Original Article

Preparation of monoclonal antibodies against mannosylated lipoarabinomannan (ManLAM), a surface antigen of BCG vaccine produced in Iran

Mohammad Taghikhani, Rasul Moukhah[1]

Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, [1]Department of Clinical Biochemistry, School of Medicine, The University of Tarbiat Modares, Tehran, Iran

Abstract

Background: Bacille Calmette–Guerin (BCG) vaccine is the only vaccine that is used against *Mycobacterium tuberculosis*, but its efficacy is limited in mycobacterium-endemic regions. One of the major antigens present on the cell envelope of the vaccine that suppresses the immune system is mannosylated lipoarabinomannan (ManLAM).

Materials and Methods: In this study, we immunized 4-week-old mice with sonicated BCG vaccine injected intraperitoneally two times at an interval of 2 weeks and with ManLAM antigen injected intravenously and then extracted the spleen cells of the immunized mice. They were fused with SP2/0 myeloma cells.

Results: Five cell line clones producing antibody against ManLAM antigens were prepared and each clone was tested for immunoreactivity against sonicated BCG and purified ManLAM by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. The clones designated H13F33E11 and H23D91G4 reacted strongly with ManLAM. Immunoblotting using monoclonal antibodies (MAbs) H13F33E11 and H23D91G4 showed that these MAbs bind to ManLAM with a molecular weight of 35 kDa.

Conclusions: In this study, we produced a monoclonal antibody of immunoglobulin G3 (IgG3) subclass. This MAb can be used for purification of ManLAM in culture media and detection of the antigen in patient's urine and for increasing the efficacy of BCG vaccine.

Key Words: BCG vaccine, lipoarabinomannan, ManLAM, mannosylated lipoarabinomannan

Address for correspondence:
Mr. Rasul Moukhah, MSc, Department of Clinical Biochemistry, School of Medical Sciences, University of Tarbiat Modares, Tehran, Iran. E-mail: r_moukhah@yahoo.com
Received: 17.02.2014, **Accepted:** 12.05.2014

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is a global problem that infects a third of the world's population, and every year about 9 million new cases of clinical tuberculosis (TB) are added to the estimate.[1] The effectiveness of the only licensed vaccine for TB, bacille Calmette–Guerin (BCG), is only partial.[2]

Since its first use as a human vaccine in 1921, BCG vaccine has been given by a variety of routes, including oral, intradermal, and percutaneous routes. Currently the intradermal route is the most commonly used and is the only method recommended by the World Health Organization (WHO). Reports show that intradermal BCG vaccination induces antibodies of the immunoglobulin G1 (IgG1), IgG2, and IgG3

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.175901

How to cite this article: Taghikhani M, Moukhah R. Preparation of monoclonal antibodies against mannosylated lipoarabinomannan (ManLAM), a surface antigen of BCG vaccine produced in Iran. Adv Biomed Res 2016;5:15.

isotypes. From 1888 to 1990, Glatmann-Freedman and Casadevall have been reviewed the protective and non-protective roles of antibody responses against Mtb infection.[3] Guirado *et al*.[4] reported that passive immunization with sera obtained from mice treated with detoxified Mtb extracts exerted significant protection. Lopez and colleagues investigated the efficacy of two monoclonal antibodies (MAbs), TBA61 against the 16-kDa antigen and TBA48 against the 38-kDa antigen, in the control of pulmonary infection.[5]

Lipoarabinomannan (LAM) is a polysaccharide with a phosphatidyl-*myo*-inositol anchors and an important target of the immune responses induced by intradermal BCG vaccination.[6] This is a major cell surface component of Mtb and has diverse biological activities in the development of mycobacterial pathogenesis and in the interaction with macrophages *in vitro*.[7] These antigens are classified into two types, mannosylated lipoarabinomannan (ManLAM) and arabinosylated lipoarabinomannan (AraLAM).[8] Due to its presence on the bacterial surface, ManLAM has also been suggested as a vaccine candidate (Glatman-Freedman *et al.*, 2004). Several studies suggest that anti-LAM antibodies may have had an important protective role in immune system. For example, the administered monoclonal antiarabinomannan antibody increased the survival of mice after challenge with Mtb.[6] In 2004, Hamasur *et al*. reported that the passive immunization of BALB/c mice with MAb (SMITB14) of IgG1 subclass to LAM can give protection against Mtb infection in BALB/c mice.[9]

