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Mannose-binding lectin enhances the attachment and phagocytosis of mycobacteria *in vitro*

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

Agnieszka Bonar^{BCF}, Magdalena Chmiela^{AD E}, Wiesława Rudnicka^{DG}
and Barbara Różalska^{ADEF}

Department of Immunology and Infectious Biology, Institute of Microbiology and Immunology, University of Łódź, Poland

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Summary

Introduction:

Phagocytosis is the critical first step in the *Mycobacterium (M.) tuberculosis*-phagocyte interaction. The process involves microbial ligands and phagocyte surface receptors. It is known that serum mannose-binding lectin (MBL), an innate immune system component, may enhance the uptake of microbes by phagocytic cells and activate the complement system. Since phagocytes are the replicative environment for mycobacteria and, as we described earlier, tuberculosis patients differ from controls in serum MBL level, we asked whether MBL plays a role in promoting *M. tuberculosis* access to phagocytic cells.

Materials and Methods:

To estimate the influence of MBL on the phagocytic process, FITC-labeled *Mycobacterium bovis* BCG was used as a model bacterium. Neutrophils from healthy individuals were used as phagocytes. Phagocytosis was performed in the presence or absence of recombinant MBL (rMBL; 2 or 20 µg/ml). The activation of complement was determined by dot-blot immune assay with monoclonal antibodies against C5b-C9.

Results:

We showed that phagocytosis of the bacteria was more intensive in the presence of human rMBL. Both attachment and ingestion of mycobacteria were enhanced when MBL and active complement components (fresh serum) were present in the medium. The dot-blot method showed that the bacteria slightly activated complement by themselves. This effect was enhanced in the phagocyte-bacteria co-cultures containing rMBL.

Conclusion:

It is possible that MBL may serve *in vivo* as one of the factors facilitating the entry of mycobacteria into phagocytes, pathogen spread, and the establishment of infection.

Key words:

tuberculosis • mycobacteria • phagocytes • collectins • mannose-binding lectin

Abbreviations:

TB – tuberculosis, **MBL** – mannose-binding lectin, **PMN** – polymorphonuclear leukocyte (neutrophils), **BSA** – bovine serum albumin, **FCS** – fetal calf serum, **FITC** – fluorescein isothiocyanate, **PBS** – phosphate-buffered saline.

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Author's address:

Prof. Barbara Różalska, Department of Immunology and Infectious Biology, University of Łódź, Banacha 12, 90-237 Łódź, Poland, fax: +48 42 665 58 18, e-mail: rozab@biol.uni.lodz.pl

INTRODUCTION

As with many other bacteria, the interaction of *Mycobacterium (M.) tuberculosis* with host immune mechanisms (innate and adaptive) determines outcome after infection^{4, 24}. *M. tuberculosis* has developed mechanisms to escape immune surveillance at many stages in the host response. This facultative intracellular pathogen may impair the recognition that the bacteria has invaded phagocytic cells, both macrophages and neutrophils^{18, 24}. Multiple host-pathogen interactions allow mycobacteria to enter phagocytes. This process is mainly mediated by the complement receptors (CRs) CR1, CR3, and CR4²². CR1 (CD35) recognizes C3b- and C4b-coated particles, whereas CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which are members of the β_2 integrin family, primarily recognize C3bi-coated particles and a variety of microbial ligands as well as other glycoproteins^{6, 7, 24}. The phagocytosis of various bacteria, including mycobacteria, may also depend on the interaction between phagocyte mannose receptors and mannose-binding lectin (MBL), recognized as an opsonin and complement-activating molecule. MBL belongs to the family of proteins involved in the innate immune system called collectins or defense collagens^{13, 14, 20}. MBL is secreted into the blood and other body fluids and is mainly produced by the liver, although the recent demonstration of MBL expression on mucosal surfaces suggests that this lectin has a general function in innate immunity^{2, 8, 10}. It is known that MBL binds to the surface of various bacteria, including mycobacteria, promoting their uptake and eradication¹⁶. Moreover, MBL, and its associated serine proteases (MASPs), can activate both the classical and alternative complement pathways^{21, 22, 24}. Thus, MBL-mediated activation of complement in this fashion may also result in C3 opsonization of bacteria^{16, 25}. MBL is also known as a molecule which modulates the degree of tissue inflammation²⁵. Since phagocytes are the replicative environment for mycobacteria and, as we described earlier, tuberculosis (TB) patients differ from controls in serum MBL level³, we asked whether mycobacteria binding to and their ingestion by polymorphonuclear leukocytes (PMNs) could be related to the high concentration of MBL.

