Anti-Apolipoprotein A-I Autoantibody: Characterization of Monoclonal Autoantibodies from Patients with Systemic Lupus Erythematosus

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ABSTRACT. ***Objective***. The autoantibody to apolipoprotein A-I (apoA-I), a major constituent of high density lipoproteins (HDL), has been detected in sera of patients with systemic lupus erythematosus (SLE). We established a series of monoclonal anti-apoA-I antibodies (MAAI) from 2 patients with SLE and report the reactivities of MAAI with oxidized HDL, anionic substances, and blood coagulation factors.

Methods. Peripheral blood B cells from patients with SLE were immortalized by Epstein-Barr virus, and B cells secreting anti-apoA-I antibodies (AAI) were fused with mouse myeloma cells. Six MAAI reactive with human apoA-I in both ELISA and immunoblotting analysis were established. The reactivities of MAAI with HDL, ssDNA and dsDNA, phospholipids such as cardiolipin (CL), and coagulation factors were examined by ELISA.

Results. Although all MAAI bound effectively to apoA-I after the protein had been denatured and transferred to the filter membrane (in immunoblotting analyses), they bound less effectively to apoA-I present in HDL. Both oxidation of HDL in the presence of Mn^{2+} and an association of apoA-I with autoxidized trilinolein strongly enhanced the binding of MAAI to apoA-I, suggesting that MAAI recognize a defined region of apoA-I, which is exposed upon interacting with oxidatively modified lipids. MAAI showed a functional heterogeneity in their cross-reactivity with self-components: some MAAI were shown to cross-react with anionic substances such as CL and ssDNA, and one MAAI was shown to bind effectively to thrombin.

Conclusion. We identified a novel family of AAI that shows preferential binding to apoA-I in oxidatively modified HDL. These AAI are composed of antibodies with heterogeneous cross-reactivities to various self-components such as anionic phospholipids, ssDNA, and thrombin. (J Rheumatol 2001;28:990–5)

Key Indexing Terms:
APOLIPOPROTEIN A-I — OXIDIZED HIGH DENSITY LIPOPROTEINS
MONOCLONAL AUTOANTIBODY — SYSTEMIC LUPUS ERYTHEMATOSUS — ELISA

Epidemiological studies have shown that high density lipoprotein (HDL) and its major protein constituent, apolipoprotein A-I (apoA-I), are protective factors against atherosclerosis and coronary artery disease[1]. It is widely accepted that HDL removes unesterified cholesterol and other lipids from peripheral tissues and delivers them to the liver for catabolism. ApoA-I is an effective cofactor for lecithin-cholesterol acyltransferase (LCAT) and also stimulates the efflux of free cholesterol from peripheral tissue, providing substrates for the LCAT reaction[2]. As a result of these activities apoA-I is suggested to play a role in regulating the cholesterol content in peripheral tissues through the reverse cholesterol transport pathway, and several studies[3-6] have suggested an inverse correlation between the level of apoA-I and the incidence of atherosclerosis. Recent studies[7,8] have shown that HDL is the major carrier of lipid hydroperoxides in human plasma, and lipids in HDL are oxidized in preference to those in low density lipoprotein (LDL) when human plasma is exposed to aqueous peroxyl radicals. Moreover, HDL can accept oxidized cholesteryl esters from LDL, by the process mediated by cholesteryl ester transfer protein, and cholesteryl ester hydroperoxides in HDL are rapidly cleared from the circulation[9]. Further, apoA-I plays a role in the reduction of the hydroperoxide derivatives of cholesteryl esters and phosphatidylcholine

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associated with human HDL[10]. These observations suggest that HDL and apoA-I play a physiological role in detoxification and exit route of oxidized lipids from circulation.

It has been reported that patients with systemic lupus erythematosus (SLE) are at increased risk for atherosclerotic and thrombotic diseases such as myocardial infarction[11-13]. Although the longterm use of corticosteroid has been suggested to be the cause of accelerated atherosclerosis[14-16], recent studies have reported that the decreased level of HDL and apoA-I in patients with SLE not ingesting corticosteroid was associated with the presence of anticardiolipin antibody[17,18].

