Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype

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Mycobacterium abscessus is an increasingly important cause of human disease; however, virulence determinants are largely uncharacterized. Previously, it was demonstrated that a rough, wild-type human clinical isolate (390R) causes persistent, invasive infection, while a smooth isogenic mutant (390S) has lost this capability. During serial passage of 390S, a spontaneous rough revertant was obtained, which was named 390V. This revertant regained the ability to cause persistent, invasive infection in human monocytes and the lungs of mice. Glycopeptidolipid (GPL), which plays a role in environmental colonization, was present in abundance in the cell wall of 390S, and was associated with sliding motility and biofilm formation. In contrast, a marked reduction in the amount of GPL in the cell wall of 390R and 390V was correlated with cord formation, a property associated with mycobacterial virulence. These results indicate that the ability to switch between smooth and rough morphologies may allow *M. abscessus* to transition between a colonizing phenotype and a more virulent, invasive form.

Received 24 October 2005

Revised 7 March 2006

Accepted 7 March 2006

INTRODUCTION

Mycobacterium abscessus causes a variety of clinical syndromes and is responsible for the majority of pulmonary infections due to rapidly growing mycobacteria (Griffith *et al.*, 1993). *M. abscessus* has also been identified as an emerging pulmonary pathogen in patients with cystic fibrosis (Cullen *et al.*, 2000; Fauroux *et al.*, 1997; Olivier *et al.*, 2003; Sermet-Gaudelus *et al.*, 2003), including those who have undergone lung transplantation (Sanguinetti *et al.*, 2001). Two important clinical questions are whether isolation of *M. abscessus*, or other non-tuberculous mycobacteria (NTM), from the sputum of a patient represents

colonization or invasive pulmonary infection, and whether lung colonization inevitably leads to the development of invasive pulmonary infection (Brown-Elliott & Wallace, 2005; Cullen *et al.*, 2000).

NTM have long been recognized as having rough and smooth colony phenotypes (Fregnan & Smith, 1962). Studies have shown that colony morphology in NTM is influenced by cell wall glycopeptidolipid (GPL) (Barrow & Brennan, 1982; Eckstein *et al.*, 2000). In *Mycobacterium smegmatis* and *Mycobacterium avium* GPLs are associated with sliding motility and the ability to form biofilms (Carter *et al.*, 2003; Recht *et al.*, 2000). Both of these characteristics are thought to play a role in bacterial colonization (Recht *et al.*, 2000). Several reports have found a correlation between colony morphology and virulence, with rough variants generally being more virulent than smooth variants (Belisle & Brennan, 1989; Collins & Cunningham, 1981; Schaefer *et al.*, 1970). These reports are consistent with

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Abbreviations: GPL, glycopeptidolipid; NTM, non-tuberculous mycobacteria; SCID, severe combined immunodeficiency; TDM, trehalose 6,6' dimycolate.

results from our previous studies in which we reported that a rough *M. abscessus* clinical isolate became attenuated after it spontaneously dissociated into a smooth variant (Byrd & Lyons, 1999). During serial passage of the smooth variant, one colony spontaneously reverted back to the rough phenotype affording a unique opportunity to examine the role of GPL in colony phenotype, and the influence of colony phenotype on biofilm formation and the ability of *M. abscessus* to cause persistent, invasive infection.

METHODS

Mycobacterial strains. The *M. abscessus* smooth strain 390S was derived from the rough strain 390R, as described previously (Byrd & Lyons, 1999; Howard *et al.*, 2002). After several passages of 390S on 7H10-OADC plates, a rough colony revertant was isolated and inoculated into broth. Aliquots of this new rough variant, which was named 390V, were stored at -70°C . Additional *M. abscessus* strains were obtained from P. Conville, Public Health Service, NIH, Bethesda, MD, USA, and had the following morphologies: strains 6639 and 8988, smooth; strains 1056 and 1475, rough; strain 8243, intermediate. *M. smegmatis* strain mc²155 was used as a control for GPL expression.

Colony morphology and growth pattern. Colony morphology was assessed on 7H11 plates. Examination for cording was performed by inoculating bacteria into 7H9 broth at a concentration of 1×10^4 bacteria ml^{-1} and culturing them at 37°C under stationary phase conditions for 7 days. Culture suspensions were spun onto glass slides using a cytocentrifuge (Shandon), and the bacteria were heat fixed and stained with crystal violet dye. Bacteria were examined at $\times 400$ magnification using a light microscope (Nikon) and photographed.

