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Characterization of the virulence of *Mycobacterium avium* complex (MAC) isolates in mice

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SUMMARY

The virulence of different isolates of MAC was studied in naturally susceptible BALB/c mice. In preliminary experiments, MAC bacteria forming smooth transparent colonies on solid media (SmT variants) were found to be virulent for BALB/c mice, causing progressive infection; smooth opaque (SmOp) were generally avirulent, being slowly eliminated from the infected organs; and rough (Rg) variants were either avirulent or as virulent as SmT variants. We chose to compare the virulence of different isolates of MAC of different origins, studying only the SmT morphotype. Strains of MAC isolated from naturally infected animals were those that most consistently caused progressive infections. AIDS patients-derived isolates were of intermediate virulence or devoid of virulence in mice. The environmental strains were eliminated from mice or did not proliferate. Strains of MAC isolated from individuals who were not infected by HIV varied in virulence from completely avirulent to highly virulent. There was no close correlation between virulence and restriction fragment length polymorphism (RFLP) type, although all highly virulent strains were of the A/I type. There was also no correlation between virulence analysed *in vivo* and the ability to grow in cultured macrophages.

Keywords virulence macrophages AIDS mycobacteria

INTRODUCTION

The incidence of MAC infections has been rising in the last 10 years. Most of this can be accounted for by the extreme susceptibility of AIDS patients to infection by these bacteria [1]. In parallel, there has also been a rise in the numbers of detected MAC infections in individuals who are not infected with HIV [2]. Whether this is a real increase in incidence of these infections in unpredisposed individuals, or a bigger awareness of these infections with a consequent better detection by the bacteriology laboratories is not yet clear. MAC bacteria are ubiquitous in nature, and have been isolated from the environment [3–5], from the stools of normal humans [6] and from infected wild animals [7]. A systematic analysis of the virulence of MAC isolates according to their origin has not been done. It is known that there is variability in their *in vivo* and *in vitro* virulence. Some of this variability can be accounted for by the different morphotypic variants of the bacterial strains. Three colonial variants of MAC have been described: smooth-transparent (SmT), smooth-opaque (SmOp), and rough (Rg). Some SmT variants studied are highly virulent for chickens and mice [8–12], SmOp variants are most often not virulent [8–12], and Rg variants have been reported to be either non-virulent [8–10] or highly virulent [10,11]. These studies have not, however, characterized the colonial morphology of the bacteria isolated from the infected animals, but only the morphology of the bacteria in the inocula used to infect the animals. It was thus not apparent if the morphology of the variants was maintained *in vivo*.

Virulence may, in addition, be independent of the morphotype studied. It is likely that different isolates thriving in distinct habitats may present distinct virulences to a mammalian host. It is also likely that the virulence of a MAC strain needed to infect a given host may vary according to its immune status; a less virulent strain may be able to infect an immunocompromised host, whereas a higher virulence may be necessary for a certain isolate to persist or proliferate in a host with intact mechanisms of defence. In order to evaluate the unclear relationships between host and MAC virulence we compared here the virulence of the different isolates of MAC in mice naturally susceptible to infection by this pathogen. We also compared the use of *in vitro* infections of cultured macrophages to evaluate virulence with the results obtained in our *in vivo*

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Table 1. Origin of the MAC isolates used in the study

Origin	Strain
Wild birds	PE9, PE17, PE22, PE23, PE27, PE29
Chicken	25291*
Armadillo	2606, 8637†
Pig	M520, M506
AIDS patients¶—stools	SK46, 1818‡, 3509
AIDS patients—sputum	SK55, 2069‡, 2459, 2466
AIDS patients—rectum	SK62
AIDS patients—blood	SK64, 1101, 1162w, 2296, 2435, 101, 1581
AIDS patients—bone marrow	2447
AIDS patients—unknown origin	2-6, 2-8, 2-29, 2-100, 2-151
Healthy human (stools)	757‡
Non-AIDS humans¶	1943, 1983, 2485, 4208§, 5299, 5791, 5816
Environment (Zaire)	4542‡, 4543‡, 5280‡

* From ATCC. Same as strain 724 from the Trudeau Mycobacterial Culture Collection.

