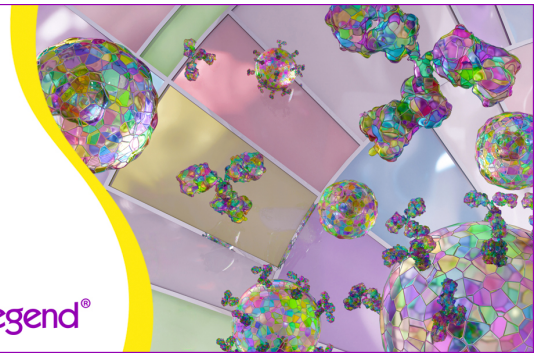


Prevent non-specific
polymer dye interaction
with True-Stain™
Multi-Fluor Buffer

Learn more ▶

BioLegend®



The Journal of Immunology

RESEARCH ARTICLE | SEPTEMBER 01 2001

Human IgA Activates the Complement System Via the Mannan-Binding Lectin Pathway¹ ✓

Anja Roos; ... et. al

J Immunol (2001) 167 (5): 2861–2868.

<https://doi.org/10.4049/jimmunol.167.5.2861>

Related Content

Different Molecular Events Result in Low Protein Levels of Mannan-Binding Lectin in Populations from Southeast Africa and South America

J Immunol (September,1998)

Different effects of native *Candida albicans* mannan and mannan-derived oligosaccharides on antigen-stimulated lymphoproliferation in vitro.

J Immunol (January,1990)

Immunologic significance of diverse specificity of monoclonal antibodies against mannans of *Candida albicans*.

J Immunol (November,1989)

Human IgA Activates the Complement System Via the Mannan-Binding Lectin Pathway¹

Anja Roos,^{2*} Lee H. Bouwman,* Daniëlle J. van Gijlswijk-Janssen,* Maria C. Faber-Krol,* Gregory L. Stahl,[†] and Mohamed R. Daha*

The recently identified lectin pathway of the complement system, initiated by binding of mannan-binding lectin (MBL) to its ligands, is a key component of innate immunity. MBL-deficient individuals show an increased susceptibility for infections, especially of the mucosal system. We examined whether IgA, an important mediator of mucosal immunity, activates the complement system via the lectin pathway. Our results indicate a dose-dependent binding of MBL to polymeric, but not monomeric IgA coated in microtiter plates. This interaction involves the carbohydrate recognition domain of MBL, because it was calcium dependent and inhibited by mannose and by mAb against this domain of MBL. Binding of MBL to IgA induces complement activation, as demonstrated by a dose-dependent deposition of C4 and C3 upon addition of a complement source. The MBL concentrations required for IgA-induced C4 and C3 activation are well below the normal MBL plasma concentrations. In line with these experiments, serum from individuals having mutations in the MBL gene showed significantly less activation of C4 by IgA and mannan than serum from wild-type individuals. We conclude that MBL binding to IgA results in complement activation, which is proposed to lead to a synergistic action of MBL and IgA in antimicrobial defense. Furthermore, our results may explain glomerular complement deposition in IgA nephropathy. *The Journal of Immunology*, 2001, 167: 2861–2868.

The complement system is an important component of host defense. Activation of the complement cascade takes place upon the interaction of complement components with a variety of pathogens, either directly or via Abs bound to pathogen Ags. Three different pathways for complement activation have been described, the classical pathway, the alternative pathway, and the lectin pathway, of which the latter is most recently identified. The lectin pathway is mainly driven by mannan-binding lectin (MBL).³

MBL, a member of the collectin family (1), is a C-type lectin present in serum as a part of a large proenzymatic complex. The MBL protein consists of three to six identical homotrimeric subunits. Each trimer is composed of a collagen-like tail part and a globular head part containing a carbohydrate recognition domain (CRD). The collagen-like part of MBL interacts with the MBL-associated serine proteases MASP-1 (2), MASP-2 (3), and MASP-3 (4). These enzymes are responsible for the complement-activating properties of the MBL complex, by the cleavage of C4, C2, and C3 (5). The CRD of MBL is able to bind in a calcium-dependent way to a number of saccharides, such as D-mannose,

L-fucose, and N-acetylglucosamine (GlcNAc) (1). MBL binding to a ligand induces activation of the MASP enzymes, leading to complement activation up to the terminal pathway. Both the structural and functional properties of MBL are strikingly similar to those of C1q, the recognition unit of the classical complement pathway.

Genetic mutations in the MBL gene are present with a high frequency in the human population (6). Until now, three different point mutations have been described that lead to the production of MBL with structural aberrations and impaired complement-activating properties (6, 7). Heterozygous and homozygous expression of these mutant alleles is associated with an enhanced incidence of a range of infections, in both children and adults (8, 9). In this spectrum of diseases, mucosal infections, occurring in the respiratory tract and the gastrointestinal tract, are common. Furthermore, mutations in the MBL gene have a significant negative impact on chronic diseases such as rheumatoid arthritis, systemic lupus erythematosus, and cystic fibrosis, resulting in an increased incidence of complicating infections and/or a worse outcome (10–12). These studies indicate the importance of the lectin pathway of complement activation in antimicrobial defense. In line of this function of the lectin pathway, MBL is able to bind directly to a number of microorganisms, via the carbohydrates expressed on their surface (1, 13). Upon binding, complement activation takes place, leading to either direct elimination via the terminal complement pathway, or opsonization and phagocytosis.

In the mucosal immune system, a major factor of defense is IgA. It is present in plasma at a concentration of ~2 mg/ml, and it is secreted at mucosal surfaces throughout the body, where it is postulated to play an important role as a defense mechanism against invading microorganisms (14, 15). Upon interaction of IgA with pathogens, the IgA molecule can have diverse effector functions, including the direct prevention of invasion of microorganisms, the interaction with the phagocytic IgA Fc receptor CD89, and complement activation.

Complement activation by IgA has been previously shown to involve the alternative, but not the classical complement pathway

*Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands; and [†]Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

Received for publication February 27, 2001. Accepted for publication June 25, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ These studies were supported by grants from the European Community (EC-Bio-tech; Bio4-CTD97-2242) and from the Dutch Kidney Foundation (no. PC95).

² Address correspondence and reprint requests to Dr. Anja Roos, Department of Nephrology, D3P, Leiden University Medical Center, Postbox 9600, 2300 RC Leiden, The Netherlands. E-mail address: A.Roos@LUMC.NL

³ Abbreviations used in this paper: MBL, mannan-binding lectin; CRD, carbohydrate recognition domain; Dig, digoxigenin; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; MASP, MBL-associated serine protease; NHS, normal human serum; VBS, Veronal-buffered saline.

(16, 17). No data are available concerning the possible involvement of the lectin pathway. A classical disease involving IgA and complement activation is primary IgA nephropathy, a common renal disease involving mesangial deposition of IgA and complement components, resulting in end stage renal failure in ~30% of the patients (18). Recent studies indicate the presence of MBL in association with IgA in the mesangial area of patients with IgA nephropathy (19, 20).

Therefore, we examined whether IgA induces complement activation via a possible interaction with MBL. Our results demonstrate activation of the lectin pathway by IgA. This novel interaction in the immune system is proposed to contribute to the roles of MBL and IgA in host defense, as well as to the pathogenesis of IgA nephropathy.