Genetic analysis of bacterial strains. *M. abscessus* 390S is lacking 14.2 kbp of DNA that is present in the original 390R strain (Howard *et al.*, 2002). We used this genomic deletion to verify the identity of our 390V strain. For genomic DNA preparation and Southern blotting, bacteria were scraped from plate cultures and lysed by bead beating using 0.1 mm silica/zirconium beads in a BioSpec Mini-BeadBeater. DNA was extracted using the PUREGENE DNA isolation kit for Gram-positive bacteria (Gentra Systems), and then recovered following phenol/chloroform extraction and 2-propanol precipitation. Southern blots were prepared and probed (Sambrook *et al.*, 1989) following the electrophoresis of 2 μg genomic DNA digested with the indicated restriction enzymes.

To further confirm the identity of the 390V strain, PCR products from the deletion region were compared among the 390 strains. For analysis of the deletion region, five primers were designed, based on the published sequence (Howard *et al.*, 2002). R5del5 (5'-GTCCTCGT-AGAAGTACCGATC-3') is located within ORF5, 220 bp upstream of the start of the deletion. R18del3 (5'-GTTCGTCGGAATGGCAC-3') is located within ORF18, approximately 240 bp downstream of the deletion. R6M3 (5'-GTTTCGAGCAACGTGCTG-3') is located within the deletion, in ORF6. MabR3int (5'-CCAGCGACGGAGTATCTC-3') and MabR3-send (5'-GCCAGACTGTAGGACATGA-3') are located in ORF3, outside of the deletion. Reactions were performed with 20 ng *M. abscessus* DNA, 1 pmol μl^{-1} appropriate primers and Promega PCR Master Mix, with an initial denaturation followed by 30 cycles of $95^{\circ}\text{C}/30\text{ s}$, $55^{\circ}\text{C}/30\text{ s}$, $72^{\circ}\text{C}/30\text{ s}$, and a final 7 min extension at 72°C .

Reversion rate. To determine the reversion rate of the 390S strain from a smooth to a rough phenotype, a single 3-day-old 390S colony was picked from a 7H11 agar plate and inoculated into 5.0 ml 7H9 broth in a small shaker flask. The culture was incubated at 37°C at 100 r.p.m. on a shaker incubator and harvested during early exponential phase growth. The entire culture volume was

divided and plated on a total of 50 Petri dishes ($150 \times 15\text{ mm}$) containing 7H11 agar, and cultured for 3 days at 37°C . Plates were then examined using a dissecting microscope, and the total number of c.f.u. and rough revertants counted. Rough revertants that were discrete colonies, not bordering or touching adjacent colonies, were graded as definite revertants. Those rough revertants that were small and touching larger smooth colonies were considered possible revertants since they may have arisen secondarily out of an adjoining smooth colony. An estimate of the reversion rate was arrived at as a range with only definite revertants representing the upper limit, and both definite and possible revertants representing the lower limit of the range.

Sliding motility. Strain motility was assessed by inoculating bacterial cells into the centre of plates prepared with M63 minimal media containing 0.3% agarose (Recht *et al.*, 2000). Plates were incubated in a humidified incubator at 37°C and colony spread was assessed after 3 days.

Biofilm formation. Bacteria in Sauton's medium were cultured in a Calgary biofilm device (Ceri *et al.*, 1999). At the indicated time points, pegs were removed from the lid of the device and placed into microcentrifuge tubes containing sterile PBS. In addition, Sauton's medium was removed from the remaining wells and replaced with fresh medium. Bacteria were dislodged from pegs by sonication for 20 s using a cup horn sonicator, and supernatants were plated on 7H11 agar to determine c.f.u. In some experiments, bacteria on pegs attached to lids were fixed for 30 min with methanol, followed by staining with 1% crystal violet dye for 1 h. Pegs were photographed using an inverted phase-contrast microscope and then destained with 100% ethanol. The A_{570} value of the crystal violet/ethanol solution was then determined (Recht *et al.*, 2000).

Lipid extraction and analysis. To characterize lipid profiles from the 390R, 390S and 390V *M. abscessus* variants, lipids from plate-grown cultures were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ [2:1, v/v; 10 ml (g wet weight bacteria) $^{-1}$]. Bacteria were extracted twice at 56°C (15 min with sonication) and once at 4°C overnight, and insoluble material was removed by centrifugation and filtration (0.20 μm PTFE filter; Millipore). Combined extracts were subjected to biphasic partitioning in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (4:2:1, by vol.). Lipids in the organic phase were stored at -20°C . GPLs are resistant to mild alkaline methanolysis, which destroys nonspecific acylglycerols (McNeil *et al.*, 1989), and alkali treatment was performed according to the method of Brennan & Goren (1979). In brief, total lipid extracts (6 mg ml^{-1}) were treated with an equal volume NaOH (0.2 M in CH_3OH , 45 min at 37°C), neutralized with glacial acetic acid, and the alkali-stable lipids subjected to biphasic partitioning.

