Virulence and drug susceptibility of *Mycobacterium celatum*

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The virulence and drug susceptibility of a clinical isolate of *Mycobacterium celatum* which showed smooth transparent (ST) and smooth opaque (SO) colonies were studied. While ST cells multiplied intracellularly and maintained their coccobacillary form in a human macrophage model of infection, SO cells formed long filaments and completely destroyed the phagocytes. In BALB/c mice, the ST variant, but not the SO variant, grew efficiently in the spleen, liver and lung. The ST variant was usually more resistant *in vitro* than the SO variant to drugs, with MIC values for clarithromycin (CLA), azithromycin (AZI), ciprofloxacin, sparfloxacin, amikacin, clofazimine, ethambutol and isoniazid being higher than those of the SO variant. In beige mice infected with the more highly virulent variant ST, CLA and AZI were the most active drugs in terms of viable count reduction in organs and mutant selection. Together, these observations indicate that the ST variant of *M. celatum* is a virulent form that can be efficiently inhibited *in vivo* by CLA and AZI.

**Keywords:** *Mycobacterium celatum*, macrophage infection, mice infection model, drug susceptibility

**INTRODUCTION**

*Mycobacterium celatum* was first described in 1993 as a new mycobacterial species biochemically indistinguishable from the *Mycobacterium avium–intracellulare* complex (MAC), with a mycolic acid pattern closely related to that of *Mycobacterium xenopi* (Butler et al., 1993). The infections caused by this organism were reported to occur mostly in persons with suppressed cell-mediated immunity, such as AIDS patients (Piersimoni et al., 1994, 1997; Gholizadeh et al., 1998; Bonomo et al., 1998; Bull et al., 1995; Tortoli et al., 1995; Zurawski et al., 1997), but infections also occurred in apparently immunocompetent hosts (Bux-Gewehr et al., 1998). Drug susceptibility testing showed that this organism was resistant to rifampicin (RMP), isoniazid (INH) and pyrazamide (Butler et al., 1993), so that regimens similar to those administered in MAC-infected patients were usually used for the treatment of *M. celatum* infections (Piersimoni et al., 1997; Gholizadeh et al., 1998; Bonomo et al., 1998; Bull et al., 1995; Tortoli et al., 1995; Zurawski et al., 1997).

Some authors (Butler et al., 1993) reported that *M. celatum* colonies were predominantly small, smooth and dome-shaped, and that flat transparent colonies were rarely observed. In other studies (Tortoli et al., 1995), colonies were found to be polymorphic and similar to those of MAC and *M. xenopi*. Colonies of strains isolated from AIDS patients were reported to be transparent in the first period of growth but creamy white and pigmented after 8–12 weeks incubation (Bull et al., 1995). The factors influencing the change in colony morphology in mycobacteria are not known, but studies on antimicrobial susceptibility and pathobiological significance of the smooth transparent (ST) and smooth opaque (SO) variants of MAC have been reported (Reddy et al., 1996); in contrast, no information on the virulence and drug susceptibility of *M. celatum* colonial variants has been published.

The purpose of this investigation was to present data on the growth *in vitro*, *ex vivo* and *in vivo* of two colonial
variants of a *M. celatum* strain isolated from an AIDS patient; in addition, the *in vitro* and *in vivo* susceptibility of the two morphotypes to antimicrobial agents is shown.

**METHODS**

Micro-organism and isolation of colonial variants. A *Mycobacterium celatum* strain (Cel 3) isolated from an AIDS patient was kindly provided by Dr C. Piersimoni (Piersimoni et al., 1997) and used throughout this study. When the strain was grown on Middlebrook 7H10 agar (Difco) at 37 °C under 5% CO₂, it showed two colonial variants with ST and SO morphology. ST and SO colonies were reisolated on 7H10 plates and incubated for 1 week, then suspended in Middlebrook 7H9 broth (Difco) and stored at −40 °C until use. Colonies were observed using a stereomicroscope or an inverted microscope; bacterial cells were stained by the Kinyoun method (Master, 1992).