Materials and Methods

Purification of MBL/MASP complexes

MBL and its associated proteases were purified from pooled plasma obtained from healthy human donors, essentially as described by Tan et al. (21). In brief, a precipitation step was performed using polyethylene glycol 3350 (Sigma, St. Louis, MO; 7% w/v). The precipitate was dissolved in TBS-T/Ca²⁺ (50 mM Tris, 0.15 M NaCl, 0.05% Tween 20, 20 mM CaCl₂, pH 7.8) and incubated for 18 h at 4°C with mannan-agarose (Sigma; equilibrated with TBS-T/Ca²⁺). After extensive washing with TBS-T/Ca²⁺/1 M NaCl, bound proteins were eluted using TBS-T containing 10 mM EDTA. Fractions containing MBL, as determined by ELISA, were pooled and concentrated. To remove contaminating Igs, the MBL preparation was absorbed using a mixed absorbent consisting of 4E8 (mAb anti-IgA, produced in the Laboratory of Nephrology, Leiden, The Netherlands) coupled to Biogel A5 (Bio-Rad, Richmond, CA), HB57 (mAb anti-IgM, hybridoma obtained from the American Type Culture Collection, Manassas, VA) coupled to Biogel A5, and protein G coupled to Sepharose (from Pharmacia, Uppsala, Sweden). The resulting MBL preparation contained negligible amounts of IgA (<0.5%), whereas IgG and IgM were undetectable. Furthermore, the MBL preparation did not contain any detectable C1q, as determined by single radial immunodiffusion and by a sensitive C1q-specific hemolytic assay. This purification method results in copurification of MASP proteins, as shown by Western blotting using rabbit anti-MASP-1 Abs, prepared as described (3), and rabbit anti-MASP-2 Abs (kindly provided by R. B. Sim, University of Oxford, Oxford, U.K.), as well as by C4 consumption assays (data not shown). The resulting MBL-MASP preparation was subjected to ELISA to determine the MBL concentration (as described below), and subsequently used in all experiments.

Purification of human IgA

IgA was purified from pooled normal human serum (NHS) or recalcified donor plasma, as described by Hiemstra et al. (16), with minor modifications. In brief, the majority of serum proteins were removed by dialysis against H₂O, and precipitation by ZnSO₄. Proteins in the supernatant were precipitated using glycine and (NH₄)₂SO₄, dialyzed against TE buffer (10 mM Tris, 2 mM EDTA, pH 7.8), and loaded on a DEAE-Sephacel column (Pharmacia). IgA was eluted with a linear salt gradient (conductivity 1–20 mS). IgA-containing fractions, as determined by ELISA, were pooled, concentrated, and further purified by gel filtration, using a Sephacryl S-300 column (Pharmacia). Veronal-buffered saline (VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 2 mM EDTA was used as a running buffer. Fractions were tested for IgA by ELISA; IgA of different molecular sizes, i.e., monomeric, dimeric, and polymeric IgA, were pooled separately on basis of its position in the elution profile. Reanalysis of these different IgA preparations indicated that this molecular size is stable: typically >90% runs at the same position in the elution profile. Contamination with IgG and IgM was below 0.4% on a weight basis, as determined by ELISA. Experiments were performed with polymeric IgA, unless otherwise indicated.

Purification of human IgG and IgM

Human IgG was purified from outdated plasma obtained from healthy donors, as described previously (22). Human IgM was purified from human serum by eglobulin precipitation and anion exchange chromatography, as described (22), followed by cation exchange chromatography, using CM-Sephadex C-50 (Pharmacia), and gel filtration, using a Sephacryl S-300 column (Pharmacia). The IgG preparation was free of any detectable IgA and IgM, and the IgM preparation was free of any detectable IgA and IgG.

Purification of functionally active C4

Freshly obtained NHS (120 ml) was adjusted with TEB buffer (10 mM Tris, 2 mM EDTA, 1 mM benzamidine hydrochloride hydrate (Sigma)) to pH 7.8 and conductivity of 4 mS. This sample was loaded on a DEAE-Sephacel column (5 × 10 cm), which was previously equilibrated with TEB buffer at pH 7.8 and mS 4. The column was extensively washed with the same buffer, followed by elution with a linear salt gradient with a conductivity from 4 to 25 mS. Fractions were tested for the presence of C4 using a hemolytic assay. In this assay, serum from C4-deficient guinea pig is used as a complement source and SRBC sensitized with rabbit anti-SRBC Abs as targets. Lytic activity of an excess amount of C4-deficient serum can be restored by addition of a limiting amount of C4. Peak fractions containing C4 (at 15 mS) were pooled and concentrated. The concentration of C4 was determined by single radial immunodiffusion.

Human serum containing wild-type or mutated MBL

Serum samples were obtained from five healthy donors having the wild-type genotype at codons 52, 54, and 57 from the first exon of the MBL gene (A genotype) as well as from six healthy donors with a homozygous (*n* = 2) or heterozygous (*n* = 4) mutation at codon 54 (B genotype) (6). These genotypes were identified by an oligonucleotide ligation assay (A. Roos, L. H. Bouwman, J. R. Muñoz, T. C. M. Zuiverloon, M. Tilanus, and M. R. Daha, manuscript in preparation) and were confirmed by DNA sequencing.

ELISA protocol

For all ELISAs, Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated using coating buffer (100 mM Na₂CO₃/NaHCO₃, pH 9.6), for 16 h at room temperature or for 2 h at 37°C. After each step, plates were washed three times with PBS containing 0.05% Tween 20. Residual binding sites were blocked by incubation with PBS containing 1% BSA. Unless otherwise indicated, all subsequent steps were incubated in PBS containing 0.05% Tween 20 and 1% BSA, for 1 h at 37°C. Detection Abs were conjugated to digoxigenin (Dig) using Dig-3-*O*-methylcarbonyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester (from Boehringer Mannheim, Mannheim, Germany), according to instructions provided by the manufacturer. Detection of binding of Abs conjugated to Dig was performed by HRP-conjugated rabbit anti-Dig Abs (Fab, from Boehringer Mannheim). Enzyme activity of HRP was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). The OD at 415 nm was measured using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT).

MBL detection ELISA

Plates were coated with 3E7 (mAb anti-MBL (mouse IgG1, kindly provided by T. Fujita, Fukushima Medical University School of Medicine, Fukushima, Japan)) at 5 μ g/ml. Samples containing MBL were incubated, followed by detection with Dig-conjugated 3E7. A calibration line was produced using pooled human serum from healthy donors with a known concentration of MBL (kindly provided by P. Garred, National University Hospital, Copenhagen, Denmark).

MBL binding to IgA or mannan

IgA was coated at 5 μ g/ml, unless otherwise indicated. Mannan (from *Saccharomyces cerevisiae*; Sigma (M7504)) was coated at 100 μ g/ml. As a negative control, BSA (Sigma) was coated at 10 μ g/ml. After blocking with BSA, MBL was incubated in BVB²⁺ (VBS, 0.5 mM MgCl₂, 1 mM CaCl₂, 0.05% Tween 20, 1% BSA, pH 7.5) for 1 h at 37°C. MBL binding was detected using Dig-conjugated 3E7. In some experiments, MBL was preincubated (30 min, 20°C) in a calcium-free buffer (BVB/MgEGTA: VBS, 5 mM MgCl₂, 0.05% Tween 20, 1% BSA, 10 mM EGTA) or in BVB²⁺ containing D-mannose, L-fucose, GlcNAc, or *N*-acetylgalactosamine (GalNAc) (obtained from Sigma), followed by addition of the mixture to the plates. Saccharides were applied at a concentration of 100 mM, unless otherwise indicated. Additional inhibition studies were performed using purified mouse mAb directed against MBL (1C10 and 3F8; Ref. 23), which were incubated together with MBL at concentrations between 2 and 20 μ g/ml.

