Subdivision of Daughter Strains of Bacille Calmette-Guérin (BCG) According to Secreted Protein Patterns

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(Received 11 March 1986; revised 14 June 1986)

In order to identify proteins secreted by live organisms, daughter strains of the Bacillus Calmette-Guérin (BCG) were grown for 4–7 d in a defined medium containing [35S]methionine. Secreted components were then separated by polyacrylamide gel electrophoresis under both denaturing and non-denaturing conditions, and analysed by autoradiography and in an Ambis beta-scanner. The results indicate 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. High-producer strains (Japanese, Brazilian and Russian) secrete very large quantities of this material, which constitutes approximately 23% of all secreted protein. These findings correlate with earlier studies in which degradation products of the protein dimer may have been identified, and with the data from patterns of cell wall lipids.

INTRODUCTION

Little is known about the protective antigens of mycobacteria because these have proved difficult to fractionate by conventional means. Recently there have been reports of the cloning of mycobacterial genomes in Escherichia coli, and screening, with monoclonal antibodies, of colonies which produce mycobacterial antigens (Young et al., 1985). The monoclonal antibodies used for this selection were, however, raised to dead bacilli, or sonicates, while there is much evidence to suggest that live bacilli evoke much better protection (reviewed by Rook, 1980; Lovik & Closs, 1984). In vitro experiments lead to similar conclusions. Thus relatively few T cell clones derived from the peripheral blood of normal donors by stimulation with sonicated Mycobacterium tuberculosis are able to recognize live bacilli (Rook et al., 1986). It is likely that such recognition is essential for efficient immunity, hence the efficacy of live vaccines. It is therefore important to identify the antigens which are secreted by live bacilli, or present on their surfaces, so that relevant antigens can be selected from the E. coli expression libraries.

We report here the use of brief (4–7 d) culture of small numbers of BCG bacilli in a defined medium containing [35S]methionine as a way of identifying proteins which are being secreted by actively metabolizing organisms rather than leaking from dead ones. The method also avoids autolysis, and appears to be both a powerful way of defining this important group of antigens, and a highly discriminative taxonomic technique.

METHODS

Strains of Mycobacterium bovis BCG. The substrains of BCG examined were obtained freeze-dried as supplied by the respective sources (Table 1).

Growth conditions and preparation of secreted proteins. BCG organisms were first cultured on Lowenstein–Jensen medium at 37 °C, and then subcultured once. All daughter strains were subcultured for about 5 d, then suspensions...
Table 1. Origin of the BCG daughter strains used in the study

<table>
<thead>
<tr>
<th>Daughter strain</th>
<th>Origin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>British (Glaxo)</td>
<td>Routine batch</td>
</tr>
<tr>
<td>Japanese (Tokyo)</td>
<td>1st WHO International Standard</td>
</tr>
<tr>
<td>Tice (Chicago)</td>
<td>Routine batch no. 39</td>
</tr>
<tr>
<td>Brazilian (Moreau strain)</td>
<td>WHO seed lot†</td>
</tr>
<tr>
<td>Czechoslovakian (Prague)</td>
<td>IABS (G)</td>
</tr>
<tr>
<td>French (Institut Pasteur, Paris)</td>
<td>IABS (H)</td>
</tr>
<tr>
<td>Russian (Moscow)</td>
<td>IABS (E)</td>
</tr>
<tr>
<td>Merieux (Institut Merieux, Lyon)</td>
<td>IABS (D)</td>
</tr>
<tr>
<td>Danish (Copenhagen)</td>
<td>Routine batch no. 121</td>
</tr>
<tr>
<td>Dakar (Institut Pasteur)</td>
<td>IABS (B)</td>
</tr>
<tr>
<td>Dutch (Bilthoven)</td>
<td>IABS (A)</td>
</tr>
<tr>
<td>Indonesian (Biofarma, Bandung)</td>
<td>Routine batch no. P595A</td>
</tr>
</tbody>
</table>

