Analysis of T Cell Responses to the β2-Glycoprotein I-Derived Peptide Library in Patients with Anti-β2-Glycoprotein I Antibody-Associated Autoimmunity

Hiroshi Ito, Sho Matsushita, Yoshiaki Tokano, Hiromichi Nishimura, Yoshihiko Tanaka, Shoji Fujisao, Hiroaki Mitsuya, Hiroshi Hashimoto, and Yasuharu Nishimura

ABSTRACT: Antiphospholipid syndrome (APS) is an autoimmune disease that accompanies anti-phospholipid antibodies measured as either anti-cardiolipin antibodies (aCL) or lupus anticoagulant. β2-glycoprotein I (β2GPI) is the most common and apparently the best-characterized antigenic target for aCL. To investigate T-cell responses to β2GPI, we stimulated PBMC of 18 APS or systemic lupus erythematosus (SLE) patients carrying anti-β2GPI and 10 healthy controls, using a peptide library covering the β2GPI sequence. We established seven CD4+ T cell lines reactive with β2GPI peptide. Three of four epitopes for patient-derived T cell lines were p244–264, whereas one T cell line from a control subject also recognized p244–264. Furthermore, there was no tendency for particular HLA class II molecules to present β2GPI peptides. However, cytokine producing patterns were significantly different between T cell lines from patients and those from healthy individuals (p = 0.028); patients’ T cells tend to exhibit higher IL-4 and lower IFN-γ responses. These T cell lines did not react to β2GPI purified from human plasma. These results indicate that β2GPI-reactive CD4+ T cells of APS/SLE patients mainly recognize cryptic p244–264 in the context of various HLA class II molecules, and exhibit Th0-Th2-type responses. Our findings may provide a clue to the pathogenesis of APS. Human Immunology 61, 366–377 (2000). © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: autoimmune disease; autoreactive T cells; β2-glycoprotein I; antiphospholipid syndrome; HLA

ABBREVIATIONS

aCL anti-cardiolipin antibodies
APC antigen presenting cell
APS antiphospholipid syndrome
β2GPI β2-glycoprotein I
HLA human leukocyte antigen

INTRODUCTION

Antiphospholipid syndrome (APS) exhibits a variety of clinical features, including arterial/venous thrombosis, recurrent spontaneous abortion/intrauterine fetal death and thrombocytopenia with the presence of anti-phospholipid antibodies. Anti-cardiolipin antibodies (aCL), one of anti-phospholipid antibodies, have been frequently detected in sera from patients with APS, systemic lupus erythematosus (SLE) and related collagen diseases. Recent studies have shown that aCL do not directly recognize the cardiolipin structure but do recognize β2-glycoprotein I (β2GPI) interacting with a
variety of negatively charged molecules such as anionic phospholipids and oxidized polystyrene surface (plates) [1, 2]. β₂GPI is the most common and best-characterized antigenic target for aCL. It has been proposed that anti-β₂GPI antibody recognizes a cryptic epitope expressed by a conformational change occurring when β₂GPI interacts with negatively charged molecules [2]. Alternatively, it has been suggested that binding of β₂GPI to anionic structures increases the density of β₂GPI, thus promoting the binding of autoantibodies to β₂GPI, because anti-β₂GPI antibodies bind to β₂GPI with low affinity in a fluid phase, and require increased density of β₂GPI for efficient binding in vitro [3, 4].