Infection studies in human macrophages. Leukocyte buffy coats obtained from healthy donors were separated over Ficoll–Hypaque (Histopaque 1077; Sigma), as previously described (Fattorini et al., 1995). Briefly, suspensions of isolated peripheral blood mononuclear cells prepared in complete RPMI 1640 medium (Life Technologies) containing isolated peripheral blood mononuclear cells prepared in complete RPMI 1640 medium (Life Technologies) containing 10% heat-inactivated (56 °C, 30 min) fetal calf serum, 25 mM HEPES and 2 mM L-glutamine were adjusted to 1 × 10⁶ cells ml⁻¹ and distributed in 0.5 ml volumes in 24-well tissue culture plates. After 2 h incubation at 37 °C under a humidified 5% CO₂ atmosphere, non-adherent cells were aspirated and the monocytes (2–4 × 10⁵ per well) were washed extensively. Monolayers were enumerated in the bacterial population was defined as the ratio between treated and untreated control mice on day 56. Besides the determination of the total number of c.f.u. from Charles River. Mice were infected intraperitoneally (i.p.) with 0.5 ml of a bacterial suspension containing 1 × 10⁶ ST or SO c.f.u. At different time points, mice were killed by cervical dislocation and 4 ml sterile PBS was injected into the peritoneum. The fluid was withdrawn and added to an equal volume of Middlebrook 7H9 broth. Suspensions were serially 10-fold diluted and plated onto 7H10 agar medium. The c.f.u. were counted after incubation of the plates for 10–14 d at 37 °C in humidified air with 5% CO₂. The organs, collected under aseptic conditions, were suspended in 7H9 medium, ground in homogenizers, briefly sonicated, and the number of c.f.u. was determined.

MIC determination by the agar dilution method. MICs were determined by the twofold agar dilution technique using Middlebrook 7H10 agar. Inocula were prepared by suspending SO or ST colonies in Middlebrook 7H9 broth. Antibiotic-containing plates with drug concentrations ranging from 64 to 0.06 µg ml⁻¹ were inoculated with 3 × 10⁵ or 3 × 10³ c.f.u. and incubated at 37 °C in plastic bags for 14 d. The MIC was defined as the lowest drug concentration at which no visible growth of the organism was observed.

Antimicrobial susceptibility in beige mice. Male beige mice (C57BL/6/bg/bg) were obtained from Jackson Laboratories. Mice were infected i.p. with 0.2 ml of a bacterial suspension containing 1 × 10⁵ ST c.f.u. (Gangadharam, 1995; Fattorini et al., 1998). One day after infection, five mice were killed, and the organs were aseptically removed, homogenized in 1.5 ml Middlebrook 7H9 broth and sonicated at −40 °C until use. Colonies were observed using a stereomicroscope or an inverted microscope; bacterial cells were stained by the Kinyoun method (Master, 1992).

**RESULTS**

Colony and cellular morphology

The morphology of SO and ST colonies and the microscopic appearance of SO and ST cells after 7 d incubation on Middlebrook 7H10 agar are shown in Fig. 1. The SO variant displayed smooth dome-shaped opaque colonies (Fig. 1a) which developed a pale yellow pigmentation after 3–4 weeks incubation; the ST variant formed smooth transparent colonies (Fig. 1b) with
Fig. 1. Microscopic appearance of ST and SO variants of *M. celatum*. (a) SO colonies, as observed by a stereomicroscope. (b) ST colonies, as observed by an inverted microscope. (c) Microscopic aspect of SO cells (Kinyoun staining). (d) Microscopic aspect of ST cells (Kinyoun staining). (e) Electron micrograph of ultrathin sections of SO cells; bar, 0.5 µm. (f) Electron micrograph of ultrathin sections of ST cells; bar, 0.5 µm.
irregular filamentous extensions at the edge. Microscopic examination of SO and ST colonies on day 7 showed that the SO variant was formed by aggregates of long branched septated cells (Fig. 1c) and the ST variant was formed by coccobacilli (Fig. 1d); after 14 d incubation, both ST and SO colonies were formed by cells similar to 7-d-old SO cells. While SO colonies were easily maintained in stable cultures in 7H10 medium, a transition of ST colonies into SO colonies (ST → SO) was observed after 3–4 weeks incubation, as shown by the appearance of opaque spots in the central area which increased in size and transformed ST into SO colonies with time.

By transmission electron microscopy, the majority of SO cells of 7-d-old cultures appeared as septated, sometimes branched, cells (Fig. 1e), while ST cells appeared as coccobacilli (Fig. 1f). In older cultures, ST cells also acquired a branched morphology similar to SO cells (results not shown).

**Growth of ST and SO variants in human macrophages**

The growth of the two colonial variants of *M. celatum* in human macrophages is shown in Fig. 2.