Analysis of complement activation by MBL

Activation of complement via MBL was assessed as follows. Plates were coated with IgA, mannan, or BSA, blocked by BSA, and, in some cases, incubated with MBL, as described above. Subsequently, plates were incubated with 2% NHS as a complement source, diluted in BVB/MgEGTA, for 1 h at 37°C. Deposition of C3 was detected by Dig-conjugated RfK22 (mAb anti-human C3 (mouse IgG1) produced at the Laboratory of Nephrology). Alternatively, C4 activation was assessed using a method

adapted from Vorup-Jensen et al. (24). For these experiments, MBL, diluted in BVB²⁺, was incubated for 1 h at 37°C and for 16 h at 4°C. Plates were washed with PBS/5 mM CaCl₂/0.05% Tween 20, and C4 was added (1 μg/ml, diluted in BVB²⁺ containing 1 mM MgCl₂ and 2 mM CaCl₂). C4 binding was detected with affinity-purified goat anti-human C4 Abs conjugated to Dig, or with Dig-conjugated C4-4A (mAb anti-C4, kindly provided by C. E. Hack, Sanquin Blood Supply Foundation, Amsterdam, The Netherlands). In some experiments, activation of C4 was assessed directly in human serum. For this assay, all washing and incubation steps were performed in the absence of Tween 20, which reduced nonspecific staining. Plates were coated with IgA, mannan, IgG (5 μg/ml), or IgM (5 μg/ml), washed with PBS, and blocked by 1% gelatin in PBS. Serum was diluted in VBS containing 2 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% gelatin, and incubated for 1 h at 37°C. Subsequently, C4 binding was detected, using PBS/1% BSA as a dilution buffer for Ab conjugates.

Statistical analysis

Differences in C4 activation between sera from two groups of donors (i.e., either MBL wild-type or MBL mutant genotype) were analyzed using a *t* test, and are considered statistically significant when *p* values are <0.05.

Results

Interaction of MBL with IgA

The binding of MBL to IgA was studied using microtiter plates coated with purified human IgA. Addition of MBL resulted in a dose-dependent binding to IgA, but not to a control coating with

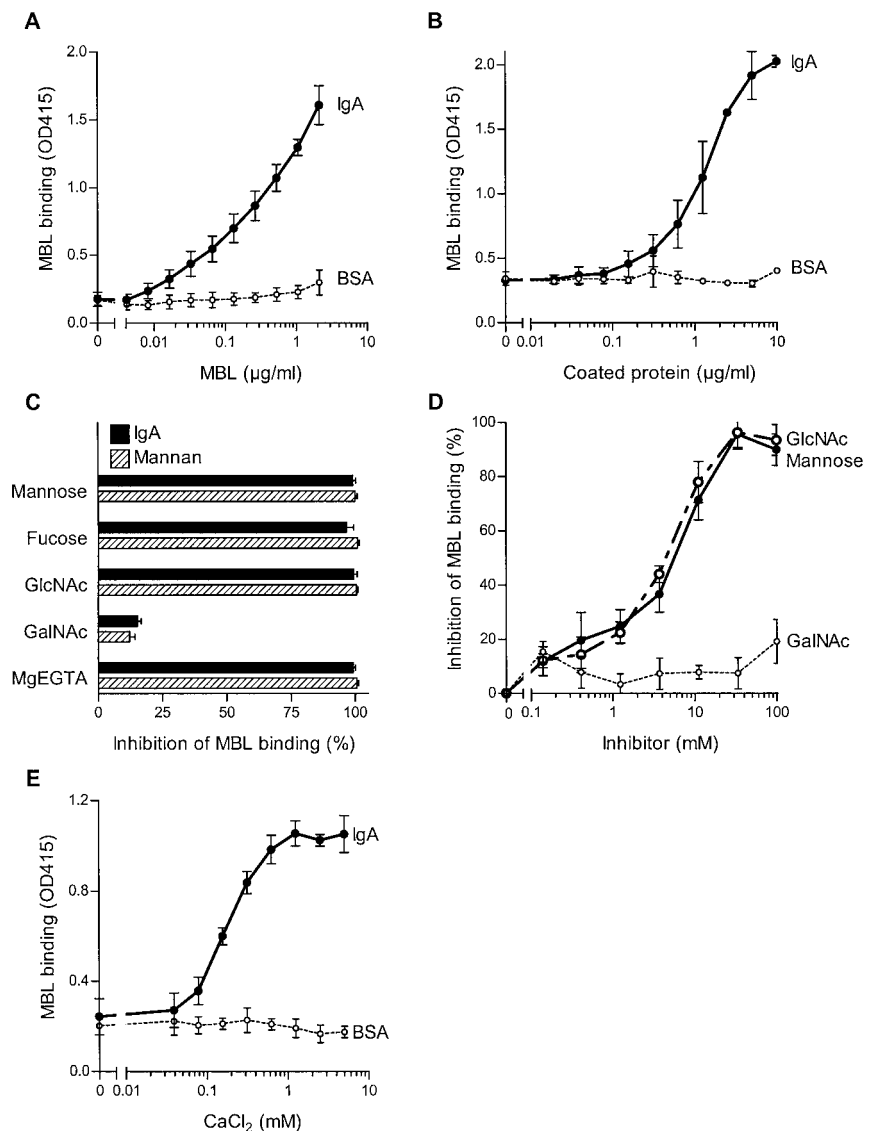
BSA only (Fig. 1A). Binding was clearly detectable at an MBL concentration of 20 ng/ml. Coating of different concentrations of IgA, followed by incubation with a fixed concentration of MBL revealed that MBL binding was maximal at an IgA-coating concentration of 5 μg/ml (Fig. 1B).

The characteristics of the interaction between MBL and IgA were studied by preincubating MBL in the presence of various inhibitors. Preincubation with D-mannose, L-fucose, or GlcNAc, but not GalNAc blocked the binding of MBL to IgA and to its major ligand mannan (Fig. 1C). This inhibition by saccharides was dose dependent: IC₅₀ values for mannose and GlcNAc were between 5 and 10 mM for binding of MBL to IgA (Fig. 1D) and to mannan (data not shown). Furthermore, incubation of MBL in a calcium-free buffer containing MgEGTA prevented binding of MBL to IgA and to mannan (Fig. 1C). Binding of MBL to IgA is calcium dependent and reaches a plateau at 1 mM CaCl₂ (Fig. 1E). This concentration was chosen for further assays. These results indicate a calcium-dependent interaction of the CRD of MBL with human IgA.

