behaviors in p38, ERK and JUN activation between aPL and IL-1/TLR suggest that the signal/receptor structure of aCL/β_2GPI should be different from that of IL-1/TLR.

Several inhibitors for p38 have been developed and investigated in animal models of inflammatory diseases, and recently some of these inhibitors, such as BIRB796 or RWJ67657, are under clinical trials. Administration of SB203580 was beneficial in a murine model of endotoxin-induced shock and collagen-induced arthritis in mice. BIRB796 inhibited endothelial activation after administration of LPS in humans (63, 64), and RWJ67657 inhibited TNF-α, IL-8 and IL-6 in humans without significant adverse effects (65).

Strategies are focused on preventing the induction of procoagulant substances by aPL. New lipophilic statins, such as fluvastatin and simvastatin, can inhibit endothelial cell activation induced by aCL/β_2GPI (66, 67) and also the TF up-regulation on endothelial cells can be inhibited by these drugs, providing an additional therapeutic tool for treatment of thrombosis in APS. Recently, dilazep, an anti-platelet agent, was reported to reduce TF induction by aPL *in vitro* (48). In addition, our findings give a clue to establish more specific treatments by down-regulating the p38 MAPK pathway, presumably contributing to better management of the affected patients.

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Abbreviations

aCL	anti-cardiolipin antibodies
aCL/β_2GPI	β_2GPI-dependent anti-cardiolipin antibodies
AP-1	activator protein-1
aPL	anti-phospholipid antibodies
APS	anti-phospholipid syndrome
FVIIa	activated factor VII
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
β_2GPI	β_2Glycoprotein I
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MyD88	myeloid differentiation protein 88
NF-κB	nuclear factor κB
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
TRAF6	TNF receptor-associated factor 6

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Significance of Valine/Leucine^{247} Polymorphism of β_2-Glycoprotein I in Antiphospholipid Syndrome

Increased Reactivity of Anti–β_2-Glycoprotein I Autoantibodies to the Valine^{247} β_2-Glycoprotein I Variant

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Objective. **To clarify the consequences of the valine/leucine polymorphism at position 247 of the β_2-glycoprotein I (β_2GPI) gene in patients with antiphospholipid syndrome (APS), by investigating the correlation between genotypes and the presence of anti-β_2GPI antibody. The reactivity of anti-β_2GPI antibodies was characterized using recombinant Val^{247} and Leu^{247} β_2GPI.**

Methods. **Sixty-five Japanese patients with APS and/or systemic lupus erythematosus who were positive for antiphospholipid antibodies and 61 controls were analyzed for the presence of the Val/Leu^{247} polymorphism of β_2GPI. Polymorphism assignment was determined by polymerase chain reaction followed by restriction enzyme digestion. Recombinant Val^{247} and Leu^{247} β_2GPI were established to compare the reactivity of anti-β_2GPI antibodies to β_2GPI between these variants. The variants were prepared on polyoxygenated plates or cardiolipin-coated plates, and the reactivity of a series of anti-β_2GPI antibodies (immunized anti-human β_2GPI monoclonal antibodies [Cof-19–21] and autoimmune anti-β_2GPI monoclonal antibodies [EY1C8, EY2C9, and TM1G2]) and IgGs purified from patient sera was investigated.**

Results. **A positive correlation between the Val^{247} allele and the presence of anti-β_2GPI antibodies was observed in the patient group. Human monoclonal/polyclonal anti-β_2GPI autoantibodies showed higher binding to recombinant Val^{247} β_2GPI than to Leu^{247} β_2GPI, although no difference in the reactivity of the immunized anti-β_2GPI between these variants was observed. Conformational optimization showed that the replacement of Leu^{247} by Val^{247} led to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction.**

Conclusion. **The Val^{247} β_2GPI allele was associated with both a high frequency of anti-β_2GPI antibodies and stronger reactivity with anti-β_2GPI antibodies compared with the Leu^{247} β_2GPI allele, suggesting that the Val^{247} β_2GPI allele may be one of the genetic risk factors for development of APS.**

The antiphospholipid syndrome (APS) is characterized by arterial/venous thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL) (1–3). Among the targets of aPL, β_2-glycoprotein I (β_2GPI), which bears epitopes for anticardiolipin antibodies (aCL), has been extensively studied (4–6). APS-related aCL do not recognize free β_2GPI, but do recognize β_2GPI when it is complexed with phospholipids or negatively charged surfaces, by exposure of cryptic epitopes (7) or increment of antigen density (8).

The significance of antigen polymorphism in the production of autoantibodies or the development of autoimmune diseases is now being widely discussed. It is speculated that amino acid substitution in antigens can lead to differences in antigenic epitopes of a given protein. In particular, β_2GPI undergoes conformational

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alteration upon interaction with phospholipids (9). β_2GPI polymorphism on or near the phospholipid binding site can affect the binding or production of aCL (anti-β_2GPI autoantibodies), the result being altered development of APS. Polymorphism near the antigenic site, or which leads to alteration of the tertiary structure of the whole molecule, may affect the binding of autoantibodies. Five different gene polymorphisms of β_2GPI attributable to a single-nucleotide mutation have been described: 4 are a single amino acid substitution at positions 88, 247, 306, and 316 (10), and the other is a frameshift mutation associated with β_2GPI deficiency found in the Japanese population (11). In particular, the Val/Leu^{247} polymorphism locates in domain V of β_2GPI, between the phospholipid binding site in domain V and the potential epitopes of anti-β_2GPI antibodies in domain IV, as we reported previously (12). Although anti-β_2GPI antibodies are reported to direct to domain I (13) or domain V (14) as well, it should be considered that a certain polymorphism alters the conformation of the molecule, affecting function or antibody binding at a distant site.

We previously reported that, in a group of British Caucasian subjects, the Val^{247} allele was significantly more frequent in primary APS patients with anti-β_2GPI antibodies than in controls or in primary APS patients without anti-β_2GPI antibodies (15), but the importance of the Val^{247} allele in patients with APS is still controversial. In this study, we analyzed the correlation between the β_2GPI Val^{247} allele and anti-β_2GPI antibodies in the Japanese population. We also investigated the reactivity of anti-β_2GPI antibodies to recombinant Val^{247} β_2GPI and Leu^{247} β_2GPI, using a series of monoclonal anti-β_2GPI antibodies and IgGs purified from sera of patients with APS. Finally, to investigate the difference in anti-β_2GPI binding to those variants, we conformationally optimized to domain V and the domain IV–V complex of β_2GPI variants at position 247, referring the crystal structure of β_2GPI.

PATIENTS AND METHODS

Patients and controls. The study group comprised 65 patients (median age 38 years [range 18–74 years]: 57 women and 8 men) who attended the Hokkaido University Hospital, all of whom were positive for aPL (IgG, IgA, or IgM class aCL, and/or lupus anticoagulant). Thirty-four patients had APS (16 had primary APS, and 18 had secondary APS), and 31 patients did not have APS (24 had systemic lupus erythematosus [SLE], and 7 had other rheumatic diseases). Among all subjects, 19 had a history of arterial thrombosis, and 6 had venous thrombosis. Of the 31 patients with a history of pregnancy, 8 experienced pregnancy complications (some patients had more than 1 manifestation of pregnancy morbidity). Anti-β_2GPI antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as β_2GPI-dependent aCL (16). IgG, IgA, or IgM class β_2GPI-dependent aCL were found in 30, 14, and 21 patients, respectively (some patients had >1 isotype), and 34 patients had at least 1 of those isotypes. Lupus anticoagulant, detected by 3 standard methods described previously (17), was found in 51 patients. The diagnoses of APS and SLE, respectively, were based on the preliminary classification criteria for definite APS (18) and the American College of Rheumatology criteria for the classification of SLE (19). Informed consent was obtained from each patient or control subject. The control group comprised 61 healthy individuals with no history of autoimmune, thrombotic, or notable infectious disease.

Determination of β_2GPI gene polymorphism. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a standard phenol–chloroform extraction procedure or the DnaQuick kit (Dainippon, Osaka, Japan). Polymorphism assignment was determined by polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion (PCR–restriction fragment length polymorphism) using *Rsa* I (Promega, Southampton, UK) as described previously (15).

Purification of patient IgG. Sera from 11 patients positive for IgG class β_2GPI-dependent aCL were collected. The mean (±SD) titer of aCL IgG from these patients was 29.0 ± 21.5 IgG phospholipid (GPL) units (range 12.4 to >98 GPL units). IgG was purified from these sera using a protein G column and the MAbTrap GII IgG purification kit (Pharmacia Biotech, Freiburg, Germany), as recommended by the manufacturer.

