NRAMP1- or cytokine-induced bacteriostasis of Mycobacterium avium by mouse macrophages is independent of the respiratory burst

M. Salomé Gomes and Rui Appelberg

Author for correspondence: M. Salomé Gomes. Tel.: +351 226074900. Fax: +351 226099157.
e-mail: sgomes@ibmc.up.pt

Restriction of the growth of Mycobacterium avium was studied in wild-type and p47phox-deficient macrophages. The ability of gamma interferon and tumour necrosis factor alpha to induce antitymbacterial activity in bone-marrow-derived macrophages or the expression of the NRAMP1-mediated resistance to M. avium were not affected by the deficiency in p47phox. The addition of exogenous iron increased mycobacterial growth in macrophages expressing a functional NRAMP1 protein or a mutant NRAMP1 protein. Reactive oxygen species are therefore not involved in the constitutive or induced anti-M. avium activities of the mouse macrophage.

Keywords: iron, antimicrobial, innate immunity

INTRODUCTION

Mycobacterium avium is a facultative intracellular pathogen that survives and proliferates inside the macrophages of susceptible hosts (Frehel et al., 1991). The capacity to grow inside macrophages implies that M. avium must be capable of either avoiding the activation of some of its antimicrobial mechanisms or resisting their consequences. One of the ways by which M. avium can avoid destruction inside macrophages is by inhibiting the production of superoxide. In fact, the production of superoxide by macrophages upon phagocytosis of M. avium is usually low (Bermudez & Young, 1989). Also, the production of higher levels of oxygen radicals was associated with strains of the mycobacterium with lower virulence (Bermudez & Young, 1989; Gangadharam & Edwards, 1984; Sarmento & Appelberg, 1996). Nevertheless, macrophages can be activated to produce increased amounts of superoxide by treatment with IFN-γ (gamma interferon) and/or TNF-α (tumour necrosis factor α), treatments which concomitantly cause M. avium growth restriction (Sarmento & Appelberg, 1996; Appelberg et al., 1992). Previous studies using scavengers of reactive oxygen species indicated that the production of superoxide was not necessary for growth restriction of the most virulent strains of M. avium, but could play a role in the control of less virulent strains (Sarmento & Appelberg, 1996; Appelberg & Orme, 1993). The interpretation of these results is made difficult by the lack of understanding about the capacity of these scavenging compounds to reach the M. avium-containing phagosomes and the fact that other molecules such as peroxidases or iron may modulate the effectiveness of the antimicrobial action of reactive oxygen species. In this context, it has been proposed that NRAMP1, a putative cation transporter expressed in the membranes of late endosomes, lysosomes and phagosomes, namely those containing M. avium inside macrophages (Gruenheid et al., 1997; Searle et al., 1998), transports iron into the bacterium-containing phagosome and causes bacterial killing via the induction of toxic reactive oxygen species (Kuhn et al., 1999; Zwilling et al., 1999). Additionally, it has been reported that mouse macrophages expressing the R allele of the Nramp1 gene can produce more superoxide than those expressing the S allele of the same gene (Denis et al., 1988), which could suggest a role for reactive oxygen species in the NRAMP1-mediated mycobacteriostasis. On the other hand, we have proposed an opposite view in that NRAMP1 pumps iron out of the phagosome, thus depriving the micro-organism of this nutrient (Gomes & Appelberg, 1998) rather than influencing the generation of Fenton-derived hydroxyl radicals. This hypothesis is also supported by the data obtained by Jabado et al. (2000).

It is now possible to readdress these questions using mice that were rendered deficient in one of the components of the NADPH oxidase, p47phox (Jackson et al.,

Abbreviations: BMMφ, bone-marrow-derived macrophages; IFN-γ, gamma interferon; SmD, smooth domed; SmT, smooth transparent; TNF-α, tumour necrosis factor α.
1995). Segal et al. (1999) have infected these mice with *M. avium* and saw no differences in susceptibility when compared to wild-type mice. However, only one strain of *M. avium* was used, and the studies were performed in vivo. In the present work, we expanded these experiments to include different strains of *M. avium*, with different virulences, and we performed the experiments with isolated macrophages so as to address the role of superoxide production in the interaction of macrophages with *M. avium*, in the absence of additional effects on other components of the immune response. Overall, our results indicate that the production of superoxide through the NADPH oxidase is not necessary either for cytokine or NRAMP1-mediated growth restriction of *M. avium* inside mouse bone-marrow-derived macrophages (BMMφ).

