Degalactosylated and/or Denatured IgA, but Not Native IgA in Any Form, Bind to Mannose-Binding Lectin

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Degalactosylated and/or Denatured IgA, but Not Native IgA in Any Form, Bind to Mannose-Binding Lectin

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Mannose-binding lectin (MBL) is produced in hepatocytes and secreted into plasma. It plays an important role in activation of complement in cooperation with MBL-associated serine proteases (MASPs) 1, 2, and 3 and works to exclude microorganisms in concert with phagocytic cells (1–3). The serum level of MBL in children is higher than in adults (4–5). A deficiency in MBL can cause a vulnerability to infection (6–8). It is well-known that there are three point mutations leading to MBL deficiency, and these occur at codons 52, 54, and 57 in exon 1 of the human MBL structural gene encoding collagen-like sequences (9–11).

MBL is known to bind to agalactosyl IgG (IgG-G0), but not to normally galactosylated (native) IgG, and to activate the lectin pathway of complement (12). It has also been reported that IgG-G0 is strongly related to the pathogenesis of rheumatoid arthritis (RA) (13, 14). Recently, it was reported that native polymeric IgA in serum is reactive with MBL and activates the complement system via the lectin pathway (15). Since coexistence of MBL with IgA has been noted in the glomerular deposits of IgA nephropathy (IgAN) (16–18), polymeric IgA with MBL-binding activity would appear to have a role in the pathogenesis of IgAN. However, recent reports have pointed out that IgD and IgE, (19) and possibly IgM (20), did not show MBL binding (21). These reports led us to investigate whether IgA is really reactive with MBL. To carry out this investigation, we collected a number of purified preparations of monoclonal and polyclonal IgA of various classes, subclasses, and allotypes and tested these for their MBL-binding ability using an ELISA system, since a remarkable clonal variability in glycosylation patterns was observed among human myeloma proteins including IgA (22, 23). Furthermore, it has been reported that the glycosylation of myeloma proteins may differ from their normal serum counterparts (23, 24), and therefore, we isolated normal serum IgA from pooled human serum in this study and examined its MBL-binding activity.

It was found in the present study that, in general, native IgA, irrespective of class, subclass or allotype, or of their monomeric or polymeric status did not show significant MBL binding. However, they became reactive with MBL when they were degalactosylated by galactosidase treatment. Furthermore, their MBL binding was enhanced by successive denaturation with acidic treatment. The activation of complement via the lectin pathway was also demonstrated with degalactosylated and/or denatured IgA but not with native IgA. A possible involvement of IgA with altered glycosylation and molecular structure in the pathogenesis of IgAN will be discussed.

Materials and Methods

Purification of MBL

Human MBL was isolated from pooled serum by means of a sequential procedure involving α-mannose Sepharose 6B affinity column chromatography, unmodified Sepharose 6B affinity column chromatography which allows for Sepharose to act as a MBL ligand (4, 25), and fast protein liquid chromatography (FPLC; Pharmacia LKB) using Superose 6 and Mono Q columns as described previously (4, 25). In brief, human serum, dialyzed against buffer A (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1.0 mM CaCl2, 0.05% NaN3) was applied to a α-mannose Sepharose 6B column previously equilibrated with the same buffer. After extensive washing of the column with the same buffer, the bound proteins were eluted with buffer B containing EDTA (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.01 M EDTA, 0.05% NaN3). The eluate was adjusted with 2 M CaCl2 to a final

