoxanthine guanine phosphoribosyl transferase; IFN-γ, interferon-gamma; iNOS, isoform of nitric oxide synthetase; NR, neutral-red cytochemical reaction; PAT, polyacyltrehaloses; RANTES, regulated upon activation normal T-cell expressed and secreted; SL, sulfolipids; TLC, thin-layer chromatography; TNF, tumor necrosis factor.

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lence factors in *M. tuberculosis* to be described. An excellent review of the determinants of virulence in *M. tuberculosis* has recently been published [2]. On the other hand, interesting observations concerning the virulence of tubercle bacilli have also been carried out by studying natural mutants. One of the most analyzed natural mutant has been the H37Ra strain.

In 1934, Steeken et al. [3] observed that the H37 strain of *M. tuberculosis* produced two distinct forms of colonies on egg plates and that one of these forms was less virulent in guinea pigs. The experiments continued until two stable strains were obtained, the virulent (H37Rv) and the avirulent (H37Ra) [3–5]. Unlike H37Rv, H37Ra was unable to produce progressive disease either in guinea pigs or in mice. Many comparative analyses between H37Ra and H37Rv were undertaken in order to search for cellular structures of tubercle bacilli related to virulence. In 1948, Dubos and Middlebrook [6] described the neutral-red cytochemical reaction (NR) that clearly distinguishes H37Rv from H37Ra. They reported that, in an alkaline aqueous solution of neutral-red, the cells of the virulent H37Rv *M. tuberculosis* strain fixed the dye and became red in color (NR positive), whereas the cells of the avirulent strain H37Ra remained unstained in the same conditions (NR negative). In the following years, NR was evaluated and the resulting studies showed coinciding results, namely, that *M. tuberculosis* complex freshly isolated strains were NR positive, whereas other mycobacteria of diverse environmental origins that were non-pathogenic for guinea pigs were, with few exceptions, NR negative [7–9].

In 1959, Middlebrook et al. [10] studied the cell wall components responsible for NR in H37Ra and H37Rv, and established a positive correlation between the content of sulfolipids (SL) and a positive NR in H37Rv. Recently, a *pks2* mutant of *M. tuberculosis* H37Rv unable to produce SL has been generated [11]. We have analyzed this mutant for NR and it is NR positive; SL are therefore not responsible for the NR positive character in H37Rv [12]. Consequently, the cell wall components responsible for this striking difference between virulent H37Rv and avirulent H37Ra strains of *M. tuberculosis* remain unknown. It is not the objective of this work to identify these components; before undertaking research on the components related to NR, we believe more evidence on the relationship between NR and virulence in *M. tuberculosis* strains needs to be obtained. With this aim in mind, we have studied an *M. tuberculosis* strain (originally both deficient in SL and NR positive) isolated from an immunocompetent tuberculosis patient [13]. After more than 1 year of passages in culture media, this strain turned NR negative. We analyzed the virulence of the two NR variants of the *M. tuberculosis* strain in immunocompetent and immunodeficient mice. We also analyzed the content of the cell wall lipids of these two NR variants.

As cell wall methyl-branched lipids have been related to virulence in *M. tuberculosis* [14], we studied NR in different *M. tuberculosis*-constructed mutants unable to produce some of these lipids.

2. Materials and methods

2.1. Bacterial strains

The *M. tuberculosis* strains used in this study were the two reference strains H37Rv (ATCC 27294) and H37Ra (ATCC 25177); the MT103 *M. tuberculosis* strain, a clinical strain isolated from an immunocompetent patient [15]; an NR negative natural mutant of the MT103 strain, renamed MT103a; the MT103-derived mutants deficient in the expression of *fadD26, fadD28*, *mmpL7* and *drrC* genes and unable to synthesize pthiocerol dimycocerosates (DIM) or translocate DIM into the cell wall [13,15]; the complemented *drrC* deficient mutant with *drrC* gene (*drrCc*), this complementation restored the localization of DIM in the cell wall [13]; the H37Rv-mutants deficient in the expression of the *pks2, msl3* and *pks12* genes and unable to synthesize SL [11], polyacyltrehaloses (PAT) [16] and DIM [17], respectively.

2.2. Neutral-red staining

Neutral-red staining was performed in a test tube, as described previously [18]. Briefly, mycobacterial strains were grown on Middlebrook 7H10 medium at 37 °C. When required, the following antibiotics were used at the specified concentrations: kanamycin (25 μg/ml) and hygromycin B (100 μg/ml). Bacterial cells were placed in screw-cap tubes containing 5 ml of 50% aqueous methanol, and washed twice for 1 h, each time at 37 °C. Following this, a solution of 0.002% neutral-red in barbital buffer (1% sodium barbital in 5% NaCl, pH 9.8) was added to the washed cells; the results were evaluated after 1 h.

2.3. Mouse model

Our study was performed using specific pathogen-free C57BL/6 and CB-17 *scid/scid* (SCID) female mice, 6–8 weeks old, which were obtained from Charles River (Bagneux cedex, France). The mice were shipped in appropriate travel conditions with the corresponding certificate of health and origin. All the animals were kept under controlled conditions in a P3 high security facility with sterile food and water ad libitum.

