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# Humoral Immune Response of Tuberculous Patients against the Three Components of the *Mycobacterium bovis* BCG 85 Complex Separated by Isoelectric Focusing

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**An isoelectric-focusing technique followed by Western blot (immunoblot) analysis was used to investigate the immunoglobulin G response of tuberculous patients against each of the three components of the *Mycobacterium bovis* BCG antigen 85 complex. The 85A component was stained by the tuberculous as well as the non-tuberculous sera. In contrast, the 85B and the 85C proteins of the complex were not stained by the control sera but were stained by 20 of 28 tuberculous serum samples.**

The technical practicability of serological testing for tuberculosis has been amply demonstrated. Most authors have used the well-known enzyme-linked immunosorbent assay technique, which is easy to perform but necessitates highly purified antigens or monoclonal antibodies to obtain a high degree of diagnostic accuracy (3).

Proteins of the antigen 85 complex from *Mycobacterium bovis* BCG are abundantly secreted into the supernatant of a variety of mycobacterial cultures (7, 12). The unseparated antigen 85 complex was previously purified by Daniel et al. and called antigen 6 (11). The results of a serological assay were promising but not confirmed, as the authors subsequently studied antigen 5 (9). The 85 complex comprises three closely related proteins, 85A, 85B, and 85C, with approximate molecular masses of 32, 30, and 33 kDa, respectively (7).

These proteins are encoded by three distinct genes for which significant differences in sequence have been observed (1, 6, 12). The genes encoding 85A and 85B have recently been identified in both *Mycobacterium tuberculosis* and *M. bovis* BCG. They are strongly conserved in both species (5). The 85B component corresponds to the alpha antigen in reference 6 and to MPB59 in the crossed-immunoelectrophoresis reference system for BCG antigens (2). The 85A component is identical to P32 of BCG in reference 4 and to MPT44 in the crossed-immunoelectrophoresis system (2). Both antigens 85A and 85B have previously been purified and used in serological tests, with a sensitivity of about 70% for each (8, 10).

Because these proteins are difficult to purify in large amounts by biochemical techniques, we analyzed the immunoreactivity of each part of the complex by using isoelectric focusing (IEF) followed by Western blot (immunoblot) analysis. This combination of methods not requiring protein denaturation was applied here for the first time to examine humoral response against mycobacterial antigens.

Serum samples were provided by a medical dispensary of Médecins sans Frontières in the Philippines. Twenty-eight patients (mean age, 43 ± 13 years) presented active tuberculosis. The diagnosis was assessed by positive auramine staining of at least one of three sputum samples and was confirmed by culture. The results were double-checked in each case. Of the 53 healthy control subjects, 10 (mean age, 35 ± 16 years) had been treated for tuberculosis during the last 3 years. Twenty-six of the control subjects were tuberculin (PPD Rt23; 2U, Copenhagen, Denmark) skin test negative (mean diameter of induration, <10 mm).

The 85 complex was purified from 14-day-old BCG culture filtrates (4). We performed vertical nondenaturing IEF on 12.3% polyacrylamide gels (150 [height] by 130 by 1.5 mm) containing a 6.25% ampholyte mixture (2.5 to 5 and 4 to 6.5 in a 3/2 ratio; Pharmacia, Uppsala, Sweden). Prefocusing was performed at 500 V · h for 30 min before sample application and focusing at 4,500 V · h for 3 h (Hoefer Scientific Instruments, San Francisco, Calif.). Three micrograms of unseparated 85 complex per centimeter (in width) of gel was added at the cathode. After equilibration, the isoelectric points of the three components on the gel were determined by Coomassie blue coloration and use of a low-isoelectric-point calibration kit (Pharmacia). The results were quite close to the values in previous studies: 4.55 for the 85A protein, 4.15 for the 85B protein, and 4.35 for the 85C protein (7). Antigen 85A corresponds to a large protein band and represents more than 90% of the total protein in the complex as estimated by gel scanning (Fig. 1).

The proteins were also electrophoretically transferred onto nitrocellulose sheets (0.2 μm; Bio-Rad Laboratories) in a pH 8.5 buffer with a Hoefer apparatus. The nitrocellulose was thereafter incubated for 1 h in pH 7.5 Tris buffer saline (TBS) containing 3% bovine serum albumin to block nonspecific protein binding. It was then cut into strips and incubated overnight with human sera diluted in TBS–0.05% Tween (TBS-T). The strips next were washed in TBS-T and incubated for another 4 h with anti-human immunoglobulin G peroxidase-conjugated rabbit immunoglobulins (Dakopatts, Copenhagen, Denmark) diluted 1/500 in TBS-T. The washing procedure was repeated three times and followed by the addition of a peroxidase substrate containing alpha-chloronaphthol (Bio-Rad Laboratories) in the presence of hydrogen peroxide. Development time was limited to 15 min. Finally, the strips were washed in distilled water and dried.

