Development of Mouse Monoclonal Antibody Anti-Ag85B Protein of *Mycobacterium tuberculosis*

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Abstract. One of the abundant biomarkers in tuberculosis (TB) infected human serum is the Ag85B antigen. The quest for protein biomarkers has been conducted to develop a diagnostic kit and vaccine for TB. Numerous studies have demonstrated that the development of monoclonal antibodies (mAbs) that recognize a specific repertoire of Mycobacterium tuberculosis antigens and the tests based on monoclonal antibodies have proved helpful in TB detection. Unfortunately, numerous mAbs currently used for TB serodiagnosis were generated elsewhere. Improving the ability to produce mouse monoclonal antibodies, specifically against Ag85B, is imperative as Indonesia is the second prevalent TB country. Therefore, a synthetic plasmid expressing Ag85B-HA fusion protein was expressed in the mammalian system. The expression of Ag85B protein in the 293T cells lysates was confirmed by western blot analysis. A specific protein band around 36 kDa appeared against antibody anti-HA. The lysate of recombinant mammalian cells was used as an inducer to generate B-cells expressing mouse monoclonal antibodies anti-Ag85B. By fusing 'PAI' mouse myeloma cells and B-cells from immunized mice, hybridoma mAb-producing cells were obtained. As many as 43 hybridomas cells were tested against recombinant Ag85B antigen by immunofluorescence assay (IFA). We found two (2) hybridomas cells specifically secreting monoclonal antibody anti-Ag85B. One hybridoma, 3C12 clone, was confirmed to have the ability to recognize Ag85B protein validated by western blot analysis. Similar to previous research, our western blot analysis shows that our monoclonal antibody can identify the antigen Ag85 at a protein size of around 30 kDa. Further analysis and characterization should be conducted to explore the potency of those monoclonal antibodies for developing TB diagnostic kits.

Keywords: monoclonal antibody · Ag85B · tuberculosis

1 Introduction

Tuberculosis is caused by Mycobacterium tuberculosis (Mtb), which is made up of pathogens that can live in both humans and animals [1]. Even though a lot of work has been done over the past 20 years to make new vaccines, drugs, and diagnostics,

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tuberculosis is still a global emergency. Nucleic acid–based amplification tests, imaging, and breath analysis of volatile organic compounds, show promise for better point-of-care rapid tests for tuberculosis [2]. These TB diagnosis methods are limited because expensive and may need help from donors to stay in place in low- and middle-income countries with high TB rates [3].

In 2009, SD Bioline invented the TB MPT64 in vitro diagnostic kit, which can recognize Mtb by detecting the mycobacterial protein 64 (MPT64) in a positive liquid culture. This test uses immunochromatography and lateral flow to identify MPT64 antigen by using a gold-labeled antibody. The SD Bioline TB MPT64 assay kit uses the difference in genomes to diagnose Mtb and Non-Mtb at a cheaper cost within 15 min, with easy-to-understand results. It's portable and available for primary health care [3].

The most common method is identifying Mtb's abundantly released protein in a growth medium. Several secreted proteins, including MPT64, the 10-kDa culture filtrate protein (CFP 10), and the antigen 85 (Ag85) complex, have been identified as potential early detection markers for tuberculosis. Antigen 85 (Ag85) complex is found in up to 30% of M. tuberculosis secreted proteins. It is compose of three similar proteins, Ag85A (32 kDa), Ag85B (30 kDa), and Ag85C (32.5 kDa), in a 2:3:1 ratio [4]. Boucau and colleagues demonstrated mycolyltransferase activity of Mtb as a screening approach with a colorimetric assay. The results showed that this assay is appropriate for high-throughput screening applications [5].

Previous studies attempted to produce monoclonal antibodies anti-Ag85, such as antibody anti-Ag85A [6] and anti-Ag85B-Hsp16.3 fusion protein [7]. However, none of those research used anti-Ag85B antibodies to establish a lateral flow test platform for detecting tuberculosis infection. In this study, we reported the development and partial characterization of monoclonal antibody anti-Ag85B generated from the mice immunized directly with the lysate of mammalian cells expressing Ag85B recombinant protein. This preliminary study was part of the project to develop a cheap and effective rapid diagnostic test for TB infection.