MATERIALS AND METHODS

Labeling of bacteria

Mycobacterium (M.) bovis BCG bacilli were grown for 14–21 days in Middlebrook 7H9 broth (Difco) + ADC (Difco) + 0.05% Tween 80. The bacilli were heat killed (120°C for 20 min) and labeled with fluo-

rescein isothiocyanate (FITC; Sigma, St. Louis, USA). Briefly, the bacilli (1×10^9 /ml) were suspended in phosphate-buffered saline (PBS) containing FITC (100 μ g/ml), vortexed for 30 s, sonicated for 15 s, and kept for 18 h at room temperature, with agitation, in the dark. After washing with PBS + 4% bovine serum albumin (BSA), the bacilli were suspended in RPMI-1640 medium with 20% fetal calf serum. The labeled bacilli could be stored at 4°C for 3 weeks without loss of fluorescence.

Human PMNs

PMNs were isolated from the peripheral blood of healthy donors and separated by centrifugation on GradiSol G (Aqua-Medica). The cells were washed and suspended in RPMI-1640 medium with 10% autologous plasma, non-inactivated or heat-inactivated.

Phagocytosis assay

Aliquots of 100 μ l of the FITC labeled bacterial suspensions (1×10^8 cells/ml) were mixed in triplicate on microplates with 100- μ l aliquots of the neutrophil suspension (1×10^6 cells/ml), prepared in 1 ml of RPMI-1640 containing 10% autologous serum without MBL, supplemented or not with 2 or 20 μ g of human recombinant MBL (rMBL; kindly donated by Dr. A. Ezekowitz, Harvard Medical School, Boston, USA). The cultures were incubated for 1 h at 37°C in 5% CO₂, the phagocytosis was stopped on ice (5 min), and the cells were centrifuged (300 $\times$ g, 5 min, 4°C). The fluorescence was measured in a Wallac 1420 counter VICTOR² (Oy Turku, Finland) at 485 nm excitation and 530 nm emission wavelengths and expressed as relative fluorescence units (RFU). The fluorescence of attached but not ingested bacilli was quenched with 0.1% trypan blue in PBS (100 μ l/well), the dye was exchanged with PBS, and the fluorescence was measured as above.

Detection of C5b-C9 complement complex

Activation of complement during phagocytosis was estimated by the immune dot-blot method using monoclonal antibodies against C5b-C9 complexes (Dako, Denmark). Two microliters of post-phagocytosis supernatants were dropped 3-fold on a BA 85 membrane (0.45 μ m; Schleicher and Schuel, Germany). Unbound membrane sites were blocked for 2 h at room temperature with 2% BSA in 50 mM Tris-HCl/200 mM NaCl, pH 7.4 (Tris-HCl/NaCl/BSA), and then washed with the same buffer without BSA (Tris-HCl/NaCl). The membranes were incubated for 18 h at room temperature with mouse monoclonal antibodies to C5b-C9 complexes diluted

1:40 in Tris-HCl/NaCl/BSA. After washing with Tris-HCl/NaCl containing 0.5% Tween 80, rabbit antibodies to mouse immunoglobulins conjugated with horseradish peroxidase (diluted 1:1000 in Tris-HCl/NaCl/BSA) were added to the membranes for 2 h and kept at room temperature. Next the membranes were washed and the color reaction was developed by adding 100 μ l of substrate for peroxidase: 4-chloro-1-naphtol (1 ml of 3 mg/ml in 5 ml of Tris-HCl/NaCl/BSA) and 30% H_2O_2 (0.5 μ l/ml). The color reaction was stopped after 20 min with H_2O . A set of appropriate controls excluding unspecific reactions was included in the study protocol. The intensity of color reaction was estimated semi-quantitatively (-/+).

Statistics

Calculations were performed using Statistica 5.5 PL (Statsoft). Student's *t*-test was applied to reveal significant differences in the groups of results. A value of $p < 0.05$ was accepted as the level of significance.

