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ORIGINAL ARTICLE

Immunogenicity of mannan derived from *Mycobacterium bovis* as a promising adjuvant in vaccine BCG

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ABSTRACT

Background and Objectives: Lipoarabinomannan is one of the components of the significant structural cell surfaces of mycobacteria and serves as an immunostimulatory factor. TNF-α and IL-12 are two examples of the anti-bacterial inflammatory cytokines that are activated and induced during infection.

Materials and Methods: In this study, mannan was extracted and processed, and then Bulb/c female mice were used in three groups, one group was given BCG vaccine, the other group was given BCG vaccine with mannan adjuvant, and a non-injected group was used as a control group. Inflammatory factors interleukin-12, TNF-α, IgG and IgM were measured in mouse serum.

Results: The levels of the inflammatory factors interleukin-12 and TNF-α in the serum isolated from mice receiving the BCG vaccine with mannan adjuvant showed a significant difference compared to the group that received only the BCG vaccine and the control group [IL-12] and , with P≤0.05.The examination of the level of IgG immune factors in these three groups revealed a significant difference. The group that received the BCG vaccine with mannan adjuvant showed a marked contrast compared to the group that received only the BCG vaccine and the control group, with P≤0.05. The level of IgM was higher in the group that received the BCG vaccine alone compared to the adjuvant vaccine group and the control group, with P≤0.05.

Conclusion: Our results indicated that mice receiving the BCG vaccine with mannan adjuvant had significantly higher serum levels of IL-12, TNF-α, and IgG than the group receiving BCG alone.

Keywords: *Mycobacterium bovis*; Adjuvant; Mannan; Immunity

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INTRODUCTION

Tuberculosis (TB) is an infection caused by bacteria. Although it usually affects the lungs, this illness can also impact other body organs (1). Tuberculosis progresses slowly in the body and its effects last for months. Tuberculosis bacteria remain inactive in the body of most people throughout their lives. In other people (for example, those with weakened immune systems), the bacteria may become active and cause tuberculosis. Because the TB vaccine is a weakened form of the bacteria (microbe) and leads to a mild disease, it will not cause TB. However, it will help the children to develop resistance (immunity) to it if they are exposed. Children and infants who receive the TB vaccine are especially well-protected against severe and uncommon forms of the disease, such as TB meningitis. Vaccination aims to produce both protective immunity and a long-lasting immune response against infections and pathogens (2, 3). Yeast, bacteria, and plants all produce mannan, a polysaccharide that can trigger opsonization and phagocytosis. Moreover, it stimulates the generation of lymphocytes and IL-1. Mannans functions as an immunostimulatory agent that can activate bone marrow dendritic cells in vitro, resulting in the release of inflammatory cytokines like TNF-α and IL-1β. It has also been demonstrated that manan causes the production of IFNγ, IL-2, and IL-12, which are signs of a Th1 response (4). Antibodies such as IgA, IgG1, and IgG2a are generated in the serum, along with IgA in various mucosal areas like saliva, tear ducts, lungs, and vagina. The glycoprotein mangan consists of mannose units linked to a protein, serving as an emulsifier with a structure resembling naturally occurring amphiphilic molecules. Adjuvants are commonly given alongside whole dead or subunit vaccines to enhance immunity (5). When choosing an adjuvant, it is crucial to take into account the particular antigen, the targeted disease, the required type of immune response, and the duration of the immune response. Adjuvants are employed to augment the immune response to vaccination antigens by heightening their immunogenicity. They effectively enhance the potency and durability of the immune response, as well as enhance the affinity between antibodies and antigens (6). Both individuals of varying ages can reap the benefits of an adjuvant's capacity to enhance cellular immunity and amplify the immune response. Consequently, through the utilization of a reduced amount of antigen in the vaccine composition, cost savings can be achieved. Mannan lectin receptors and other members of the C-type lectin family associated with the mannose receptor bind to mannan, leading to the initiation of complement activation, opsonization, and phagocytosis. Mannan also triggers the generation of IL-1, caspase I activation, and the formation of inflammatory pores. Given that C-type lectin receptors facilitate endocytosis, mannan and its derivatives have been employed as vaccine adjuvants to direct antigens towards antigen-presenting cells (APC) (7, 8). The objective of this research was to evaluate the immunogenic properties of mannan extracted from *Mycobacterium bovis* as a potential adjuvant in BCG vaccine in Bulb/c mice.