2 Materials and Methods

2.1 Ethical Statement

BALB/c mice were kept and handled in the animal facility of The Center for Pharmaceutical and Medical Technology (BPPT) in Indonesia. The Medical Faculty of University Indonesia Committee on the Ethics of Animal Experiments authorized the entire experiment protocol (Permit Number: KET-476/UN2.F1/ETIK/PPM.00.02/2019).

2.2 Cells

PAI myeloma cells and 293T cells were grown in RPMI 1640 (Gibco) medium with 10% FBS and 1% penicillin-streptomycin. They were maintained in an incubator at 37 °C with 5% CO_2.

2.3 Construction of Recombinant Plasmid PcDNA3.1-HA-Ag85B

The database sequence of Ag85B gene from a *Mycobacterium tuberculosis* Beijing strain was used as a template for synthesized and codon optimization. The genes were inserted into pcDNA3.1-HA and named as pcDNA3.1-HA-Ag85B (Fig. 1A). Digestion by *Hind*III and *Xba*I restriction enzymes was carried out to confirm the construct of the synthetic plasmid was purchased at Genescript.

2.4 Expression of Ag85B Recombinant Protein

Twenty μg of plasmid pcDNA3.1-HA-Ag85B was transfected into $3x10^6$ cells 293T cells followed by incubation at 37 °C with 5% CO_2 for 2 days. Cells were lysed in 200 μl of phosphate saline buffer pH 7.4 and used western blot analysis or for mice immunization.

2.5 Generating Hybridoma

Mouse monoclonal antibody prepared following the fusion hybridoma procedure (Köhler & Milstein, 1975). Two balb/c mice (4–6 weeks) were immunized with 200 μl of lysed transfected cells via the intraperitoneal route. Boosters were given two times every two weeks with the same dose. Splenocytes were collected from immunized mice one week after the last boosting, and the fusion was performed with mouse myeloma PAI cells. The fused cells were grown on a selective DMEM-HAT medium and screened the hybridomas by Immunofluorescence assay (IFA).

2.6 Immunofluorescence Assay (IFA)

The 293T cells were seeded in 96 well plates, and transfected on the next day with 0.5 μg/well of pcDNA3.1-HA-Ag85B, followed by incubation at 37 °C for 2 days. After transfection, cells were fixed with 3.7% formaldehyde followed by incubation with supernatant of hybridoma or commercial antibody anti-HA (ThermoScientific) as primary antibodies and goat anti-mouse IgG-Alexa Fluor 488 (ThermoScientific) as the secondary antibody. The plates were observed under a fluorescence microscope and green fluorescent cells indicate the interaction of mAbs with Ag85B protein.

2.7 Western Blot Analysis of Recombinant Protein Ag85B

Anti-HA commercial monoclonal antibody and hybridoma supernatant were used to detect the recombinant protein Ag85B using Western blot analysis. The 293T cell lysate was loaded into 10% acrylamide gels, and then transferred to PVDF membranes. The membranes were incubated at 4 °C for overnight, followed by a 1-h incubation with anti-mouse IgG antibody conjugated to IRdye-800 CW. The expression of the Ag85B was observed by western blot imager (LI-COR Odyssey Clx).

