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Overlapping humoral autoimmunity links rheumatic fever and the antiphospholipid syndrome

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***Objective*: Rheumatic fever (RF) and the antiphospholipid syndrome (APS) are autoimmune diseases that share similar cardiac and neurological pathologies. We assessed the presence of shared epitopes between M protein, N-acetyl-β-D-glucosamine (GlcNAc) and β2 glycoprotein-I (β2GPI), the pathogenic molecules engaged in these autoimmune conditions.**

***Methods*: Sera from the APS patients were affinity-purified on β2GPI and β2GPI-related peptide columns. Sera from RF patients were affinity-purified on protein G column. The β2GPI and M protein-related peptides were prepared by conventional solid-phase peptide synthesis. The enzyme-linked immunosorbent assay direct binding and inhibition studies were performed on the RF and APS sera for the presence, and cross-reactivity, of antibodies against β2GPI, β2GPI-related peptides, streptococcal M protein, M-derived peptides and GlcNAc.**

***Results*: Antibodies (Abs) to β2GPI were found in 24.4% of 90 RF patients. Antibodies against various β2GPI-related peptides were found in 1.1–36.7% of the patients. The immunoglobulin G sera from RF patients possessed significant anti-β2GPI activity, while sera from APS patients contained a considerable anti-streptococcal M protein as well as anti-GlcNAc activity. Furthermore, affinity-purified anti-β2GPI and anti-β2GPI-related peptide Abs from APS patients cross-reacted with streptococcal M protein and M5 peptide, while β2GPI and β2GPI-related peptides inhibited anti-streptococcal M protein activity from RF patients. The results were confirmed by immunoblot analyses. The β2GPI also inhibited anti-GlcNAc activity from APS patients with chorea.**

***Conclusions*: The results of our study, showing a considerable overlap of humoral immunity in RF and APS, support a hypothesis that common pathogenic mechanisms underlie the development of cardiac valve lesions and Central Nervous System abnormalities in both diseases.**

KEY WORDS: Streptococcal infection, Anti-β2GPI Abs, Anti-M-protein Abs, Carditis, Chorea.

Introduction

Rheumatic fever (RF) and subsequent rheumatic heart disease (RHD) represent a relatively common connective tissue disease, caused by *Streptoccocus pyogenes* infection in 3–4% of susceptible and untreated children and adolescents [1]. Carditis affects 30–45% of RF patients and is the most serious manifestation of the disease, leading to valvular lesions and development of RHD. Other major features of RF include migratory polyarthritis, erythema marginatum, subcutaneous nodules and Sydenham's chorea [2]. By and large, autoimmune diseases have long been considered a shadow following infectious diseases. Among the major antigens recognized during a wide variety of bacterial, viral and parasitic diseases, many belong to conserved protein families, sharing extensive sequence identity or conformational fits with host molecules, namely molecular mimicry. Therefore, molecular mimicry, primarily between streptococcal M protein and self-structures, has been thought to be a leading mechanism for the development of acute rheumatic fever (ARF) after streptococcal pharyngitis [3–5]. Patients with RF have elevated levels of circulating autoantibodies directed against streptococcal antigens from bacterial cell wall, such as M protein, N-acetyl-β-D-glucosamine (GlcNAc) polysaccharide and other not well-defined streptococcal antigens. These antibodies cross-react with human proteins having coiled-coil structures, such as myosin, tropomyosin and valvular proteins, as well as the surface of human neuronal cells, hence, they may have a major role in the pathogenesis of RHD as well as Sydenham's chorea [5–12].

The classical 'Hughes Syndrome'—antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid antibodies (aPL) which bind target molecules mainly via β-2-glycoprotein-I (β2GPI), and/or lupus anticoagulants, associated with recurrent fetal loss, thromboembolic phenomena, thrombocytopaenia, heart (Libman–Sacks endocarditis) and neurological disorders [13–19]. The common denominator for all systemic features in APS is the association with the presence of aPL directed mainly to β2GPI molecule, a heavily glycosylated membrane-adhesion glycoprotein, present in blood plasma at a concentration of 150–300 μg/ml [20, 21]. β2GPI exhibits several properties *in vitro* that define it as an anticoagulant [22, 23], and it has a role in the clearance of apoptotic bodies from the circulation [24, 25]. The β2GPI molecule was found to be immunogenic and induce experimental APS model *in vivo* [26–28]. During the last years, the infectious origin of APS has proved to be one of the explanations for generation of anti-β2GPI antibodies (Abs) by sharing molecular mimicry with common bacteria or with

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cytomegalovirus-derived synthetic peptide [29, 30]. Previous studies linked the presence of aPL with significant valvular heart lesions in patients with APS [17, 31, 32]. The pathological spectrum of valvular lesions found in these patients is indistinguishable from that found in chronic RHD, and includes non-infective verrucous vegetations (Libman–Sacks endocarditis), thickening of valve cusps and, occasionally, significant valvular dysfunction—either regurgitation, stenosis or both without evidence of vegetations [33, 34]. Immunoglobulins (Ig), later shown as anti-phospholipid Abs, in association with complement were shown to be localized on defective valves derived from APS patients [35, 36]. The frequency of valvular lesions in APS appears to be quite high, with up to 63% of APS patients revealing at least one valvular abnormality on echocardiography [13]. The pathogenesis of valvular abnormalities in APS is not entirely clear, but it is well-accepted that aPL play a pathogenic role in the development of these lesions [17, 31, 32]. There are also many case reports and small series of patients with chorea associated with aPL and APS [37, 38]. In view of the similarities in clinical, pathological and echocardiographical presentation between RF and APS, we sought to evaluate possible immunological mechanisms shared by the two diseases.