† Armadillo-derived mycobacterium (ADM)-3 belonging to a new species [26].

‡ Isolates classified as MAC according to traditional methods [13] but Gen-Probe-negative [14].

§ Recognized by Gen-Probe as *Mycobacterium intracellulare*.

¶ No information was available on the clinical status of AIDS patients or on the clinical history of HIV$^-$ humans.

model. We used a mouse model, and discuss the relevance of the results obtained with it as regards the extrapolation of the results to the human infection.

MATERIALS AND METHODS

Bacteria

The origins of the *Myco. avium* isolates used in this study are shown in Table 1. Bacterial strains were either inoculated into Middlebrook 7H9 medium (Difco, Detroit, MI) containing 0·04% Tween 80 (Sigma, St Louis, MO) for the preparation of the inocula, or streaked onto Middlebrook 7H10 agar medium (Difco) for the screening of specific morphotypes. When different morphotypes were detected, colonies were picked and cultured in 7H9 broth. Bacteria were grown for 1–2 weeks, collected by centrifugation, suspended in a small volume of saline–0·04% Tween 80, briefly sonicated to disperse bacterial clumps, and diluted. Aliquots were frozen at −70°C until use. One aliquot was used to make the quantification of the number of colony-forming units (CFU) and evaluate the colonial morphology. Before inoculation in mice, the aliquots were thawed at 37°C and diluted in saline–Tween 80 if necessary. Strains 2.6, 2.8, 2.29, 2.100, 2.151 and 101 were kindly provided by Dr Ian M. Orme (Colorado State University CO); strain 101 was originally from Dr L. Young (Kuzell Institute, CA) and strain 2.151 morphotypes had been previously cloned and characterized by Dr John Belisle (Colorado State University). The other strains were identified by traditional methods [13] and with commercial DNA probes (Gen-Probe Inc., San Diego, CA). The tests were performed according to the directions in the literature supplied with the kits of AccuProbe test [14]. The typing of the isolates was done by restriction fragment length polymorphism (RFLP) as described elsewhere [15].

Animals

BALB/c mice *Myco. avium*-susceptible [16]) were obtained from a local breeder (Instituto Gulbenkian de Ciência, Oeiras, Portugal). Mice were kept under standard hygiene conditions and were used at about 8 weeks of age.

Study of the infection in vivo

Mice were inoculated in the lateral tail vein with 0·25 ml of the bacterial suspension containing 10^6 or 10^8 CFU of MAC. At different time intervals, mice were killed by cervical dislocation and their livers and spleens were aseptically removed, homogenized in a 0·04% Tween 80 solution in distilled water, and serial dilutions of the homogenates were plated onto 7H10 medium. Colonies were counted and characterized after incubation at 37°C for 10–15 days.

Study of the infection in vitro

Bone marrow-derived macrophages were prepared as described [17,18] and infected with the different isolates of MAC. Peritoneal cells were collected from the peritoneal cavities of mice inoculated with 1 ml of 10% sodium caseinate (Sigma) 4 days earlier. Three million cells were plated per well of 24-well tissue culture plates and allowed to adhere for 2 h. Non-adherent cells were removed by washing with warm Hanks' balanced salt solution (HBSS; GIBCO, Paisley, UK) and the adherent exudate macrophages were then infected. Macrophage monolayers were allowed to phagocytose the bacteria for 4 h, extensively washed and incubated in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 10% fetal calf serum (FCS; GIBCO), 10 mM HEPES buffer (GIBCO) and no antibiotics. Bacterial growth was evaluated at different time-points of infection by lysing the macrophage monolayers with saponin (0·1% final concentration), serially diluting the lysate in Tween 80 (0·04% in water) and plating in 7H10

medium. Data were represented as growth indices which were calculated by subtracting the mean log number of CFU at day 0 of infection from that found on day 11.