2.3.1. Bacteria and infection

M. tuberculosis strains were grown in Proskauer Beck medium containing 0.01% Tween 80 to mid-log phase and stored at –70 °C in 2 ml aliquots. The mice were placed in the exposure chamber of an airborne infection apparatus (Glascol Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with 7 ml of a *M. tuberculosis* suspension at a previously calculated concentration to provide an approximate uptake of 20 viable bacilli into the lungs. Four mice were used for every time-point in each experimental group. The numbers of viable bacteria in the left lung and spleen homogenates and broncoalveolar liquid (BAL) in weeks 3, 9, 18 and

22 were followed over time by plating serial dilutions on nutrient Middlebrook 7H11 agar (Biomedics s.l., Madrid, Spain) and counting bacterial-colony formation after 21 days incubation at 37 °C. Special care was taken not to include hiliar lymph nodes at the time of removing the left lung so as not to artificially increase the CFUs value. BAL was obtained by a gentle intratracheal injection of 1 ml phosphate-buffered saline and the lungs were immediately extracted after euthanasia by means of a halothane overdose (Zeneca Farma, Pontevedra, Spain).

2.3.2. Animal health

The mice were weighed once a week. They were supervised daily under a protocol paying attention to weight loss, apparent good health (bristled hair and wounded skin) and behavior (signs of aggressiveness or isolation). Animals were euthanized with a halothane (Fluothane, Zeneca Farma) overdose so as to avoid any suffering. Sentinel animals were used to check specific pathogen-free conditions in the facility. Tests for 25 known mouse pathogens were all negative. All experimental proceedings were approved and supervised by the Animal Care Committee of the *Germans Trias i Pujol* University Hospital in accordance with European Union Laws for the protection of experimental animals.

2.3.3. mRNA quantification

The procedures are also described elsewhere [19]. In short, total RNA from the middle right lobe of C57BL/6 mice was extracted using a commercial phenol–chloroform method, RNAzol (Cinna/Biotecx, Friendswood, IX, USA). After a DNase treatment with a DNA-free kit (Ambion, Woodward Austin, USA), a denaturing agarose gel was used to assess the stability of RNA. Five micrograms of RNA was reverse transcribed using a Superscript RT kit (Gibco BRL, Grand Island, NY, USA) following the manufacturer's recommendations to obtain cDNA. The quantitative analysis for IFN-γ, RANTES, iNOS and TNF was performed using a LightCycler™ System (Roche Biochemicals, Idaho Falls, ID, USA). A real-time PCR was carried out in glass capillaries to a final volume of 10 μl in the presence of 1 μl of 10× reaction buffer (Taq Polymerase, dNTPs, $MgCl_2$, SYBRGreen, Roche Biochemicals), 1 μl of cDNA (or water as a negative control, which was always included), $MgCl_2$ to a final concentration of 2–5 mM and primers to a final concentration of 0.5 μM were also added. A single peak was obtained for each PCR product by melting the curve analysis, and only one band of the estimated size was observed in the agarose gel. Hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression was analyzed for every target sample to normalize efficiency in cDNA synthesis and RNA loading. A ratio based on the HPRT mRNA expression was obtained for each sample.

2.3.4. Survival

SCID mice were infected in order to determine the virulence of the two strains of MT103 in relation to the survival of the mice. Mice were sacrificed according to an end-point protocol. In this protocol, the most decisive parameter used to decide when to sacrifice an animal was weight. Once animals had lost 35% of their highest weight, they were then sacrificed. The significance of differences between survival times was determined using the Mantel–Haenszel test.

2.3.5. Histology and morphometry

Procedures have been described in previous works [19]. Briefly, two right-lung lobes from each C57BL/6 mouse were fixed in buffered formalin and subsequently embedded in paraffin. Every sample was stained with hematoxilin–eosin. For the histometry, sections 5 μm thick from each specimen were stained with hematoxilin–eosin and photographed at 6× using a Stereoscopic Zoom SMZ800 microscope (Nikon, Tokyo, Japan) and a Coolpix 990 digital camera (Nikon). Sections of eight lung lobes were studied in each case. A sequence of appropriate software programs was used, Scion Image (Scion Corporation, Frederick, MD, USA) and Photoshop 5.0 (Adobe Systems Incorporated, San José, CA, USA), to determine the area of each single lesion and the total tissue area in photomicrographs at each time point. Sections were blindly evaluated in order to attain a more objective measurement.

2.4. Analysis of lipids

For lipid analysis, mycobacterial strains were grown on Middlebrook 7H10 agar at 37 °C. Non-covalently attached lipids were extracted first with chloroform/methanol (1:2 [vol/vol]) and then with chloroform/methanol (2:1 [vol/vol]). Pooled organic extracts, containing the lipids, were partitioned with chloroform/methanol/water (8:4:2 [vol/vol/vol]); the organic phase was separated and evaporated to dryness. Mycolic acids were extracted by saponification and methylated with diazomethane, as previously described [20].

