HYBRIDOMA
Volume 29, Number 3, 2010

DOI: 10.1089/hyb.2009.0097

A Novel Monoclonal Antibody Against A60 Antigen of *Mycobacterium bovis* Bacillus Calmette-Guerin

Salar Bakhtiyari,[1] Karimeh Haghani,[2] Elham Farhadi,[3] Mohammad Soukhtanloo,[4] Nima Rezaei,[5] and Mohammad Taghikhani[1]

Mycobacterium contains several immunologically active substances, which play a principal role in mycobacterial diseases. The majority of the highly antigenic proteins present in mycobacterial homogenates are components of the A60 complex.In this study, A60 antigen was prepared from cytoplasm of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). Cytoplasm was fractionated by passage through the column of sepharose 6B and ConA-sepharose 4B. After purification of spleen cells of the immunized mice, the cells were fused with SP2/0 myeloma cells. Four clone cell lines producing antibody against A60 antigens were established and each clone was tested for immunoreactivity against purified A60 by ELISA and immunoblotting. The clone designated DEB7 reacted strongly with A60. Immunoblotting using MAb DEB7 showed that this MAb binds to a single protein of A60 subunit with a molecular weight of 65 kDa. This subunit of A60 *M. bovis* recognized by DEB7 MAb could be used to increase the sensitivity and specificity of immunoassay or other potential roles in mycobacterium infection.

Introduction

Tuberculosis is one of the oldest documented communicable diseases. Mycobacteria, the cause of this infection, contain numerous immunologically active compounds, which play a principal role in mycobacterial infections. These compounds are derived from the complex cell wall with a matrix of polysaccharides and peptides, or from the cytoplasm.[1] Cytosolic antigens involved in the induction of protective immunity against tuberculosis could be used as new candidate molecules for diagnosis or vaccines. Two types of antigens are found within the cytoplasm: ribosomes contain an rRNA core with associated proteins, and the thermostable macromolecular anti-complex contains both free and bound lipids, as well as proteins and polysaccharides.[2–4] Thermostable macromolecular antigen (TMA), which is present in all mycobacteria, has been the object of extensive investigation.[5]

A60 antigen, or the TMA of *Mycobacterium bovis*, is composed of about 30 components, the majority of which are present in mycobacterial homogenates. A60 is known to trigger both humoral and cellular immune reactions.[5,6] The immunodominance of A60 in tuberculosis has been quantified.[7] Only small components of A60 are species specific and not shared by *Mycobacterium leprae* and *Mycobacterium avium*.[8,9]

The purpose of this study was to prepare a monoclonal antibody against A60 antigen for further application in detection of the immunodominant *M. bovis* and species-specific immunoassay and to purify the non-recombinant and recombinant antigen 60 from bacterium culture for further use in antibody detection against A60 in tuberculosis patients' sera.

Materials and Methods

Materials

Polyethylene glycol (PEG; molecular weight 1500), RPMI 1640, streptomycin, penicillin, hypoxanthin-aminopetrin-thymidine (HAT), hypoxanthin-aminopetrin,[6] bovin serum albumin (BSA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), peroxidase labeled goat anti-mouse IgG (Fab specific), and other chemicals were all purchased from Sigma Chemical (St. Louis, MO). Fetal calf serum (FCS) was obtained from Gibco (Grand Island, NY). 96-well plates and other plastic wares were obtained from NUNC (Roskilde, Denmark). Calmette-Guerin strain of *M. bovis*

[1]Department of Clinical Biochemistry, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran. [2]Department of Biochemistry and Biophysics, Faculty of Sciences, Tarbiat Modares University, Tehran, Iran.
[3]Department of Hematology, Iranian Blood Transfusion Organization (IBTO), Tehran, Iran.
[4]Department of Clinical Biochemistry, Faculty of Medicine, Mashhad Medical University, Mashhad, Iran.
[5]Growth and Development Research Center, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran.

(BCG), myeloma cell line Sp2/0 origin, and Balb/c mice were purchased from the Pasture Institute of Iran (Tehran).

Bacterial culture

The Calmette-Guerin strain of *M. bovis* BCG was grown at 37°C, 5% CO_2 in broth media. Bacteria were collected by centrifugation (1000 *g*, 1 h) and cell pellets inactivated by incubation with 0.5% formaldehyde, then washed and suspended in phosphate buffer (pH 7.4, 20 mM), and finally stored at −20°C.