RESULTS

The origin of the different isolates is described in Table 1. The initial cultures arriving in our laboratory were composed of either pure morphotypes or combinations of two or three distinct morphotypes. There was no predominant pattern, nor any association of a given pattern with a particular origin of isolation. The characteristic morphologies are shown in Fig. 1. SmT colonies were translucent with a smooth, bright surface; SmOp colonies were dome-shaped and opaque; Rg colonies had wrinkled surface and borders, and were opaque. Some colonies would acquire pigmentation with time, but no extensive characterization of pigment production was done since virulence was not associated with pigment production in the first strains screened.

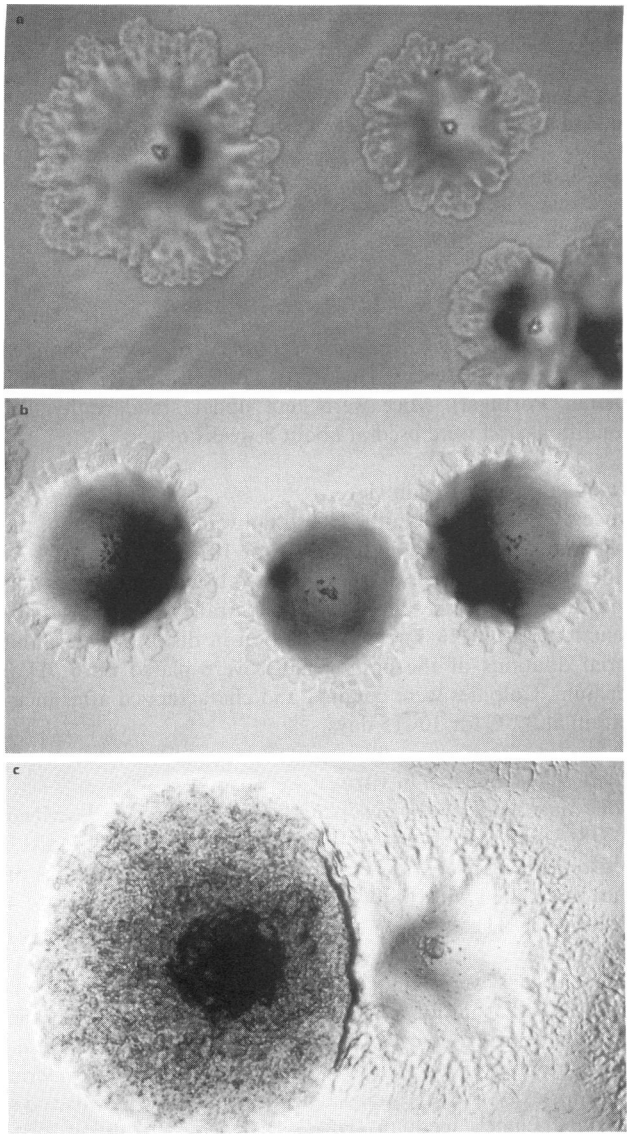

Fig. 1. Colony morphology of the variants used in the study. Middlebrook 7H10 agar plates seeded with the bacteria were incubated for 2 weeks and photographed with under illumination. (a) Smooth-transport (SmT) colonies. (b) Smooth-opaque (SmOp) colonies. (c) One rough (Rg) (left) and one SmT (right) colonies.

