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Fluorochrome-linked immunoassay for functional analysis of the mannose binding lectin complement pathway to the level of C3 cleavage

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Abstract

The humoral response to invading pathogens is mediated by a repertoire of innate immune molecules and receptors able to recognize pathogen-associated molecular patterns. Mannose binding lectin (MBL) and ficolins are initiation molecules of the lectin complement pathway (LCP) that bridge innate and adaptive immunity. Activation of the MBL-dependent lectin pathway, to the level of C3 cleavage, requires functional MASP-2, C2, C4 and C3, all of which have been identified with genetic polymorphisms that can affect protein concentration and function. Current assays for MBL and MASP-2 lack the ability to assess activation of all components to the level of C3 cleavage in a single assay platform. We developed a novel, low volume, fluorochrome linked immunoassay (FLISA) that quantitatively assesses the functional status of MBL, MASP-2 and C3 convertase in a single well. The assay can be used with plasma or serum. Multiple freeze/thaw cycles of serum does not significantly alter the assay, making it ideal for high throughput of large sample databases with minimal volume use. The FLISA can be used potentially to identify specific human disease correlations between these components and clinical outcomes in already established databases.

Keywords

MBL; quantification; assay

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Introduction

Clinically, MBL moderates disease severity in both its 'traditional' and 'non-traditional' capacities. In its 'traditional' role, MBL deficiencies have been linked to recurrent respiratory infections (Cedzynski et al., 2004; Gomi et al., 2004; Zhang et al., 2005; Kaur et al., 2006), meningococcal disease (van Emmerik et al., 1994; Bax et al., 1999; Kuipers et al., 2003; Bathum et al., 2006) and *P. aeruginosa* infection in burn patients (Moller-Kristensen et al., 2006). Alternatively, MBL recognition of self-antigens has been demonstrated in sterile inflammatory processes including systemic lupus erythematosus (Ohlenschlaeger et al., 2004; Seelen et al., 2005b; Calvo-Alen et al., 2006; Font et al., 2007), thoracoabdominal aortic aneurysm repair (Fiane et al., 2003), IgA nephritis (Endo et al., 1998; Roos et al., 2001; Hisano et al., 2001; Roos et al., 2006), rheumatoid arthritis (Garred et al., 2000; Saevarsdottir et al., 2001; Gupta et al., 2005; Burton and Dwek, 2006), atherosclerosis (Madsen et al., 1998; Hegele et al., 2000; Spence and Norris, 2003; Sjöholm et al., 2006), diabetes (Hansen et al., 2004; Hansen et al., 2003; Bouwman et al., 2005; Hovind et al., 2005), cancer (Baccarelli et al., 2006; Scudiero et al., 2006) and coronary artery disease (Best et al., 2004; Saevarsdottir et al., 2005). The dichotomous nature of this C-type lectin to recognize both foreign- and/or self-antigens under varying conditions is the focus of intense research, as the appropriate balance of functional MBL and LCP-activation may predict outcomes in various disease states.

Several assays are available for the quantification of MBL, either functionally (mannan assay) or non-functionally (sandwich ELISA) (Petersen et al., 2001; Thiel et al., 2002; Roos et al., 2003; Takahashi et al., 2005; Seelen et al., 2005a). However, these assays cannot fully investigate the functional status of the early lectin pathway cascade proteins in a single analysis to the level of C3 cleavage. Further, activation of the alternative complement pathway directly via MBL (e.g., in the absence of MASP-2) has been described recently and can not be assessed by available assays. The novel FLISA described here can analyze the concentration of functional MBL and key components of LCP activation in serum and citrated (3.2%) plasma. Functional MBL, the functional capabilities of MASP-2 (e.g., by observing C4b deposition), the functional formation of the C3 convertase (e.g., observing C3b deposition and thus the functional status of C2) on microtiter plates are analyzed concurrently in a single well. This technique utilizes multi-channel infrared technology (LICOR Biosciences, Lincoln, Nebraska) based on detection with a panel of directly labeled antibodies, giving three-endpoint specificity with a minimum volume requirement of sample (<2 µl total) for analysis. Data from this FLISA can therefore quantitatively determine functional MBL and the associated activation of the MBL-dependent LCP through the level of C3 convertases. This assay can quickly analyze large databases of serum/plasma samples from human disease populations to detect potential correlations between these early complement components and clinical outcomes.

