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Differential microorganism-induced mannose-binding lectin activation

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Abstract

Mannose-binding lectin (MBL) is a serum complement factor playing a dominant role in first-line defense. When MBL binds to specific sugar moieties on microorganisms, the lectin complement pathway (LCP) is activated. Changes in the *mbl* gene and promotor may result in MBL with less activity, predisposing the individual to recurrent infections. Using a functional MBL assay, we investigated at what concentration different microbes activated MBL. Less than 1 colony-forming unit (CFU) of *Neisseria meningitidis* groups B and C still activated MBL, which may be ascribed to filterable blebs. *Nocardia farcinica* and *Legionella pneumophila* activated MBL well, which raises new questions about host susceptibility. In contrast to other research, *Pseudomonas aeruginosa* activated the LCP potently.

Keywords: Mannose-binding lectin; Lectin pathway; Functional assay; Microorganism

1. Introduction

Mannose-binding lectin (MBL) is an ancient complement protein discovered only a few decades ago [1,2]. Being the leading protein of the lectin complement pathway (LCP), it belongs to the family of calcium-dependent collagenous lectins (collectins) [3]. This C1q-like molecule has three identical sugar-binding domains with specificity for mannose, *N*-acetyl-D-glucosamine (GlcNAc), and fucose, but not for antibodies. When one or more of these sugars are present on a microbial surface, binding and subsequent activation of MBL will take place, which indirectly leads to C3b and C3bi deposition on the microbial surface as well.

The LCP comprises MBL and its associated serine proteases, and complement components C4, C2, and C3. The latter three components are shared with the phylogenetically younger classical complement pathway, which becomes activated when microorganisms bind immunoglobulins. MBL activation results in direct microbial opsonization and/or microbial lysis via membrane attack complexes [4], a property that makes the lectin complement pathway part of the innate immune system.

Certain microorganisms appear to be ideal substrates for MBL binding and subsequent lectin pathway activation. E.g., the fungal cell wall is relatively rich in mannan (polymerized mannose) [5], the mycobacterial cell wall rich in lipoarabinomannan (LAM) [6], and GlcNAc constitutes an important building stone of peptidoglycan of bacterial cell walls [7]. Bacterial capsules also contain a variety of polysaccharides including GlcNAc, mannose, fucose, and derivatives thereof, which suggests that certain capsular types may activate the lectin pathway as well. In fact, MBL binding to various bacterial, fungal, viral, and parasitic agents has been demonstrated by means of flow cytometry [8,9], radio-immunoassay [10,11], enzyme-linked immunosorbent assay (ELISA) [11,12,13], or electron microscopy [14]. MBL binding has been tested functionally using a C4b deposition assay [15,16]. However, discrepancies in MBL binding and activation by microbes may be found, depending on the method used.

Recently, we devised a novel, hemolytic assay for the estimation of functional MBL, based on the phenomena of *Saccharomyces cerevisiae*-induced MBL activation and subsequent bystander lysis of chicken erythrocytes [17].

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MBL was the rate-limiting factor in that assay, making the test highly specific for MBL. In the present study, we used the same assay to compare MBL activation by different microorganisms.

2. Materials and methods

2.1. Human pooled serum

Blood collected from healthy workers of our laboratory was allowed to clot for 60 min before centrifugation ($1500\times g$) at room temperature for 10 min. Sera were then pooled (human pooled serum; HPS), and stored in 100-μl aliquots at −70°C until further use.

2.2. MBL-deficient serum

Serum from an MBL-deficient subject was used as MBL-reagent serum. This serum was also stored in 100-μl aliquots at −70°C.

2.3. Microbial strains

The microbial strains tested in the functional MBL assay comprised a selection of defined strains, including those from the American Type Culture Collection (ATCC) and the British National Culture Type Collection (NCTC). The Gram-positive bacterial strains tested included: *Staphylococcus aureus* Cowan I, *Micrococcus lysodeikticus*, *Enterococcus faecalis* ATCC 29212, unencapsulated *Streptococcus pneumoniae* strain Rx1 (kindly provided by Dr. Larry McDaniel, Jackson, MI, USA), and group A β-hemolytic *Streptococcus* strain NY-5, which is a strong exotoxin SPE-A1 producer [18].