Establishment of the experimental protocol

To test and compare the virulence of different isolates, a particular morphotype has to be used, otherwise different proportions of distinct colonial variants in the inocula may account for the differences in two different isolates. To assess which of the three morphotypes had consistently the higher virulence and could thus be used to compare different bacterial strains, we made preliminary experiments either injecting bacterial inocula prepared directly from the sample received or, alternatively, selected a few isolates from which different morphotypes had been isolated, and tested them separately. The results are shown in Figs 2 and 3. Inocula prepared from the stock culture contained different proportions of SmT, SmOp and Rg morphotypes. In Fig. 2 we show the proliferation of four strains of *Myco. avium* whose inocula had not been selected for the presence of isolated morphotypes. Of these four strains, only one (strain 2435) contained a mixture of colonial variants that could be easily identified in the inocula. Strain 2447 was composed of SmT variant, and strains 2459 and 2466 of SmOp variants. When SmOp variants were inoculated (strains 2459 and 2466) we could detect the emergence of the SmT forms after 1 month of infection. All four of the SmT variants were able to persist or proliferate in the infected organs. Only the SmOp variant of strain 2435 was able to proliferate, whereas the SmOp variants of strains 2459 and 2466 were slowly eliminated. In subsequent studies we have never found any other strains which showed proliferation of the SmOp variant (not shown). It was not possible to ascertain whether the emergence of the SmT variants of strains 2459 and 2466 was due to their presence in the inocula in such low frequencies that their detection was not possible or, alternatively, whether they were revertants from the SmOp variants. In a second set of experiments we studied the proliferation of purified morphotypes of three different strains of *Myco. avium* (Fig. 3). All SmT morphotypes were able to proliferate even though they showed differences in their virulence. The two SmOp variants were eliminated, as was one of the Rg variants from strain 25291. However, both another Rg morphotype of this latter strain and the Rg variant isolated from strain 2.151 were able to proliferate *in vivo*. We thus decided to select SmT morphotypes from the different strains for further studies aimed at comparing virulence between isolates.

Virulence of MAC according to the origin of the strain

We studied the *in vivo* proliferation of 41 strains of MAC derived from infected animals, AIDS patients, non-AIDS-infected human individuals, and from the environment. The results are shown in Fig. 4. The isolates originating from infected animals (wild birds, chickens, pigs and armadillo) were highly virulent for mice, with two exceptions out of the 11 strains studied (these latter two were both isolated from pigs). These highly virulent isolates proliferated from 3.18 to 5.77 $\log_{10}$ units in 100 days in the livers of the infected mice. Isolates from AIDS patients exhibited lower virulence in BALB/c mice. Some of the strains (11/19) were able to