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3 Abbreviations used in this paper: MBL, mannose-binding lectin; MASP, MBL-associated serine protease; IgAN, IgA nephropathy; FSC, free secretory component; sIgA, secretory IgA; Biot-MBL, biotinylated MBL; FPLC, fast protein liquid chromatography; IEP, immunoelectrophoresis; paB, polyclonal Ab; Rb, rabbit; RA, rheumatoid arthritis.
concentration of Ca\(^{2+}\) at 25 mM. The resulting precipitates, mainly composed of serum amyloid P component, were removed by centrifugation and the supernatant was loaded onto an unmodified Sepharose 6B column equilibrated with buffer A. The column was eluted stepwise with buffer A, 0.05 M N-mannose in buffer A, and finally buffer B. The fractions eluted with 0.05 M mannose were further fractionated with the FPLC system, first on a Superose 6 column (running buffer: 50 mM Tris-HCl (pH 8.0), 1 mM NaCl, 1 mM EDTA, 0.05% NaN\(_3\), 0.05% Tween 20) and then on a Mono Q column (running buffer: 50 mM Tris-HCl (pH 8.0), linear salt gradient 0.1–1.0 M NaCl, 0.05% NaN\(_3\)). Fractions containing MBL, as determined by ELISA (4), were pooled and concentrated. To remove contaminating IgS and MASP-1/3, the MBL preparation was passed through a tandem affinity column consisting of: 1) anti-human-MASP-1/3 mAb (25, 26) (IEP; a gift of Dr. M. Tsujimura, Fukuoka Red Cross Blood Center, Fukuoka, Japan) Sepharose; 2) anti-human-IgM polyvalent Ab (pAb; DakoCytomation) Sepharose; and 3) protein G (Pharmacia) Sepharose; 4) anti-human-IgA pAb (DakoCytomation) Sepharose; and 5) anti-human-MBL mAb (27) (3E7; a gift of Prof. M. Matsushita, Department of Applied Biochemistry and Institute of Biochemistry, Fukuoka University, Fukuoka, Japan) Sepharose. Elution was performed with buffer B containing EDTA in the closed system within the above five columns overnight at 4°C. The five columns were then separated, and the MBL-containing fraction was eluted from the anti-human-MBL mAb (27) (3E7; a gift of Prof. M. Matsushita) Sepharose. Elution was then separated, and the IgA fraction was eluted from the anti-human-IgA column by 0.1 M Gly-HCl (pH 2.0) and immediately neutralized with Tris buffer and incubated for 2 h at room temperature. It was dialyzed extensively against PBS, and the biotinylated MBL was performed by ELISA. In brief, microtiter plates (Maxi-sorp; Nunc) were coated with various Igs or MBL in PBS containing 0.05% NaN\(_3\) (PBSN) for 16 h at room temperature or for 2 h at 37°C. After incubating, the microtiter plates were washed three times with PBS-T (PBS containing 0.1% Tween 20), and were blocked by 250 μl/well of 1% BSA (Sigma-Aldrich) in PBS for 1 h at 37°C or overnight at room temperature, and stored at 4°C until use. For solid phase Igs and liquid-phase MBL, Igs were coated on wells at 5 μg/ml, unless otherwise indicated. The Ig-coated microtiter plates thus prepared were then treated with biotinylated MBL (a source of MBL) in a Ca\(^{2+}\)-containing dilution buffer (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 2 mM CaCl\(_2\), containing 0.01% thimerosal, 0.1% BSA, and 0.1% Tween 20) or in an EDTA-containing dilution buffer (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 10 mM EDTA containing 0.01% thimerosal, 0.1% BSA, and 0.1% Tween 20) and incubated for 2 h at room temperature. After washing with PBS-T, 100 μl of biotinylated anti-MBL mAb (3E7) were added to each well and incubated overnight at 4°C. In some experiments, instead of MBL or serum as a source of MBL as the loading sample, Biot-MBL (5 ng/ml, unless otherwise indicated) dissolved in 25 μl of DMSO and added to the MBL (2.5 mg/500 μl) in the 0.05 M NaHCO\(_3\) (pH 8.5), and the mixture was incubated for 4 h at room temperature. It was dialyzed extensively against PBS, and the biotinylated MBL (Biot-MBL) thus prepared was stored at 4°C with 0.05% NaN\(_3\).