When macrophages were infected with ST cells, an increase from $1 \pm 0.3 \times 10^8$ c.f.u. (ml macrophage lysate)$^{-1}$ on day 0 (about one mycobacterium per macrophage) to $4 \pm 2 \times 10^8$ c.f.u. (ml macrophage lysate)$^{-1}$ on day 3 (three to four mycobacteria per macrophage; Fig. 2a) and $13 \pm 3 \times 10^8$ c.f.u. (ml macrophage lysate)$^{-1}$ on day 7 (about 10 mycobacteria per macrophage) was observed, as determined by both c.f.u. counts and Kinyoun staining. ST cells maintained their coccobacillary shape and did not alter the viability of the phagocytes (1, 2 and 5 % dead macrophages were seen on day 0, 3 and 7, respectively). The macrophages infected with the ST variant were about 30, 40 and 70 % on day 0, 3 and 7, respectively, and the numbers of...
extracellular c.f.u. (ml supernatant)$^{-1}$ were $0.1 \pm 0.02 \times 10^5$, $0.4 \pm 0.1 \times 10^5$ and $2.0 \pm 0.4 \times 10^5$ on day 0, 3 and 7, respectively.

SO cells also multiplied efficiently within the macrophage, but the pattern of growth was different from that of ST cells. On day 0, the number of intracellular c.f.u. was $2 \pm 0.4 \times 10^5$ c.f.u. (ml macrophage lysate)$^{-1}$, with about 50% of macrophages infected and one to three mycobacteria per macrophage; about 1% dead macrophages was seen and the c.f.u. number in the supernatant was $0.2 \pm 0.04 \times 10^5$ ml$^{-1}$. After 3 d, a rapid growth of SO mycobacteria in macrophages in the form of long filaments invading the cytoplasm (Fig. 2b) and completely destroying the monolayers after 7 d was observed.

By electron microscopy it was shown that the bacteria were located exclusively inside phagosomes (Fig. 3). Usually these vacuoles contained some ST cells (from one to four) on day 0 (Fig. 3a) and a single bacterium on day 3 (Fig. 3b). Ferritin labelling (Fig. 3d) revealed that even though lysosome–phagosome fusion had occurred, ST cells showed no visible signs of degradation. The labelling also showed that the bacteria were surrounded by a 45–80-nm-thick electron-translucent zone resembling a capsule which separated the bacterial wall from the ferritin layer.

Single SO branched cells were observed inside separate vacuoles on day 0 or 3 (Fig. 3c). Ferritin labelling revealed that SO cells were surrounded by an irregularly shaped layer with short filaments protruding outward (Fig. 3e). No mycobacteria of either variant were apparently found outside vacuoles.

**Growth of ST and SO variants in BALB/c mice**

The kinetics of *M. celatum* growth in BALB/c mice is shown in Fig. 4. After i.p. infection, about 4% of the ST inoculum and 2% of the SO inoculum was recovered in the spleen, liver and lung on day 1; furthermore, from 6 to 7% of ST cells and <1% of SO cells were found in the peritoneum. The ST variant multiplied in the spleen, liver and lung up to day 14, then a containment of the infection was observed. In infected mice, bacterial growth was associated with a 20% increase in the lung weight on day 7, and 40% and 24% increase, respectively, in the spleen and liver weights on day 14, in comparison with uninfected mice; after 2 weeks, organ enlargements slowly decreased but never returned to the uninfected mice levels. SO c.f.u. decreased by 80% in the liver and by 88% in the spleen and lung until day 14, then slightly increased in the spleen and lung but not the liver; after this time, colonies with intermediate SO–ST morphology were observed in the viable count plates. SO variant infection was associated with a lower increase in organ weights up to day 14, in comparison with ST variant infection (14% increase in lung on day 7 and 29% and 10% increase in the spleen and liver weights, respectively, on day 14), followed by a rapid return to the uninfected mice levels. In the peritoneum, SO cells were cleared more rapidly than ST cells in the first day of infection, then a rapid decrease in ST and SO c.f.u. occurred.

**Antimicrobial susceptibility in vitro**

In vitro susceptibility of ST and SO variants to different drugs is shown in Table 1. The ST variant was more resistant than the SO variant to CLA, AZI, CIP, SPA (sparfloxacin), AMI, CLO (clofazimine), EMB and INH, with MIC values ranging from 0.25 to 8 µg ml$^{-1}$ for the ST variant and from 0.06 to 0.5 µg ml$^{-1}$ for the SO variant. The highest MIC differences between the ST and SO variants were observed for the fluoroquinolones CIP and SPA, with the ST variant being 32 and 16 times more resistant than the SO variant, respectively. Among other drugs, the ST variant was eight times more resistant than the SO variant to CLA, AZI and EMB, four times to CLO and INH, and two times to AMI. A noticeable exception was observed for the rifamycins RMP and RFB, for which the MIC values were 16 and 0.5 µg ml$^{-1}$ for the ST variant, respectively, and 64 and 2 µg ml$^{-1}$ for the SO variant, respectively.