Monoclonal anti-β_2GPI antibodies. Two types of anti-β_2GPI monoclonal antibodies were used. Cof-19, Cof-20, and Cof-21 are mouse monoclonal anti-human β_2GPI antibodies obtained from immunized BALB/c mice, directed to domains V, III, and IV of β_2GPI, respectively. These monoclonal antibodies recognize the native structure of human β_2GPI (12).

EY1C8, EY2C9, and TM1G2 are IgM class autoimmune monoclonal antibodies established from patients with APS (20). These antibodies bind to domain IV of β_2GPI, but only after interaction with solid-phase phospholipids or with a polyoxygenated polystyrene surface. EY1C8 and EY2C9 were established from a patient whose genotype of β_2GPI was heterozygous for Val/Leu^{247}. The genotype of the patient with TM1G2 was not determined.

Preparation of recombinant β_2GPI. As previously reported, genes were expressed in *Spodoptera frugiperda* Sf9 insect cells infected with recombinant baculoviruses (12). A full-length complementary DNA of human β_2GPI coding Val^{247} was originally obtained from Hep-G2 cells (21), and the valine residue was replaced by leucine, using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI). The sequence of the primers for a mutant Val^{247}→Leu (GTA→TTA) is as follows: 5′-GCATCTTGTAAATTACCTGTGAAAAAAG-3′. A DNA sequence of the mutant was verified by analysis using ABI Prism model 310 (PE Applied Biosystems, Foster City, CA).

Binding assays of monoclonal anti-β_2GPI antibodies and purified IgGs to the recombinant β_2GPI (cardiolipin-coated plate). The reactivity of a series of monoclonal anti-β_2GPI antibodies and IgG fractions (purified from the sera of APS patients positive for IgG class anti-β_2GPI) against 2 β_2GPI variants was investigated using an ELISA. ELISAs were performed using a cardiolipin-coated plate as previously reported (16) but with a slight modification. Briefly, the wells of Sumilon Type S microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were filled with 30 μl of 50 μg/ml cardiolipin (Sigma, St. Louis, MO) and dried overnight at 4°C. After blocking with 2% gelatin in phosphate buffered saline (PBS) for 2 hours and washing 3 times with 0.05% PBS–Tween, 50 μl of 10 μg/ml recombinant β_2GPI and controls were distributed and incubated for 30 minutes at room temperature. Wells were filled with 50 μl of serial dilutions of monoclonal antibodies (Cof-19–21, EY1C8 and EY2C9, and TM1G2) or purified patient IgG (100 μg/ml), followed by incubation for 30 minutes at room temperature. After washing 3 times, 50 μl of alkaline phosphatase–conjugated anti-mouse IgG (1:3,000), anti-human IgM (1:1,000), or anti-human IgG (1:6,000) was distributed and incubated for 1 hour at room temperature. The plates were washed 4 times, and 100 μl of 1 mg/ml p-nitrophenyl phosphate disodium (Sigma) in 1M diethanolamine buffer (pH 9.8) was distributed. Optical density (OD) was read at 405 nm, with reference at 620 nm. One percent fatty acid–free bovine serum albumin (BSA) (A-6003; Sigma)–PBS was used as sample diluent and control.

Binding assays of monoclonal anti-β_2GPI antibodies to recombinant β_2GPI (polyoxygenated plate). Anti-β_2GPI antibody detection assay using polyoxygenated plates was performed as previously reported (22), with minor modifications. Briefly, the wells of polyoxygenated MaxiSorp microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated with 50 μl of 1 μg/ml recombinant β_2GPI in PBS and incubated overnight at 4°C. After blocking with 3% gelatin–PBS at 37°C for 1 hour and washing 3 times with PBS–Tween, 50 μl of monoclonal antibodies, diluted with 1% BSA–PBS, were distributed and incubated for 1 hour at room temperature. The following steps were taken, in a similar manner.

Conformational optimization of domain V and the domain IV–V complex in human β_2GPI variants at position 247. A conformation of domain V in the valine variant at position 247 was first constructed from the crystal structure of the leucine variant (implemented in Protein Data Bank: 1CIZ) (23). Replacement of leucine by valine at position 247 was performed using the Quanta system (Molecular Simulations, San Diego, CA), and the model was optimized by 500 cycles of energy minimization by the CHARMm program (24), with hydrophilic hydrogen atoms and TIP3 water molecules (25). Molecular dynamics simulation (5 psec) of the model was then performed with 0.002 psec time steps. The cutoff distance for nonbonded interactions was set to 15Å, and the dielectric constant was 1.0. A nonbonded pair list was updated every 10 steps. The most stable structure of each domain in the dynamics iterations was then optimized by 500 cycles of energy minimization. The final structures of domain V consisted of 2,616 atoms, including 603 TIP3 water molecules, and had a total energy of -1.63×10^4 kcal/mole with a root-mean-square force of 0.869 kcal/mole.

Molecular models of a domain IV–V complex (leucine and valine variants at position 247) were further constructed by considering the location of the oligosaccharide attachment site in domain IV, the location of epitopic regions of the Cof-8 and Cof-20 monoclonal antibodies, the junction between domains IV and V, and molecular surface charges of both domains. These models were again optimized by molecular dynamics simulation and by energy minimization as described above. The final structures of the complex in the leucine and valine variants consisted of 3,773 and 3,778 atoms, respectively, including hydrophilic hydrogen atoms and 806 and 808 TIP3 water molecules, respectively, and had total energy of -2.07×10^4 and -2.03×10^4 kcal/mole with a root-mean-square force of 0.985 and 0.979 kcal/mole, respectively.

Statistical analysis. Correlations between the allele frequencies and clinical features such as the positiveness of β_2GPI-dependent aCL were expressed as odds ratios (ORs) and 95% confidence intervals (95% CIs). P values were determined by chi-square test with Yates' correction. P values less than or equal to 0.05 were considered significant.

RESULTS

Val/Leu^{247} polymorphism of β_2GPI and the presence of β_2GPI-dependent aCL. As shown in Table 1, the Leu^{247} allele was dominant in the population of healthy Japanese individuals, compared with Caucasians, which is consistent with a previous report (26). Japanese patients with anti-β_2GPI had a significantly increased frequency of the Val^{247} allele, compared with Japanese patients without anti-β_2GPI ($P = 0.0107$) or Japanese controls ($P = 0.0209$).

The binding of autoimmune anti-β_2GPI to recombinant Val^{247} and Leu^{247} β_2GPI. Representative binding curves using cardiolipin-coated plates and polyoxygenated plates are shown in Figure 1. Regardless of the type of plates, Cof-20 bound equally to valine and leucine variants of β_2GPI (Figures 1a and c), in any concentration of Cof-20. The binding curves of Cof-19 and Cof-21 were similar to that of Cof-20 (results not

Table 1. Frequency of the Val^{247} allele of β_2GPI in patients with APS*

Group	Japanese	British Caucasians
Patients with anti-β_2GPI	23/68 (33.8)†	48/56 (85.7)‡
Patients without anti-β_2GPI	9/62 (14.5)	39/58 (67.2)
Controls	23/122 (18.9)	55/78 (70.5)

* Values are the number (%). β_2GPI = β_2-glycoprotein I; APS = antiphospholipid syndrome.
† $P = 0.0107$ versus patients without anti-β_2GPI (odds ratio [OR] 3.01, 95% confidence interval [95% CI] 1.26–7.16), and $P = 0.0209$ versus controls, by chi-square test (OR 2.15, 95% CI 1.09–4.23).
‡ $P = 0.204$ versus patients without anti-β_2GPI (OR 2.92, 95% CI 1.16–7.39), and $P = 0.0396$ versus controls, by chi-square test (OR 2.51, 95% CI 1.03–6.13).