Total lipid extracts or alkali-stable lipids (which consist primarily of GPLs) were resuspended at 10 $\mu\text{g}\ \mu\text{l}^{-1}$ in $\text{CHCl}_3/\text{CH}_3\text{OH}$ and spotted onto aluminium-backed silica gel-60 TLC plates (EM Science). Lipids were resolved in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (100:14:0.8 or 65:25:4, by vol.). Plates were sprayed with 10% CuSO_4 in 8% phosphoric acid or H_2SO_4 (50% in CH_3OH) to visualize all lipids. To detect glycolipids with 6-deoxyhexoses plates were sprayed with 1-naphthol (Sigma; 3% with 10% H_2SO_4 in CH_3OH). Plates were charred with a heat gun until spots with hues characteristic of the different lipid classes appeared (Rhoades *et al.*, 2003). GPLs charred to a golden-yellow colour with H_2SO_4 or to an orange-pink colour with 1-naphthol/ H_2SO_4 . Plates were also sprayed with orcinol (0.5% with 15% trichloroacetic acid in water-saturated n-butanol) to detect aldehydes, but there were no differences from the colours of the spots that had been sprayed with H_2SO_4 (data not shown).

Infection of human monocytes. A previously developed assay for growth of *M. abscessus* variants in human monocytes was used (Byrd & Lyons, 1999) with slight modification. Human monocyte

monolayers in Iscove's medium/1% normal human serum were infected with *M. abscessus* strains for 90 min, followed by three washes with Iscove's medium/1% normal human serum. A time zero count of monocyte-associated c.f.u. was plated and then 60 µg amikacin ml⁻¹ was added to the tissue culture media to kill any remaining extracellular bacteria. After 48 h *M. abscessus* c.f.u. from cell lysates were plated and the remaining wells washed twice with Iscove's medium/1% normal human serum to remove amikacin, followed by readdition of medium to the wells. At 72 h c.f.u. from cell lysates were plated again. Middlebrook 7H9 broth (Difco) was used for dilution of cell lysates prior to plating for c.f.u. Middlebrook 7H11 agar (Difco) plates (100 × 15 mm bacteriological Petri dishes) were used for plating c.f.u. from infected monolayers. In addition, nuclear counts in replicate wells were determined, and c.f.u. standardized to 10⁵ nuclei to account for any differences in monocyte number in the different monolayers, as has been described previously (Byrd, 1997). Viability was analysed in replicate infected wells by Trypan blue exclusion at each time point to ensure that the variants did not have a differential effect on monocyte viability independent of bacterial growth, as has been described previously (Byrd, 1997).

Murine pulmonary infection assay. *M. abscessus* variants were used to infect SCID mice as previously described (Byrd & Lyons, 1999), with the exception that 50 µl fluid drops containing a bacterial suspension (10⁵ c.f.u.) of *M. abscessus* 390R, 390S or 390V were inoculated intranasally rather than intratracheally. Immediately after infection, and at various time points thereafter, mice were sacrificed, and the lungs and spleens removed and placed in 3.0 ml PBS. The organs were homogenized and dilutions plated in triplicate on Middlebrook 7H11 agar.

The experimental protocol used in these studies was approved by Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

Statistics. Data were compared using Student's *t*-test. Data were considered significant with a *P* < 0.05.

RESULTS

Detection of a rough revertant (390V) from *M. abscessus* 390S

The *M. abscessus* smooth variant, 390S, is a spontaneous mutant obtained from the rough clinical isolate, 390R (Byrd & Lyons, 1999). Although the phenotypes of rough and smooth variants were stable over multiple passages, we obtained a single rough colony following routine plating of the smooth variant, and cultured it for further analysis. The rough revertant, named 390V, has a colony morphology similar to that of the original 390R isolate, but it has a drier, more wrinkled appearance (Fig. 1a–c). All 390 strains had identical growth rates in liquid media (data not shown).