The lipidic extracts were analyzed using thin-layer chromatography (TLC) on 20 × 20 Silica Gel 60 TLC plates (Merck, Germany). The following solvent systems were used for the chromatographic development of glycolipids: chloroform/methanol (99:1, 95:5 and 85:15 [vol/vol]) and chloroform/methanol/water (30:8:1 and 60:35:8 [vol/vol/vol]). Carbohydrate-containing compounds were visualized by spraying the TLC plates with 1% anthrone (Sigma, USA) in H_2SO_4, followed by heating at 120 °C. Molybdenum oxide (1.3%) in sulfuric acid 4.2 M (Molybdenum blue reactive, Sigma, USA) was used to reveal phosphorous-containing substances. Apolar lipids were analyzed by two-dimensional TLC using petroleum ether (b.p. 60–80 °C)/ethyl acetate (98:2 [vol/vol], three times), in the first direction, and petroleum ether (b.p. 60–80 °C)/acetone (98:2 [vol/vol]), in the second direction. Methyl mycolates were analyzed by TLC using petroleum ether (b.p. 60–80 °C)/diethyl ether (85:15 [vol/vol], three times). Apolar lipids and methyl mycolates were visualized by spraying TLC with 10% molybdophosphoric acid (Merck, Germany) in ethyl alcohol (w/v) and heating at 120 °C for 15 min. Previously purified lipids were used as TLC standards.

3. Results

3.1. Neutral-red staining

Neutral-red became yellow when added to barbital buffer pH 9.8. However, the cells of H37Rv and MT103 immediately took on a red coloration with the staining solution. This NR positive reaction was also observed in *pks2, msl3, pks12* mutants and in the *drrCc* complemented strain (Fig. 1 and Table 1). No appreciable differences were observed in the intensity of the red coloration between the NR positive strains, and the cells submerged in the staining solution retained their red coloration for several days. However, the H37Ra and the *fadD26, fadD28, mmpL7* and *drrC* mutants were NR negative (Fig. 1 and Table 1). The cells pertaining to these strains kept their natural color in the staining solution even after several hours of the staining reaction. The strains used in this work were subcultured in 7H10 medium and retested four times for NR. All strains proved to be very stable for NR and we observed no loss in staining intensity.

We determined to continue the NR experiments for a longer period of time with H37Ra, H37Rv and MT103, and to stop the experiments with the other strains. H37Rv proved to be very stable for NR over 2 years, and again we observed no loss in staining intensity. Over the same period of time, H37Ra also did not turn red. However, MT103 became NR negative after 18 months of passages on culture media (Fig. 1). This MT103 NR negative strain did not recuperate the capacity to become red in the subsequent cultures. We termed this natural mutant MT103a. After infecting mice, the MT103a cells recovered from infected mouse tissue continued to be NR negative.

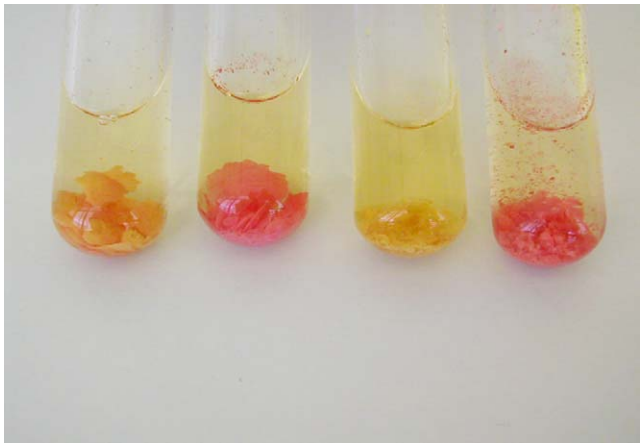

Fig. 1. Neutral-red staining in test tube; from left to right, MT103a, MT103, H37Ra and H37Rv strains.

Table 1
Relation between the result of the neutral-red reaction and the presence of methyl-branched lipids in the cell wall of *M. tuberculosis* strains

M. tuberculosis strains [a]	Neutral-red reaction	Presence of methyl-branched lipids in the cell wall		
		DIM	SL	PAT
H37Rv	+	+	+	+
pks2	+	+	–	+
msl3	+	+	+	–
pks12	+	–	+	+
H37Ra	–	+	–	–
MT103	+	+	–	+
MT103a	–	–	–	+
fadD26	–	–	–	ND
fadD28	–	–	–	ND
mmpL7	–	–	–	ND
drrC	–	–	–	ND
drrCc [b]	+	+	–	ND

[a] See Section 2 for the description of the characteristics of each strain.
[b] *drrCc* is the MT103 *drrC* mutant complemented with the *drrC* gene, this complementation restored the localization of DIM in the cell wall.

DIM, pthiocerol dimycocerosates; SL, sulfolipids; PAT, polyacyltrehaloses; + indicates the presence of the glycolipid or a NR positive reaction; - indicates the absence of the glycolipid or a NR negative reaction; ND indicates data no available.

3.2. Virulence in mice

Considering the virulence of the MT103 and MT103a strains, the results clearly demonstrate that MT103 has a higher capacity for virulence, shown by a lower mean for survival days in SCID mice (29.7 ± 0.7 vs. 55.4 ± 2.5 days) (Fig. 2). In C57BL/6 mice, MT103 obtains a higher bacillary concentration, especially in week 3 post-infection (Fig. 3). Interestingly, there was no difference in the BAL bacillary concentration between both *M. tuberculosis* strains, and thus this data are not related to an increase of virulence.