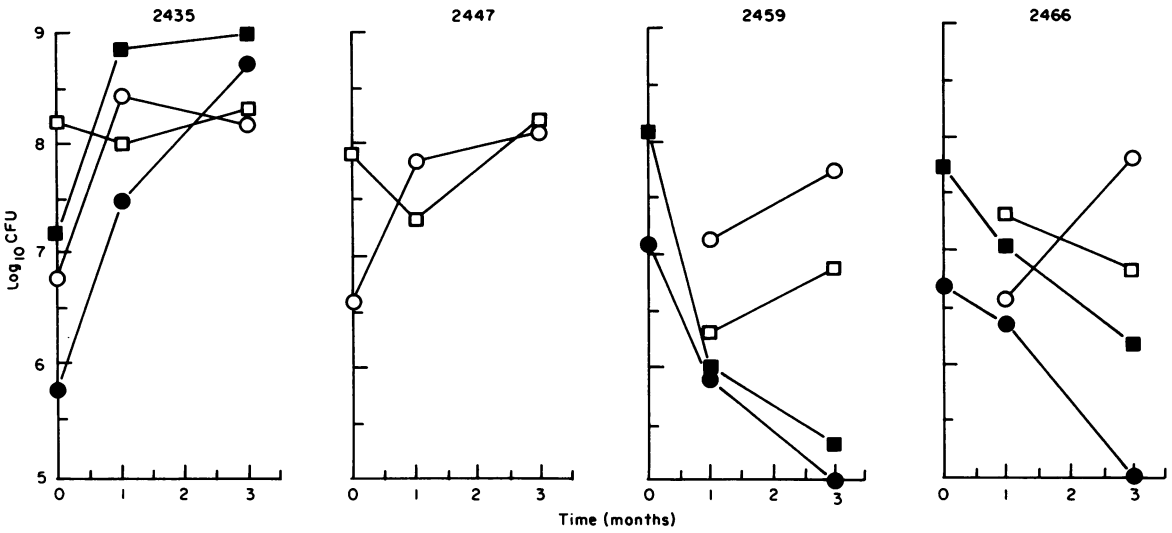

Fig. 2. Colony-forming unit (CFU) counts in the spleen (circles) and liver (squares) of BALB/c mice infected with four isolates of *Mycobacterium avium* from AIDS patients (isolate numbers 2435, 2447, 2459, and 2466). Closed symbols represent the smooth-opaque (SmOp) variant counts, and open symbols those of the smooth-transparent (SmT) variants.

proliferate even though maximal growth did not exceed 2·29 $\log_{10}$ units in 100 days in the livers of the infected mice. The other eight strains did not grow, or were slowly eliminated from the livers (growth index from 0·00 to −2·81 $\log_{10}$ units in 100 days). The isolates from human patients not infected by HIV varied in virulence, including two strains with virulence similar to the animal isolates (growth index of 3·15 and 4·13 $\log_{10}$ units in 100 days), four strains with intermediate virulence (growth indices of 0·39, 0·71, 0·99 and 1·06 $\log_{10}$ units in 100 days) and two strains that were progressively eliminated from the infected livers. The three environmental strains were avirulent, being eliminated from the livers of infected mice. Although some SmOp revertants were occasionally detected after plating the organs from infected mice, they were a minority and were not associated with the fate of the microorganism, i.e. the percentage of SmOp colonies was not higher in the avirulent compared with more virulent strains.

A selection of 23 strains covering the different groups of isolates was then analysed according to the RFLP types previously described [18]. All the highly virulent strains were

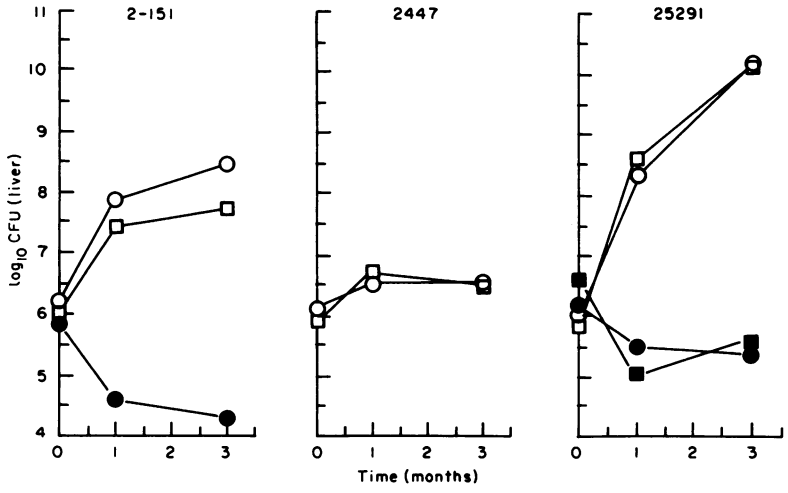

Fig. 3. Colony-forming unit (CFU) counts in the liver of BALB/c mice inoculated with cloned pure smooth-transparent (SmT) (○), smooth-opaque (SmOp) (●), or rough (Rg) (□, ■) colonial variants.