Materials and Methods

Patient Samples and Standard Serum Components

Serum and/or plasma samples were collected from informed consent donors, following IRB approval, from the Brigham and Women's Hospital population, Boston, MA. Individual serum samples were pooled to create a standardized human serum stock (PLS) for assay evaluation and validation. Each PLS batch was assigned a lot number, and frozen in aliquots at -80°C for future FLISA use. Serum samples representing various homozygotic MBL haplotypes are from informed consent donors from the Brigham and Women's Hospital, and the Texas Heart Institute, Baylor College of Medicine, Houston, Texas.

The MBL content of the PLS was standardized using MBL-deficient serum and a quality controlled, purified MBL protein standard (Staten Serum Institut, Copenhagen, Denmark). Purified and quantified complement components C4b and C3b (>95% purity; Complement

Technologies Inc., San Diego, CA) were used as quality control samples and to standardize the downstream activated components of the MBL-dependent LCP in the PLS.

Antibodies

All labeled antibodies utilized long-wavelength near-infrared fluorochromes IRDye® 800CW or IRDye® 700DX (Rockland Immunochemicals, Gilbertsville, PA) or AlexaFluor 680 (Molecular Probes/Invitrogen, Carlsbad, California). Fluorochrome to protein conjugation was performed by Rockland Immunochemicals, or according to manufacturer's instructions for dye labeling (IRDye® 800 Conjugation kit, Rockland Immunochemicals). Optimal dilutions for FLISA labeled antibodies were defined by our laboratory in pilot studies. Antibodies used for conjugation are as follows: IRDye800 labeled 2A9 [mouse anti-human MBL (Collard et al., 2000)], goat anti-human C4 (Cappel/ICN Pharmaceuticals, Aurora, Ohio); AlexaFluor 680 mouse anti-human C3 clone 7C12 (Tosic et al., 1989), and donkey anti-goat IgG IRDye800 (Cappel/ICN). Optimal concentrations of each antibody were determined in pilot studies.

Plate Preparation

High binding, 384-well, clear, flat bottom microplates (Corning Incorporated, Corning, NY) were coated with 25 µl per well of 0.5 mg/ml mannan (Sigma, St. Louis, MO) in sodium carbonate/bicarbonate buffer pH 9.8. Plates were incubated overnight at 4°C. The plates were then blocked with 110 µl per well of 3% BSA in PBS without calcium or magnesium (Sigma) for two hours at room temperature. Plates were then washed three times with PBS/Tween 20 (0.5%), once with PBS, and once with veronal buffered saline (VBS). Excess volume was removed from the plates, which were covered and stored at -30°C.