**METHODS**

**Bacteria.** *Mycobacterium avium* strain 25291, smooth transparent (SmT) variant, was obtained from the American Type Culture Collection (Manassas, VA). Strains 2447 SmT and 2-151 smooth domed (SmD; opaque) were isolated from AIDS patients and given to us by F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium and J. Belisle, Colorado State University, USA, respectively. Strain 1983 SmT was isolated from an HIV-negative patient.

All mycobacteria were grown in Middlebrook 7H9 Broth (Difco) with 0.04% Tween 80 (Sigma). Cultures were harvested during exponential phase, centrifuged, washed in saline with Tween 80, briefly sonicated and stored in aliquots at −70 °C until used.

**Animals.** *p47*phox-deficient mice were bred at the IBMC (Instituto de Biologia Molecular e Celular) facilities from breeding pairs kindly provided by Drs Steven Holland and Braham Segal, from the National Institutes of Health, Bethesda, MD, USA (Jackson et al., 1995). These mice were kept in HEPA (high efficiency particulate air)-filter-bearing cages and fed sterilized food and water. Mice were initially genotyped for the *Nramp1* gene as indicated in the text below. For each condition tested, three culture wells were used. The results presented correspond to the mean and standard deviation of these three wells.

**RESULTS**

**Growth of different strains of *M. avium* inside murine BMMφ; effect of IFN-γ and TNF-α**

To evaluate the role played by superoxide production in the growth restriction of *M. avium* inside murine macrophages, we used BMMφ from mice that do not express the *p47* component of the phagocyte oxidase (*p47*phox−/−) and infected them with one of four different strains of *M. avium*, as described in Methods. The infected macrophages were then cultured in medium alone or were treated with 100 U IFN-γ or 50 U TNF-α per culture well per day, both singly and in combination, during the first 4 days of infection. At different time points after infection, the cells were lysed and the number of bacteria quantified as c.f.u. in agar medium. As *p47*phox−/−, expressing macrophage controls, we used BMMφ from either 129Sv or C57Bl/6 mice, according to the *Nramp1* allele expressed by the *p47*phox−/− macrophages, as determined by genomic PCR analysis. Either IFN-γ or TNF-α given alone caused growth inhibition of all four strains of *M. avium* tested in both types of macrophages (*p47*phox−/− or *p47*phox+/−). The combination of the two cytokines was the most efficient stimulus, inducing mycobacteriostasis of the most viru-
SmT or 2-151 SmD. Moreover, phagocyte oxidase—
with IFN-γ
M
Nramp1
using p47
on mycobacterial proliferation. It is clear from these
kinetics of growth of the four
the four strains, 2-151 SmD. In Fig. 1, we show the
log10 increase c.f.u. per well
Time (days)
NT 56.00
8.00
4.00
0.00
–4.00
–8.00
2-151 SmD
1983 SmT
2447 SmT
25291 SmT
Namp1 S
Namp1 R

Fig. 1. Effect of IFN-γ and TNF-α treatment on the growth kinetics of different M. avium strains inside p47phox−/− macrophages expressing either the S or the R allele of the Nramp1 gene or from C57Bl/6 (p47phox−/−, Namp1R) or 129Sv (p47phox−/−, Namp1S) control mice (squares). Macrophages were infected with 10^6 c.f.u. per well of each of the M. avium strains shown (25291 SmT, 2447 SmT, 1983 SmT and 2-151 SmD) and were either left untreated (white symbols) or were treated daily with 100 U recombinant murine IFN-γ ml^{-1} plus 50 U recombinant murine TNF-α ml^{-1} until day 4 of infection (black symbols). The graph shows the mean ± one standard deviation of the log_10 increase in c.f.u. per well obtained from three wells for each condition. Each of the experiments was done at least twice. One representative experiment is shown.