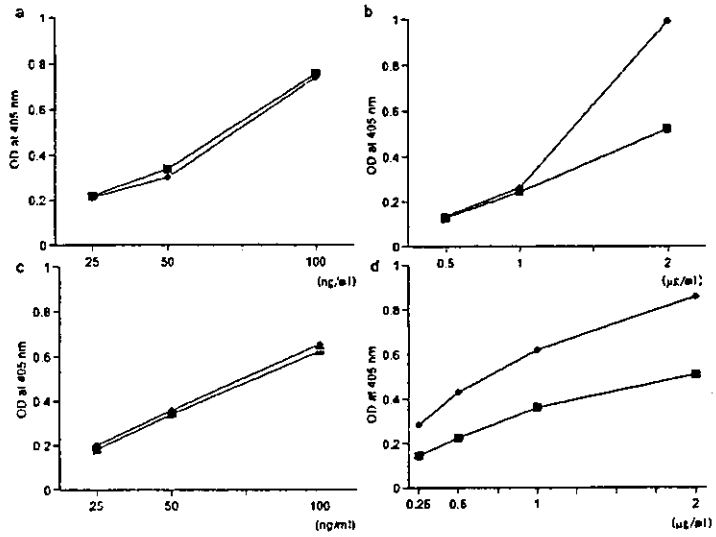

Figure 1. Representative binding curves of monoclonal anti-β_2-glycoprotein I (anti-β_2GPI) antibodies to recombinant valine/leucine^{247} β_2GPI. **a**, Binding curve of Cof-20 using cardiolipin-coated plate. **b**, Binding curve of EY2C9 using cardiolipin-coated plate. **c**, Binding curve of Cof-20 using polyoxygenated plate. **d**, Binding curve of EY2C9 using polyoxygenated plate. Binding to Val^{247} β_2GPI and Leu^{247} β_2GPI are indicated with diamonds and squares, respectively. OD = optical density.

shown). In contrast, EY2C9 showed stronger binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI (Figures 1b and d). EY1C8 and TM1G2 also showed stronger binding to Val^{247} β_2GPI. Figure 2a shows the binding of the monoclonal antibodies, on cardiolipin-coated plates, in the following concentrations: for Cof-19–21, 100 ng/ml;

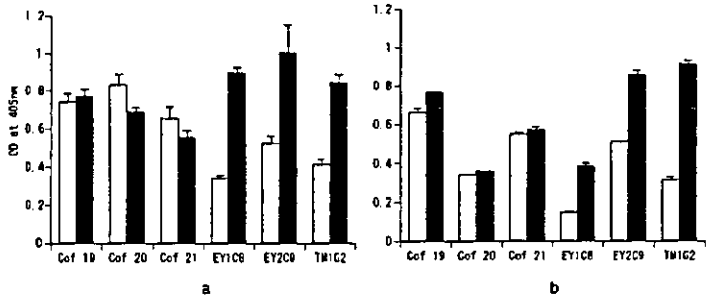

Figure 2. Reactivity of anti-β_2-glycoprotein I (anti-β_2GPI) antibodies to β_2GPI variants. **a**, The binding of monoclonal anti-β_2GPI antibodies to the recombinant valine/leucine^{247} β_2GPI was investigated using enzyme-linked immunosorbent assay (ELISA) on cardiolipin-coated plates. Concentrations of antigens and antibodies were as follows: for recombinant β_2GPI, 10 μg/ml; for Cof-19–21, 100 ng/ml; for EY1C8 and EY2C9, 2 μg/ml; for TM1G2, 5 μg/ml. **b**, The binding of monoclonal anti-β_2GPI antibodies to the recombinant Val/Leu^{247} β_2GPI was investigated using ELISA on polyoxygenated plates. Concentrations of antigens and antibodies were as follows: for recombinant β_2GPI, 1 μg/ml; for Cof-19–21, 50 ng/ml; for EY1C8 and EY2C9, 2 μg/ml; for TM1G2, 5 μg/ml. Results were presented as the optical density (OD) at 405 nm. Open columns indicate binding activity to Leu^{247} β_2GPI, and solid columns indicate binding activity to Val^{247} β_2GPI. Bars show the mean and SD.

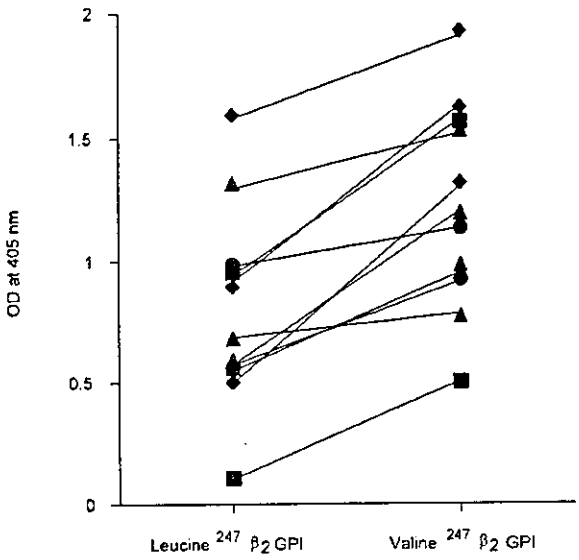

Figure 3. Reactivity of purified IgG from patients (100 μg/ml) to recombinant Val/Leu^{247} β_2-glycoprotein I (β_2GPI) (10 μg/ml), presented as the optical density (OD) at 405 nm. Squares, circles, and triangles indicate patients homozygous for the Leu^{247} allele, homozygous for the Val^{247} allele, and heterozygous for the Val/Leu^{247} allele, respectively. Diamonds indicate patients whose genotypes were not available.

for EY1C8 and EY2C9, 1 μg/ml; and for TM1G2, 2.5μg/ml. In contrast with the close reactivity of Cof-19, Cof-20, and Cof-21 between Val^{247} β_2GPI and Leu^{247} β_2GPI, autoimmune monoclonal antibodies (EY1C8, EY2C9, and TM1G2) showed higher binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI. The autoimmune monoclonal antibodies also showed a higher binding to Val^{247} β_2GPI directly coated on polyoxygenated plates (Figure 2b). IgG in sera collected from 11 patients (100 μg/ml) also showed higher binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI on cardiolipin-coated plates, regardless of the patients' genotypes (Figure 3).

Conformational alteration by leucine replacement by valine at position 247. Each domain V conformation in 2 variants at position 247 is shown in Figure 4a. The root-mean-square deviations for matching backbone atoms and equivalent atoms in the leucine and valine variants were 0.76 and 1.11 Å, respectively. The largest shift was observed at Val^{303}, one of the residues located on the backbone neighboring position 247. The shift seemed to be caused by weak flexibility of side chains consisting of Val^{247}, Pro^{248}, and Val^{249} and the electrostatic interactions between Lys^{250}, Lys^{251}, Glu^{307}, and Lys^{308}.

The molecular models of the IV–V complex in leucine and valine variants are shown in Figure 4b. The root-mean-square deviations for matching these backbone atoms and equivalent atoms were 1.72 and 2.03 Å, respectively. Electrostatic interactions and hydrogen bonds between Asp^{193} and Lys^{240}/Lys^{250}, Asp^{222} and Lys^{305}, and Glu^{228} and Lys^{308} appeared in the IV–V complex, but the interaction between Glu^{228} and Lys^{308} was disrupted by the leucine replacement by valine, because direction of the Lys^{308} side chain was significantly changed in the complex. As a result, Trp^{235} of domain IV, located on the contact surface with domain V, was slightly shifted.

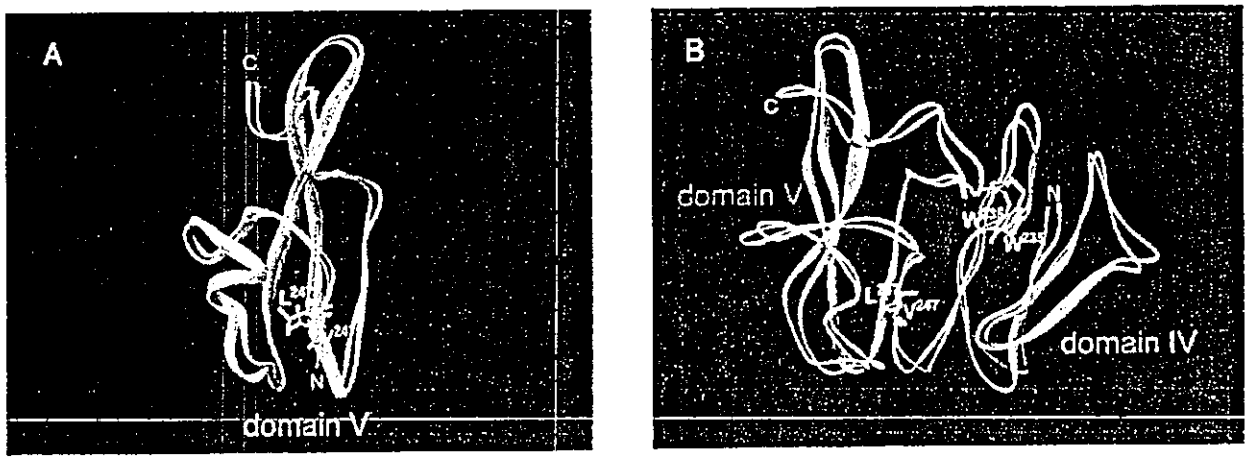

Figure 4. Conformational alterations in domain V (A) and in the domain IV–V complex (B), replacing leucine by valine at position 247. Structure of the valine (light blue) and leucine (white) variants was shown by a ribbon representation with the secondary structure.