Looking at mRNA expression, MT103 induces earlier and higher levels of IFN-γ and iNOS, reflecting the higher bacillary concentration (Fig. 4). However, the TNF and RANTES expression in MT103a reach the maximum level at week 18, reflecting the late increase in bacillar concentration when compared with MT103. Figs. 5 and 6 also reflect the higher virulence of MT103 as is able to induce a larger pulmonary infiltration.

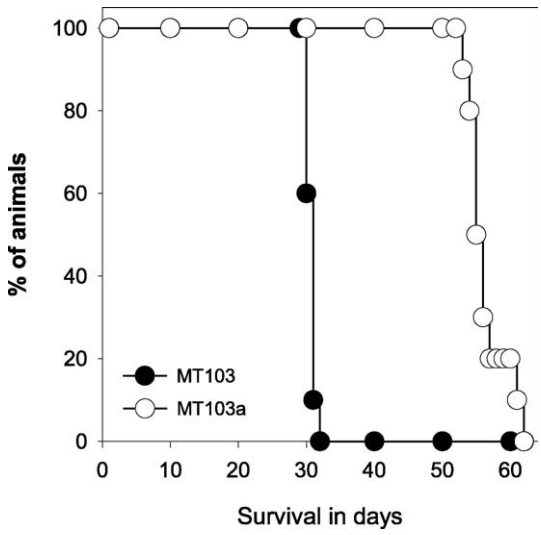

Fig. 2. Survival of SCID mice (10 per group) infected by aerosol. Differences were significant, according to the Mantel–Haenszel test.

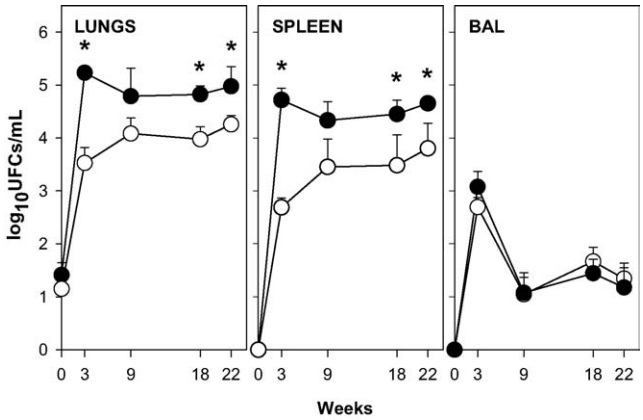

Fig. 3. Evolution, in C57BL/6 mice, of lung, BAL and spleen bacillary concentrations for each isolate. Data represent the average and standard deviation of $\log_{10}$ CFUs obtained for each isolate. Concentrations are expressed as the mean ± S.D. for four mice. Full and open symbols represent values of MT103 and MT103a strains, respectively. Differences between means were compared using the Student's *t*-test and are marked by *, when significant ($P < 0.05$).

3.3. Lipid patterns

The content of methyl-branched lipids of H37Rv (*pks2, msl3, pks12*) and MT103 (*fadD26, fadD28, mmpl7, drrC, drrCc*)-derived mutants, was previously described [11,13,15–17] (Table 1).

When the MT103 strain became NR negative, we extracted and compared the lipid pattern of the MT103 strain (NR positive) and its natural mutant, the MT103a strain (NR negative). Both strains presented the typical mycolates for this species, i.e. α-mycolates, keto and methoxy mycolates (data not shown).

With regard to the non-covalently attached lipids, the two strains contained similar patterns of trehalose dimycolates, phospholipids and phospatidylinositol mannosides (data not shown), but differed in the content of methyl-branched lipids. When the MT103 strain became NR negative, it lost the capacity to synthesize DIM (Fig. 7 and Table 1) and did not recover this capacity in subsequent cultures. No other changes in the lipid content were observed between MT103 and its natural DIM mutant (MT103a strain). After infecting mice, the MT103a cells recovered from infected mouse tissue continued to be unable to synthesize DIM.

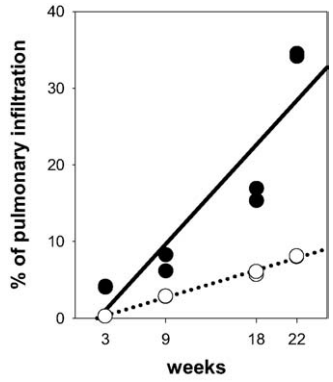

Fig. 5. Evolution, in C57BL/6 mice, of granulomatous infiltration in the lungs. Data show the ratio of the granuloma-involved area compared to the overall tissue area in two lung lobes for each mouse. Full and open symbols represent values of MT103 and MT103a strains, respectively. Data show duplicates of the area obtained from two pulmonary lobes in four mice. Linear regressions were determined using the Pearson product moment correlation coefficient ($P < 0.05$ in all cases).

4. Discussion

In the past, a positive NR was related to virulence in *M. tuberculosis* basically due to three features: i) all the *M. tuberculosis* recent isolates were NR positive, ii) the virulent *M. tuberculosis* H37Rv strain was NR positive, whereas their avirulent variant, H37Ra, was NR negative, and iii) mycobacteria from diverse environmental origins that were non-pathogenic for guinea pigs were, with few exceptions, NR negative [6–9].