MBL-dependent LCP FLISA

All FLISA analyses were performed using the Odyssey and/or Aeries Infrared Imaging systems (LICOR), and all sample processing for plates utilized a fully automated liquid handling and plate washing system (BIO-TEK Instruments Inc., Winooski, VT). Prepared 384-well mannan plates (protocol above) were thawed at room temperature (25°C) for 15 minutes. Diluted plasma (see preparation below) and/or serum were then added to plates at 15 µl per well and serially diluted using the Precision 96/384 well Microplate Pipetting System (BIO-TEK Instruments Inc.). Sample diluent for serum or plasma samples was fortified veronal buffered saline (VBS⁺⁺; 143 mM NaCl, 5 mM Barbitol Sodium Salt, 5 mM MgCl₂, 5 mM CaCl₂). Buffers alone were added to mannan wells as a background control. Plates were incubated at 37°C for 30 minutes and then washed with Buffer A: (145 mM NaCl, 10 mM HEPES, 10 mM CaCl₂, 490 µM MgCl₂, 0.05% Tween) four times (Elx405 Microplate Washer with Bio-Stack Microplate Stacker; BIO-TEK Instruments Inc.). MBL was detected by addition of 15 µl per well of 1:500 IRDye800 2A9 (anti-MBL) diluted in Buffer A incubated for one hour at 25°C, washed (3x with Buffer A) and then read on the Odyssey/Aeries Infrared Imaging system (LICOR) at 800nm. Following this first reading, plates were soaked for 15 minutes with 100 µl/well of Buffer B: (50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 0.05% Tween-20, 100 mM D(+)-mannose at pH 7.8), followed by 1x wash with Buffer B. Plates were read again on the Aeries/Odyssey Infrared Imaging system (LICOR) at 800nm to establish baseline fluorochrome emittance following removal of MBL, and the primary antibody against MBL. Next, 15 µl per well of goat anti-human C4 at 1:500 (diluted in Buffer A) was added to plates and incubated for one hour at 25°C. Following a 4x wash with Buffer A, 15 µl per well of 1:1000 IRDye800 donkey anti-goat IgG, and 1:3000 AlexaFluor 680 7C12 mouse anti-human C3 (diluted in Buffer A) were incubated for one hour at 25°C, washed (4x with Buffer A) and read on the Aeries/Odyssey Infrared Imaging system (LICOR) concurrently at 700 nm and 800 nm. Measurement of MBL (ng/ml) is interpolated from the serum or plasma dilution series,

and MBL-dependent activation products (pg) are evaluated at 3% serum unless otherwise stated.

For inhibition studies, mAb 3F8 specific for human MBL (Collard et al., 2000) and anti-human factor D antibody (Stahl et al., 2003) at the concentrations indicated were added to serum at 25°C for 30 minutes prior to plate incubation.

Plasma preparation

Serum and citrated (3.2%; BD Vacutainer, Franklin Lakes, NJ) plasma samples were obtained from the same three donors. Citrated plasma (100 µl) was mixed 1:1 with VBS⁺⁺ buffer supplemented to 16.3 mM calcium chloride to induce coagulation as described (Poller et al., 2002). Serum and coagulated plasma samples were allowed to clot overnight at 4°C. Serum was separated from fibrin clots by centrifugation and then used in the FLISA.

Freeze - thaw effects

Serum samples were aliquoted and subjected to multiple rounds of freezing (−80°C) and thawing to room temperature. Freeze/thaw samples were then subjected to FLISA analysis.

Statistics

Samples were averaged in triplicate and expressed as mean ± the standard error (SE). PLS integrated intensities were averaged in triplicate per plate and in quadruplicate per assay with inter-assay CV values expressed as a percentage. A power analysis was performed to select the appropriate sample of representative haplotypes to achieve statistical significance for functional MBL evaluation. Significance was defined as $p < 0.05$.

Results

The LCP, including the MBL-dependent LCP is outlined in Figure 1. As mannan is a major ligand for MBL, evaluation of MBL binding on mannan-coated plates can therefore demonstrate functional MBL levels in serum or plasma samples. MBL binding was detected using a directly labeled IRDye800 anti-MBL mAb (2A9) visualized on the 800 nm infra-red channel. A pooled human serum standard (PLS) was standardized by using MBL-deficient serum (Staten Serum Institut; MBL B/B also recognized as O/O) reconstituted with a quantified purified human MBL standard (Staten Serum Institut). Inter-assay coefficient of variation was 4%. Correlation between detected units of integrated intensity (II) at 800 nm and ligand binding was performed using a linear regression model to establish MBL-dependent binding in ng/ml (Figure 2a). The lower limit of detection was 10 ng/ml. Specificity of functional MBL binding was demonstrated by blocking with mAb 3F8 (Figure 2b) or D-mannose (data not shown). Using the PLS to establish functional MBL levels, triplicate values of individual donor serum sample diluted in binding buffer were averaged and expressed as ng/ml (Figure 2c, DN 1-4).

Following analysis of the functional MBL concentrations present, the plate was then regenerated by removing non-covalently bound MBL/MASP-2 complexes (and their associated detector antibodies). The regeneration buffer removes the Ca^{2+} required for MBL complex binding and competitively inhibits MBL binding by addition of D-mannose. Following regeneration, infra-red emittance on the 800 nm channel returned to background levels. Because activated components of LCP activation downstream of MBL/MASP-2 (i.e. C3b and C4b) are covalently bound, regeneration of the analysis platform increases the analysis from a single endpoint assay to a 3-endpoint analysis of MBL-dependent LCP activation from a single serum sample.

