Nicked β_2-glycoprotein I: a marker of cerebral infarct and a novel role in the negative feedback pathway of extrinsic fibrinolysis

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β_2-Glycoprotein I (β_2-GPI) is proteolytically cleaved by plasmin in domain V (nicked β_2-GPI), being unable to bind to phospholipids. This cleavage may occur in vivo and elevated plasma levels of nicked β_2-GPI were detected in patients with massive plasmin generation and fibrinolysis turnover. In this study, we report higher prevalence of elevated ratio of nicked β_2-GPI against total β_2-GPI in patients with ischemic stroke (63%) and healthy subjects with lacunar infarct (27%) when compared to healthy subjects with normal findings on magnetic resonance imaging (8%), suggesting that nicked β_2-GPI might have a physiologic role beyond that of its parent molecule in patients with thrombosis. Several inhibitors of extrinsic fibrinolysis are known, but a negative feedback regulator has not been yet documented. We demonstrate that nicked β_2-GPI binds to Glu-plasminogen with K_D of 0.37×10^{-6} M, presumably mediated by the interaction between the fifth domain of nicked β_2-GPI and the fifth kringle domain of Glu-plasminogen. Nicked β_2-GPI also suppressed plasmin generation up to 70% in the presence of tissue plasminogen activator, plasminogen, and fibrin. Intact β_2-GPI lacks these properties. These data suggest that β_2-GPI/plasmin-nicked β_2-GPI controls extrinsic fibrinolysis via a negative feedback pathway loop. (Blood. 2004; 103:3766-3772)

Introduction

β_2-Glycoprotein I (β_2-GPI), also known as apolipoprotein H, is a phospholipid-binding plasma protein. Phospholipid-bound β_2-GPI is one of the major target antigens for antiphospholipid antibodies[1-3] present in patients with antiphospholipid syndrome (APS), an autoimmune disorder characterized by arterial/venous thrombosis and pregnancy morbidity.[4] β_2-GPI has 5 homologous short consensus repeats, designated as domains I to V. Domains of β_2-GPI structurally resemble each other, except that domain V has an extra C-terminal loop and a positively charged lysine cluster. In 1993, Hunt et al[5] reported that β_2-GPI is proteolytically cleaved between Lys317 and Thr318 in domain V (nicked β_2-GPI), being unable to bind to phospholipids. This cleavage is generated by factor Xa or by plasmin, with plasmin being more effective.[6]

A large number of reports have detailed the in vitro properties of β_2-GPI as a natural anticoagulant/procoagulant regulator by inhibiting phospholipid-dependent reactions, such as prothrombinase and tenase activity on platelets or phospholipid vesicles,[7,8] factor XII activation,[9] and anticoagulant activity of activated protein C.[10,11] Apart from specific hemostatic functions, β_2-GPI activates lipoprotein lipase,[12] lowers the triglyceride level,[13] binds to oxidized low-density lipoprotein to prevent the progression of atherosclerosis,[14] and binds to nonself particles or apoptotic bodies to allow their clearance.[15-17] Little attention has been given to the functions of the nicked form of β_2-GPI because its phospholipid-binding activity was thought to exert the physiologic or pathologic functions of β_2-GPI.

Fibrinolytic reactions involve the formation of plasmin from the zymogen plasminogen and the hydrolytic cleavage of fibrin to fibrin degradation products by plasmin. Plasminogen, a 92-kDa glycoprotein, is present in plasma at a concentration of approximately 2 μM.[18] Plasminogen consists of 7 domains: one N-terminal peptide, 5 kringle domains bearing a lysine-binding site (LBS) with the capacity to bind fibrin as well as antifibrinolytic proteins carrying lysine, and one serine protease domain.[19] Plasmin conversion from plasminogen by tissue plasminogen activator (tPA) is a key event in extrinsic fibrinolysis for the thrombolysis against intravascular blood clots. Plasmin is one of the most potent enzymes and has a variety of biologic activities; thus, the regulation of plasmin generation and activity is important to maintain the homeostatic balance in vivo. In particular, an excess of fibrinolytic activity can lead to life-threatening bleeding events. Physiologic inhibitors of extrinsic fibrinolysis include α_2-antiplasmin (α_2-AP)[20] and plasminogen activator inhibitor 1 (PAI-1).[21] These inhibitors regulate fibrinolysis through different mechanisms.

Nicked β_2-GPI has been identified by sandwich enzyme-linked immunosorbent assay (ELISA) in plasma of patients with disseminated intravascular coagulation (DIC)[22] or leukemia,[23] both conditions characterized by massive thrombin generation and fibrinolytic

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turnover. To investigate the biologic and clinical significance of nicked β_2-GPI in a disease characterized by a lower level of thrombin generation and fibrin turnover than DIC, we evaluated the cleavage ratio of β_2-GPI in plasma of patients with ischemic stroke and the results are presented herein. Further, we investigated the role of nicked β_2-GPI in extrinsic fibrinolysis and demonstrate for the first time that nicked β_2-GPI binds to plasminogen. We also describe the inhibitory effect of nicked β_2-GPI on the fibrin surface where plasminogen is proteolytically activated into plasmin. Because β_2-GPI may be cleaved in vivo by plasmin during thrombus formation and thrombolysis, these phenomena represent a novel negative feedback loop in extrinsic fibrinolysis where β_2-GPI plays a key role.

Patients, materials, and methods

Study patients

The study population comprised 62 patients with history of ischemic stroke diagnosed by magnetic resonance imaging (MRI) performed at the time of admission to the Azabu Neurosurgical Hospital (female-to-male ratio, 12:50; mean age, 68 ± 9 years). Blood samples were obtained from the patients at least 6 months after their last occlusive event.

We also investigated 130 age- and sex-matched apparently healthy subjects with no history of cerebral infarct who consented to join the study. All subjects underwent a cerebral MRI at the Neuroradiology Department at Mitsui Memorial Hospital and images were analyzed by an experienced neuroradiologist. According to the MRI findings the healthy subjects were divided into 2 groups: 52 with lacunar infarcts (female-to-male ratio, 20:32; mean age 67 ± 9 years) and 78 without any abnormality (female-to-male ratio, 26:52; mean age, 66 ± 6 years). Blood sampling was performed at the same time of the MRI scan. All the patients and healthy volunteers provided informed consent according to Declaration of Helsinki principles.

Blood collection

Venous blood was collected in tubes containing one-tenth volume of 0.105 M sodium citrate and was centrifuged immediately at 4°C. Plasma samples were depleted of platelets by filtration then stored at −70°C until use.

Materials

Monoclonal antibodies. To measure the plasma levels of nicked or total β_2-GPI, we used 2 monoclonal antibodies, 1 monoclonal anti–nicked β_2-GPI antibody (NGPI-60) that specifically reacts against nicked β_2-GPI and the other monoclonal anti-β_2-GPI antibody (NGPI-23) that equally reacts with nicked and intact β_2-GPI.[23]

An IgG mouse monoclonal antihuman β_2-GPI antibody directed to domain III of human β_2-GPI (Cof-22) was used for the purification of nicked β_2-GPI and evaluation of the binding of nicked β_2-GPI to immobilized Glu-plasminogen.[24] Cleavage of β_2-GPI by plasmin did not affect the binding of Cof-22 to β_2-GPI because the epitope of Cof-22 antibody on β_2-GPI molecule resides on domain III (data not shown).

Antihuman plasminogen antibodies directed to kringles 1 to 3 or against kringle 4 were obtained from American Diagnostica (Greenwich, CT).

Proteins. β_2-GPI was purified from human plasma, as described.[25] Nicked β_2-GPI was prepared as reported[26] with slight modifications that included an additional purification step; β_2-GPI was treated with human plasmin (Calbiochem Novabiochem, La Jolla, CA) at 37°C for 3 hours, at a molar ratio of β_2-GPI/plasmin of 8:1. Plasmin-treated β_2-GPI was first purified on a Cof 22–Sepharose column and subsequently on a heparin-Sepharose column. The heparin nonbinding fraction was collected and further purified by ion-exchange chromatography using Mono-Q column (Pharmacia Biotech, Uppsala, Sweden). Purified β_2-GPI was reduced using 2-mercaptoethanol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), appearing as a single band smaller than that of the intact one (data not shown).

The domain V–deleted mutant protein (domains I-IV) of β_2-GPI was expressed using a baculovirus system as reported.[24] This mutant β_2-GPI does not include the cleavage site for plasmin.

Glu-plasminogen was purified from the plasma of healthy Japanese donors using chromatography on lysine-Sepharose 4B (Pharmacia Biotech) and diethylaminoethyl (DEAE) Sephadex A-50 (Pharmacia Biotech). Plasminogen kringles 1 to 3 fragment, plasminogen kringle 4 fragment, and mini-plasminogen, which consists of the kringle 5 and serine protease domain of plasminogen, were obtained from Technoclone (Vienna, Austria). Recombinant tPA (2-chain, Duteplase) was obtained from Sumitomo Pharmaceutical (Osaka, Japan). ϵ-Aminocaproic acid (EACA) was purchased from Sigma Chemical (St Louis, MO).

Methods

Measurement of plasma levels of nicked β_2-GPI. Plasma levels of nicked β_2-GPI were determined by a sandwich ELISA as previously described with slight modifications.[23] Briefly, polystyrene microtiter plates were coated with 100 μL monoclonal anti–nicked β_2-GPI antibody (NGPI-60) in 50 mM Tris (trishydroxymethylaminomethane)–HCL, pH 7.5, containing 0.15 M NaCl and incubated overnight at 4°C. Wells were washed 3 times with 0.5 M NaCl containing 0.05% Tween 20 and 100 μL citrated plasma samples diluted 5-fold in 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and 0.05% Tween 20 (sample buffer) were added. After 2 hours of incubation at room temperature and washing 3 times, 100 μL biotinylated F(ab')$_2$ fragment of monoclonal anti-β_2-GPI (NGPI-23; 2 μg/mL) was added to each well, followed by 1 hour of incubation. Then, 100 μL alkaline phosphatase (ALP)–conjugated streptavidin (Zymed, San Francisco, CA) at a 1:1000 dilution in sample buffer was added to each well. After another 1 hour of incubation and 3 times washing, 200 μL substrate (1 mg/mL p-nitrophenylphosphate disodium [Sigma Chemical] in 1 M diethanolamine buffer [pH 9.8]) was added. Optical density (OD) was read at 492 nm with reference at 620 nm using an ELISA plate reader. The plasma levels of nicked β_2-GPI were determined from a standard curve constructed with citrated plasma spiked with known amounts of purified nicked β_2-GPI.

Measurement of plasma levels of total β_2-GPI. Plasma levels of total β_2-GPI were determined by a sandwich ELISA using F(ab') fragment of NGPI-23 as the capture antibody and biotinylated antihuman β_2-GPI rabbit IgG as the tag antibody as previously reported.[23] Plasma samples of 50 μL (8000-fold diluted) were added to the wells containing the immobilized antibody. The ALP-conjugated streptavidin (Zymed) was then added and bound ALP was determined as described ("Measurement of plasma levels of nicked β_2-GPI"). The amounts of total β_2-GPI in plasma were calculated from a calibration curve constructed with known amounts of purified β_2-GPI. A nicked β_2-GPI ratio was calculated in all samples using the formula: (plasma nicked β_2-GPI/plasma total β_2-GPI) × 1000.

Other laboratory investigations. The same plasma samples were tested for thrombin-antithrombin (TAT) complexes, plasmin-antiplasmin (plasmin inhibitor) complex (PPI), and D-dimers (DDs) by latex agglutination assay using commercial kits LPIAACE TAT, LPIAACE PPI, LPIAACE D-D dimer (Dia-Iatron, Tokyo, Japan), according to the manufacturer's instructions.

