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PURIFICATION AND MOLECULAR ANALYSIS OF BCG ANTIGEN 60

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ABSTRACT

Tuberculosis remains as an important socioeconomical and medical problem throughout the world and especially in Iran. Early and timely diagnosis of pulmonary and extrapulmonary tuberculosis is vital to initiate prompt treatment. Current diagnostic methods are either slow or lack enough sensitivity or specificity.

Several mycobacterial antigens are involved in the complex interaction with the immune system of the host. Their identification is important for both diagnosis and protection against mycobacteria. Antigen 60 (A60) is a thermostable antigen found in the cytosol of *M. bovis* and *M. tuberculosis*. An ELISA test using A60 is designed for diagnosis of tuberculosis with satisfactory results. In previous studies, A60 has also showed a protective effect against experimental infections and useful immunotherapeutic effects in promotion of cancer development.

In the present work we tried to purify A60 from the cytoplasm of BCG. A60 was purified by exclusion gel chromatography using sepharose 4B. A60 was recognized by bidimensional immunoelectrophoresis with anti-BCG and anti-A60 antiserum, where it appears as the less mobile component. In agarose electrophoresis, A60 showed only one band but in immunodiffusion it showed two immunoprecipitinogen lines with anti-BCG anti-serum. In analyzing with dot blotting, both cytoplasm and cell wall of BCG showed positive reaction with anti-A60 anti-serum. When A60 was fractionated by SDS-PAGE and analyzed by western blot using anti-A60 antibody, 65,46, 40, 38 and 35 KDa protein fractions were identified.

It is concluded that A60 is a macromolecular antigen of BCG with a molecular weight of 10^6-10^7 Da and is a lipoprotein-polysaccharide complex which contains several proteins. A60 is present in both cytoplasm and cell wall of BCG and can easily be purified from BCG vaccine using exclusion chromatography by sepharose 4B, to be used for designing diagnostic tests for TB.

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Keywords: Antigen 60 (A60), BCG , Size Exclusion Chromatography.

INTRODUCTION

Tuberculosis has a high incidence in developed countries as well as in undeveloped countries. Early and timely diagnosis of pulmonary as well as extrapulmonary tuberculosis is vital to initiate prompt treatment. The commonly used diagnostic tests for tuberculosis such as sputum examination of acid fast bacilli, culture of sputum or other fluids, the tuberculin skin test and radio-

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logical investigation don't achieve the required diagnostic sensitivity.[1] Enormous efforts have been made to develop a simple, quick, sensitive and specific biochemical marker for early diagnosis of tuberculosis, based either on detection of antibodies produced against a battery of mycobacterial antigens or detection of mycobacterial antigens in serum and other body fluids by employing either ELISA tests or molecular techniques.[1]

Mycobacteria contain numerous immunologically active substances which play a dominant role in mycobacterial diseases. One group of mycobacterial antigens that has been the object of extensive investigation is the thermostable macromolecular antigen complexes (TMA). The best known members of this group are antigen 60 (A60) of *Mycobacterium bovis* and *Mycobacterium tuberculosis*.[2] A60 is the main thermostable antigen of several preparations such as the old tuberculin (OT) and purified protein derivative (PPD), which are widely used as diagnostic reagents in human medicine.[3] A60-based serological assays and an enzyme linked immunosorbent assay (ELISA) have been developed for use in tuberculosis diagnosis.[4]

A60 has approximately equal portions of carbohydrates, proteins and lipids. It is known to trigger humoral as well as cellular immune reactions.[5] 85% of the antimycobacterial antibodies in patients sera were directed against A60.[6] The high immunogenicity of A60 introduce it as a proper candidate for developing specific diagnostic immunoassays.[7,8] In previous studies A60 has also shown protective effects against experimental infections[9,10] and useful immunotherapeutic effects in promotion of cancer development.[11,12,13,14,15]

MATERIAL AND METHODS

Bacteria

M. bovis BCG was the Calmette-Guerin Master Seed lot 1173-p2 lot c from the Pasteur Institute of Iran. Bacteria were grown in Sutton medium and incubated at 37°C. Bacterial colonies were collected and densed in Birco system and stored at -20°C.