Preparation of A60

Purification of A60 was carried out as described by Cocito.[2] Briefly, 50 mL of phosphate buffer were added to 1 g of cell pellet of bacteria. After prolonged stirring (8 h, 4°C), centrifugation (30000 *g*, 20 min, 4°C) was performed to separate the extracellular matrix from the cell pellets. The pellets were suspended in minimum volume of Tris-HCl buffer (50 mM, pH 7.4), NH_4Cl (40 mM) and magnesium acetate (15 mM). The pellet was sonicated 2 h in an ice bath. Homogenates cells were centrifuged at 25,000 *g* for 20 min at 4°C to remove unbroken cells. The supernatant contained whole cytoplasm proteins. A60 was prepared from the cytoplasm of *M. bovis* BCG by exclusion gel chromatography on sepharose 6B columns (Pharmacia, Uppsala, Sweden).[2] A60 protein content was measured by the method of Bradford[10] using bovine serum albumin as standard protein. The purity of the A60 was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).

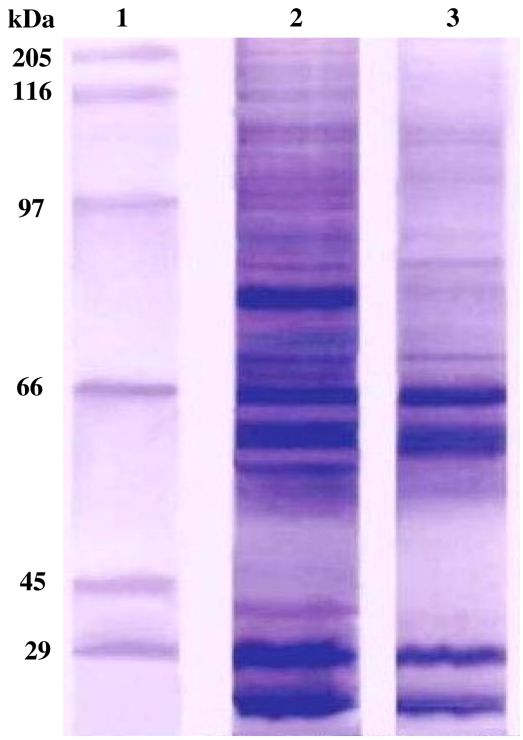

FIG. 1. SDS-PAGE in a vertical slab gel. Lane 1, protein molecular weight marker; lane 2, sonicated *M. bovis* BCG; lane 3, purified A60 using sepharose 6B column.

Monoclonal antibody production

Preparation of myeloma cells. Myeloma cells were cultured with 8-azaguanine to ensure their sensitivity to the hypoxanthine-aminopterin-thymidine (HAT) selection medium used after cell fusion. A week before cell fusion, myeloma cells were grown in 8-azaguanine. Cells must have high viability and rapid growth. The HAT medium allows only the fused cells to survive in culture.

Fusion of myeloma cells with immune spleen cells. Six-week-old female Balb/c mice were given 50 μg of purified A60 antigen in complete Freund's adjuvant intraperitoneally. The next inoculations were performed with half of the antigen in incomplete Freund's adjuvant biweekly in the same way for 2 months. Blood samples were taken from tail vena 10 days after the fifth immunization and tested for antibody production by direct ELISA. The two mice exhibiting the highest antibody titers and sensitivity to A60 were selected. Three days before fusion, the antigen was given intravenously. The two mice were sacrificed 3 days after the final injection, and their spleens removed for use in hybridoma production. Spleen cells were fused with SP2/0 myeloma cells using polyethylene glycol 1500 as the fusing agent, according to the method of Kohler and Milstein[11] and the cells were grown in HAT and HT media. The cells were maintained in HAT until macroscopic colonies were observed and the myeloma controls had disappeared. The HAT medium was then replaced with hypoxanthine-thymidine medium. The content in each well was screened for anti-A60 reactivity by indirect ELISA and positive ones were cloned by twice limiting dilution on the feeder layer in 96-well plates. Four clone cell lines producing antibody against A60 antigen were established in one fusion. The immunoglobulin isotype was determined by the isotyping strip kit.

Indirect ELISA

96-well plates were coated with 5 μg/mL of A60 at 37°C overnight in carbonate buffer (pH 8.6). Nonspecific binding was blocked with 1% bovine serum albumin (BSA) in PBS buffer (pH 7.5) for 1 h at 37°C. After washing, plates were incubated for 1 h at 37°C with antiserum of mice or supernatant of hybridoma cells. Finally, the plates were washed as before and were incubated with anti-mouse IgG horseradish peroxidase (HRP) conjugate for 1 h at 37°C. After washing, color developed with 3,3′, 5,5′- tetramethyl benzidine (TMB) and was stopped with 2 N HCl. The absorbance was determined in 450 nm.