We have shown that natural autoantibodies to apoA-I (AAI) were readily detected in sera of normal BALB/c mice[19]. Merrill, *et al* have isolated a gene for apoA-I from a mouse cDNA library, screened with serum from a SLE patient who had a cerebrovascular accident[20]. Subsequently they showed the frequent production of AAI in patients with SLE and primary antiphospholipid syndrome (APS) in which sera from 32.5% and 22.9% of patients with SLE and primary APS, respectively, were positive for anti-apoA-I antibodies[21]. To further characterize the reactivities of AAI in patients with SLE, we have established a series of monoclonal anti-apoA-I antibodies (MAAI) from peripheral B cells of the patients. We found that MAAI show a preferential binding to apoA-I in oxidatively modified HDL, and cross-reactivities of MAAI with various self-components have also been analyzed.

MATERIALS AND METHODS

Materials. Lipid. Phosphatidylserine (from bovine brain), phosphatidylethanolamine (from *Escherichia coli*), phosphatidylinositol (from bovine liver), sphingomyelin (from bovine brain), and phosphatidic acid (PA, from egg yolk) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Phosphatidylcholine from egg was prepared by chromatography on aluminium oxide neutral and Iatrobeads[22]. Trilinolein was purchased from NuChek Prep (Elysian, MN, USA). Cardiolipin (CL) from bovine heart was purchased from Avanti Polar Lipids.

Coagulation factors. Prothrombin (II) was purchased from Enzyme Research Lab (South Bend, IN, USA) and Factor Xa from Danex Biotek (Mundelstrup, Denmark). Bovine factor V was purified from barium-absorbed bovine plasma using the method of Dahlbäck[23]. Thrombin (IIa) from bovine plasma was purchased from Mochida Pharmaceutical Co. (Tokyo, Japan).

HDL. Whole blood obtained by venipuncture from a healthy volunteer was collected into a Vacutainer tube containing citrate phosphate dextrose (Terufakkusu-SC407J8, Terumo Co., Tokyo, Japan). Blood cells settled after 1500 rpm centrifugation for 15 min at 4°C. HDL (d = 1.063 to 1.21 g/ml) were isolated by sequential ultracentrifugation in NaBr solution[24]. Isolated HDL were extensively dialyzed at 4°C in the dark against Tris buffered saline (TBS, 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl) containing 0.01% NaN_3. HDL were stored at 4°C.

ApoA-I. The apoA-I was isolated from ether/ethanol delipidated human HDL by Sephacryl S-200 gel filtration column chromatography in urea buffer (100 mM Tris-HCl buffer, pH 8.6, containing 1 mM EDTA and 8 M urea) as described[25].

Sera. Sera from patients with SLE (n = 39) and healthy volunteers (n = 18) were collected, stored at –80°C and thawed at 37°C before use. The diagnosis of SLE was established by rheumatologists at Tokyo Medical University Hospital. Patients with SLE were classified according to the American College of Rheumatology preliminary criteria.

ELISA. ELISA was performed at room temperature as follows. In brief, each well of microtiter plates (Immulon 2, Dynatech Laboratories, Guyacourt, France) was coated for 2 h with either a fixed amount of lipids or various amounts of antigens. For lipids 50 μl of lipid antigens in ethanol (10 μM) prepared by evaporation at room temperature was plated onto each microwell of another plate (Immulon 1, Dynatech). The wells were blocked with TBS (10 mM Tris HCl buffer, pH 7.4, 150 mM NaCl) containing 3% bovine serum albumin (3% BSA-TBS) for 2 h and were incubated with sera, culture supernatants, or MAAI diluted with 1% BSA/TBS for 2 h. After washing 5 times with TBS, the bound antibody was detected by incubating the wells with biotinylated anti-human IgM or IgG (Zymed Laboratories, San Francisco, CA, USA), followed by incubation with horseradish peroxide (HRP) conjugated streptavidin (Zymed). The enzyme substrate (o-phenylenediamine) was added to each well and the reaction was stopped by the addition of 50 μl of 4 N H_2SO_4 after incubation for 10 min. Optical density at 492 nm (OD492) was determined for each well in the ELISA reader (MTP-32 Microplate Reader, Corona Electric, Tokyo, Japan).