Preparation and fractionation of bacterial homogenates

Bacteria were suspended in 150 mM NaCl, 10 mM Na phosphate buffer pH=7.4 (PBS) and were disrupted and homogenized in a hyperation homogenizer (15000 bar at 4°C). Homogenates were centrifuged first for 30 min at 4°C and then for 15 min to sediment the cell wall. The supernatant contained whole cytoplasm.

Column chromatography of A60 and fractions

Mycobacterial cytoplasm was fractionated on columns of sepharose 4B which were eluted with PBS buffer.

Electrophoretic techniques

Crossed immunoelectrophoresis was performed according to the technique of Closs et al. (1980) on glass plates (5×7cm) covered with 2 gel slabs made of 1% agarose gel in 0.02 M Tris-barbital buffer pH=8.6. Antigen solution was applied to the origin and run for 2h at 200V at 15°C in the first dimension. Bands were cut and transferred to the final plate, on which superior gel were placed. The upper gel contained 200 µL of anti-BCG antiserum. In crossed immunoelectrophoresis with intermediate gel, the intermediate gel contained either agarose only (control), or agarose plus 200 µL anti-A60 antiserum. Electrophoresis in the second dimension was run at 80V for 18h at 15°C. Slants were washed, stained with Coomassie brilliant blue and photographed (according to Closs et al's method).[16] Fractionation of A60 fragments was carried out with SDS-PAGE.

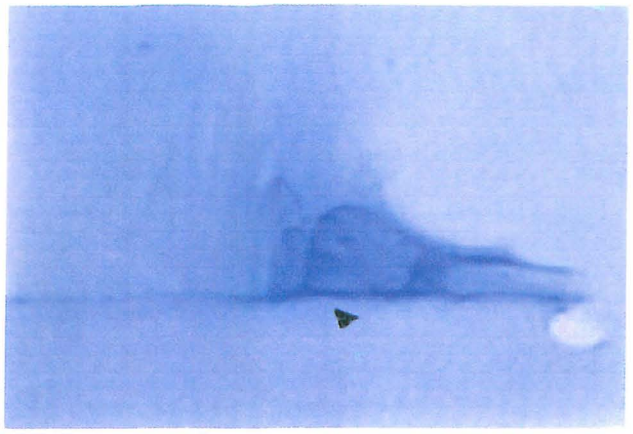
Immunodiffusion

Double diffusion precipitation test was performed on glass plates covered with 1% (W/V) agarose in 85%(W/V) NaCl, 0.05M Na phosphate buffer, pH=7.2. 20 µL anti- BCG antiserum was placed in the central well and 20 µL antigen samples in the peripheral wells. Plates were kept in humidity chamber for 24h at 20°C. Plates were washed, stained with Coomassie brilliant blue and photographed.

Dot blot and Western blot

For dot blot 10 µL A60 and other column fractions were spotted on nitrocellulose membrane. Spots were blocked with blocking buffer (BSA 1% , Tween 20 0.5%, PBS, pH=7.2). Anti-A60 antiserum (1/1000 dilution) was placed on the spots for 1h of incubation at 37°C. Membrane was washed with washing buffer (Tween 20 0.5%, PBS) and incubated for 1h with secondary antibody (dilution of 1/1000 peroxidase labeled anti-human IgG) and washed with washing buffer before being stained with diamine benzidine (DAB) in the presence of hydrogen peroxidase.

For Western blot analysis, A60 and cytoplasm of *M. bovis* BCG were fractionated by electrophoresis on 6-16% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE), in parallel with molecular weight markers. Electrophoresed components were electrophoretically transferred to the nitrocellulose membrane blocked with blocking buffer. The membrane was incubated for 1h with primary antibody (Anti-60, 1/1000 dilution) at 37°C and for 1h with secondary antibody (1/1000 dilution anti-human IgG-HRP) and washed with washing buffer before being stained with diaminobenzidine (DAB) in the presence of hydrogen peroxidase.