Patients and methods

Patients and control subjects

Ninety patients with RHD, followed for a period of 2–5 yrs by a cardiologist from the Heart Institute at the University of São Paulo, Brazil, had a previous history of RF, defined according to modified Jones' criteria [2] and echocardiographically documented valvular heart disease, and 24 patients had previous episodes of chorea. Forty-two APS patients were evaluated, all of them fulfilled the 1997 revised Sapporo criteria for the APS [39]. The sera collection procedures were approved by the Heart Institute Ethics Committee (HC-FMUSP) and informed consent was obtained from patients.

Antibodies

ILA-1 mAb. An anti-β2GPI mAb originated from an APS patient [40]. This mAb was able to activate endothelial cells via enhancing tissue factor release, adhesion of monocyte and adhesion molecules expression (ICAM-I, VCAM-I, E-selectin), and induce experimental APS *in-vivo*. Peptide B was identified by this mAb from a phage display peptide library and could neutralize ILA-1 biological function [40].

Anti-N-acetyl-β-D-glucosamine mAb and preparation of N-acetyl-β-D-glucosamine–BSA antigen. Anti-streptococcal mAb 3B6 was specific for N-acetyl-β-D-glucosamine (GlcNAc) [41]. The antigen for 3.B6 mAb N-acetyl-glucoseamine was conjugated to bovine serum albumin (BSA) by a two-step reaction as previously described [9, 10]. P-aminiphenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma, St Louis, MO) was activated by an equimolar amount of glutaric dialdehyde in 0.1M sodium carbonate buffer, pH 9, for 30 min at 20°C, and then mixed with BSA (Sigma) in the same buffer. The mixture was incubated 1 h at 20°C with subsequent dialysis against 0.05M Tris-HCl buffer, pH 8.5. The conjugate was applied onto a DEAE-Sephacryl column (Pharmacia Biotech, Norden AB Sollentuna, Sweden), equilibrated with 0.05M Tris-HCl buffer, pH 8.5, and then was eluted by step gradient of NaCl in the same buffer.

Affinity-purifications

Affinity-purification of the polyclonal anti-β2GPI Abs. β2GPI was affinity-purified from fresh plasma on a commercial heparin column (Pharmacia). The purity was confirmed by SDS-PAGE gel and immunoblot. β2GPI was conjugated to Cyanogen Bromide (CNBr)-activated sepharose and a β2GPI column was constructed. The human anti-β2GPI Abs were affinity-purified from five APS patients' sera on the β2GPI column. Sera from the APS patients were loaded onto the β2GPI column. Following extensive washing, the bound Abs were eluted with glycine-HCl 0.2 M pH 2.5, neutralized with 2M Tris and dialysed against Phosphate Buffer Saline (PBS).

Affinity-purification of the polyclonal anti-β2GPI/peptide D Abs. Peptide D ^{275}DKVSFFCKNKEKKC^{289} was coupled to CNBr-activated sepharose and used to construct the peptide D column. Sera from five APS patients positive to peptide D were loaded onto the column. Glycine-HCl 0.2 M pH 2.5 was used to elute the bound immunoglobulin, neutralized and dialysed.

Origin of IgG from RF patients. Sera from five RF patients were affinity-purified on protein G column (Pharmacia). Following extensive washing, the bound Abs were eluted with glycine-HCl 0.2 M pH 2.5, neutralized with 2 M Tris and dialysed against PBS. Immunoglobulin G (IgG) affinity-purified from healthy donor on protein G column (Pharmacia), was used as negative control.

Western blot analyses

Proteins (β2GPI or M protein) were separated in a 10% SDS-PAGE by carefully placing 5 μg of protein in each lane. Nitrocellulose membrane (Invitrogen Life Technologies) was used to transfer the proteins, and the membrane was blocked with 5% skimmed milk [in TBS 1% (pH 7.4) and 0.01% Tween-20]. The appropriate immunoglobulins (anti-β2GPI, IgG from RF patients, IgG control) were added to the relevant strips and incubated for 2 h at room temperature. Following extensive washings, the blots were incubated 1 h at room temperature with anti-peroxidase, and the reaction was detected using appropriate substrate.

The synthetic peptides used in this study. The following β2GPI-related peptides were used in the study: peptide A: ^{58}LKTPRV^{63} (P58–63) [40], peptide B: ^{208}KDKATF^{213} (P208–213) [40], peptide C: ^{133}TLRVYK^{138} (P133–138) [40], peptide D: ^{275}DKVSFFCKNKEKKC^{289} (P275–289) [42, 43]. Scrambled forms of the studied β2GPI-related synthetic peptides were used as negative controls: scrambled peptide A (scA): RLTVKP, sc-peptide B: FKTKDA, sc-peptide C: VTRYLK, sc-peptide D: KFKDEFKSKCNK, sc-peptide E: PKSVCQKRV GRTQLASQVIV.

The following M protein peptides were used from the *N*-terminal portion: ^{11}QRAKEALDKYELENH^{25} (P11–25), ^{62}LER KTAELTSEKKEHEAENDK^{82} (P62–82), ^{81}DKLKQQRDTLST QKET^{96} (P81–96) [44], ^{111}TQELANKQQESKENEKALN^{130} (P111–130), ^{131}ELLEKTVKDKIAKEQENKET^{150} (P131–150), ^{183}LDETVKDKLAKEQKSKQNI^{201} (P183–201) [45], ^{163}ETIGT LKKILDETVK^{177} (P163–177), ^{191}LAKEQKSKQNIGALKQE LAK ^{210} (P191–210) [44, 45]. Scrambled form of M5 peptide, KLKADQSEKIQDENVKTKL, was used as a control for the inhibition assays.