**MBL-binding experiments**

The binding of MBL to immobilized Igs or the binding of IgA to immobilized MBL was performed by ELISA. In brief, microtiter plates (Maxisorb; Nunc) were coated with various Igs or MBL in PBS containing 0.05% NaN\(_3\) (PBSN) for 16 h at room temperature or for 2 h at 37°C. After incubating, the microtiter plates were washed three times with PBS-T (PBS containing 0.1% Tween 20), and were blocked by 250 μl/well of 1% BSA (Sigma-Aldrich) in PBSN for 1 h at 37°C or overnight at room temperature, and stored at 4°C until use. For solid phase Igs and liquid-phase MBL, Igs were coated on wells at 5 μg/ml, unless otherwise indicated. The Ig-coated microtiter plates thus prepared were then treated with biotinylated MBL (a source of MBL) in a Ca\(^{2+}\)-containing dilution buffer (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 2 mM CaCl\(_2\), containing 0.01% thimerosal, 0.1% BSA, and 0.1% Tween 20) or in an EDTA-containing dilution buffer (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 10 mM EDTA containing 0.01% thimerosal, 0.1% BSA, and 0.1% Tween 20) and incubated for 2 h at room temperature. After washing with PBS-T, 100 μl of biotinylated anti-MBL mAb (3E7) were added to each well and incubated overnight at 4°C. In some experiments, instead of MBL or serum as a source of MBL as the loading sample, Biot-MBL (5 ng/ml, unless otherwise indicated) dissolved in 25 μl of DMSO and added to the MBL (2.5 mg/500 μl) in the 0.05 M NaHCO\(_3\) (pH 8.5), and the mixture was incubated for 4 h at room temperature. It was dialyzed extensively against PBS, and the biotinylated MBL (Biot-MBL) thus prepared was stored at 4°C with 0.05% NaN\(_3\).

**Purification of human serum IgA**

Human serum IgA was purified from normal human recalcified donor plasma as described by Hiemstra et al. (30) with modifications. In brief, the majority of serum proteins were removed by dialysis against H\(_2\)O and followed by precipitation with ZnSO\(_4\). Proteins in the supernatant were precipitated using glycine and (NH\(_4\))\(_2\)SO\(_4\), dialyzed against 20 mM phosphate buffer (pH 7.6) containing 2 mM EDTA, and loaded onto an FPLC system fitted with a Mono Q column. IgA was eluted with a linear salt gradient (0–40 mM NaCl) in the same buffer as for dialysis. Two major IgA-containing fractions, each eluting at a low or high NaCl concentration (we termed the former fraction C (cathodic) and the latter fraction A (anodic)), as determined by dot blotting, were pooled, concentrated, and further purified by gel filtration using an FPLC Superose 6 column. PBS (20 mM phosphate buffer (pH 7.0) and 0.15 M NaCl) containing 2 mM EDTA was used as running buffer. Fractions were tested for IgA by dot blotting, and further pooled separately based on their elution position (we designated the monomeric fraction as M and the polymeric one as P). Thus, four IgA fractions, i.e., IgA-C-M, IgA-C-P, IgA-A-M, and IgA-A-P, were obtained. Each IgA fraction was then applied to a tandem affinity column, consisting of four Sepharose columns each coupled with 1) an anti-human-α2 macroglobulin (DakoCytomation), 2) an anti-human-IgM (DakoCytomation), 3) IgA mAb S2, and 4) an anti-human-IgA mAb TBS (10 mM Tris-HCl buffer (pH 8.0) and 0.175 mM NaCl) containing 2 mM EDTA and 0.05% NaN\(_3\) was used as the running buffer, which was passed through the four-column system overnight at 4°C. The four columns were then separated, and the IgA fraction was eluted from the anti-human-IgA Sepharose column by 0.1 M Gly-HCl (pH 2.0) and immediately neutralized with Tris buffer and incubated for 2 h at room temperature. For the evaluation of MBL-binding activity with immobilized Igs, values were calculated from the absorbance of MBL bound to immobilized IgA2m(2)(Kur) (5 μg/ml) which was set as an internal reference Ig on every microtiter plate, and was arbitrarily assigned a value of 1000 U. The
units observed do not necessarily reveal concentration with linearity but are substituted for OD.

**Inhibitory activity of Igs against the binding of MBL to immobilized mannan**

Mannan (2 μg/ml) was coated on the microtiter plate and blocked. Ten microliters per well of serum (used as a source of MBL) diluted to 1/10 with Ca²⁺-containing dilution buffer and 90 μl/well of Igs (as inhibitors), each serially diluted with the same buffer, were simultaneously added to the mannan-coated wells. As a positive inhibitor, serially diluted mannan was used and added instead of Igs in the reaction. MBL bound to the immobilized mannan was assessed using biotinylated anti-MBL Ab as described above. The percentage of inhibition of MBL binding to immobilized mannan was calculated and was plotted against the concentration of the Igs (or mannan) added.