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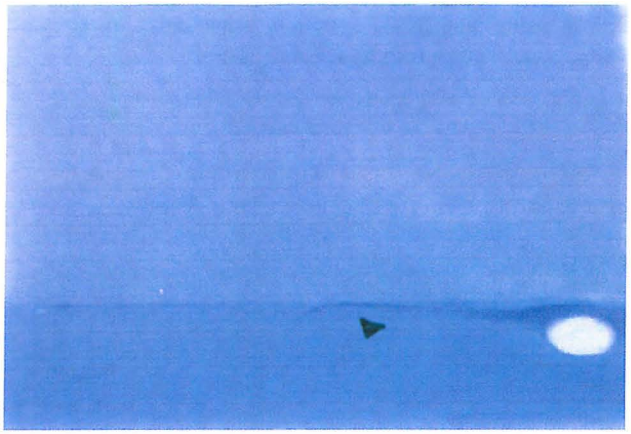
2a



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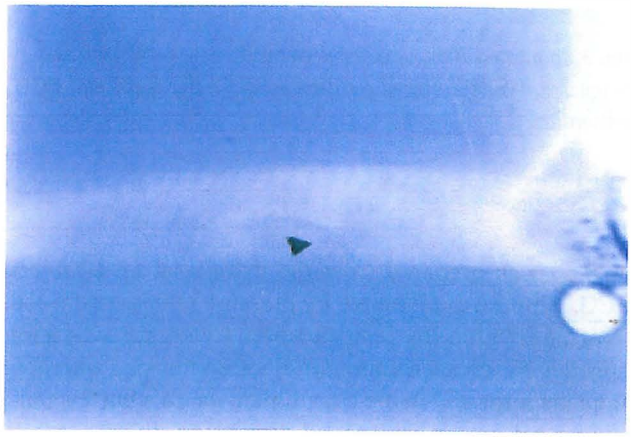

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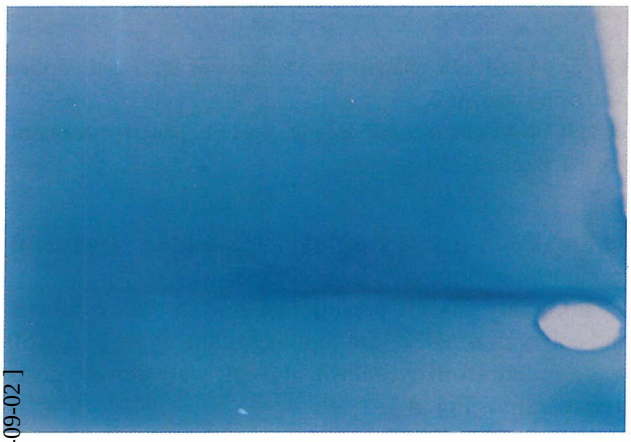

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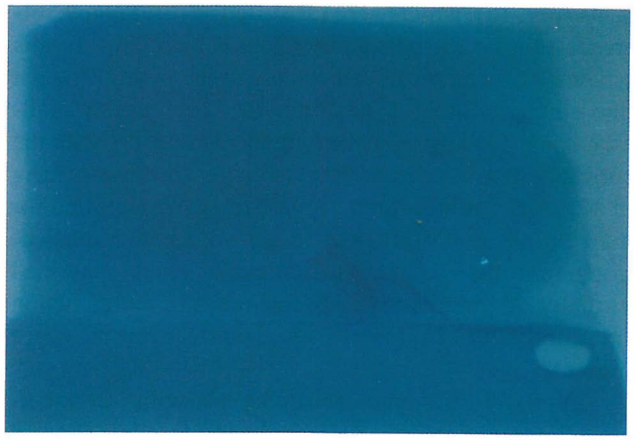

2c

Fig. 1. Crossed immuno-electrophoresis.
a: Cytoplasm of BCG, b: Exclusion fraction of chromatography column containing A60, c: Inclusion fraction of chromatography column containing other cytoplasmic components.

Fig. 2. Crossed immuno-electrophoresis with intermediate gel (CIE), a: Cytoplasm of BCG, b: Exclusion fraction of chromatography column containing A60, c: Inclusion fraction of chromatography column containing other cytoplasmic components.