**Antimicrobial susceptibility in vivo**

The antimicrobial susceptibility of ST variants to drugs in the beige mice model is shown in Fig. 5. The organisms efficiently multiplied in untreated control mice, as shown by a c.f.u. increase of approximately $3 \log_{10}$ in the spleen and liver and $2 \log_{10}$ in the lung. Compared with controls, all drugs tested caused a statistically significant c.f.u. reduction ($P < 0.05$) on day 28 and 56, with the exception of RMP and RFB in the lungs; however, as expected, RMP and RFB also showed poor activity in the spleens and livers (reduction of less than $2 \log_{10}$ in c.f.u. numbers on day 56). Although CIP was more effective that RMP and RFB, a slow and constant c.f.u. increase in all organs studied was observed on both day 28 and day 56. EMB was only bacteriostatic in the lung up to the end of the observation time but showed a better activity in the spleen and liver (more than $3 \log_{10}$ reduction in comparison with the control on day 56). AMI and INH showed an activity comparable with EMB in the spleen and liver, but with a remarkable regrowth in the lung after day 28. AZI and CLA showed the best activity in all organs with $4 \log_{10}$ reduction on day 56 in the spleen and liver in comparison with controls, and more than $2 \log_{10}$ reduction in the lung. After 56 d infection, mutants with MIC values corresponding to eight times the wild-type ST MIC were observed in untreated and drug-treated mice, respectively, at the following frequencies: RMP, $1 \times 10^{-5.18}$ and $1 \times 10^{-6.66}$; RFB, $1 \times 10^{-7.18}$ and $1 \times 10^{-8.64}$; CIP, $1 \times 10^{-7.39}$ and $1 \times 10^{-8.58}$; EMB, $1 \times 10^{-6.83}$ and $1 \times 10^{-4.74}$; INH, $1 \times 10^{-6.55}$ and $1 \times 10^{-4.29}$. No mutants were detected after therapy with CLA, AZI or AMI.

**DISCUSSION**

The existence of different colonial variants in mycobacteria was recognized a long time ago; however, the pathobiological significance of colony morphology has
Fig. 3. Ultrathin sections of human macrophages infected with the ST (a, b, d) or SO (c, e) variant of M. celatum. The bacteria were confined inside phagosomes. Usually the vacuoles contained some ST bacteria (from one to four) on day 0 (a) and a single bacterium on day 3 (b). Ferritin labelling (d) showed the occurrence of phagosome–lysosome fusion but
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*Fig. 4.* Time course of ST and SO variants of *M. celatum* in the organs and peritoneal cavity of BALB/c mice inoculated i.p. with $10^7$ c.f.u. Each point represents the mean value of five mice and the standard deviation. ●, ST variant; ○, SO variant.

not been extensively studied (Reddy et al., 1996; Schaefer et al., 1970; Rastogi et al., 1989).

The strain of *M. celatum* used in the present investigation showed SO colonies similar to those of MAC (Butler et al., 1993; Tortoli et al., 1995) and ST colonies with a ‘moustached’ appearance. In MAC, ST > SO transition has been attributed to various reasons, including metabolic starvation (McCarthy, 1974) and decreased temperature (Woodley & David, 1976). The observation that in *M. celatum* ST > SO transition at 37 °C occurred in the third to fourth week supports the possibility that also in this organism starving conditions favour the growth of SO cells, which are nutritionally less demanding than cells of the ST type.

When human macrophages were infected with ST cells, mycobacteria multiplied efficiently inside the phagocytes. A different pattern was displayed by SO cells, as shown by the formation of long filaments inside the macrophage which rapidly invaded and destroyed them. Similar observations were reported in MAC-infected HeLa cells (Brosbe et al., 1962), but, to our knowledge, not in MAC-infected macrophages, in which mycobacteria were found to lie inside the limits of the phagocytes (Meylan et al., 1990) and never exhibited a tendency toward branching (Crowle et al., 1986). The *M. celatum* pattern is more similar to that observed in cells infected with *Nocardia asteroides*, in which aggregates of filaments were formed in glial cell cultures.