Enzymatic cleavage of C4, with consequent C4b deposition onto the mannan plate demonstrates functional MASP-2 activity (Figure 1). Although circulating levels of MASP-2 have been evaluated using a sandwich ELISA (Moller-Kristensen et al., 2003), functional levels of MASP-2 associated with functional MBL complexes at physiological MBL concentrations (Sorensen et al., 2007), or in patient samples in a single assay have yet to be established. Following the regeneration step, MBL/MASP-2 complex dependent C4b and C3b deposition on mannan plates from human serum were analyzed at 800nm and 700nm, respectively, after incubation with the detection antibodies described in the *Materials and Methods* section.

Using purified, quantified amounts of human C4b as a standard, we then determined the amount of MBL-dependent C4b that was bound to the mannan coated plates following incubation of the PLS. As shown in Figure 3a a log-linear relationship between the integrated intensity of activated PLS deposited C4b and known amounts of C4b was observed. These data were used to standardize the PLS and its ability to deposit known amounts of C4b onto mannan-coated plates. Inter-assay coefficient variation was 10%. Specificity for MBL-dependent C4b deposition was further verified by the ability of mAb 3F8 to dose-dependently inhibit C4b deposition (Figure 3b). Serum samples prepared in the previous step for MBL analysis (Figure 2c, DN 1-4) were further evaluated in the same well for MBL-dependent C4b deposition against the PLS using a log-linear regression fit and expressed as MBL-dependent C4b in pg (Figure 3c).

The classical/lectin C3 convertase, C4b2a, is formed by MBL/MASP-2 cleavage of complement protein C2, concurrent with C4 cleavage and deposition of C4b (Figure 1). Formation of the C3 convertase demonstrates the capacity of the MBL-dependent LCP to activate the complement cascade once MBL binds to its target ligand. Thus, C3b deposition signifies formation of a functional C3 convertase and as such indirectly allows for evaluation of functional C2 in a serum/plasma sample (Figure 1). The dual channel technology of this LCP assay allows for the multiplicity of endpoint evaluation using the 700 nm channel to assess MBL-dependent C3 convertase activity by observation of C3b deposition onto the mannan coated plate.

Similar to the methods used for the standardization for C4b, known purified, quantified amounts of C3b were used as standards and a log-linear relationship between the integrated intensity of activated PLS deposited C3b was observed on the 700 nm channel (Figure 4a). These data were used to standardize the PLS and its ability to deposit known amounts of C3b onto mannan-coated plates. Inter-assay coefficient variation was 5%. Specificity for MBL-dependent C3b deposition was further verified by the ability of mAb 3F8 to dose-dependently inhibit C3b deposition (Figure 4b). Serum samples prepared in the previous step for MBL analysis (Figure 2c, DN 1-4) and C4b analysis (Figure 3c) were further evaluated in the same well for MBL-dependent C3b deposition against the PLS using a log-linear regression fit and expressed as MBL-dependent C3b in pg (Figure 4c).

To confirm the precision of this MBL-dependent LCP assay, donors of known MBL haplotype (Table I) representing a broad range of functional MBL expression were analyzed for MBL and MBL-dependent C4b and C3b deposition. As observed in Figure 5a measurement of functional MBL confirms the previously reported levels of functional MBL observed in these haplotypes (Steffensen et al., 2000; Terai et al., 2003). Expanding upon these past observations, the MBL-dependent LCP activation and subsequent C4b (Figure 5b) and C3b (Figure 5c) deposition associated with these known MBL-haplotypes are quantified using this novel assay system. Activation of the LCP by the functional MBL complex (e.g., functional MASP-2) reveals a quantifiable direct proportional increase of functional MBL binding and C4b deposition (Figure 5d). We show for the first time the capacity for these various MBL

haplotypes to also deposit C3b in the same well as C4b. Previously, correlative relationships between MBL haplotype and C4b or C3b deposition in the mannan assay required multiple assays (Roos et al., 2003). Using the present mannan assay, a quantifiable proportional increase of functional MBL binding and C3b deposition was observed (Figure 5e). The MBL-dependent LCP phenotype can be summarized by evaluating MBL binding, MBL-dependent C4b and C3b deposition together (Figure 5f). This 3D graph demonstrates that functional MBL, dictated by individual MBL haplotypes, appears to drive the activation and deposition of effector LCP components.