The Gram-negative bacterial strains included were: *Legionella pneumophila* NCTC 11233, *Helicobacter pylori* (patient isolate), *Escherichia coli* ATCC 25922, *Salmonella typhimurium* strains M206 and the M206 Re mutant (with LPS lacking the O-antigenic side chain, composed only of keto-deoxyoctonic acid and lipid A) [19], and *Yersinia enterocolitica* serotype O:3. Four *Neisseria gonorrhoeae* strains differing in piliation and opacity protein (F62, N300 (piliated, Opa-negative), N303 (piliated, Opa_{50}), and N392 (piliated, Opa_{30})) [20,21] were a kind gift from Dr. M. Dehio, Tübingen, Germany, and *Neisseria meningitidis* groups B and C (patient isolates) were also included in our assay for MBL activation.

Non-fermenting Gram-negative strains tested were: *Burkholderia cepacia* ATCC 25417, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 10145, and a mucoid *P. aeruginosa* isolate cultured directly from the sputum of a cystic fibrosis patient.

The actinomycete *Nocardia farcinia* was a clinical isolate. The National Institute for the Environment and Public Health (RIVM, Bilthoven, the Netherlands) provided the *Mycobacterium bovis* BCG strain. *Candida albicans* ATCC 14053 was obtained commercially. Baker's yeast (*S. cerevisiae*) was used as the reference organism at a concentration of 6×10^6 yeast cells ml^{-1}.

2.4. Microbial cultures

Strains were cultured according to standard procedures [22]. *L. pneumophila* was cultured on BCYE agar (Oxoid, Basingstoke, UK), gonococci on GC agar (Becton Dickinson, Cockeysville, MD, USA), both meningococcal strains on Columbia blood agar (Oxoid), *H. pylori* on Belo Horizonte medium (Oxoid), *B. cepacia* on *B. cepacia* agar (Oxoid), the yeasts *C. albicans* and *S. cerevisiae* on Sabouraud agar (Merck, Darmstadt, Germany), and *M. bovis* BCG in Dubos liquid medium (Difco, Le Pont du Claix, France) supplemented with glycerin (Merck) and bovine albumin (Sigma, Zwijndrecht, the Netherlands). All other bacterial strains were cultured on Columbia blood agar.

For use in the functional assay, the microorganisms were suspended in veronal-buffered saline with 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{++}), until an absorbance value of 1.0 at 660 nm was reached. The microbial suspensions were serially diluted (1/2 up to $1/6.4\times10^7$). In order to study activation by subcellular products of meningococci, meningococcal suspensions were filtered through a 0.22 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA). Bacterial concentrations at OD 1.0 varied from 1.5×10^8 colony-forming units (CFU) per ml for *Neisseria* species to 1.1×10^9 for *B. cepacia*. Microorganisms were enumerated microscopically by the Thoma slide counting chamber method (W. Schreck, Hofheim, Germany).

2.5. Functional microtiter assay for MBL activation by microorganisms

The functional MBL test used to study MBL activation by microorganisms was derived from a recently developed assay based on *S. cerevisiae*-induced bystander C5b6-mediated hemolysis of chicken erythrocytes (ChE) [17]. In that assay, serum to be tested for functional MBL was serially diluted ($10^{-0.5}$). A fixed amount of MBL-activating baker's yeast cells (3.0×10^5 cells/well), MBL-deficient serum (1.5 μl/well) providing all complement components except MBL, and target cells (1×10^7 ChE/well) were added. The conditions were chosen in such a way that MBL was the rate-limiting factor in that assay.