When we obtained an NR negative natural mutant from the MT103 *M. tuberculosis* strain, we realized that we had an excellent opportunity to confirm whether the co-relation between virulence and positive NR was true. The evolution of *M. tuberculosis* infection in the aerosol model of murine tuberculosis is a useful tool for determining the virulence of different strains [21,22]. In this case, a SCID mice has been

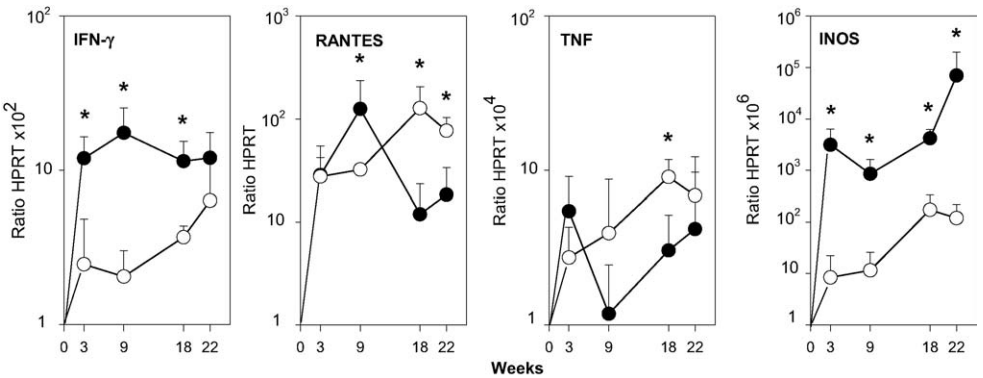

Fig. 4. Evolution, in C57BL/6 mice, of mRNA expression of Th1 response-related cytokines TNF and iNOS. Results are presented as the mean values and S.D. of the ratio for each chemokine or cytokine, and the HPRT value for the corresponding sample. Full and open symbols represent values of MT103 and MT103a strains, respectively. The difference between means was determined using the Student's test and is marked by *, when significant ($P < 0.05$).

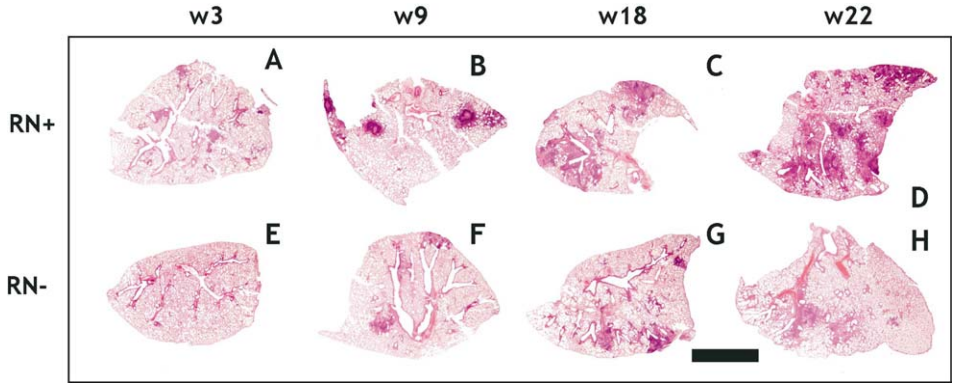

Fig. 6. Evolution, in C57BL/6 mice, of infiltration induced by MT103 (A–D) and MT103a (E–H) infected mice in post-infection weeks 3 (A, E), 9 (B, F), 18 (C, G) and 22 (D, H). Microphotographs were taken with a Nikon stereoscopic microscope SMZ800 (Nikon) at 10×. Bar represents 8 mm.

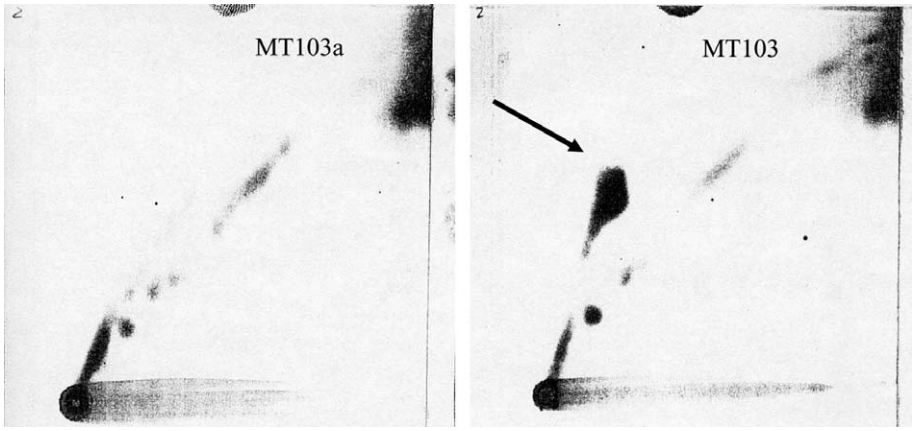

Fig. 7. Two-dimensional TLC of lipidic extracts obtained from MT103a and MT103. The arrow marks the spot corresponding to DIM.