M streptococcal peptides were synthesized by the 'tea bag' method using t-BOC chemistry (Laboratory of Immunology, Heart Institute, São-Paulo, Brazil) and were checked by mass spectrometry and purified by high pressure liquid chromatography (HPLC). The β2GPI peptides were prepared by conventional solid-phase peptide synthesis, using an ABIMED AMS-422 automated solid-phase multiple peptide synthesizer (Langfeld, Germany). For purity determination, analytical reversed-phase HPLC was performed using a prepacked Lichrosphere-100 RP-18 column (Merck, Darmstadt, Germany).

Peptide biotinylation. Resin-bound peptides of 11 mg (Wang-Resin, Calbiochem-Novabiochem AG, Lufelfingen, Switzerland) were suspended in *N*-methyl-2-pyroidone (NMP). Fifteen mmol of biotin-*N*-hydroxysuccinimide (Sigma Chemical Co., St Louis MO, USA) and 15 nmol of di-isopropylethylamine were added to the peptide mixture. After 16 h, the biotinylated peptides were deprotected and cleaved from the resin by a cleavage mixture containing 5% triethylsilan (Fluka Chemicals, Buchs, Switzerland), 5% water and 90% trifluoroacetic acid. The cleaved peptides were precipitated with ice-cold peroxide-free ether and the pellet was dissolved in water and subsequently lyophilized. Biotinylated peptides were purified by HPLC using 0.1% trifluoroacetic acid in 20% H_2O in acetonitrile.

Direct binding of sera or IgG from RF patients, or anti-β2GPI to β2GPI

Ninety-six wells-enzyme-linked immunosorbent asssay (ELISA) plates (Maxisorp, Nunc, Kamstrup, Roskilde, Denmark) were coated with β2GPI 1 μg/ml in PBS. Coated plates were blocked with 3% BSA, after which human sera from RF patients and from healthy controls, at different dilutions 1:200–1:6500 or affinity-purified IgG at concentration of 0–50 μg/ml were added for 2 h at room temperature. The binding was probed by goat anti-human IgG conjugated to alkaline phosphatase (Jackson, Research Laboratory Inc., West Grove, Pennsylvania, USA) and appropriate substrate. The colour reaction was read in Titertrek ELISA reader (SLT- Labistruments, Austria) at optical density (OD) of 405 nm. Positive binding was defined as OD higher than mean + 2 S.D.

Direct binding of sera or anti-β2GPI to M protein

Ninety-six-well ELISA plates (Maxisorp, Nunc, Kamstrup, Roskilde, Denmark) were coated with a recombinant M protein (Guilherme L) 5 μg/ml in PBS. Coated plates were blocked with 3% BSA, after which human sera from APS patients at different dilutions 1:200–1:6500 or affinity-purified anti-β2GPI at concentration of 0–50 μg/ml were added for 2 h at room temperature. The binding was probed as described above.

Direct binding of Ig to the studied peptides

The binding of the anti-β2GPI Abs or sera from RF patients or IgG affinity-purified from RF patients was determined by ELISA. Ninety-six-well ELISA plates were coated with streptavidin 5 μg/ml in $NaHCO_3$ 0.05M pH 9.5 overnight at 4°C. The plates were blocked with 3% BSA for 1 h at 37°C and exposed to biotinylated peptides for 2 h at room temperature followed by a second blocking procedure with 3% BSA. The tested immunoglobulins were added at different concentrations for 2 h incubation at room temperature. The immunoglobulin binding to the peptides was probed with anti-human-IgG conjugated to alkaline phosphatase followed by the addition of appropriate substrate.

Inhibition of binding of sera or affinity-purified IgG from APS and RF patients to the different peptides

The cross-reactivity of binding of anti-β2GPI Abs from APS or total IgG affinity-purified from RF patients to β2GPI or M protein was confirmed by: (i) direct binding of the APS sera or affinity-purified anti-β2GPI Abs to M protein and its synthetic peptides; (ii) direct binding of the RF sera or affinity-purified IgG to β2GPI and its synthetic peptides and (iii) inhibition assays. β2GPI molecule, β2GPI-related synthetic peptides, group A streptococcal M protein and M protein synthetic derivatives (listed earlier) were used as inhibitors. Affinity-purified anti-β2GPI Abs from five APS patients or total RF-IgG from five patients, at 50% binding to β2GPI or M-protein, respectively, were pre-incubated (overnight at 4°C) with different concentrations of β2GPI related synthetic peptides, M protein and its related synthetic peptides. The reaction mixture was then transferred to β2GPI or M protein-coated ELISA plates. The binding was probed with anti-human-Fc conjugated to alkaline phosphatase (Jackson) and appropriate substrate. The percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \frac{[(\text{OD affinity} - \text{purified IgG}) - (\text{OD affinity} - \text{purified IgG with inhibitor})]}{\text{OD (affinity} - \text{purified IgG)} \times 100}.$$

Results

Anti-β2GPI targeting of RF sera

Ninety patients with RHD were studied, all of them fulfilled the modified Jones' criteria [2]. Patients' characteristics are presented in Table 1. Twenty-two RHD patients tested positive for anti-β2GPI Abs (24.4%, $P < 0.001$ *vs* controls) (Table 2). Antibodies against peptides B and D were also significantly elevated compared with controls ($P < 0.001$) (Table 2). IgG was affinity-purified from five representative sera from RF patients. The anti-β2GPI binding of the RF sera at dilution of 1:400 was significantly elevated and ranged between 0.450 ± 08 and 0.832 ± 0.101 OD at 405 nm, in comparison with OD of 1.714 ± 0.124 OD at 405 nm APS patients' sera ($P < 0.004$), or 0.118 ± 0.047 OD at 405 nm of sera from an healthy individual ($P < 0.001$), at the same concentration (Fig. 1A). The binding of sera derived from patients with RF to β2GPI behaved in a dose-dependent manner (Fig. 1A). The data were