To verify that 390V was a derivative of 390S and not an accidental contaminant from another rough strain, we analysed 390V for the R/S-D1 deletion, which is the 14.2 kbp deletion found in the 390S genome (Howard *et al.*, 2002). Although this region is intact in the parental 390R strain (Fig. 2a) (Howard *et al.*, 2002), our previous study showed that some rough clinical isolates have extensive deletions within this region, indicating that the deletion in 390S was unlikely to be responsible for the change to a smooth

Fig. 1. Growth characteristics of *M. abscessus* variants 390R (a, d, g), 390S (b, e, h) and 390V (c, f, i). (a–c) Seven-day-old colonies of *M. abscessus* variants on 7H11 agar viewed from the top of the colony demonstrating rough and smooth morphologies. (d–f) Two-day-old colonies photographed through the bottom of the culture plates. Both 390R (d) and 390V (f) demonstrated colony morphology consisting of irregular, parallel, linear cords, which were not present in 390S (e). (g–i) *M. abscessus* variants were grown at 37 °C for 7 days in 7H9 broth media. Cytospin preparations of infected cultures were heat fixed and stained with crystal violet. *M. abscessus* 390R (g) and 390V (i) demonstrated serpentine cording while 390S bacteria (h) remained as a single cell suspension.

colony morphology. However, the R/S-D1 deletion did provide a suitable target for confirming that the 390V rough revertant was derived from 390S. Therefore, we hybridized a Southern blot with a DNA probe (Fig. 2a) that overlaps one end of the deletion, and found that 390V and 390S shared the same band pattern (Fig. 2b, lanes 2, 3, 5, 6), which differed from the 390R band pattern (Fig. 2b, lanes 1, 4).

To confirm that the limits of the deletion in 390V were the same as in 390S, we used a series of primers in PCR analysis (Fig. 2a, c). 390V and 390S showed the same PCR amplification pattern with primer sets. In particular, amplification using primers 1 and 3, which anneal to regions flanking opposite sides of the deletion, yielded a 460 bp product with both 390V and 390S, but not with 390R because the 14 kbp region between the two primers would not be amplified under the conditions used (Fig. 2c, lanes 5–8). These data show that 390V is likely to be a rough revertant from 390S.

Examining the spontaneous reversion of 390S to a rough phenotype under controlled conditions indicated a reversion rate in the range of 1 in 10⁵ to 1 in 10⁶. This lends

Fig. 2. *M. abscessus* 390V is derived from 390S. (a) Location of primers and probe used to analyse 390V. The bar labelled 390R indicates the 25 kbp section of the 390R genome previously described (Howard *et al.*, 2002). The lightly shaded central area of this bar represents R/S-D1, which is the 14.2 kbp region deleted in 390S. Short vertical lines denote 2 kbp divisions. Black circles denote *Sst*I restriction sites. The black bar labelled probe shows the location of the 9 kbp *Bam*HI fragment used in the Southern blotting analysis. Numbered arrows indicate the positions of primers used in PCR (see Methods for sequences): 1, R5del5; 2, R6M3; 3, R18del3; 4, MabR3-int; 5, MabR3-5end. Note that in 390V and 390S, the region between primers 1 and 3, which is marked by a dotted line, is approximately 460 bp due to the deletion. (b) Southern blotting of *M. abscessus* 390V DNA. Genomic DNA was digested with *Pvu*II (lanes 1–3) or *Sst*I (lanes 4–6), electrophoresed on a 0.8% agarose gel and analysed by Southern blotting. A radiolabelled 9 kbp *Bam*HI fragment, which overlaps part of the R/S-D1 deletion, was used as the probe. Lanes 1, 4, 390R; lanes 2, 5, 390S; lanes 3, 6, 390V. Size markers (in kbp) are indicated on the left. (c) PCR analysis of the R/S-D1 region of 390V. Genomic DNA from the *M. abscessus* 390 strains was compared by PCR using three primer sets. Samples were electrophoresed on a 1% agarose gel containing ethidium bromide. 390V and 390S showed the same amplification patterns. Lanes 1–4, primers 1 and 2, which anneal next to, and within, the deletion, respectively; lanes 5–8, primers 1 and 3, which anneal to opposite flanks of the deletion; lanes 9–12, primers 4 and 5, which anneal within an ORF adjacent to the deletion. Lanes 1, 5, 9, water control; lanes 2, 6, 10, 390V; lanes 3, 7, 11, 390S; lanes 4, 8, 12, 390R. M, 1 kbp ladder (Invitrogen), molecular mass sizes (in kbp).

further support to the evidence that our rough variant 390V arose from 390S.

***M. abscessus* 390R and the 390V revertant exhibit cord formation while the 390S strain does not**

Mycobacterium tuberculosis and several NTM grow in what have been described as ropes, bundles or serpentine cords of acid-fast bacilli in liquid media or on agar plates (Middlebrook *et al.*, 1947; Lorian, 1966). A particular rough colony phenotype is associated with cord formation in NTM. This type of colony is characterized microscopically by numerous irregular parallel filaments that form ridges and grooves throughout the colony (Fregnan & Smith, 1962). A comparison of the colony morphology of our *M. abscessus* strains to that described in these studies indicates that both 390R and 390V exhibit the rough colony phenotype associated with cording (Fig. 1d–f). Importantly, both *M. abscessus* 390R and *M. abscessus* 390V also form

cords when grown in broth, but *M. abscessus* 390S lacks this ability (Fig. 1g–i).