also used to study the ability of both *M. tuberculosis* strains to overcome the innate immunity, as SCID mice are not able to trigger a specific immune response [23,24]. In this regard, mice infected with MT103 had a lower survival rate when compared with those infected with MT103a, thus reflecting that a lack of MT103a virulence is related to its inability to overcome the unspecific response. Lung and spleen bacillary concentration reached in the C57BL/6 mice also reflects a higher ability of MT103 to better settle in these organs (Fig. 3). This difference is even more evident at the acute phase of the infection (week 3), probably as a consequence of the higher influence of the unspecific immunity in MT103a. The lack of differences obtained in the BAL is not a new issue. It has previously been described when assessing the virulence of *M. tuberculosis* clinical strains [25]. On the other hand, comparison among mice DBA/2 and C57BL/6 strains with different degrees of resistance against *M. tuberculosis*, susceptible and resistant, respectively, did show a higher concentration in DBA/2 [19]. Recently, the study of new parameters such as mRNA pulmonary local expression for cytokines related to the bactericidal activity generated against the bacilli like IFN-γ and TNF; the induction of iNOS; and the expression of chemokines linked to the induction of Th1 responses, has offered new tools for supporting virulence studies in this model [25]. In this regard, the data obtained reflects the higher bacillary concentration reached by MT103, triggering a high IFN-γ and iNOS expression from the beginning, with not much difference on TNF levels (Fig. 4). This was the usual scenario, previously observed [19], as MT103 behaves similar to a reference strain such as H37Rv. However, this is not the case with a hypervirulent strain such as Beijing, for instance, which is able to decrease the IFN-γ expression [26]. Furthermore, granulomatous infiltration of the lungs adds a valuable parameter closely related to the pathogenic capacity of the strains, as mice finally die due to the almost complete infiltration of the lungs [19,21]. Data shown in Figs. 5 and 6 clearly demonstrate the higher infiltration induced by MT103, and thus its higher pathogenicity, when compared with MT103a. Therefore from our comparative studies using MT103 and MT103a, we can conclude that the NR negative character is related to a loss of virulence.

In accordance with this, the other NR negative strains in this study, H37Ra and MT103-derived mutants *fadD26, fadD28, mmpL7* and *drrC* (see Table 1 and experimental procedures for a description of the mutant strains), have been described by other authors as attenuated in a mouse model [4,15]. However, not all the attenuated strains of *M. tuberculosis* were NR negative. We have studied a *pks12* mutant that has been reported as attenuated in mice and found this to be NR positive [17].

Lipid analyses revealed that when MT103 turned NR negative, it lost the capacity to synthesize DIM (Fig. 7). These complex lipids have been related to virulence in recent studies. Constructed mutants from H37Rv, Erdman and MT103 *M. tuberculosis* strains unable to synthesize DIM or unable to correctly locate these lipids in the cell wall were found attenuated in different tuberculosis mice models [15,17,27].

In this work, we have studied the NR in certain *M. tuberculosis*-constructed mutants (see Table 1). One of these was an H37Rv DIM deficient strain presenting a disruption of the *pks12* gene [17]. Recently, Matsunaga et al. [28] reported that the *pks12* gene is involved in the formation of mannosyl-β-1-phosphomycoketides, rather than DIM, and suggested that the *pks12* mutant deficient in the production of DIM (the strain used in this study) is also unable to synthetize mannosyl-β-1-phosphomycoketides [28]. The same author questions whether the attenuation of DIM-less mutants obtained from the Erdman and MT103 *M. tuberculosis* strains [15,27] was due only to DIM loss.

The objective of the present study is to test whether NR is a marker of virulence and not to carry out research into DIM locus, or into the role of DIM as a virulence factor. However, we believe that we have found a valuable DIM-less natural mutant. Further characterization of the nature of the spontaneous mutation presented by the MT103a strain, causing this defect in the biosynthesis of DIM, would contribute to a better understanding of this interesting lipid.

At this point, it should be recalled that the MT103 wild strain was originally deficient in SL; the MT103a and MT103-derived mutants (*fadD26, fadD28, mmpL7* and *drrC*) therefore lack two methyl-branched lipids, DIM and SL (Table 1).

In order to ascertain whether the absence of DIM alone is enough to cause the loss of the neutral-red activity, or whether the additional absence of SL or other methyl-branched lipids such as PAT is necessary, we performed the NR in H37Rv-derived mutants *pks2, msl3*, and *pks12*, which are deficient in SL, PAT and DIM, respectively.

We found that all these mutants were NR positive. Thus the absence of SL, PAT or DIM is not enough to lose the neutral-red activity (Table 1). However, all the strains studied in this work that lacked two types of the methyl-branched lipids were NR negative. As we have shown in Table 1, these were: H37Ra, which does not produce SL and PAT; MT103a, which does not produce SL and DIM; and the MT103-derived mutants that are unable to produce SL and DIM or translocate DIM to their cell wall. In accordance with these results, the complemented *drrC* mutant (*drrCc*) that recovered the capacity to locate DIM in the cell wall [13] also recovered its neutral-red positive character.