ELISA for binding of intact or nicked β_2-GPI to plasminogen. The binding of nicked or intact β_2-GPI was investigated by ELISA. Fifty microliters of Glu-plasminogen (10 μg/mL) in phosphate-buffered saline (PBS), pH 7.4, was distributed in each well of a Sumilon Type S microtiter ELISA plate (Sumitomo Bakelite, Tokyo, Japan) and incubated overnight at 4°C. After washing twice with PBS and blocking with 2% gelatin-PBS for 1 hour at 37°C, 50 μL of serial dilutions of intact or nicked β_2-GPI in 1% bovine serum albumin (Sigma Chemical)–PBS (1% BSA-PBS) were placed in each well. Plates were incubated for 1 hour at room temperature and washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween), then 50 μL/well Cof-22 (100 ng/mL) in 1% BSA-PBS was distributed. After incubation and washing as above, 50 μL/well of ALP-conjugated anti-mouse IgG (Sigma Chemical), diluted 1:2000 in 1% BSA-PBS, was put into each well, followed by incubation. Substrate (100 μL) was distributed after washing 4 times with PBS-Tween and incubated. OD was read at 405 nm with reference at 620 nm.

The role of plasminogen LBS in binding to nicked β_2-GPI was evaluated by a competitive ELISA adding serial dilutions of EACA, a lysine analog, into the nicked β_2-GPI solution.

Kinetic assay for molecular interaction between nicked β_2-GPI and plasminogen. Real-time analysis for molecular interaction between nicked β_2-GPI and Glu-plasminogen was performed using an optical-biosensor, IAsys system (Affinity Sensors, Paramus, NJ). Biotinylated Glu-plasminogen was immobilized on the wall of a biotin cuvette (Affinity Sensors) via streptavidin (Sigma Chemical). After blocking with 0.01% BSA-PBS and washing with PBS, various concentrations (up to 4 μM) of native or nicked β_2-GPI were placed in the cuvette and ligand bound to the plasminogen-coated surface was detected. Obtained data were fitted using linear regression to find the intercept and gradient. This analysis was used to determine the association rate constant (k_{ass}) and dissociation rate constant (k_{diss}), from the variation of the on-rate constant (k_{on}) with ligand concentration. According to the equation; $k_{on} = k_{diss} + k_{ass}$ [ligand], K_D and K_A are determined as follows; $K_D = k_{diss}/k_{ass}$ and $K_A = k_{ass}/k_{diss}$.

Inhibition ELISA. To identify the nicked β_2-GPI-binding site on Glu-plasminogen, the inhibition of Glu-plasminogen binding by fragments of plasminogen was examined. Fifty microliters of nicked β_2-GPI (0.2 μM) diluted in PBS was put into each well of a MaxiSorp microtiter plate (Nalge Nunc International, Roskilde, Denmark) and incubated overnight at 4°C. After washing twice with PBS and blocking with 2% gelatin-PBS for 1 hour at 37°C, serial dilutions of inhibitor (BSA, plasminogen kringle 1-3, plasminogen kringle 4, or mini-plasminogen) were added (50 μL/well) followed by overnight incubation at 4°C. After washing with PBS-Tween, 10 μg/mL Glu-plasminogen was then added (50 μL/well) and incubated for 30 minutes at room temperature, and plates were washed 3 times with PBS-Tween. To compare the inhibitory effect between kringle 1 to 3 and mini-plasminogen, a monoclonal antikringle 4 antibody (American Diagnostica) was used to detect bound Glu-plasminogen, whereas a monoclonal antikringle 1 to 3 antibody (American Diagnostica) was used to compare the inhibition of mini-plasminogen with that of kringle 4. After incubation with these monoclonal antibodies, bound Glu-plasminogen on nicked β_2-GPI was evaluated by ALP-conjugated antimouse IgG, followed by substrate addition as described ("ELISA for binding of intact or nicked β_2-GPI to plasminogen").

Inhibitory effect of nicked β_2-GPI on the binding of plasminogen to fibrin. To investigate whether nicked β_2-GPI interferes with the binding of Glu-plasminogen to immobilized fibrin in a liquid phase or not, the following experiment was done. Each well of a Sumilon Type S microtiter plate (Sumitomo Bakelite) was coated with soluble fibrin monomer (5 μg/mL) and incubated at 4°C overnight, followed by washing with PBS-Tween and blocking with 2% gelatin-PBS at 37°C. Biotinylated Glu-plasminogen (5 μg/mL in 1% BSA-PBS) was preincubated with different concentrations of intact or nicked β_2-GPI for 1 hour at room temperature and added to the wells in triplicate. After incubation for 1 hour at room temperature, each well was washed with PBS-Tween. ALP-conjugated streptavidin was diluted to 3000 times in PBS and distributed to the wells. After 1 hour of incubation and washing, substrate was added and absorbance was measured as described.

Effects of intact or nicked β_2-GPI on tPA activity: chromogenic assay. In the presence of fibrin, tPA can effectively activate plasminogen to plasmin. Because we speculated that nicked β_2-GPI might interfere with this activation step by binding to plasminogen, chromogenic assay measuring plasmin generation was introduced in the presence of tPA, Glu-plasminogen, fibrin monomer, and β_2-GPI. The effect of intact/nicked β_2-GPI on the activity of plasmin generated was evaluated using a parabolic rate assay. The activity of tPA was measured in a chromogenic assay as described[27] with some modifications. A mixture of the same volume of 50 U/mL tPA in PBS and 1 M acetate buffer (pH 3.9) was incubated for 5 minutes at room temperature, then diluted 1:160 with assay buffer (50 mM Tris-HCl, pH 8.8, 100 mM NaCl, and 0.01% Triton X-100). Then 100 μL of the diluted tPA solution was incubated in a Sumilon Type S microtiter plate with 100 μL detection reagents consisting of Glu-plasminogen and plasmin-sensitive substrate (Glu-plasminogen [70 μg/mL] and 0.6 mM chromogenic substrate S-2251 [Chromogenix, Möndal, Sweden] in assay buffer) with intact or nicked β_2-GPI and 2 μL/well soluble fibrin monomer (3.3 mg/mL, in 3.5 M urea). The final concentrations of intact/nicked β_2-GPI were 0, 0.25, and 0.5 μM. Domain I to IV of β_2-GPI mutant or BSA served as the negative control. After incubation at 37°C for 12 hours, the activity of plasmin generated was determined by measuring absorbance at 405 nm using a microplate reader (model 3550; BioRad, Hercules, CA). A standard curve was generated using serial dilutions of tPA. The plasmin generation in this system was expressed as corresponding tPA activity (U/mL).

Effects of intact or nicked β_2-GPI on tPA activity: fibrin plate assay. To exclude the possibility that nicked β_2-GPI affected S-2251 cleavage without interacting with fibrinolytic factors, fibrinolysis was evaluated by conventional fibrin plate assays. Fibrin was layered on a plastic plate 10 cm in diameter, using the same volumes of 0.2% plasminogen-free fibrin (Sigma Chemical), 1% agarose, and 200 μL/plate thrombin, 20 U/mL. Then, 6 μL of the diluted tPA solution ("Effects of intact or nicked β_2-GPI on tPA activity: chromogenic assay") was incubated with the same volume of Glu-plasminogen (70 μg/mL) in assay buffer, with intact or nicked β_2-GPI (up to 0.5 μM). After 36 hours of incubation at 37°C, the area of lysis rings was measured. A standard curve was generated from serial dilutions of tPA.

Statistical analysis. Statistical evaluation was performed by the *t* test, Fisher exact test, χ^2 test, or Spearman rank correlation as appropriate. *P* values less than .05 were considered statistically significant.

Results

Levels of nicked β_2-GPI in plasma samples

The plasma levels of nicked β_2-GPI ratio are shown in Figure 1. A normal level of nicked β_2-GPI ratio was derived from the apparently healthy subjects without any MRI abnormality, the mean plus 1 SD representing the upper limit of normal. A higher prevalence of elevated nicked β_2-GPI ratio was found in patients with ischemic stroke (63%, 39 of 62) and healthy subjects with lacunar infarct (27%, 14 of 52) when compared to healthy subjects with normal MRI findings (8%, 6 of 78). Relative risks of having stroke or asymptomatic lacunar infarction were approximated by odds ratio (95% CI), 20.3 (7.6-54.2) and 4.4 (1.6-12.4), respectively.

The prevalence of elevated levels of markers of thrombin generation and fibrinolytic turnover in our population are shown in Figure 2. A statistically significant correlation was observed between levels of PPI and nicked β_2-GPI ratio in plasma of healthy subjects with lacunar infarct ($r^2 = 0.31$, $P = .02$). No correlations were found between nicked β_2-GPI ratio and DDs or TAT complexes in any of the groups.

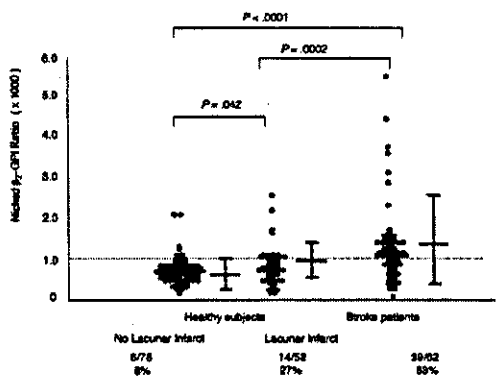

Figure 1. Plasma levels of nicked β_2-GPI. Total and nicked β_2-GPI plasma levels were determined by ELISA. A nicked β_2-GPI ratio, (plasma nicked β_2-GPI/plasma total β_2-GPI) × 1000, was established in all the samples. The dashed line indicates the mean + 1 SD of the ratio in healthy subjects without lacunar infarct. *P* values were calculated using *t* test.

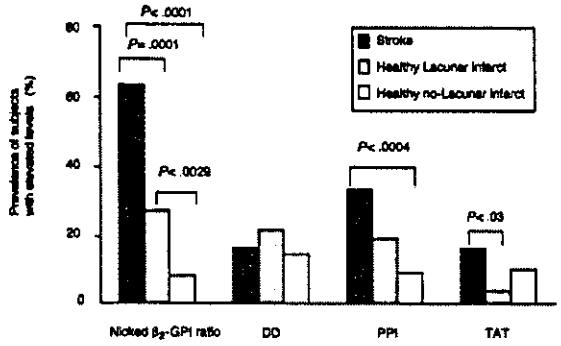

Figure 2. Prevalence of abnormally elevated plasma levels of nicked β_2-GPI and of markers of thrombin generation/fibrinolytic turnover in our population. Plasma levels of D-dimers (DD), plasmin-antiplasmin complex (PPI), and thrombin-antithrombin complexes (TAT) were determined in all the subjects as described in "Patients, materials, and methods."

In the apparently healthy subjects group (n = 130), plasma nicked β_2-GPI ratio significantly correlated with age ($r^2 = 0.483$, $P < .0001$; Figure 3). Therefore, plasma measurement of nicked β_2-GPI might be a useful screening tool in the assessment of patients at risk of ischemic stroke.

Binding of nicked β_2-GPI to Glu-plasminogen

The binding of up to 0.4 μM nicked β_2-GPI to solid-phase Glu-plasminogen occurred in a dose-dependent manner, whereas the same concentrations of intact β_2-GPI did not bind to Glu-plasminogen (Figure 4A). The binding of Cof-22 to β_2-GPI was not affected by the cleavage of β_2-GPI. Molecular interaction between intact or nicked β_2-GPI and plasminogen was investigated using an optical biosensor. Nicked β_2-GPI showed a large extent of binding to immobilized Glu-plasminogen, whereas intact β_2-GPI did not show any specific binding (Figure 4B). The data of k_{on} at different concentrations of nicked β_2-GPI were fitted using linear regression, determining k_{ass} as 0.0006 $M^{-1}s^{-1}$ and k_{diss} as 0.0022 s^{-1} (Figure 4C). Accordingly, K_D and K_A were determined as 0.37×10^{-6} M and 2.70×10^6 M^{-1}, respectively.

Inhibition of binding of Glu-plasminogen to nicked β_2-GPI by the fragments of plasminogen or by EACA

The binding of Glu-plasminogen (10 μg/mL) to immobilized nicked β_2-GPI, but not to native β_2-GPI, was demonstrated by ELISA. For the inhibition assay, the fragments of plasminogen (mini-plasminogen or kringle 4) as the inhibiting factors were added to the wells coated with nicked β_2-GPI, and bound Glu-plasminogen was detected using a monoclonal antikringle 1 to 3 antibody. Mini-plasminogen, but not kringle 4, inhibited the binding between Glu-plasminogen and nicked β_2-GPI (Figure 5A). Kringle 1 to 3 fragment or mini-plasminogen was added as inhibitor and bound Glu-plasminogen was detected using a monoclonal antikringle 4 antibody. Glu-plasminogen binding to nicked β_2-GPI was dose dependently inhibited by mini-plasminogen but not by kringle 1 to 3 fragment (Figure 5B). The fifth domain or the catalytic domain of Glu-plasminogen, therefore, was predicted to mediate its binding to nicked β_2-GPI.

