The Secreted Antigens of Mycobacterium tuberculosis and Their Relationship to Those Recognized by the Available Antibodies

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Proteins secreted by strains of Mycobacterium tuberculosis during short-term, zinc-sufficient batch culture were identified in order to define antigens likely to be relevant to the pathogenesis of human disease. [35S]Methionine-labelled proteins in supernatants of 4–7 d cultures were separated by PAGE under both denaturing and non-denaturing conditions, and the position of labelled material was determined. Secreted protein patterns of M. tuberculosis were quite similar to those of Bacillus Calmette-Guérin (BCG) but differed by the absence of the 46 kDa dimeric protein specific to BCG and by the presence in large amounts of a 23 kDa protein which, when denatured, gave 13 kDa subunits. This 13 kDa subunit protein constituted up to 20% of secreted proteins in classical strains of M. tuberculosis of phage type B but was not detected in phage type I strains from South India. This may be relevant to the different pathogenicity of these strains. Western blot analysis showed that antigens defined in supernatants of short-term (3 d) cultures of M. tuberculosis constituted a small subset of those seen in supernatants of organisms cultured for longer periods. One of the secreted proteins has the interesting property of binding to fibronectin. The available monoclonal antibodies and antisera have been used to identify lines on immunoblots corresponding to the secreted/released antigens of M. tuberculosis. The present findings suggest that there are major secreted antigens to which antibodies do not yet appear to have been produced experimentally.

INTRODUCTION

Numerous investigators, using classical separation techniques or molecular biological approaches, have identified and isolated individual antigens of Mycobacterium tuberculosis which may be involved in stimulation or suppression of the human immune response during infection (reviewed by Rook, 1987). The long-term objectives of such studies are the selection of antigens for use in the production of more efficient vaccines. Recently, mouse monoclonal antibodies (MAbs) directed against mycobacterial antigens have been characterized (Engers, 1985, 1986) and used to screen for expression of these antigens in recombinant DNA libraries (Young et al., 1985a, b). The antigens which are immunodominant for murine B cells are, however, not necessarily the important ones for human T-cell responses. Furthermore, the protective antigens of a successful parasite may not be spontaneously immunodominant. Thus many T-cell clones which recognize sonicated mycobacteria fail to recognize live ones (Rook et al., 1986). Conversely, there is evidence suggesting that live mycobacteria secrete certain

Abbreviations: BCG, Bacillus Calmette-Guérin; MAb, monoclonal antibody.
antigens and these might be relevant for protective immunity. Such secreted antigens were identified from daughter strains of *Bacillus Calmette-Guérin* (BCG) grown for 4–7 d in a defined medium containing \[^{35}\text{S}]\text{methionine} (Abou-Zeid *et al.*, 1986).

The principal aims of this study were to identify the proteins secreted by actively metabolizing *M. tuberculosis* using short-term cultures (Abou-Zeid *et al.*, 1986), and to determine whether these potentially important antigens are amongst those identified by the currently available MAbs and antisera.

In addition, we looked for the presence in culture supernatants of antigens binding to fibronectin since mycobacteria were shown to attach to fibronectin-coated surfaces (Ratliff *et al.*, 1988). Fibronectin is a glycoprotein widely distributed on cell surfaces, connective-tissue matrices and basement membranes (Hynes & Yamada, 1982), and excretion by *M. tuberculosis* of molecules binding to these sites could sensitize the tissues to the immunopathological attack by the immune response directed against the organism. We also determined whether the secretion of these proteins was a result of zinc deficiency, in view of the reported effects of zinc on protein secretion (De Bruyn *et al.*, 1981).

**METHODS**

*Mycobacterial strains. M. tuberculosis* (H\textsubscript{3}R\textsubscript{v} and a clinical isolate) and *M. bovis* BCG Glaxo were obtained from the collection of Dr J. L. Stanford, Middlesex Hospital Medical School, London. Three South Indian strains of *M. tuberculosis* belonging to phage type I, and three Western-type strains of phage type B, typed for a previous study (Grange *et al.*, 1978), were also included.