GPL is abundantly expressed in the *M. abscessus* 390S strain but is minimal in both the 390R and 390V revertants

Since smooth and rough colony morphologies are associated with the presence and absence, respectively, of GPLs in *M. avium* and *M. smegmatis* (Barrow & Brennan, 1982; Billman-Jacobe *et al.*, 1999; Eckstein *et al.*, 2000), we examined whether GPL was expressed by our variants using TLC.

Preliminary TLC results obtained using the same methods used to study *M. smegmatis* GPLs (Recht & Kolter, 2001) indicated that 390R, 390V and other rough *M. abscessus* variants (strains 1056 and 1475) lacked lipid bands that were present in 390S and other smooth variants (strains 6639 and 8988), as well as a variant with an intermediate

phenotype (strain 8243). These bands aligned with GPLs from *M. smegmatis* (data not shown), suggesting that only the smooth *M. abscessus* strains expressed GPLs. To further define the cell wall lipids of our 390R, 390V and 390S *M. abscessus* variants, lipid extracts were compared by TLC after spraying with sulfuric acid. The total lipid profiles of the 390R and 390V strains were very similar, containing trehalose mycolates, phosphatidylinositol mannosides and other phospholipids; these lipids were classified based on their mobility and characteristic hues, purple, brown-orange and yellow, respectively, as compared to extracts from *Mycobacterium bovis* BCG and *M. tuberculosis* (data not shown) (Rhoades *et al.*, 2003). The proportions of the lipids in the 390R and 390V extracts were also similar except for the most polar of the trehalose dimycolates (Fig. 3a, b, asterisk), which was more abundant in the 390R extract. These classes of lipids were also present in the 390S extract except for one of the trehalose monomycolates (Fig. 3b, small arrow) and an unidentified wax (Fig. 3b, large arrowhead). Notably, the 390S extract contained numerous additional lipids that charred to a gold-yellow hue with sulfuric acid (Fig. 3b). These lipids exhibited R_F values similar to or greater than those of the trehalose mycolates

(Fig. 3b, square bracket). The colour and mobility of the extra lipids are similar to those of apolar GPLs of *M. avium* (McNeil *et al.*, 1989), and it has been reported that *M. abscessus* expresses GPLs (Lopez-Marin *et al.*, 1994). To provide further evidence that these lipids are *M. abscessus* GPLs, the plates were sprayed with 1-naphthol to detect deoxyhexoses found in GPLs, and extracts were also subjected to mild alkaline methanolysis to which GPLs are resistant (McNeil *et al.*, 1989; Torrelles *et al.*, 2002). 1-naphthol turned the extra lipids of the 390S extract to a characteristic orange-pink (Fig. 3c), and the only other lipids detected were the trehalose mycolates, which have a different deoxyhexose that charred to a lavender hue. Upon mild alkaline methanolysis most lipids, including the phospholipids and trehalose mycolates, were degraded; however, the extra lipids in the 390S extract remained (Fig. 3c). The shift in mobility of the saponified lipids was likely due to partial deacetylation caused by the methanolysis treatment. Interestingly, lipids that aligned with these were also present at very low proportions in alkali-treated 390R and 390V extracts, indicating that GPLs are made by all three strains. These results show that, in addition to trehalose glycolipids and phospholipids expressed by all

Fig. 3. TLC analysis of the lipid profiles of *M. abscessus* strains. Equal weights (150 µg) of total lipid extracts and alkali-stable extracts (saponified) from 390R, 390S and 390V strains of *M. abscessus* were resolved in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ [65:25:4 (a) or 100:14:0.8 (b, c), by vol.] and visualized with H_2SO_4 (a, b) or 1-naphthol (c). (a) All extracts contained trehalose mycolates (TM), phospholipids (PL) and phosphatidylinositol mannosides (PIM) in similar proportions, except for an abundant TDM (asterisk). The 390S extract contained additional gold-yellow-charring lipids that resist saponification. (b) Resolution of the less polar lipids revealed the presence of multiple additional lipids (square bracket) that constituted a large proportion of the S extract and much smaller proportions of the other extracts. TDM (asterisk) was present in the 390S extract, co-migrating with the additional lipids; however, the 390S extract lacked a polar TM (small arrow) and an unidentified wax (arrowhead). (c) Deoxyhexose-containing lipids (as detected by 1-naphthol) were present in all extracts. 390R and 390V extracts contained mostly lavender-coloured TMs; whereas the 390S extracts contained abundant orange-pink-coloured lipids that were alkali-resistant. The change in mobility of these lipids was likely due to deacetylation from the saponification.

three strains, the 390S strain expresses a significantly higher proportion of GPLs. As 390R was the parental strain to 390S, and 390V was derived from 390S, our results indicate that high level GPL expression is a reversible phenotype.