When the binding of nicked β_2-GPI (10 μg/mL) to solid-phase Glu-plasminogen was tested in the presence of different concentrations of EACA, the binding between nicked β_2-GPI and immobilized Glu-plasminogen was abolished in a dose-dependent manner (Figure 5C). Accordingly, LBS on plasminogen might mediate the binding of nicked β_2-GPI to Glu-plasminogen.

Binding of plasminogen to fibrin interfered with by nicked β_2-GPI

We also investigated whether nicked β_2-GPI has an effect on the binding of Glu-plasminogen to immobilized fibrin monomer using an ELISA system. After preincubation with nicked β_2-GPI, but not with intact β_2-GPI, Glu-plasminogen showed decreased binding activity to soluble fibrin monomer (Figure 5D).

Effects of nicked β_2-GPI on extrinsic fibrinolysis

The amidolytic activity of newly generated plasmin was evaluated as tPA activity (U/mL) in a chromogenic assay. The activity increased with the concentration of tPA (data not shown). When nicked β_2-GPI was added, the tPA activity decreased in a dose-dependent manner (Figure 6A). Intact β_2-GPI at 0.25 μM did not suppress the fibrinolytic activity, whereas intact β_2-GPI in a higher concentration (0.50 μM) slightly suppressed the fibrinolytic activity. The same amount of BSA or the recombinant domain I to IV of β_2-GPI did not affect the tPA activity.

The fibrinolytic activity of generated plasmin was measured as tPA activity (U/mL) in a fibrin plate assay. Fibrinolytic activity was suppressed by nicked β_2-GPI at 0.25 and 0.50 μM. Intact β_2-GPI at 0.50 μM also slightly inhibited the fibrinolytic activity. However, 0.25 μM intact β_2-GPI did not affect the fibrinolytic activity of tPA (Figure 6B).

Discussion

In the first part of this study, we demonstrated that plasma levels of nicked β_2-GPI were elevated in patients with ischemic stroke, indicating an elevated degree of fibrin turnover, but lower than that of DIC where thrombin and plasmin are massively generated.

In fact, nicked β_2-GPI was detected in large quantities in plasma of patients with DIC, a pathologic state characterized by marked increase of plasma PPI.[22] We observed a strong correlation between plasma levels of nicked β_2-GPI and those of PPI in the healthy individuals showing lacunar infarcts on MRI, suggesting that nicked β_2-GPI may rather reflect "minor" plasmin generation. In the presence of larger plasmin generation, the correlation between nicked β_2-GPI and PPI may be lost,[23] presumably due to the consumption of α_2-AP. In individuals with MRI abnormalities the prevalence of increased nicked β_2-GPI ratio was higher than that of PPI, DDs, and TAT complexes (46%, 27%, 19%, and 11%, respectively). Thus, the detection of nicked β_2-GPI may

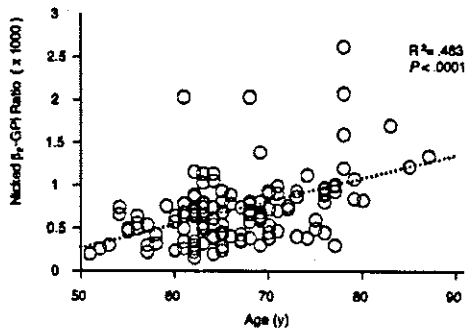

Figure 3. Correlation between plasma levels of nicked β_2-GPI and age in apparently healthy subjects. Nicked β_2-GPI was measured by a sandwich ELISA. The dotted line represents the regression curve. Each circle shows nicked β_2-GPI ratio and age in each subject.

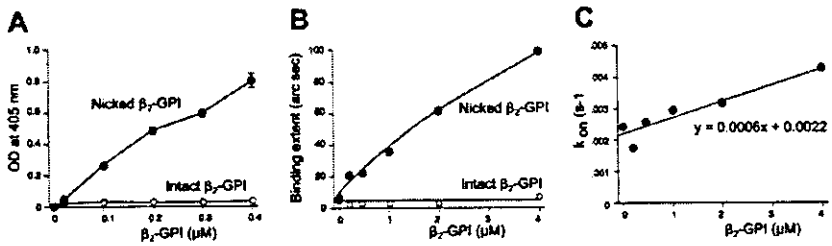

Figure 4. Binding of intact/nicked β_2-GPI to Glu-plasminogen. (A) Binding of intact or nicked β_2-GPI to immobilized Glu-plasminogen was evaluated by ELISA using mouse monoclonal anti-β_2-GPI antibody Cof-22. Closed circles indicate the dose-dependent binding of nicked β_2-GPI to Glu-plasminogen, whereas open circles indicate that intact β_2-GPI is unable to bind to Glu-plasminogen. (B-C) Kinetic plot showing molecular interaction between Glu-plasminogen and intact or nicked β_2-GPI. Intact β_2-GPI or nicked β_2-GPI binding to Glu-plasminogen was detected using IAsys, an optical biosensor as described in "Patients, materials, and methods." Binding extent (arc sec) was compared between intact and nicked β_2-GPI (B). Obtained on-rate constant (k_{on}) for nicked β_2-GPI was plotted and fitted using linear regression to find the intercept and gradient (C). A formula for determining the association rate constant (k_{ass}) and dissociation rate constant (k_{diss}) is as follows: $k_{on} = k_{diss} + k_{ass}$ [ligand]. Error bars indicate SDs.

represent a more sensitive marker of vascular lesions than PPI, DDs, or TAT complexes.

In support of this concept is the correlation between nicked β_2-GPI ratio and age in the apparently healthy subjects, suggesting that "minor" plasmin generation might be associated with subclinical or early clinical atherosclerosis. It is widely accepted that atherosclerosis is associated with endothelial cell activation and minor plaque rupture leading to small thrombus formation, secretion of t-PA, and plasmin generation, ultimately cleaving β_2-GPI. Indeed, nicked β_2-GPI can be generated on the surface of activated endothelial cells or platelets.[23]

In the second part of this study, we investigated the properties of nicked β_2-GPI in vitro to evaluate the biologic significance of our observations. We showed that nicked β_2-GPI specifically binds to Glu-plasminogen and inhibits extrinsic fibrinolysis in vitro. In contrast, neither domain I to IV of β_2-GPI nor intact β_2-GPI revealed such functions. The administration of intact β_2-GPI in higher concentrations also suppressed plasmin generation, perhaps owing to the nicked β_2-GPI produced by the newly generated plasmin. Under clinical conditions characterized by massive plasmin generation such as DIC or acute thrombosis, plasmin is generated by tPA released from activated endothelial cells with thrombus formation, and plasmin cleaves β_2-GPI on the thrombus, changing the properties of β_2-GPI. We propose that β_2-GPI is a precursor of plasmin-nicked β_2-GPI, a physiologic inhibitor of fibrinolysis.

The crystal structure of human β_2-GPI has been defined.[28,29] Bouma et al[28] proposed that a large positively charged patch in domain V binds to anionic surfaces with a flexible and partially hydrophobic loop inserted into the lipid layer. According to the conformation of the nicked domain V, as predicted from the x-ray structure of the intact domain V and confirmed by heteronuclear magnetic resonance, the nicked C-terminal loop is tightly fixed by electrostatic interaction with enhanced stability, the result being neutralization of the positive charge of the lysine cluster.[26,30]

Glu-plasminogen, a full-length protein, is the naturally circulating form of plasminogen. Kringle 5 of Glu-plasminogen has a higher affinity for intact fibrin.[31,32] LBS in kringle 5 of Glu-plasminogen mediates its binding to N-terminal lysine on fibrin, an event essential to initiate fibrinolysis reactions. This initial binding of Glu-plasminogen to fibrin induces a conformational change from a "closed" to an "open" form, thus promoting accessibility to plasminogen activators such as tPA or urokinase.[19] On the fibrin surface, generated plasmin cleaves the single-chain tPA into the 2-chain tPA, a more active form, providing a positive feedback for plasmin generation. Plasmin simultaneously degrades fibrin and makes C-terminal lysine of fibrin more accessible to plasminogen via kringles 1,[33,34] 2, and 3,[35] thus accelerating fibrinolysis.

According to the results of the inhibition studies using plasminogen fragments or EACA (Figure 5), and comparison of the effect on plasmin generation between nicked β_2-GPI and domain I to IV of β_2-GPI (Figure 6A), it would be indicated that the binding of nicked β_2-GPI to Glu-plasminogen is mediated by the interaction between the lysine-cluster patch in domain V of the nicked β_2-GPI and LBS on the plasminogen kringle 5,[36] although it still may be possible that an excess amount of EACA interacts with the catalytic domain of Glu-plasminogen. The conformational difference between intact and nicked β_2-GPI is critical for its binding to

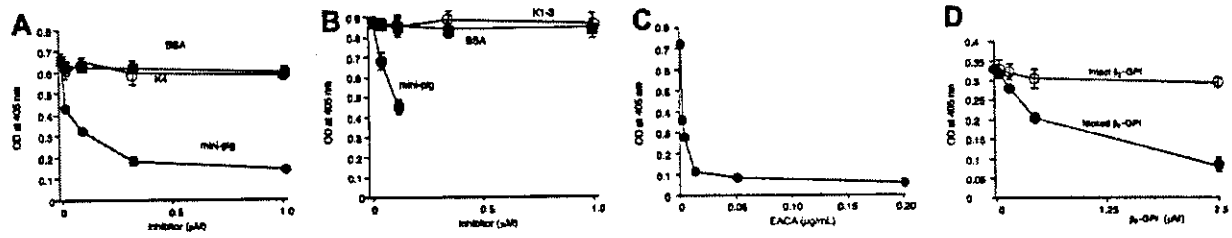

Figure 5. Identification of the binding site of Glu-plasminogen to β_2-GPI by inhibition ELISA using plasminogen fragments. (A) Binding of Glu-plasminogen to immobilized nicked β_2-GPI was tested by ELISA in the presence of possible inhibitors. After nicked β_2-GPI immobilization onto microtiter plates, different concentrations of kringle 4 of plasminogen (○) or mini-plasminogen (that consists of kringle 5 and catalytic domain of plasminogen; ●) were added as inhibitors. BSA (■) served as control. After incubation and washing, Glu-plasminogen (10 μg/mL) was added and bound Glu-plasminogen was determined using kringle 1- to 3-specific mouse monoclonal antiplasminogen antibody. (B) For the inhibition ELISA kringle 1 to 3 of plasminogen (○) or mini-plasminogen (●) served as inhibitors. Glu-plasminogen bound to immobilized β_2-GPI was detected using kringle 4-specific mouse monoclonal antiplasminogen antibody. Assays were run in triplicate. (C) Competitive ELISA using EACA, a lysine homologue. Binding of nicked β_2-GPI (0.2 μM) to immobilized Glu-plasminogen was tested by ELISA using Cof-22 antibody in the presence of various concentrations of EACA (0-0.20 μg/mL). (D) Soluble fibrin monomer (5 μg/mL) was coated on the surface of a microtiter plate and blocked. Biotinylated Glu-plasminogen (5 μg/mL) was preincubated with intact or nicked β_2-GPI and added to the wells. After incubation and washing, ALP-conjugated streptavidin was used for detection. Assays were run triplicate. Error bars indicate SDs. K indicates kringle; mini-plg, mini-plasminogen.