Immunoblotting

The fractionation of sonicated *M. bovis* BCG was performed in a vertical slab gel unit according to Laemmli,[12] using 12% separating gels and 5.0% stacking gels. After electroblotting on the nitrocellulose paper (NCP), the unreacted sites on paper were blocked with a 2% solution of BSA in 10 mM PBS (pH 7.5) for 1 h at room temperature. The NCP was then incubated with the appropriate dilution (1:200) of MAb in the same buffer for 2 h. The NCP was washed three times with PBS. Goat anti-mouse IgG conjugated to HRP was then added and incubated for 1 h at room temperature. After incubation, the NCP was washed three times with PBS. The reaction bands

Table 1. Clones Selected for Further Experiments After Fusion and First and Second Limiting Dilution

Selected clones after fusion		*Selected clones after first limiting dilution*		*Selected clones after second limiting dilution*	
Clone	*OD at 450 nm*	*Clone*	*OD at 450 nm*	*Clone*	*OD at 450 nm*
A10	1.62	AG8	1.53	AGF5	1.65
D5	2.32	DE6	2.72	DEDEB7[a]	2.68
F3	1.56	FB3	1.69	FBE11	1.64
G11	1.72	GC10	1.57	GCA8	1.65
NC (SP2/0)	0.27	NC (SP2/0)	0.31	NC (SP2/0)	0.23

OD, optical density; NC, negative control.
[a]Clone selected for further experiments.

were visualized with hydrogen peroxide and 3,3′-Diaminobenzidine (DAB).

Production of ascites containing mouse monoclonal antibodies

Six-week-old Balb/c mice were injected intraperitoneally with sterile paraffin oil (0.5 mL/mouse). A second injection of $2\times10^5 - 10^6$ hybridomas in sterile incomplete RPMI-1640 was administered on day 11 by intraperitoneal injection. After 7 to 12 days, ascites (5–10 mL/mouse) containing mouse monoclonal antibodies were extracted and purified by ammonium sulfate precipitation and protein A-sepharose chromatography.[13]

Antigen purification by affinity chromatography

The purified MAbs from ascites fluids were coupled to cyanogen bromide-activated sepharose 4B (Pharmacia) at a ratio of 5 mg of antibody to 1 mL gel in 0.25 M sodium bicarbonate buffer (pH 9.0) containing 0.5 M sodium chloride for 2 h at room temperature. The gel particles were

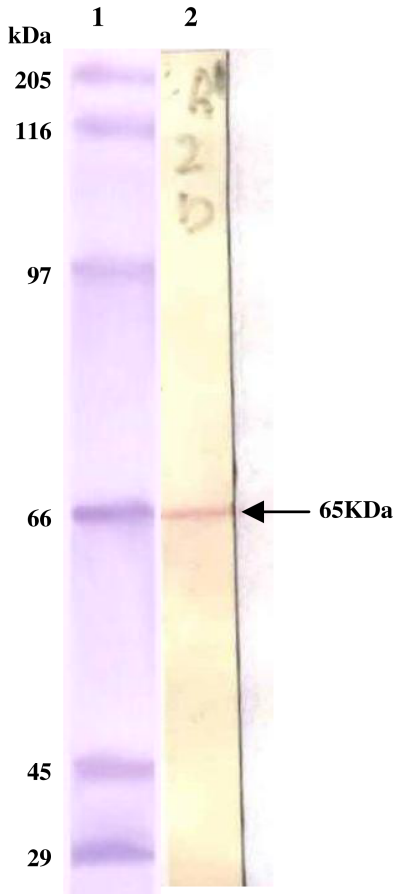

FIG. 2. Immunoblotting of A60 complex using MAbs produced by DEB7 clone. This MAb recognizes a 65 kDa protein component of A60 (lane 2). Lane 1, protein molecular weight marker.

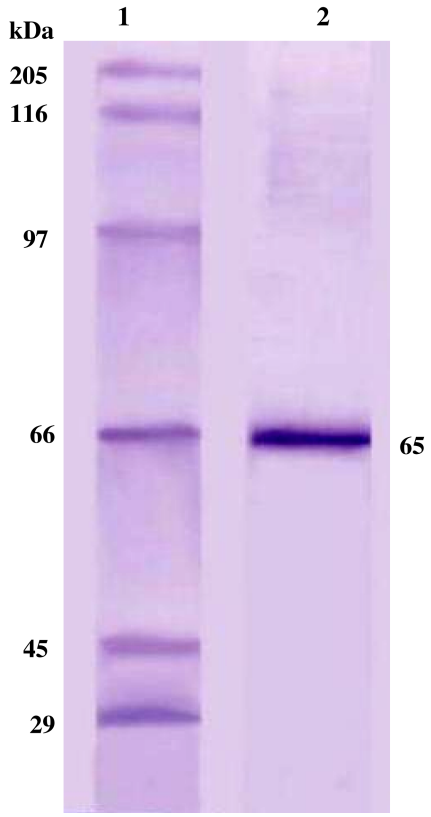

FIG. 3. SDS-PAGE in a vertical slab. Lane 1, protein molecular weight marker; lane 2, purified 65 kDa protein component of A60 using affinity chromatography on sepharose 4B.