**Secreted protein antigens** \[^{35}\text{S}]\text{methionine}-labelled proteins in culture supernatants of mycobacteria were obtained in exactly the same way as described previously for *M. bovis* BCG (Abou-Zeid *et al.*, 1986). In brief, bacteria were grown as suspensions in modified methionine-free Eagle's minimum essential medium (Flow Laboratories) with 10 \(\mu\)Ci (370 kBq) \[^{35}\text{S}]\text{methionine} (800 Ci mmol\(^{-1}\), Amersham) added in a final volume of 100 \(\mu\)L. Cultures were grown for 4 and 7 d in an atmosphere of 5% (v/v) \(\text{CO}_2\) with the \[^{35}\text{S}]\text{methionine} present throughout. The influence of zinc on the secretion of proteins by actively metabolizing organisms was studied by adding 0.5 or 5 \(\mu\text{M-Zn}^{2+}\) to the medium of certain cultures. Cultures were then passed through Millipore filters (Millex GV, 0.22 \(\mu\)m) and filtrates were stored at \(-70\,^\circ\text{C}\). Supernatants of a short-term (3 d) culture of a clinical isolate of *M. tuberculosis* were prepared by the same method, but without labelled methionine, then concentrated 10-fold and desalted by gel filtration on a PD-10 column (Pharmacia). For the preparation of a 21 d culture filtrate of the same strain, a pellicle of *M. tuberculosis* was grown on Sauton medium, and bacteria were then removed by sequential filtration through Whatman No. 1 paper and Millipore filters (0.2 \(\mu\)m). The protein concentrations in short-term (3 d) and long-term (21 d) culture filtrates were, respectively, 0.15 and 0.75 \(\text{mg ml}^{-1}\).

**MAbs and antisera.** MAbs against *M. tuberculosis*, characterized at a WHO workshop (Engers, 1986), were kindly provided by Dr D. B. Young, MRC Tuberculosis and Related Infections Unit, London, with the exception of MAbs F23-49 and SA-12 which were supplied, respectively, by Dr P. Klatser, Royal Tropical Institute, Amsterdam, and Dr P. Minden, Scripps Clinic, La Jolla, Calif. (Table 1). Rabbit antisera against proteins MPB 64, MPB 70 and the BCG 85 complex of *M. bovis* BCG (Harboe *et al.*, 1986; Wiker *et al.*, 1986) were generous gifts from Dr M. Harboe, Institute of Immunology and Rheumatology, Oslo.

**PAGE.** Radiolabelled proteins in culture supernatants were resolved by PAGE in different conditions as described by Abou-Zeid *et al.*, (1986). SDS-PAGE was done using a discontinuous buffer system (Laemmli, 1970), on slab gels of a 5–15% (w/v) acrylamide gradient or of 12.5% acrylamide. Native proteins were examined in a non-denaturing system (ND-PAGE) with 7, 9 or 11% acrylamide, and by two-dimensional electrophoresis involving separation by ND-PAGE on a 9% acrylamide gel in one direction followed by a second separation by SDS-PAGE on a 12.5% acrylamide gel at right-angles to the first. Gels were then dried and \[^{35}\text{S}]\text{labelled protein was detected by autoradiography using an X-ray film or by scanning with an Ambis beta-scanner (Smith, 1985).**

**Western blotting.** Proteins in 3 and 21 d culture supernatants of *M. tuberculosis* were separated by SDS-PAGE on slab gels of 12.5% acrylamide and transferred to nitrocellulose paper using a semi-dry electroblotter as described by Kyllönen & Pihlajaniemi (1981). Localization of proteins was achieved by staining with Aurodye, a colloidal gold solution (Janssen).

For immunoblotting analysis, unstained nitrocellulose membranes were soaked in 1% (w/v) BSA in PBS (0.15 M-sodium chloride, 0.15 M-sodium phosphate, pH 7.2) with 0.05% (v/v) Tween 20 to block any free protein-binding sites. After blocking, nitrocellulose strips were incubated with MAbs or with rabbit antisera overnight at 4 °C and probed, respectively, with peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit immunoglobulins (DAKO) at a 1 in 1000 dilution for 2 h at room temperature. For the identification of fibronectin-binding antigens in culture supernatants, immunoblots were incubated with fibronectin (100 \(\mu\text{g ml}^{-1}\)) for 2 h at 37 °C.
Secreted antigens of *M. tuberculosis*

Table 1. Reactivity of MAbs with sonicate and culture supernatants of *M. tuberculosis*