β₂GPI is a plasma protein consisting of 326 amino acids with a molecular mass of 50 kDa and is heavily glycosylated [5–7]. β₂GPI is present in plasma at 200 μg/ml or less [8], and possesses several in vitro properties, which qualifies it for an anticoagulant. It has been shown that β₂GPI inhibits the contact-phase activation system of blood coagulation [9], prothrombinase activity [10], and ADP-induced platelet aggregation [11]. It was suggested that anti-β₂GPI antibodies can bind to protein C via β₂GPI and interfere with the anticoagulant activity of the protein C [12]. Anti-β₂GPI antibody of autoimmune patients can be distinguished from aCL found in patients with infectious diseases which reacts with cardiolipin in the absence of β₂GPI [13]. However, it was reported that aCL in patients with acute human parvovirus B19 infection exceptionally shows specificity to β₂GPI, thus sharing characteristic of aCL found in SLE patients [14]. It was also reported that the presence of anti-β₂GPI IgG [15] and anti-β₂GPI IgA [16] is associated with thrombosis in patients with SLE, and the presence of anti-β₂GPI IgM correlates well with a history of pregnancy loss [17].

Various HLA class II alleles are reportedly associated with patients carrying anti-β₂GPI antibodies [18, 19]. One of the HLA-DR4 haplotypes (HLA-DR4-DQB1*0302) was seen to be associated with anti-β₂GPI antibodies in Mexican American patients and, to a lesser extent, in white American patients. The frequency of HLA-DR53 (DRB4*0101) was significantly increased in Mexican American patients with anti-β₂GPI antibodies. The frequency of an HLA-DR13 haplotype (DRB1*1302-DQB1*0604/5) was also significantly increased in patients with anti-β₂GPI antibodies in black Americans. In Japanese populations, SLE patients with anti-β₂GPI IgG were associated with DRB1*0901, as compared to those without anti-β₂GPI IgG, although the corrected P value was not significant. Association of HLA-DR5 (possibly DRB1*1201) with the aCL-positive primary APS in Mexican patients was also reported [20]. Shoenfeld’s group reported that treatment of BALB/c mice with anti-CD4 antibodies suppressed the development of experimental APS [21]. They also demonstrated that whole bone marrow cells were able to transfer experimental APS in BALB/c mice, whereas T cell-depleted bone marrow cells could not do so [22]. These studies indicate an essential role for CD4+ T cells in the development of APS, and HLA class II-restricted CD4+ T cells responding to β₂GPI in peripheral blood mononuclear cells (PBMC) of patients with APS were reported [23].

We previously stimulated PBMC of SLE patients with anti-β₂GPI IgG using β₂GPI purified from human plasma, but β₂GPI-reactive T cell lines could not be established. In the present study, we stimulated PBMC of subjects affected with APS or SLE that carry anti-β₂GPI IgG, using a peptide library that covers almost the entire length of the β₂GPI sequence. We established CD4+ T cell lines reactive with β₂GPI peptides and analyzed T cell epitopes, restriction HLA molecules and cytokines produced by these T cells.

MATERIALS AND METHODS

Subjects
Peripherally venous blood samples were obtained, with informed consent, from 18 Japanese patients of APS or SLE (1 primary APS, 4 APS secondary to SLE, 10 SLE, 3 lupus-like disease, 3 men, 15 women, mean age 43.0 years, range 19–66). All patients were positive for anti-β₂GPI IgG (anti-β₂GPI-dependent cardiolipin IgG). The antibodies were measured using anti-CL:β₂GPI ELISA kits (Yamasa Shoyu Co., Choshi, Japan) at SRL (Tokyo, Japan) or BML, Inc. (Kawagoe, Japan). Two of the five APS patients had arterial thrombosis; three complained of recurrent spontaneous abortions. SLE was diagnosed on the basis of the 1982 revised criteria for the classification of SLE published by the American College of Rheumatology (ACR). Lupus-like disease patients fulfilled 2 or 3 findings included in the ACR criteria for SLE, with positive finding of antinuclear antibody. Most of patients were treated with corticosteroids and some were treated with immune suppressive agents such as azathioprine. Peripheral venous blood samples obtained from 10 unrelated healthy controls, without anti-β₂GPI IgG, (4 men, 6 women, mean age 25.8 years, range 22–32) were also studied.