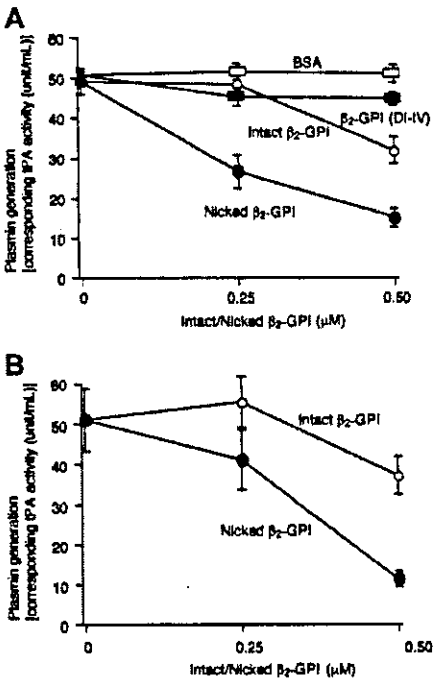

Figure 6. Inhibitory effect of nicked β_2-GPI on plasmin generation. (A) Plasmin generation was measured by parabolic rate assay using synthetic substrate S-2251 in the presence of tPA, Glu-plasminogen, and fibrin monomer. Nicked β_2-GPI (●), intact β_2-GPI (○), β_2-GPI domain I-IV mutant (■), or BSA (□) was added to the reaction in the indicated concentrations. After 12 hours of incubation, absorbance at 405 nm was measured and expressed as tPA activity (U/mL) using tPA as standard. (B) Fibrinolytic activity was measured using fibrin plate assay. Solution reaction containing tPA, Glu-plasminogen, and nicked (●) or intact β_2-GPI (○) were placed onto fibrin plates. After 36 hours of incubation, the ring area of lysis was measured. Assays were performed in triplicate. Error bars indicate SDs. D indicates domain.

phospholipid or plasminogen. The lysine-cluster patch in domain V of nicked β_2-GPI may gain accessibility for the LBS of Glu-plasminogen, whereas the C-terminal loop of intact β_2-GPI may interfere with interactions of LBS and the Glu-plasminogen kringle 5.

The fibrinolytic system is regulated at different levels, either at plasminogen activation or at enzymatically active plasmin. Many factors, including α_2-AP, α_2-macroglobulin, α_1-antitrypsin, inactivated C1, PAI-1, and PAI-2, prevent the overactivation of the fibrinolytic system. The most potent inhibitors are α_2-AP and PAI-1[37]; the former binds to a component of kringle 1 to 3 of plasminogen[38] and can neutralize the generated plasmin more rapidly than α_2-macroglobulin.

Fibrinolysis initiates on binding of kringle 5 of plasminogen to lysine residues on fibrin followed by the binding of kringle 1 to 3 of plasminogen to lysine residues on the cleaved fibrin. α_2-AP does not bind to kringle 5 of plasminogen, hence, does not seem to affect the first interaction. Based on the observation that nicked β_2-GPI interferes the binding between Glu-plasminogen and fibrin monomer (Figure 5D), it is likely that the binding of nicked β_2-GPI to Glu-plasminogen affects the first step of fibrinolysis at least and exerts an inhibitory function in the fibrinolytic system via different mechanisms from that of α_2-AP.

In conclusion, first we have demonstrated that plasma levels of nicked β_2-GPI can be a sensitive marker of cerebral ischemic events and we suggest that plasma measurement of nicked β_2-GPI might be a useful screening tool in the assessment of patients at risk of ischemic stroke. Second, we propose that nicked β_2-GPI is a physiologic inhibitor of fibrinolysis and that plasmin cleavage of β_2-GPI is part of the negative feedback pathway of extrinsic fibrinolysis.

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Anti-mannose binding lectin antibodies in sera of Japanese patients with systemic lupus erythematosus

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SUMMARY

Mannose-binding lectin (MBL) is a key element in innate immunity with functions and structure similar to that of complement C1q. It has been reported that MBL deficiency is associated with occurrence of systemic lupus erythematosus (SLE). We hypothesized that anti-MBL antibodies, if present, would affect the occurrence or disease course of SLE, by reduction of serum MBL levels, interference of MBL functions, or binding to MBL deposited on various tissues. To address this hypothesis, we measured the concentration of anti-MBL antibodies in sera of 111 Japanese SLE patients and 113 healthy volunteers by enzyme immunoassay. The titres of anti-MBL antibodies in SLE patients were significantly higher than those in healthy controls. When the mean + 2 standard deviations of controls was set as the cut off point, individuals with titres of anti-MBL antibodies above this level were significantly more frequent in SLE patients (9 patients) than in controls (2 persons). One SLE patient had an extremely high titre of this antibody. No associations of titres of anti-MBL antibodies and (i) genotypes of MBL gene, (ii) concentrations of serum MBL, or (iii) disease characteristics of SLE, were apparent. Thus, we have confirmed that anti-MBL antibodies are indeed present in sera of some patients with SLE, but the significance of these autoantibodies in the pathogenesis of SLE remains unclear.

Keywords Lupus/ systemic lupus erythematosus autoantibodies MBL C1q polymorphisms

INTRODUCTION

Both genetic and environmental factors are important in the development of systemic lupus erythematosus (SLE), a systemic autoimmune disease of unknown origin [1,2]. With respect to genetic background, deficiencies in components of the classical pathway of complements (C1q, C1r, C1s, C4 or C2) are known to be major predisposing risk factors for SLE [3–6]. In complement deficiencies, an abnormal clearance of not only immune complexes [3], but also apoptotic cells, has been suggested as contributive towards the occurrence of SLE [7]. Inappropriate levels of apoptotic nuclei are suggested to be a source of autoantigens in SLE [8].

Mannose-binding lectin (MBL) comprises a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q [9]. The MBL gene is located on the long arm of chromosome 10 at 10q11.2-q21 and contains 4 exons [10]. Several polymorphisms have been reported for the MBL gene, and a large interindividual difference in serum MBL concentration among test subjects is caused by the possession of variant alleles. Codon 52, 54 and 57 polymorphisms are all on exon 1, and the presence of any of the minority alleles results in a significant reduction of the serum MBL concentration. Furthermore, homozygosity for minority alleles results in almost complete deficiency of serum MBL [11,12]. This has been attributed to increased degradation of the mutated protein [12]. In the promoter region of the MBL gene, polymorphisms are reported at positions −550, −221 and + 4, and they also greatly influence the levels of serum MBL [13,14]. MBL mediates lectin-dependent activation of the complement pathway [9], and plays an important role in host defense against microorganisms by phagocytosis. Individuals lacking this protein could develop severe episodes of bacterial infections from early life [15–17].

Recently, several studies have suggested that MBL deficiency, or low serum MBL levels caused by polymorphisms in the structural portion or promoter region of the MBL gene, may be associated with occurrence of SLE [18–22]. Two possible explanations for the associations between MBL deficiency and the occurrence of SLE are suggested. Firstly, MBL can bind to and initiate uptake of apoptotic cells by macrophages [23], and an abnormal

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clearance of apoptotic cells caused by MBL deficiency may result in the overexpression of autoantigens. Alternatively, viral infection is believed to be one of causes of SLE [24–26], and MBL deficiency may lead to more frequent infections. On the other hand, deposits of MBL were found in glomerular tissues of SLE patients [27,28], and D-mannose and N-acetylglycosamine, both possible ligands for MBL, are present in the salivary glands of patients with Sjögren's syndrome [29]. In this situation, MBL may have a pathogenic role during the course of SLE.

It has been reported that autoantibodies to C1q are associated with hypocomplementemia and glomerulonephritis [30]. If autoantibodies to MBL, a molecule similar to C1q in structure and functions, are present in patients with SLE, they may: reduce MBL levels; interfere with MBL functions; or bind to MBL deposited to various diseases. We investigated whether anti-MBL antibodies are indeed present in sera of Japanese patients with SLE.

PATIENTS AND METHODS

Patients and controls

Samples used for the study were taken from 111 Japanese patients with SLE, at Division of Rheumatology, Department of Internal Medicine, University Hospital of Tsukuba, Japan. All patients fulfilled the 1997 American College of Rheumatology (ACR) Classification Criteria for SLE. Patients with drug-induced lupus were excluded. The study was approved by the local ethics committee, and written informed consent was obtained from all participants of this study. Medical information including clinical manifestations, and laboratory data were collected simultaneously with sampling. Samples from 113 Japanese healthy volunteers served as controls.

Detection of immunoglobulin G (IgG) binding to MBL

Sumilon S plates (Sumitomo Bakelite, Tokyo Japan) were coated overnight at 4°C with 100 μl/well of recombinant MBL [31] in a carbonate/bicarbonate-buffer (pH 9·6) at a concentration of 1 μg/ml. The plates were washed three times with tris-buffered saline (TBS, pH 7·4) containing 0·05% Tween-20 (TBS/Tw). Unoccupied binding sites were blocked by incubation with 1% bovine serum albumin (BSA) in TBS for 1 h at 37°C. One hundred μl/well of serum samples diluted to 1 : 50 in TBS/Tw containing 0·3% BSA and 1 mM EDTA were added to the wells, and the plates were incubated overnight at 4°C. EDTA was included to inhibit the Ca^{2+} dependent binding of MBL to carbohydrates present on the Fc portion of IgG. All samples were analysed in triplicates. After incubation, 100 μl/well alkaline phosphate (AP)-conjugated goat antihuman IgG, specific for Fab fragment (Sigma, St Louis, MO, USA) diluted 1 : 5000 in TBS/Tw, was added to each well. The microtiter plates were incubated for 1 h at room temperature. Subsequently, alkaline phosphate substrate (Sigma) was added to each well. The plates were incubated for 2 h at room temperature. Optical densities (OD) were measured at 405 nm. The concentration of IgG reactive with MBL is expressed in units/ml of serum (U/ml), where the concentration in a standard sample was defined as 1000 U/ml. Standard curves were generated in all assays performed.

Inhibition assays

Anti-MBL positive sera diluted to 1 : 50 were preincubated with TBS or recombinant MBL at concentrations from 0·1563 μg/ml to 10 μg/ml at room temperature for 1 h. The samples were then put onto MBL-coated plates, and IgG binding to MBL was measured as described above.

Typing of the MBL gene

Genomic DNA was purified from peripheral blood leucocytes using the DnaQuick DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan), and stored at −30°C. Typing of the MBL gene allele was performed by using the polymerase chain reaction- restriction fragment length polymorphism method according to the methods of Madsen *et al.* [11]. The wild-type allele was designated as allele A, and codon 54 substitution (glycine to aspartic acid) was designated as allele B. Previous studies have shown that codon 52 and 57 polymorphisms are not present or extremely rare in the Japanese population [32,33].

Measurement of the serum MBL concentration by enzyme immunoassay

Serum concentration of MBL was measured by a specific enzyme immunoassay utilizing two rabbit polyclonal anti-MBL antibodies as described previously [31]. All samples were stored at −80°C and no previous freeze/thaw was done.

Statistics

Mann–Whitney *U*-test, Fisher's exact test, chi-square analysis and Spearman's rank correlation test were used. *P*-values of <0·05 were considered to be statistically significant.

RESULTS

Detection of autoantibodies to MBL in patients with SLE

Titers of IgG reactive with human MBL in patients with SLE were significantly higher than those in healthy controls; $P < 0.0001$, median MBL concentration ± standard deviation (s.d.); 47·4 ± 49·3 and 30·6 ± 29·2, in SLE patients and healthy controls, respectively (Fig. 1). The assay was performed in the presence of EDTA in order to inhibit the binding between the carbohydrate recognition domain of MBL and carbohydrates on the Fc portion of IgG. Furthermore, selected samples were digested with pepsin and $F(ab')_2$ fragments were purified. $F(ab')_2$ fragments did bind to MBL coated plates, indicating that IgG–MBL interaction detected in this assay is indeed antigen-antibody binding (results not shown). We found a patient with an extremely high level of serum anti-MBL, and the titre of anti-MBL antibodies in the serum of this patient was designated 1000 U/ml. The number of subjects having a titre of more than 2 sd. above the average of healthy controls (89·5, indicated by dotted line in Fig. 1) was 9 of the patients with SLE, and 2 of the healthy controls. This difference was statistically significant ($P = 0.0341$ by Fisher's exact test).

A titration curve could be adequately drawn using serial dilutions of the standard serum (Fig. 2a). In addition, adding excess amounts of recombinant MBL to diluted standard serum inhibited the binding of IgG to solid phase MBL in a dose dependent manner (Fig. 2b).