Production of MAAI. We obtained blood from 2 patients with SLE who showed elevated serum titer of IgG antibody against apoA-I. Peripheral blood mononuclear cells were isolated by Ficoll- Hypaque (density 1.077 g/cm^3) (Organon Teknika, Turnhout, Belgium) gradient centrifugation at 400 × g for 30 min at 20°C, were washed twice in 20% fetal calf serum (FCS)-RPMI 1640. Then 1.5×10^6 mononuclear cells in 8 ml of 20% FCS-RPMI 1640 were added in 3 ml of Epstein-Barr virus-containing supernatant obtained from the B85/8 marmoset cell line, and were cultured in 20% FCS-RPMI 1640 containing 0.2% cyclosporine (Sigma, St. Louis, MO, USA) at 37°C in 5% CO_2. The transformed B lymphocytes were cultured in 96 well microtiter plates at 1.56×10^4 cells per well. The culture medium was screened for AAI by ELISA. The myeloma cell line P3-X63-Ag8-653 (obtained from the Japanese Cancer Research Resources Bank, Tokyo) was seeded (2×10^5 cells/ml) in T-25 flasks (Falcon, Samta, SC, USA) and cultured every 3 to 4 days in 5 μM ouabain-containing synthetic medium GIT (Nihon Pharmaceutical Co., Tokyo, Japan). The surviving cells were subsequently grown in T-75 flasks. The resulting line, P3-X63-Ag8-653, was found to be ouabain resistant. For fusion, 4×10^6 of transformed B cells and 4×10^6 of P3-X63-Ag8-653 cells were washed in Dulbecco's modified Eagle's medium (DMEM; Nissui, Japan) and then mixed in a 50 ml tube. After centrifugation at 150 × g for 10 min at room temperature, cells were fused essentially as described by Roder, *et al*[26] by using 50% polyethylene glycol solution (PEG 4000; Merck, Darmstadt, Germany) in DMEM. Fused cells were washed 3 times with 20% FCS-RPMI 1640 and were cultured in 96 well microtiter plates at 4×10^4 cells with 20% FCS-RPMI 1640 per well. From 24 h after fusion, cells were cultured sequentially in 100 μM hypoxanthine/400 nM aminopterin/16 μM thymidine (HAT medium; Flow Laboratories, Burlingame, CA, USA) containing 5 μM ouabain on days 1 to 14, 100 μM hypoxanthine/16 μM thymidine on days 14 to 21, and finally GIT. Hybridoma supernatants were screened for antibodies that were reactive with apoA-I, and those bound to BSA (Sigma) on solid phase were avoided. Hybridomas producing AAI were selected for cloning by 5 rounds of limiting dilution in 96 well plates, and 6 clones were established. IgM antibodies were purified to homogeneity by ammonium sulfate precipitation followed by gel filtration column chromatography (TSK G4000 SWXL, Toso Co., Tokyo, Japan).

Effect of oxidized lipid on binding of MAAI to apoA-I. To study the effect of oxidized lipid on the interaction between MAAI and apoA-I, the wells of an Immulon 1 plate were coated with trilinolein (1 nmol/well) in ethanol and were autoxidized by incubating at 37°C for 12 h[19]. The wells were then incubated with apoA-I (0.25 μg/well) and MAAI (C7A2: 0.5 μg/well, E10A9: 0.1 μg/well) for 2 h at room temperature. The bound MAAI was detected by biotinylated anti-human IgM and HRP conjugated streptavidin

as described above. In some experiments probucol antioxidant (2.5 pmol/well) was included in the trilinolein solution, and the wells were incubated at 37°C for 12 h.