In the present assay, MBL-activating *S. cerevisiae* was substituted by other microorganisms. The different microbial concentrations were added in a 50-μl volume per well. A checkerboard titration was done as follows: HPS (as the source of MBL) was serially ($10^{-0.5}$) diluted, starting with a dilution of 10% HPS in VBS^{++} per well (final concentration 3.3% HPS) in the horizontal lines of the microtiter

plate, while the serially diluted microorganisms were added to the vertical rows. Per microtiter plate, a 5-ml mixture was made containing 150 μl MBL-deficient serum and 10^9 ChE in VBS^{++}. A 50-μl sample was taken from this mixture and added to each test well. Then, the plate was incubated at 37°C for 1 h, and spun for 10 min in a microtiter centrifuge. Thereafter, 50-μl samples of each supernatant were transferred to a flat-bottom plate containing 200 μl water per well. Hemoglobin release was measured in an ELISA reader operating at a wavelength of 405 nm.

Percentages of hemolysis were calculated using controls for 100% (water lysed) and 0% (buffer incubated) hemolysis. The percentages were then transformed according to the equation published by Borsos and Rapp [23]:

$$Z = -\ln(1-\text{fraction of erythrocytes lysed})$$

in which Z is the mean number of active sites per chicken erythrocyte. Human pooled serum (HPS) in EGTA-VB (8 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid with 2.5 mM Mg^{2+}) was used to test alternative pathway activation by the various microbes. Direct hemolysis of the erythrocytes by microbial products was excluded by incubating microorganisms and erythrocytes together with MBL-deficient serum only. Microbial MBL activation was related to the MBL activity of 3×10^5 *S. cerevisiae* cells/well in a 50-μl volume, added to a HPS dilution series in the same microtiter plate as the microbe tested. The microbial concentration giving rise to a Z value of 0.6 (calculated by $Z_{\text{microorganism}}/Z_{S.\ cerevisiae} = 0.6$) was used as a means to rank the microorganisms based on their MBL-activating capacities. All experiments were repeated at least twice.

2.6. Specificity of microbial MBL activation

To test the specificity of microbe-induced MBL activation, experiments were also executed in the presence of serial (1/3) dilutions of either D(+)-mannose (Sigma) or *N*-acetyl-D-glucosamine (Serva, Heidelberg, Germany). The highest saccharide concentration tested was 2.5 mg per well. MBL-containing human pooled serum (20%; 25 μl) samples were preincubated with the saccharide (25 μl) at 37°C for 30 min. After incubation of the saccharides with HPS, microbial cells were added. Sugar-inhibitable hemolysis was considered MBL mediated.

3. Results

A functional MBL assay was used to compare MBL activation by different microorganisms. In the test, microbial numbers as well as the MBL concentration were varied. Curves obtained were mostly straight parallel lines, showing less hemolysis with decreasing numbers of bacteria. Incidentally, optimum curves were obtained. By determining the intersection of the straight lines with the horizontal line $Z = 0.6$, the number of microorganisms activating MBL in the assay could be compared (Table 1).

In order of decreasing MBL-activating abilities, the following microbes were ranked: *N. meningitidis* groups B and C, *Nocardia farcinica*, the four gonococcal strains (showing equal MBL-activating capacities irrespective of pili or opacity protein), *S. typhimurium*, the Re mutant of *S. typhimurium* (lacking the O-antigenic side chain, theoretically having less MBL-activating sites), *M. bovis* BCG, mucoid and non-mucoid *P. aeruginosa* (showing no difference on outcome between mucoid and non-mucoid strains), *C. albicans*, *L. pneumophila*, *Y. enterocolitica*, *M. lysodeikticus*, *S. aureus* Cowan I, *E. coli*, and *H. pylori*. The *E. faecalis* strain did not activate MBL at all.

The competitive, inhibitory effect of MBL-specific saccharides on lectin pathway activation is shown for the reference organism *S. cerevisiae* and for the unencapsulated Gram-positive bacterium *M. lysodeikticus*. *S. cerevisiae*-induced MBL activation could be counteracted by adding to the sera to be tested 2.5 mg/well or 1/3 dilutions of this suspension of D(+)-mannose, the carbohydrate substrate with the greatest affinity for MBL (Fig. 1a). *N*-acetyl-D-glucosamine competed with *M. lysodeikticus* for MBL binding in the same manner, as is shown in Fig. 1b. MBL activation by all other microbes tested could be inhibited by the saccharides too.