<table>
<thead>
<tr>
<th>MAb</th>
<th>IgG subclass</th>
<th>Mycobacterial specificity*</th>
<th>Molecular mass of protein antigen (kDa)</th>
<th>Reactivity with:</th>
<th>Reactivity with:</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sonicate</td>
<td>3 d culture supernatant</td>
<td>21 d culture supernatant</td>
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<tr>
<td>L7</td>
<td>IgG1</td>
<td>CR-L</td>
<td>71</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TB-78</td>
<td>IgG1</td>
<td>CR-L</td>
<td>65</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TB-71</td>
<td>IgG2a</td>
<td>Mt-C</td>
<td>38</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SA1-D2D</td>
<td>IgG1</td>
<td>CR-B</td>
<td>23</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F23-49</td>
<td>IgG2a</td>
<td>Mt-C</td>
<td>14</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TB-68</td>
<td>IgG1</td>
<td>CR-L</td>
<td>12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SA-12</td>
<td>IgG2a</td>
<td>CR-L</td>
<td>12</td>
<td>+</td>
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* CR-L, limited cross-reactivity (i.e. reacting with a restricted number of the mycobacterial strains tested); Mt-C, *M. tuberculosis* complex (i.e. reacting with *M. tuberculosis* virulent or avirulent strains, *M. bovis* BCG and *M. africanum*); CR-B, broad cross-reactivity (i.e. reacting with a majority of the strains tested).

Bound fibronectin was detected with peroxidase-conjugated rabbit anti-fibronectin immunoglobulins (DAKO) at a 1 in 500 dilution.

**Molecular mass determination.** A mixture of $^{14}$C-labelled protein markers (Amersham) was used for the determination of molecular mass by SDS-PAGE. Two Ambis computer programs were used to calculate the molecular mass and to quantify the amount of radioactivity incorporated in a specific band (expressed as a percentage of the total in that lane) (Smith, 1985). A mixture of standard protein markers (MW-SDS-200, Sigma) were used for the determination of molecular mass of unlabelled proteins. Determination of the molecular mass of native proteins in a non-denaturing system is described elsewhere (Abou-Zeid et al., 1986).

**RESULTS**

**SDS-PAGE protein patterns of *M. tuberculosis*.** $[^{35}$S]$\text{Methionine}$-labelled proteins in culture supernatants of *M. tuberculosis* and *M. bovis* BCG Glaxo were separated by SDS-PAGE on acrylamide gradient gels and the corresponding autoradiograms are shown in Fig. 1(a). Patterns of two strains of *M. tuberculosis*, a laboratory strain H$_{37}$Rv and a clinical isolate from Britain, were identical. Both strains secreted large amounts of a protein with a subunit molecular mass of 13 kDa. The band corresponding to the 13 kDa protein was quantified in relation to the total radioactivity present in the lane and constituted up to 20% of the secreted proteins. In contrast, the 13 kDa protein band was weak in the pattern of the BCG strain (Fig. 1a). The majority of proteins secreted were the same for BCG and *M. tuberculosis* with the exception of the protein band with subunit molecular mass of 23 kDa which was not found in the supernatants of *M. tuberculosis*. Addition of 0.5 or 5 μM-Zn$^{2+}$ to culture medium did not affect bacterial growth and did not modify the protein content of the culture supernatant (Fig. 1a). Short-term bacterial cultures in Sauton medium gave protein patterns similar to those obtained from cultures grown in Eagle’s minimum essential medium (data not shown).

The secreted protein patterns of South Indian strains of *M. tuberculosis* (phage type I) were quite different from those of phage type B classical strains (Fig. 1b). The 13 kDa protein band which was abundant in the supernatants of the classical strains (and of H$_{37}$Rv, which is also phage type B, Fig. 1a), was not detected in those of South Indian strains of phage type I.

**Nature of the 13 kDa protein by two-dimensional PAGE.** A culture supernatant of *M. tuberculosis* was resolved by electrophoresis in a non-denaturing system. The molecular mass of the native protein giving the 13 kDa band on SDS-PAGE was 23 kDa, as determined by electrophoresis of culture filtrates on a set of gels of 7, 9 and 11% acrylamide concentrations. In the two-dimensional electrophoretic pattern (Fig. 2), there was a single strong spot obtained
Fig. 1. Autoradiographs showing SDS-PAGE profiles of 35S-labelled proteins in mycobacterial culture (4 d) supernatants. Labelled supernatants were separated on slab gels of a 5–15% acrylamide gradient (a) and 12.5% acrylamide (b). (a) Lanes 1, 3 and 5 are, respectively, culture supernatants of M. bovis BCG Glaxo, M. tuberculosis H37Rv and a clinical isolate of M. tuberculosis grown with 5 μM-Zn2+. Lanes 2, 4 and 6 are culture supernatants of the same strains shown in lanes 1, 3 and 5, but grown without Zn2+. (b) Lanes 1 and 2 are culture supernatants of M. tuberculosis H37Rv (phage type B) obtained from two different sources, and lane 3 is culture supernatant of a South Indian strain of M. tuberculosis (phage type I). The positions of molecular mass markers are shown. The 13 kDa protein secreted in large quantities by some strains of M. tuberculosis is arrowed.

from the 23 kDa protein and corresponding to the 13 kDa protein. The 23 kDa protein represented about 20% of the native secreted proteins and consisted of 13 kDa subunits in denaturing conditions.