**Inhibitory activity of monosaccharides against the binding of MBL to immobilized Igs**

Fifty microliters per well of serum (used as a source of MBL) and 50 μl/well of monosaccharides (100 mM) serially diluted with Ca²⁺-containing dilution buffer were simultaneously added to the Ig-coated wells. MBL bound to the immobilized Ig was assessed as described above, and was plotted against the concentration of the monosaccharide added; i.e., D-mannose (Man), N-acetyl-D-galactosamine (GlcNAc), N-acetyl-D-glucosamine (GlcNAc), N-galactose (Gal), and D-mannose (Man), N-acetyl-D-galactosamine (GlcNAc), N-acetyl-D-glucosamine (GlcNAc), N-galactose (Gal), or D-mannose (Man). Gal was purchased from Wako and other monosaccharides were monosaccharides from Sigma-Aldrich.

**Effect of acidic treatment of Igs on their MBL binding**

Wells coated with various Igs were pretreated with 100 μl/well of acidic buffer (0.1 M Gly·HCl (pH 2.0), containing 0.15 M NaCl) for 1 h at 37°C. After washing with PBST, Biot-MBL (5 ng/ml) was added to the wells, and the bound MBL was determined as indicated above.

**Degalactosylation of Igs**

Degalactosylation of the Igs was performed by digesting them with an enzyme mixture consisting of neuraminidase (EC 3.2.1.18) and β-D-galactosidase (EC 3.2.1.23) (both enzymes from streptococcus 6644K; Seikagaku) in sodium acetate buffer (50 mM, pH 5.5) containing 10 mM MnCl₂ (12, 35–37). In practice, 100 μl of the enzyme mixture containing neuraminidase at 10 μU/ml and β-D-galactosidase at 1 μU/ml were added to the Ig-coated wells and incubated at 37°C for 48 h. Efficacy of the enzyme concentration and incubation time in the degalactosylation of Ig were assessed in separate experiments. The lack of proteolytic degradation of Igs during the above degalactosylation procedure was confirmed by following experiments. One human IgG (Sigma-Aldrich) and four myeloma IgA samples, each 100 μg, were incubated in 100 μl of the above enzyme mixture at 37°C. A 20-μl aliquot of the reaction mixture was sampled every 24 h until 72 h, and the reaction was terminated by treatment at 56°C for 10 min (12). The Igs treated with the identical procedure without addition of the enzymes were served as controls. The possible proteolytic degradation was assessed by 7.5% SDS-PAGE without 2-ME.

**Analysis of complement activation by MBL bound to Igs**

Activation of complement through the binding of MBL to serum IgA or immunoglobulin G was assessed as follows. Microtiter plates were coated with Igs at 5 μg/ml, blocked by BSA and then incubated at 4°C for 1 day with serial dilutions of normal human serum as a source of MBL, MASPs, and complement component C4 in a Ca²⁺-containing buffer supplemented with 1 mM MgCl₂. IgA2m(2)(Kur) coated on each microtiter plate was used as a reference-Ig for MBL binding. After washing the microtiter plates, Clq deposition was detected by a biotinylated Rb anti-human-C1q pAb (Serotec AHC001) followed by HRP-conjugated streptavidin (Nichirei), and C4b deposition, by a Rb anti-human-C4c pAb (DakoCytometry A0065) followed by biotinylated goat-anti-Rb IgG (Nichirei), and HRP-conjugated streptavidin (Nichirei). Color development of the plates was performed as above. The amount of C4b deposition was assessed as above. In each experiment, MBL-MASPs isolated in the present study and a commercially available purified human C4 (Calbiochem) were used and added instead of Igs in the reaction. MBL bound to the immobilized mannan was assessed using biotinylated anti-MBL Ab as described above.