The finding that *N. meningitidis* groups B and C still

Table 1
Ranking of microorganisms according to their MBL-activating capacities

Microbial strain	Concentration at $Z = 0.6$ (CFU)
N. meningitidis group B	1
N. meningitidis group C	1
N. farcinica	5
N. gonorrhoeae F62	50
N. gonorrhoeae N300	50
N. gonorrhoeae N303	50
N. gonorrhoeae N392	50
S. typhimurium M206	75
S. typhimurium M206 Re mutant	300
M. bovis BCG	400
S. cerevisiae	1350
P. aeruginosa ATCC 27853	5000
P. aeruginosa ATCC 10145	5000
P. aeruginosa mucoid (CF)	5000
C. albicans ATCC 14053	3×10^5
L. pneumophila NCTC 11233	5×10^6
Y. enterocolitica	3×10^7
M. lysodeikticus	3×10^7
S. aureus Cowan I	3×10^8
H. pylori	3×10^8
E. coli ATCC 29522	3×10^8
E. faecalis	no activation at all

Z value stands for the amount of hemolysis in the functional assay, showing the active sites per erythrocyte. CFU: colony-forming units.

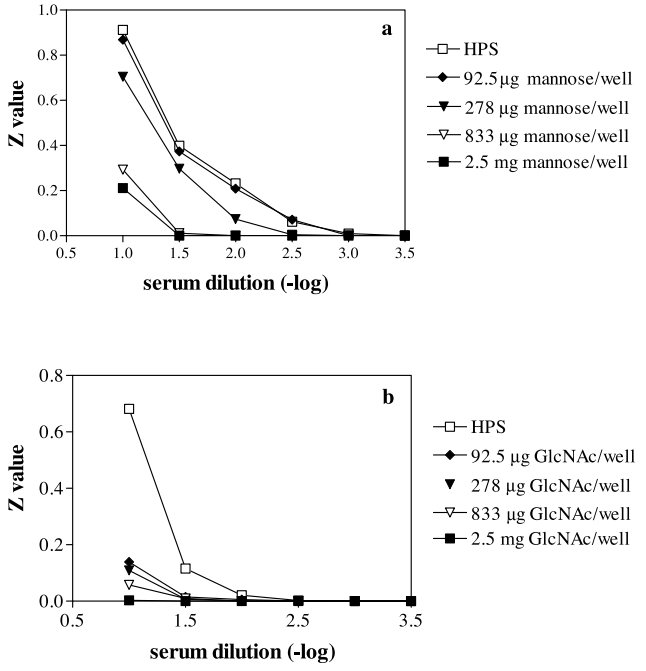

Fig. 1. Competition of carbohydrates and bacteria for MBL binding. a: Mannose inhibition of *S. cerevisiae*-induced MBL activation. b: *N*-acetyl-D-glucosamine (GlcNAc) inhibition of *M. lysodeikticus*-induced MBL activation.

caused MBL-mediated hemolysis despite low bacterial concentrations demanded additional experimental information. After passing the meningococcal suspensions through a microbial filter, no differences were seen between the amount of MBL-induced hemolysis caused by the unfiltered suspension and the filtered one (not shown).

Some microbial strains produced hemolysins that lysed the chicken erythrocytes. Therefore, these strains could not be evaluated in the functional MBL assay. This was true for group A β-hemolytic streptococcus strain NY-5, *B. cepacia* ATCC 25417, and the heat-killed, unencapsulated *S. pneumoniae* strain Rx1.

4. Discussion

Many experiments regarding complement activation by different microorganisms were carried out either before the definite recognition of the alternative pathway (around 1980) [24], or when the lectin pathway was yet to be discovered (before 1989). This necessitates reevaluation of the capacities of different microorganisms to activate the three complement pathways. The ancient lectin pathway, activation of which was the subject of this study, leads to antibody-independent microbial coating with C3b, and ingestion in cells of the monocyte phagocyte lineage or, in case of e.g. *Neisseriae*, in terminal complement pathway (TCC)-mediated microbial lysis. As multiple Fc receptor polymorphisms of polymorph nuclear (PMN) leukocytes severely complicate opsonic studies, the focus of this paper is restricted to functional activation of MBL by microorganisms.