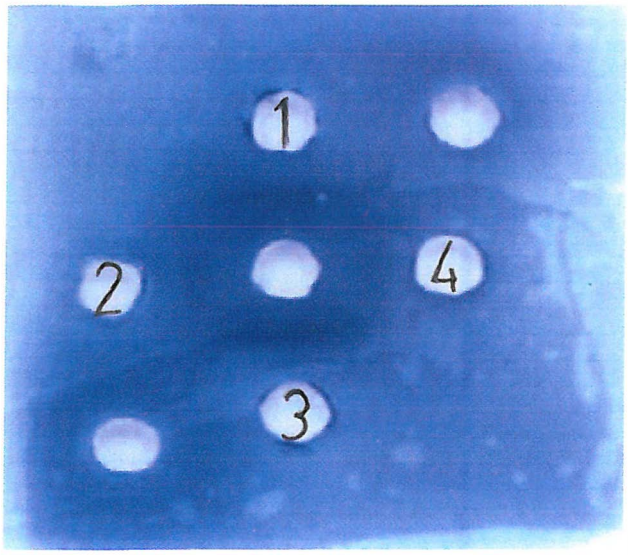

Fig. 3. Immunodiffusion analysis of purified A60. Wells 1 and 3: cytoplasm, Wells 2 and 4: purified A60, Center well: anti-BCG antiserum.

RESULTS

A60 was prepared from the cytoplasm of *M. bovis* BCG, which was disrupted by pressure release and freed from the envelope by centrifugation. Cytoplasm was fractionated by crossed immuno-electrophoresis, whereby antigens migrated in the first dimension in a buffer-containing gel and in the second dimension, in an agarose containing anti-BCG antiserum. Coomassie brilliant blue staining disclosed the presence of some thirty components (Fig. 1a), which were identified according to a reference scheme introduced by Closs et al (1980). In this scheme A60 complex corresponds to the precipitinogen line which is closest to the origin. Its Rf is thus the smallest among BCG cytoplasm components reacting with the correspondent antiserum. Exclusion gel chromatography of *M. bovis* cytoplasm on sepharose 4B was applied for preparation of A60. Figure 1b shows the immunoelectrophoretic pattern of exclusion peak, which contained virtually all cytoplasmic A60, whereas in the inclusion fraction (Fig. 1c) most cytoplasmic proteins were present.

In crossed immunoelectrophoresis with intermediate gel containing anti-A60, exclusion fraction showed one precipitinogen line in intermediate gel (Fig. 2b) whereas in the inclusion fraction (Fig. 2c) other cytoplasmic components were seen. Fig. 1a shows the CIE pattern of BCG cytoplasm.

Cytoplasm of BCG and A60 were analyzed by the double immunodiffusion technique using anti-BCG antiserum. A60 yielded two strongly diffusing bands whereas the cytoplasm yielded several immunoprecipitinogen lines (Fig. 3).

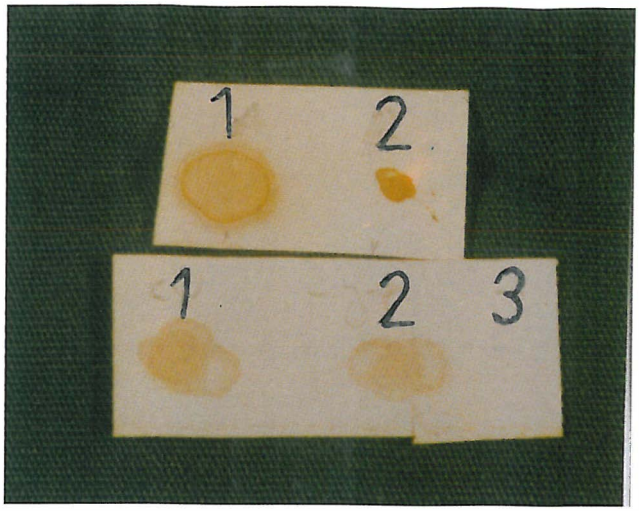

Fig. 4. Dot blot analysis of cytoplasm and cell wall of BCG.
Up row using anti-BCG, down row using anti-A60, 1: cytoplasm, 2: cell wall, 3: BSA as negative control.