Immunoblotting analysis. Proteins from human HDL were separated by sodium dodecyl sulfate-polyacrylamide gel elecrophoresis (SDS-PAGE) with the use of 12.5% acrylamide gel and transferred to nitrocellulose membrane at 2 mA/cm^2 for 60 min in 25 mM Tris, 192 mM glycine at room temperature using the protein transfer system (Bio-Rad, Richmond, CA, USA). The nitrocellulose membrane with blotted protein was blocked by incubation with 3% BSA/TBS at room temperature for 2 h, and was then incubated with 1% BSA/TBS and MAAI (5 μg/ml) for 2 h at room temperature. Subsequently, the membrane was incubated with biotinylated anti-human IgM (Zymed) for 2 h, followed by incubation with horseradish peroxidase-streptavidin for 1 h. The binding of MAAI to apoA-I on the membrane was identified by coloring with the use of 4-chloro-1-naphthol as substrate.

Binding of MAAI to HDL and oxidized HDL. HDL were oxidized by incubating with 1 mM $MnCl_2$ $4H_2O$ at 37°C for 48 h then dialyzed at 4°C in the dark against TBS. Binding of MAAI to HDL was examined by a competitive ELISA in which MAAI were preincubated with several concentrations of HDL or oxidized HDL, and the binding of MAAI to the plate-coated apoA-I was measured by ELISA. In this assay the wells of the microtiter plates (Immulon 2; Dynatech) were coated with 50 μl (0.25 μg/well) of purified human apoA-I at room temperature for 2 h. After washing, the wells were blocked with 3% BSA/TBS for 2 h. The mixtures containing 40 μl of MAAI (1.5 μg/ml) and the same volume of serial concentrations of either HDL or oxidized HDL (1.125 ~ 72 μg/ml) were preincubated 1 h at room temperature. Fifty microliter aliquots of the mixture were transferred to the apoA-I coated wells and then incubated 2 h. The amounts of bound MAAI were determined by serial incubations with biotinylated anti-human IgM and HRP conjugated streptavidin.

Measurement of proteins. Protein concentrations of lipoprotein and apoprotein were determined by the Lowry method[27]. The BCA protein assay method (Pierce, Rockford, IL, USA) was used for protein estimation of MAAI. BSA was used as a standard for these methods.

RESULTS

Binding of MAAI to oxidized HDL and apoA-I with trilinolein. AAI in sera from patients with SLE (n = 39) and healthy volunteers (n = 18) was examined by ELISA. Titers of IgM and IgG AAI in sera from healthy volunteers were 0.18 ± 0.10 (OD492; mean ± standard deviation, SD) and 0.05 ± 0.03, respectively. High titers of IgM antibody (OD492 > 0.4) were detected in 6 (15.3%) of 39 patients with SLE. High titers of IgG antibody (OD492 > 0.2) were detected in 3 (7.7%) of 39 patients with SLE (data not shown). Six IgM MAAI (C10LD1, YC3G12, C7A2h, E10A9, C7A2, and IB3) reactive with apoA-I in both ELISA and immunoblotting were established from 2 SLE patients with high titers of IgG AAI. In ELISA, all clones bound significantly to apoA-I and no cross-reaction with BSA was observed (data not shown). In immunoblotting analyses, all clones bound specifically to the 28 kDa protein of HDL as well as to purified human apoA-I (Figure 1).

Next we examined the reactivity of MAAI to apoA-I present in HDL. We employed a competitive inhibition ELISA in which MAAI were first incubated with purified HDL and then binding of MAAI to the plate-coated apoA-I was determined by ELISA. As shown in Figure 2, no significant inhibition was observed when MAAI were preincubated with native HDL, suggesting that MAAI bound less effectively to apoA-I present in HDL. We found that the binding of MAAI to the plate-coated apoA-I was effectively inhibited by HDL when the purified HDL was incubated at 37°C in the presence of 1 mM $MnCl_2$ for 48 h. Results obtained with 2 clones, C7A2 and E10A9, are shown in Figure 2. No significant inhibition was observed with the HDL incubated in the absence of $MnCl_2$, suggesting that the oxidative modification of HDL markedly enhanced the interaction between MAAI and apoA-I present on HDL.