Associations between levels of anti-MBL antibodies, and MBL gene genotypes or serum concentrations of MBL in patients with SLE

Serum MBL concentrations reflected the MBL genotype of the individual in accordance with previous reports (Fig. 3) [11,12].

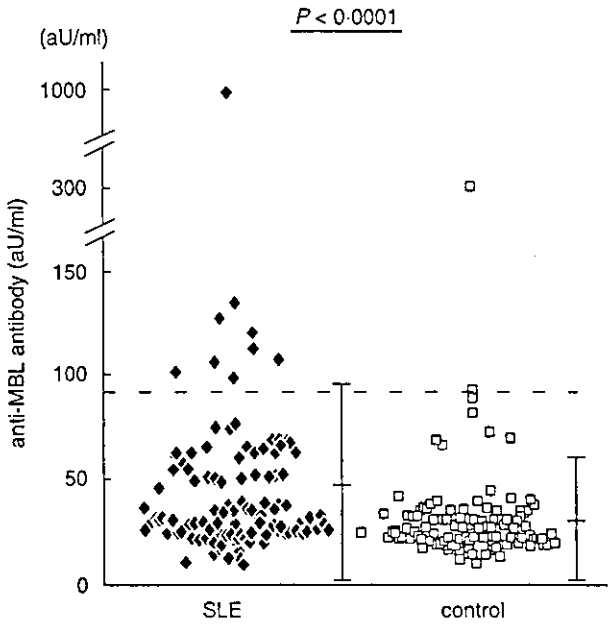

Fig. 1. Autoantibodies to mannose-binding lectin (MBL) in serum samples. Anti-MBL antibodies were measured in 111 samples from patients with systemic lupus erythematosus (SLE) and in 113 samples from healthy controls, in the presence of EDTA (1 mM). Dotted line indicates 2 standard deviation (s.d.) above average in healthy controls. *P*-value by Mann–Whitney *U*-test. aU, arbitrary units.

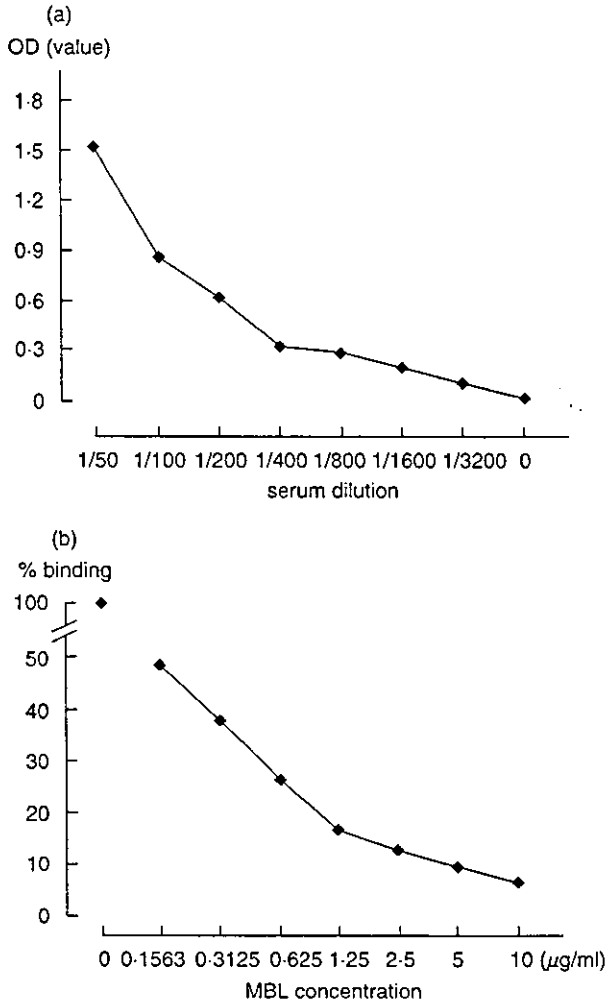

Fig. 2. Titration curve and inhibition assay for autoantibodies to mannose-binding lectin (MBL). (a) Titration curve for anti-MBL antibodies using serial dilutions of the standard serum in the presence of EDTA (1 mM). (b) Inhibition assay for anti MBL antibodies adding excess amount of recombinant MBL to diluted standard serum in the presence of EDTA (1 mM).

Serum MBL concentrations in SLE patients were not significantly different from those in healthy individuals ($P = 0{\cdot}5296$). Among individuals with the same genotype, SLE patients tended to have higher MBL concentrations than controls, but without statistical significance (AA; $P = 0{\cdot}3385$, AB; $P = 0{\cdot}5556$, BB; $P = 0{\cdot}1573$ by Mann–Whitney's *U*-test).

We next examined whether genotypes of the MBL gene in patients with SLE are associated with levels of anti-MBL antibodies (Fig. 4). Titres of anti-MBL antibodies tended to be lower in patients with allele B (AA; $60{\cdot}15 \pm 133{\cdot}3$, AB; $50{\cdot}10 \pm 26{\cdot}95$, BB; $38{\cdot}23 \pm 18{\cdot}88$), but no significant differences were observed.

Finally, we compared the serum concentrations of MBL and titres of anti-MBL antibodies in patients with SLE. We found no significant relationship between them (Fig. 5).

Relationships between the presence of anti-MBL antibodies in sera, and clinical characteristics or disease parameters of SLE

We investigated whether patients having anti-MBL antibodies at titres above 2 sd. of the average in healthy controls had some significant clinical characteristics (Table 1). No significant associations were observed. However, patients with higher serum concentration of anti-MBL antibodies tended to have a lower occurrence of anti-DNA antibodies, although statistical significance was not achieved. The incidence of infections requiring hospitalization during their course of SLE was not significantly higher in patients with higher serum concentration of anti-MBL antibodies.

We next analysed whether or not titres of anti-MBL antibodies are associated with various disease parameters of SLE in 111 SLE patients. Anti-DNA antibodies and total IgG tended to be positively related with anti-MBL antibodies, but statistical significance was not achieved. No other correlation was observed (Table 2).

DISCUSSION

In this study, we found the presence of autoantibodies against MBL in some patients with SLE. This is in accordance with the study by Seelen *et al.* [34], which was published very recently.

We confirmed that we were indeed detecting anti-MBL antibodies by; addition of EDTA in the enzyme immunoassay, thereby inhibiting the Ca^{2+} dependent binding of carbohydrate recognition domain on MBL to carbohydrates on IgG; digesting IgG with pepsin, and confirming that the binding region of IgG was on $F(ab')_2$; and detecting an inhibition of aqueous MBL to the binding of IgG to solid phase MBL. These methods and results are similar to those reported by Seelen *et al.* [34], except that we did detect dose dependent inhibition by our inhibition assay. The reason for this discrepancy is unclear, but may possibly be due to

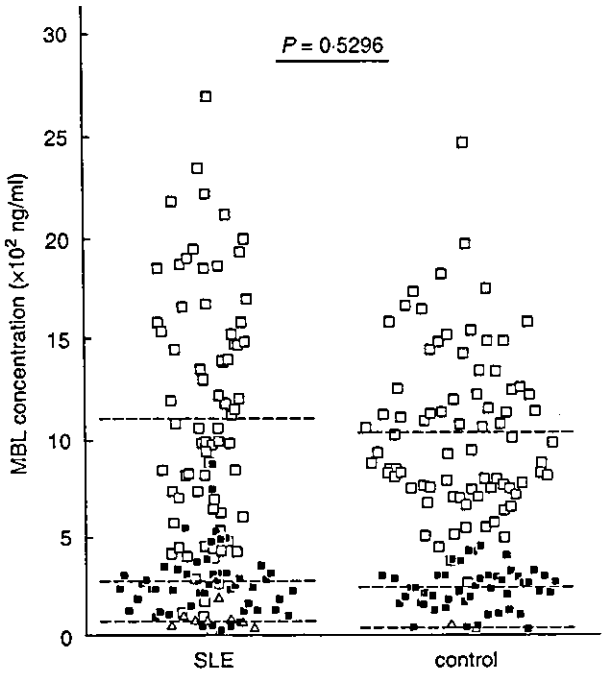

Fig. 3. Serum mannose-binding lectin (MBL) concentrations in 111 patients with systemic lupus erythematosus (SLE) and 113 healthy controls. Subjects with homozygosity for the codon 54 wild-type allele (□), subjects with heterozygosity for the codon 54 variant (■), and subjects with homozygosity for the codon 54 variant allele (Δ) are indicated in both patients with SLE and healthy controls. Dotted lines indicate average of titres of serum MBL concentrations in each genotype on both groups. *P*-value by Mann–Whitney *U*-test.

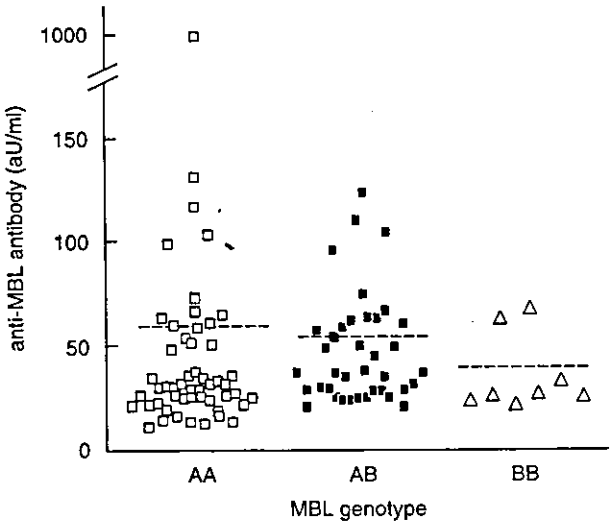

Fig. 4. Association between genotypes of the mannose-binding lectin (MBL) gene and levels of anti-MBL antibodies in patients with systemic lupus erythematosus (SLE). AA; homozygosity for the codon 54 wild-type allele, AB; heterozygosity for the codon 54 variant, BB; homozygosity for the codon 54 variant allele. Dotted lines indicate average of titres of anti-MBL antibodies in each genotype. aU, arbitrary units.

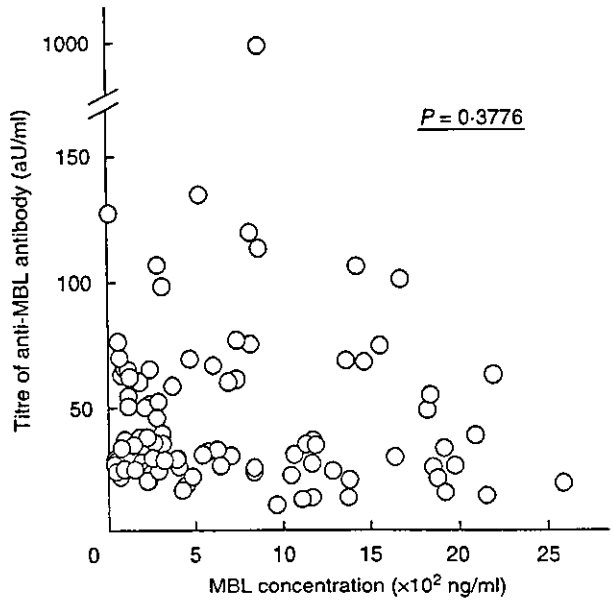

Fig. 5. Association between titres of anti mannose-binding lectin (MBL) antibodies and concentrations of MBL in systemic lupus erythematosus (SLE) patients. *P*-value by Spearman's rank correlation test. aU, arbitrary units.