lent strains and bacterial killing of the least virulent of
the four strains, 2-151 SmD. In Fig. 1, we show the
kinetics of growth of the four M. avium strains tested
inside C57Bl/6 or p47phox−/− macrophages expressing the S allele of Nramp1, and the effect of IFN-γ plus TNF-α
on mycobacterial proliferation. It is clear from these
data that the absence of a functional phagocyte oxidase
did not increase the permissiveness of murine BMMφ to
M. avium, even of the low virulence strains, like 1983
SmT or 2-151 SmD. Moreover, phagocyte oxidase-
deficient macrophages were equally capable of inhibiting the growth or even killing M. avium upon stimulation with IFN-γ plus TNF-α. Similar results were obtained using p47phox−/− macrophages expressing the R allele of Nramp1, in parallel with macrophages derived from 129Sv mice (Fig. 1). Superoxide production was measured as phorbol myristate acetate-stimulated cytochrome c reduction. The combination of IFN-γ and

TNF-α induced the production of superoxide in wild-type macrophages (in higher amounts in 129Sv than in C57Bl/6 macrophages), while this production could not be detected in p47phox−/− macrophages (data not shown).

Role of superoxide production on the NRAMP1
protein induced growth restriction

It has been proposed that NRAMP1 transports iron into
the bacterium-containing phagosome and causes bac-
terial killing via the Fenton catalysis of toxic reactive
oxygen species (Goswami et al., 2001; Kuhn et al.,
1999; Zwilling et al., 1999). This hypothesis implies that
the growth restriction activity of NRAMP1 is depen-
don, or at least highly favoured by, the presence of a
functional phagocyte oxidase in the phagosomal mem-
brane, as a source of superoxide. To challenge this
hypothesis and since we had p47phox−/− littermates
expressing either the Nramp1 or S alleles, we compared
the growth of M. avium inside these different types of
macrophages. As shown in Fig. 1, macrophages from
Namp1R mice were consistently more effective at
controlling the proliferation of virulent strains than
macrophages from Namp1S mice. No expression of this
phenotype was observed with the non-virulent 2-151
SmD strain. The deficiency in the phagocyte oxidase did
not reduce the ability of macrophages from Namp1S
mice to control the growth of M. avium and indeed in
some experiments, such as the case here with strain 2447
SmT, some improvement in the anti-mycobacterial
activity of these macrophages was observed upon
reduction of p47phox. These results strongly suggest
that NRAMP1-mediated growth restriction does not
involve the production of oxygen radicals.

Effect of iron on the intramacrophagic growth of M. avium

As said previously, some authors claim that NRAMP1
pumps iron into the phagosome and that contributes to
M. avium killing through generation of hydroxyl
radicals. We tested the effects of iron addition to
macrophages infected with M. avium and whether those
effects were dependent on the presence of a functional
NADPH oxidase. As shown in Fig. 2, the addition of
iron caused an increase in the intra-macrophagic growth
of M. avium, rather than inhibition. As expected, the
same stimulatory effect was seen in macrophages lacking
either a functional phagocyte oxidase or a functional
NRAMP1 protein. Similar results were also obtained
when ferrous sulfate was used as the source of iron.

DISCUSSION

Strains of M. avium differ widely in their virulence as
accepted by the capacity to grow in mice or mouse-
derived macrophages (Pedrosa et al., 1994). One of the
factors suggested to be involved in these differences is
the capacity to inhibit the induction of superoxide
production during phagocytosis and/or the resistance to
superoxide and other reactive oxygen species (Sarmento