TABLE 1. Patients' characteristics

RF patients' characteristics	
Age (yrs, mean ± S.D.)	14.4 ± 3.8
Number of RF acute attacks (mean ± S.D.)	1.4 ± 0.6
Males:females	33:57
Documented acute carditis (%)	69.5
Polyarthritis (%)	33.3
Chorea (%)	29.3
APS patients' characteristics	
Age (yrs, mean ± S.D.)	40.0 ± 13.7
Males:females	4:38
Recurrent abortions (%)	33.3
Thrombotic episodes (%)	85.7
Stroke (%)	45.2
Epilepsy (%)	9.5
Chorea (%)	4.8
Cardiac valvular disease (%)	33.3
Livedo reticularis (%)	11.9
Thrombocytopaenia (%)	21.4

TABLE 2. Prevalence of anti-β2GPI and its derivatives in sera of RF patients

Antibodies against	RF patients ($n = 90$)	Controls ($n = 100$)	*P*-value
β2GPI	22 (24.4%)	4 (4.0%)	<0.001
Peptide A ^{58}LKTPRV^{63}	5 (5.6%)	2 (2.0%)	NS
Peptide B ^{208}KDKATF^{213}	33 (36.7%)	3 (3.0%)	<0.001
Peptide C ^{133}TLRVYK^{138}	1 (1.1%)	4 (4.0%)	NS
Peptide D ^{275}DKVSFFCKNKEKKC^{289}	16 (17.8%)	3 (3.0%)	<0.001

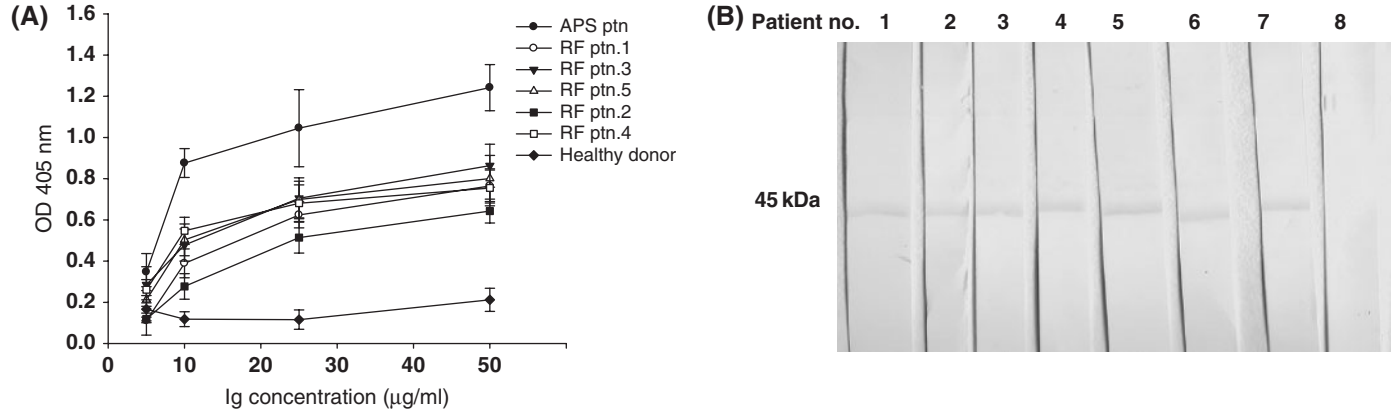

FIG. 1. (**A**) Anti-β2GPI binding of affinity-purified IgG from five RF patients. Affinity-purified IgG from five RF patients was introduced to β2GPI-coated plates at different concentrations. The data are presented as mean ± S.D. in OD at 405 nm, of three repetitive experiments. (**B**) Anti-β2GPI binding of affinity-purified IgG from five RF patients by western blot. β2GPI, run on 12% SDS-PAGE, transferred to nitrocellulose, was probed with affinity-purified IgG from five RF patients (lines 1–5), anti-β2GPI IgG affinity-purified from APS patients (lines 6, 7) and control IgG (line 8), were introduced to β2GPI on the nitrocellulose.

TABLE 3. Prevalence of anti-M protein and its derivatives in sera of APS patients

Ab to	APS patients ($n=42$)	Healthy individuals ($n=100$)	P-value
M protein	7 (16.6%)	2 (2.0%)	$P<0.001$
^{11}QRAKEALDKYELENH^{25}	1 (2.3%)	1 (1.0%)	NS
^{62}LERKTAELTSEKKEHEAENDK^{82}	4 (9.5%)	–	$P<0.02$
^{81}DKLKQQRDTLSTQKET^{96}	2 (4.8%)	1 (1.0%)	NS
^{111}TQELANKQQESKENEKALN^{130}	3 (7.1%)	–	NS
^{131}ELLEKTVKDKIAKEQENKET^{150}	4 (9.5%)	–	$P<0.02$
^{163}ETIGTLKKILDETVK^{177}	4 (9.5%)	1 (1.0%)	$P<0.02$
^{183}LDETVKDKLAKEQKSKQNI^{201}	6 (14.2%)	1 (1.0%)	$P<0.001$
^{191}LAKEQKSKQNIGALKQELAK^{210}	2 (4.8%)	1 (1.0%)	NS

confirmed by immunoblot (Fig. 1B). As seen in line 1–5, IgG which was affinity-purified from five representative sera from RF patients bind to β2GPI in the same manner as anti-β2GPI affinity-purified from two APS patients' sera. Irrelevant IgG did not bind β2GPI.