For the purpose stated above, we modified a recently developed, functional MBL assay, based on the principle of yeast-induced, C5b6-mediated bystander hemolysis of chicken erythrocytes [17]. The hemolytic assay, specific for MBL by using MBL-deficient reagent serum, combines *S. cerevisiae*-induced MBL pathway activation with bystander hemolysis of chicken erythrocytes. The assay is easier to perform than complement fixation assays, and very reproducible. As shown previously, the influence of classical complement pathway activation is minimized in hemolytic assays when using diluted serum of less than 5% [25]. Competition of MBL with carbohydrates is a measure of the remaining complement activity induced by the HPS used (Fig. 1). The influence of antibodies on classical pathway activation in this MBL assay was studied with *S. cerevisiae*, using serum from an MBL-deficient subject; the effect of this IgG- and IgM-containing serum was negligible [17].

By substitution of baker's yeast, the MBL-activating capacity of other microbes could be evaluated. The most striking finding was that, depending on the microbe tested, either straight lines or optimum curves were obtained (*x*-axis: number of microorganisms; *y*-axis: *Z* value, or number of active sites per cell). The 'optimum curve' phenomenon can be explained by microbe-induced depletion of early complement components C2 and C4 upon testing higher microbial concentrations, resulting in less hemolysis than expected.

Several of our results differ dramatically from those found by others for several microorganisms tested. For example, very weak or absent MBL binding by *P. aeruginosa* was found by other researchers [15,26], and functional MBL activation by *P. aeruginosa* strains ($n = 7$) could not be detected in a functional C4-binding assay [15]. *P. aeruginosa* belongs to the non-fermenting group of Gram-negative bacteria involved in severe pulmonary infections in cystic fibrosis (CF) patients, resulting in a shortened life-span in those patients carrying variant MBL alleles [27]. MBL activation as shown in our assay provides a better explanation for the severe infections in MBL-deficient CF patients.

Extremely low concentrations (≤ 1 CFU) of groups B and C meningococci still activated MBL to a *Z* value of 0.6 in the functional assay. A new finding is that, even after filtration of the bacterial suspensions, unambiguous MBL activation was found, pointing towards a soluble MBL-activating factor. A candidate factor may be the 'blebs', vesicles formed by parts of the meningococcal outer membrane. Blebs, consisting partly of LPS, are secreted in serum and cerebrospinal fluid within seconds in vitro as well as in vivo [28]. Although previous experiments showed that MBL can bind to sialylated meningococcal LPS [29,30], and to opacity and porin B proteins (M.M.

Estabrook, D.J. Jack, N.J. Klein and G.A. Garvis, Abstracts 5th International Workshop on C1, and collectins, abstr. II-3, 2001), our findings show the importance of a soluble, filterable factor in meningococcal pathology, which raises new questions, and may explain the increased susceptibility of MBL-deficient persons to meningococcal disease [31].

Gonococcal pili and opacity protein variations do not contribute to differences in the lectin pathway-activating capacity. Also, the ability of gonococci to activate MBL functionally is less pronounced when compared to meningococci.

S. cerevisiae, the first reported microorganism associated with MBL [1], is a yeast of low pathogenicity. Its potent MBL-activating capacity can be related to the high polysaccharide content of the cell wall, which consists mainly of polymers of *N*-acetylglucosamine (chitin), glucose (β-glucan), and mannose (mannan) [5]. The cell wall of *C. albicans* resembles that of *S. cerevisiae*, but the two yeast species differ in pathogenicity [5]. Surprisingly, more *C. albicans* cells were needed to achieve the same amount of hemolysis when compared to *S. cerevisiae*. Whereas clinical disease with *S. cerevisiae* is rare, *Candida* species cause mucosal and systemic disease in both normal and immunocompromised patients. Immunity to *Candida* infections is complement related [32], but also dependent on the quantity and quality of peripheral phagocytes and on T-cell function.