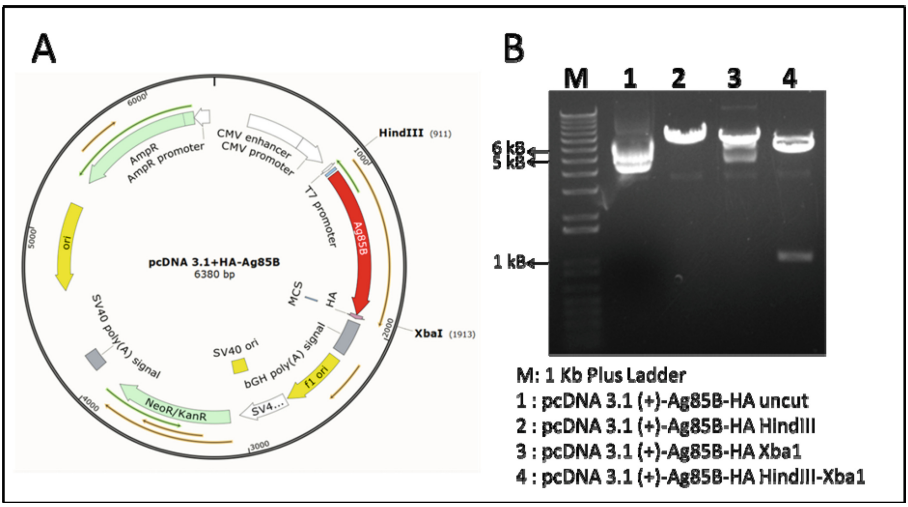

Fig. 1. Plasmid recombinant pcDNA3.1-HA-Ag85B. (A) Construct of the plasmid pcDNA3.1-HA and insert AG85B by *in silico* analysis. (B) Confirmation of plasmid recombinant by double digestion of restriction enzymes *HindIII* and *XbaI*.

2.8 SDS Page

The secreted monoclonal antibody from the hybridoma supernatant was analyzed using 10% gel acrylamide of SDS PAGE. As much as 100 ul of supernatant was mixed with 20 ul of 6X loading dye followed by boiling for 10 min. The mixture of supernantant and loading dye was loaded into acrylamide gels. The samples were run at a constant voltage of 130 V for 100 min. Membranes were stained with coomassie brilliant blue.

3 Results

3.1 Expression of Recombinant Plasmid PcDNA3.1-HA-Ag85B

The size of recombinant plasmid pcDNA3.1-HA-Ag85B is 6380 bp (Fig. 1A). Double digestion with *Hind*III and *Xba*I of the recombinant plasmid was predicted to produce 2 parts, the backbone pcDNA3.1-HA and insert gene Ag85B, respectively. Analysis of agarose gel showed that the plasmid recombinant was digested completely into 2 bands with a size of 5.4 kb, which corresponds to pcDNA3.1-HA, and another band with a size around 1 kb for insert gene Ag85B (Fig. 1B).

Monoclonal antibody anti-HA confirmed the expression of Ag85B recombinant protein in both IFA and western blot analysis. The transfected 293T cells showed strong fluorescence compared (Fig. 2A) to non-transfected cells (Fig. 2B). Snapgene software was used to predict the size of recombinant Ag85B protein in pcDNA3.1-HA. A clear band around 36 kDa was obtained against the anti-HA monoclonal antibody on the PVDF membrane (Fig. 2C). This result is similar to the prediction analysis.