Anti-M protein targeting by sera originated from APS patients

Forty-two APS patients were evaluated, 38 females and four males, mean age 40.0 ± 13.7 yrs, mean follow-up period was 7.5 ± 4.3 yrs. Patient's characteristics are presented in Table 1. As demonstrated in Table 3, 16.6% of the APS patients' sera significantly recognized M protein as well as the peptides located at positions 62–82, 131–150 and 163–177. The most prominent recognition was noticed for the M peptide ^{183}LDETVKDKL AKEQKSKQNI^{201} (14.2%, $P<0.001$). Significant M protein binding by affinity-purified anti-β2GPI Abs from five APS patients' sera ($P<0.02$), in a dose-dependent pattern, is demonstrated in Fig. 2a. The data were confirmed by immunoblot (Fig. 2b). Affinity-purified anti-β2GPI from five representative sera from APS patients bound to M protein at the same manner as IgG affinity-purified from sera originated from two patients with RF. Irrelevant IgG did not bind M protein.

Anti-β2GPI Abs affinity-purified from APS patients cross-react with M protein and M5 peptide (P183–201)

The cross-reactivity of anti-β2GPI with M protein was documented by inhibition assays. Figure 3 presents an inhibition of 44 ± 3% in the binding of affinity-purified anti-β2GPI to β2GPI by M protein, and 29 ± 3% by M5 peptide (P183–201) at a concentration of 25 μg/ml. This inhibition of binding by M protein was significant when compared with the binding of anti-β2GPI (85 ± 6%) ($P<0.02$) and in the presence of scrambled peptides (6 ± 2%) ($P<0.001$). Non-significant difference in the percentage of inhibition was noticed when β2GPI-related peptide B or M5 peptide (P183–201) were used as inhibitors ($P>0.05$). These data were strengthen by the strong inhibitory potential of M5 peptide (P183–201) to inhibit the binding of affinity-purified anti-β2GPI/peptide D to β2GPI-related peptide D, $P<0.001$ as compared with scrambled form of the peptides (Fig. 4). The difference in β2GPI/peptide D (84 ± 6%) and M5 peptide (69 ± 5%) as inhibitors was non-significant ($P>0.05$). Furthermore, M5 peptide (P183–201) could significantly abrogate the binding of anti-β2GPI/peptide B mAb to β2GPI/peptide B (e.g. 58 ± 6% inhibition in comparison with 7 ± 2% using scrambled peptide M5, $P<0.002$, as shown in Fig. 5.

β2GPI and β2GPI-related peptides B and D inhibited the binding of RF-IgG to M protein

M protein inhibited the binding of RF-IgG to M protein by 84 ± 6% at a concentration of 25 μg/ml, while the M5 peptide ^{183}LDETVKDKLAKEQKSKQNI^{201} inhibited by 62 ± 6% (Fig. 6). β2GPI and β2GPI-related peptide B ^{208}KDKATF^{213} or peptide D ^{275}DKVSFFCKNKEKKC^{289} decreased the binding of RF-IgG to M protein by 31 ± 4, 36 ± 3 and 42 ± 2%, respectively ($P>0.05$ each *vs* the other, and $P<0.001$ in comparison with scrambled peptide < 10%, concentration of 25 μg/ml) (Fig. 6).

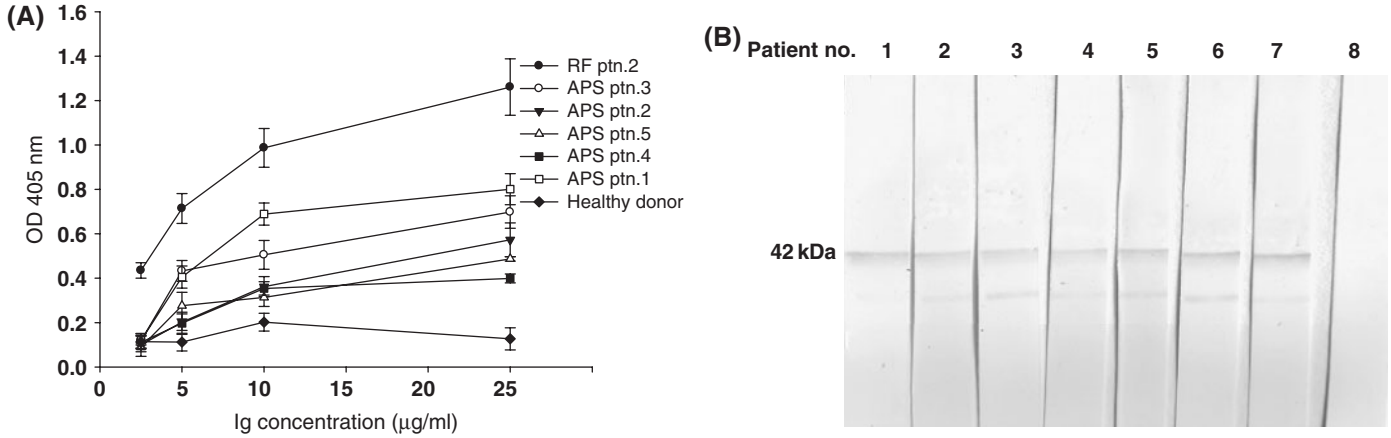

FIG. 2. (**A**) M protein binding by affinity-purified anti-β2GPI Abs from five APS patients. The anti-β2GPI IgG were introduced to M protein-coated ELISA plates at different concentrations. The data are presented as mean ± S.D. in OD at 405 nm of three repetitive experiments. (**B**) M protein binding by affinity-purified anti-β2GPI Abs from five APS patients by immunoblot: M protein run on SDS-PAGE, transferred to nitrocellulose and probed with anti-β2GPI IgG affinity purified from five APS patients (lines 1–5), and two total IgG affinity-purified from RF patients (lines 6, 7). Control IgG as negative control is presented on line 8.