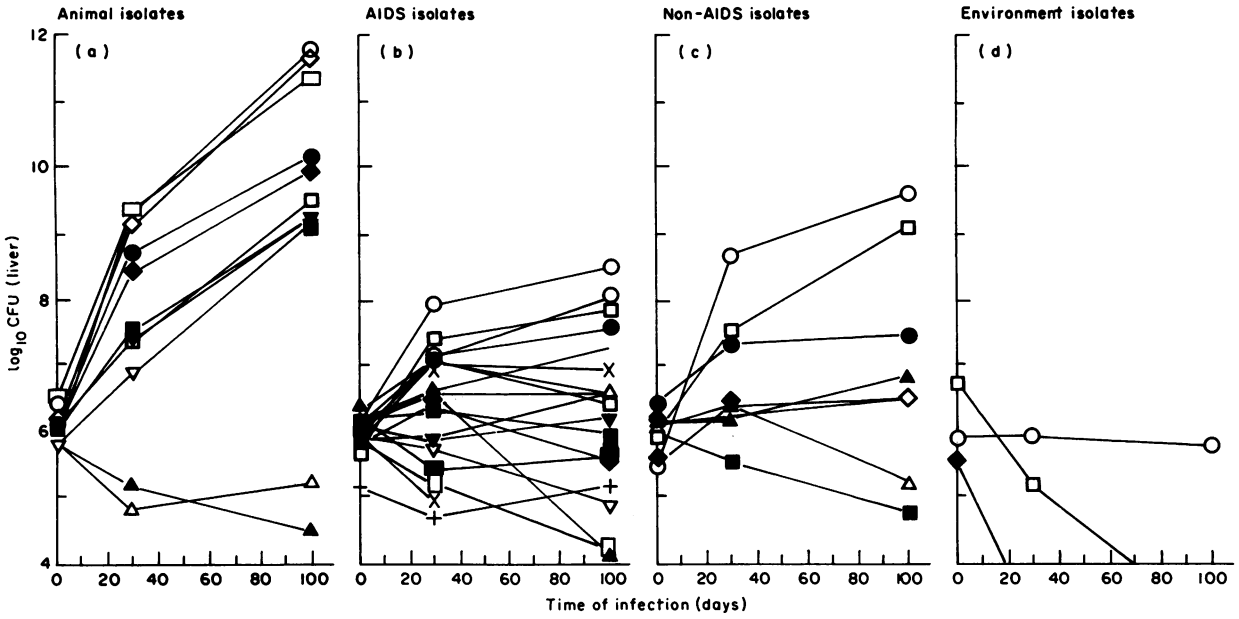

Fig. 4. Growth of a collection of MAC strains of smooth-transparent (SmT) morphotype with four distinct origins (animal, AIDS patients, non-AIDS humans, and environment) in the livers of BALB/c mice. (a) ○, 2606; ◆, PE9; ●, PE17; ◇, 25291; ■, PE27; □, PE23; ▲, M520; △, M506; ▽, PE29; ▼, PE22; ▭, 8637. (b) ●, 1818; ○, 2435, 2151; ▽, 1101; ◇, 1162w; ■, 3509; □, 101; ▲, 2447; △, SK64; ▼, SK46; ▭, SK55; ▬, SK62; ×, 2.8, 2.100; ▯, 2.29; ▮, 2.6; —, 1581; ⊡, 2296; ◆, 2069. (c) ○, 1943; ◆, 5299; ●, 2485; ◇, 757; ■, 1983; □, 5791; ▲, 4208; △, 5816. (d) ○, 4542; ◆, 5280; □, 4543.

of the A/I type, whereas the strains with intermediate virulence and the avirulent strains showed any of the RFLP types (A, H, as well as two avirulent strains with the A/I type) (Table 2).