Recently, a proposed interaction of MBL with the alternative pathway (AP) has been evaluated (Selander et al., 2006). Further, the AP mediated amplification of optimal MBL-dependent LCP-opsinophagocytosis has been demonstrated (Brouwer et al., 2006). We evaluated C4b and C3b deposition in this assay at 3–25% serum concentrations (Figure 6). As shown in Figure 6a, C4b deposition is entirely dependent on MBL, as MBL inhibition with mAb 3F8, but not factor D inhibition, completely inhibits C4b deposition at all the serum concentrations evaluated. In contrast, C3b deposition (Figure 6b) at 13% and 25% serum is dependent on MBL and alternative pathway interactions, as MBL (3F8) and factor D (anti-fD) inhibition completely inhibited C3b deposition, whereas inhibition of each of these two molecules individually was ineffective. At serum concentrations of 3% and 6%, inhibition of MBL, but not factor D effectively inhibited C3b deposition. Interestingly, in an MBL-deficient (low) donor (DN1), it is clear that MBL-dependent LCP activation (e.g., C4b and C3b deposition) is compromised in comparison to MBL-normal or high expressers (see Figure 3c and Figure 4c). However, evaluating this same donor in the window of MBL-dependent AP amplification (i.e., 25% serum), it is clear that C4b deposition remains relatively compromised (Figure 3c vs. 6c) yet C3b deposition recovers to MBL-normal levels (Figure 4c vs. 6d). Thus, this assay addresses a potential role of the MBL-dependent pathway to interact with the alternative pathway to increase C3b deposition in MBL-deficient or MBL-low expresser patients.

Many clinical database samples are present as serum and/or plasma and frozen at -80°C , which may or may not have been thawed at one time or another. The quality of these samples and their use in this assay is critically important, as the complement system could possibly be compromised by freeze/thaw cycles. In order to evaluate the potential use of serum, as well as plasma, samples were collected from the same individual as described in *Materials and Methods*. As shown in Table 2, plasma samples could be adequately evaluated in the FLISA. After addition of calcium, plasma values for MBL, C3b and C4b deposition tended to be higher than serum samples, but these values did not reach statistical significance. Serum samples subjected to multiple freeze/thaw cycles demonstrated similar values for the measured parameters even after five cycles. Thus, this assay can be used with serum or plasma (e.g., following re-calcification). Further, serum samples exposed to as many as five freeze/thaw cycles can also be used in this assay.

Discussion

We describe a novel assay that detects the functional status of the MBL-dependent portion of the LCP through the level of C3 cleavage utilizing a high-throughput liquid handling system, together with infra-red technology. This is the first multi-tiered LCP assay prototype that can successfully analyze the key components of early LCP activation from a single sample in a single assay. This analysis can be performed for serum samples and plasma samples (e.g., following re-calcification). Complete MBL-dependent LCP function to the level of C3 convertase assembly and C3 cleavage can be evaluated in a single, small volume sample of serum or plasma, making this assay quick, time and cost efficient.

Initial LCP assay technology demonstrated the correlation of MBL with activation and deposition of C4 and C3 complement fragments at low serum concentrations, without reconstitution of individual components, yet with non-quantifiable endpoints and in separate analytical steps (Super et al., 1990). Current assays still require additional sample and evaluation of additional endpoints (C4 or C3) in separate experimental steps (Roos et al., 2003), or additional sample and reconstitution with exogenous C4 (Petersen et al., 2000; Petersen et al., 2001). Importantly, the present assay evaluates the physiological contributions of the patient's own C3 and C4, including interaction with MASP-2, formation of the C3 convertase, and subsequent C3 activation and C3b deposition in a single sample analysis.