MATERIALS AND METHODS

Manan extraction. After cultivating the B. B. bacillus, the separation process of isolating the B. B. bacillus from the Suton culture medium (via filtration) is employed to prepare the BCG cake using Birko. In this research, the prepared biomass of *Mycobacterium bovis*, commonly known as BCG cake, was obtained in a ready form from the BCG production department of the Institut Pasteur Research Production Complex in Iran. Mycobacterium mannan was isolated and purified through a series of steps. Initially, lipids were extracted using a chloroform-methanol ratio of 1:2. Subsequently, for LAM/LM purification, cells were resuspended in deionized water and underwent centrifugation twice at 6760 rpm for 20 minutes at 25°C. The biomass was then separated from the supernatant in an 18,000 rpm centrifuge at 25°C for 1 hour. Contaminants such as glucans, proteins, DNA, and RNA were eliminated through enzymatic digestion using α-amylase, trypsin, DNase 1, and RNase treatments, followed by dialysis. Next, 90% phenol was added to the liquid containing lipoglycan, and the mixture was incubated with shaking at 68°C for 1 hour. The aqueous phase was separated from the phenol layer by low-speed centrifugation, and the phenolic phase was extracted again with an equal volume of water. The two aqueous extracts were combined, and after extraction with chloroform, the residual phenol was eliminated. The resulting lipoglycans (LAM/LM) were resuspended in a buffer of 0.2 M NaCl, 0.25% sodium deoxycholate (w/v), 1 mM EDTA, and 10 mM Tris, pH 8.0, followed by purifica-

tion through a Sephadex G75 column.

Blood sampling and serum separation. Twenty-four Balb/c female mice (3 weeks) were selected and divided into three groups of eight. One group was given the BCG vaccine together with Mannan, another group was given the BCG vaccine alone, and the third group was considered as a control. Twenty-eight days after the initial injection blood samples were taken from all groups. Serum samples were separated from blood samples and after centrifugation at 3000 rpm, the level of IgG and IgM antibodies, as well as the level of IL-12 and TNF-α, were analyzed in different groups.

Determiation of serum IgG, IgM level. The IgG and IgM mouse ELISA kit, manufactured by Abcam in the United Kingdom, is a laboratory technique used to quantitatively measure mouse IgG in serum, plasma, and supernatant from cell cultures. This assay utilizes plate strips coated with a specific antibody that binds to mouse IgG. Standards and samples are then added to the wells, allowing the IgG in the sample to bind to the immobilized antibody. The wells are subsequently washed, and an HRP-conjugated anti-mouse IgG detector antibody is introduced. After removing any unbound detector antibody, a TMB substrate solution is added to the wells, resulting in the development of a color that is directly proportional to the amount of IgG bound. The intensity of the developing blue color is measured at 600 nm. Alternatively, the reaction can be halted using the Stop Solution, which changes the color from blue to yellow. The intensity of the yellow color can then be measured at 450 nm and reported as Pg/ml.

Determination of serum IL-12 level. The Mouse IL-12 Solid Phase Sandwich ELISA kit from Thermo Fisher Scientific in Poland utilizes a pair of matched antibodies to quantify the target of interest. Initially, a target-specific antibody is immobilized in the microplate wells provided. Subsequently, samples, standards, or controls are introduced into these wells and bind to the immobilized antibody. A second antibody (detector) is then added to form a sandwich, followed by the addition of a substrate solution. This solution reacts with the enzyme-antibody-target complex, generating a detectable signal. The intensity of this signal, measured at a wavelength of 450 nm, is directly correlated with the concentration of the target in the original sample and is expressed as Pg/ml.

Determination of serum TNF-α level. The Mouse TNFα solid-phase sandwich ELISA kit from Thermo Fisher Scientific in Poland utilizes a matched antibody pair to quantify the target of interest. Initially, a target-specific antibody is coated onto the microplate wells. Following this, samples, standards, or controls are introduced into the wells and bind to the immobilized antibody. The sandwich structure is completed by the addition of a second detector antibody. Subsequently, a substrate solution is introduced, which interacts with the enzyme-antibody-target complex to generate a detectable signal. The strength of this signal is directly correlated with the concentration of the target in the original specimen and is reported in Pg/ml.

SDS-PAGE. A volume of 10 µL of 3× sample buffer (SB) was introduced to 19 µL of the sample intended for analysis by SDS-PAGE. The composition of the sample buffer consisted of 140 µl 12% SDS, 65 µl 3 M DTT, 300 µl H_2O, and 3765 µl 7× sample buffer, which was prepared by combining 7 mL 1 M Tris pH 6.8, 8.3 mL 89% Glycerol, 4 mg Coomassie Brilliant Blue G, and 3.1 mL H_2O. Subsequently, the samples were subjected to boiling at a temperature of 90°C for a duration of 5 minutes, unless otherwise specified. For electrophoresis, a 20-µL portion of each sample was loaded onto SDS-PAGE gels with a thickness of 0.75 mm and containing 15% acrylamide. The gels were then run at a voltage range of 80-100 V.