* IABS indicates that the strains were used in a collaborative study organized by the International Association of Biological Standardization (Report, 1977).
† From the WHO International Reference Centre for BCG seed lots and Control of BCG Products, Copenhagen. Prepared by the Copenhagen technique.

were washed three times with Earle's balanced salt solution (Gibco Europe) and resuspended in methionine-free Eagle's minimum essential medium (Flow Laboratories) supplemented with 0.2% glutamic acid, 0.2% asparagine and 0.005% ferric ammonium citrate. Bacterial suspensions (100 µl) containing 2 × 10^8 organisms and 10 µCi (370 kBq) [^{35}S]methionine (Amersham) were added in triplicate to wells of flat-bottomed microtitre trays (Nunc).

The trays were incubated for 4–7 d at 37 °C in a CO₂ incubator; the extent of bacterial proliferation during this culture period was not monitored. The cultures were then filtered on Millipore filters (Millex-GV, 0.22 µm) and the filtrates, which were free of bacteria, were kept at −70 °C until electrophoresis.

Polyacrylamide gel electrophoresis (PAGE). Radioactively labelled proteins in 50 µl samples of culture supernatants were resolved by PAGE in three different conditions.

(1) SDS-PAGE. Electrophoresis was carried out according to the discontinuous buffer system of Laemmli (1970) on slab gels which consisted of 12.5% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel. The 0.8% bisacrylamide was replaced by 0.6% acrylaide plus 0.2% bisacrylamide to prevent the gel cracking on drying. After freeze-drying, radiolabelled samples were dissolved in sample buffer [0.0625 M-Tris/HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% bromophenol blue] and boiled for 2 min.

(2) Non-denaturing (ND)-PAGE. Native proteins were examined in a non-denaturing system based on the method of Davis (1964) with 7, 9 or 12% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel. Samples were dissolved in sample buffer [0.0625 M-Tris/HCl pH 6.8 containing 10% (w/v) sucrose and 0.001% bromophenol blue].

(3) Two-dimensional PAGE. A two-dimensional electrophoresis has been developed involving separation in a non-denaturing system (ND-PAGE) in one direction followed by second separation in a dissociating system (SDS-PAGE) at right angles to the first. After the first-dimension run, the slab gel was sliced into strips which were kept frozen at −20 °C in aluminium foil. For the second-dimension separation, gel strips were thawed and equilibrated for 45 min with 10 ml treatment buffer (equivalent to the sample buffer described in the SDS-PAGE section but without tracking dye). The gel strip was then carefully placed on top of a gel which consisted of a 12.5% (w/v) acrylamide separating gel and a 4% (w/v) acrylamide stacking gel set without a comb to 2 cm from the top of the plates. A few ml of a 1% (w/v) agarose solution were pipetted around the gel strip. Electrophoresis was performed on a vertical slab gel unit (Hoefer), at constant current using 20 mA per gel for 40 min, then the current was increased to 30 mA per gel and the electrophoresis continued until the dye reached the bottom of the gel.

Processing of gels. If the proteins were only to be fixed the gel was placed overnight in 40% (v/v) methanol and 10% (v/v) acetic acid in water. If the proteins were to be stained, the gel was placed overnight in 0.1% Coomassie brilliant blue solution made in 40% (v/v) methanol and 10% (v/v) acetic acid. The gel was then destained by shaking with a solution of 10% (v/v) methanol and 10% (v/v) acetic acid. Gels were then fluor-impregnated with Amplify (Amersham) and dried onto Whatman 3MM paper using a slab gel dryer (Bio-Rad). Gels were either exposed to an X-ray film for 3 d at −70 °C or scanned for beta-particle emission using an Ambis beta-scanner (Smith, 1985).

Molecular mass determination. A mixture of ¹⁴C-labelled protein markers (Amersham) was used for the determination of molecular mass by SDS-PAGE. Molecular mass markers were myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). Dried gels were scanned for 4 h on an Ambis beta-scanner. Two Ambis computer programs were used,
Secreted proteins of BCG

Fig. 1. SDS-PAGE profiles of 35S-labelled proteins in culture supernatants of the following BCG strains: 1, French; 2, Tice; 3 Czechoslovakian; 4, Russian; 5, Brazilian; 6, Japanese. The positions of molecular mass markers are shown on the left. The 23 kDa protein secreted in large quantities by producer strains is arrowed.