DISCUSSION

This study shows the positive correlation between the Val^{247} β_2GPI allele and anti-β_2GPI antibody production in a Japanese population, confirming the correlation observed in a British Caucasian population in our previous report (15). A positive correlation between the Val^{247} allele and the presence of anti-β_2GPI antibodies was also reported in Asian American (26) and Mexican patients (27). However, this correlation was not observed in other American populations (26) or in patients with thrombosis or pregnancy complications in the UK (28). This discrepancy may be the result of the difference in the frequency of the Val^{247} allele among races, or the difference in the background of investigated patients. Another possibility is that the relationship between the Val^{247} allele and thrombosis in Caucasians may be controversial due to underpowered studies or to differences in the procedure used to detect anti-β_2GPI antibodies. Methods for the detection of anti-β_2GPI antibodies differ among laboratories. For example, cardiolipin-coated plates or oxygenated plates are used in some methods, whereas unoxygenated plates are used in others. In addition, bovine β_2GPI is used instead of human β_2GPI in some assays. The antibodies used for standardization also differ, although monoclonal antibodies such as EY2C9 and HCAL (29) have been proposed as international standards of calibration materials.

β_2GPI is a major target antigen for aCL, and, according to our previous investigation, B cell epitopes reside in domain IV and are considered to be cryptic and to appear only when β_2GPI interacts with negatively charged surfaces such as cardiolipin, phosphatidylserine, or polyoxygenated polystyrene surface (7), although other studies indicate that the B cell epitopes are located on domain I (13) or domain V (14). According to another interpretation for the specificity of aCL, increment of the local antigen density on the negatively charged surface also contributes to anti-β_2GPI detection in ELISA (8,30). Studies on the crystal structure of human β_2GPI revealed that the lysine-rich site and an extended C-terminal loop region on domain V are crucial for phospholipid binding. Position 247 is located at the N-terminal side of domain V, and, around this position, Lys^{242}, Ala^{243}, and Ser^{244} were suggested to play a role in the interaction between domains IV and V (9,23,31).

Although the Val/Leu^{247} polymorphism may not be very critical for the autoantibody binding, the amino acid substitution at this point was revealed to affect the affinity of monoclonal aCL established from patients with APS and that of purified IgG from patients positive for β_2GPI-dependent aCL. We conformationally optimized to domain V and the domain IV–V complex of β_2GPI variants at position 247, referring the crystal structure of β_2GPI. IgG aCL was screened using the standardized aCL ELISA, in which both the Leu^{247} and the Val^{247} allele of β_2GPI are contained as antigen. Although biochemical characteristics and structure are similar between valine and leucine, the replacement of Leu^{247} by Val^{247} leads to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction (Figure 4). It is likely that the structural alteration affects the affinity between anti-β_2GPI autoantibodies and the epitope(s) present on its molecule. One explanation for this phenomenon is that this β_2GPI polymorphism affects the electrostatic interaction between domain IV and domain V or the protein–protein interaction, resulting in differences in the accessibility of the recognition site by the autoantibodies, or the local density of β_2GPI.

Another possible explanation of the correlation between the Val/Leu^{247} polymorphism of β_2GPI and anti-β_2GPI antibodies is T cell reactivity. Ito et al (32) investigated T cell epitopes of patients with anti-β_2GPI autoantibodies by stimulating patients' PBMCs with a peptide library that covers the β_2GPI sequence. Four of 7 established CD4+ T cell clones reacted to peptide fragments that include amino acid position 244–264, then position 247 is included among the candidate epitopes. Arai et al (33) found preferred recognition of peptide position 276–290 by T cell clones from patients with APS. They also found high reactivity to peptide 247–261 in one patient. We speculate that a small alteration in the conformation arising from the valine/leucine substitution at position 247 may affect the susceptibility to generate autoreactive T cell clones in patients with APS.

Our results in this study indicate that the Val/Leu^{247} polymorphism affects the antigenicity of β_2GPI for anti-β_2GPI autoantibodies, and that the Val^{247} allele can be a risk factor for having autoantibodies against this molecule. Therefore, the Val/Leu^{247} variation of β_2GPI may be crucial for autoimmune reactivity against β_2GPI. We further show the significance of the Val/Leu^{247} polymorphism of β_2GPI in the strength of the binding between β_2GPI and anti-β_2GPI autoantibodies. The significance of antigen polymorphisms in the production of autoantibodies or in the development of autoimmune diseases is not well understood. To our knowledge, this report is the first to present a genetic polymorphism of

autoantigen directly affecting its interaction with autoantibodies.

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REVIEW

Specificities, Properties, and Clinical Significance of Antiprothrombin Antibodies

Olga Amengual, Tatsuya Atsumi, and Takao Koike

Introduction

Antibodies against phospholipid-binding proteins, such as β_2-glycoprotein I (β_2GPI) and prothrombin, are associated with the clinical manifestations of the antiphospholipid syndrome. In the last decade, there has been increasing interest in antiprothrombin antibodies (APTs). The immunologic and functional properties of these antibodies have been investigated, and they have been found to vary widely, depending mainly on their affinity for human prothrombin. Recently, antibodies against phosphatidylserine–prothrombin complex (APS/PT) have been detected, and these antibodies, rather than antibodies against prothrombin alone (APT-A), are closely associated with antiphospholipid syndrome and lupus anticoagulant (LAC). Despite increasing knowledge about their mechanisms of action, the clinical significance of APTs has not yet been established, and the question of whether antibodies to particular phospholipid-binding plasma proteins or phospholipid–protein complexes lead to different clinical presentations and/or thrombogenic mechanisms needs to be addressed.

Antiphospholipid antibodies (aPL): from antibodies against "phospholipids" to antibodies against "phospholipid-binding proteins"

Antiphospholipid antibodies are immunoglobulins associated with a variety of clinical phenomena, including arterial and venous thrombosis, pregnancy morbidity, neurologic disorders, and thrombocytopenia. The term "antiphospholipid syndrome" is used to link these clinical manifestations to the persistence of aPL, and it is recognized as one of the most common causes of acquired thrombophilia (1,2).

It has been shown that aPL, despite their name, are not directed against anionic phospholipids, as had previously been thought, but are a part of a large family of autoantibodies against phospholipid-binding plasma proteins or phospholipid–protein complexes (3,4). The most common and best characterized antigenic targets of these antibodies are β_2GPI (5–7) and prothrombin (8). Other phospholipid-binding proteins, including high and low molecular weight kininogens (9), annexin V (10), and protein C and protein S (11,12), may be important targets as well. Since most of these antigens are involved in blood coagulation, some aPL may hamper the regulation of the coagulation system, providing an explanation of the high incidence of thrombotic events in patients with antiphospholipid syndrome.

β_2GPI is a phospholipid-binding protein that has been studied extensively. β_2GPI plays an important role in the binding of aPL to phospholipids. In 1990, three groups of investigators, Galli et al (5), McNeil et al (6), and Matsuura et al (7), independently reported that anticardiolipin antibodies (aCL), which were associated with the antiphospholipid syndrome, were not directed against CL alone but required a plasma protein (β_2GPI) as a cofactor to bind CL in enzyme-linked immunosorbent assay (ELISA) plates. β_2GPI bears the epitopes for aCL binding that are exposed when β_2GPI binds to negatively charged phospholipids or irradiated plastic plates (13). Several studies have highlighted the significance of using anti-β_2GPI antibodies as an alternative ELISA method that has higher specificity for the diagnosis of antiphospholipid syndrome than does the conventional aCL ELISA (14–16).

Prothrombin, another major phospholipid-binding protein, was first reported to be a possible cofactor for LAC in 1959 (17). In subsequent years,

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prothrombin has attracted considerable interest, and several groups have dealt with APTs in an attempt to clarify their immunologic characteristics and the role they may play in thrombosis in patients with antiphospholipid syndrome. It has been shown that APTs detected by ELISA represent a heterogeneous family of antibodies that includes APT-A and APS/PT. This review summarizes recent information on the prothrombin molecule and on the specificities and properties of APTs.