IgA activates the complement system via the lectin pathway

To assess whether the interaction of MBL with IgA induces complement activation, activation of C4 and C3 was studied by

FIGURE 1. Binding of MBL to immobilized IgA. Microtiterplates were coated with either IgA or BSA, as indicated. MBL binding was detected by ELISA. **A**, MBL was added to coated IgA (5 μg/ml) or BSA at concentrations as indicated. Data represent mean ± SD from four independent experiments. **B**, IgA was coated at various concentrations, and binding of a fixed concentration of MBL (2 μg/ml) was detected. Data represent mean ± SD from two independent experiments. **C**, Wells were coated with mannan or IgA, and incubated with MBL (0.1 μg/ml on mannan; 1 μg/ml on IgA) either under standard assay conditions (control) or in the presence of MgEGTA, D-mannose, L-fucose, GlcNAc, or GalNAc, as indicated in *Materials and Methods*. The percentage inhibition of MBL binding to IgA or mannan was calculated using the following formula: $100 - (100 \times (\text{MBL binding (+ inhibitor)} - \text{MBL binding to BSA}) / (\text{MBL binding (control)} - \text{MBL binding to BSA}))$, using OD values measured at 415 nm. None of the inhibitors affected the background binding of MBL to BSA. Mean and SD are shown of triplicate wells of a representative experiment. Similar results were obtained in five independent experiments. **D**, Similar experiment as shown in **C**, but the inhibitors were applied at different concentrations, as indicated (mean ± SD from two independent experiments). **E**, Wells were incubated with MBL (1 μg/ml) in the presence or absence of different concentrations of CaCl₂, as indicated. Data represent mean ± SD from two of three similar experiments.



ELISA. For experiments studying activation of C3, NHS diluted in a MgEGTA-containing buffer was used as a complement source. The use of MgEGTA prevented activation of the classical pathway and the lectin pathway in the complement source that may occur irrespective of the MBL that was previously bound to the coating, resulting in low background levels. Binding of MBL to IgA and to mannan, which was first achieved in the presence of calcium, was clearly detectable after a 1-h incubation with serum in the presence of MgEGTA (Fig. 2, *A* and *B*), as has been previously reported for the binding of MBL to mannan-coated erythrocytes (25). Using these conditions, binding of purified MBL-MASP complexes induced a concentration-dependent deposition of C3 on coated IgA (Fig. 2*C*) and on coated mannan (Fig. 2*D*) upon addition of serum. Coated mannan requires ~10 times less MBL than coated IgA to induce the same level of MBL binding and C3 deposition (Fig. 2, *A* and *C* vs *B* and *D*).

MBL binding to IgA and mannan also resulted in activation of C4. After binding of MBL-MASP complexes to either IgA (Fig. 2*E*) or mannan (Fig. 2*F*), addition of purified C4 resulted in a dose-dependent deposition of C4 on the coating. C4 activation was detectable at MBL concentrations between 0.01 and 1 ng/ml, depending on the coating used. MASP enzymes required for activation of C4 are present in the MBL preparation, as demonstrated by

its direct ability to induce C4 consumption in the fluid phase (data not shown).

The results presented above strongly suggest that activation of C3 and C4 is induced by the binding of MBL and associated MASPs to IgA or mannan. To further establish this, the MBL preparation was preincubated on coated IgA or mannan in the presence of inhibitors, followed by addition of a complement source and analysis of deposition of C3 (Fig. 3*A*) and C4 (Fig. 3, *B* and *C*). As expected for an MBL-dependent mechanism, preincubation of MBL with mannose, fucose, and GlcNAc, but not GalNAc, blocked the activation of C3 and C4, both on IgA and mannan. Inhibition of C4 activation by mannose and GlcNAc was dose dependent, and 50% inhibition was reached at saccharide concentrations between 5 and 10 mM, both on IgA (Fig. 3*C*) and on mannan (data not shown). Similar dose-response relationships were observed for saccharide inhibition of C3 activation (data not shown).

Incubation of MBL on mannan performed in the presence of MgEGTA completely inhibited subsequent C3 and C4 activation (Fig. 3, *A* and *B*). On IgA, similar results were obtained for C4 deposition (Fig. 3*B*), whereas deposition of C3 was inhibited for a major part, but not completely (Fig. 3*A*).

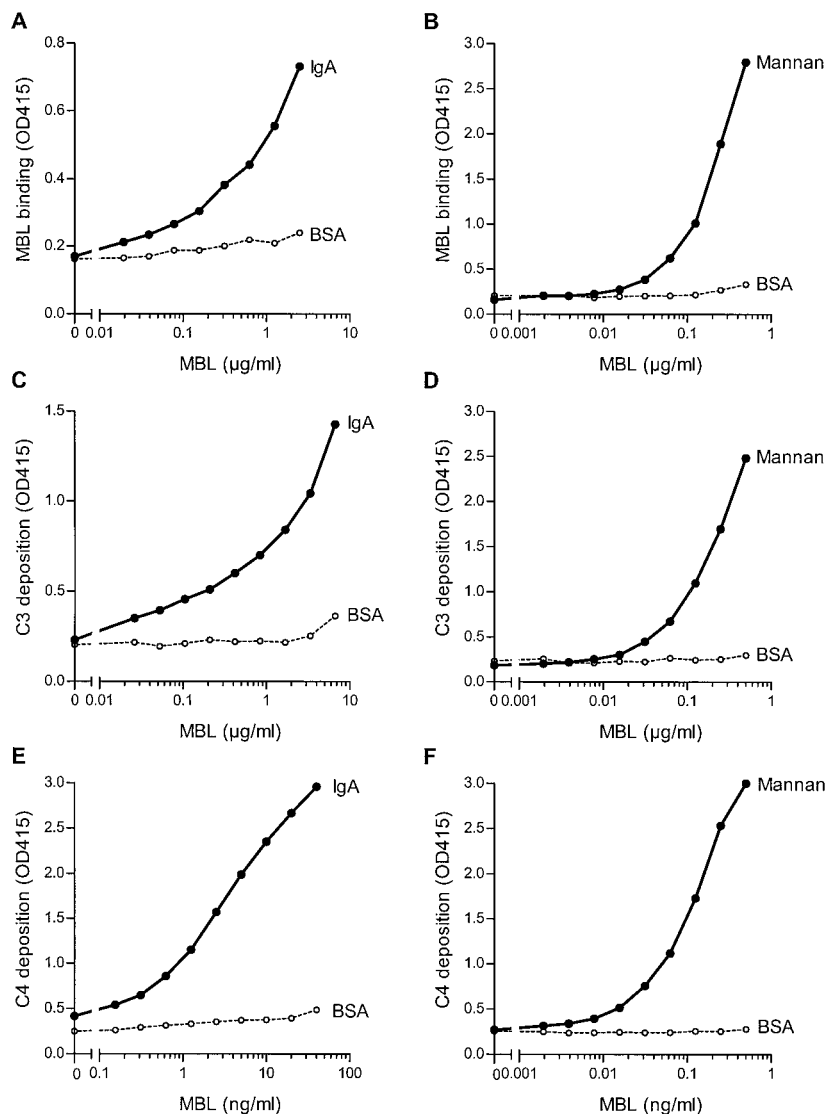
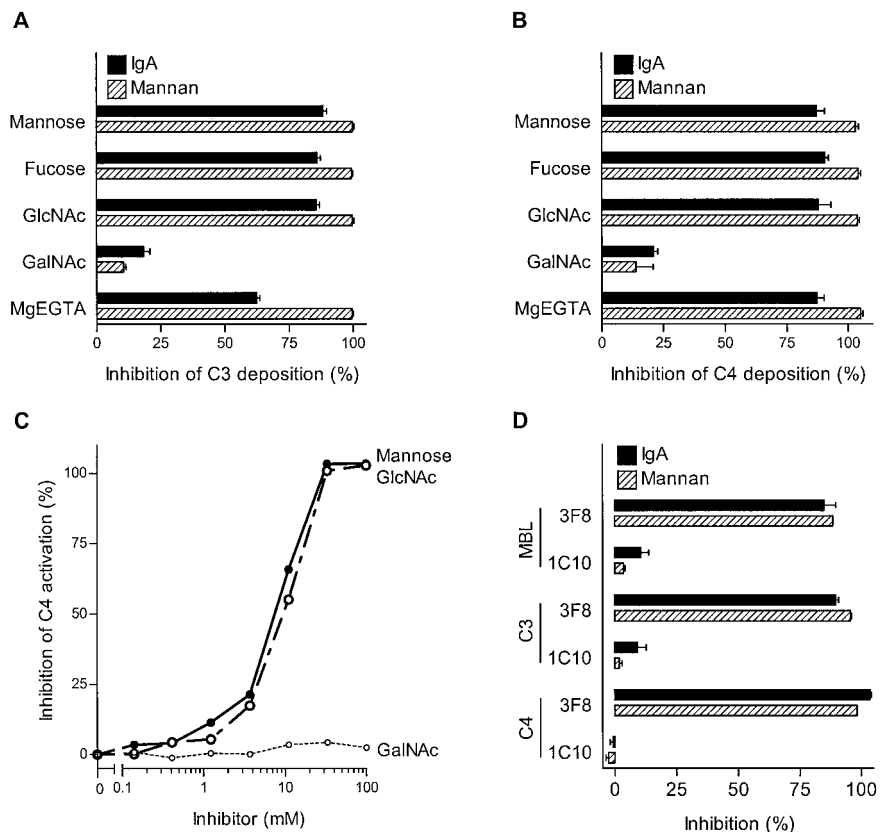