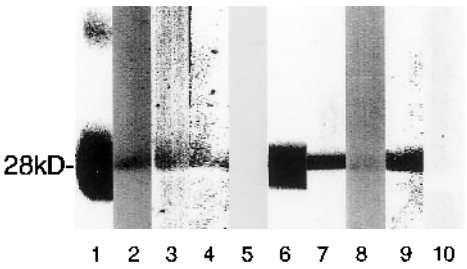

Figure 1. Binding of MAAI to the 28 kDa HDL protein and apoA-I: 10 μg of HDL (lanes 1–5) and 3 μg of purified human apoA-I (lanes 6–10) were subjected to SDS-PAGE on 12.5% acrylamide gels. The proteins were transferred to the nitrocellulose membrane, followed by immunoblotting analysis with MAAI. Typical results obtained with MAAI (5 μg/ml), C10LD1 (lanes 2, 7), E10A9 (lanes 3, 8), and C7A2h (lanes 4, 9) are shown. Lanes 1 and 6 show Coomassie brilliant blue staining of the protein and lanes 5 and 10 represent the background staining without MAAI.

It has been reported that autoantibodies to delipidated apoA-I were detected by the use of oxidized and/or gamma irradiated plates such as Immulon 2, which may mimic a negatively charged phospholipid environment[21,28]. In the present ELISA, MAAI bound effectively to apoA-I coated onto the surface-treated Immulon 2 plates[29], while they did not show any significant binding to apoA-I coated onto the Immulon 1 plates. We found that MAAI bound effectively to apoA-I when the wells of Immulon 1 plates were first coated with trilinolein (1 nmol/well) in ethanol solution and were autoxidized by incubating at 37°C for 12 h[19], followed by incubation with apoA-I and MAAI (Figure 3). The MAAI binding to apoA-I was significantly reduced when a small amount of probucol antioxidant (2.5 pmol/well) was included in the trilinolein solution (Figure 3). In contrast, a xenogenic anti-human monoclonal antibody, clone A/11 (Chemicon International, Temecula, CA, USA), established by immunization of BALB/c mice with purified human apoA-I, bound effectively to apoA-I irrespective of the antioxidant employed for the lipid coating. The decrease of A/11 binding to apoA-I was not caused by the difference in the amount of antioxidant present on the plates. These observations suggest that the interaction of oxidized lipids with apoA-I increased the immunoreactivity of apoA-I with MAAI.

Cross-reactivity of MAAI. Since some autoantibodies raised

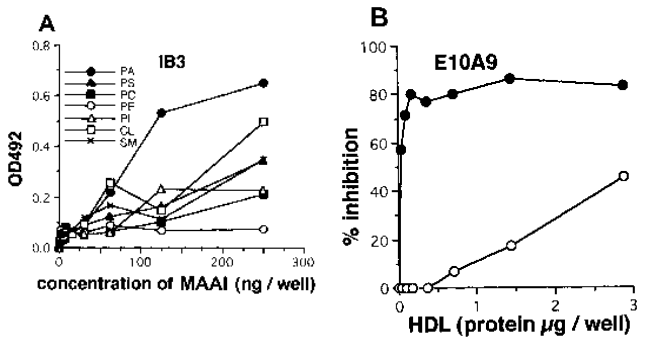

Figure 2. Inhibition of binding of MAAI to apoA-I by oxidized HDL. MAAI [C7A2 (A), E10A9 (B)] were preincubated with various amounts of native HDL (○) or oxidized HDL (●) for 1 h at room temperature. The mixture was transferred to wells preincubated with purified human apoA-I (0.25 μg/well) for 2 h. MAAI binding to apoA-I was detected by biotinylated anti-human IgM and HRP streptavidin. Inhibition of MAAI binding is shown as percentage inhibition of the binding of MAAI to apoA-I in the presence of native or oxidized HDL compared with the binding of MAAI to apoA-I in the absence of native or oxidized HDL.