Peptides and Purified β₂GPI
The 15–21 mer peptides with a 10- to 15-residue overlap were designed from the human β₂GPI sequence reported by Steinkasserer et al. [6], synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Co., Kyoto, Japan), based on the 9-fluorenylmethylxycarbonyl strategy and purified using C18 re-
verse-phase high-performance liquid chromatography (Waters, Milford, MA, USA). Peptides used for primary stimulation of PBMC are listed in Table 1. Purified $\beta_2$GPI was kindly provided by Yamasa Shoyu Co. (Choshi, Japan). Nature of $\beta_2$GPI was checked by SDS-polyacrylamide gel electrophoresis before use in T cell proliferation assays.

**Generation of T Cell Lines and Clones Reactive to $\beta_2$GPI Peptides**

T cell lines reactive to human $\beta_2$GPI peptides were established from PBMC of four patients and two healthy controls. PBMCs ($1.5 \times 10^5$/well) were incubated with 0.1–0.2 $\mu$M each of human $\beta_2$GPI peptide mixtures with 2.5 $\mu$g/ml MAR4 (anti-CD29 mAb; Pharmingen, San Diego, CA, USA), which we earlier found to increase efficiency in establishing autoreactive T cells [24], in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 $\mu$g/ml of streptomycin, 50 $\mu$M 2-mercaptoethanol (2-ME) for inhibition of a formation of S-S bonds between Cys-residues of the peptides, and 10% heat-inactivated normal human plasma in 96-well flat-bottomed culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). After 7–9 days, irradiated (3,000 cGy) autologous PBMC ($1.5 \times 10^5$/well) pulsed for 6 h with a human $\beta_2$GPI peptide mixture (0.5 $\mu$M each) were added to the culture wells carrying T cell blasts, with human recombinant interleukin-2 (rIL-2) (50 U/ml) and human recombinant interleukin-4 (rIL-4) (10 U/ml) for maintenance and expansion. Reactivity against the mixture of overlapping peptide was determined and T cells were expanded weekly by feeding as described above. When two or more culture wells carried peptide-reactive T cells, aliquots of growing cultures were combined as a bulk T cell line, for each donor.

Cloning was done in Terasaki plates (Nunc, Roskilde, Denmark) by limiting dilution at 0.33 T cells per well in the presence of irradiated (3,000 cGy) autologous PBMC or restriction HLA class II carrying allogeneic PBMC ($3 \times 10^4$/well) pulsed with specific peptide (10 $\mu$M), and in the same medium, as described above, with human rIL-2 (50 U/ml) and human rIL-4 (10 U/ml). Growing microcultures (2–10% of all culture wells) were then expanded at weekly intervals first in a 96-well plate, and then in a 24-well plate by feeding with irradiated feeder cells ($1.5 \times 10^5$/well) pulsed with a specific peptide (10 $\mu$M), in the presence of human rIL-2 and human rIL-4.

The cell surface markers of T cells were analyzed on a FACScan, using anti-CD4, anti-CD8 (Pharmingen, San Diego, CA, USA), and anti-T cell receptor $\alpha/\beta$ (Becton Dickinson, San Jose, CA, USA) mAbs conjugated with either fluorescein isothiocyanate or phycoerythrin.

**Quantitation of $\beta_2$GPI Peptide-specific Responses of T Cells**

Antigen-specific proliferation of the T cell lines was assayed by culturing the T cells ($3 \times 10^5$/well) in 96-well flat-bottom culture plates in the presence of either