Prothrombin molecule

Prothrombin (factor II) is a vitamin K–dependent glycoprotein synthesized in the liver and present at a concentration of ~100 μg/ml in normal plasma. Its gene spans 21 kbp on chromosome 11 (18). Mature human prothrombin that circulates in blood consists of a single-chain glycoprotein of 579 amino acid residues with a molecular weight of 72 kd, including 3 carbohydrate chains and 10 γ-carboxyglutamic acid residues (19). During its biosynthesis in the liver, prothrombin undergoes γ-carboxylation. These γ-carboxyglutamic residues, known as the GLA domain, are located on fragment 1 of the prothrombin molecule. The GLA domain is essential for the calcium-dependent binding of phospholipid to prothrombin, which in time, is necessary for the conversion of prothrombin to biologically active α-thrombin. The GLA domain is followed by a kringle domain containing 2 kringle structures and a carboxyl-terminal serine protease. Kringle is a structure with 3 characteristic intradisulfide bonds that has been identified in a number of proteins, including prothrombin (20). Kringles of each protein have distinct functions in many cell types and interact with substrate cofactor receptors (21). In the prothrombin molecule, the kringle domain is involved in the binding of thrombin to fibrin (18).

Prothrombin is physiologically activated by the prothrombinase complex (activated factor X, factor V, calcium, and phospholipids). Once negatively charged phospholipids bind prothrombin, prothrombinase complex converts prothrombin to thrombin, which triggers fibrinogen polymerization into fibrin (22). In addition, thrombin binds thrombomodulin on the surface of endothelial cells and activates protein C, which then exerts its anticoagulant activity by digesting factor V and depriving the prothrombinase complex of its most important cofactor. Because of this negative-feedback pathway, prothrombin/thrombin behaves as an "indirect" anticoagulant.

Prothrombin activation catalysis by prothrombinase complex constitutes an ordered sequential reaction that proceeds via the cleavage at Arg^{322}–Ile^{323} and results in the liberation of meizothrombin, a catalytically active intermediate composed of fragment 1+2 and A and B chains of thrombin linked by a disulfide bond. Further cleavage at Arg^{273}–Thr^{274} results in the formation of α-thrombin (23) (Figure 1). Factor Xa alone catalyzes the activation of human prothrombin by proteolytic cleavage at Arg^{273}–Thr^{274}, which results in the liberation of fragment 1+2 and prethrombin 2. Prethrombin 2 is composed of the A and B chains of thrombin with the bond at Arg^{322}–Ile^{323} intact. Further proteolysis at this bond yields α-thrombin (Figure 1). One of the most potent enzymes known, α-thrombin not only converts fibrinogen to fibrin, but also acts on factors V, VIII, and XIII, protein C, platelets, and endothelial cells (24).

Antiprothrombin antibodies

Anticoagulant or procoagulant characteristics of APTs. The involvement of prothrombin as a cofactor for a circulating anticoagulant was first considered in 1959 by Loeliger (17). The addition of normal plasma to that of a patient with LAC increased the degree of coagulation inhibition. A low plasma level of prothrombin was also found, suggesting that the cofactor associated with the expression of LAC activity was most likely prothrombin. In 1960, Rapaport et al (25) described the case of a child with LAC who underwent recurrent episodes of bleeding. Further investigations showed a severe prothrombin deficiency, a prolonged prothrombin time, and a prolonged activated partial thromboplastin time. During the 1980s, more research was performed to clarify the hypoprothrombinemia in patients with LAC, and in 1983, Bajaj et al (26) were the first to ascertain the presence of prothrombin-binding antibodies in patients with LAC and severe hypoprothrombinemia. They postulated that hypoprothrombinemia results from the rapid clearance of prothrombin–APT complexes from the circulation.

In 1984, Edson et al (27) demonstrated prothrombin–APT complexes by counterimmunoelectrophoresis (CIE) in the plasma of patients with LAC but without severe hypoprothrombinemia. These findings were confirmed and extended by Fleck et al (28), who found that 74% of the population with LAC under study had prothrombin–APT complexes by CIE and showed that APT had LAC activity. Subsequently, circulating prothrombin–APT complexes were also observed in patients with LAC and with normal prothrombin levels.

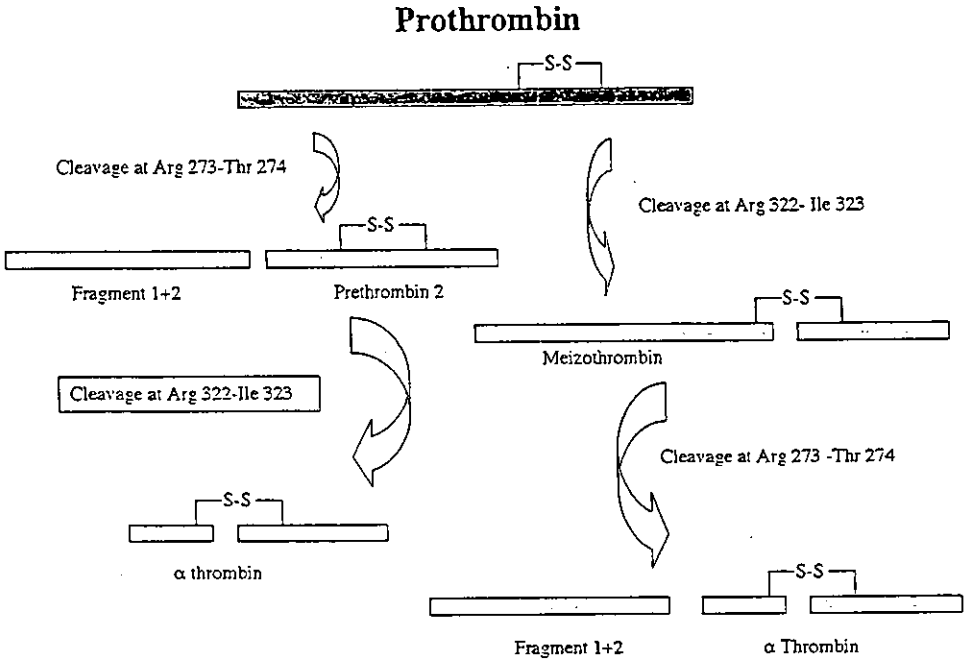

Figure 1. Conversion of human prothrombin to α-thrombin. **Left,** Activated factor Xa catalyzes the activation of human prothrombin by proteolytic cleavage at Arg^{273}–Thr^{274}, resulting in the liberation of fragment 1+2 and prethrombin 2. Prethrombin 2 is composed of the A and B chains of thrombin with the disulfide bond (S–S) at Arg^{322}–Ile^{323} intact. Further proteolysis at this bond yields α-thrombin. **Right,** Prothrombinase complex activates human prothrombin in an ordered sequential reaction via cleavage at Arg^{322}–Ile^{323} and results in the liberation of meizothrombin, followed by cleavage at Arg^{275}–Thr^{274}, which leads to the formation of α-thrombin.

In 1991, Bevers et al (8) highlighted the importance of APT in causing LAC activity when they studied 16 patients with both aCL and LAC. After incubation with CL-containing liposomes, the LAC activity remained in the supernatant from 11 patients. These 11 samples demonstrated LAC activity in a phospholipid-bound prothrombin–dependent manner. Later, Oosting et al (11) showed that LAC inhibited endothelial cell–mediated prothrombinase activity and that the IgG fraction containing LAC activity bound to phospholipid–prothrombin complex.

It was shown that most LACs depend on the presence of phospholipid-bound prothrombin and phospholipid-bound β_2GPI, and the anticoagulant properties of APTs have been studied by several groups. Permpikul et al (29) purified IgG fractions from 10 patients with LAC, showing that LAC activity was due to APTs in at least 9 of the samples. Some investigators (30,31) reported the existence of two types of circulating APTs which can be distinguished on the basis of their effects in coagulation assays: 1) functional, which cause LAC activity, and 2) nonfunctional, which do not contribute to the LAC activity. The differences in effects are probably caused by differences in epitope specificities between the different APTs (32).