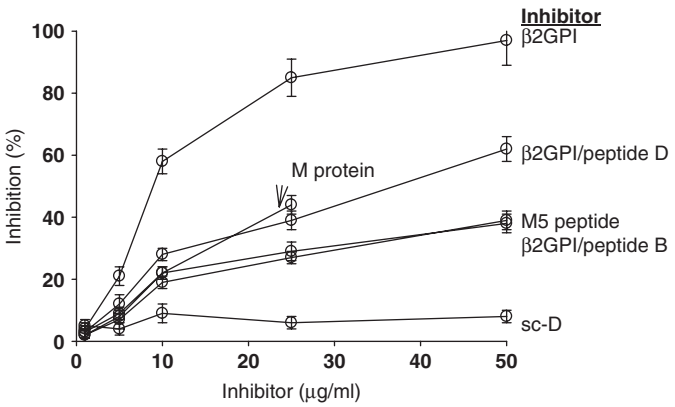

FIG. 3. Inhibition of binding of anti-β2GPI to β2GPI by β2GPI M protein and its synthetic derivatives. Anti-β2GPI mAb at concentration of 10 μg/ml was incubated with β2GPI, β2GPI-related peptide B, M protein, M5 peptide (P183–201) or scrambled form of peptide D (which separately had no inhibitory effect) at 0–50 μg/ml. The β2GPI binding by anti-β2GPI, following incubation with the inhibitors was calculated as percentage of inhibition. Data are presented as mean ± S.D. of three repetitive experiments.

Anti-β2GPI affinity-purified from two APS patients with chorea cross-react with GlcNAc

RF patients have circulating anti-GlcNAc Abs with reactivity towards cardiac myosin and neuronal cells [10, 11]. Figure 7 shows that anti-β2GPI affinity-purified from sera of two APS patients with chorea also recognize GlcNAc in a dose-dependent manner (e.g. 0.410 ± 0.06 OD at 405 nm, 0.341 ± 0.07 OD at 405 nm at concentration of 1 μg/ml, respectively). The anti-GlcNAc activity of the anti-β2GPI was significant in comparison with 0.05 ± 0.05 OD at 405 nm of anti-β2GPI purified from an APS patient with no chorea and no anti-GlcNAc activity, $P < 0.001$.

To further evaluate the cross-reactivity between the anti-β2GPI and anti-GlcNAc mAb from a RF patient, inhibition experiments were conducted. As described in Fig. 8, IgG affinity-purified from two APS chorea-positive patients bound GlcNAc by 5.1 times less or 4.1 times less when inhibited by GlcNAc, in comparison with inhibition by six times of the binding of GlcNAc by anti-GlcNAc mAb, $P < 0.002$ in comparison with the binding of control IgG from APS patient without chorea. Control antigen BSA was not inhibitory to the Abs. β2GPI (at high concentration of 0.5 mg/ml), abrogated the binding of anti-GlcNAc mAb 3.B6, and of APS/chorea-positive IgG, to GlcNAc by 41.5, 42.4 and 47.4%, respectively. This inhibition was significant ($P < 0.02$), in comparison with 90.6, 75.4 and 80% inhibition of 3.B6 by GlcNAc in the presence of GlcNAc, or $P < 0.001$ in comparison with IgG from chorea-negative APS.

Discussion

In the present study, we demonstrate a considerable overlap of antibody specificities in RF and APS. About 24% of the RHD patients had anti-β2GPI Abs. Antibodies against β2GPI-related peptides were also common. We showed that not only IgG sera from RF patients possess significant anti-β2GPI activity, but also sera from APS patients contain a considerable anti-streptococcal M protein, as well as anti-GlcNAc activity. Furthermore, affinity-purified anti-β2GPI and anti-β2GPI-related peptide Abs from APS patients cross-reacted with streptococcal M protein and M5 peptide, while β2GPI and β2GPI-related peptides B and D inhibited anti-M protein activity of RF patients. β2GPI also inhibited anti-GlcNAc activity from APS patients with chorea.

The role of streptococcal infection in the aetiopathogenesis of RF is well-established [46–50]. Although there is little evidence for direct involvement of group A streptococci in the affected tissues of ARF patients, there is a large body of epidemiological and immunological data indirectly implicating group A streptococcus in the initiation of the disease process. It is well-known that outbreaks of RF closely follow epidemics of either streptococcal sore throats or scarlet fever, adequate antibiotic treatment of a documented streptococcal pharyngitis markedly reduces the incidence of subsequent RF, and appropriate antimicrobial prophylaxis prevents the recurrences of disease in known ARF patients [51]. In contrast to RF, the relationship between infectious agents and development of APS has only recently been recognized. The aPL have been documented in a large number of infectious diseases, including viral, bacterial, spirochetal and parasitic infections [52]. Although the incidence and clinical significance of β2GPI-dependent aPL in infectious diseases remains largely unknown, it is possible that infections might trigger the development of pathogenic anti-β2GPI Abs, conceivably via molecular