Functional MBL, MBL complex-dependent C4b and C3b activation and deposition from plasma or serum samples are evaluated in this assay on mannan-coated plates using a pooled serum standard (PLS). The PLS was batched, aliquoted and assigned a lot designation thereby establishing intra-assay consistency as reported in the *Materials and Methods*. A common PLS for multiple endpoint analysis adds to the already efficient and cost-effective analysis platform by essentially combining three individual protein standards into one sample. The PLS is specific for MBL (Figure 2), MBL-dependent C4 activation (Figure 3), and formation of MBL-dependent C3 convertase (Figure 4), as the anti-MBL mAb 3F8 can abrogate MBL binding and the subsequent C4b and C3b deposition. The PLS was also evaluated against a standard serum control commonly used in other MBL assay protocols, and independently confirmed the functional MBL content as 1µg/ml (data not shown).

Available assay techniques have correlated functional MBL in ng/ml to patient haplotype (Madsen et al., 1995) (Dean et al., 2006), therefore a representative panel of MBL haplotypes (Table 1) was selected and evaluated for functional MBL in confirmation of the described MBL-dependent LCP FLISA (Figure 5a). Evaluated MBL levels were within the standard error of the means (SE) of the previously reported values as indicated in Table 1. Thus, we have verified that MBL concentrations associated with the FLISA correspond to those that have been previously reported by other assay measurements.

Other assay platforms evaluating MBL-dependent C3 activation have looked at MBL-dependent AP amplification or a 'bypass' pathway of MBL/C3 interaction (Selander et al., 2006; Brouwer et al., 2006), although MBL-dependent C3 activation was not quantitated in either assay. In addition to the direct evaluation of MBL-specific LCP activation, the FLISA can address the mechanisms of alternate MBL-dependent complement activation recently proposed (Selander et al., 2006; Brouwer et al., 2006). It is clear that direct MBL-dependent C4b2a activation of the LCP is driven by functional MBL as shown in Figures 5 and 6, and as published (Selander et al., 2006). However, as shown in Figure 6d the capacity for MBL-dependent AP amplification may salvage the potential for downstream complement activation at the level of C3 convertases with respect to MBL deficient or 'low' expressers.

Potential limitations of the FLISA

Many clinical sample databases consist of plasma and/or serum samples, which are frozen and may have been thawed at least one time. We demonstrate that the FLISA can use serum as well as citrated plasma after addition of calcium and removal of the fibrin clot by centrifugation. Plasma samples tended to have higher values, but this would be expected from coagulation of whole blood to make serum. Further, we demonstrate that as many as five freeze/thaw cycles have no significant effect on the values obtained for MBL, C3b and C4b deposition in this assay. Thus, this assay is well suited for rapid and cost-effective evaluation of large database collections of serum/plasma samples of human disease.

Although this assay is designed for use with probes that are evaluated on far infra-red wavelengths (700 and 800nm respectively) using the LICOR imaging system, the assay technology is not limited to this detection system. As discussed in the *Materials and Methods*, optimal concentrations of detection antibodies were determined in pilot studies performed in our laboratory. By adjusting the detection antibody with other commercially available probes (e.g. signal emission on alternative wavelengths) other imaging platforms are easily adapted to perform this analysis, for example, using an immunofluorescent plate reader. This adaptation simply requires that optimal concentrations of detection probes be calibrated.

We recognize that patients with hypercatabolism of complement cause reduced complement levels that may influence this assay, particularly the level of C4, as well as hereditary C4 deficiency. These diseases include lupus erythematosus, serum sickness, certain glomerulonephritides, chronic active hepatitis, and others. Thus, C4b deposition in the FLISA could be limited by C4 deficiency or consumption, and not functional MASP-2 capacity. Complete deficiency of C4 is well documented, although rare. Addition of exogenous C4 to a C4b-deposition negative patient sample, a technique that has been described previously (Petersen et al., 2000; Petersen et al., 2001), and performing a second evaluation for MBL/MASP-2 dependent C4b deposition could be used for this rare occurrence. Alternatively, functional and quantitative MASP-2 levels may be evaluated independently as a fourth endpoint using a novel anti-MASP-2 mAb, which is currently in development in our laboratory.