**Immunoblotting of culture supernatants of M. tuberculosis.** Aurodye staining of Western blots of culture filtrates of M. tuberculosis (Fig. 3) showed that antigens secreted or released by organisms grown for 3 d (lane 2) appeared to be a small subset of those seen in supernatants of old cultures (lane 1) where leakage from dead organisms becomes significant. Western blot analysis of the reactivity of the available MAbs (shown in Table 1 and Fig. 3) with culture supernatants of M. tuberculosis indicated that the antigens recognized by some of the MAbs in sonicate preparations were also present amongst those which are secreted, but this was not so for TB-78 or TB-68, which bound only to antigens in the sonicate. Anti-MPB 64 and anti-BCG 85 polyclonal antibodies bound to protein antigens with molecular masses of 27 and 29–31 kDa, respectively. No reactivity of anti-MPB 70 antibodies to any proteins in the culture filtrate of M. tuberculosis was observed. Fibronectin bound to a 30 kDa protein in 3 and 21 d culture supernatants and also to proteins in the range 58–60 kDa in the 21 d culture filtrate.

**DISCUSSION**

Definition of the secreted antigens of M. tuberculosis is a prerequisite for the study of the role of these components in protective immunity or immunopathology in man. We have previously described how this group of antigens can be identified in supernatants of BCG strains cultured briefly in the presence of [35S]methionine (Abou-Zeid et al., 1986).

We have shown here that strains of M. tuberculosis, including clinical isolates and laboratory strains, also secrete a number of proteins during the first few days of incubation. However, the patterns of secreted proteins differed between strains from Britain and South India. The former
Secreted antigens of M. tuberculosis

Fig. 2. Two-dimensional electrophoretic pattern of the culture (4 d) supernatant of M. tuberculosis. The first dimension, non-denaturing gel, which is shown above the second dimension, denaturing gel, was run at the same time as the one which was loaded for the second dimension. The molecular mass of the heavy line seen in the non-denaturing gel was determined to be 23 kDa. It is apparent that the native 23 kDa protein became a single 13 kDa spot in the SDS gel.

strains secreted large quantities of a 23 kDa protein which, when denatured, gave 13 kDa subunits. This protein appeared to be a dimer although the molecular mass estimated for the native protein differed slightly from the sum of the mass of the two monomers. The 13 kDa subunit protein was not detected in the supernatant of South Indian strains of phage type I. Phage typing of M. tuberculosis by Bates & Mitchison (1969) divided strains into three main types; A, B and I (intermediate). Strains of phage type A and B correspond to the classical guinea-pig virulent tubercle bacillus. Type A strains are distributed worldwide while type B strains are more restricted to Europe and the North American subcontinent. Type I strains mostly correspond to a variant with low virulence for the guinea-pig, which is particularly common in South India. The present findings support a previous suggestion (Grange et al., 1978) that strains of type I are of a distinct group within the species M. tuberculosis.

It has been shown that M. tuberculosis var. bovis (BCG) cultured in Zn²⁺-deficient Sauton medium gave a low growth yield and an increased protein content in the culture supernatant (De Bruyn et al., 1981). However the addition of increasing amounts of Zn²⁺ (0.5 and 5 μM) to our culture medium did not modify the secreted protein patterns of M. tuberculosis or BCG. This suggests that the concentration of Zn²⁺ in our medium was adequate without the supplements, and was not a factor determining the patterns we have observed. Indeed these patterns appear to be relatively stable. A recent study on the effects of the very varied manufacturing procedures
Fig. 3. Western blot analysis of culture supernatants of *M. tuberculosis*. (a) Proteins in culture supernatants (lane 1, 21 d; lane 2, 3 d) of *M. tuberculosis* were separated by SDS-PAGE on slab gels of 12.5% acrylamide and transferred to nitrocellulose paper by electroblotting. Reference lanes were stained with Aurodye, a colloidal gold solution. The reactivities of MAbs (L7, TB-71, SA1.D2D, F23-49 and SA-12), of anti-MPB 64 and anti-BCG 85 antisera, and of fibronectin (FN) with immunoblots of culture supernatants of *M. tuberculosis* are shown on the right, and molecular masses of protein antigens detected are indicated. (b) Reactivity of MAbs with 3 d culture supernatant of *M. tuberculosis*. Lane 1, L7; lane 2, TB-71; lane 3, SA1.D2D; lane 4, SA-12.

used in the commercial preparation of BCG vaccines showed no changes in patterns of proteins secreted by BCG substrains cultured in different centres (Abou-Zeid *et al.*, 1987a).