Analysis of virulence in in vitro *cultured macrophages*

To compare our *in vivo* model of analysis of virulence with such testing in an *in vitro* model, we infected macrophages from the same mouse strain with a selected number of MAC strains which had shown a broad variation in *in vivo* proliferation. Both bone marrow-derived (Fig. 5a) and freshly explanted peritoneal macrophages (Fig. 5b) were used. There was no correlation between the ability of the different isolates of MAC to grow *in vivo* and their ability to proliferate in cultured macrophages (Fig. 5a, b). Furthermore, the results obtained with the two macrophage populations were also different, mainly due to the broader range of growth indices in bone marrow-derived macrophages (Fig. 5a) compared with the closely similar growth indices in peritoneal macrophages (with the exception of one strain) (Fig. 5b). MAC strains were distributed in four different trends of behaviour: those that were either avirulent (strains 1983, 2069 and 4542) or virulent (strains 101, 5791, 8637 and PE9) in both the *in vivo* and *in vitro* (with bone marrow macrophages) assays, those that were able to grow *in vitro* but not *in vivo* (strains 1101, 2447, 3509 and 4208), and the most unexpected group of those strains able to proliferate *in vivo* but not in cultured macrophages (strain 2606) (Fig. 5a). Interestingly, this latter strain was the only strain showing a high proliferation in peritoneal macrophages (Fig. 5b).

Table 2. Characterization of the restriction fragment length polymorphism (RFLP) type of MAC strains according to origin and virulence

Origin	Virulence*	A	H	A/I	Total of strains
Environment	–	Ø	3	Ø	3
AIDS patients	±	5	1	Ø	6
AIDS patients	–	4	1	Ø	5
Animal	+++	Ø	Ø	6	6
Animal	–	1	Ø	1	2
Non-AIDS	–	Ø	Ø	1	1

* Virulence was determined from data from Fig. 4; strains were classified as avirulent (–) when they were eliminated from the infected livers during the experiment, as intermediate (±) when they proliferated but always less than 3·0 $\log_{10}$, and as highly virulent (+ + +) when they proliferated $\geqslant$3·0 $\log_{10}$ in the infected livers during the experiment.

DISCUSSION

Mycobacterium avium complex bacteria are found in nature [3–5] and appear to colonize healthy humans without causing any symptoms [6]. It is, however, an important agent of secondary infection in immunocompromised individuals such as AIDS patients, leading to increased morbidity and reducing the survival in these patients [19]. It may also infect apparently healthy individuals, being in this case more often found among elderly females [2]. Finally, it can also infect wild and domestic animals [7]. In spite of some studies on virulence of this

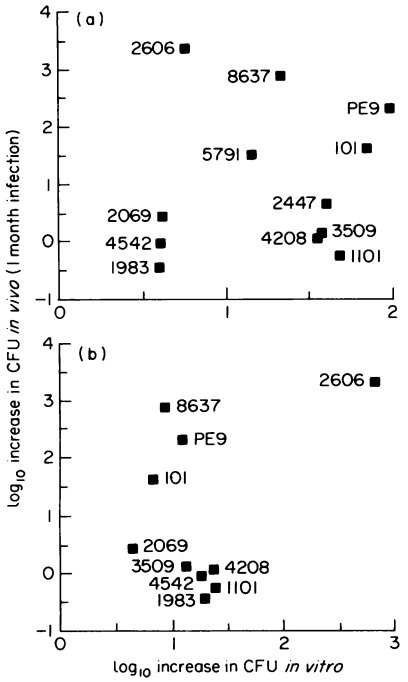

Fig. 5. Relation between *in vivo* growth (log_{10} difference in viable counts between day 0 and day 30 of infection) and *in vitro* growth in macrophages (log_{10} difference between day 0 and day 11) either derived *in vitro* from bone marrow (a) or from the peritoneal cavity (b). CFU, colony-forming units.