Neutral-red is a phenazine dye that possesses indicator properties. It is yellow in alkaline conditions, shifting to red in acidic solutions and turning blue in a strong acid environment. The color change from yellow to red responds to a protonation in the nitrogen of the central benzene [29] (Fig. 8). The neutral-red staining solution (1% sodium barbital in 5% NaCl, pH 9.8) is yellow, but the *M. tuberculosis* strains fix

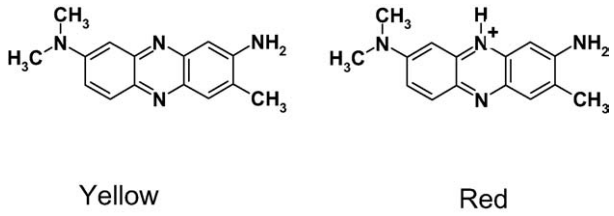

Fig. 8. Ionization forms of neutral-red. In an alkaline environment (pH > 9), the stain is in its basic form (yellow). In an acid environment, the stain is in its red monovalent cationic form.

and reduce the dye, and their cells are stained red. Thus, a positive NR indicates the presence of compounds with reducing power on the surface of *M. tuberculosis* cells.

It has been reported that *M. tuberculosis* has a complex envelope. From the inside to the outside we found: (i) a cell wall composed of three covalently attached macromolecules: peptidoglycan, arabinogalactan and mycolic acids, (ii) a variety of non-covalently attached lipids, and (iii) a hydrophilic capsule of polysaccharides, proteins and lipids. It has been proposed that the methyl-branched lipids belonging to the class of non-covalently attached lipids interact with mycolic acids and with components in the capsule, attaching the components of the capsule to the surface of the mycobacteria [30–33]. As methyl-branched lipids are not located on the surface of *M. tuberculosis*, they are probably not directly involved in the reduction of neutral-red. Furthermore, DIM and PAT are neutral molecules. Only SL are charged and could give protons to neutral-red, but *M. tuberculosis* strains that lack SL continued to be NR positive. These lipids are, therefore, not directly responsible for the reduction of neutral-red. As we have mentioned above, one of the roles of methyl-branched lipids could be to act as an interface between the hydrofobic environment created by mycolic acids and the hydrophilic capsule. We can therefore hypothesize that the loss of more than one class of methyl-branched lipids would result in the non-attaching of the components in the capsule responsible for the reducing power of the external surface of *M. tuberculosis*.

However, it is necessary to bear in mind that the reduction of neutral-red implies the absorption of the dye, so the same compounds that absorb the dye need not necessarily be the same as those that reduce it.

In summary, the results obtained in this study confirm that i) the external surface of *M. tuberculosis* acts as a reducing agent; ii) this reducing power is altered when more than one class of methyl-branched lipid lacks *M. tuberculosis* strains in the cell wall; and iii) the external surface reducing power is related to virulence in *M. tuberuclosis* strains, and this reducing power can be measured by NR.