**Analytical conditions**

The binding of MBL to IgA was assessed in two different ways: with one method, a specific amount of IgA was coated on wells which were then loaded with different amounts of purified MBL (Fig. 1A) or serially diluted serum (as a source of MBL) (Fig. 1B), and with the other method, the IgA were coated on wells in different amounts and loaded with a specific amount of purified MBL (Fig. 1C) or by a specific amount of serum (Fig. 1D). In each experiment in which the monosacch. Igs were immobilized, significant MBL binding was detected only in the case of IgA2m(2) dimer (Kur) and only when the reaction was performed in a Ca²⁺-containing buffer. When these experiments were conducted in a buffer containing EDTA, MBL binding was completely abolished (Fig. 1A, A and B), indicating that the binding of MBL to the immobilized IgA2m(2)(Kur) was Ca²⁺ dependent, a behavior consistent with that of C-type lectin. Other monoclonal Igs, including IgA, IgG, IgM, and IgE, did not show any significant binding. These results suggested that most monoclonal Igs, including IgA other than IgA2m(2)(Kur), do not bind to MBL, and that IgA2m(2)(Kur) is an exceptional one in this respect. To ascertain this, we obtained purified polyclonal serum IgA preparations with four different molecular forms (C-M, C-P, A-M, and A-P; see Materials and Methods) and tested their MBL binding in the same way as had been done for the monoclonal Igs. As can be seen in Fig. 2, while the positive control IgA2m(2)(Kur) exhibited a strong ability to bind to MBL, no MBL binding was detected with polyclonal serum IgA preparations, irrespective of their molecular forms. Thus, in the following experiments, results of serum polymeric IgA A-P were presented as a representative data for polyclonal serum IgA, since data obtained with the four serum IgA preparations were essentially the same among them.

**Effect of Igs on the binding of MBL to mannan**

The preceding MBL-binding experiments were conducted using liquid-phase MBL and solid-phase Igs, and vice versa. Thus, one may claim that this situation is far from natural state, i.e., in the circulation or on the tissues. To overcome this claim, we examined the effect of Igs on the binding of MBL to immobilized mannan. As can be seen in Fig. 3, the binding of MBL to immobilized mannan was distinctly inhibited by IgA2m(2)(Kur), indicating the occurrence of MBL binding to IgA2(2)(Kur) both in the liquid phase through its carbohydrate recognition domain. No such inhibitory effect was observed for any other Igs so far tested. These results indicate that the use of immobilized Igs as MBL ligand was appropriate in the evaluation of MBL-binding ability of Igs. To enforce the latter notion further, we made additional experiments comparing the binding of variable amount of Biot-MBL (diluted to half stepwise
from 20 ng/ml) to immobilized IgA2m(2)(Kur) with that to immobilized mannan. In these experiments, we prepared five different immobilized IgA2m(2)(Kur) microtiter plates coated at 0.625, 1.25, 2.5, 5, and 10 μg/ml, respectively, and also five immobilized mannan microtiter plates coated at 1.25, 2.5, 5, 10, 20, and 100 μg/ml, respectively, and tested for their binding with variable amounts of Biot-MBL. When the ODs (Biot-MBL binding) obtained for each immobilized IgA2m(2)(Kur) concentration were plotted against those for immobilized mannan, they lied on a straight line with a quite high correlation coefficient ($r = 0.993–1.000$, mean: 0.998, in 30 combinations), indicating that the binding of MBL to immobilized Igs is quite comparable to that to immobilized mannan. In Fig. 4, we showed a graph in the case of IgA2m(2)(Kur) coated at 1.25 μg/ml and mannan at 5 μg/ml as a representative one.

**Effect of monosaccharides on the binding of MBL to IgA2m(2)(Kur)**

The binding of MBL to IgA2m(2)(Kur) was apparently inhibited by monosaccharides in the order of N-acetyl-D-glucosamine > L-fucose, D-mannose > D-glucose > D-galactose > N-acetyl-D-galactosamine (Fig. 5A). Fig. 5B shows the same monosaccharide inhibition test in which the IgA2m(2)(Kur) was replaced with degalactosylated and acid-denatured IgM (Sigma-Aldrich) with MBL-binding activity (see Effect of degalactosylation and subsequent acidic denaturation on MBL binding below). The inhibition pattern was essentially the same as that observed with IgA2m(2)(Kur). These results were in agreement with previous reports on the saccharide selectivity of MBL (38, 39).