(1) the molecular weight program, and (2) the quantitation program. For (1), the position of each 14C-labelled protein standard was marked and the molecular mass added in to the data. A curve was then computed to serve as an internal standard for the determination of unknown molecular masses of bands in other lanes, such as the BCG lane. For (2), an individual lane was again screened, each required band was marked and radioactivity in the band was calculated as a percentage of the total in that lane.

The molecular mass of native proteins was determined in a non-denaturing system by a modification of the methods of Bryan (1977) and Davis (1964). Protein standards (Sigma) ranged from 14 kDa to 132 kDa. For each protein, logarithms of electrophoretic mobilities on a set of gels of various polyacrylamide concentrations were plotted against the percentage gel concentration. From these plots, individual slopes were determined for each protein and the logarithm of the negative slope was plotted against the logarithm of the molecular mass of each protein standard: a linear plot was produced from which the molecular mass of the unknown protein was calculated.

RESULTS

SDS-PAGE

Autoradiograms of [35S]methionine-labelled proteins in culture supernatants of BCG substrains are shown in Figs 1 and 2. All the BCG daughter strains used in this study secreted proteins during growth, and the patterns clearly fell into two main subgroups differing by the presence or absence of a single protein. Representatives of the Brazilian, Japanese and Russian daughter strains secreted this protein in large amounts. It was present in small amounts in the supernatants of the British and Merieux daughter strains, but was not detected in those of the Dakar, Danish, Dutch, Indonesian, French, Czechoslovakian and Tice daughter strains.

The molecular mass of the protein secreted in large amount by the Brazilian, Japanese and Russian substrains was determined from the data shown in Fig. 3. The molecular mass of this major protein was computed to be 23434 Da in denaturing gels and the protein is referred to as the 23 kDa protein.
Fig. 2. SDS-PAGE profiles of $^{35}$S-labelled proteins in culture supernatants of the following BCG strains: 1, Indonesian; 2, Danish; 3 and 4, Dutch, batches A224 and A530; 5, Dakar; 6 and 7, Merieux batches D051 and D946; 8, British. The positions of molecular mass markers are shown on the left.

Fig. 3. Molecular mass determination by use of the beta-scanner. Molecular mass standard (ST) markers and BCG supernatants were run in parallel lanes on an SDS gel which was then dried. The gel was scanned, a histogram was produced (left of figure) from the standards and a molecular mass calibration curve was drawn automatically (not shown). The BCG histogram was placed on the monitor (right of figure), the position of the peak was marked with an arrow and following a 'read' instruction, the positions of the standards were screened against the histogram together with a molecular mass evaluation of the arrowed peak.
The band corresponding to the 23 kDa protein was quantified by the beta-scanner in relation to the total radioactivity present in the lane: it constituted up to 22–23% of the secreted proteins of the Brazilian, Japanese and Russian daughter strains and 3–4% of those of the British and Merieux daughter strains.

**Non-denaturing PAGE**

Proteins secreted by live BCG daughter strains were also examined by electrophoresis in a non-denaturing system. Lanes corresponding to Brazilian, Japanese and Russian BCG substrains contained once again a strong band, which was absent from the culture supernatants of those BCG daughter strains that did not secrete the 23 kDa protein (not shown). The molecular mass of the native protein giving this strong band was 46 kDa, as determined by electrophoresis of culture supernatants of Brazilian and Japanese substrains on a set of gels of 7, 9 and 12% (w/v) acrylamide concentrations (described in Methods). This 46 kDa protein represented about 20% of the native secreted proteins, as had been found for the 23 kDa protein by SDS-PAGE.