We previously reported that BCG daughter strains can be subdivided into two groups according to their secretion of a 46 kDa protein dimer consisting of two similar 23 kDa subunits (Abou-Zeid *et al.*, 1986). This 46 kDa protein, which constitutes up to 23% of the secreted proteins of the high-producer strains, is present in smaller amounts (4%) in the culture supernatant of the British Glaxo strain. The 23 kDa band was missing from the SDS-PAGE profile of *M. tuberculosis* supernatants although the majority of protein lines appear to be shared with BCG.
Secreted antigens of M. tuberculosis

Aurodye staining of Western blots of culture supernatants of M. tuberculosis demonstrated that antigens secreted by actively metabolizing organisms constituted a small subset of those present in supernatants of old cultures (3–5 weeks), when products of leakage from dead organisms become prominent. We suspect that the existence of some antigens which are secreted, but only present in small quantities inside dead bacilli, may partly explain the fact that live vaccines are better than killed ones since these antigens are likely to be relevant to rapid recognition of bacilli by cells of the immune system. Some of the antigens characterized by the available MAbs appear to be among those which are secreted. However the cross-reactive protein with a molecular mass of 65 kDa recognized by TB-78 MAb was not present in culture filtrates and appears to be a cytoplasmic antigen. This is compatible with the recent demonstration that this antigen probably belongs to a family of RNA-stabilizing proteins (Young et al., 1987), the function of which is presumably intracellular. A recent report that this antigen is present in supernatants of BCG grown under Zn2+-deficient conditions (De Bruyn et al., 1987) raises the interesting possibility that if zinc levels are sufficiently low in vivo, particularly inside macrophages, additional antigens may become secreted, and therefore rapidly available to the immune system.

The MAb TB-68, reactive with a protein antigen with a molecular mass of 14 kDa, failed to bind to immunoblots of M. tuberculosis supernatant, and the F23-49 MAb, which binds to a protein of the same molecular mass and to the same recombinant protein (Husson & Young, 1987), bound to a protein in the 21 d supernatant but not to any in the 3 d sample. Therefore, this is unlikely to be a true secreted protein.

We have recently shown that the 46 kDa dimeric protein antigen secreted in large amounts by some BCG daughter strains corresponds to protein MPB 70 present in long-term culture filtrates (Abou-Zeid et al., 1987b). Western blot analysis with anti-MPB 70 antibodies revealed no cross-reactivity between the 23 kDa subunit protein of BCG and the 13 kDa subunit protein of M. tuberculosis.

Reactivity of anti-MPB 64 antibodies with a 27 kDa protein antigen in the culture filtrate of a clinical isolate of M. tuberculosis confirmed earlier findings by immunoprecipitation and Western blotting (Harboe et al., 1986; Abou-Zeid et al., 1987b). Protein MPB 64 occurred in the same BCG substrains as MPB 70, but unlike the latter, showed cross-reactivity with M. tuberculosis H37Rv and Aoyama B.

Incubation with anti-BCG 85 antiserum, which recognizes a complex of three antigens (29–31 kDa) abundant in BCG culture fluid (Wiker et al., 1986), showed that this complex cross-reacts with antigens present in the culture fluid of M. tuberculosis. The relationship to the BCG 85 complex of the fibronectin-binding protein from M. tuberculosis which has a similar molecular mass (30 kDa), is under investigation. It has been shown that supernatants from BCG cultures inhibited the binding of BCG to fibronectin-coated surfaces (Ratliff et al., 1988). The identification of fibronectin-binding antigens in culture supernatants of M. tuberculosis could be of great relevance to the understanding of the immunopathology of the disease.

The present study highlights the fact that there are major secreted antigens of M. tuberculosis to which MAbs do not yet appear to be available, though some of them have properties, such as an affinity for fibronectin, which suggest that they may be physiologically important.

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