Table 1. Disease characteristics of 111 patients with systemic lupus erythematosus (SLE) categorized by positivity of anti-mannose-binding lectin (MBL) antibody

	Positive (n = 9)	Negative (n = 102)	P-value
Malar rash	3	44	0·7309
Discoid lupus	0	13	0·5951
Photosensitivity	1	22	0·6821
Oral ulcers	2	20	0·9999
Arthritis	5	59	0·9999
Serositis	4	22	0·2099
Renal disorder	1	29	0·4399
Neurological disorder	0	9	0·9999
Haematologic disorder			
Haemolytic anemia	0	8	0·9999
leukopenia	4	52	0·7422
lymphopenia	4	48	0·9999
thrombocytopenia	1	27	0·4447
Anti-ds DNA Ab	4	74	0·1225
Anti-Sm Ab	0	8	0·9999
Antiphospholipid Ab	3	18	0·3673
ANA	8	95	0·5033
Infections requiring hospitalization	3	29	0·7155

Anti-MBL antibody positive was defined as having a titre higher than mean +2 s.d. of 113 healthy individuals. Serositis, pleuritis or pericarditis; renal disorder, proteinuria or cellular casts; neurological disorder, seizures or psychosis; Anti-ds DNA Ab, anti-double strand DNA antibody; Anti-Sm Ab, anti-Sm antibody; Antiphospholipid Ab, antiphospholipid antibody. P = AA + AB versus BB by chi-square analysis.

Table 2. Associations of titres of anti-mannose-binding lectin (MBL) antibody and various disease parameters of systemic lupus erythematosus (SLE) in 106 SLE patients

Disease parameters of SLE	*P*-value[a]
Anti-DNA antibody	0·2173
C3	0·8844
C4	0·2131
CH50	0·7919
IgG	0·0665
IgA	0·9026
IgM	0·1637

[a]Spearman's rank correlation test.

the nature of anti-MBL antibodies in individual patients, or concentrations or conformations of MBL used in the assays.

Similarities in structure and function exist between MBL and C1q, and it is known that C1q-deficient or anti-C1q antibody positive individuals have a high probability of developing SLE [5,30,35,36]. It has been reported that MBL deficiency may be associated with the occurrence of SLE [18–22], although deficiency of MBL is not an extremely high risk factor, in contrast to deficiencies of other complement molecules such as C1q. The presence of autoantibodies against MBL may cause similar pathological conditions to those found in MBL deficiency, as with the case of anti-C1q antibodies. In this context, it is noteworthy that a previous study has shown that anti-C1q antibodies do not recognize MBL [37], which suggests that anti-MBL and anti-C1q antibodies are not identical.

In accord to previous studies, serum MBL concentrations were closely associated with the MBL genotypes of the individuals studied (Fig. 3). However, in this study, no significant differences in serum MBL concentrations were observed between SLE patients and healthy controls, when individuals with the same genotype were compared. This is different from the study by Seelen *et al.* [34], where they found that serum MBL concentrations were higher in SLE patients than in controls. This difference may be due to differences in MBL genotype distributions or disease activities of SLE in the individuals studied, or other unknown factors.

We next asked whether there is any association between levels of anti-MBL antibodies and MBL genotypes. No such correlation was observed (Fig. 4). However, levels of anti-MBL antibodies in patients having genotype AB were higher than those in patients with genotype AA, if we excluded one patient with genotype AA with an extremely high level of anti-MBL antibodies (Fig. 4). In addition, some genotype BB patients had anti-MBL antibodies (Fig. 4). We went on to study the relationship between serum MBL concentration and levels of anti-MBL antibodies. There was no statistically significant relationship (Fig. 5). These findings support the notion that elevated serum MBL is not a causative factor for anti-MBL antibody production, and other factors should contribute to the production of these autoantibodies. One possible factor is the production of mutated MBL protein in genotype AB or BB individuals. Individuals with genotype AB or BB produce a mutated MBL protein which is degraded in sera, since they are unable to form a stable oligomerized structure [12,38]. These degraded MBL protein products may have a role in the occurrence of anti-MBL antibodies. However, at this point, this remains only a speculation. Other factors must be important as well, since some patients with genotype AA also have anti-MBL antibodies. Many questions need to be solved, before the mechanisms of autoantigen recognition and autoantibody production including anti-MBL antibodies could be clarified.

We examined the disease characteristics of SLE in anti-MBL antibodies positive patients (Table 1). There were no significant relationships between the possession of a significantly high titre of anti-MBL antibodies, and the characteristics or parameters of SLE. This is in accord with the report by Seelen *et al.* [34], which showed no difference between anti-MBL levels in sera of patients with active disease and inactive disease, especially concerning renal involvement. However, among patients having high titre of anti-MBL antibodies, smaller number of patients tended to have anti-DNA antibodies, and more patients (3 of 9 patients, 33%) developed intestinal pneumonitis, which usually occur in less than 10% of SLE patients [39]. Thus, we felt that some cases had somewhat atypical features of SLE. Whether this is only a coincidence or not is unclear. A study of larger number of patients should be done to clarify the clinical significance of anti-MBL antibodies in SLE.

It has been reported that individuals lacking MBL are prone to severe episodes of bacterial infections from early life [15–17]. A recent study has shown that presence of MBL minority alleles is a risk factor for infection in patients undergoing bone marrow transplantation [40]. It is also reported that the MBL deficiency, resulting from the possession of the variant alleles of the MBL gene, is a risk factor in patient receiving immunosuppressive therapy [19,20]. Although we anticipated that decreased MBL function caused by anti-MBL antibodies might lead to more frequent infections during the course of SLE, we could not find, in the present study, any significant associations between the presence of anti-MBL antibodies and the occurrence of infections requiring hospitalization after initiation of therapy of SLE. The effect of anti-MBL antibodies to increased susceptibility to infections in individuals under immunosuppressive therapy may not be as large as that caused by MBL gene polymorphisms. Since only 9 patients had significantly high titre of serum anti-MBL antibodies, a larger study is necessary to confirm this observation.

In conclusion, we detected anti-MBL antibodies in sera of patients with SLE. However, we could not find any significant relationships with MBL genotype, clinical characteristics and parameters of SLE in this study. Further studies are necessary to elucidate the actual functions of autoantibodies to MBL in the pathogenesis of SLE, and to determine the value of measuring these autoantibodies in clinical practice.

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T Cell Receptor Repertoire of T Cells in the Kidneys of Patients With Lupus Nephritis

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Objective. **To determine the role of T cells in the pathogenesis of lupus nephritis (LN).**

Methods. **Renal biopsy specimens from 12 patients with systemic lupus erythematosus were used for the experiments. We analyzed T cell receptor (TCR) Vβ1–20 family genes on intrarenal T cells and on peripheral blood lymphocytes (PBLs) by nested reverse transcriptase–polymerase chain reaction (PCR) and Southern blot analysis. Nucleotide sequence was determined in the third complementarity-determining region of the TCR Vβ gene in expanded T cells. Messenger RNA (mRNA) expression levels of Th1 and Th2 cytokines on infiltrating T cells were measured by nested PCR.**

Results. **The repertoire of TCR Vβ in intrarenal T cells was relatively restricted compared with that in PBLs. The TCR Vβ8 and TCR Vβ20 genes were preferentially expressed in 6 of 12 patients (50%) and the TCR Vβ9 and TCR Vβ14 genes were expressed in 5 of 12 patients (42%). Junctional sequences of complementary DNA encoding the TCR Vβ8 and TCR Vβ20 genes in intrarenal T cells showed oligoclonal expansion, indicating antigen-driven stimulation. Interleukin-4 (IL-4) and IL-10 mRNA were highly expressed on intrarenal T cells, while interferon-γ mRNA was not detected.**

Conclusion. **Our findings suggest that T cells infiltrating the kidneys of patients with LN may recognize restricted epitopes on antigens and function as Th2-type T cells.**

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Lupus nephritis (LN) is characterized by the infiltration of mononuclear cells, mainly CD4+ T cells, and the deposition of immune complexes within the glomeruli (1). The immune deposits are caused by pathogenic anti-DNA autoantibodies and autoantigen complex. This pathogenic anti-DNA autoantibody response is dependent on CD4+ Th cells (2–6). The third complementarity-determining region (CDR3) of the T cell receptor (TCR) β chains expressed by these pathogenic Th clones bears a recurrent motif of anionic residues, suggesting it is specific for autoantigens with cationic residue.

Studies have indicated that the restricted oligoclonal T cells may play an important role in the development of various diseases, including multiple sclerosis, rheumatoid arthritis, and Sjögren's syndrome (7,8). Funauchi et al (9) reported that the levels of interleukin-2 (IL-2) and interferon-γ (IFNγ) (Th1 cytokines) were lower, while those of IL-4 and IL-10 (Th2 cytokines) were higher, in peripheral blood mononuclear cells (PBMCs) of patients with systemic lupus erythematosus (SLE) than in those of healthy subjects. Viallard et al (10) showed that IL-10 production by PBMCs was significantly higher in patients with SLE than in healthy controls, while IL-1 and IFNγ contents did not differ between SLE patients and controls. Richaud-Patin et al (11) reported high gene expression of IL-4, IL-6, IL-10, and tumor necrosis factor α in SLE patients compared with healthy subjects, while the expression of IL-1β, IL-2, and IFNγ genes was low or undetectable in PBMCs of SLE patients. These findings indicate that the preferential increase in cytokine production from Th2 cells relative to that by Th1 cells might be associated with polyclonal B cell activation seen in SLE.

In the present study, we investigated the role of T cells in the pathogenesis of LN by comparing the TCR Vβ repertoires in intrarenal T cells and peripheral blood lymphocytes (PBLs) of patients with LN, using nested reverse transcriptase–polymerase chain reaction (RT-

PCR). Our results showed that the TCR Vβ repertoire is relatively restricted in the kidneys. In addition, the junctional sequence of TCR Vβ genes from the kidney demonstrated that TCR Vβ8– and TCR Vβ20–positive T cells expanded oligoclonally and there were some conserved amino acids in the CDR3 of TCR Vβ genes. These findings suggest that limited nephritogenic antigens might activate T cells, resulting in the development of nephritis in patients with SLE.

PATIENTS AND METHODS

Patients and renal biopsies. Twelve patients with LN were referred to Tsukuba University Hospital, Toho University Sakura Hospital, and Shimoshizu National Hospital, and all met the criteria for diagnosis of SLE (12). They consisted of 2 men and 10 women, ages 17–93 years (mean 42.8), and all patients were in the active stage of SLE with LN. A percutaneous renal biopsy obtained in each patient prior to the administration of any medications and nutritional remedies showed marked mononuclear cell infiltration in the glomeruli. According to World Health Organization (WHO) criteria, 2 patients (SLEK2 and SLEK11) had class III nephritis and 10 had class IV nephritis (SLEK3–10, SLEK12, and SLEK13). Typing of HLA–DR and HLA–DQ alleles was performed by PCR combined with dot-blot hybridization using sequence-specific oligonucleotide probes, based on the protocol of the Eleventh Histocompatibility Workshop (13). The study protocol was approved by the Human Ethics Review Committee of Tsukuba University, and a signed consent form was obtained from each patient.

Histopathologic and immunohistochemical analyses. Tissue samples from the kidneys of patients with LN were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. For immunostaining, a portion of a sample was snap frozen, and cryostat sections were cut and stained with anti-CD3, anti-CD20, anti-CD4, or anti-CD8 monoclonal antibodies (mAb; Becton Dickinson, Mountain View, CA). Cryostat sections were incubated with biotinylated rabbit anti-mouse Ig (Dako, Glostrup, Denmark), then with StreptABComplex/horseradish peroxidase (Dako), and finally with a peroxidase substrate.

RNA preparation and analysis of TCR Vβ gene use by PCR. Renal biopsy samples and PBLs of patients with LN were lysed for 10 minutes at room temperature in 20 μl of lysis buffer (40 m*M* Tris HCl [pH 8.5], 60 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol [DTT], 0.5% Nonidet P40 [NP40], and 0.05 units/μl of RNasin [Promega, Madison, WI]), and total RNA was prepared. Total RNA was reverse-transcribed using 8 μl of 5× buffer (100 m*M* Tris HCl [pH 8.5], 150 m*M* KCl, 7.5 m*M* $MgCl_2$, 25 m*M* DTT, and 0.5 mg/ml bovine serum albumin [nuclease-free; Wako, Osaka, Japan]), 2 μl of oligo$(dT)_{15}$ (50 pmoles/μl), 2 μl of dNTPs (2 m*M*) (Gibco BRL Life Technologies, Gaithersburg, MD), 0.3 μl Moloney murine leukemia virus reverse transcriptase (60 units) (Gibco BRL), and diethyl pyrocarbonate–distilled deionized H_2O in a total volume of 40 μl. The reaction was carried out at 37°C for 1 hour. The reaction mixture encoding complementary DNA (cDNA) was used for first-round PCR analysis. PCR was conducted using the method described by Sumida et al (7).