Methods for detecting APTs: APT-A versus APS/PT. The first techniques used for screening APTs were double diffusion, CIE (26–28,33), and assays based on the impairment of prothrombin activation by APTs (11,34). In 1995, Arvieux et al (35) showed that APTs could be detected by a standard ELISA. This ELISA used prothrombin as antigen coated onto irradiated plates (APT-A ELISA). The behavior of APTs in ELISA closely resembles that of anti-β_2GPI. In fact, APTs cannot be detected when prothrombin is immobilized on nonirradiated plates (35), but binding is observed if prothrombin is immobilized on a suitable anionic surface, adsorbed on gamma-irradiated plates, or exposed to immobilized anionic phospholipids. In 1996, Matsuda et al (36) demonstrated that APS/PT could be detected in LAC-positive patients. Later, Galli et al (30) reported that the ELISA system with PS-bound prothrombin (APS/PT ELISA) was more efficient in demonstrating the presence of APT than the

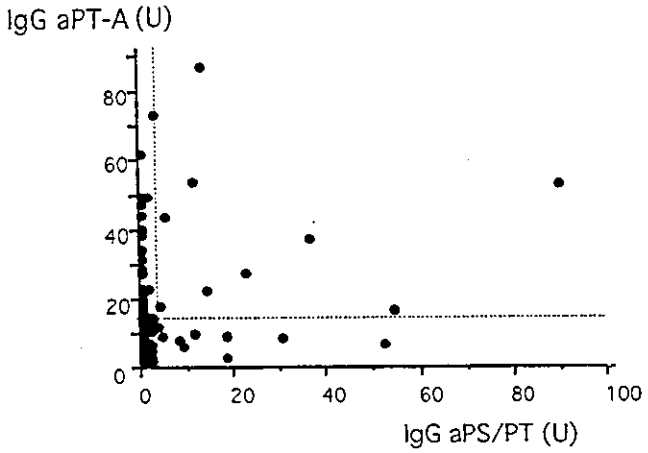

Figure 2. Relationship between IgG titers of antiprothrombin antibodies against prothrombin alone (aPT-A) and antibodies against phosphatidylserine–prothrombin complex (aPS/PT). The titers of IgG aPT-A and IgG aPS/PT in patients with antiphospholipid syndrome or other autoimmune diseases were determined by enzyme-linked immunosorbent assay. The dashed line indicates the cutoff for positivity. Many patients with only 1 positive determination were found. U = units.

system using prothrombin alone coated on high-binding plates (APT-A ELISA), suggesting the existence of two types of APTs.

Our group (37) recently reported that the detection of APS/PT strongly correlated with the presence of LAC. Most investigators have used only the APT-A ELISA, presuming that both systems of detection (APT-A and APS/PT ELISAs) detect the same antibodies. However, we showed that there was no correlation of APS/PT and APT-A titers in patients with antiphospholipid syndrome or other autoimmune diseases (Figure 2). Human monoclonal APS/PT, derived from a patient with antiphospholipid syndrome, did not bind to prothrombin immobilized on oxidized plates (38). Therefore, the APS/PT assay detects, at least in part, a population of antibodies different from APT-A, and these antibodies have significant clinical relevance because of their correlations with clinical features of antiphospholipid syndrome and with the presence of LAC. Table 1 summarizes the historical background of research on APTs.

Hematologic properties of APTs

The mechanisms by which APTs cause LAC activity are not yet clearly understood. Pierangeli et al (39) have suggested that APTs cause prolongation of in vitro clotting by inhibiting the conversion of prothrombin into thrombin. However, it seems unlikely that APTs prolong the clotting times by hampering the activation of prothrombin through binding near the activation sites in the molecule, since such a mechanism would not explain the neutralizing effects of high phospholipid concentrations.

Recently, Simmelink et al (40) showed that addition of affinity-purified APTs from LAC-positive plasma to normal plasma induced LAC activity, and that LAC

Table 1. Historical background of research on antiprothrombin antibodies (APTs)*

Author, year (ref.)	Findings
Loeliger, 1959 (17)	Prothrombin proposed as a possible cofactor for LAC
Rapaport et al, 1960 (25)	Plasma coagulation disturbances in SLE could result from a combination of a plasma inhibitor and hypoprothrombinemia
Bajaj et al, 1983 (26)	Presence of prothrombin-binding antibodies in patients with LAC and hypoprothrombinemia
Edson et al, 1984 (27)	Prothrombin–APT complexes demonstrated by CIE in the plasma of patients with LAC and without severe hypoprothrombinemia
Fleck et al, 1988 (28)	Circulating prothrombin–APT complexes observed in patients with LAC and normal PT levels
Bevers et al, 1991 (8)	Importance of APT in causing LAC activity
Oosting et al, 1993 (11)	LAC inhibited endothelial cell–mediated prothrombinase activity; IgG fraction containing LAC activity bound to phospholipid–prothrombin complex
Arvieux et al, 1995 (35)	APT could be detected by a standard ELISA (APT-A ELISA)
Matsuda et al, 1996 (36)	APS/PT could be detected in LAC-positive patients
Galli et al, 1997 (30)	APT recognized prothrombin more efficiently in ELISA using PS-bound prothrombin
Atsumi et al, 2000 (37)	Strong correlation between APS/PT and antiphospholipid syndrome

* LAC = lupus anticoagulant; SLE = systemic lupus erythematosus; CIE = counter immunoelectrophoresis; ELISA = enzyme-linked immunosorbent assay; APT-A = antibodies against prothrombin alone; APS/PT = antibodies against phosphatidylserine–prothrombin complex.

activity was neutralized upon increasing the phospholipid concentration. They also showed that complexes of prothrombin and APT with LAC activity inhibited both prothrombinase and tenase complex. Thus, in the procoagulant pathway, APTs might increase the affinity of prothrombin for negatively charged phospholipids, thereby competing with clotting factors for the available catalytic phospholipid surface, a mechanism similar to that of anti-β_2GPI. The model, based on increased affinity of protein–antibody complexes (β_2GPI or prothrombin) for negatively charged phospholipids, can explain why LAC activity caused by both anti-β_2GPI and APT can be neutralized by the addition of extra phospholipids. Furthermore, in their investigation of the anticoagulant activity of the protein C system, Galli et al (41) demonstrated the dominant inhibitory effect on activated protein C activity of anti-β_2GPI compared with the effect of APT using isolated total IgG fraction.

Immunologic characteristics of APTs

APTs bind to prothrombin coated on gamma-irradiated (35) or activated polyvinyl chloride ELISA plates (APT-A) (12,30) or exposed to immobilized PS (APS/PT) (30). Recently, it has been shown that APTs also recognize prothrombin when it is bound to hexagonal (II) phase phosphatidylethanolamine, a neutral phospholipid used as an excess of phospholipids in the clotting assay to confirm the presence of LAC (42).

APTs may be directed against cryptic epitopes or neoepitopes (antigens) exposed when prothrombin binds to anionic phospholipids, and/or they may be low-affinity antibodies binding bivalently to immobilized prothrombin. However, experimental evidence has not clearly established whether (or which) APTs are anti-neoepitopes or low-affinity antibodies. Wu and Lentz (43) observed that human prothrombin undergoes a conformational change upon binding to PS-containing surfaces in the presence of calcium. On the other hand, Galli et al (30) demonstrated that prothrombin was recognized more efficiently when it was bound to PS-coated ELISA plates using calcium ions and that APTs could be of low affinity. They suggested that prothrombin complexed with PS could allow clustering and better orientation of the antigen, offering optimal conditions for antibody recognition.

A high percentage of APTs have species specificity for the human protein (8), and a minority of them react with bovine prothrombin (44). The epitope(s) recognized by APTs remains to be defined. Rao et al (45), used purified IgG preparations from LAC-positive patients and demonstrated binding of APT to prethrombin 1 and fragment 1 as well as to the whole prothrombin molecule. However, none of the antibodies reacted with immobilized thrombin. These findings suggest that dominant epitopes are likely to be located near the phospholipid-binding site of the prothrombin molecule, although they may have heterogeneous distribution. Recently, Akimoto et al (46) investigated the epitope distribution of APTs using recombinant prothrombin fragments. They demonstrated that 61.5% of APTs were dominantly directed to fragment 1 of prothrombin and 38.4% to the prethrombin site (fragment 2 plus α-thrombin).

Clinical significance of APTs

The clinical significance of APTs has not yet been established (47). A number of clinical studies have investigated the clinical relevance of APTs detected by ELISA using prothrombin alone as antigen (APT-A ELISA), and these have been recently summarized by Galli (48). Some of these studies showed positive correlations between APT-A and some of the clinical features of antiphospholipid syndrome.

