Antibody bound to the surface antigen MPB83 of *Mycobacterium bovis* enhances survival against high dose and low dose challenge

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**Abstract**

Tuberculosis caused by infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis* is a significant disease of man and animals. Whilst cellular immunity is the major immunological component required for protection against these organisms, recent reports have suggested that monoclonal antibodies can modify infection with *M. tuberculosis*. To test whether the same was true for *M. bovis* infection, we determined the effect of preincubation of *M. bovis* with a monoclonal antibody on subsequent intravenous infection of mice. Antibodies bound to the surface of *M. bovis* increased the survival time of mice infected with *M. bovis* and changed the morphology of granulomas and the distribution of acid-fast bacilli in the lung. These studies suggest that antibodies directed to the surface of virulent mycobacteria can modulate their virulence in vivo.

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1. Introduction

Tuberculosis (TB) of humans remains one of the most devastating global infectious diseases and it is currently estimated that two million people die of the disease each year [1]. *Mycobacterium bovis* also infects humans although the global burden of *M. bovis* infection in humans is unknown. However, because of its wider host-range, *M. bovis* causes significant economic losses to the livestock industry and threatens global biodiversity of wildlife [2–6].

Currently it is believed that cellular immunity is the major component required for protection against *Mycobacterium tuberculosis* and *M. bovis* whereas the role of antibodies in protective immunity remains controversial (reviewed in [7]). Nevertheless evidence is emerging to suggest that monoclonal antibodies can modify various aspects of mycobacterial infections. Several strains of mice, including IFN-γ and major histocompatibility complex (MHC) Class II deficient mice, receiving *M. tuberculosis* coated with a monoclonal antibody specific for arabinomannan survived longer than controls [8]. Also, administration of *M. tuberculosis* coated with monoclonal antibodies specific for Heparin-binding Hemagglutinin Adhesin (HBHA) [9] to mice via the intranasal route reduced CFU in the spleen but not in the lung suggesting that anti-HBHA antibodies interfered with mycobacterial dissemination [10].

Our research group has had a long-standing interest in a 26 kDa antigen of the *M. tuberculosis* complex since it was first identified as a serodominant antigen during natural *M. bovis* infection of badgers and cattle [11–15]. This antigen, subsequently purified and named MPB83 [16] is highly expressed by *M. bovis* in vitro. MPB83 is post-translationally modified by the mycobacterium to yield a glycosylated, lipoylated form associated with the...
cell wall and surface accessible, as well as a glycosylated non-lipoylated form which is released into the bacterial culture filtrate [17–20]. Studies in small animal models have identified MPB83 as a protective antigen against \( M. \) \( \text{bovis} \) but not \( M. \) \( \text{tuberculosis} \) challenge [21–23].

Here, we report that binding a specific IgG2b monoclonal antibody that recognises a surface-exposed epitope of MPB83 [20] to the surface of \( M. \) \( \text{bovis} \) increased the survival of mice infected with \( M. \) \( \text{bovis} \) and influenced the host tissue response to infection. This study suggests a potentially protective role for antibodies directed to MPB83, which could be mediated through the immune response and/or through interference with the function of MPB83 in vivo.

2. Materials and methods

2.1. Bacteria and media

The strain of \( M. \) \( \text{bovis} \) used in this study (AF2122/97) was isolated from a tuberculin test reactor cow in 1997, cultured at VLA Weybridge and stored as frozen aliquots at \(-80^\circ\text{C}\). The genome of this strain was recently fully sequenced [24]. Bacterial enumeration was performed on Middlebrook 7H10 agar containing 4.16 mg/ml sodium pyruvate and 10% (v/v) Middlebrook OADC enrichment.