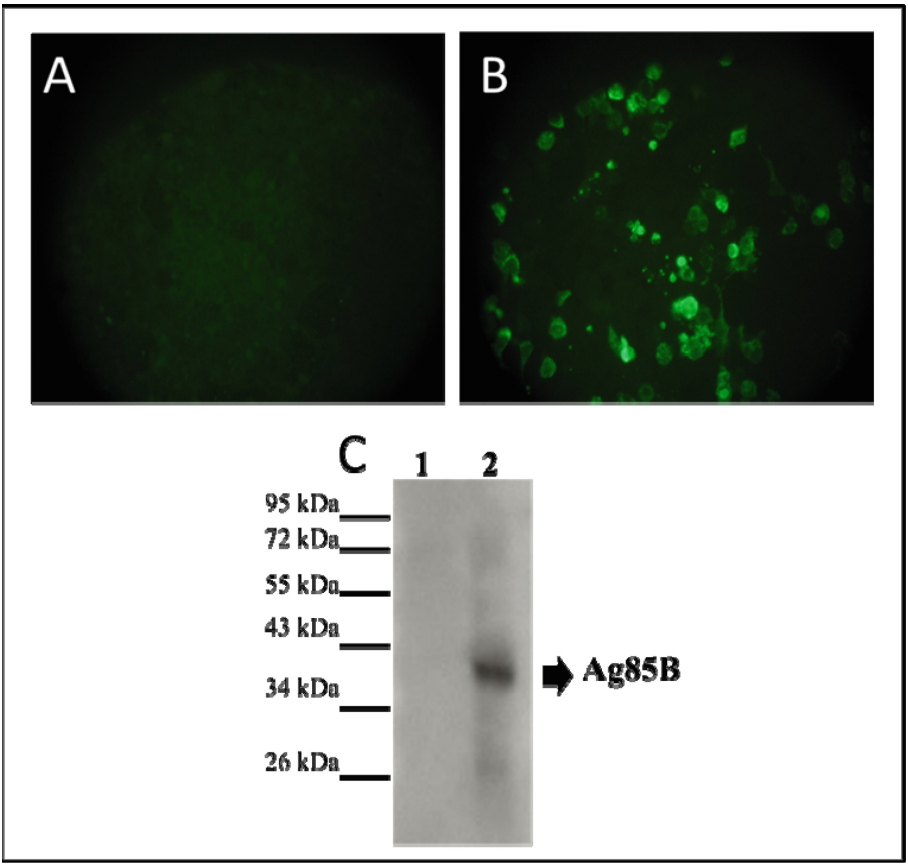

Fig. 2. Expression of recombinant Ag85B protein. (A) 293T cells (mock cells) without plasmid recombinant (B) Transfected 293T cells detected in IFA with anti-HA monoclonal antibody (C) Western blot analysis of lysate of transfected cells, detected with anti-HA monoclonal antibody.

3.2 Screening of Hybridoma of Anti-Ag85B Monoclonal Antibody

After fusion, the hybridomas are cultivated with selective HAT media for 5–6 weeks. Around 43 hybridoma clones were determined for further screening of the monoclonal antibodies specific to Ag85B protein. Supernatants from the selected hybridomas were used as the first antibody to detect the recombinant protein from transfected 293T cells in an immunofluorescence assay. Two clones showed reactivity to transfected cells compared to non-transfected cells as negative control (Fig. 3A). By western blot analysis, one clone, 3C12, was further analyzed to evaluate its ability to recognize the Ag85B protein. A band around 30 kDa appeared from the lysate of 293T transfected cells with pcDNA3.1-HA-Ag85B (Fig. 3B).