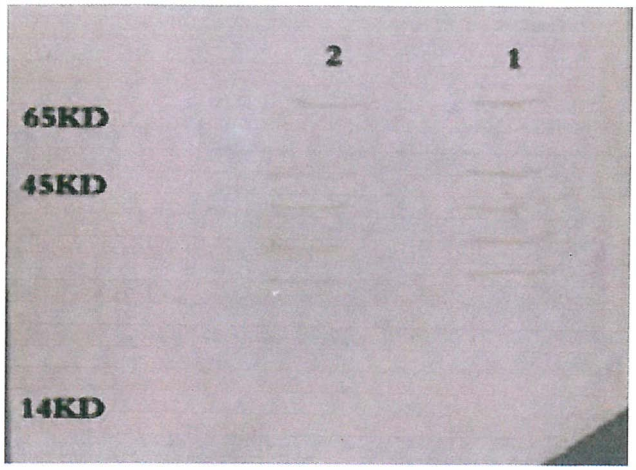

Fig. 5. Western blot analysis of cytoplasm of BCG and purified A60 using anti-A60.
Lane 0: BSA as marker and negative control, Lane 1: cytoplasm, Lane 2: purified A60.

For analyzing the presence of A60 in the cytoplasm and cell wall of BCG, dot blot technique using anti-A60 was applied. Both cytoplasm and cell wall showed positive reaction (Fig. 4).

A more precise identification of the components of A60 was obtained by dissociation of the complex and fractionation by polyacrylamide gel electrophoresis. After electrophoretic transfer to nitrocellulose membrane, A60 components were analyzed by Western blot technique using anti-A60 antibody and four single bands corresponding to 35, 38, 40, 46, 65 KDa proteins were recognized (Fig. 5).

DISCUSSION

Mycobacteria interact with the immune system of the host through numerous antigens. In the fight against mycobacteriosis, identification of these antigens may play an important role for both diagnosis and protection. Application of a reference system based on crossed immunoelectrophoresis allowed the separation of some 30 antigens of *Mycobacterium tuberculosis* and in analyzing of *M. bovis* cytoplasm has pointed towards antigen 60 as being the less mobile polymer in the crossed immunoelectrophoresis plates.[16] Several mycobacterial antigens have been purified, some of which have been proven to be of value in mycobacterial serology and cutaneous testing. One of the most important groups of these antigens is TMA (Thermostable Macromolecular Antigens). The pronounced recognition of TMA by sera from patients with leprosy and tuberculosis suggests an immunodominant role of these components. 85% of the antimycobacterial antibodies in patients' sera were directed against A60, the TMA complex of *M. bovis*, indicating that the majority of *M. bovis* components that react with tuberculosis patients' sera were present in the A60 complex.[6]

The present work describes the purification of A60 from the cytoplasm of *M. bovis* BCG working seed lot 1173-P2 lotC which was prepared from Pasteur Institute of Iran. Exclusion chromatography using sepharose 4B was applied for this purpose. The appearance of this antigen in the exclusion volume of sepharose 4B column (Fig. 2B) agrees with data reported in another work (Cocito et al. 1986) yielding an estimate value of about 10^6-10^7 Da for the main lipopolysaccharide component of the A60 molecule.[4] In last researches sepharose 6B was used for this purpose with similar results.[4]

In dot blot analyzing both cell wall and cytoplasm of BCG showed positive reaction suggesting the presence of A60 in both components of the bacteria. The occurrence of the 65, 46, 40, 38 and 35 KDa proteins in A60 was confirmed by using anti-A60 antiserum in A60 western blot. These data show that A60 contains several antigenic determinants that react with anti-A60 antiserum.

Its parietal location, high molecular mass and complex composition of some 30 proteins, polysaccharides and lipids may explain the high immunogenicity of A60 which introduce it as a proper candidate for developing specific diagnostic immunoassays.[6,7]

The dissociation of A60 into its constituent parts will probably be the prerequisite for comprehensive knowledge of the role of this antigen and of its constituents. In the future vaccinal strains of mycobacteria might be prepared by specifically deleting or inactivating sequences encoding the polypeptides used for the immunological tests. It would thus be possible to vaccinate without inducing positivity in diagnostic immunoassays.

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