2.2. Incubation of \( M. \) \( \text{bovis} \) with antibodies

A monoclonal antibody (MBS43 of IgG2b isotype) with specificity to MPB83 [25] was chosen on the basis of its ability to bind to MPB83 on the surface of BCG Tokyo [20]. The antibody was concentrated 30-fold using a 5000 molecular weight cut-off VivaSpin 20 centrifugal concentrator unit (VivaScience Ltd., Binbrook, UK). Concentrated MBS43 was then dialysed against PBS containing 0.05% (v/v) Tween 20 (Sigma, Poole, UK) (PBSTw20) using a PD10 dialysis column (Amersham Pharmacia Biotech). The dialysed concentrated MBS43 was filter-sterilised and the concentration of antibody found to be 1 mg/ml by radial immunodiffusion.

An isotype-matched control (MOPC) was obtained from Sigma (M74644) and resuspended in PBSTw20 to a concentration of 1 mg protein per ml. A single vial of \( M. \) \( \text{bovis} \) 2122/97 was diluted in PBSTw20 and sonicated briefly. The sonicated cells were split into three equal volumes and incubated with the concentrated MBS43 or resuspended control (MOPC) at a ratio of 1 CFU to 10 ng of antibody. PBSTw20 was added to the third aliquot to achieve the same dilution. All three preparations were incubated for 3 h at RT on a rotating wheel to ensure thorough mixing. Bacterial cells were pelleted by centrifugation, washed once in PBSTw20, and sonicated briefly prior to injection into mice.

2.3. Inoculation of mice

Female BALB/c mice weighing between 16 and 19 g and free of intercurrent infection were obtained from Charles River UK Ltd., Margate, UK. For the high dose challenge experiment, groups of eight mice received one of the three \( M. \) \( \text{bovis} \) preparations intravenously, representing an \( M. \) \( \text{bovis} \) challenge of \( 10^4 \) CFU. An additional three mice were left unchallenged as controls. All mice were weighed frequently from day 10 of the experiment. Mice were killed when an individual had lost 20% of its starting weight (the humane end-point) or at the end of the experiment if still alive. The lungs and spleen were removed to saline buffered formalin. The experiment was terminated 38 days after \( M. \) \( \text{bovis} \) challenge due to closure of the animal facilities.

For the low dose experiment, three groups of 36 mice each were inoculated intravenously with 1500 CFU \( M. \) \( \text{bovis} \) prepared as before. Another group of 14 mice were left unchallenged as controls. Six mice from each of the three infected groups were killed 1 h and 2, 7, 14 and 27 days after infection. The liver, spleen and lungs from four of the six mice were taken for bacteriology, whilst these organs plus the left kidney and heart were taken from the remaining two mice and placed in saline buffered formalin. Two control mice were killed at the same time-points and organs taken into formalin only. The six mice remaining in each group after day 27 were weighed regularly and killed at the humane end-point. The organs from these mice were taken into formalin only. The experiment was terminated after 141 days.

2.4. Bacteriology and histopathology

Organs were homogenised in 5 ml sterile distilled water. Viable counts were performed on serial dilutions of the macerate and examined after five weeks incubation at 37 °C for growth of mycobacteria. The limit of detection was 25 CFU. Formalin-fixed tissues were blocked in a standard way for each mouse and paraffin wax-embedded. At least two sections were taken for each organ and duplicates of each section stained with haematoxylin & eosin and Ziehl Neelsen. Selected sections were stained with van Giesson. Sections were examined blinded to which time-point and treatment the section corresponded.

2.5. Statistical analyses

Appropriate statistical tests were chosen and data analysed using the InStat software package (version 4.00, GraphPad, San Diego, CA), with the exception of the \( \chi^2 \) test, for which Analyse-it\textsuperscript{TM} (version 1.65, Analyse-It Software, Ltd, UK) was used. For CFU data the log10 values were used for analysis.
3. Results

3.1. Mice survived challenge with a high dose of M. bovis incubated with anti-MPB83 antibody

*M. bovis* incubated prior to challenge with MBS43 but not with isotype-matched control antibodies (MOPC) or PBSTw20 was found to be of reduced virulence when $10^4$ CFU were injected intravenously into BALB/c mice. The reduction in virulence was expressed in terms of the weights of individual mice (Fig. 1) and the percentage of animals surviving to the end of the experiment (Fig. 2). These differences were not attributable to any toxic effect of antibody treatment on *M. bovis* as the viability and concentration of each preparation was found to be equal by plating a sample of each onto solid medium (data not shown). All mice receiving the MBS43-treated *M. bovis* were alive at the termination of the experiment at day 38. In comparison, all mice that received *M. bovis* in PBSTw20 were killed by day 35. By the Logrank test of significance between survival curves, the MBS43-treated *M. bovis* group was significantly different from the MOPC ($p < 0.01$) and PBSTw20 ($p < 0.0001$) groups. The median survival time for the PBSTw20 group was 33 days compared with 35 days for the MOPC group. The survival curves for these groups were not significantly different ($p = 0.19$).