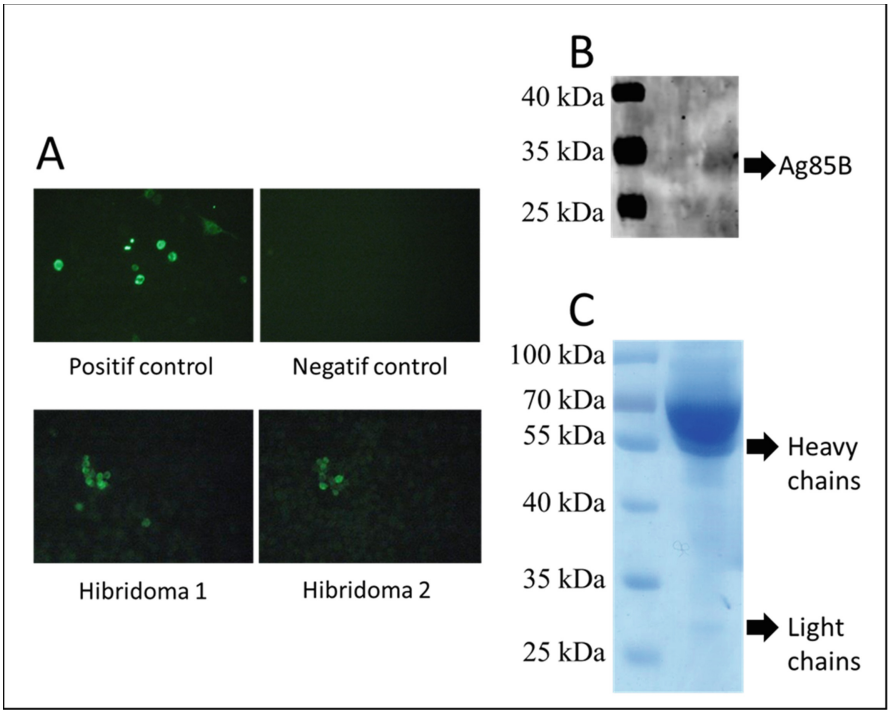

Fig. 3. Screening hibridomas anti-Ag85B. (A) In the first screening of hybridomas, 2 hybridomas could detect Ag85B in 293T transfected cells. (B) Clonal 3C12 recognizes Ag85B protein from the lysate of transfected cells. (C) Two molecules represented as heavy and light chains of monoclonal antibody were appeared from the supernatant of 3C12 clonal.

3.3 Monoclonal Antibody Anti-Ag85B in a Hybridoma Supernatant

The monoclonal antibody anti-Ag85B in the hybridoma supernatant culture was verified using SDS PAGE analysis. Two bands correspond to heavy chains (~50 kDa), and light chains (~25 kDa) of antibody monoclonal appeared clearly from the supernatant of clone 3C12 (Fig. 3C).

4 Discussion

Secreted proteins from Mycobacterium tuberculosis could be used to diagnose both active tuberculosis and latent tuberculosis infection [8]. The serodiagnostic capability of the antigens, which include 38kD, 16kD, ESAT6, MTB48, MTB81, and Ag85B, has been investigated [9–12]. Mycobacterium tuberculosis secretes the most abundant Ag85 complex secretory protein, Ag85B, a useful diagnostic and immunoprotective tool [4, 7]. Prior research in the pathological diagnosis of tuberculosis, immunohistochemistry (IHC) detection of Ag85B has shown to be a straightforward approach with higher sensitivity than Ziehl-Neelsen staining [13]. Recombinant protein can also be employed in the clinic for serodiagnosis, as demonstrated by the ability of human antibodies to

identify Ag85B-Hsp16.3 fusion protein. However, in healthy people vaccinated with the BCG, these antigens may share antigenic epitopes; hence, detection of antibody responses may be concealed [7].

The Ag85B antigens of Mtb are absent in healthy individuals, hence mAbs that recognize a specific repertoire may boost positive detection and reduce false-positive results [7]. In this study, we set up an experiment to generate mouse monoclonal anti-Ag85B. We expressed Ag85B recombinant protein in mammalian 293T cells and used the lysate cell as an inducer to raise the specific antibody against the antigen in Balb/c mice. These cells have several advantages over other host expression systems. Efficiently transfected with commercial transfection reagents, able to express large quantities of recombinant proteins, and capable of post-translation modification of compatible recombinant and bioactive proteins with humans are several advantages of the mammalian expression system [14].

After fusion, we recovered two out of 43 hybridomas with reactivity against recombinant Ag85B protein by IFA (Fig. 3A) and western blot (Fig. 3B). Immunofluorescence assay was employed to evaluate the functionality of the monoclonal antibodies since this assay could evaluate the native form of the target antigen in situ [15]. One of the most commonly used techniques in cell and molecular biology is western blotting. Researchers can identify individual proteins from a complicated mixture of proteins isolated from cells using a western blot technique. Separation based on size, transfer to a solid substrate, and tagging the target protein with the appropriate primary and secondary antibody are the three components of this approach [16]. Similar to previous research, our western blot analysis shows that our monoclonal antibody can identify the antigen Ag85 at a protein size of roughly 30 kDa [7].

5 Conclusion

Using hybridoma technology, two monoclonal antibodies specific to MTb Ag85B antigen have been obtained. The antibodies recognized the Ag85B antigen expressed in mammalian cell cultures in both the immunofluorescence assay and western blot analysis. These findings make our antibodies a potential biological material that could be used in developing a TB detection system.

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