***M. abscessus* 390S exhibits sliding motility and biofilm formation not demonstrated by 390R or 390V**

The ability of smooth strains of *M. smegmatis* and *M. avium* to slide across the surface of motility agar (Martinez *et al.*, 1999) and form biofilms is due to the presence of GPL (Recht *et al.*, 2000; Recht & Kolter, 2001). Smooth and intermediate strains of *M. abscessus*, including 390S, were motile and formed a thin film of growth spreading 1–2 cm from the centre of the plate, while the rough strains were non-motile (data not shown). In addition, the 390S variant had the capability to adhere to and grow on the surface of polystyrene pegs in the Calgary biofilm device, while both 390R and 390V lacked this ability (Fig. 4). These results indicate that 390S is able to form biofilms and is consistent with our finding of GPL expression by this variant.

The *M. abscessus* 390V revertant is able to persist and multiply in human monocyte monolayers

To investigate the relationship between colony morphology and persistent infection, we infected primary human monocytes with similar numbers of 390R, 390S and 390V, and compared their growth over a 3-day period. As in previous experiments (Byrd & Lyons, 1999), 390S declined in number over 3 days, whereas 390R caused persistent, progressive infection. Importantly, 390V showed a similar pattern as 390R, with overlapping numbers of c.f.u. in monocytes at day 3 (Fig. 5). These results strongly suggest that the ability of 390R and 390V to persistently infect human monocytes is a consequence of their rough surface phenotype.

The *M. abscessus* 390V revertant causes invasive infection in the lungs of mice

To determine whether the ability of 390R to cause persistent, invasive infection in mouse lungs (Byrd & Lyons, 1999) was regained by the 390V revertant we infected SCID mice and examined the growth of *M. abscessus* strains in the lungs over a 4-week period. Comparison of the 390S strain to the 390V strain shows that the revertant causes persistent, invasive infection (Fig. 6). At 28 days, no c.f.u. were recovered from the lungs of any of the four mice infected with the 390S strain. In contrast, at this time point, c.f.u. were recovered from the lungs of each of the four mice infected with either the 390R or the 390V strain. Consistent with the ability of 390V to cause invasive disease was the finding that both 390R (2 out of 4 mice, mean = 310 c.f.u.) and 390V (3 out of 4 mice, mean = 140 c.f.u.) were found in the spleens of mice at 21 days. In contrast, no c.f.u. from

Fig. 4. *M. abscessus* 390S demonstrates biofilm-forming capability, which is lacking in the 390R and 390V variants. (a) *M. abscessus* variants were inoculated into wells (2×10^4 c.f.u. per well) in a Calgary biofilm device consisting of a 96-well tray covered with a lid containing peg inserts. The cultures were incubated at 37 °C on a rotary shaker (150 r.p.m.) with the media in the wells replaced once at 48 h. The pegs were removed from the lids and sonicated in PBS at 48 and 96 h. Supernatants were plated to determine c.f.u. Data are the mean of quadruplicate determinations at 48 and 96 h. ○, 390R; △, 390S; ▲, 390V; *, 390S versus 390R and 390V, $P < 0.01$, *t*-test. (b) *M. abscessus* variants were inoculated as above. At 48 h the bacteria from pegs attached to lids were fixed and stained with crystal violet dye. Bacteria were then destained with ethanol and the A_{570} value of the ethanol solution determined. Data are the mean of quadruplicate determinations at 48 h. *, 390S versus 390R and 390V, $P < 0.01$, *t*-test. (c) The tips of crystal violet-stained *M. abscessus* pegs from (b) were photographed using an inverted phase-contrast microscope ($\times 100$ magnification) prior to destaining. 390S demonstrates a relatively uniform layer of bacteria on the tip of the peg, whereas 390R and 390V demonstrate only occasional dark-staining bacterial aggregates.

the 390S strain were found in the spleens of any mice throughout the course of infection.