Some roles of this antigen are direct interaction with the host and participating in the intracellular survival of mycobacteria, and triggering innate and adaptive immune responses, including the activation of CD1b-restricted T cells; also, this antigen has an immunosuppressive role in the immune system.[10]

The purpose of this study was to produce a MAb against ManLAM antigen to be used for the evaluation of the immunological roles of ManLAM, purification of it in bacterium culture, and its detection in the TB patients' urine samples.

MATERIALS AND METHODS

Animal housing and surgical procedures were carried out in accordance with the Animal Care and Use Committee laws of Tarbiat Modares University (Tehran, Iran) to minimize the suffering of animals and the number of animals.

Calmette–Guerin strain of *Mycobacterium bovis* (BCG), myeloma cell line of SP2/0 origin, and BALB/c mice were purchased from the Pasture Institute of Iran. Polyethylene glycol (PEG; M_W 1500), RPMI 1640, streptomycin, penicillin, hypoxanthine–aminopterin–thymidine (HAT), hypoxanthine–aminopterin, bovine serum albumin (BSA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), peroxidase-labeled goat anti-mouse IgG (Fab specific), ManLAM antigen, and other chemicals were all purchased from Sigma Chemical Company (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Gibco (Grand Island, NY, USA). Also, 96-well plates and other plasticware were obtained from NUNC (Roskilde, Denmark).

Bacterial culture

The Calmette–Guerin strain of *M. bovis* BCG was grown at 37°C, 5% CO2 in broth media. Bacteria were collected by centrifugation (1000 g, 1 h), and cell pellets were 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 myeloma cells

Myeloma cells were cultured in the presence of 30 μg/ml 8-azaguanine to ensure their sensitivity to the HAT medium (hypoxanthine-aminopterin-thymidine) used as selection medium after cell fusion. A week before cell fusion, myeloma cells were grown in 8-azaguanine (5×10^5 myeloma cells were prepared per fusion). The HAT medium allowed only the fused cells to survive in culture.

Immunization and fusion of myeloma cells with immune spleen cells

Approximately 5 μg/ml of killed BCG was prepared for injection by emulsification with FIA. six female BALB/c mice (four-week-old) were intraperitoneally vaccinated with 0.5 ml of mixture of sonicated BCG (1 ml) vaccine prepared in phosphate-buffered saline (PBS; 10 μg/ml) and FIA (1 ml). As the final boost, the same doses of sonicated BCG vaccine were given 4 weeks later and then the sera from the mice were tested for IgG by enzyme-linked immunosorbent assay (ELISA) after 2 and 4 weeks. Finally, a mouse that exhibiting the highest antibody titers and has sensitivity to ManLAM was selected. Exactly 0.2 ml of ManLAM antigen (4 μg/ml) prepared in PBS was injected intravenously after 2 weeks, 5 days before fusion. The same dose of antigen was injected intravenously. The mouse was sacrificed 5 days after the final injection and the spleens from the immunized mice were removed and forced through a mesh screen (mesh size 50) to be used in hybridoma production. Spleen cells were fused with SP2/0 myeloma cells using PEG 1500 as the fusing agent, according to the method

of Kohler and Milstein,[11] and the cells were grown in HAT and HT (hypoxanthine-thymidine) 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-ManLAM reactivity by In-Directed ELISA and the positive ones were cloned by twice limiting dilution on the feeder layer in 96-well plates. Two cell line clones producing antibody against ManLAM antigen were established in one fusion. The immunoglobulin isotype was determined by isotyping the strip kit.