RESULTS

The high serum levels of MBL in most of the patients with TB, demonstrated in our earlier report³, prompted us to ask whether and how MBL influences the mycobacterium/phagocyte interaction. Phagocytosis was estimated by fluorescence assay with a model bacterium, FITC-labeled *M. bovis* BCG, and PMNs from healthy individuals as phagocytes. The intensity of binding and the ingestion of *M. bovis* BCG bacilli by PMNs were observed in medium with 10% autologous serum containing 2 or 20 μ g/ml of rMBL. We showed that phagocytosis (attachment + ingestion) of the bacteria was more intensive, in a dose-dependent manner, in the presence of rMBL (2 or 20 μ g/ml) and complement (non-inactivated serum; Fig. 1). The intensity of phagocytosis of *M. bovis* in medium without MBL, expressed as RFU, was lower (343 ± 43) than in medium containing 2 or 20 μ g/ml of rMBL, i.e. 662 ± 48 and 755 ± 74 , respectively (the differences were statistically significant: $p = 0.008$ and $p = 0.0009$). Both attachment and ingestion of the mycobacteria were enhanced. In the presence of 20 μ g/ml of rMBL, the increase in fluorescence units from 104 ± 65 to 412 ± 69 (attachment) and from 216 ± 33 to 342 ± 93 (ingestion) was significant ($p = 0.049$ and $p = 0.0051$, respectively; Fig. 1). In contrast, without active complement in the medium (heat-inactivated serum), the phagocytosis (attachment + ingestion) of *M. bovis* BCG by neutrophils was only slightly enhanced by the presence of 20 μ g/ml MBL, although the difference was not signifi-

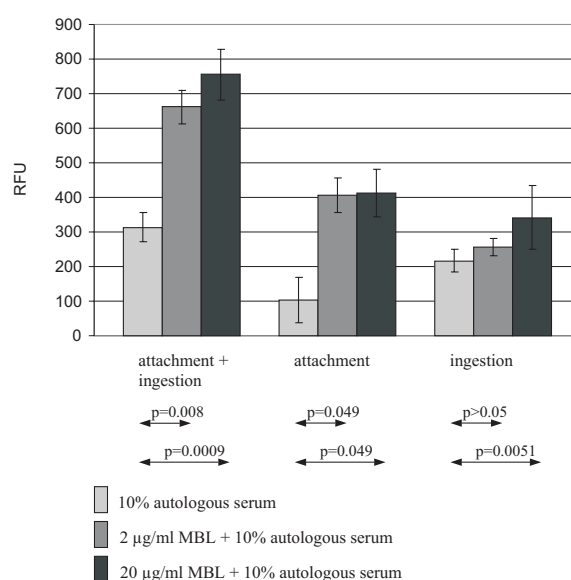


Figure 1. MBL-mediated enhancement of the phagocytosis of *M. bovis* BCG by neutrophils. Bacteria/phagocytes co-culture in the presence of MBL (2 or 20 μ g/ml) and complement (fresh autologous serum)

cant ($p = 0.28$). Further analysis, by quenching of extracellular fluorescence, showed that neither attachment nor ingestion of the mycobacteria were significantly enhanced (data not shown).

The activation of complement in all kinds of post-phagocytosis supernatants was determined by the dot-blot immune assay with monoclonal antibodies against C5b-C9 complex. The semi-quantitative (+/+/+/++) dot-blot evaluation results are presented in Fig. 2. It was observed that mycobacteria weakly activated complement by themselves (+), although this effect was enhanced in the phagocyte-bacteria co-cultures containing rMBL 2 μ g/ml (++) and 20 μ g/ml (+++).

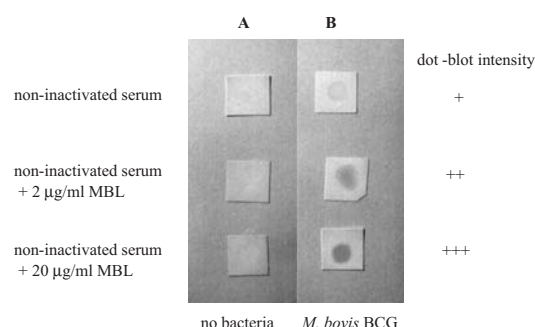


Figure 2. Detection of C5b-C9 complement complexes in post-phagocytosis supernatants by the immunoenzymatic dot-blot method using monoclonal anti-C5b-C9 antibodies. **A** – supernatants of phagocyte cultures without bacteria, **B** – supernatants of phagocyte: *M. bovis* BCG co-cultures, collected after 1 h of incubation at 37°C.

DISCUSSION

In this paper we show that phagocytosis of *M. bovis* BCG by human PMNs is enhanced by the presence of a high concentration of the serum collectin MBL and active complement components in the medium. The idea for testing such a possibility started from the observation that significantly higher MBL levels were found in TB patients than in healthy individuals. Mean concentrations of MBL in these groups were, respectively, 15.8 and 12.6 µg/ml. The highest MBL levels (>20 µg/ml) were found in 39.7% of TB patients but in only 20.3% of controls³. Therefore we wanted to investigate whether a high MBL serum concentration could be a relative disadvantage for the host with regard to TB and if it plays a role in mycobacterium/phagocyte interaction. Our data demonstrated such a possibility.