FIGURE 2. Binding of MBL to IgA induces complement activation. Wells were coated with IgA, mannan, or BSA, and in the first step incubated with MBL-MASP complexes in various concentrations, as indicated, followed by addition of a complement source in the second step. For *A–D*, the second step consisted of NHS (2% in VBS/BSA/Tween 20/MgEGTA), which was followed by detection of binding of MBL (*A* and *B*) or deposition of C3 (*C* and *D*). Alternatively, purified C4 was added in the second step, followed by detection of C4 binding (*E* and *F*). Please note that MBL concentrations on the *x*-axis in *E* and *F* are in nanogram per milliliter. The results are representative for at least three independent experiments.

FIGURE 3. Complement activation is dependent on MBL binding. Plates were coated with mannan or IgA, followed by incubation with MBL in the presence of inhibitors, as described in Fig. 1C. *A*, MBL was incubated at 1 and 0.1 $\mu\text{g/ml}$ on IgA and mannan, respectively, followed by addition of NHS (2% in VBS/BSA/Tween 20/MgEGTA) and assessment of C3 deposition. *B*, MBL was incubated at 2 and 0.1 ng/ml on IgA and mannan, respectively, followed by addition of C4 and assessment of C4 binding. Results in *A* and *B* are mean \pm SD of one of two or three triplicate experiments. *C*, Similar experiment as shown in *B*, but the saccharides were applied at different concentrations, as indicated. *D*, MBL was incubated on IgA and mannan in the presence or absence of mAb anti-MBL (3F8 and 1C10), as indicated. Binding of MBL and activation of C3 and C4 were assessed as described in Fig. 1 and in *A* and *B*, respectively. Anti-MBL Abs were used at 20 $\mu\text{g/ml}$ (MBL-binding experiments) or at 2 $\mu\text{g/ml}$ (complement activation experiments). Results represent the mean \pm SD of triplicate experiments. Inhibition was calculated as described in Fig. 1C.



To further prove the MBL dependence of C3 and C4 activation on IgA and mannan, additional blocking studies were performed using mAb anti-MBL. Two different MBL-specific mAb were used (Fig. 3D): 3F8 that blocks MBL-mediated complement activation and, as a control, 1C10 that binds to MBL, but does not block its function (23). The mAb 3F8 totally inhibited the binding of MBL as well as the activation of C3 and C4 on IgA and on mannan, whereas mAb 1C10 did not have any effect.

Complement activation is known to be predominantly a function of polymeric IgA (26). We tested the different molecular sizes of IgA for their ability to activate the lectin pathway. Polymeric IgA is superior to dimeric IgA in activation of the lectin pathway (Fig. 4). No significant activation could be detected by monomeric IgA.

Previous studies have shown that IgA can activate the alternative pathway in serum in the presence of MgEGTA (16). Only the

alternative pathway, but not the classical pathway nor the lectin pathway, can proceed in the absence of Ca^{2+} . To examine the combined contribution of the lectin pathway and the alternative pathway to activation of C3 by IgA, complement activation was studied with or without a preincubation with MBL. Serum incubated in the presence of MgEGTA, but in the absence of MBL, induced a clear deposition of C3 on IgA (Fig. 5), in agreement with previously published data (27). Preincubation with MBL in a calcium-containing buffer enhanced the deposition of C3 dose dependently. Deposition of C3 was reduced to background levels when EDTA was present in the complement source (data not shown).

Serum containing mutated MBL has a partial defect in the activation of C4 by mannan and by IgA

MBL derived from individuals with mutations in exon 1 of the MBL gene has an impaired ability to activate the complement

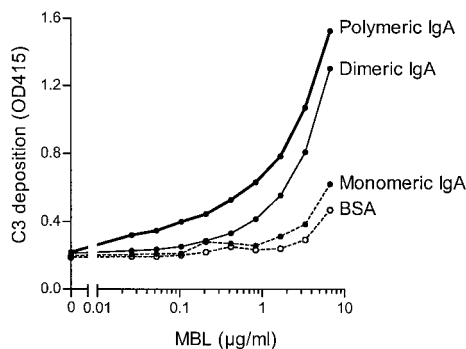


FIGURE 4. Lectin pathway activation is mainly a function of di- and polymeric IgA. Plates were coated with monomeric, dimeric, and polymeric IgA. MBL in different concentrations and NHS (2% in VBS/BSA/Tween 20/MgEGTA) were successively incubated, and C3 binding was detected. One of two similar experiments is shown.

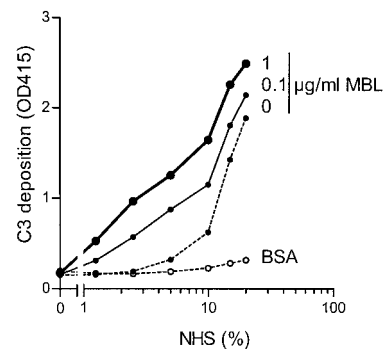


FIGURE 5. IgA activates both the lectin pathway and the alternative pathway. Plates were coated with IgA or BSA, as indicated, and incubated in the presence or absence of MBL, followed by NHS in different concentrations in VBS/BSA/Tween 20/MgEGTA. C3 deposition was detected. Similar results were obtained in two experiments.