mycobacterial species, little is known on the virulence pattern according to the host populations it infects. Here we analysed the ability of 41 different strains of MAC to proliferate in naturally susceptible mice. Since MAC forms colonies on clear solid media that can be morphologically distinguished into three groups (SmT, SmOp, and Rg variants) with associated variation in their virulence against chickens and mice [8–12], we made a preliminary analysis of the morphotype-associated virulence in mice and chose to study the SmT variants on the basis of their most consistent ability to grow in mice. We showed here that animal isolates are most often highly virulent for mice, whereas AIDS-derived isolates had intermediate virulence or were devoid of the capacity to proliferate in mice. Among the isolates from non-AIDS patients, a higher heterogeneity was found, with a virulence spectrum ranging from highly virulent to avirulent strains. None of the three environmental isolates was able to infect mice. Our data suggest that animal isolates might have been selected for high virulence, since they probably encountered healthy hosts with no immune defect, whereas isolates from AIDS patients had a lower requirement for virulence since they were infecting immunocompromised hosts. In this respect, it is important to stress that the proliferation of a highly virulent animal strain of MAC in mice was not affected by T cell depletion [20], nor was reduced by either vaccination with bacille Calmette–Guerin (BCG) or adoptive transfer of protective T cells ([20], Appelberg and Castro, unpublished observations). On the other hand, T cell depletion, namely of the $CD4^+$ T cell subpopulation, in mice led to enhanced growth of an AIDS-derived isolate, and protection could be detected after adoptive transfer of immune T cells as well as after BCG immunization [20,21]. The high heterogeneity among non-AIDS isolates may suggest a heterogeneity in the immune status among the infected individuals. We postulate that the highly virulent isolates may be infecting individuals with no apparent predisposing condition, whereas the less virulent strains may have been isolated from humans with some immune defect. We are analysing more MAC isolates from non-AIDS patients and trying to compare the virulence with the clinical history of the infected individuals to test this hypothesis. Finally, the fact that the environmental isolates are not virulent for mice could suggest that these are adapted to growth outside mammalian cells and that they could only infect immunocompromised hosts. Alternatively, they could change virulence and become infective for immunocompetent humans and other animals.

We have recently found an association between virulence in mice and the presence of an insertion sequence, IS901 [22]. The strains of MAC harboring this IS element are typed by RFLP into a distinct pattern named A/I. Here we analysed the RFLP patterns of 23 of the strains studied and found that the highly virulent strains were all of the A/I type. However, two avirulent strains also exhibited the same RFLP type. Thus, although the presence of IS901 may lead to virulence, the finding of an A/I type does not predict virulence. In fact, the avirulent Rg variant of strain 25291 still retains the A/I restriction pattern as found in the virulent Rg and SmT variants (data not shown). Whether the IS element is a determinant of virulence will be decided by genetic manipulation of the microorganisms. The present results also support the previous findings [22] that the MAC strains of type A (as well as type H) have an intermediate virulence or are avirulent in mice.

The analysis made *in vivo* did not correlate with an analysis made with either peritoneal or bone marrow-derived macrophages. Since T cells do not seem to exert a detectable protective effect before the first month of infection [20,21], our results suggest that factors other than the ones involved in the interaction of the mycobacterium and the macrophage are involved in an early determination of the fate of the infection. An additional candidate in determining bacterial growth may be natural killer (NK) cells, since they have been shown to be involved in an early defence mechanism against MAC infection [23]. In this respect we found that beige mice are more susceptible to a *Myco. avium* isolate of intermediate virulence, whereas the most virulent isolates proliferate in beige mice at almost the same rate as in immunocompetent mice (R. Appelberg, unpublished observations). Although some strains maintained their relative virulences in both the *in vivo* and *in vitro* assays, some were not able to proliferate *in vivo* despite proliferating *in vitro*. These strains may trigger the protective activity of some non-macrophagic cells whose activity cannot be detected in the *in vitro* assay using bone marrow-derived macrophages. Finally, there are those isolates of MAC that do grow *in vivo* but not *in vitro*; we suggest that the *in vitro* maturation of the macrophages may have induced some antimycobacterial activity on these cells that is not induced *in vivo*. The growth of MAC in peritoneal macrophages was more uniform than that observed in bone marrow-derived macrophages, and is similar to the uniformity shown in other works using human macrophages [24]. Our results suggest that analysis of virulence of mycobacteria performed in cultured

macrophages, namely in human monocytes [24,25], may give an erroneous estimate of mycobacterial virulence.

In conclusion, the study of the interactions between different MAC isolates and mice may reveal important aspects of MAC physiology underlying virulence characteristics which may give us insights on future novel therapeutical approaches.

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