The role of PMNs in resistance to pathogenic mycobacteria was neglected; however, it was recently suggested that they perform a scavenging function in mycobacteria granuloma and that their secretory products have the ability to enhance macrophage bactericidal activity²⁴. Phagocytosis of bacteria is the result of multiple interactions between the microorganisms and host cells. These interactions depend on ligation of phagocytic receptors by microbial structures or opsonic molecules. It is known that for *M. tuberculosis* the first critical stage of the interaction with macrophages and PMNs comprises adhesion of the bacilli to the phagocyte membrane^{6, 7, 24}. This process may be mediated by many components of normal serum, among which MBL seems to play an important role, as it binds to the phagocyte's mannose receptor, driving lectin phagocytosis^{2, 10}. Moreover, many reports identified MBL as a complement activator which facilitates opsonin phagocytosis^{21, 22}. Phagocytosis can be mediated by MBL in two ways: complement activation and opsonic C3 deposition, or through an intrinsic effect of MBL itself. Additional opsonic factors (i.e. immunoglobulins) do not always seem to be necessary for MBL to increase phagocytosis; however, the presence of opsonins which do improve pathogen binding would be required for an MBL-mediated effect of internalization^{2, 8, 10, 24, 25}.

We have shown that in the presence of MBL, mycobacteria caused complement activation and their attachment to neutrophils increased. These processes led to enhanced ingestion of bacteria by the phagocytes, proving earlier observations of de Miranda Santos et al.⁵, Garred et al.⁹, and others^{1, 8}, who proposed a dual role for MBL which explains the wide range of levels of this collectin seen in a popu-

lation. While low concentration has been associated with recurrent or severe infections in children and adults caused by extracellular pathogens, a high concentration may enhance targeting of intracellular organisms at host phagocytes, the milieu preferred by these pathogens. In the light of our results it is clear that MBL itself promotes the attachment of mycobacteria to neutrophils, and this effect, as well as ingestion of the bacteria, were enhanced in the presence of MBL and active complement components. This suggests that the lectin pathway of complement activation mediated by MASP serine proteases is important in the mycobacteria-phagocyte interaction. Such enhanced binding of *M. tuberculosis* to host cells may represent a risk factor for clinical TB. Thus, genetic polymorphisms of the MBL gene associated with increased production of MBL have been suggested to be a relative disadvantage in mycobacterial infections^{9, 15, 19, 21, 23}. However, it should be stressed that there are multiple mechanisms for the uptake of *M. tuberculosis* by phagocytes, involving a number of different receptors which lead to different modes of signal transduction and immune activation. The possible role of MBL-mediated phagocytosis in this process and its real importance *in vivo* remains to be shown, since individuals with a relatively low MBL level developed TB^{3, 17}. On the other hand, as described by Hoal van Helden et al.¹¹ and others^{9, 12, 19, 24}, the heterozygosity for MBL variant alleles, which encode low serum MBL levels, is associated with protection against clinical TB, mainly in its disseminated form or tuberculous meningitis. In this respect, MBL may be the factor modulating the inflammatory response initiated by mycobacteria, as has been recently reported²³. Besides MBL, other collectins, e.g. lung surfactant proteins SP-A, SP-D, are known to be molecules important in innate immunity to mycobacteria^{12, 20, 25}. A recent report published by Kudo et al.¹² indicated that both pulmonary collectins, SP-A and SP-D, significantly increased phagocytosis of bacteria by alveolar macrophages, whereas in the presence of MBL the uptake of bacteria was not significantly increased. Similarly, MBL added to the culture medium in our experiments at a concentration 20 µg/ml, in the absence of complement, did not significantly enhance the attachment or ingestion of mycobacteria by PMNs.

Because *M. tuberculosis* can survive and multiply in phagocytic cells, the collectin-mediated attachment and, probably, entry to the host cells may lead to differences in signal transduction and cell activation and may be an important pathway to escape intracellular killing. It is suggested, however, that collectins, and probably other pattern recognition receptors, will

usually only encounter their ligands in the context of other receptors. Thus, in the light of the fact that various components and processes build up innate

immunity to mycobacteria, it is clear why such great interindividual differences in outcome after infection are noted^{6, 14, 24, 25}.

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