no visible signs of ST bacterial degradation were seen; the labelling also revealed that the organisms were surrounded by an electron-translucent zone resembling a capsule (arrow). SO vacuoles contained a single bacterium on day 0 or 3 (c) with numerous septa and marked branching. In ferritin-labelled macrophages (e), the bacterial cell appeared surrounded by short strands of filamentous material (arrows). Bars, 0.5 µm.
Table 1. Activity of antimicrobial agents against ST and SO colonial variants of *M. celatum*

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (µg ml⁻¹) of:</th>
<th>ST variant</th>
<th>SO variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>1.00</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Azithromycin</td>
<td>4.00</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>8.00</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>2.00</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>1.00</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Clofazimine</td>
<td>0.25</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>4.00</td>
<td>0.30</td>
<td></td>
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<tr>
<td>Isoniazid</td>
<td>2.00</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>16.00</td>
<td>64.00</td>
<td></td>
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<tr>
<td>Rifabutin</td>
<td>0.50</td>
<td>2.00</td>
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</table>

(Beaman & Beaman, 1993) and human macrophages (unpublished observations of our group).

The ST variant was more virulent than the SO variant in the host, as reported for MAC (Reddy et al., 1996; Schaefer et al., 1970). In contrast to what was observed in cultured macrophages, the growth of SO bacteria was rapidly contained in mice, thus indicating the presence of an intact immune system is an essential requirement for SO variant control. However, the formation of intermediate ST–SO colonies which occurred during the progress of the infection in organs infected with the SO variant indicates that the ST phenotype is selected for the maintenance of infection. In comparison with what is known for MAC (Woodley & David, 1976), *M. celatum* shows an increased resistance to RMP and RFB. ST colonies are, in general, more resistant than SO colonies to many of the drugs tested; in contrast, it is interesting that in the SO variant the resistance to RFB and RMP was higher than in the ST variant.

Some discrepancies in the susceptibility of the clinical isolates of *M. celatum* have been reported (Piersimoni et al., 1997; Tortoli et al., 1995) in comparison with initial studies (Butler et al., 1993). This was believed to be due to either differences in the methods of testing or selection of strains representing different clones in the bacterial populations (Piersimoni et al., 1997; Tortoli et al., 1995). Our observations support the latter hypothesis and indicate that the presence of different proportions of ST and SO variants or intermediate forms in a bacterial population may affect drug susceptibility results; *in vitro* propagation or storage of clinical strains for an unspecified amount of time could be a cause of the differences in drug susceptibility results observed by various authors.

Therapy of *M. celatum* infections in AIDS patients is usually based upon administration of three to four anti-MAC agents, including CLA, AZI, RFB, CIP, AMI, EMB and CLO (Piersimoni et al., 1994, 1997; Gholidzadeh et al., 1998; Bonomo et al., 1998; Bull et al., 1995; Tortoli et al., 1995; Zurawski et al., 1997; Masur, 1993). Our data showed that, in the beige mouse model, CLA, AZI and EMB are the most active antimicrobial agents among those tested, even if only a bacteriostatic activity was seen in the lung. However, while no drug-resistant mutants could be detected in the spleen of mice treated with CLA and AZI, many mutants were found in...
EMB-treated mice. Overall, our data indicate that CLA and AZI, which are drugs recommended as first-choice agents for therapy of MAC infections in AIDS patients (Masur, 1993), could also be active against M. celatum infections in humans. The observation that CLA-containing regimens were usually beneficial for treatment of these infections in HIV-positive patients (Piersimoni et al., 1997; Tortoli et al., 1995; Zurawski et al., 1997) is in keeping with our results. As expected, RMP, which showed high MIC values against both ST and SO variants, was not effective in vivo; unfortunately, RFB, which appeared to be more active than RMP in vitro, was also ineffective in mice. The latter observation is in keeping with a recent paper (Gholizadeh et al., 1998) in which the occurrence of M. celatum infection in two HIV-positive patients treated prophylactically with RFB was reported. As for CIP, this drug is considered to be active in vitro against M. celatum strains (Butler et al., 1993; Bonomo et al., 1998; Bull et al., 1995; Tortoli et al., 1995) but, unexpectedly, showed low efficacy in mice. The discrepancy may be explained with the knowledge that, in vitro, the ST variant is 32 times more resistant than the SO variant to CIP. These observations clearly emphasize the need to use freshly isolated colonies for testing in vitro drug susceptibility of M. celatum.

Overall, our results indicate that the two colonial variants, ST and SO, of M. celatum can show differential cellular morphology, growth in macrophages, virulence in mice and drug susceptibility, and that CLA and AZI are, in the beige mouse model, the most effective drugs against the virulent ST variant.

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