Since MBL binding leads to opsonization and internalization in monocytes and monocyte-derived macrophages, it can be theorized that normal serum MBL levels predispose to more severe disease with intracellular pathogens, which survive and replicate in monocytes and macrophages. This theory has been confirmed in patient studies for tuberculosis in HIV-negative individuals [33–35] and for Ethiopeans suffering from leprosy, which is caused by *Mycobacterium leprae* [36]. Also, a direct correlation has been found between high MBL levels and the development of invasive (visceral) leishmaniasis, which is caused by the intracellular protozoa *Leishmania chagasi* [37]. As expected, very low concentrations of the intracellular microorganism *M. bovis* BCG activated MBL in our assay. This can be explained by vivid MBL binding to the mycobacterial cell wall components peptidoglycan and lipoarabinomannan (LAM). The role of MBL in e.g. *Mycobacterium avium* intracellular infections in HIV patients, demands further research.

The intracellular pathogen *L. pneumophila* activates the lectin pathway, which may represent a quick means of transport of the bacterium into pulmonary macrophages after inhalation by the host. In the macrophages, bacterial replication takes place within 2–4 h [38], which may result in severe pneumonia. MBL deficiency may protect against severe legionellosis, which is currently under investigation.

Our study confirms the strong MBL-activating ability of the intracellular *Salmonella* species. The decreased MBL-activating capacity in the functional assay of the Re mutant can be explained by the shorter LPS side chain, composed only of lipid A and keto-deoxyoctonic acid. Other authors confirmed that MBL binds avidly to *S. typhimurium* core LPS structures, and that this type of binding is abrogated by the addition of O-antigen [39].

Until now, no study has looked at the role of innate complement proteins and the pathogen *N. farcinica*, which causes pulmonary infections beside brain abscesses in patients with dysfunctional cellular immunity. One would expect that nocardial infections would be more common, knowing that many people carry variant MBL alleles. However, this microorganism grows slowly and is usually killed by macrophages and T-lymphocytes [40].

The pathogen *Y. enterocolitica* activates MBL in our assay, a finding demanding further research. After ingestion of meat and refrigerated food, this pathogen causes gastroenteritis and, rarely, reactive arthritis. The role of MBL in *H. pylori*-associated gastritis, gastric ulceration and lymphoma (MALT-type B-cell) also remains to be elucidated. Recent research showed that *H. pylori* activated the classical pathway in the absence of specific antibodies, at least via LPS [41], but the specific lectin pathway was not studied. However, as *H. pylori* activated MBL in the functional assay, it is more likely that the lectin pathway is the major complement pathway involved in opsonization of the bacterium.

Testing the MBL-activating capacities of microorganisms in fixed circumstances may not be representative for what happens in the host, and it is more likely that our functional assay approaches the more complex in vivo situation. Therefore, ranking of microorganisms in terms of MBL activation can give a different impression from the ranking of microbes in terms of MBL binding.

Several bacteria show α- or β-hemolytic activity when cultured on sheep blood agar, and this quality makes these strains unsuitable for testing in the functional MBL assay using chicken erythrocytes. For instance, *Streptococcus pyogenes* contains powerful hemolysins. The same holds true for *B. cepacia*, and the unencapsulated *S. pneumoniae* strain.

In conclusion, our functional MBL assay can be used to evaluate MBL binding and activation by most microbes, providing new insight in the double role of MBL in genetic susceptibility to microbial diseases. The present study shows that extremely low concentrations of meningococci lead to profound MBL activation, as do filterable meningococcal products. Contradictory to other in vitro studies, *P. aeruginosa* does activate MBL, providing experimental evidence for the clinical outcomes in MBL-deficient CF patients. New insight was gained in the activation of MBL by *L. pneumophila*, *N. farcinica* and *H. pylori*, raising exiting new questions as to the role of this ancient protein in (intracellular) infection.

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