**Analysis of MBL binding to various Igs**

Among Igs so far tested, only one, IgA2m(2)(Kur), exhibited significant MBL binding as discussed above. We then collected several other batches of monoclonal and polyclonal Igs including commercially available IgG, IgM, and colostral IgA, and tested whether any of these showed MBL binding. In these experiments, each Ig was coated onto a microtiter plate at 5 μg/ml and reacted with a fixed amount of Biot-MBL (5 ng/ml). The relative value of the binding of MBL to each immobilized Ig was calculated from the value for IgA2m(2)(Kur) which was used as the internal reference and assigned 1000 arbitrary units. As can be seen in Table I (no Tx), among the number of monoclonal Igs tested, only IgA2m(2)(Kur) exhibited apparent MBL binding. Other isotypes of monoclonal Igs, such as IgG(Rak), IgM(Loy), IgD(Hok), and IgE(Des), showed no significant ability to bind to MBL. As for the polyclonal Igs, a relatively high MBL binding was observed with...
some sIgA preparations but not with others including a commercial colostral sIgA (Sigma-Aldrich). Our serum IgA preparations had no MBL-binding capacity whatsoever. In addition, FSC, a component of sIgA, was almost incapable of binding to MBL as well.

**Effect of acidic denaturation of Igs on their MBL binding**

It was recently reported that slgA did not bind to MBL in its native form but became reactive when the slgA was pretreated with acidic buffer, a procedure known to unfold/denature protein’s structure and expose its carbohydrate residues (40). Thus, we treated the immobilized Igs with acidic buffer (pH 2) and tested them for their ability to bind to MBL (Table I, Acid). All slgA preparations showed an increase in MBL binding after the acidic treatment, consistent with the above report (40). IgA2m(2)(Kur) also exhibited an increase of MBL binding which was >2.5 times greater than that before the treatment. However, most of the other IgA including polyclonal serum IgA did not show significant increases in MBL binding, and only certain IgA1 preparations exhibited sizable increases. Monoclonal IgG and IgM, and polyclonal IgG also showed increases in MBL binding to some extent. FSC failed to bind to MBL, even after acidic treatment.

**Effect of degalactosylation of Igs on their MBL binding**

It has been reported that agalactosyl IgG (IgG-G0), but not native IgG, has MBL-binding activity and is thought to play a role in the pathogenesis of RA (12). This gave rise to an idea that degalactosylation might elicit MBL-binding activity in Igs. We took five different isotypes of monoclonal Igs, IgGr(Rak), IgM(Loy), IgE(Des), IgA1 m (Smi), and IgA2m(1) m (Asb), as representatives, and examined their time course changes in MBL binding after digestion with an enzyme mixture of neuraminidase and β-1,4-galactosidase. Every Ig tested showed an increase in MBL binding after degalactosylation, although the extent of increase varied among Igs. However, between IgA subclasses, members of the IgA2 subclass showed greater increases than those of IgA1. Every slgA showed a strong increase in MBL binding after degalactosylation. FSC, which was almost negative for binding before digestion, acquired very strong MBL-binding activity after digestion.

**Effect of degalactosylation and subsequent acidic denaturation on MBL binding**

The preceding results showed that degalactosylation of Igs induced or enhanced their MBL binding. Then, the degalactosylated Igs were further treated with acidic buffer to expose carbohydrate residues and tested for their changes in MBL binding (Table I, DeGal + Acid). In every degalactosylated Ig tested, the acidic treatment induced striking increases in MBL binding, although the extent of the increase varied. A similar phenomenon was observed in FSC. As for the polyclonal serum IgA, an apparent increase in MBL binding after degalactosylation and acidic denaturation was also observed as shown in Fig. 6.