3157

Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
reactive oxygen species production for the macrophage virulence, to readdress the question of the importance of macrophage-generated nitric oxide (Gomes et al., 1999), the present data show that mouse macrophages must have oxygen and nitrogen reactive species-in-dependent mechanisms that are activated by IFN-γ and caused the bacteriostasis or killing of this opportunistic pathogen. The mechanisms involved in the resistance of pathogenic mycobacteria to macrophage-generated reactive oxygen species are not completely elucidated. There is no functional OxyR system in Mycobacterium tuberculosis (Sherman et al., 1995) although the expression of catalase-peroxidase correlates with resistance against hydrogen peroxide (Manca et al., 1999). Also, the presence of cyclopropanated mycolic acids seems to be important for resistance against hydrogen peroxide, since the transcription of Mycobacterium smegmatis with a gene involved in the biosynthesis of these molecules renders the bacterium more resistant to hydrogen peroxide (Yuan et al., 1995). However, M. avium is more resistant to hydrogen peroxide than M. tuberculosis (Gangadharam & Pratt, 1984), suggesting that the former mycobacterial species may have additional scavenger mechanisms to deal with oxidative stress.

Unlike M. tuberculosis, however, M. avium proliferation in vivo is under the control of the Nramp1 gene (Appelberg & Sarmento, 1990; Medina et al., 1996). NRAMP1 is a transmembrane protein expressed in endosomal and phagosomal membranes of macrophages, that contributes to inhibition of growth of several intracellular pathogens, including Mycobacterium bovis, M. avium, Leishmania donovani and Salmonella typhimurium (Gruenheid & Gros, 2000). Two alleles of the Nramp1 gene occur naturally in laboratory mouse strains. Only the wild-type or R allele encodes a functional protein, while the S allele is presumably not expressed or encodes a non-functional protein (Gruenheid & Gros, 2000). NRAMP1 mediates pleiotropic effects, ranging from major histocompatibility complex expression to superoxide production or phagosome acidification (Gruenheid et al., 1997; Denis et al., 1988; Hackam et al., 1998). It is not clear how these effects contribute to the growth restriction of intracellular pathogens. A large number of genes with high homology to Nramp1 have been recently characterized, both from mammals and from microorganisms (Gruenheid & Gros, 2000). These proteins seem to be implicated in divalent cation transport, namely Fe³⁺ and Mn²⁺ (Gruenheid & Gros, 2000; Jabado et al., 2000). We have previously reported data supporting the hypothesis that the mycobacteriostatic action of NRAMP1 is due to iron-depletion of the pathogen-containing phagosome (Gomes & Appelberg, 1996). In the present work, we studied four M. avium strains, covering a wide spectrum of virulence, to readdress the question of the importance of reactive oxygen species production for the macrophage resistance against M. avium, using a new tool, p47(phox)-/- mice. These mice fail to respond with a respiratory burst when appropriately triggered, e.g. with phorbol esters or particles (Jackson et al., 1993 and our unpublished observations). Despite this enzymic deficiency in an antimicrobial pathway, macrophages from the p47(phox)-/- mice were able to respond to IFN-γ and TNF-α by decreasing the growth of all the M. avium strains tested. In the case of the least virulent strain, 2-151 SmD, the cytokine treatment led to bacterial killing. The degree of growth restriction or killing was the same as was observed with wild-type macrophages of similar genetic background. In the case of strain 2-151 SmD, the lack of a functional NADPH oxidase did not lead to an increase in bacterial growth, showing that the lack of virulence is not explained by susceptibility to reactive oxygen species.

Since we have already shown that M. avium is resistant to macrophage-generated nitric oxide (Gomes et al., 1999), the present data show that mouse macrophages must have oxygen and nitrogen reactive species-independent mechanisms that are activated by IFN-γ and TNF-α and cause the bacteriostasis or killing of this opportunistic pathogen. The mechanisms involved in the resistance of pathogenic mycobacteria to macrophage-generated reactive oxygen species are not completely elucidated. There is no functional OxyR system in Mycobacterium tuberculosis (Sherman et al., 1995) although the expression of catalase-peroxidase correlates with resistance against hydrogen peroxide (Manca et al., 1999). Also, the presence of cyclopropanated mycolic acids seems to be important for resistance against hydrogen peroxide, since the transcription of Mycobacterium smegmatis with a gene involved in the biosynthesis of these molecules renders the bacterium more resistant to hydrogen peroxide (Yuan et al., 1995). However, M. avium is more resistant to hydrogen peroxide than M. tuberculosis (Gangadharam & Pratt, 1984), suggesting that the former mycobacterial species may have additional scavenger mechanisms to deal with oxidative stress.