3.2. Antibody treatment influenced the long-term survival but not the bacterial load in *M. bovis* infected mice

We repeated the experiment using a lower dose of *M. bovis* to produce a more chronic duration of disease. Fig. 3 shows the percentage of mice surviving to the end of the experiment. No unchallenged mice died during the 141 days of the experiment. In contrast, mice challenged with *M. bovis* were killed before the end of the experiment although their survival was increased by the pre-incubation of *M. bovis* with MBS43. Treatment of
M. bovis with MBS43 significantly increased the median survival time of mice exposed to the low dose challenge by 38 days compared with the PBSTw20 group and by 49 days compared with the MOPC group (Fig. 3). This represents an increase in survival of 41% and 60%, respectively. By the Logrank test the survival curve for the MBS43 group was significantly different to that of the PBSTw20 group \((p < 0.005)\) and the MOPC group \((p < 0.02)\). The survival curves for the PBSTw20 and MOPC groups were not significantly different from one another \((p = 0.97)\).

In addition, mice were killed at intervals throughout the experiment to determine whether survival was correlated with bacterial load and/or pathology in major organs. A group of mice were killed 1 h after infection in order to test whether the presence of antibodies on the surface of the mycobacteria influenced their tissue distribution and hence the course of disease. The seeding of each organ was the same between groups with the following exceptions: liver; MOPC vs. PBSTw20 \((p < 0.05, \text{Kruskal–Wallis with Dunn’s post test})\) and lung; MBS43 vs. PBSTw20 \((p < 0.01, \text{Kruskal–Wallis with Dunn’s post test})\). From day 2 onwards there were no significant differences in the growth of M. bovis both in number and rate, between all groups (data not shown).

3.3. Antibody treatment influenced the formation of granulomas in the infected lung

Lung lesions consisted of diffuse or nodular granulomatous consolidation of the lung parenchyma, differing in severity between groups. The most significant difference was in the estimation of normal lung parenchyma present in each section (Fig. 4). The MBS43 treatment group had twice the estimated median percentage of normal lung parenchyma compared with the PBSTw20 and MOPC control groups. This difference was statistically significant \((p < 0.05, \text{Kruskal–Wallis with Dunn’s post test})\). Photomicrographs showing the representative lung histopathology for each group in this experiment are shown in Fig. 5. Histopathological

\[\text{Fig. 4. Quantitation of the pulmonary pathology reduced by antibody treatment. An assessment of the percentage of normal parenchyma present in the lungs of mice was made following intravenous infection with } 10^9 \text{ CFU (high dose) M. bovis pre-incubated with either a monoclonal antibody (MBS43) specific to the surface antigen MBP83, an isotype-matched control antibody (MOPC), or PBS containing 0.05% (v/v) Tween 20 (PBSTw20). Group size = eight. Mice were killed 38 days after infection or at a defined humane end-point and the lungs removed to formalin. For each mouse, the fixed tissue was blocked in a standard way and two sections cut and stained with H&E. A pathologist then examined the sections in a blinded fashion. The box defines the 75th and 25th percentiles with the median value shown as a line. The whiskers define the maximum and minimum values. Significance was tested using the Kruskal–Wallis non-parametric ANOVA with Dunn’s post test.}\]