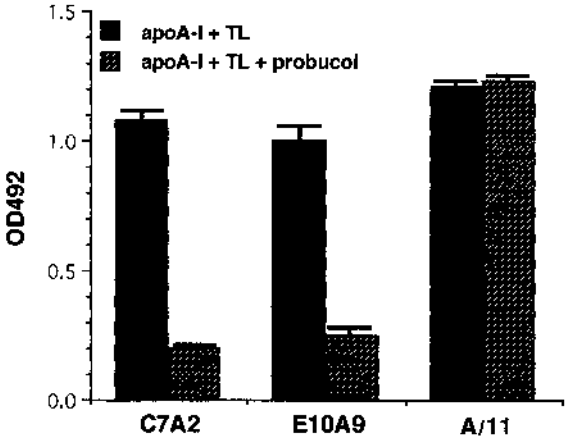

Figure 3. Effect of autoxidized trilinolein (TL) in on binding of MAAI to apoA-I. Wells of Immulon 1 microtiter plates were coated with trilinolein in ethanol (1 nmol/well) and the lipid was autoxidized for 12 h at 37°C in the presence (shaded bar) or absence (black bar) of probucol (2.5 pmol/well). After incubation with BSA (0.5 μg/well), they were incubated with MAAI (C7A2: 0.5 μg/well, E10A9: 0.1 μg/well) and A/11 (0.1 μg/well). Bound MAAI and A/11 were detected by biotinylated anti-human IgM and HRP streptavidin. A xenogenic anti-human Mab, A/11, was used as a control. Results are shown as the mean ± SEM of 3 different experiments.

from patients with connective tissue diseases such as SLE are reported to cross-react with various self-components such as dsDNA and ssDNA, anionic substances, and serum proteins[30,31], cross-reactivities of MAAI with various self-antigens were examined in ELISA. We found that MAAI showed some functional heterogeneity in reactivity with various self-substances. Among 6 MAAI, 2 clones (IB3, C7A2h) reacted significantly with anionic phospholipids such as phosphatidic acid and CL. Two clones (C10LD1, YC3G12) showed an extensive cross-reaction with ssDNA, but neither of them reacted with dsDNA. IB3 was also shown to bind effectively to thrombin, but not to other blood coagulation factors, IIa, V, and Xa. No significant cross-reaction with blood coagulation factors was observed with other MAAI. The reactivity profiles of IB3 with anionic phospholipids and blood coagulation factors are shown in Figure 4. These results indicate that MAAI are composed of autoantibodies with heterogeneous cross-reactivities to various self-components such as ssDNA, anionic phospholipids, and thrombin.

DISCUSSION

All 6 of the MAAI established from 2 patients with SLE bound effectively to apoA-I after the protein had been denatured and transferred to the filter membrane (in immunoblotting analysis), whereas they bound less effectively to apoA-I present in HDL. Oxidation of HDL in the presence of Mn^{2+} strongly enhanced the binding of MAAI to HDL, suggesting that the oxidative modification of HDL increased the immunoreactivity of apoA-I to MAAI. The plasma form of human apoA-I consists of a single 243 amino acid polypeptide[32] and secondary structure analysis indicated that apoA-I contains a series of 22 amino acid amphipathic α-helical repeats that form stable complexes with phospholipids[33,34]. Several studies[35,36] have shown that the conformational structure of apoA-I is highly dependent on the composition and concentration of surface lipids, phospholipids, and cholesterol in HDL, and modifications of the lipid composition of HDL alter the immunoreactivity of apoA-I with monoclonal anti-apoA-I antibody raised against defined regions of the protein. Marcel, *et al* showed that the lipid peroxidation of HDL changes the expression of specific epitopes of apoA-I, resulting in the increase of immunoreactivity of the protein[37]. They suggested that lipid peroxidation could specifically cross-link with apoA-I and change its conformation and antigenicity. We found that the binding of MAAI to apoA-I was significantly enhanced when apoA-I had formed a complex with autoxidized trilinolein, suggesting that MAAI could recognize an epitope of apoA-I that may be exposed by interacting with the oxidized lipid.