**Analysis of complement activation by Ig-bound MBL**

Complement activation by MBL bound to immobilized Ig was assessed using IgA2m(2)(Kur) as the positive control for MBL binding, and polyclonal serum IgA and IgG as the negative controls. In this experiment, appropriately diluted normal human serum was used as a source of MBL, MASPs, and complement components. As shown in Fig. 7, deposition of C4b (Fig. 7B) but not C1q (Fig. 7A) was indeed observed with IgA2m(2)(Kur) having MBL-binding activity but none was seen with polyclonal serum IgA lacking MBL binding, indicating the occurrence of complement activation via MBL, the lectin pathway, in the case of IgA2m(2)(Kur). Furthermore, as shown in Fig. 7C, when the degalactosylated and/or acid-denatured polyclonal serum IgAs were used as the immobilized Igs, C4b deposition appeared, and the amount of C4b deposition was fairly correlated with their MBL-binding ability (see Table I), again indicating the activation of complement via MBL. As for serum IgG, prominent depositions of C1q and C4b were observed, indicating the activation of the classical complement activation pathway.
Because the above experiments assessing C4 activation by MBL were performed using human serum as a source of MBL, MASPs, and C4, one may claim a possible contribution of ficolins, other serum lectins having complement activation capacity, to the present results. To rule out this possibility, we made the following experiments using isolated MBL-MASPs and purified human C4 instead of whole serum: various IgA preparations were coated on the microtiter plate. After degalactosylation and subsequent acidic denaturation, they were reacted with isolated MBL-MASPs and

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Table I. Summary of data on the binding of MBL to various IgAs

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<tr>
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<tr>
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Because the above experiments assessing C4 activation by MBL were performed using human serum as a source of MBL, MASPs, and C4, one may claim a possible contribution of ficolins, other serum lectins having complement activation capacity, to the present results. To rule out this possibility, we made the following experiments using isolated MBL-MASPs and purified human C4 instead of whole serum: various IgA preparations were coated on the microtiter plate. After degalactosylation and subsequent acidic denaturation, they were reacted with isolated MBL-MASPs and purified human C4. The C4b deposition and MBL bound to the IgAs were assessed, respectively, and levels of C4b deposition were plotted against those of bound MBL. As shown in Fig. 8, the amount of C4b deposition positively correlated with that of the IgA-bound MBL ($r = 0.862$, $p < 0.001$), confirming the activation of C4 through the MBL bound to the degalactosylated and acid-denatured IgAs. It was also apparent that IgA2 induced a higher level of C4 activation via MBL than did the IgA1 subclass.

Table I. Summary of data on the binding of MBL to various IgAs before and after treatment with acidic buffer, after degalactosylation only (DeGal), and after degalactosylation and subsequent acidic treatment (DeGal + Acid). The binding of MBL to various IgAs was assessed by addition of Biot-MBL (5 ng/ml) to immobilized IgAs (5 g/ml). The relative MBL-binding activity of IgA was expressed as Units (U), as deduced from that of IgA2m(2)(Kur) which was arbitrarily assigned 1000 U. Note that the 1000 U was represented by bold characters in this table. No Tx, no treatment; DeGal, degalactosylation; Acid, acidic treatment; DeGal + Acid, degalactosylation and subsequent acidic treatment; m, monomer; d, dimer; p, polymer; hp, high polymer; -, not known. The given name of myeloma protein or origin of sample is shown in parentheses.
Discussion

Our present results clearly showed that IgA, irrespective of subclass or allotype, or whether in a monomeric or polymeric form, do not bind to MBL, as other classes of Ig do not (12, 19, 20). This was especially the case with our preparation of normal serum IgA. This result contradicts the finding of a previous report that serum polymeric IgA binds to MBL (15). The reason for this discrepancy is not known. However, it is possible that their purified IgA was degraded or denatured during purification and/or during storage, and hence became reactive with MBL, as demonstrated in the present denaturation study. In our experience, Igs of the polymeric type such as polymeric IgA, IgM, or IgG are more liable to aggregate during storage or become insoluble after lyophilization, strongly suggestive of some deterioration of their tertiary structure.