A monoclonal antibody (VIII H2, dilution 1/50) known to react with an epitope present on the three antigens and a

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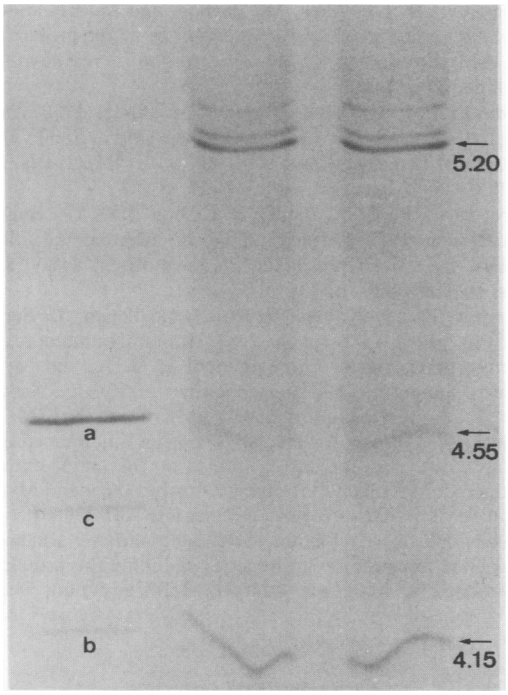


FIG. 1. IEF of the antigen 85 complex (lane 1, 85A [a], 85B [b], and 85C [c]) and of isoelectric point markers (lanes 2 and 3). Coomassie blue R250 stain was used.

rabbit anti-mouse peroxidase-conjugated antiserum (Dakopatts) diluted 1/500 in TBS-T were used to identify the antigens after separation by IEF. The three antigen bands were found to be stained by the monoclonal antibody, their degrees of coloration varying with the amount of antigen present on the nitrocellulose strip (Fig. 2 and 3).

Titrations of control and tuberculous sera were performed. The degrees of coloration for each band decreased in parallel, showing that there were no dilution artifacts. The optimal working dilution was determined to be 1:100. A serum of well-known reactivity was included in each experimental run to verify the results. All of the 53 control and 28 tuberculous serum samples were tested twice.

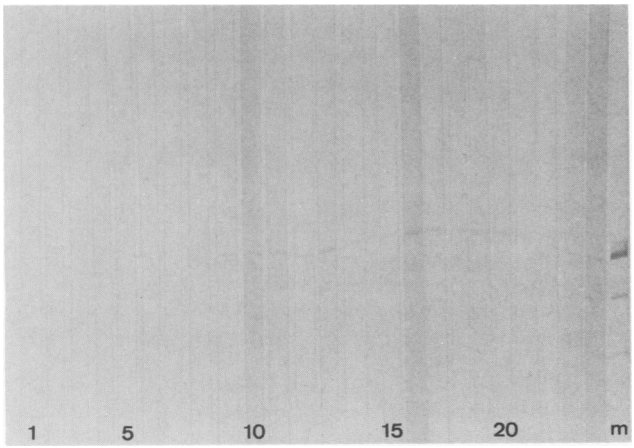


FIG. 2. Western blot analysis of the antigen 85 complex components after IEF separation. Results for 24 control serum samples are shown. The strip in lane m was incubated with a monoclonal antibody that reacts to an epitope common to the three antigens.

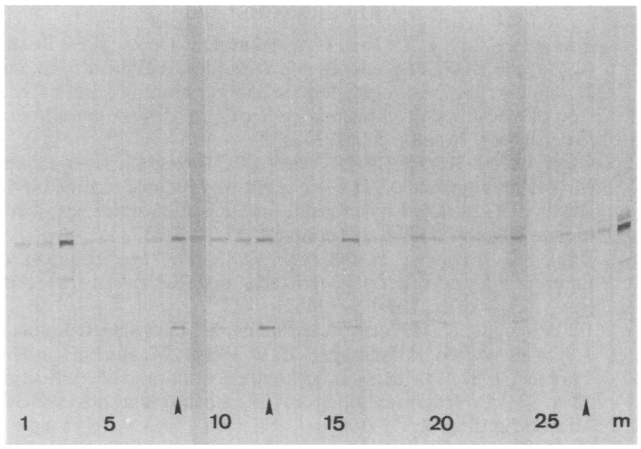


FIG. 3. Western blot analysis of 28 tuberculous serum specimens. The strip in lane m was incubated with a monoclonal antibody that reacts to an epitope common to the three antigens. Twenty serum specimens recognized antigen 85B, 85C, or both in addition to 85A. Three serum samples (arrows) definitely recognized component 85B better than component 85A. One serum sample (lane 3) clearly reacted with component 85C but only weakly with 85B.

Control sera weakly recognized the antigen 85A but did not recognize the 85B and 85C components (Fig. 2). In contrast, a different pattern of antigen recognition was observed when tuberculous sera were tested (Fig. 3). All of these sera recognized the 85A band. In more than half of the cases, the degree of staining for the tuberculous sera was higher than for the control sera. Twenty of twenty-eight (71%) tuberculous serum specimens recognized either 85B or 85C or both components of the complex. There was no relationship between the degree of 85A staining and recognition of the two other 85 components, but in all cases of pronounced 85A staining, 85B and 85C were also recognized. In a singular case, the 85A and 85C bands were both strongly colored while the 85B band was weakly stained.

A pattern of recognition similar to the one observed when the monoclonal antibody was used would be expected if epitopes shared by the three antigens were involved in the humoral immune reaction. This was not the case here, since in three serum samples, antigen 85B was recognized better than antigen 85A, suggesting the presence on this protein of a highly immunogenic epitope specifically recognized by some individuals affected by active tuberculosis.

It thus appears that a qualitative analysis of the humoral response directed against each of the three components of the 85 complex allows better distinction between healthy subjects (some healed of tuberculosis) and patients with active tuberculosis.

We further conclude that antigen 85A (P32) seems to be the least specific component for the serodiagnosis of active tuberculosis.

Using monoclonal antibodies, we intend to better characterize specific epitopes present on the 85B and 85C components.

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