\[\text{Fig. 5. Appearance of the pulmonary pathology influenced by antibody treatment. Representative micrographs of formalin fixed lung show the difference between groups with respect to the amount of normal lung parenchyma present (a–c) and the cellular composition of granulomas in the same section (d–f). Mice were infected intravenously with } 10^9 \text{ CFU (high dose) M. bovis pre-incubated with either PBSTw20 (a and d), MOPC (b and e), or MBS43 (c and f). Sections were stained with H&E. Size bar represents 100 μM or 10 μM for the low and high magnification images, respectively. Each low magnification section contains the median percentage of normal lung parenchyma for that group. The higher magnification sections show the abundance of lymphocytes within the lung granulomas typical of the MOPC (e) and MBS43 (f) groups, compared with their scarcity in the granulomas of the PBSTw20 group (d). Macrophages are abundant in all sections. The mice shown were killed on day 32 (PBSTw20 and MOPC) or day 38 (MBS43).}\]
changes were observed in most of the spleens in the high dose experiment, although no significant difference in the pathology was observed between groups.

The progression of pathology in the organs of all mice infected with the low dose of \textit{M. bovis} was examined in detail but could not account for the increased survival of the mice infected with \textit{M. bovis} pre-treated with MBS43. However, detailed examination of the lung pathology supported the observations made in the high dose experiment. No pathology was observed in the lungs until day 14 post-challenge, and then only in the PBSTw20 group. All mice had lesions in their lungs by day 27 and those of the MBS43 group were smaller and more nodular and compact compared with either the PBSTw20 or MOPC groups (data not shown). As for the high dose experiment, the MBS43 treatment group had significantly more normal lung parenchyma compared with the PBSTw20 control group (median of 90% versus 72%, \(p < 0.05\), Kruskal–Wallis with Dunn’s post test). However, the MOPC control group also had a median normal lung percentage of 90%, but because of the small sample size and variation between animals this was not statistically different from the PBSTw20 group. Similar numbers of AFB were apparent histologically in the lungs, liver and spleen of all mice at day 27, consistent with the bacteriology data obtained from the tissue homogenates.

3.4. Antibody treatment influenced the distribution of AFB in the lung

Sections from the left cranial and the left and right caudal lobes stained with Ziehl Neelsen were blinded and examined by a pathologist and the presence of AFB in granulomas and the interstitial tissue recorded. The distribution of AFB in these sections is shown in Table 1. Only those sections that included AFB in the majority of fields were included for analysis. All AFB in the lungs of the MBS43 group, and most of the AFB in the MOPC group were located within lung granulomas, whereas the AFB in the PBSTw20 group were located throughout the lung tissue. The difference between the MBS43 and PBSTw20 groups was statistically significant (\(P = 0.007\), Fisher’s exact test). Eight sections of the MBS43 group had no AFB in any of the granulomas present, compared with only one section from the MOPC group and none from the PBSTw20.

4. Discussion

The role antibodies play in the protective host response to intracellular pathogens such as mycobacteria is a subject of much debate (reviewed in [26]). One conclusion from a 10 year investigation of 176 patients with primary hypogammaglobulinaemia was that such patients were more likely to contract TB than normal people [27,28]. A recent comprehensive review of experimental data gives further support to a protective role for antibodies against TB [7]. Studies involving pre-incubation of monoclonal antibodies that bind surface antigens of \textit{M. tuberculosis} have also suggested a protective role for antibodies in the mouse model [8]. Similarly, we have demonstrated that an IgG2b monoclonal antibody directed to MPB83, a surface-exposed antigen of \textit{M. bovis} and \textit{M. tuberculosis}, can significantly reduce the virulence of \textit{M. bovis} for mice if incubated with the organism prior to challenge. The protective effect was not mediated through a change in the viability of \textit{M. bovis}, either in vitro or in vivo. Furthermore, the antibody treatment did not induce clumping of the bacteria. Neither was protection mediated through a classical opsonisation effect resulting in preferential uptake of the mycobacteria by the liver, as was the case for mycobacterial LAM [29], pneumococcal [30] and Brucella [31,32]. This was important to establish since the liver has been demonstrated to contain the growth of virulent mycobacteria better than either the lung or spleen [33,34]. In fact, the distribution and growth of \textit{M. bovis} in the liver, lungs and spleen was not influenced by incubation with either specific or control antibodies. Instead, the protective effect of MBS43 antibody was only revealed histologically, where the lungs of these mice were observed to contain smaller, more nodular