Unlike M. tuberculosis, however, M. avium proliferation in vivo is under the control of the Nramp1 gene (Appelberg & Sarmento, 1990; Medina et al., 1996). NRAMP1 is a transmembrane protein expressed in endosomal and phagosomal membranes of macrophages, that contributes to inhibition of growth of several intracellular pathogens, including Mycobacterium bovis, M. avium, Leishmania donovani and Salmonella typhimurium (Gruenheid & Gros, 2000). Two alleles of the Nramp1 gene occur naturally in laboratory mouse strains. Only the wild-type or R allele encodes a functional protein, while the S allele is presumably not expressed or encodes a non-functional protein (Gruenheid & Gros, 2000). NRAMP1 mediates pleiotropic effects, ranging from major histocompatibility complex expression to superoxide production or phagosome acidification (Gruenheid et al., 1997; Denis et al., 1988; Hackam et al., 1998). It is not clear how these effects contribute to the growth restriction of intracellular pathogens. A large number of genes with high homology to Nramp1 have been recently characterized, both from mammals and from microorganisms (Gruenheid & Gros, 2000). These proteins seem to be implicated in divalent cation transport, namely Fe³⁺ and Mn²⁺ (Gruenheid & Gros, 2000; Jabado et al., 2000). We have previously reported data supporting the hypothesis that the mycobacteriostatic action of NRAMP1 is due to iron-depletion of the pathogen-containing phagosome (Gomes & Appelberg, 1996). In the present work, we studied four M. avium strains, covering a wide spectrum of virulence, to readdress the question of the importance of reactive oxygen species production for the macrophage resistance against M. avium, using a new tool, p47(phox)-/- mice. These mice fail to respond with a respiratory burst when appropriately triggered, e.g. with phorbol esters or particles (Jackson et al., 1993 and our unpublished observations). Despite this enzymic deficiency in an antimicrobial pathway, macrophages from the p47(phox)-/- mice were able to respond to IFN-γ and TNF-α by decreasing the growth of all the M. avium strains tested. In the case of the least virulent strain, 2-151 SmD, the cytokine treatment led to bacterial killing. The degree of growth restriction or killing was the same as was observed with wild-type macrophages of similar genetic background. In the case of strain 2-151 SmD, the lack of a functional NADPH oxidase did not lead to an increase in bacterial growth, showing that the lack of virulence is not explained by susceptibility to reactive oxygen species.

Since we have already shown that M. avium is resistant to macrophage-generated nitric oxide (Gomes et al., 1999), the present data show that mouse macrophages must have oxygen and nitrogen reactive species-independent mechanisms that are activated by IFN-γ and TNF-α and cause the bacteriostasis or killing of this opportunistic pathogen. The mechanisms involved in the resistance of pathogenic mycobacteria to macrophage-generated reactive oxygen species are not completely elucidated. There is no functional OxyR system in Mycobacterium tuberculosis (Sherman et al., 1995) although the expression of catalase-peroxidase correlates with resistance against hydrogen peroxide (Manca et al., 1999). Also, the presence of cyclopropanated mycolic acids seems to be important for resistance against hydrogen peroxide, since the transcription of Mycobacterium smegmatis with a gene involved in the biosynthesis of these molecules renders the bacterium more resistant to hydrogen peroxide (Yuan et al., 1995). However, M. avium is more resistant to hydrogen peroxide than M. tuberculosis (Gangadharam & Pratt, 1984), suggesting that the former mycobacterial species may have additional scavenger mechanisms to deal with oxidative stress.

