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# Characterization of the Secreted Antigens of *Mycobacterium bovis* BCG: Comparison of the 46-Kilodalton Dimeric Protein with Proteins MPB64 and MPB70

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**Western blot analysis showed that the 46-kilodalton (kDa) dimeric protein antigen secreted in large amounts by some daughter strains of *Mycobacterium bovis* BCG corresponded to protein MPB70 present in long-term culture filtrates of the Japanese substrain. The 46/23-kDa antigen is the most abundant protein in supernatant from a 5-day culture but is masked by leaked products in old culture supernatants. No similarities were found between the 46-kDa protein and MPB64, a protein with the same strain distribution, or with the antigen of similar molecular mass recognized by monoclonal antibody SA1.D2D.**

Because of their potential importance for protective immunity, we recently identified the secreted proteins of daughter strains of *Mycobacterium bovis* BCG, which were grown for 4 to 7 days in a defined medium containing [$^{35}$S]methionine (2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of culture supernatants of 12 BCG daughter strains fell into two main subgroups differing by the presence or absence of a 46-kilodalton (kDa) protein dimer consisting of two 23-kDa subunits. The 46-kDa protein constituted up to 23% of the secreted proteins of the high-producer strains Brazilian, Japanese, and Russian. It was present in small amounts in the supernatants of the British and Merieux daughter strains but was not detected in those of the Czechoslovakian, Dakar, Danish, Dutch, French, Indonesian, and Tice strains.

Nagai et al. (6) had previously isolated and purified protein MPB70 (18 kDa) from a 4- to 5-week culture filtrate of the Japanese strain because of its high concentration (10% of total protein content). The strain distribution of the MPB70 protein is the same as that of the 46-kDa dimer described above. Moreover, in both skin test and lymphoproliferative studies MPB70 was strictly BCG specific and elicited responses only in guinea pigs sensitized with high-producer strains (5; K. Haslov, A. B. Andersen, and M. W. Bentzon, Scand. J. Immunol., in press).

MPB64 is another distinct immunogenic protein in the culture filtrate of the Japanese strain (4). Its reported molecular mass is 23 kDa, and it occurs in the same BCG substrains as MPB70 but, unlike MPB70, it also occurs in *Mycobacterium tuberculosis* $H_{37}R_v$ and Aoyama B (4, 5). The MPB64 and MPB70 proteins showed no immunologic similarity, and there was no homology in the N-terminal amino acid sequence.

In the present study, we have resolved the confusion by comparing the immunochemical properties of proteins MPB64 and MPB70 with those of the secreted 46-kDa dimer and also checked for a possible relationship to the cross-reactive mycobacterial antigen of similar molecular mass recognized by monoclonal antibody SA1.D2D. [$^{35}$S]methionine-labeled proteins in the culture supernatant of the Brazilian BCG substrain were obtained as described by Abou-Zeid et al. (2). Supernatants of brief (5-day) cultures of the Brazilian and the British strains were prepared by the same method, but without labeled methionine, concentrated 10-fold, and desalted by gel filtration on a PD 10 column (Pharmacia, Uppsala, Sweden). Purified MPB70 protein and 4-week-old culture filtrate of the Japanese strain were kindly provided by S. Nagai, Osaka City University Medical School, Osaka, Japan. Production of rabbit anti-MPB70 and anti-MPB64 antisera has been described previously (3). The broadly cross-reactive monoclonal antibody SA1.D2D (7) reacting with a protein antigen with a subunit molecular weight of 23,000 was obtained from D. B. Young, Tuberculosis and Related Infections Unit, London.

SDS-PAGE, Western blotting (immunoblotting), staining of blots, autoradiography, and molecular weight estimations were performed as described previously (1, 2). For immunoblotting analysis, unstained nitrocellulose membranes were soaked in 1% (wt/vol) bovine serum albumin in phosphate-buffered saline with 0.05% (vol/vol) Tween 20. After blocking was done, nitrocellulose strips were incubated in the rabbit antiserum diluted to 1:50 and probed with a 1:1,000 dilution of peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO P217). Monoclonal antibody SA1.D2D was used at 1:7,500, and binding was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin at a 1:1,000 dilution (DAKO P260).

The autoradiogram of the SDS-PAGE profile of $^{35}$S-labeled proteins in the culture supernatant of the Brazilian BCG substrain (Fig. 1, lane A1) looked identical to the Aurodye-stained Western blot of concentrated supernatant from a 5-day culture, which should undergo minimal autolysis (lane B1). As reported previously (2), the secreted protein pattern of the British strain, a low producer of the 46/23-kDa protein, differed from that of the Brazilian strain by the presence of small amounts of that protein (lanes B1 and B2). Comparing lanes B1 and B2 with lane B3, proteins in supernatants of 5-day cultures appeared to be a small subset of those present in the usual supernatant of old cultures, where leakage of material from dead organisms becomes important. A Western blot of purified MPB70 protein (lane B4) gave a strong band with the same molecular mass as the 23-kDa protein identified in the short-term supernatant. The