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Number of sections with AFB</th>
<th>Inside granulomas</th>
<th>Outside granulomas</th>
<th>Absent from granulomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBS43c (24)</td>
<td>12</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>PBSTw20 (15)</td>
<td>15</td>
<td>12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MOPC (12)</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*The distribution of AFB between groups was statistically significant (\(P = 0.008\), \(\chi^2\) test). Difference in distribution compared with PBSTw20 group was tested by Fisher’s exact test.

*Only those sections that included AFB in the majority of fields are given.

\(n\) = Total number of sections examined.

\(P = 0.007\).

\(P = 0.114\).
granulomas, a greater area of normal lung parenchyma compared with controls, and a significant increase in the proportion of AFB contained within the granulomas compared with the surrounding tissue. These findings are reminiscent of those observed previously for *M. tuberculosis* pre-incubated with an IgG3 monoclonal antibody specific for arabinomannan [8].

The protective effect of antibody pre-treatment could derive from differences in the way the immune response is primed on first exposure to the mycobacteria. Previous studies have shown that the presence of antibodies on antigen surfaces influences antigen presentation to CD4 T cells. For example, Coughlan et al. [35] demonstrated that antibody-mediated antigen uptake via Fc gamma receptors on dendritic cells resulted in functional augmentation of antigen presentation via the MHC Class II pathway and T cell proliferation. If this were the case here, it would explain why the protective effects are seen beyond the period of time for which the antibody is likely to be present. Similarly, the mechanism responsible for the reduction in pathology induced by antibodies to MPB83 may be the result of differences in the response of macrophages to opsonised mycobacteria. Oposonising antibodies directed to mycobacterial antigens can promote the expression of pro-inflammatory cytokines such as TNFα and IL-6 by human macrophages [36,37]. Whilst this has been proposed as a mechanism for enhanced immunopathology in human TB, the production of TNFα in response to virulent mycobacteria is also critical for protection [38-40]. In fact, it was proposed 25 years ago that macrophages displaying Fc receptors for IgG2b-antigen aggregates could influence the duration and pathological characteristics of the granulomatous response [41]. More recent studies have implicated chemokines such as chemokine ligand 2 (monocyte chemoattractant peptide 1) in this process [42-45]. It is possible, therefore, that the presence of oposonising antibodies may cause inflammation to focus better on the mycobacteria as cells migrate into the lung at the initial stages of granuloma formation. This could explain why in the MBS43 treatment group, AFB were found exclusively within the granulomas (Table 1). The partial protective effects seen with the MOPC control, including the effect on AFB distribution, may be due to non-specific binding of IgG2b antibodies to the surface of the mycobacteria, or possibly through carry-over of complement component C5 in the inoculum. Complement C5 is implicated in the formation of granulomas in murine TB [46,47] and murine ascites fluid can be rich in C5 [48].

The recent solution of the structure of MPB70 [49] suggests that both MPB70 and the highly homologous MPB83 are involved in the binding of *M. bovis* to host cell proteins. Such specific interactions could modulate host cell behaviour to the advantage of the pathogen, perhaps through the stimulation of signalling pathways. This type of function has recently been identified for a glycosylated cell wall constituent of *M. tuberculosis*, which has been shown to block the maturation of the Mycobacterium-containing phagosome by interfering with intracellular trafficking events [50]. It is therefore possible that antibody bound to MPB83 might interfere with the function of MPB83 in vivo. Alternatively, as suggested by Teitelbaum et al. [8], monoclonal antibody binding to the mycobacterial surface may neutralize the toxic or immunosuppressive effects of mycobacterial polysaccharides and glycolipids. Whatever the protective mechanism at work, the ability of a monospecific antibody to significantly alter the course of *M. bovis* infection suggests the significance of local antibody responses should not be overlooked in the understanding of TB pathogenesis or in the pursuit of improved vaccines for TB.

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