DISCUSSION

Our results demonstrate that a single clinical isolate of *M. abscessus* has the ability to revert from a smooth, non-invasive, biofilm-forming phenotype (390S) to a rough,

Fig. 5. *M. abscessus* 390V has regained the ability to persist in human monocytes. Human monocyte monolayers were infected with the *M. abscessus* 390R (○), 390S (△) or 390V (▲) strains. Bacterial c.f.u. in cell lysates were determined at the indicated intervals and corrected to the number of monocytes in the monolayers. Monolayers were >98% viable at all time points in all groups. Data are the mean of duplicate determinations at time 0, and quadruplicate determinations at 2 and 3 days. *, 390R and 390V versus 390S, $P < 0.05$, *t*-test; **, 390R and 390V versus 390S, $P < 0.01$, *t*-test.

invasive, non-biofilm-forming phenotype (390V). These findings have potentially important implications for understanding the pathogenesis of *M. abscessus* lung infection.

The results of our study are consistent with studies of NTM that have compared rough and smooth colony variants, and found that rough variants tend to be more virulent in experimental infection models. It has been reported that rough forms of *Mycobacterium kansasii* persist longer than

Fig. 6. *M. abscessus* 390V has regained the ability to cause persistent infection in mice. SCID mice ($n=4$ per group) were intranasally inoculated with *M. abscessus* 390R (white bars), 390S (crosshatched bars) or 390V (black bars) strains. At the indicated time intervals the mice were sacrificed, and the total lung bacterial c.f.u. determined. The data represent the mean $\pm$ SD. *, 390S versus 390V, $P < 0.05$, *t*-test; **, 390S versus 390V, $P < 0.05$, *t*-test.

smooth variants in experimentally infected mice (Belisle & Brennan, 1989; Collins & Cunningham, 1981). The relationship between virulence and colony phenotype is more complicated in *M. avium* strains. Rough variants of *M. avium* are generally pathogenic for mice and chickens, whereas smooth opaque variants are generally non-pathogenic (Schaefer *et al.*, 1970). However, a smooth transparent colony phenotype is associated with increased virulence (Reddy *et al.*, 1994), and a rough transparent colony phenotype with even greater virulence (Kansal *et al.*, 1998). Although our *M. abscessus* 390S variant lacks the ability to cause invasive infection it exhibits both sliding motility and biofilm formation, which are postulated to play a role in surface colonization (Martinez *et al.*, 1999; Recht *et al.*, 2000). Progressive infection by NTM is often preceded by colonization of various anatomic sites. In addition, colonization of medical devices by NTM can lead to subsequent serious infection, for example catheter-related bacteraemia (Brown-Elliott & Wallace, 2002). Both sliding motility and biofilm formation are dependent on GPL expression (Recht *et al.*, 2000; Recht & Kolter, 2001). Consistent with these observations, our results demonstrate abundant expression of GPL by the 390S variant but minimal expression by the original 390R isolate and the 390V revertant. It is thus likely that GPL is responsible for the sliding motility and biofilm formation that we have observed in the 390S variant.

GPLs are found in the outermost portion of the mycobacterial cell envelope of NTM (Barrow *et al.*, 1980; Furuchi & Tokunaga, 1972; Goren *et al.*, 1972; Ortalo-Magne *et al.*, 1996) and contain antigenic determinants for a number of mycobacterial species (Brennan & Goren, 1979; Camphausen *et al.*, 1985; Lopez-Marin *et al.*, 1994). The GPL molecule typically consists of a tripeptide-amino alcohol core with an amide linked long chain fatty acid. This lipopeptide core is substituted with 6-deoxytalose and *O*-methylated rhamnose to generate the non-specific core GPLs found in many species of NTM (Brennan & Goren, 1979). These non-specific GPLs are further modified by the addition of oligosaccharides to produce antigenic serovar specific GPLs in some mycobacterial species (Brennan & Nikaido, 1995; Lopez-Marin *et al.*, 1994).

M. abscessus has been found to possess five major GPLs, which are also found in *Mycobacterium chelonae* and exhibit cross-reactivity with *M. chelonae* antiserum (Lopez-Marin *et al.*, 1994). The differences between these five groups are due to differences in the location and/or number of the acetyl and sugar moieties. Only two *M. abscessus* isolates were analysed in the report by Lopez-Marin *et al.* (1994) and it was not noted whether these isolates had a rough or a smooth phenotype. Based on our results it is likely that these isolates expressed the smooth phenotype.

Phenotypic change associated with GPL expression has been described previously. Spontaneously occurring *M. avium* mutants lacking GPL were identified by a change from a smooth to a rough colony phenotype. Loss of *M. avium* GPL

can occur as a result of deletion of large genomic regions encoding GPL synthesis proteins (Eckstein *et al.*, 2000). A gene encoding a mycobacterial peptide synthetase designated *mps* is required for assembly of the lipopeptide core of GPL in *M. smegmatis* (Billman-Jacobe *et al.*, 1999). We have identified an *mps* gene homologue in our three *M. abscessus* variants that has strong similarity to the *mps* gene of *M. smegmatis* and *M. avium* (unpublished data). This suggests that all three mycobacterial species utilize similar mechanisms for GPL synthesis. However, whereas deletions were responsible for observed differences in *M. avium* colony morphotypes (Eckstein *et al.*, 2000), the ability of the *M. abscessus* 390 isolate to bidirectionally change colony phenotype suggests a reversible mechanism, rather than a deletion of genes involved in GPL synthesis. In addition, the expression of small amounts of GPL by our rough variants also argues against deletion.