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References

[1] World Health Organization, 2002, Tuberculosis fact sheet no. 104. [Online.]. http://www.who.int/mediacentre/factsheets/who104/en/.
[2] I. Smith, *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence, Clin. Microbiol. Rev. 16 (2003) 463–496.
[3] W. Steeken, W.H. Oatway, S.A. Petroff, Biological studies of the tubercle bacillus III. Dissociation and pathogenicity of the R and S variants of the human tubercle bacillus (H37), J. Exp. Med. 60 (1934) 515–544.
[4] G. Middlebrook, The experimental analysis of virulence of tubercle bacilli, Bull. N. Y. Acad. Med. 26 (1950) 498–506.
[5] W. Steenkenand, L.U. Gardner, History of H37 strain of tubercle bacillus, Am. Rev. Tuberc. 54 (1946) 62–66.
[6] R.J. Dubos, G. Middlebrook, Cytochemical reaction of virulent tubercle bacilli, Am. Rev. Tuberc. 58 (1948) 698–699.
[7] P. Haudoroy, M. Cevey, Importance des réactions cyto-chimiques pour la détermination de la virulence des mycobactéries et intérêt pour la classification, Ann. Inst. Pasteur (Paris) 84 (1953) 1034–1035.
[8] M.P. Haudoroy, M.Y. Postenak, Sur une réaction permettant de distinguer les mycobactéries virulentes des mycobactéries avirulentes, C. R. Hebd. Seances Acad. Sci. 228 (1949) 781–782.
[9] D. Hughes, M. Moss, M. Hood, M. Henson, Virulence of *Mycobacterium tuberculosis*: evaluation of a test using neutral red indicator, Am. J. Clin. Pathol. 24 (1954) 621–625.
[10] G. Middlebrook, C.M. Coleman, W.B. Schaefer, Sulfolipid from virulent tubercle bacilli, Proc. Natl. Acad. Sci. USA 45 (1959) 1801–1804.
[11] T.D. Sirakova, A.K. Thirumala, V.S. Dubey, H. Sprecher, P.E. Kolattukudy, The *Mycobacterium tuberculosis pks2* gene encodes the synthase for the hepta- and octamethyl-branched fatty acids required for sulfolipid synthesis, J. Biol. Chem. 276 (2001) 16833–16839.
[12] N. Andreu, I. Gibert, T. Sirakova, P.E. Kolattukudy, M. Luquin, Neutral red staining of cells of a sulfolipid-deficient *Mycobacterium tuberculosis pks*2 mutant proves that sulfolipids are not responsible for this cytochemical reaction, J. Clin. Microbiol. 42 (2004) 1379–1380.
[13] L.R. Camacho, P. Constant, C. Raynaud, M.A. Lanéelle, J.A. Triccas, B. Gicquel, M. Daffe, C. Guilhot, Analysis of the pthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*, J. Biol. Chem. 276 (2001) 19845–19854.
[14] M. Daffé, P. Draper, The envelope layers of mycobacteria with reference to their pathogenicity, Adv. Microb. Physiol. 39 (1998) 131–203.
[15] L.R. Camacho, D. Ensergueix, E. Perez, B. Gicquel, C. Guilho, Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis, Mol. Microbiol. 34 (1999) 257–267.
[16] V.S. Dubey, T.D. Sirakova, P.E. Kolattukudy, Disruption of *msl3* abolishes the synthesis of mycolipanoic and mycolipenic acids required for polyacyltrehalose synthesis in *Mycobacterium tuberculosis* H37Rv and causes cell aggregation, Mol. Microbiol. 45 (2002) 1451–1459.
[17] T.D. Sirakova, V.S. Dubey, H.J. Kim, M.H. Cynamon, P.E. Kolattuhudy, The largest open reading frame (*pks12*) in the *Mycobacterium tuberculosis* genome is involved in pathogenesis and dimycocerosyl phthiocerol synthesis, Infect. Immun. 71 (2003) 3794–3801.
[18] C.Y. Soto, N. Andreu, I. Gibert, M. Luquin, Simple and rapid differentiation of *Mycobacterium tuberculosis* H37Ra from *M. tuberculosis* clinical isolates through two cytochemical tests using neutral red and nile blue stains, J. Clin. Microbiol. 40 (2002) 3021–3024.
[19] P.J. Cardona, S. Gordillo, J. Díaz, G. Tapia, I. Amat, A. Pallarés, C. Vilaplana, A. Ariza, V. Ausina, Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with *Mycobacterium tuberculosis*, Infect. Immun. 71 (2003) 5845–5854.
[20] M. Daffé, M.A. Laneélle, C. Asselineau, V. Lévy-Frébault, H. David, Intérêt taxonomique des acides gras des mycobactéries : proposition d'une méthode d'analyse, Ann. Microbiol. 134B (1983) 241–256 (Inst Pasteur).
[21] R.J. North, A.A. Izzo, Mycobacterial virulence. Virulent strains of *Mycobacterium tuberculosis* have faster in vivo doubling times and are better equipped to resist growth-inhibiting functions of macrophages in the presence and absence of specific immunity, J. Exp. Med. 177 (1993) 1723–1733.
[22] D.J. Ordway, M.G. Sonnenberg, S.A. Donahue, J.T. Belisle, I.M. Orme, Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice, Infect. Immun. 63 (1995) 741–743.
[23] R. Manganelli, L. Fattorini, D. Tan, E. Iona, G. Orefici, G. Altavilla, P. Cusatelli, I. Smith, The extra cytoplasmic function sigma factor sigma(E) is essential for *Mycobacterium tuberculosis* virulence in mice, Infect. Immun. 72 (2004) 3038–3041.
[24] R.J. North, A.A. Izzo, Granuloma formation in severe combined immunodeficient (SCID) mice in response to progressive BCG infection. Tendency not to form granulomas in the lung is associated with faster bacterial growth in this organ, Am. J. Pathol. 142 (1993) 1959–1966.
[25] P.J. Cardona, S. Gordillo, I. Amat, J. Díaz, J. Lonca, C. Vilaplana, A. Pallares, R. Llatjos, A. Ariza, V. Ausina, Catalase-peroxidase activity has no influence on virulence in a murine model of tuberculosis, Tuberculosis (Edinb.) 83 (2003) 351–359.
[26] B. López, D. Aguilar, H. Orozco, M. Burger, C. Espitia, V. Ritacco, L. Barrera, K. Kremer, R. Hernandez-Pando, K. van Huygen, D. Soolingen, A marked difference in pathogenesis and immune responses induced by different *Mycobacterium tuberculosis* genotypes, Clin. Exp. Immunol. 133 (2003) 30–37.
[27] J.S. Cox, B. Chen, M. McNeil, W.R. Jacobs, Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice, Nature 402 (1999) 79–83.
[28] I. Matsunaga, A. Bhatt, D.C. Young, T.Y. Cheng, S.J. Eyles, G.S. Besra, V. Briken, S.A. Porcelli, C.E. Costello, W.R. Jacobs, D.B. Moody, *Mycobacterium tuberculosis pks12* produces a novel polyketide presented by CD1c to cells, J. Exp. Med. 200 (2004) 1559–1569.
[29] R.D. Lillie, Quinone-imines dyes, azines, in: R.D. Lillie (Ed.), Conn's Biological Stains, ninth ed, The Williams and Wilkins Company, Baltimore, 1977, pp. 376–392.
[30] P.J. Brennan, H. Nikaido, The envelope of mycobacteria, Annu. Rev. Biochem. 64 (1995) 29–63.
[31] A. Lemassu, M. Daffé, Structural features of the exocellular polysaccharides of *Mycobacterium tuberculosis*, Biochem. J. 297 (1994) 351–357.
[32] D.E. Minnikin, Lipids: Complex lipids, their chemistry, biosynthesis and roles, in: C. Ratledge, J. Stanford (Eds.), The Biology of Mycobacteria, Academic Press, London, 1982, pp. 95–184.
[33] D.E. Minnikin, L. Kremer, L.G. Dover, G.S. Besra, The methyl-branched fotifications of *Mycobacterium tuberculosis*, Chem. Biol. 9 (2002) 545–553.