Enzyme-linked immunosorbent assay

Flat-bottomed 96-well polyvinyl chloride plates were coated with 100 ml of BCG (5 μg/ml) and incubated at 37°C overnight in carbonate buffer (pH 8.6). The plates were washed with PBS containing 0.05% tween 20 (PBS-T) and blocked with 1% BSA in PBS buffer (pH 7.5) at 37°C for 1 h. After washing, the plates incubated for 0.5 h at 37°C with antiserum of mice or supernatant of hybridoma cells. Finally, the plates washed as before and incubated with anti-mouse IgG horseradish peroxidase (HRP) conjugate for 1 h at 37°C. After washing, color was developed with 3,3',5,5'-tetramethyl benzidine (TMB) and stopped with 1 N HCl. The absorbance was determined at 450 nm. ELISA for ManLAM was performed as BCG.

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 0.5% stacking gels. After electroblotting on the nitrocellulose paper (NCP), the nonreactive 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. Gout 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 were visualized with hydrogen peroxide and 3,3'-diaminobenzidine (DAB).

Production of ascites containing mouse MAbs

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

Antigen purification by the affinity chromatography method

The purified MAbs from ascites fluids were coupled to cyanogen bromide–activated sepharose 4B (Pharmacia, Uppsala, Sweden) 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 then made to react 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. Exactly 106.5 ml of sonicated *M. bovis* was first dialyzed in the same buffer overnight and subsequently passed through the column at a flow rate of 0.5 ml/min. Afterward, this column was washed extensively with the Tris-HCl buffer until a stable baseline was obtained. The bound fraction was eluted with 3 M sodium thiocyanate, pooled, and dialyzed against 0.15 M phosphate buffer (pH 7.4), and the protein concentration was measured by the Bradford method and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Hybridizing and screening hybridoma clone for the MAb

After hybridization, the detection of hybrids was performed by ELISA which showed 13 wells with positive hybridoma clones. At the end of the first month , eight clones died and five clones secreted the antibody. After being cultured, detected, screened, and subcloned, five positive hybridoma clones (designated H13F33E11, H23D93G4, H13F32C9, H23D93F11, and H23D91G4), which could secrete MAbs against ManLAM antigen and BCG, were obtained. The H23D91G4 clone strongly reacted with ManLAM antigen in ELISA [Table 1].

Class and subclass identification of MAb against ManLAM antigen

The class and subclass of MAb that was secreted by H23D91G4 hybridoma was IgG_3.

Immunoblotting result

The result of sonicated *M. bovis* BCG and ManLAM antigen immunoblotting with IgG_3 MAb showed that this MAb recognized a single ManLAM component in *M. bovis* BCG and ManLAM antigen with a molecular weight of 35 kDa [Figure 1].

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 (against BCG)	OD at 450 nm (against ManLAM)	Clone	OD at 450 nm (against BCG)	OD at 450 nm (against ManLAM)	Clone	OD at 450 nm (against BCG)	OD at 450 nm (against ManLAM)
H1	2.15	2.31	H1E8	1.53	1.23	H13F32F3	1.95	2.07
H2	2.38	2.40	H13F3	2.72	2.40	H15F33E11	2.15	1.46
H3	0.35	0.18	H21D9	1.69	1.36	H23D91G4*	2.60	2.68
H4	1	0.13	H23D9	2.06	2.30	H23D9B12	2.57	2.61
H5	0.12	0.4	H43D5	0.5	0.7	H23D9C32	2.13	2.17
H6	0.84	0.78	H54C2	1.02	0.9	H23B9C40	2.40	2.59
NC (SP2/0)	0.27	0.24	NC (SP2/0)	0.31	0.29	NC (SP2/0)	0.23	0.24

*Selected clone for further experiments. OD: Optical density, NC: Negative control