Puurunen et al (49) reported the presence of APT-A in 34% of patients with systemic lupus erythematosus (SLE) and found positive correlations of both APT-A and anti-β_2GPI with deep vein thrombosis in this population. Sorice et al (50) described a higher frequency of APT-A in patients with SLE and antiphospholipid syndrome than in those without antiphospholipid syndrome and demonstrated a significant correlation between the presence of these antibodies and the clinical features of antiphospholipid syndrome. Bertolaccini et al (51) reported positivity for APT-A in 58 of 207 patients with SLE and observed that 53% of patients with APT-A had a history of thrombotic events (odds ratio [OR] 2.5, 95% confidence interval [95% CI] 1.4–4.6). Furthermore, in a series of 177 patients with SLE or primary antiphospholipid syndrome, Muñoz-Rodriguez et al (52) found that thrombotic events were more prevalent in patients with APT-A (45% versus 28%; $P = 0.02$) and that these antibodies represented an independent risk factor for arterial thrombosis (OR 2.4, $P = 0.04$). Additionally, those investigators found an association between APT-A and thrombocytopenia in patients with primary antiphospholipid syndrome (OR 6.7, $P = 0.007$). Using multivariate logistic regression analysis, Nojima et al (53) demonstrated that the coexistence of IgG APT-A and LAC activity represented a risk factor for venous thromboembolism in patients with

Table 2. Antiprothrombin antibodies directed against prothrombin alone (APT-A) and associations with autoimmune disorders*

Author, year (ref.) [no. of patients]	Population diagnosis or features	Prevalence of APT-A, %	Clinical associations with APT-A
Horbach et al, 1996 (31) [175]	SLE	38 (IgG), 18 (IgM)	No correlation with thrombosis
Pengo et al, 1996 (12) [22]	aPL positive	50	No correlation with thrombosis
Puurunen et al. 1996 (49) [139]	SLE	34	Deep venous thrombosis
D'Angelo et al, 1997 (55) [110]	Clinical suspicion of autoimmune diseases	51	No correlation with thrombosis
Forastiero et al, 1997 (56) [233]	aPL positive	26	No correlation with thrombosis
Galli et al, 1997 (30) [59]	aPL positive	58	No correlation with thrombosis
Swadzba et al, 1997 (58) [127]	SLE/lupus-like disease	28 (IgG), 29 (IgM)	No correlation with thrombosis
Bertolaccini et al, 1998 (51) [207]	SLE	28	Thrombosis
Guerin et al, 1998 (57) [295]	Autoimmune/infectious diseases	59	No correlation with thrombosis; correlation with antiphospholipid syndrome
Inanç et al. 1998 (59) [7]	SLE	43	No correlation with thrombosis
Sorice et al, 1998 (50) [59]	SLE, primary antiphospholipid syndrome	25	Clinical features of antiphospholipid syndrome
Lakos et al, 2000 (54) [70]	Systemic autoimmune diseases	33	Thrombosis
Muñoz-Rodriguez et al, 2000 (52) [177]	SLE, primary antiphospholipid syndrome	47	Thrombosis, arterial thrombosis, thrombocytopenia
Nojima et al. 2001 (53) [124]	SLE	52	Venous thrombosis

* SLE = systemic lupus erythematosus; aPL = antiphospholipid antibodies.

SLE (OR 19.1, 95% CI 4.7–77.1). Lakos et al (54), who evaluated a population of 70 patients with systemic autoimmune disorders, claimed high specificity of APT-A for the diagnosis of antiphospholipid syndrome.

Conversely, Pengo et al (12) found no correlation between the presence of APT-A and thrombosis in 22 patients with aPL and a history of at least one thromboembolic event. Horbach et al (31), in a large population of patients with SLE, found that both IgG and IgM APT-A were more frequent in patients with a history of venous thrombosis, but the correlation was not significant when examined by multivariate analysis. Horbach et al showed that LAC was the strongest risk factor for thrombosis and that neither APT-A nor anti-β_2GPI gave additional information concerning a thrombotic risk in SLE. Similar data were obtained by D'Angelo et al (55) using samples from 110 inpatients referred for evaluation of their aPL status, 32 of whom had a history of thrombotic events. D'Angelo et al found that only the presence of LAC, but not aCL, anti-β_2GPI, or APT-A, was significantly associated with a history of previous thrombosis (OR 15.8, 95% CI 4.3–59.6, $P < 0.001$). Forastiero et al (56), in a group of 233 patients with LAC and/or aCL, showed that APT-A were related to venous thrombosis; however, the multivariate analysis demonstrated that anti-β_2GPI, but not APT-A, were the only independent risk factor for venous thrombosis in those patients. Galli et al (30) detected APT-A in 58% of patients with aPL, but the overall prevalence of these antibodies was similar in patients with and without a history of thrombosis (95% versus 86%, respectively).

Guerin et al (57) described the presence of APT-A in a variety of disorders, including antiphospholipid syndrome, SLE, and conditions associated with a high rate of false-positive results in conventional aPL tests. Those investigators observed lack of specificity of APT-A for antiphospholipid syndrome and failed to demonstrate an association between the presence of these antibodies and thrombosis; however, they found a correlation between APT-A and antiphospholipid syndrome, suggesting that these antibodies might be associated more with other clinical manifestations, such as fetal loss or thrombocytopenia. Swadzba et al (58) did not find a correlation between IgG APT-A and thrombosis in SLE, although they found a high prevalence of the IgM isotype in patients with lupus-like disease and a history of thrombotic events. Additionally, in a longitudinal study of 7 patients with SLE and thrombotic events, Inanç et al (59) demonstrated that APT-A were far less predictive of thrombosis than were anti-β_2GPI.

In a study of patients without autoimmune disease, Vaarala et al (60) found that high levels of APT-A conferred high risk of myocardial infarction or cardiac death in middle-aged men, and Palosuo et al (61) found a close relationship between high APT-A levels and deep venous thrombosis and pulmonary embolism in middle-aged men. However, Eschwege et al (62) showed no correlation between APT-A and thrombotic events in

a large population of unselected patients with a history of venous thrombosis. In that study, LAC positivity was the only aPL test result that was strongly associated with the severity of thrombosis. Table 2 summarizes the clinical associations of APT-A in autoimmune disorders.

Taken together, the controversial nature of the available data regarding APT-A, the lack of a well-standardized assay, and the fact that the majority of the studies are retrospective make it difficult to make definite conclusions regarding the clinical significance of these antibodies. More "cross-sectional" and/or prospective clinical studies are warranted to establish the clinical relevance of APT-A.

We have reported the detection of APS/PT and have shown that the results obtained depend closely on the assay performed (37). The presence of APS/PT, but not APT-A, correlated significantly with the clinical manifestations of antiphospholipid syndrome (OR 8.29, 95% CI 3.03–22.71, $P < 0.0001$ versus OR 1.89, 95% CI 0.71–5.06, P not significant). Furthermore, APS/PT were more closely associated with LAC than were APT-A. Thus, APS/PT are a marker for antiphospholipid syndrome, and their detection may help to confirm the presence of LAC.

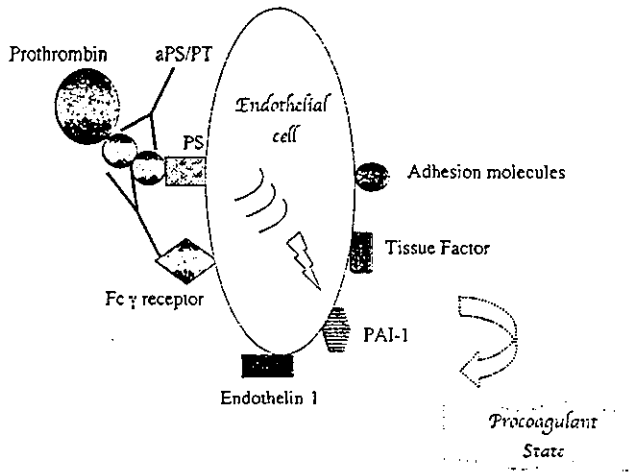

Figure 3. Proposed mechanism of action of antiprothrombin antibodies at the endothelial cell level. After an activation signal, anionic phospholipids (phosphatidylserine [PS]) are exposed on the endothelial cell. Prothrombin binds those phospholipids on the cell surface, and circulating antibodies against PS–prothrombin complexes (aPS/PT) recognize the epitope(s) on the prothrombin molecule and bind to them. Those antibodies (or immune complex) activate endothelial cells via specific pathways or via Fcγ receptors. This induces the release of procoagulant substances, which leads to coagulation activation, platelet aggregation, and thrombosis. PAI-1 = plasminogen activator inhibitor 1.

Pathogenic role of APTs

The pathophysiologic mechanisms of APTs are not completely known. However, there is increasing evidence that they play a role in the hypercoagulable state of antiphospholipid syndrome.

The antigens are present in plasma or on cell surfaces exposed to plasma, and they are therefore accessible to circulating antibodies. Some effects on endothelial cells have been proposed: 1) APTs inhibit thrombin-mediated endothelial cell prostacyclin release and hamper protein C activation (3); 2) APTs could recognize the prothrombin–anionic phospholipid complex on the endothelial cell surface, thus activating endothelial cells and inducing procoagulant substances via prothrombin (63); or 3) APTs could increase the affinity of prothrombin for negatively charged phospholipids (45).