Unlike M. tuberculosis, however, M. avium proliferation in vivo is under the control of the Nramp1 gene (Appelberg & Sarmento, 1990; Medina et al., 1996). NRAMP1 is a transmembrane protein expressed in endosomal and phagosomal membranes of macrophages, that contributes to inhibition of growth of several intracellular pathogens, including Mycobacterium bovis, M. avium, Leishmania donovani and Salmonella typhimurium (Gruenheid & Gros, 2000). Two alleles of the Nramp1 gene occur naturally in laboratory mouse strains. Only the wild-type or R allele encodes a functional protein, while the S allele is presumably not expressed or encodes a non-functional protein (Gruenheid & Gros, 2000). NRAMP1 mediates pleiotropic effects, ranging from major histocompatibility complex expression to superoxide production or phagosome acidification (Gruenheid et al., 1997; Denis et al., 1988; Hackam et al., 1998). It is not clear how these effects contribute to the growth restriction of intracellular pathogens. A large number of genes with high homology to Nramp1 have been recently characterized, both from mammals and from microorganisms (Gruenheid & Gros, 2000). These proteins seem to be implicated in divalent cation transport, namely Fe³⁺ and Mn²⁺ (Gruenheid & Gros, 2000; Jabado et al., 2000). We have previously reported data supporting the hypothesis that the mycobacteriostatic action of NRAMP1 is due to iron-depletion of the pathogen-containing phagosome (Gomes & Appelberg, 1996). In the present work, we studied four M. avium strains, covering a wide spectrum of virulence, to readdress the question of the importance of reactive oxygen species production for the macrophage resistance against M. avium, using a new tool, p47(phox)-/- mice. These mice fail to respond with a respiratory burst when appropriately triggered, e.g. with phorbol esters or particles (Jackson et al., 1993 and our unpublished observations). Despite this enzymic deficiency in an antimicrobial pathway, macrophages from the p47(phox)-/- mice were able to respond to IFN-γ and TNF-α by decreasing the growth of all the M. avium strains tested. In the case of the least virulent strain, 2-151 SmD, the cytokine treatment led to bacterial killing. The degree of growth restriction or killing was the same as was observed with wild-type macrophages of similar genetic background. In the case of strain 2-151 SmD, the lack of a functional NADPH oxidase did not lead to an increase in bacterial growth, showing that the lack of virulence is not explained by susceptibility to reactive oxygen species.
NRAMP1-induced mycobacteriostasis and superoxide

1998). Other authors claim that NRAMP1 transports iron from the cytosol into the pathogen-containing phagosome and that this would contribute to bacterial killing by stimulating the production of hydroxyl radicals from less toxic reactive oxygen species, namely superoxide and hydrogen peroxide (Goswami et al., 2001; Kuhn et al., 1999; Zwilling et al., 1999). If the bacteriostatic activity of NRAMP1 were to be due to hydroxyl formation, then it would be hampered in macrophages lacking NADPH oxidase, the enzyme responsible for the production of superoxide. The data presented here show that this is not the case. The Nramp1-mediated resistance was not affected by the mutation induced in the oxidase system as the addition of exogenous iron to macrophages expressing a functional NRAMP1 protein blocked antimicrobial activity instead of promoting it as the previous hypothesis would have predicted. In some experiments, the deficiency in the phagocyte oxidase was even able to increase the antimicrobial activity of macrophages expressing the functional NRAMP1 molecule although the mechanism involved was not investigated here.

In summary, our data show that restriction of growth of M. avium by macrophages is independent of the generation of reactive oxygen species through the respiratory burst NADPH oxidase. This is true for the antimycobacterial mechanisms induced by macrophage-activating cytokines such as IFN-γ and TNF-α as well as for the constitutive antimycobacterial mechanism mediated by the NRAMP1 protein.

ACKNOWLEDGEMENTS

We thank Drs Steven Holland and Braham Segal for their gift of breeding pairs of p47phox-deficient mice.

This work was supported by contract 13232/1998 from the PRAXIS XXI programme.

REFERENCES


Segal, B. H., Doherty, T. M., Wynn, T. A., Cheever, A. W., Sher, A.


Received 4 March 2002; revised 10 May 2002; accepted 14 May 2002.