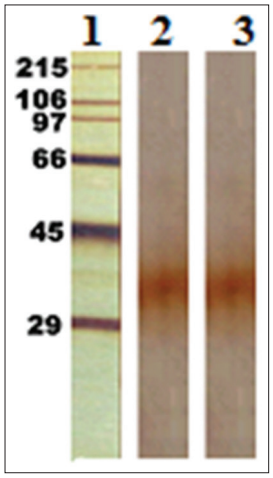

Figure 1: Immunoblotting of sonicated BCG (lane 2) and ManLAM (lane 3) using MAbs produced by H23D91G4 clone. These MAbs recognize a 35-kDa glycolipid component of ManLAM (lane 2). Protein molecular weight marker (lane 1)

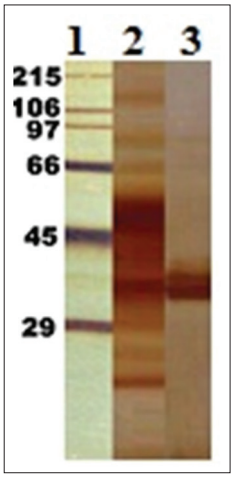

Figure 2: SDS-PAGE in a vertical slab gel. Protein molecular weight marker (lane 1). Sonicated *M. bovis* BCG (lane 2). Purified ManLAM using sepharose 6B column (lane 3)

Separation of antigen by solid-phase affinity chromatography

From 106.5 mg of *M. bovis* sonicate applied to the H23D91G4-sepharose 4B column, 1.37 mg (1.28%) was eluted with 3 M sodium thiocyanate. Staining of SDS-PAGE–fractionated sample (20 μg) revealed a 35-kDa band [Figure 2].

DISCUSSION

The only TB vaccine currently available is an attenuated strain of *M. bovis*, termed BCG, which has variable and limited efficacy in TB-endemic regions. LAM antigen, with a molecular weight of 17–37 kDa,[14,15] a major cell-surface component of Mtb, is composed of a mannan core linked to a linear arabinan chain to which oligoarabinosyl side chains are attached.[16] It was established that most of these side chains of the LAM from the virulent strain of Mtb are capped with either mono-, di-, or trimannosyl residues. This LAM is termed mannosylated and named ManLAM. This capping is missing from the LAM on AraLAM isolated from rapidly growing strains of mycobacteria.[17] Moreover, the reducing end of this AraLAM mannan core was found to linked to a phosphatidyl-*myo*-inositol anchor.[16]

It was reported that mannose-capped lipoarabinomannans (ManLAMs) from *M. bovis* BCG and Mtb inhibited interleukin (IL)-12 production by human dendritic cells. The inhibitory activity was abolished by the loss of the mannose caps.[18] So, it is supposed that the ManLAM of BCG might be one of the causes that lead to limited efficacy of the vaccine *in vivo*. It is of interest to explore whether any new inhibitor of ManLAM of BCG could improve immunogenicity of the BCG vaccine *in vivo*.[19,20]

In this study, we used 4-week-old mice because at this age, the immune system of mice is comple tely developed. In this study against others that use of composition of antigen and complete adjuvant in the first and second injection, we used of killed BCG vaccine and incomplete adjuvant, because of the complete adjuvant property of BCG, abundance of ManLAM on the surface of BCG vacci ne, and low immunogenicity of ManLAM alone. The disadvantages of this method are its high cost and time. Thus, in order to produce the antibody in a

concentration of milligrams, the first and second injections were administered intraperitoneally. In the next step, for the better stimulation of memory cells producing antibody against ManLAM, the mice were injected with purified ManLAM intravenously.

So far, several MAbs of IgM and IgG3 [5c11 (IgM), 4f11 (IgM), and 9d8 (IgG3)] against ManLAM antigens have been introduced.[17,21] However, cross-reactivity occurs with other mycobacteria that are not suitable for use in diagnosis. So, research is ongoing to find suitable clones.

Because of the glycolipidic structure of ManLAM, the results of SDS-PAGE in a vertical slab gel and immunobloting of sonicated BCG and ManLAM are smear like, while the bands in SDS-PAGE of proteins are clearly identified.