We are now analyzing the epitopes for MAAI. Our preliminary results suggest that the binding site of MAAI was localized to within residues 9–50 in the amino terminal end of the protein (unpublished observations). Further analysis of the epitope and the molecular species of oxidized lipids that enhanced the immunoreactivity of apoA-I will provide additional insight into the interaction between AAI and apoA-I.

Analysis of patient sera by Dinu, *et al* indicated the association between the presence of anti-apoA-I antibodies and antiphospholipid antibodies, as suggested by the relatively high prevalence of anti-apoA-I antibodies in patients with

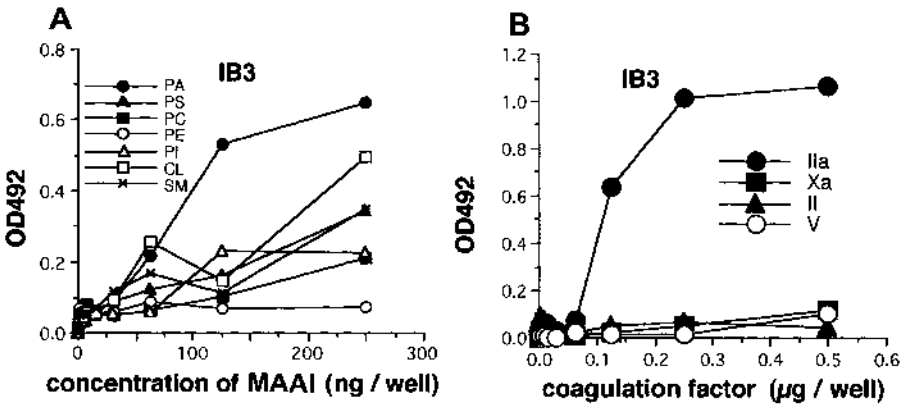

Figure 4. Cross-reactivities of MAAI (IB3). Wells of microtiter plates were coated with various amounts of either phospholipids (PA, CL, PS, PI, PC, PE, and SM) (A) or blood coagulation factors (II, IIa, V, and Xa) (B). Bound IB3 (0.25 μg/well) was detected with biotinylated anti-human IgM followed by incubation with HRP streptavidin. PA: phosphatidic acid, PS: phosphatidylserine, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol, CL: cardiolipin, SM; sphingomyelin.

primary antiphospholipid antibody syndrome and the fact that the majority of patients with anti-apoA-I antibodies also have antiphospholipid antibodies[21]. We have shown that some MAAI show an extensive cross-reaction with anionic phospholipids and ssDNA. One MAAI, IB3, was also shown to bind effectively to thrombin, but not significantly to other blood coagulation factors such as prothrombin, V, and Xa. These results indicate that AAI are composed of autoantibodies with heterogeneous cross-reactivities.

Our study revealed no significant statistical correlation between the AAI titers in sera of patients with SLE and those of other autoantibodies such as antinuclear antibodies, anti-DNA antibodies, anti-cardiolipin $ß_2$-glycoprotein I antibody, and lupus anticoagulant, suggesting that MAAI may be a distinct family of antiphospholipid antibodies.

We determined reactivities of a novel family of monoclonal anti-apoA-I antibodies. MAAI consisted of antibodies with heterogeneous cross-reactivities to thrombin or anionic substances such as CL and ssDNA, and showed preferential binding to apoA-I in oxidatively modified HDL. These MAAI may give us new insight into the lipoprotein metabolism of patients with SLE.

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