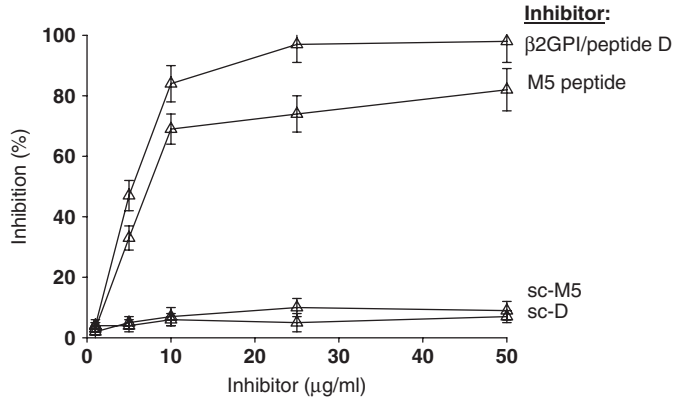

FIG. 4. M5 peptide (P183–201) inhibits the binding of anti-β2GPI/peptide D to β2GPI/peptide D. Affinity-purified anti-β2GPI Abs on β2GPI peptide D column was incubated with peptide D, scrambled peptide D (sc-D), M5 peptide, or sc-M5 peptide and later tested for peptide D binding by ELISA. The data are presented as mean ± S.D. in OD at 405 nm of three repetitive experiments.

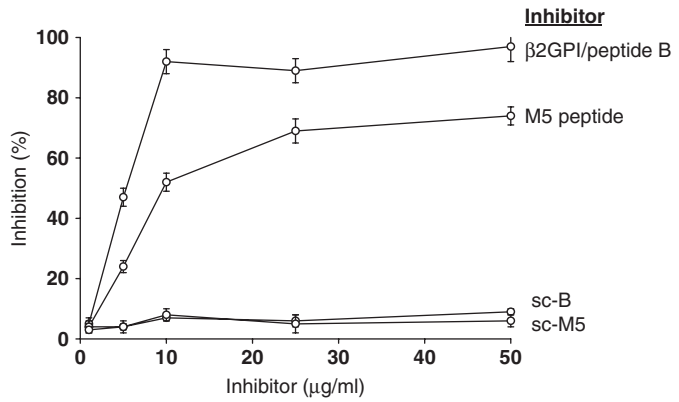

FIG. 5. M5 peptide (P183–201) inhibits the binding of anti-β2GPI-mAb to β2GPI related peptide. ILA-1 anti-β2GPI mAb binding to β2GPI-peptide B was incubated with M5 peptide (P183–201) and later tested for β2GPI-peptide-B binding by ELISA. Scrambled form of peptide-B (sc-B) or peptide-M5 (sc-M5) were used as negative controls. The data are presented as mean ± S.D. in OD at 405 nm, of three repetitive experiments.

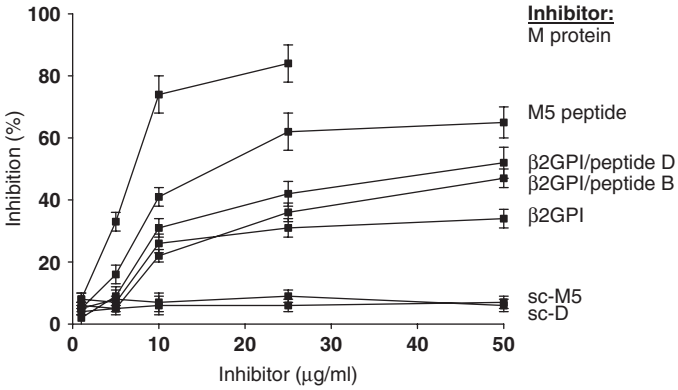

FIG. 6. M protein inhibited the binding of RF-IgG to M protein by M protein, β2GPI and its synthetic derivatives. RF/IgG, at 50% binding to M protein was exposed to different concentrations of M protein, M5 peptide, β2GPI, β2GPI-related peptide B or D, and scrambled form of the peptides M5, B and D were used as controls. Data are presented as mean ± S.D. of three repetitive experiments.

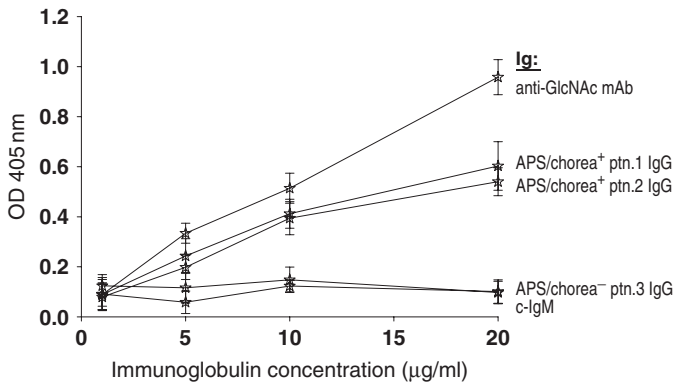

FIG. 7. Anti-β2GPI binding to GlcNAc. Anti-β2GPI affinity-purified from sera derived from two APS patients with chorea, were incubated on ELISA plates coated with GlcNAc. The data are presented as mean ± S.D. in OD at 405 nm, of three repetitive experiments.