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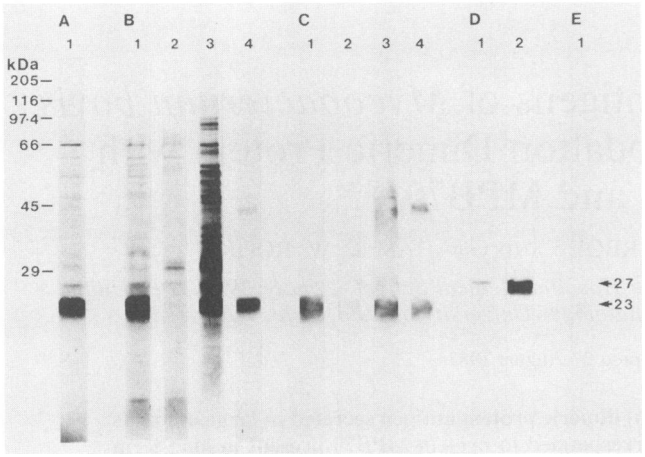


FIG. 1. Mycobacterial proteins in culture supernatants of BCG strains were separated by SDS-PAGE on slab gels of 12.5% acrylamide and then transferred to nitrocellulose paper by electroblotting. (A) Autoradiogram of [$^{35}$S]methionine-labeled proteins in 5-day culture supernatant of the Brazilian strain. (B) Immunoblots stained with Aurodye, a colloidal gold solution. Lanes: 1, 5-day supernatant of a culture of the Brazilian strain; 2, 5-day supernatant of a culture of the Glaxo strain; 3, 4-week culture filtrate of the Japanese strain; 4, purified MPB70 protein. (C) Immunoblots incubated with a rabbit anti-MPB70 antiserum. Lanes 1 to 4 correspond to the antigen preparations stained in panel B. (D) Immunoblots of supernatants of the Brazilian strain (lane 1) and of the long-term culture filtrate of the Japanese strain (lane 2) incubated with a rabbit anti-MPB64 antiserum. (E) Immunoblot of supernatant of the Brazilian strain incubated with monoclonal antibod, SA1.D2D, recognizing a 23-kDa cross-reactive protein. The positions of molecular mass markers are shown on the left.

preparation of MPB70 protein appeared to contain a minor protein with a molecular mass of approximately 42 kDa.

Western blots of the various antigen preparations were incubated with a polyclonal anti-MPB70 antiserum, and the binding patterns are shown in lanes C1 to C4. The antiserum reacted strongly with the 23-kDa subunits of the protein antigen secreted or released by the high-producer strains (Brazilian and Japanese), and the intensity of the reaction was proportional to the amount of antigen present, since it reacted poorly with the supernatant of the British substrain. Although the antibody activity pattern showed only one single band in the short-term supernatants, the antiserum reacted also with a protein antigen with a subunit molecular weight of 42,000 in the preparation of MPB70 and with antigens in the range of 40 to 50 kDa in the old supernatant. No binding of anti-MPB70 antibodies to the culture filtrate of *M. tuberculosis* was observed. Protein staining and immunoblotting with anti-MPB70 antiserum on blots of purified MPB70 and 4-week culture supernatant of the Japanese strain demonstrated that MPB70 protein is identical to the 46/23-kDa dimeric protein. MPB70 protein was reported to be a monomer with a molecular weight of 18,000 by using the method of gel filtration, but recently Haslov et al. (in press) and S. Nagai (personal communication) have determined the molecular mass by SDS-PAGE and found 22 kDa, a value similar to the subunit molecular weight of 23,000. The presence of the protein antigen with a subunit molecular weight of 43,000 in the preparation of MPB70 is unexpected, especially since the purified protein was eluted as a single peak on a Sephadex G-50 column (6). Supernatants of brief cultures do not contain it, as shown by the reactivity of the antiserum on immunoblots.

Monoclonal antibody SA1.D2D recognizing an antigen with a subunit molecular weight of 23,000 reacted poorly with the culture supernatant of the Brazilian strain. Therefore, the 23-kDa antigen recognized by this antibody appears to be distinct from the MPB70 22- to 23-kDa subunit. Moreover, our results confirm that MPB70, unlike the antigen recognized by SA1.D2D, is confined to BCG strains (3).

Anti-MPB64 antibodies bound only to a protein antigen with a subunit molecular weight of 27,000 (lanes D1 and D2). Our estimate of 27 kDa for the molecular mass of MPB64 is close to the value of 26 kDa determined recently by S. Nagai (personal communication) using SDS-PAGE. The anti-MPB64 antiserum also bound to an antigen with a subunit molecular weight of 27,000 on an immunoblot of culture filtrate of *M. tuberculosis* (data not shown). Our data, which showed no similarity between the 46/23-kDa dimer antigen and MPB64, confirmed earlier findings by immunoprecipitation (4).

The present results link two parallel studies undertaken in different laboratories on antigens of defined specificity from BCG. We have established that the MPB70 antigen corresponds to the secreted 46/23-kDa protein antigen, whereas the latter expressed no immunochemical similarities with MPB64 or with the protein recognized by SA1.D2D.

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