Statistical method. Statistical analyses were conducted utilizing GraphPad Prism statistical software version 8 (GraphPad Software, San Diego, CA, USA). Descriptive analysis was presented as mean ± standard deviation or median. The normality of the variables was assessed using the Shapiro-Wilk test. Student's t-test was employed to compare variables with normal distribution between two groups, while the Mann-Whitney U test was used for variables without normal distribution. A significance level of $P < 0.05$ was deemed statistically significant.

RESULTS

Checking the impurity of purified manan protein. To ensure the purification step of mannan, 12% polyacrylamide gel was used. Since the structure of man-

nan is a polysaccharide and under normal conditions electrophoresis is used to separate proteins, no band should be observed in the wells of purified and standard mannan samples. Therefore, two standard mannan samples (Sigma-Aldrich) and mannan extracted from *Mycobacterium bovis* strain and a 20kD protein molecule sample (Sigma-Aldrich) were investigated. The results of gel electrophoresis show that the standard sample of mannan (well 1) and the extracted mannan (well 2) do not contain protein (Fig. 1).

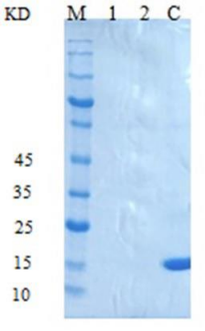

Fig. 1. Examination of the purity of purified mannan in terms of the presence of protein on a 12% SDS-PAGE gel. Standard mannan sample (1), extracted mannan (2) and control sample (C) and molecular weight marker (M).

Investigating the effect of manan on IgG and IgM levels. The animals that received the vaccine together with mannan exhibited a significant amount of IgG in their blood serum (61.58 ± 5.89 pg/ml) compared to the mice that received the vaccine alone and the control group (36.51 ± 4.57 vs. 7.68 ± 2.12 pg/ml) (P=0.01, Fig. 2A). Additionally, the level of IgM in the blood serum of the mice that received the BCG vaccine showed a significant difference compared to the groups receiving the vaccine with mannan and the control group (1.62 ± 0.57 vs. 1.38 ± 0.12 vs. 0.98 ± 0.15; P=0.02, Fig. 2B).

Investigating the effect of manan on the amount of cytokines. The amount of IL-12 and TNF-α in the serum of mice receiving mannan + BCG vaccine compared to BCG vaccine was significantly higher, according to the results of measuring the amount of various cytokines on the serum collected from the test-subject mice. The values for TNF-α levels (208 ± 9.40 for BCG plus mannan vs. 146.9 ±15.83 pg/ml for BCG vaccine alone; P=0.03) and IL-12 levels (195.4 ± 9.86 pg/ml vs. 145 ± 4.57 pg/ml) were statistically significant (P=0.02) (Fig. 3).

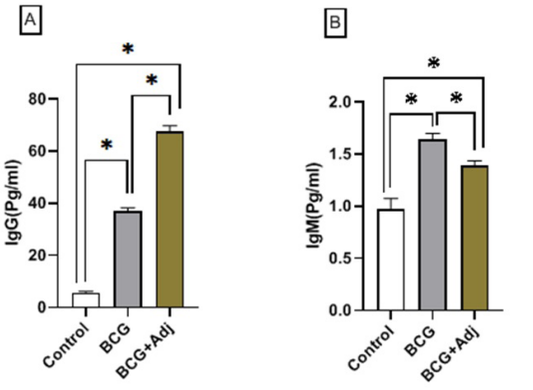

Fig. 2. IgG (A) and IgM (B) concentrations in blood serum in mice following injection of BCG vaccine alone, and mannan (Adj) + BCG vaccine, in comparison with the control group. Statistically significant differences were expressed as * for $P \leq 0.05$.

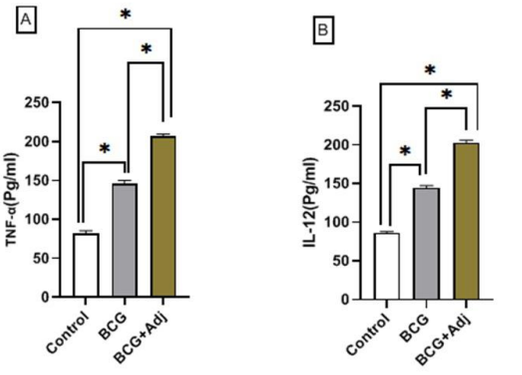

Fig. 3. The diagram of TNF-α (A) and IL-12 (B) in serum samples taken from mice shows a significant difference in the level of IL-12 and TNF-α in the group receiving BCG vaccine with manan adjuvant. Statistically significant differences were expressed as * for $P \leq 0.05$.