mimicry, thus promoting the development of APS, particularly in predisposed individuals. Indeed, we recently demonstrated a high homology between β2GPI-related hexapeptide, also employed in the current study and peptidic domains of *Streptococcus pyogenes* [30, 52]. Furthermore, studies on experimental APS models proved that molecular mimicry between β2GPI-related synthetic peptides and structures within bacteria, viruses and tetanus toxoid are a cause for experimental APS [29, 30]. Recently, we also demonstrated a possible link to the infectious origin of Libman–Sacks endocarditis [53]. The similarity between anti-β2GPI and anti-M5, as well as anti-GlcNAc activity, as found in our study, may point that certain strains of group A streptococcal infection might have a causative role not only in RF but also for APS development. Indeed, several previous reports showed the presence of aPL in streptococcal infection. Ardiles *et al*. [54] reported a prevalence of 48% of aCL in patients with acute post-streptococcal glomerulonephritis and 33% in streptococcal impetigo patients without renal involvement. A serological follow-up was performed with a second sample taken about 7 months later for the patients initially positive on IgG testing showing persistence in nine out of 12 patients. In another study, raised titres of aCL were detected in eight of 13 patients with post-streptococcal reactive arthritis [55] which were, however, independent of β2GPI and were not accompanied by thrombotic episodes. Controversies exist regarding the prevalence of aPL in RF patients. Previous studies of anti-group A streptococcal cross-reactive mAbs demonstrated reactivity with cardiolipin [56]. Figueroa *et al*. [57] reported a high rate of aCL in a group of 55 RF patients. Eighty percent of the patients were positive for aCL during acute RF attack *vs* 40% when the disease was inactive. Furthermore, a significant association was found between IgM-aCL and rheumatic valvular disease [57]. On the other hand, Ilarraza *et al*. [58] did not find anticardiopipin (aCL) in the sera of 31 RHD patients as well as in six patients with acute RF. Similarly, Narin *et al*. [59] found no significant difference in aCL levels between patients with RF or streptococcal pharyngitis and healthy controls. Diniz *et al*. [60] failed to identify aCL in 56 children with ARF and chorea, similar to the results reported by Asherson *et al*. [61]. Differences in the detection of anti-CL and anti-PL may be due to assay conditions and the detection sensitivity of the assays used. Until the assays for aCL and other aPL are standardized, comparison of different outcomes in different laboratories is difficult.

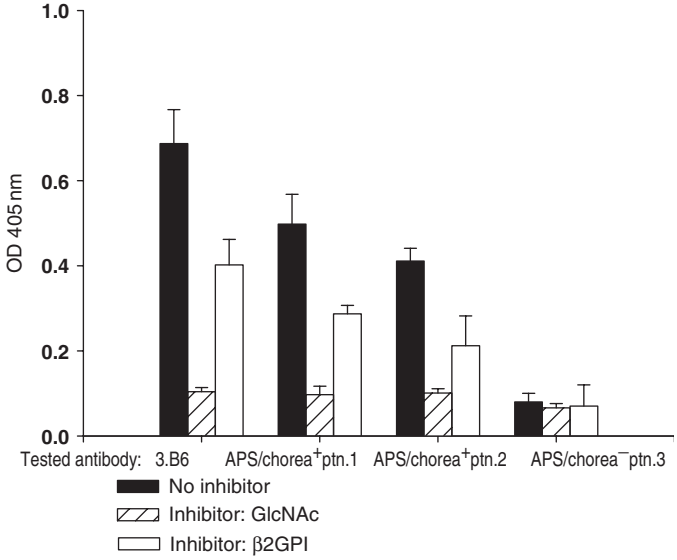

FIG. 8. GlcNAc and β2GPI inhibit the binding of IgG affinity-purified from two APS chorea-positive patients. 3B6 mAb, total IgG affinity-purified from three APS patients (#1, #2 chorea-positive, #3 chorea-negative), were incubated with GlcNAc or with β2GPI. The binding of the immunoglobulin, post-incubation with the inhibitors, was tested on GlcNAc-coated ELISA plates. The data are presented as mean ± S.D. in OD at 405 nm, of three repetitive experiments.

The pathogenic mechanisms involved in cardiac as well as other target organ diseases in RF has been widely investigated. Molecular mimicry was demonstrated regarding both humoral and cellular immune responses. Anti-streptococcal antibodies cross-react with several human tissues, including the heart, skin, brain, glomerular basement membrane, and striated and smooth muscles [50, 62]. Cross-reactive antibodies in the heart tissue may then bind to valvular endothelium leading to inflammation, cellular infiltration and valve deformities [10]. Once activated, the valvular endothelium expresses increased amounts of adhesion molecule VCAM-1, which facilitates the binding/adhesion of T-cells and consequently extravasation into the valves, leading to the cycle of scarring, neovascularization and infiltration of lymphocytes [63]. The mechanisms by which anti-β2GPI exert tissue damage in APS have also been widely investigated. Anti-β2GPI Abs were found to activate monocytes leading to tissue factor release and activate endothelial cells via induction of adhesion molecule expression including E-selectin, ICAM-I and VCAM-I [64–66]. The anti-β2GPI Abs were found to react with their antigen in association with a member of the TLR/IL-1 toll like receptor membrane on endothelial cells and directly induce activation [35]. Recently, it was suggested that endothelial cell activation induced by anti-β2GPI is initiated by cross-linking or clustering of annexin-A2 on the endothelial surface [67]. In contrast to RF, however, the pathogenesis of valvular abnormalities in APS is yet largely unknown. It has been postulated that aPL directly cause valvular or endothelial injury unrelated to severity of the disease. Ziporen *et al.* [32] have shown positive staining for human immunoglobulins and for complement compounds in the sub-endothelial layer along the surface of the leaflets and cusps. Amital *et al.* [31] reported similar findings with deposition of aCL in the sub-endothelial layer of the valve. These findings clearly indicate that the deposition of aPL on the valves resembles the deposition of immune complexes in the dermo-epidermal junction or in the kidney basement membrane in patients with SLE. As postulated by Hojnik *et al.* [17], the above data suggest that aPL play a pathogenic role in the development of valvular lesions rather than being elicited by the antigens expressed in the damaged valve tissue. The results of the present study, showing a considerable overlap between anti-β2GPI and anti-M protein or anti-GlcNAc Abs, support a hypothesis that common pathogenic mechanisms underlie the development of cardiac valve lesions and CNS abnormalities in both RF and APS.

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