Amplification was briefly performed with *Taq* polymerase in 50 μl of standard buffer using 10 μl of cDNA with 50 pmoles each of 20 different TCR Vβ primers and Cβ primer. The sequences of the TCR Vβ and Cβ primers were obtained from previously published data (7). Oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA). Denaturing was performed at 94°C for 1.5 minutes, annealing was performed at 60°C for 1 minute, and extension was performed at 72°C for 1 minute, for 30 cycles on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Aliquots (2 μl) of the first-round PCR products were used for second-round PCR, carried out using nested 5′ primers specific for 20 different TCR Vβ family genes and a 3′ primer for the TCR Cβ gene. One-fifth of the second-round PCR product was subjected to 2% agarose gel electrophoresis and visualized by ethidium bromide staining, and then confirmed by Southern blot analysis using a digoxigenin-labeled PCR product encoding the TCR Cβ gene as described previously (14).

Cloning and junctional sequencing of TCR Vβ genes. PCR products encoding the TCR Vβ8 and TCR Vβ20 genes were ligated to plasmids using the TA cloning kit (Invitrogen, Carlsbad, CA), transformed into competent INVαF′ *Escherichia coli* cells, and grown under appropriate conditions. After selection of TCR Cβ–positive colonies, plasmid DNA was purified by alkaline lysis for DNA sequencing. Nucleotide sequences were analyzed with an ABI 377 automated sequencer (Applied Biosystems).

Expression of cytokine messenger RNA (mRNA). Complementary DNA from the kidneys and PBLs of SLE patients with LN was used for PCR, with primers specific for IFNγ (forward primer 5′-TGTTACTGCCAGGACCCATAT-3′ and reverse primer 5′-TCAGCTTTTCGAAGTCATCTC-3′), IL-4 (forward primer 5′-CTTCCCCCTCTGTTCTTCCT-3′ and reverse primer 5′-TTCCTGTCGAGCCGTTTCAG-3′), and IL-10 (forward primer 5′-ATCAAGGCGCATGTGAACTC-3′ and reverse primer 5′-AGAGCGCCAGATCCGATTTT-3′). Amplification was performed at 94°C for 1.5 minutes, 55°C for 1 minute, and 72°C for 1 minute, for 30 cycles. The PCR products were electrophoresed in 1% agarose gels containing 0.5 mg/ml ethidium bromide. The expression of these cytokines was confirmed by Southern blot analysis (IFNγ 194 bp, IL-4 317 bp, IL-10 291 bp).

Statistical analysis. Data were analyzed by one-way analysis of variance and post hoc analysis calculated by Fisher's protected least significance difference method. *P* values less than 0.05 were considered significant.

RESULTS

Infiltration of CD4+ T cells into the kidney. Histopathologic examination of the kidneys of patients with LN showed marked infiltration of mononuclear cells within the glomeruli (Figure 1A). Immunohistochemical studies using mAb against CD3, CD20, CD4, and CD8 demonstrated that the infiltrating cells were mainly CD3+ T cells and, of those, the majority were CD4+ T cells and CD8+ T cells (Figure 1B).

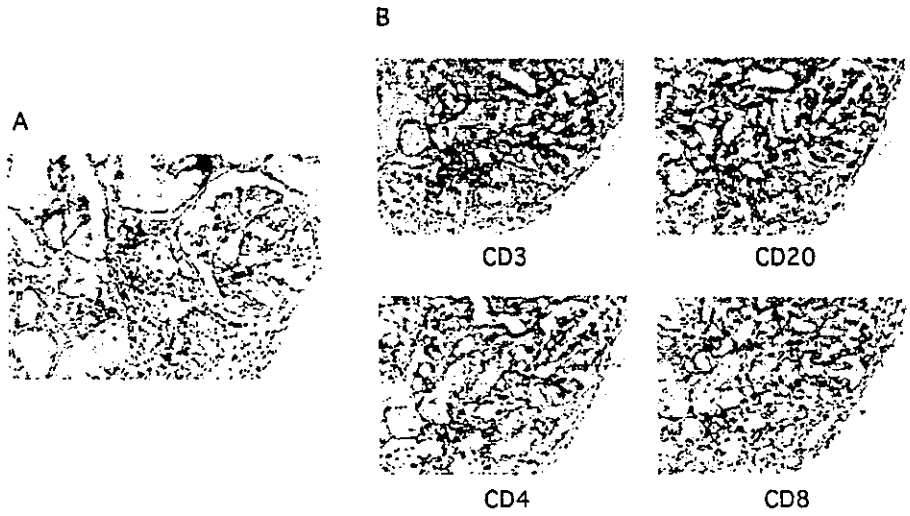

Figure 1. Infiltration of CD4+ T cells into the kidneys of patients with lupus nephritis. **A,** Histopathologic examination (hematoxylin and eosin staining), showing infiltration of mononuclear cells within the glomeruli. **B,** Immunohistochemical studies using monoclonal antibodies against CD3, CD20, CD4, and CD8, indicating that most of the infiltrating cells were CD4+ T cells. (Original magnification, × 200).

Restricted repertoire of TCR Vβ gene on intrarenal T cells. To analyze the mechanism of LN in patients with SLE, the TCR repertoire of intrarenal T cells in kidney samples was examined by PCR and Southern blot analysis. Renal biopsy specimens from 12 patients with LN were used for analysis. As controls, we used PBL samples from the same individuals. As shown in Figure 2, 1–8 TCR Vβ genes were detected in the kidneys of patients. The TCR Vβ8 and TCR Vβ20 genes were each preferentially expressed in 6 of 12 patients (SLEK4, SLEK5, SLEK9, SLEK11–13; and SLEK2, SLEK7, SLEK8, SLEK11–13, respectively) ($P < 0.05$).

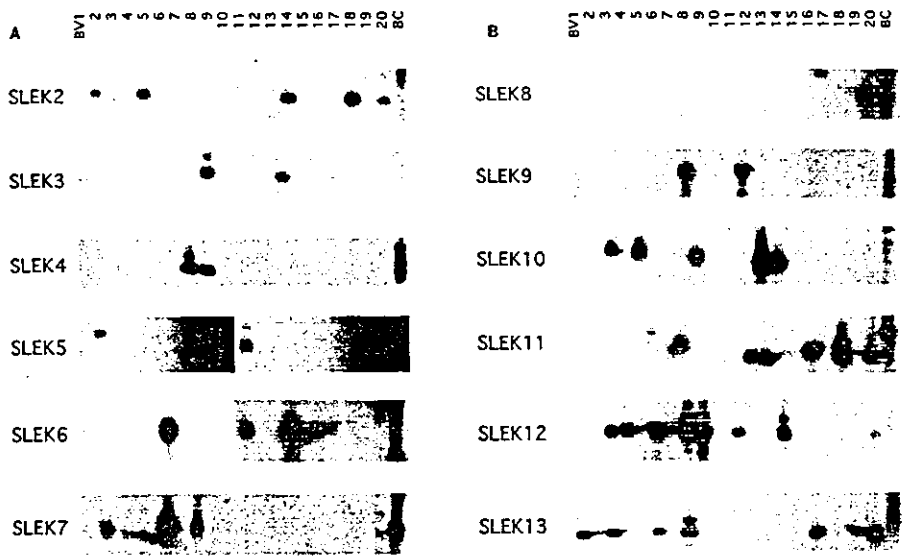

Figure 2. Restricted use of the T cell receptor (TCR) Vβ gene on intrarenal T cells of patients with lupus nephritis (LN). Renal biopsy specimens (A) and peripheral blood lymphocytes (B) from 12 patients with LN (SLEK2–13) were used for polymerase chain reaction (PCR). The TCR Vβ repertoire was examined by family PCR Southern blot analysis.

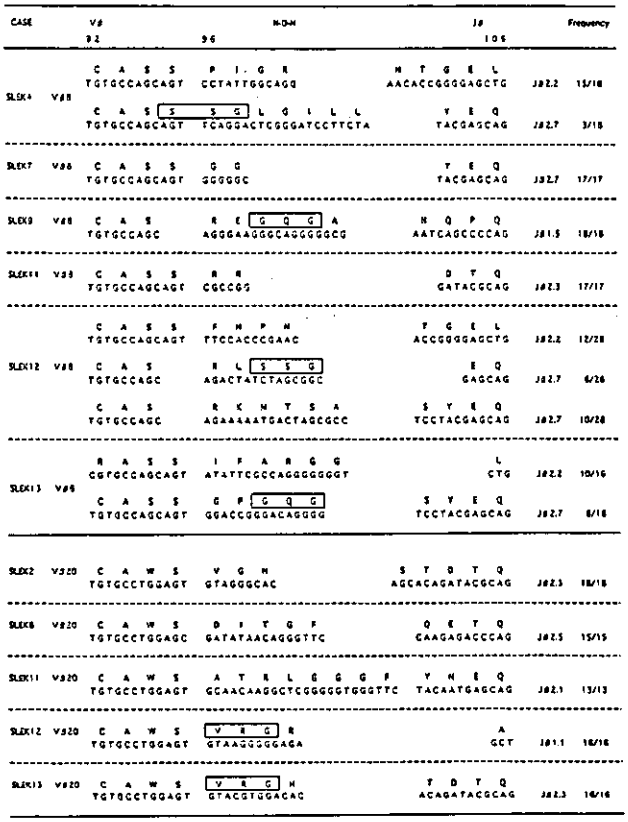

Figure 3. Junctional sequences of TCR Vβ gene in T cells from kidneys of patients with LN. The single-letter amino acid codes of the 3′ position of TCR Vβ, third complementarity-determining region, and the 5′ position of the J region are given. The conserved sequences are boxed. Frequency is the number of positive clones divided by the total number of clones. See Figure 2 for definitions.

The TCR Vβ9 and TCR Vβ14 genes were each expressed in 5 of 12 patients (SLEK3–5, SLEK10, and SLEK12; and SLEK2, SLEK3, SLEK6, SLEK10, and SLEK12, respectively). In contrast, all TCR Vβ genes were detected in PBL, and there was no predominant expression of particular TCR Vβ genes, indicating a heterogeneous TCR Vβ repertoire. These results suggest that the repertoire of the TCR Vβ genes on T cells that infiltrate the kidneys of patients with LN is restricted compared with PBLs.