DISCUSSION

Immunological adjuvants are substances that, by lengthening and modulating the immune response memory, increase and amplify an antigen's ability to elicit an immune response. Adjuvants approved for use in human vaccines possess inherent instability under *in vivo* conditions and necessitate multiple vaccine injections (9-11). The dominance of alum as the primary adjuvant can be attributed, in part, to the limited availability of resources and funding for adjuvant development. Conversely, adjuvants

such as MF59, which are oil emulsions, have been sparingly employed exclusively in Europe (12). The high safety of alum makes it necessary, in accordance with regulatory standards, to demonstrate the safety and tolerance of novel adjuvants in addition to their efficacy. As a result, the regulatory obstacles are greatly diminished when known safe and acceptable chemicals are used (13, 14). Carbohydrates generally exhibit excellent biocompatibility and low toxicity, unless under extraordinary conditions. They play a vital role in signaling within the body's immune system. These substances are efficiently metabolized or excreted (15). The likelihood of producing toxic metabolites or long-lasting tissue deposits with aluminum salts, a disease known as macrophagic myofascial, is also almost eliminated by the use of carbohydrate adjuvants. Utilizing carbohydrates as an adjuvant has this problem as one of its benefits (16, 17).

Mannan is one of the carbohydrates that has drawn a lot of interest because of its beneficial qualities. A bioactive and biodegradable polysaccharide, this carbohydrate is made by yeast, bacteria, and plants. Mannan and its derivatives offer a lot of potential for application in drug delivery systems due to a variety of features, including biocompatibility, non-toxicity, solubility, potential variability, and intrinsic biological activity (18, 19). The four distinct types of mannan found in nature each feature a skeleton made of (20, 21) connected mannoses (linear mannan) or a mixture of glucose and mannose (glucomannan), with occasionally linked galactose residues as side chains. Mannose and glucose residues in this polymer's primary structure are occasionally acetylated at carbons 2 or 3 to form (1, 4) (galactomannan/galactoglucomanan).

Mycobacteria have identified three distinct lipoarabinomannan (LAM) families. LAMs initiate several signaling pathways that regulate apoptosis and IL-12 production in macrophages and dendritic cells. ManLAM, in particular, can hinder the activation of these pathways induced by agonists such as lipopolysaccharide (LPS) or bacterial infection, as it does not induce IL-12 production and apoptosis. Despite being relatively low in immunogenicity, the cell wall peptidoglycan or lipopolysaccharide of Gram-negative bacteria enhances the immune response against co-distributed antigens. The adjuvant activity of these components is modulated by toll-like receptor (TLR) activation, which triggers danger signals in the host's defense system (22). Usually, LAM is an anti-inflammatory substance, whereas LM is a pro-inflammatory substance. However, LAM molecules conjugated to a phosphatidyl-inositol moiety induce inflammation and increase IgG and IgM levels. Additionally, it has been discovered that, depending on the strain, LM isolated from various mycobacterium strains might have an immunogenic or immunosuppressive impact. Additionally, LM from the Tokyo-12 strain of *Mycobacterium bovis* BCG has immunomodulatory activities and promotes the development of Th1 cells, an immune response to intracellular pathogens (23). The investigation in our study focused on exploring the immunological characteristics of mannan as an adjuvant in the BCG vaccine. The findings from our research reveal a noteworthy increase in the IgG level within the blood serum of mice who were administered the vaccine containing mannan, in comparison to those who received the BCG vaccine alone. However, the IgM level was lower in the group injected with the adjuvant vaccine than in the group receiving the vaccine alone. In appearance, in line with previous research, the increase in IgG is related to the antibodies produced against LAM/ManLAM (24). Furthermore, by analyzing the cytokine levels in the serum obtained from the mice under examination, a notable rise in the levels of IL-12 and TNF-α was observed in the group that received the mannan + BCG vaccine. The adjuvant present in the BCG vaccine plays a role in stimulating the expression of IL-12. Moreover, the presence of mannan in dendritic cells and macrophages triggers the CD4+ lymphocytes to express and secrete IFN-α through its interaction with TLR. Additionally, it induces the expression of IL-12 and TNF-α in CD8+ cells, causing both cell types to differentiate towards the Th1 side (25).

CONCLUSION

The use of mannan in the formulation of the BCG vaccine generally increases the stimulation of the humoral and cellular immune system, and as a result, lower doses were used using the aforementioned formulation. The bacilli could still offer a vaccine as effective as the existing one, yet safer with fewer side effects. By conducting tests and studies, a final decision can be reached regarding the optimal quantities of live mass and mannan consumption.

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