In the present study, we produced MAb (H23D91G4) that recognizes ManLAM with 35 kDa in immunoblotting. Our MAb reacted only with the 35-kDa fraction of LAMs, and this MAb is likely to be species specific of *M. bovis* and Mtb. As a future direction, this MAb can be used for purification of ManLAM antigen from bacterium culture used for antibody detection against ManLAM in TB patients' urine.

To conclude, according to our data, the ManLAM on the cell surface of *M. bovis* was identified by H23D91G4. MAb could be used to increase the immunogenicity of vaccine. Other studies focusing on the *in vivo* and *in vitro* immunosuppressive effects of purified antigen on the immune system are required to find the other applications of the purified antigen.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: Estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 1999;282:677-86.
2. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, *et al*. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 1994;271:698-702.
3. Fard RM, Moslemy M, Golshahi H. The history of modern biotechnology in Iran: A medical review. J Biotechnol Biomater 2013;3:1-5.
4. Glatman-Freedman A, Casadevall A. Serum therapy for tuberculosis revisited: Reappraisal of the role of antibody-mediated immunity against *Mycobacterium* tuberculosis. Clin Microbiol Rev 1998;11:514-32.
5. Guirado E, Amat I, Gil O, Díaz J, Arcos V, Caceres N, *et al*. Passive serum therapy with polyclonal antibodies against *Mycobacterium tuberculosis* protects against post-chemotherapy relapse of tuberculosis infection in SCID mice. Microbes Infect 2006;8:1252-9.
6. de Vallière S, Abate G, Blazevic A, Heuertz RM, Hoft DF. Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. Infect Immun 2005;73:6711-20.
7. Prinzis S, Chatterjee D, Brennan PJ. Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. J Gen Microbiol 1993;139:2649-58.
8. Venisse A, Berjeaud JM, Chaurand P, Gilleron M, Puzo G. Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG. Determination of molecular mass by laser desorption mass spectrometry. J Biol Chem 1993;268:12401-11.
9. Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, *et al*. The heparin-binding hemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. Nature 2001;412:190-4.
10. Torrelles JB, Sieling PA, Zhang N, Keen MA, McNeil MR, Belisle JT, *et al*. Isolation of a distinct *Mycobacterium tuberculosis* mannose-capped lipoarabinomannan isoform responsible for recognition by CD1b-restricted T cells. Glycobiology 2012;22:1118-27.
11. Hamasur B, Haile M, Pawlowski A, Schroder U, Kallenius G, Svenson SB. A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab') fragment prolong survival of mice infected with *Mycobacterium tuberculosis*. Clin Exp Immunol 2004;138:30-8.
12. Köhler G, Milstein C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur J Immunol 1976;6:511-9.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
14. Nigou J, Zelle-Rieser C, Gilleron M, Thurnher M, Puzo G. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: Evidence for a negative signal delivered through the mannose receptor. J Immunol 2001;166:7477-85.
15. Lawn SD, Frimpong EH, Nyarko E. Evalutoin of a commercial immunodiagnostic kit incorporating lipoarabinomannan in the serodiagnosis of pulmonary tuberculosis in Ghana. Trop Med Int Health 1997;2:978-81.
16. Schlesinger LS. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J Immunol 1993;150:2920-30.
17. Hamasur B, Källenius G, Svenson SB. A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. FEMS Immunol Med Microbiol 1999;24:11-7.
18. Venisse A, Rivière M, Vercauteren J, Puzo G. Structural analysis of the mannan region of lipoarabinomannan from Mycobacterium bovis BCG. Heterogeneity in phosphorylation state. J Biol Chem 1995;270:15012-21.
19. Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry 1978;15:429-36.
20. Briken V, Porcelli SA, Besra GS, Kremer L. Mycobacterial lipoarabinomannan and related lipoglycans: From biogenesis to modulation of the immune response. Mol Microbiol 2004;53:391-403.
21. Miller RA, Buchanan TM. Production and characterization of a murine monoclonal antibody recognizating a shared mycobacterial polysaccharide. Int J Lepr Other Mycobact Dis 1984;52:461-7.