Junctional sequences of TCR Vβ genes on intrarenal T cells. To examine the amino acid sequences of the TCR Vβ region, cDNA clones encoding the TCR Vβ8 and TCR Vβ20 genes from T cells infiltrating the kidneys of 6 patients (SLEK4, SLEK7, SLEK9, SLEK11–13) and 5 patients (SLEK2, SLEK8, SLEK11–

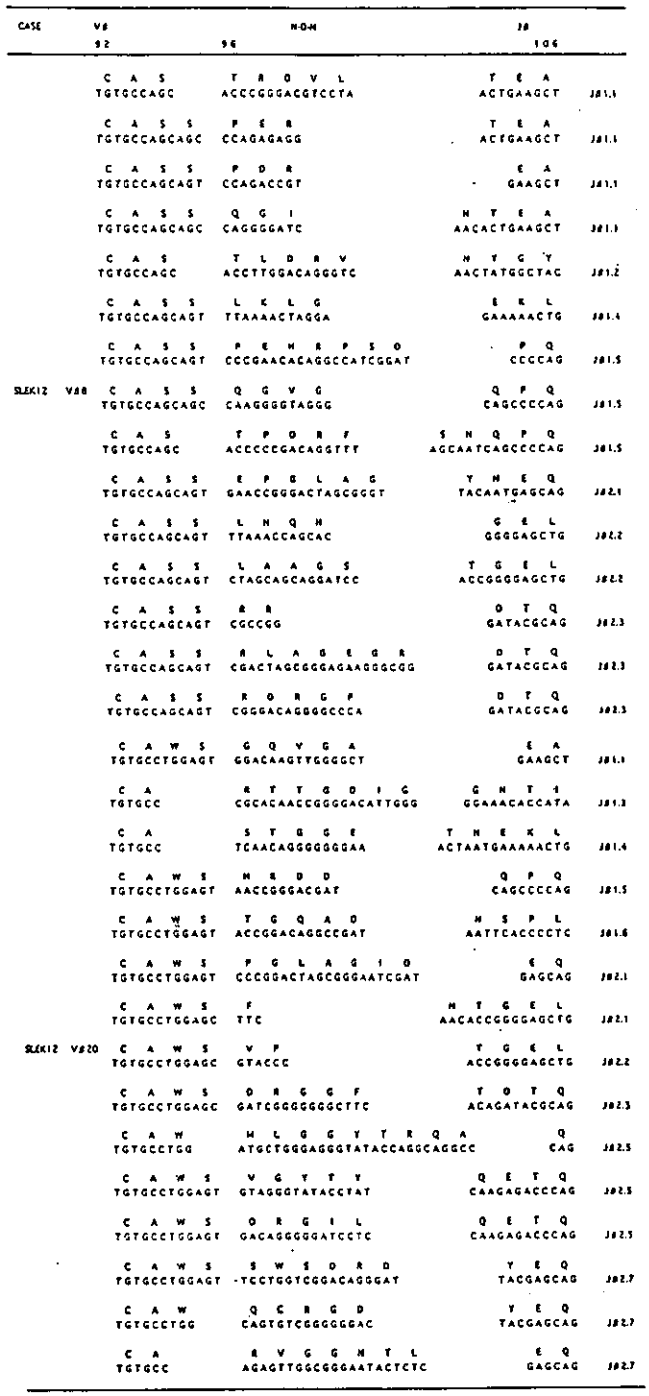

Figure 4. Junctional sequences of TCR Vβ gene in T cells from peripheral blood lymphocytes (PBLs) of a patient with LN. Representative sequences of TCR Vβ8 and TCR Vβ20 in T cells from PBLs of patient SLEK12. The single-letter amino acid codes of the 3′ position of TCR Vβ, CDR3, and the 5′ position of the J region are given. The frequency of each clone is the same. See Figure 2 for other definitions.

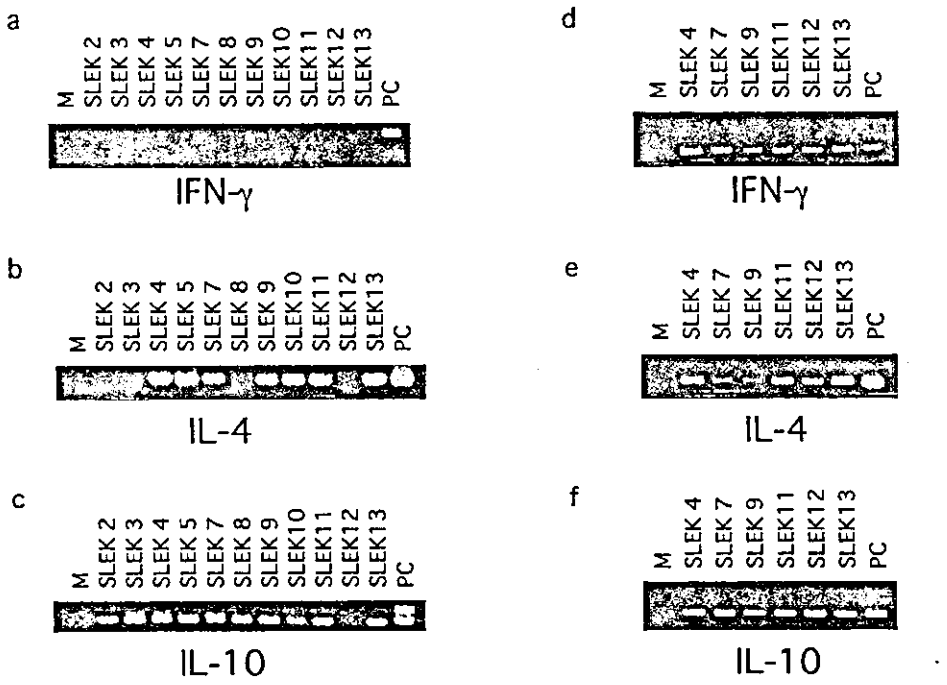

Figure 5. Expression of cytokine genes in patients with lupus nephritis. Ethidium bromide staining. **a**, **b**, and **c**, Interferon-γ (IFNγ), interleukin-4 (IL-4), and IL-10 mRNA expression, respectively, in kidneys from systemic lupus erythematosus (SLE) patients. **d**, **e**, and **f**, IFNγ, IL-4, and IL-10 mRNA expression, respectively, in peripheral blood lymphocytes from SLE patients. M = molecular size marker; PC = positive control cDNA clone.

13) respectively, were cloned and sequenced. As seen in Figure 3, junctional sequences of cDNA encoding the TCR Vβ8 and TCR Vβ20 genes on infiltrating T cells showed that these cells expanded oligoclonally, indicating an antigen-driven stimulation. The CDR3 of the infiltrating T cell clones contained conserved amino acid motifs. The SSG motif was found in TCR Vβ8 from patients SLEK4 and SLEK12; the GQG motif was found in TCR Vβ8 from patients SLEK9 and SLEK13; and the VRG motif was found in TCR Vβ20 from patients SLEK12 and SLEK13. In contrast, no conserved amino acids were detected in the CDR3 of TCR Vβ8 and TCR Vβ20 genes in PBLs from the same patient (SLEK12). The frequencies of SSG, GQG, and VRG in the CDR3 of TCR Vβ8 and TCR Vβ20 from kidneys of SLE patients were significantly high ($P < 0.05$). These results suggest that intrarenal T cells might recognize the epitopes of an autoantigen in the kidney.

Junctional sequences of TCR Vβ genes from T cells infiltrating into peripheral blood T cells. To compare intrarenal and peripheral TCR gene usage, TCR Vβ8 and TCR Vβ20 genes were examined in peripheral blood- and kidney-derived T cells from 6 individual patients with LN. Figure 4 shows data from a representative patient. There was heterogeneous TCR gene usage in the peripheral blood of this individual, with 15 distinct sequences recovered. In contrast, infiltrating T cells from the same patient showed marked clonal restriction, with only 3 sequences recovered from 28 clones. None of the latter sequences matched sequences identified from peripheral blood cDNA. These findings are consistent with the notion that antigen-driven clonal expansion results in restricted heterogeneity of the intrarenal T cells.

Expression of cytokine mRNA on intrarenal T cells. To examine the function of T cells in 11 kidneys (SLEK2–5, SLEK7–13), the expression levels of IFNγ, IL-4, and IL-10 mRNA were analyzed by the RT-PCR method. Levels of expression of mRNA for these cytokines in 6 PBL samples (SLEK4, SLEK7, SLEK9, SLEK11–13) were analyzed as controls. Intrarenal T cells showed a low expression level of IFNγ mRNA and high expression levels of IL-4 and IL-10 mRNA (Figure 5). In contrast, T cells in peripheral blood showed high

expression levels of IFNγ mRNA as well as IL-4 and IL-10. These results suggested that T cells in the kidney are Th2-type cells.

DISCUSSION

Our results show a restricted repertoire of TCR Vβ genes (Vβ8, 9, 14, and 20) in intrarenal T cells but a diverse and heterogeneous repertoire of TCR Vβ in PBLs of patients with SLE, as analyzed by the family PCR method. Recently, Massengill et al (15) demonstrated a bias in TCR Vβ gene use in infiltrating T cells in children with recent-onset LN. They examined TCR gene use on T cells in kidneys from 4 children and showed the expression of different TCR Vβ gene families. The predominant T cells were TCR Vβ18, TCR Vβ2 and TCR Vβ6, TCR Vβ17 and TCR Vβ22, and TCR Vβ6, TCR Vβ9, and TCR Vβ10 in the 4 children. The results of junctional sequence analysis described in their report demonstrated the presence of oligoclonal expansion of TCR Vβ families. Thus, our findings are consistent with those of the above study.

The sequences of the junctional regions of TCR Vβ8 and TCR Vβ20 analyzed in our study demonstrate the presence of oligoclonal expansion of TCR Vβ families, indicating antigen-driven stimulation. Moreover, there were some conserved amino acids in the CDR3 of clonally expanded T cells. These findings suggest that T cells infiltrating the kidneys of LN patients may recognize limited epitopes of antigens. What are the autoantigens recognized by T cells in the kidneys? Although there are no reports on the antigens for T cells infiltrating the kidney in SLE patients with LN, kidney-specific antigens may be the candidate antigens. Mostoslavsky et al (16) reported that anti-DNA autoantibodies cross-react with glomerular structural proteins, the acidic actin-binding protein, and α-actinin, suggesting that kidney dysfunction in SLE may be enhanced by protein–nucleic acid antigenic mimicry. It is possible that intrarenal T cells in LN recognize glomerular structural proteins, such as protein–nucleic acid, presented on the HLA-DR molecule after tissue destruction through antigen–antibody interaction. On the other hand, there is no direct evidence for the presence of glomerular structural protein specific for T cells in the kidney. Since LN is characterized by interstitial nephritis as well as proliferative glomerular nephritis, autoantigens, such as 3M1, a tubular basement membrane protein (17), might induce interstitial T cell infiltration in LN. Establishment of T cell lines from the local region of LN should shed some light to elucidate the antigens that induce T cell infiltration.

It has been proposed that an imbalance of Th1- and Th2-type T cells in the peripheral blood of patients with SLE is associated with the pathogenesis of the disease (9–11,18–20). Funauchi et al (9) found that cytoplasmic IL-2 and IFNγ levels were low and IL-4 and IL-10 levels were high on peripheral mononuclear cells of patients with SLE compared with those of healthy subjects. They predicted that the deviation of Th1 to Th2 cells in the periphery might be associated with polyclonal B cell activation in patients with SLE. In contrast, Akahoshi et al (19) demonstrated a strong predominance of Th1 cells in peripheral blood in lupus patients with WHO class IV nephritis. Using immunohistochemistry methods, Masutani et al (20) analyzed the expression of IFNγ and IL-4 on intrarenal T cells as well as peripheral blood from SLE patients with diffuse proliferative LN and showed a dominance of the Th1 response. They suggested that the peripheral blood Th1: Th2 ratio directly reflects the local histopathologic findings.

On the contrary, our study indicates that infiltrating T cells in the kidneys could produce Th2-type T cell cytokines such as IL-4 and IL-10. These findings suggest the following 2 possibilities. One is that Th2-type T cells in the kidneys might play a role in the generation of LN. The other is that Th2-type T cells accumulate in the kidneys to improve nephritis as regulatory T cells. The discrepancy between our results and the findings of Masutani et al on cytokine expression of intrarenal T cells might be due to the sensitivity of the methods used for detection of cytokines. The PCR method is more sensitive than immunohistochemistry; however, further examination of cytokine expression in the intrarenal T cells at the protein level as well as the mRNA level is warranted.

In murine models of SLE, such as (NZW × C57BL/6.Yaa)F_1, the constitutive expression of the IL-4 transgene by B cells completely prevents the development of lethal lupus-like glomerulonephritis (21). Overexpression of IL-4 results in modulation of the Th1-dominant autoimmune response in peripheral blood, resulting in a decrease of Th1-mediated IgG3 and IgG2a. Although there are no reports of cytokine production by intrarenal T cells in murine models of SLE, modification of Th1 to Th2 in the periphery might be useful as a therapeutic strategy in LN in the murine model.

In conclusion, the presentation of antigens in the context of HLA causes migration of T cells into the

glomerulus and interstitium, resulting in T cell–mediated kidney inflammation in SLE patients. Identifying antigen(s) that are recognized by T cells is also important in elucidating the molecular pathomechanisms of LN. Analysis of the cytokine profile in the present study showed the predominance of Th2-type T cells. These findings suggest two distinct strategies for therapy. The cytokine shift from Th2 to Th1 may be desirable to repair glomerulonephritis in patients with SLE, if Th2-type T cells function as pathogenic T cells, and modification from Th1 to Th2 may be hopeful for therapy in the case of regulatory Th2-type T cells. Establishment of T cells infiltrating into kidneys should shed light on the function of Th2-type T cells and selection of Th1/Th2 therapy in LN.

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