Similar to C4, there is the possibility that C3b deposition in a MBL-dependent individual is limited by hereditary C3 or C2 deficiency and/or consumption. These deficiencies/consumptions however can be adequately evaluated by presently available clinical assays or as described above by adding back the potentially limiting complement component(s) (e.g., C2 and C3) to the “deficient” sample. In addition, multiple time points over time in an acute disease setting using this FLISA have clearly shown consumption of these complement components and their eventual return to ‘normal’ levels over a period of a week or more (manuscript in preparation). The low volume required for this assay and the ease of running hundreds of samples at one time, makes this assay advantageous for long term studies of complement consumption to the level of C3 cleavage via the MBL-dependent lectin complement pathway. Additionally, since one of the design purposes of this assay was for screening large databases of particular diseases, these clinical conditions are part of the patient’s history and/or have already been excluded from the clinical trial.

Summary

The present MBL-dependent LCP FLISA extends what is currently understood about functional MBL by describing quantitatively the MBL-dependent LCP activation to the level of C3 convertases. Furthermore, we demonstrate that MBL-dependent LCP activation is influenced by the MBL genotype. Because the MBL haplotype alters LCP activation through cleavage and deposition of C3b, we describe for the first time a complete analysis of MBL-dependent complement activation in a MBL-dependent LCP activation phenotype to the level of C3 cleavage in a single assay. This analysis includes the evaluation of MBL-dependent AP amplification, which may play a role in otherwise low MBL expresser individuals. This mechanism of MBL-dependent salvage should be considered with respect to the selective pressures that retains a high frequency of *MBL2* mutation in the population. MBL-deficient/low patients may be protected when it comes to self-antigen recognition following sterile challenge (i.e. I/R injury), yet MBL-dependent AP amplification may impart protection to MBL-deficient individuals by ‘alternatively’ activating the LCP following pathogenic challenge. Utilization of this high-throughput MBL-dependent LCP FLISA will further our understanding of LCP activation in disease.

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Figure 1. The Lectin Complement Pathway

This figure demonstrates the key components in LCP activation that can be analyzed using the MBL-dependent LCP FLISA. Oval outlines delineate the endpoints directly analyzed by the assay.

Figure 2. Quantitation of MBL in the standard (PLS)

A pooled human serum standard (PLS) was standardized by using MBL-deficient serum reconstituted with a human MBL standard (Staten Serum Institut). Correlation between detected units of integrated intensity (II) at 800nm and ligand binding was performed using a linear regression model to establish MBL-dependent binding in ng/ml (panel a). Specificity of functional MBL binding was demonstrated by blocking with mAb 3F8 (panel b). Using the PLS to establish functional MBL levels (panel c), triplicate values of each donor (DN 1 = donor 1, DN 2 = donor 2, DN 3 = donor 3, DN 4 = donor 4) serum sample diluted in binding buffer are expressed as mean $\pm$ SE.

Figure 3. Quantitation of MBL-dependent C4b deposition

The amount of MBL-dependent C4b that was bound to the mannan coated plates following incubation of the PLS was revealed as a log-linear relationship between the integrated intensity of activated PLS deposited C4b and known amounts of C4b (panel a). Specificity for MBL-dependent C4b deposition was verified by inhibition with mAb 3F8 (panel b). Using the PLS to establish MBL-dependent C4b levels (panel c), triplicate values of each donor (DN 1 = donor 1, DN 2 = donor 2, DN 3 = donor 3, DN 4 = donor 4) serum sample diluted in binding buffer are expressed as mean $\pm$ SE of the PLS for C4b (horizontal bars) and MBL (vertical bars).