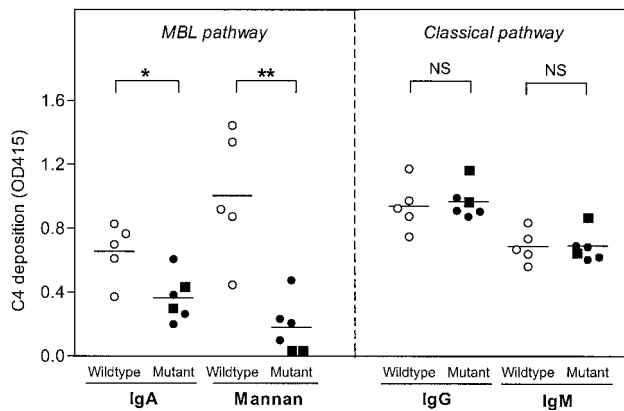


FIGURE 6. C4 activation in serum with wild-type or mutated MBL. Plates were coated with IgA, mannan, IgG, or IgM. Sera obtained from donors with a wild-type ($n = 5$) or mutant MBL genotype (heterozygous ($n = 4$; circles) or homozygous ($n = 2$; squares)) were incubated in a calcium and magnesium-containing buffer (dilution 1/80 (IgA, mannan), 1/160 (IgG), or 1/40 (IgM)), and C4 activation was assessed. Background OD values obtained in the absence of serum were subtracted. The mean of each group is indicated by a horizontal line. Differences between mutant and wild-type sera were evaluated by a t test. *, $p = 0.015$; **, $p = 0.001$.

system (7). To examine whether this defect also hampers complement activation by IgA, serum from MBL wild-type donors ($n = 5$) was compared with that from donors with a mutant genotype (homozygous ($n = 2$) or heterozygous ($n = 4$) point mutations at codon 54) in a C4 activation assay. In comparison with the control group, serum from donors having MBL gene mutations induced significantly less C4 activation both on IgA ($p = 0.015$) and on mannan ($p = 0.001$) (Fig. 6). However, when plates were coated with human IgG or IgM, as activators of the classical complement pathway, both groups of sera induced a similar level of C4 activation.

Discussion

The present study demonstrates that the CRD of MBL can bind to IgA and thereby activate the complement system via the lectin pathway. We propose that this interaction between the lectin pathway and IgA may function as a novel link between the innate and the adaptive immune system. Furthermore, the interaction between MBL and IgA is expected to contribute to mesangial complement deposition in IgA nephropathy.

Our results demonstrate a calcium-dependent interaction of the CRD of MBL with IgA. This binding was evident at concentrations well below the mean MBL plasma concentration in healthy individuals, which is $\sim 1.5 \mu\text{g/ml}$. The carbohydrate specificity of the MBL-IgA interaction is similar to that of the interaction of MBL with mannan, and is consistent with the known specificity of MBL (1). Binding of MBL to IgA induced lectin pathway activation, as demonstrated by activation of C4 and C3. Complement activation on mannan- and IgA-coated plates was inhibited by incubating the MBL preparation in the presence of mannose, in the absence of calcium, or in the presence of an MBL-blocking mAb, which is fully consistent with an MBL-dependent mechanism.

Although it has been demonstrated that MASP-1 can directly activate C3 in the fluid phase (5), we could not detect deposition of activated C3 when mannan-coated plates were incubated consecutively with MBL/MASP complexes and purified C3 (data not shown), in agreement with data reported by Vorup-Jensen et al. (24). This discrepancy is most likely due to differences in the experimental settings, including the method of detection of C3 acti-

vation and the concentration and activity of MASP-1 present. In contrast, activation of C4 was readily detectable after incubation with purified C4 under similar conditions, both on IgA and on mannan. Activation of C3 was demonstrated when NHS was used as a complement source. In the latter experiment, we show that MBL binding to plate-coated IgA or mannan is preserved during a 1-h incubation step with a calcium-free buffer, although calcium is required to establish the primary interaction of MBL with its ligands. Similar characteristics are known for the binding of MBL to mannan-coated erythrocytes (25). The MBL-MASP interaction, which is required for complement activation, is also stable in a calcium-free environment (24).

C3 activation by IgA was also demonstrated when serum was used as a complement source in the absence of calcium and without preincubation with MBL. Calcium-independent C3 activation is consistent with activation of the alternative pathway by IgA, which is in agreement with previously published data (16, 27). Apparently, different complement activation pathways cooperate to induce activation of C3 by IgA.

To further establish a role for the lectin pathway in complement activation by IgA in whole serum, we compared sera obtained from donors having either a wild-type or a mutant MBL genotype for their ability to activate C4. Our data indicate that sera from donors having heterozygous or homozygous mutations in the first exon of the MBL gene are partially deficient in activation of C4 both by IgA and by mannan. Because C4 activation by the classical pathway is similar in both groups, the observed differences in mannan- and IgA-induced C4 activation cannot be based on differences in the classical pathway activity or the concentration of active C4. Therefore, these data are strongly suggestive for the involvement of the lectin pathway in C4 activation by IgA in whole serum.

Complement activation by IgA has been subject of investigation already during several decades. It is generally agreed that IgA cannot activate the classical complement pathway (14). Activation of the alternative complement pathway by IgA is supported by both in vitro (27–29) and in vivo observations (30), as well as by the present study. It has been argued that complement activation by IgA has to rely on studies using artificially modified or presented IgA (14). However, complement activation has also been demonstrated upon binding of IgA to its natural Ag. For example, xenoreactive human IgA Abs can induce complement-mediated lysis of pig endothelial cells in a calcium-independent way (28). Furthermore, binding of human serum IgA to *Streptococcus pneumoniae* induces neutrophil-mediated bacterial killing that was complement dependent and proceeded in the presence of MgEGTA (29).

A strong suggestion for the activation of complement by human IgA in vivo is present in patients with IgA nephropathy. IgA nephropathy is a common glomerular disease characterized by mesangial deposition of IgA and complement components (31). Furthermore, deposition of C4 and C4-binding protein was shown in 30 and 60% of cases, respectively, whereas only 6% showed the presence of C1q (31). Alternative pathway activation by IgA may explain the deposition of C3, but not that of C4 in IgA nephropathy. Therefore, activation of C4 by IgA via the lectin pathway, as demonstrated in the present study, may very well be the mechanism of C4 activation in IgA nephropathy. This hypothesis is strongly supported by the deposition of MBL in association with IgA in the mesangial area of patients with IgA nephropathy (19, 20) and patients with Henoch-Schönlein purpura (32). The latter disease is also characterized by mesangial deposition of IgA and complement.

Lectin pathway activation by IgA was most prominent for polymeric IgA, followed by dimeric IgA and monomeric IgA. Similar

differences have been previously reported for activation of the alternative pathway by rat and human IgA (16, 26, 29). In addition, polymeric IgA shows enhanced binding to the phagocytic IgA FcR CD89 (33) and to human mesangial cells (34). The stronger effector functions of polymeric IgA have a beneficial role for the defense functions of IgA (29, 35, 36). In accordance, circulating Ag-specific IgA produced upon primary pathogen contact predominantly consists of polymers (29). The polymeric nature of mesangial IgA in IgA nephropathy (37) will most likely contribute to the development of renal damage, involving complement activation and mesangial cell activation.