**Two-dimensional PAGE**

In order to demonstrate the presence of two subunits in the 46 kDa protein secreted by one group of live BCG daughter strains, a sample of culture supernatant of the Brazilian substrate was run in the first dimension in a non-denaturing system on a 7% (w/v) acrylamide gel. The slab gel was then sliced and the corresponding gel strip, after equilibration in SDS buffer, was placed on the top of a 12.5% (w/v) acrylamide gel containing SDS for the second dimension. In the two-dimensional electrophoretic pattern (Fig. 4), there was one single strong spot obtained from the 46 kDa protein and corresponding to the 23 kDa protein demonstrated previously by SDS-PAGE.
DISCUSSION

All 12 BCG daughter strains studied secreted a number of proteins in the first days of incubation, the majority of which were the same for all strains. The strains could, however, be divided into two groups according to the secretion of large quantities of a 46 kDa protein, which is a dimer composed of two 23 kDa subunits. The 46 kDa protein constitutes up to 23% of the secreted proteins of the high producer strains, Brazilian, Japanese and Russian, and is present in smaller amounts (4%) in culture supernatants of British and Merieux strains. It is interesting to note that the Merieux strain was derived from the British strain. This 46 kDa dimer was not secreted by Tice, Danish, French, Czechoslovakian, Dakar, Dutch or Indonesian strains.

The findings of this study indicate the same sub-groups of BCG daughter strains as some previous investigations. Minnikin et al. (1984) showed a division of BCG daughter strains according to the mycolic acid patterns obtained by thin-layer chromatography. Most of the BCG samples studied (Chinese, Danish, British, French and Czechoslovakian) produced a simplified mycolic acid pattern composed of α-mycolates and ketomycolates except the Brazilian and the Swedish strains, which also contained methoxymycolates.

Immunoprecipitation analyses undertaken by others have revealed antigenic differences among BCG daughter strains used in the vaccine preparations. Lind (1960) found that the Brazilian, Russian and Swedish daughter strains contained an antigen (factor c) which was not demonstrable in the culture filtrates of the Danish and French strains. By using the rocket-line immunoelectrophoresis technique, Stavri et al. (1981) showed that the Brazilian, Japanese, Romanian and Russian strains shared an antigen (antigen 15) not detected in the Danish, French and Czechoslovakian strains. Finally, using crossed immunoelectrophoresis and radio-immunoassay inhibition tests, Harboe & Nagai (1984) showed that high concentrations of an antigen, MPB70, were present in the Brazilian, Japanese, Russian and Swedish strains, while a hundred times lower concentrations were detected in the supernatants of Danish, British, French and Tice substrains.

The relationship of our studies to these earlier findings is not yet certain. Nagai et al. (1981) used supernatants from cultures 4–5 weeks old, and detected a protein (MPB70) with the same strain distribution as the 46 kDa dimer which we describe here. They suggested that it was a monomer with a molecular mass of 18 kDa on SDS gels. In subsequent studies another protein (MPB64) was detected with the same strain distribution, and a molecular mass of 23 kDa (Miura et al., 1983), like the denatured form of the 46 kDa dimer. Our assays, performed with 4–7 days supernatants from cultures which undergo minimal autolysis, show that only a small amount of protein in the 18–23 kDa (non-denaturing conditions) range is secreted by any of the BCG strains studied, and no protein other than the 46 kDa dimer is both heavily secreted by strains producing MPB70 and MPB64, and virtually absent from the others. Thus it seems likely that MPB70 and MPB64 are degradation products of the 46 kDa dimer. Moreover the use of 4–7 days supernatants minimizes leakage from dead bacilli of products not normally secreted, and this could account for our estimate that the 46 kDa dimer constitutes a remarkable 23% of all the protein secreted by high producer strains. This estimate is more than double that of Nagai et al. (1981).

The significance of such massive secretion of a single protein is not clear, especially since not all strains secrete it. Its synthesis could be plasmid coded: plasmids are known to occur in mycobacteria (Crawford et al., 1981; Labidi et al., 1984). On the other hand, the gene coding for synthesis of this protein may be present in all strains, but have become derepressed only in some of them, owing to mutations affecting regulation.

This work was supported by a grant from the UNDP/World Bank/WHO programme for vaccine development.

REFERENCES


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