Figure 4. Quantitation of MBL-dependent C3b deposition

The amount of MBL-dependent C3b that was bound to the mannan coated plates following incubation of the PLS was revealed as a log-linear relationship between the integrated intensity of activated PLS deposited C3b and known amounts of C3b (panel a). Specificity for MBL-dependent C3b deposition was verified by inhibition with mAb 3F8 (panel b). Using the PLS to establish MBL-dependent C3b levels (panel c), triplicate values of each donor (DN 1 = donor 1, DN 2 = donor 2, DN 3 = donor 3, DN 4 = donor 4) serum sample diluted in binding buffer are expressed as mean $\pm$ SE of the PLS for C3b (horizontal bars) and MBL (vertical bars).

Figure 5. Functional MBL, and MBL-dependent LCP activation, correlated to MBL genotype
 Serum samples from donors with the indicated MBL haplotype were evaluated for functional MBL (panel a), C4b deposition (panel b) and C3b deposition (panel c). Differences between medians of haplotype groups for MBL, MBL-dependent C4b, and MBL-dependent C3b are statistically significant; Kruskal-Wallis ANOVA on ranks ($p < 0.001$). There is a direct correlation to functional MBL concentrations with C4b deposition (panel d) and C3b deposition (panel e). The MBL-dependent LCP phenotype can be summarized by evaluating MBL binding, MBL-dependent C4b and C3b deposition together (panel f).

Figure 6. MBL-dependent AP amplification

MBL-dependent C4b deposition evaluated in 3–25% serum is inhibited by 3F8 and not anti-factor D (panel a). Alternatively, MBL-dependent AP ‘tick over’ amplification occurs approaching physiologic serum concentration (13 and 25%), as anti-human MBL mAb 3F8 together with anti-human factor D mAb can inhibit C3b deposition (panel b), but neither of these mAb can completely inhibit independently. Analysis of MBL-dependent AP amplification at 25% serum shows that although MBL-dependent C4b deposition remains compromised in DN1 (MBL-low/deficient) compared to DN 4 (MBL-high) (panel c), deficiency in MBL-dependent LCP activation can be rescued at the level of C3 convertases (panel d) when DN 1 is compared with DN 4 at 25% serum concentration. Figures 3c and 4c represent the same donors at 3% serum and demonstrated MBL-dependent C4b and C3b deposition, respectively.

Table 1

Represented MBL Haplotypes

Haplotype (Homozygotes)	Previously Reported MBL (ng/ml)*	MBL-LCP Assay MBL (ng/ml)	n	SE
HYPA/HYPA	2003 (n=54)	2028	18	364
HYPD/HYPD	61 (n=1)	2	9	1
LXPA/LXPA	118 (n=4)	292	12	104
LYPA/LYPA	1738 (n=2)	2053	4	461
LYPB/LYPB	22 (n=2)	13	12	5
LYQA/LYQA	1899 (n=5)	1739	18	383
LYQC/LYQC	0	0	3	0

* from R. Steffensen et al., Journal of Immunological Methods 241 (2000) 33–42 and Terai et al., Eur. J. Imm., 33 (2003) 2755–2763

Table 2

Comparison of plasma and serum samples.

	MBL (ng/ml)	C4b (pg)	C3b (pg)
Serum	1505±418	2797±1417	10302±4351
Plasma	2732±576	3552±619	21325±6791

Serum and plasma samples were taken from three individuals (n=3). Plasma samples were re-calcified as described in Methods. The samples were then subjected to the FLISA as described. While there was a tendency to have higher values for each of the measured parameters in the plasma samples, no significant difference was observed. Values = mean±SE

Table 3

Effects of freezing and thawing on sera samples.

0	Rounds of freeze/thaws				
	1	2	3	4	5
MBL(ng/ml)					
1418±314	1344±333	1239±269	1635±436	1431±395	1424±265
C4b (pg)					
3118±935	2469±911	2337±776	1562±547	2713±1102	2828±1031
C3b (pg)					
33800±2551	32836±2844	34424±3556	34408±3601	34379±3300	32906±3326

Sera samples were collected from four individuals and run in the FLISA as fresh (0) samples or frozen to -80°C and thawed to room temperature from 1 – 5 times and run again in the FLISA. Data above are means±SE for n=4 determinations. No significant differences were observed for MBL, C4b or C3b at any of the individual points.