In contrast to the *M. abscessus* 390S variant, both 390R and 390V variants form cords in broth medium that are morphologically identical to those of *M. tuberculosis* (Attorri *et al.*, 2000). The mechanism for *M. abscessus* cording likely involves trehalose 6,6' dimycolate (TDM). Our TLC results suggest that TDM is present in each of our three variants; however, further compositional analysis of these bands is under way to determine their chemical structure. Recent studies indicate that the fine chemical structure of TDM determines the cording phenotype, and that TDM molecules with cording capability enhance the virulence of *M. tuberculosis* (Glickman *et al.*, 2000; Rao *et al.*, 2005). Thus, cord formation by *M. abscessus* may in part be responsible for the ability of the 390R and 390V variants to persist and cause invasive infection. The lack of cording by the 390S variant may be due to localization of GPL to the outermost portion of the *M. abscessus* cell wall preventing the interaction of TDM molecules from contiguous bacteria necessary for cording to occur. A similar hypothesis has been proposed to account for the difference in virulence between smooth and rough strains of *M. kansasii* (Belisle & Brennan, 1989). Alternatively the attenuation of the 390S variant could in some way be related to our finding that one of the trehalose monomycolates and an unidentified wax are absent from the 390S variant, but present in both the 390R and 390V variants.

M. abscessus has emerged as an important cause of infection caused by NTM (Cullen *et al.*, 2000; Fauroux *et al.*, 1997; Griffith *et al.*, 1993; Howard & Byrd, 2000; Olivier *et al.*, 2003; Sanguinetti *et al.*, 2001; Sermet-Gaudelus *et al.*, 2003). Since GPL expression by NTM has been postulated to play a role in environmental colonization (Recht *et al.*, 2000), and cord formation is required for mycobacterial persistence in infected hosts (Glickman *et al.*, 2000; Rao *et al.*, 2005), the ability to switch phenotypes may allow *M. abscessus* to transition between a colonizing organism and an invasive human pathogen. There have been no studies correlating *M. abscessus* phenotype with clinical outcome. However, one report of a severe *M. abscessus*

pulmonary infection in a cystic fibrosis patient who had received a lung transplant documented that the infecting organism had a rough colony phenotype (Sanguinetti *et al.*, 2001).

The ability to transition between smooth and rough colony phenotypes could have particular relevance to *M. abscessus* pulmonary infection in cystic fibrosis patients. The altered pulmonary physiology of these patients makes them particularly susceptible to colonization by biofilm-forming bacteria such as *Pseudomonas aeruginosa* (Boucher, 2004). Chronic lung colonization by such bacteria causes a host inflammatory reaction that damages the lungs over time (Chmiel *et al.*, 2002). Colonization of the lungs of these patients with *M. abscessus* may be favoured by strains with a smooth colony phenotype expressing GPL. Since *M. abscessus* 390S has a reversion frequency from smooth to rough in the range of $1:10^5$ – 10^6 , it is conceivable that a rough cord-forming variant could emerge from cystic fibrosis patients chronically colonized with a smooth variant expressing GPL. Our results raise the possibility that the emergence of such a variant expressing minimal GPL could herald the onset of more aggressive, invasive pulmonary infection. Such an emergence was recently demonstrated in a study involving *Burkholderia cenocepacia*. Importantly, the emergent non-biofilm-forming strain was able to persist at a much higher level in the lungs of normal mice, demonstrating that loss of biofilm-forming capability may be associated with expression of factors that increase virulence (Conway *et al.*, 2004).

In conclusion, our study provides new insights into the pathogenesis of infection caused by *M. abscessus*, and establishes a framework for assessing the clinical correlation between *M. abscessus* infection and colony phenotype, particularly with regard to the roles of GPL expression and cord formation.

ACKNOWLEDGEMENTS

Financial support: UTHCT Research Council Seed Grant (S. T. H. and X. P.), NIH grant GM58213 (R. K.), NIH grant HL55776 (C. R. L.), Department of Veterans Affairs and DeSouza Research Award from the American Lung Association (T. F. B.). We thank Liana Ponce for excellent technical assistance.

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