It has been reported that rabbit MBL can bind to IgM from several xenogeneic species (41), and that rat serum MBL can bind to human IgM (42), but there is no evidence that MBL binds to autologous IgM of any species (21, 43) including that of humans (20). Furthermore, it has been reported that human MBL does not bind to human IgD, IgE (19), or native, nondegialized IgG (12). Our present results also substantiated the lack of reactivity of human MBL with autologous IgG, IgA, IgM, IgD, and IgE. It seems natural that MBL does not bind to autologous components, including Igs as well as other components of serum. We also clearly showed in the present study that the Igs became reactive with MBL when they were treated with exoglycosidase and/or acidic denaturation. Treatment of Igs with exoglycosidase, including neuraminidase and β-galactosidase, removes the terminal sialic acid and galactose from carbohydrate chains making mannoside, N-acetyl glucosamine and/or fucose, which are all MBL ligands available for MBL binding. Possible examples of structures that have undergone this process include agalactosyl IgG (IgG-G0) (12), desialylated gp120 of HIV (44) and desialylated lipo-oligosaccharide of Neisseria meningitidis (45). Our IgA2m(2)(Kur) which exhibited a significant amount of MBL binding in its used form would be yet another such example, putatively due to its abnormal glycosylation and/or denatured structure. The IgA2m(2) molecule has five N-linked carbohydrates in its α-chains (46), and the IgA2m(2) dimer (Kur) has thus theoretically 20 carbohydrates in its molecule. It is possible that a small part of N-linked carbohydrate chains in the IgA2m(2)(Kur) are degalactosylated and are located on the molecule accessible to MBL binding. That might be the reason why the IgA2m(2)(Kur) bound to MBL in its used untreated form and still enhanced for its MBL-binding ability by further degalactosylation and/or further denaturation.

Acidic denaturation is presumed to unfold the tertiary structure of Igs and render their oligosaccharides much more accessible to MBL. Although the present study substantiated the increase in the MBL-binding activity of acid-denatured Igs, a similar observation...
has already been reported for acid-denatured sIgA (40), guanidine-
denatured IgD and IgE (19), and also for heat-denatured IgG (47). Thus, it is conceivable that MBL recognizes and binds to glyco-
proteins, including IgA, with aberrant glycosylation and/or dena-
turation, and possibly plays a role in the exclusion of altered self-
components such as aberrantly glycosylated IgG and immune complexes (21).

The ratio of IgG-G0 in normal human and in RA patients’ sera has been reported to be 25 and 50%, respectively (13). However, it has been reported that IgG in normal human serum does not bind and activate the MBL lectin pathway, whereas IgG in RA patient’s serum does so (48). A similar binding result was observed using a plant lectin, PVL (49). These phenomena have been thought to be due to differences in carbohydrate presentation between IgG-G0 in RA patients and that in normal serum (12). The two oligosaccha-
rides in the Fc region of normal serum IgG are buried between the two CH2 domains and highly conserved (50). In fact, it was re-
ported that some plant lectins did not react with normal IgG but become reactive with the IgG after heat aggregation, indicating that alteration of IgG tertiary structure by denaturation lead the carbohydrate moieties to become accessible to the lectins (51). It thus could be assumed that IgG-G0 of the RA patients but not of normal individuals has altered tertiary structure with exposed carbo-
hydrate moiety. The artificially prepared IgG-G0 showed MBL-binding must have loosened tertiary structure (50). This assumption could be further supported by the fact that artificially prepared IgG-G0 glycoforms are continuously taken up by mac-
rophages through the mannan receptor (37). Concerning the IgA N-glycosylation, it has been reported that, compared with IgG, the N-glycan structures on IgA are more completely processed and the percentage of G0-glycan in normal serum IgA is only 1.3% (24). This strongly suggests that the serum IgA preparations used in our experiment have a very small amount of G0-glycan, not enough to show significant MBL binding.

As shown in the present study, MBL binding as well as C4 activation were apparently greater with IgA2 than with IgA1 after exoglycosidase treatment. This could be simply explained by a difference in the number of exoglycosidase treatment. This could be simply explained by a

activation were apparently greater with IgA2 than with IgA1 after

show significant MBL binding.

IgA deposits of the IgA1 subclass alone contained no MBL, while

gial IgA in IgAN (18). This latter report claimed that mesangial

assumed to be responsible for codeposition of MBL with mesan-
gial IgA with MBL codeposition must be conducted, as has been done for the O-linked glycosylation in the IgA1 deposits (63), to eluci-
date the role of MBL in the pathogenesis of IgAN.

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Disclosures

The authors have no financial conflict of interest.

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