At present it is unknown which part of the IgA molecule is involved in binding to the CRD of MBL. IgA is a heavily glycosylated molecule (reviewed in Ref. 15). Several variants in the sugar composition have been described, among which high mannose type *N*-linked glycan chains (38). Especially the latter glycosylation variant may be a likely candidate to serve as a ligand for MBL. Interestingly, circulatory IgA in patients with IgA nephropathy shows an abnormal glycosylation, characterized by a decreased galactosylation of *O*-linked sugar chains (39). Patients with rheumatoid arthritis produce increased levels of a certain glycoform of IgG that lacks the terminal galactose moieties on the *N*-terminal glycan chains. This G₀-IgG has been shown to bind MBL (40). The hypothesis that altered glycosylation of IgA contributes to complement activation in IgA nephropathy is presently under investigation.

Binding of IgA to microorganisms enables its interaction with phagocytes via the phagocytic Fc α receptor CD89 (29). Together with complement activation, this may result in pathogen elimination, involving CD89 and complement receptors. MBL binding may directly contribute to phagocytosis via MBL receptors (41, 42). In this respect, it is conceivable that complement receptors and IgA receptors act together in pathogen elimination (29). In a similar way, classical pathway activation via IgG Abs works in concert with Fc γ receptors (43).

Based on a number of studies in individuals with MBL gene mutations, the prominent role of MBL in innate immunity has been well appreciated. The protective role of MBL against infection can be explained by the direct binding of MBL to microorganisms (1, 13). MBL binding to IgA may be an additional protective mechanism against microorganisms to which MBL does not bind directly. In situations in which preexposure to a pathogen has taken place, such as after vaccination or during chronic infection, MBL may act in concert with IgA to maintain host integrity. Such a mechanism is conceivable, for example, in cystic fibrosis, in which chronic lung infections often lead to irreversible pulmonary damage and death. Expression of MBL variant alleles in patients with cystic fibrosis is associated with a severely reduced life span (12).

Although MBL is an important defense factor of the immune system, it may also play an unfavorable role in disease progression. This is proposed for rheumatoid arthritis, and is suggested by the presence of MBL in renal biopsies from patients with IgA nephropathy, Henoch-Schönlein purpura, systemic lupus erythematosus, and poststreptococcal glomerulonephritis (19, 20, 32, 40, 44). IgA nephropathy is the leading cause of end stage renal disease worldwide. Therefore, the IgA-binding function of MBL described in the present study is also likely to play a dual role in immunity. On the one hand, it may link the innate and the adaptive immune system and thereby protect the individual against invading pathogens. In this respect, Ab-mediated complement activation can be considered as an additional parallel between MBL and C1q. In contrast, it may enhance the proinflammatory effects of IgA deposition in the glomerulus, ultimately leading to renal injury.

Acknowledgments

We thank N. Klar-Mohamad and R. N. van der Geest for excellent technical assistance. We are also grateful to Dr. T. Fujita (Fukushima, Japan), Dr. P. Garred (Copenhagen, Denmark), Dr. C. E. Hack (Amsterdam, The Netherlands), Dr. C. van Kooten (Leiden, The Netherlands), Dr. R. B. Sim (Oxford, U.K.), and Dr. M. Tilanus (Utrecht, The Netherlands) for critically reading the manuscript and providing valuable reagents and advice.

References

- Turner, M. W. 1996. Mannose binding lectin: the pluripotent molecule of the innate immunity. *Immunol. Today* 17:532.
- Sato, T., Y. Endo, M. Matsushita, and T. Fujita. 1994. Molecular characterization of a novel serine protease involved in activation of the complement system by mannose-binding protein. *Int. Immunol.* 6:665.
- Thiel, S., T. Vorup-Jensen, C. M. Stover, W. Schwaebler, S. B. Laursen, K. Poulsen, A. C. Willis, P. Eggleton, S. Hansen, U. Holmskov, et al. 1997. A second serine protease associated with mannan-binding lectin that activates complement. *Nature* 386:506.
- Dahl, M. R., S. Thiel, A. C. Willis, T. Vorup-Jensen, T. Christensen, S. V. Petersen, and J. C. Jensenius. 2000. Mannan-binding lectin associated serine protease 3 (MASP-3): a new component of the lectin pathway of complement activation. *Immunopharmacology* 49:79 (Abstr.).
- Matsushita, M., S. Thiel, J. C. Jensenius, I. Terai, and T. Fujita. 2000. Proteolytic activities of two types of mannose-binding lectin-associated serine protease. *J. Immunol.* 165:2637.
- Garred, P., H. O. Madsen, and A. Svejgaard. 1996. Genetics of human mannan-binding protein. In *Collectins and Innate Immunity*. R. A. B. Ezekowitz, K. N. Sastry, and K. B. M. Reid, eds. Springer-Verlag, Heidelberg, p. 139.
- Super, M., S. D. Gillies, S. Foley, K. Sastry, J. E. Schweinle, V. J. Silverman, and R. A. B. Ezekowitz. 1992. Distinct and overlapping functions of allelic forms of human mannose binding protein. *Nat. Genet.* 2:50.
- Summerfield, J. A., M. Sumiya, M. Levin, and M. Turner. 1997. Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. *Br. Med. J.* 314:1229.
- Summerfield, J. A., S. Ryder, M. Sumiya, M. Thursz, A. Gorchein, M. A. Monteil, and M. W. Turner. 1995. Mannose binding protein gene mutations associated with unusual and severe infections in adults. *Lancet* 345:886.
- Graudal, N. A., H. O. Madsen, U. Tarp, A. Svejgaard, G. Jurik, H. K. Graudal, and P. Garred. 2000. The association of variant mannose-binding lectin genotypes with radiographic outcome in rheumatoid arthritis. *Arthritis Rheum.* 43:515.
- Garred, P., H. O. Madsen, P. Halberg, J. Petersen, G. Kronborg, A. Svejgaard, V. Andersen, and S. Jacobsen. 1999. Mannose-binding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. *Arthritis Rheum.* 42:2145.
- Garred, P., T. Pressler, H. O. Madsen, B. Frederiksen, A. Svejgaard, N. Hoiby, M. Schwartz, and C. Koch. 1999. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. *J. Clin. Invest.* 104:431.
- Neth, O., D. L. Jack, A. W. Dodds, H. Holzel, N. J. Klein, and M. W. Turner. 2000. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. *Infect. Immun.* 68:688.
- Russell, M. W., M. Kilian, and M. E. Lamm. 1999. Biological activity of IgA. In *Mucosal Immunology*. P. L. O. Ogra and J. Mestecky, eds. Academic Press, San Diego, p. 225.
- Mestecky, J., I. Moro, and B. J. Underdown. 1999. Mucosal immunoglobulins. In *Mucosal Immunology*. P. L. O. Ogra and J. Mestecky, eds. Academic Press, San Diego, p. 133.
- Hiemstra, P. S., A. Gorter, M. E. Stuurman, L. A. Van Es, and M. R. Daha. 1987. Activation of the alternative pathway of complement by human serum IgA. *Eur. J. Immunol.* 17:321.
- Bogers, W. M., R. K. Stad, L. A. Van Es, and M. R. Daha. 1991. Immunoglobulin A: interaction with complement, phagocytic cells and endothelial cells. *Complement Inflamm.* 8:347.
- Emancipator, S. N., J. Mestecky, and M. E. Lamm. 1999. IgA nephropathy and related diseases. In *Mucosal Immunology*. P. L. O. Ogra and J. Mestecky, eds. Academic Press, San Diego, p. 1365.
- Endo, M., H. Ohi, I. Ohsawa, T. Fujita, M. Matsushita, and T. Fujita. 1998. Glomerular deposition of mannose-binding lectin (MBL) indicates a novel mechanism of complement activation in IgA nephropathy. *Nephrol. Dial. Transplant.* 13:1984.
- Matsuda, M., K. Shikata, J. Wada, H. Sugimoto, Y. Shikata, T. Kawasaki, and H. Makino. 1998. Deposition of mannan binding protein and mannan binding protein-mediated complement activation in the glomeruli of patients with IgA nephropathy. *Nephron* 80:408.
- Tan, S. M., M. C. Chung, O. L. Kon, S. Thiel, S. H. Lee, and J. Lu. 1996. Improvements on the purification of mannan-binding lectin and demonstration of its Ca²⁺-independent association with a C1s-like serine protease. *Biochem. J.* 319:329.
- Roos, A., M. Essers, D. van Gijlswijk-Janssen, N. V. Bovin, and M. R. Daha. 2001. Both IgG and IgM anti-pig antibodies induce complement activation and cytotoxicity. *Xenotransplantation* 8:3.