then reacted with ethanolamine (1 M) for 2 h at room temperature and washed alternately with sodium acetate (pH 4.0) containing 0.5 M sodium chloride and coupling buffer for four cycles. The washed gels were then stored in 0.1 M Tris-HCl buffer (pH 8.4) containing 0.5 M NaCl and 0.1% sodium azide at 4°C. 106.5 mL sonicated *M. bovis* was first dialyzed in same buffer overnight, subsequently passed through column at a flow rate of 0.5 mL/min. This column was then washed extensively with the Tris-HCl buffer until a stable baseline was obtained. The bound fraction was eluted with 3 M sodium tiocyanate, pooled and dialyzed against 0.15 M phosphate buffer (pH 7.4). Protein concentration was measured by the Bradford method and analyzed by SDS-PAGE.

Results

Preparation of sonicated M. bovis BCG and A60

The protein concentration of sonicated *M. bovis* BCG and purified A60 by sepharose 6B chromatography was measured by the method of Bradford, and was found to be 4.23 mg/mL and 1.47 mg/mL, respectively. The results of fractionated 20 μg of sonicated *M. bovis* BCG and purified A60 are shown in Figure 1.

Hybridization and screening of hybridoma clone for monoclonal antibody

After hybridization, the detection of hybrids was performed by ELISA, which showed four wells in which the positive hybridoma colonies were present. After being cultured, detected, screened, and subcloned, four positive hybridoma colonies (designated DEB7, AGF5, FBE11, and GCA8), which could secrete MAbs against A60 antigens, were obtained. The DEB7 clone strongly reacted with A60 in indirect ELISA (Table 1).

Subclass identification of monoclonal antibody against A60 antigen

The subclass of monoclonal antibody, secreted by DEB7 hybridoma, was IgG2a, while λλ light chain was a component of the antibody.

Immunoblotting results

The results of sonicated *M. bovis* BCG immunoblotting with DEB7 monoclonal antibody showed that this MAb recognizes a single protein component in *M. bovis* BCG with a molecular weight of 65 kDa (Fig. 2).

Separation of antigen by solid-phase affinity chromatography

From 106.5 mg of *M. bovis* sonicate applied to the DEB7-sepharose 4B column, 1.37 mg (1.28%) were eluted with 3 M sodium tiocyanate. Staining of SDS-PAGE-fractionated sample (20 μg) revealed a 65 kDa band (Fig. 3).

Discussion

Many immunologically active compounds are involved in mycobacterial infections. Identification of these antigens may play an important role for both diagnosis and protection.[14] The main active components of cytoplasm of mycobacteria are the thermostable macromolecular antigens.[15] The immunodominance of TMA complex has prompted the development of highly specific and sensitive immunoassay for mycobacterium detection.[16] The best known component of this complex in *M. bovis* and *M. tuberculosis* is antigen 60.[4,15]

In the present study, we produced monoclonal antibody (DEB7) that recognizes a specific subunit of A60 with 65 kDa in immunoblotting (Fig. 2). Other MAbs recognize different immunodominant antigens such as 65, 40, 38, 35, 19, and 14 kDa of A60 subunits, while some of these subunits share with other mycobacterium strains.[8] Our MAb reacted only with the 65 kDa subunit, and it is likely that this immunodominance is species specific to *M. bovis* and *M. tuberculosis*. This MAb can be used for purification of nonrecombinant and recombinant antigen 60 from bacterium culture used for antibody detection against A60 in tuberculosis patients' sera. The results of immunoaffinity chromatography and characterization of the MAb confirmed these findings.

In conclusion, according to our data, the 65 kDa subunit of A60 *M. bovis* identified by DEB7 MAb could be used to increase the sensitivity and specificity of immunoassay or other potential applications in the diagnosis of mycobacterium infection. Other studies, including *in vivo* and *in vitro* effects of purified antigen on T-cell activity and reactivity with biological fluid of tuberculosis patients, are needed to meet some other applications of the purified antigen.

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Address correspondence to:
Mohammad Taghikhani, Ph.D.
Department of Clinical Biochemistry
Faculty of Medical Sciences
Tarbiat Modares University
P.O. Box 14115-331
Tehran
Iran

E-mail: taghi_mo@modares.ac.ir

Received: November 22, 2009
Accepted: January 5, 2010