Field et al (64) extended the last of these observations and reported that a murine monoclonal APT and 7 IgG LACs enhanced the binding of prothrombin to phospholipid vesicles (75:25 phosphatidylcholine:PS) in a concentration-dependent manner. They also demonstrated that the monoclonal antibody and 4 of 6 IgG LACs from patients with a history of thrombosis increased thrombin production by purified prothrombinase components in a flow system. These in vitro observations have been reported in vivo, showing that thrombin production is increased in patients with LAC (65) or aPL (66). The precise mechanisms by which IgG LACs enhanced interaction of prothrombin with phospholipid vesicles are not known, although Field et al showed that antibody bivalency was essential for this effect, as was the elevated microenvironmental concentration of prothrombin on the surfaces of the phospholipid vesicles (67). They demonstrated that this enhanced binding was due to a decrease in the "off rate" for prothrombin interaction with phospholipid vesicles in the presence of LAC.

Some aPL bind to endothelial cells, suggesting that aPL may interact with endothelial cells and thus promote thrombosis (68,69). APTs can bind to immobilized PS in the presence of calcium and prothrombin (28,29), and a LAC IgG preparation can enhance the binding of prothrombin to endothelial cells and increase thrombin generation on these cells (45), suggesting that APT may concentrate prothrombin on cell surface phospholipids and thus lead to a hypercoagulable state. Zhao et al (70) generated and characterized 1 monoclonal IgG APT (from a patient with antiphospholipid syndrome) that showed LAC activity. This monoclonal IgG en-

hanced the binding of prothrombin to damaged endothelial cells and shortened plasma coagulation times in the presence of LAC. Therefore, a highly purified monoclonal APT can behave like LAC in endothelial cell-based coagulation assays and can paradoxically increase the rate of thrombosis in patients with antiphospholipid syndrome.

It has been suggested that aPL alone are unlikely to be triggering agents for thrombosis in patients with antiphospholipid syndrome and that aPL might promote and sustain thrombosis initiated by other factors. APTs may promote thrombosis by facilitating prothrombin interactions with damaged blood vessel walls and by promoting thrombin generation in flow, leading to a hypercoagulable state and consequently to a thrombotic tendency (Figure 3).

APTs could bind platelets and produce thrombocytopenia. However, antibodies directed toward specific platelet glycoproteins have been detected in patients with aPL in a proportion similar to that reported for patients with idiopathic thrombocytopenic purpura (71), suggesting that the cause of thrombocytopenia in patients with aPL is similar to that of idiopathic thrombocytopenic purpura.

So far, the pathogenic role of APTs in morbidity during pregnancy has not been clarified. Further investigations are needed to determine whether these antibodies play any role in the pathogenesis of these common features of the antiphospholipid syndrome.

Finally, other congenital or acquired factors may contribute to the final thrombotic risk in patients with aPL. These factors include prothrombin gene mutation, factor V "Leiden," hyperhomocystinemia, elevated plasma levels of prothrombin, factor VII, and von Willebrand factor, and decreased protein C and protein S activities.

Conclusion

APTs are frequently found in patients with aPL, but their immunologic characteristics and mechanisms of action are not completely understood. The evaluation of the clinical relevance of APTs is influenced by many variables and depends on the applied method of detection. APS/PT are more closely associated with antiphospholipid syndrome and LAC than are APT-A. The determination of APS/PT in clinical practice, in conjunction with other aPL, may improve the likelihood of recognizing antiphospholipid syndrome. Knowledge of the behavior of specific aPL would aid in defining specific thrombogenic pathways and improve the management of patients with antiphospholipid syndrome.

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Case report

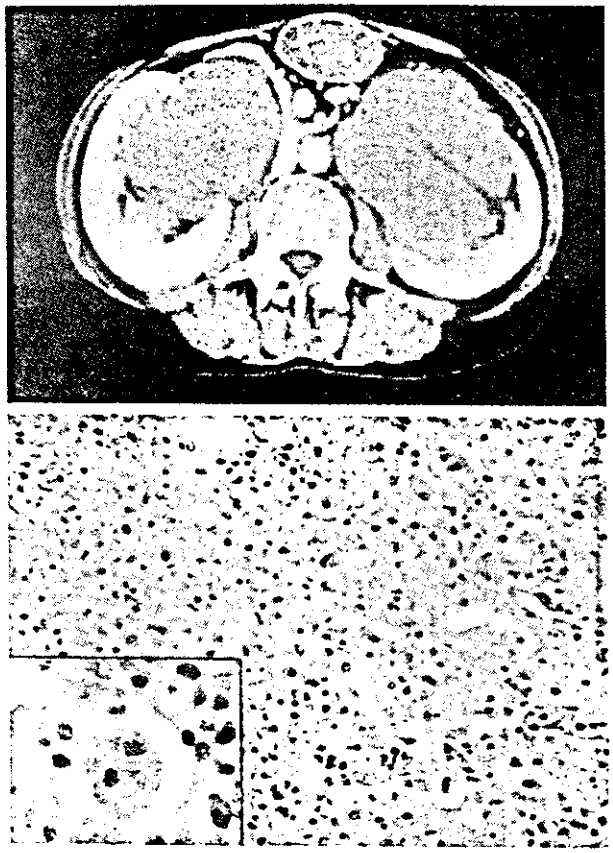
Uveitis, pancarditis, haemophagocytosis, and abdominal masses

Yoshie Sakai, Tatsuya Atsumi, Tomoo Itoh, Takao Koike

A 43-year old woman was admitted to our department in April, 1997, with a 3-month history of fatigue, fever, and polyarthralgia. She had been healthy except for chronic bilateral uveitis since 1992. She was feverish and had an apical systolic ejection murmur. There was no lymphadenopathy, nor oral or genital ulcers. Her ESR was 81 mm/h, C-reactive protein 12·2 mg/dL, haemoglobin 8·4 g/dL, leucocyte count $1{\cdot}11\times10^9$/L, and she had hypergammaglobulinaemia. Lipid profile was normal. Antinuclear antibodies, rheumatoid factor, anti-streptolysin antibodies, blood cultures, and Mantoux test were negative. Thoracoabdominal computed tomography was normal. Gallium-67 scintigraphy showed massive uptake in the ventricles, right thigh, and both knees and ankles. Doppler ultrasound showed minimal aortic and mitral regurgitation and transthoracic echocardiogram showed a small pericardial effusion and no vegetations. In May, 1997, she underwent mitral valve replacement due to severe mitral regurgitation from cord rupture, and pancarditis was diagnosed. A few days later, she developed thrombocytopenia (platelet count $2{\cdot}0\times10^4$/L) and bone-marrow aspiration showed massive proliferation of histiocytes and haemophagocytosis with negative cultures of aspirate. With a diagnosis of haemophagocytotic syndrome, steroid pulse therapy was started (methylprednisolone 1g daily for three days). Improvement was noted within one month. In July, 1997, she had a perforation of her ileum and a diagnosis of Behçet's disease was considered. She was treated with ciclosporin and steroids and was well until May, 2001, when she had fever and malaise.

Abdominal computed tomograms showed low-density parapelvic masses (8×10 cm) (figure, top). A biopsy specimen obtained at laparotomy showed proliferation of histiocytes with plasma cells and characteristically prominent lymphocytophagocytosis by histiocytes (figure, bottom). Most histiocytes were S-100 protein positive and CD1a negative on immunohistochemical staining.[1] Rosai-Dorfman disease (RDD) was diagnosed and a retrospective review of the biopsy material of mitral valve and bone marrow showed the same characteristic histiocytes. Three courses of steroids and chemotherapy with etoposide (VP-16) successfully reduced the masses. She was in remission for ten months until September, 2002, when she had fever and an increase in the size of her abdominal masses. Treatment with steroids and etoposide led to clinical improvement. When last seen in December, 2002, she was well.

In 1969, Rosai and Dorfman described sinus histiocytosis with massive lymphadenopathy[2], a benign disease characterised by fever, lymphadenopathy, leucocytosis, high ESR, and polyclonal hypergammaglobulinaemia. Extranodal sites are involved in 43% of patients. The name RDD is used in cases with prominent non-nodular involvement.[3,4] Our patient had pancarditis as the first manifestation of RDD, but the diagnosis of RDD was not made until she developed kidney involvement. RDD usually resolves spontaneously or with a low dose of steroids, but some cases require an intensive therapeutic approach.[5] Awareness of RDD and adequate histochemical staining would make the diagnosis more straightforward and lead to correct management.



Top: **Abdominal computed tomogram**
There are bilateral parapelvic masses.
Bottom: **Biopsy specimen of parapelvic mass**
There is a dense infiltration of histiocytes, lymphocytes, and plasma cells. The histiocytes were large with abundant eosinophilic cytoplasm and contain erythrocytes and lymphophagocytes (emperipolesis).

Yoshie Sakai, Tatsuya Atsumi, and Takao Koike were the clinicians in charge of the patient. Tomoo Itoh did the histological investigations. All the authors participated in writing the report. None of the authors has a conflict of interest and there was no funding.

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