23. Collard, C. D., A. Väkevä, M. A. Morrissey, A. Agah, S. A. Rollins, W. R. Reenstra, J. A. Buras, S. Meri, and G. L. Stahl. 2000. Complement activation after oxidative stress: role of the lectin complement pathway. *Am. J. Pathol.* 156:1549.
24. Vorup-Jensen, T., S. V. Petersen, A. G. Hansen, K. Poulsen, W. Schwaeble, R. B. Sim, K. B. Reid, S. J. Davis, S. Thiel, and J. C. Jensenius. 2000. Distinct pathways of mannan-binding lectin (MBL)- and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. *J. Immunol.* 165:2093.
25. Suankratay, C., X. H. Zhang, Y. Zhang, T. F. Lint, and H. Gewurz. 1998. Requirement for the alternative pathway as well as C4 and C2 in complement-dependent hemolysis via the lectin pathway. *J. Immunol.* 160:3006.
26. Rits, M., P. S. Hiemstra, H. Bazin, L. A. Van Es, J. P. Vaerman, and M. R. Daha. 1988. Activation of rat complement by soluble and insoluble rat IgA immune complexes. *Eur. J. Immunol.* 18:1873.
27. Hiemstra, P. S., J. Biewenga, A. Gorter, M. E. Stuurman, A. Faber, L. A. Van Es, and M. R. Daha. 1988. Activation of complement by human serum IgA, secretory IgA and IgA1 fragments. *Mol. Immunol.* 25:527.
28. Schaapherder, A. F. M., H. G. Gooszen, M. T. J. W. Te Bulte, and M. R. Daha. 1995. Human complement activation via the alternative pathway on porcine endothelium initiated by IgA antibodies. *Transplantation* 60:287.
29. Janoff, E. N., C. Fasching, J. M. Orenstein, J. B. Rubins, N. L. Opstad, and A. P. Dalmasso. 1999. Killing of *Streptococcus pneumoniae* by capsular polysaccharide-specific polymeric IgA, complement, and phagocytes. *J. Clin. Invest.* 104:1139.
30. Stad, R. K., D. J. van Gijlswijk-Janssen, L. A. Van Es, and M. R. Daha. 1994. Complement depletion abolishes IgA-mediated glomerular inflammation in rats. *Exp. Nephrol.* 2:182.
31. Miyazaki, R., M. Kuroda, T. Akiyama, I. Otani, Y. Tofuku, and R. Takeda. 1984. Glomerular deposition and serum levels of complement control proteins in patients with IgA nephropathy. *Clin. Nephrol.* 21:335.
32. Endo, M., H. Ohi, I. Ohsawa, T. Fujita, M. Matsushita, and T. Fujita. 2000. Complement activation through the lectin pathway in patients with Henoch-Schönlein purpura nephritis. *Am. J. Kidney Dis.* 35:401.
33. Van Zandbergen, G., C. Van Kooten, N. K. Mohamad, T. J. Reterink, J. W. de Fijter, J. G. van De Winkel, and M. R. Daha. 1998. Reduced binding of immunoglobulin A (IgA) from patients with primary IgA nephropathy to the myeloid IgA Fc-receptor, CD89. *Nephrol. Dial. Transplant.* 13:3058.
34. Reterink, T. J. F., W. E. M. Schroeijers, L. A. Van Es, and M. R. Daha. 1996. Dimeric and polymeric IgA, but not monomeric IgA, enhance the production of IL-6 by human renal mesangial cells. *Mediat. Inflamm.* 5:191.
35. Renegar, K. B., G. D. Jackson, and J. Mestecky. 1998. In vitro comparison of the biologic activities of monoclonal monomeric IgA, polymeric IgA, and secretory IgA. *J. Immunol.* 160:1219.
36. Stubbe, H., J. Berdoz, J. P. Kraehenbuhl, and B. Corthésy. 2000. Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. *J. Immunol.* 164:1952.
37. Valentijn, R. M., J. Radl, J. J. Haayman, B. J. Vermeer, J. J. Weening, R. H. Kauffmann, M. R. Daha, and L. A. Van Es. 1984. Macromolecular IgA in the circulation and mesangial deposits in patients with primary IgA nephropathy. *Contrib. Nephrol.* 40:87.
38. Endo, T., J. Mestecky, R. Kulhavy, and A. Kobata. 1994. Carbohydrate heterogeneity of human myeloma proteins of the IgA1 and IgA2 subclasses. *Mol. Immunol.* 31:1415.
39. Hiki, Y., A. Tanaka, T. Kokubo, H. Iwase, J. Nishikido, K. Hotta, and Y. Kobayashi. 1998. Analyses of IgA1 hinge glycopeptides in IgA nephropathy by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Am. Soc. Nephrol.* 9:577.
40. Malhotra, R., M. R. Wormald, P. M. Rudd, P. B. Fischer, R. A. Dwek, and R. B. Sim. 1995. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat. Med.* 1:237.
41. Sim, R. B., S. K. Moestrup, G. R. Stuart, N. J. Lynch, J. Lu, W. J. Schwaeble, and R. Malhotra. 1999. Interaction of C1q and the collectins with the potential receptors calreticulin (cC1qR/collectin receptor) and megalin. *Immunobiology* 199:208.
42. Tenner, A. J., S. L. Robinson, and R. A. B. Ezekowitz. 1995. Mannose binding protein (MBP) enhances mononuclear phagocyte function via a receptor that contains the 126,000 M_r component of the C1q receptor. *Immunity* 3:485.
43. Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 17:593.
44. Ohsawa, I., H. Ohi, M. Endo, T. Fujita, M. Matsushita, and T. Fujita. 1999. Evidence of lectin complement pathway activation in poststreptococcal glomerulonephritis. *Kidney Int.* 56:1158.