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Amino acid sequences of synthetic overlapping peptides derived from human $\beta_2$GPI and used for primary stimulation of PBMC</th>
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<tr>
<td>p1–21:</td>
<td>GRTCPKPDLPSTVPLKTF</td>
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<td>p10–30:</td>
<td>LPESTVVLKTFTEYPGEITY</td>
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<td>p19–39:</td>
<td>KTFYEPGEITEYSCPKPGYVSR</td>
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<td>p28–47:</td>
<td>ITYSCPKPGYVSRRGMRKIFIC</td>
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<td>p57–57:</td>
<td>VSRRGMRKIFICPLGTLGLWPINT</td>
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<tr>
<td>p46–61:</td>
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<tr>
<td>p51–68:</td>
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<td>p118–134:</td>
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<td>p124–138:</td>
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<td>p127–147:</td>
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<td>p137–156:</td>
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<td>p163–183:</td>
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<td>p172–191:</td>
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<td>p181–199:</td>
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<td>p190–210:</td>
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<td>p199–219:</td>
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<td>p208–228:</td>
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<td>p217–267:</td>
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<td>p226–246:</td>
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<td>p235–255:</td>
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<td>p244–264:</td>
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<td>p253–272:</td>
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<td>p262–282:</td>
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<td>p271–291:</td>
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<td>p280–295:</td>
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<tr>
<td>p285–300:</td>
<td>EKCKSYTEDAQCIDGT</td>
</tr>
<tr>
<td>p289–309:</td>
<td>SYTEDAQCIDGDTIEVPCKCFKE</td>
</tr>
<tr>
<td>p298–318:</td>
<td>DGETIEVPCKCFKEHSSLAFWK</td>
</tr>
<tr>
<td>p307–324:</td>
<td>FEHSSLAFWKT</td>
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</table>

Because some peptides contain cystein residue(s), 2-ME was added to the stock solution of peptides to prevent formation of disulfide-bonds between cysteine residues. PBMC were stimulated with the presence of 50 $\mu$M 2-ME. Because of difficulty in synthesizing peptide whose C-terminus residue is C or P, $^{32}$P and $^{32}$C were omitted.
individual soluble peptide (10 μM) or medium described above, and 6,000 cGy-irradiated autologous PBMC (1.5 × 10^6/well). Cells were cultured for 72 h in the presence of 1 μCi/well of [3H]-thymidine during the final 18-h period, and the incorporated radioactivity was measured by liquid scintillation counting. Soluble purified β2GPI were also tested for antigenicity. To determine restriction molecules for antigen presentation, the T cell lines were cultured with irradiated autologous PBMC, with or without saturating amounts of either anti-HLA class II monoclonal antibodies (mAbs) HU-4 (anti-HLA-DRB1 + DRB5 IgG2a monomorphic) [25], L243 (anti-HLA-DRB1 + DRB4 IgG2a monomorphic, reactivity to DRB3 and DRB5 yet to be determined) [26], HU11 (anti-HLA-DQ4 + DQ5 + DQ6 IgG2a) [27], HU18 (anti-HLA-DQ7 + DQ8 + DQ9 IgG2a) [28] and B7/21 (anti-HLA-DR1 I gG1 monomorphic) [25]. Peptide-pulsed, mock-pulsed allogeneic PBMC or mouse L cells transfected with HLA class II genes were also used as APC. The L-B19D7 cell line transfected with the DRA + DRB1*0403 genes, the L/KR1-3-A10 cell line transfected with the DRA + DRB1*0901 genes, the LARB1-1212 cell line transfected with the DRA + DRB1*1502 genes, the LR6.2/52a cell line transfected with the DRA + DRB3*0101 genes, the L-DRw53-Dw15 cell line transfected with the DRA + DRB4*0103 genes and the L-DR2(AB5) cell line transfected with the DRA + DRB5*0102 genes were established. Autologous PBMC pre-pulsed with the mixture of β2GPI-derived peptides were used as feeder cells for these T cell lines and some T cell lines were cloned for further studies. AK2, NT5, and KM27 were derived from patients, as well as SM21 derived from a control subject responded to a β2GPI peptide p244–264. OAA derived from a patient responded to a β2GPI peptide p154–174, IT090 and SM2 derived from controls responded to β2GPI peptide p226–246 and p64–83, respectively (Fig. 1). SM2 and SM21 responded to p64–83 and p244–264, respectively; the response pattern, when combined, is shown in Fig. 1. Thus, epitopes for the four independent β2GPI peptide-reactive T cell lines derived from patients were mapped in two β2GPI fragments. However the epitopes for three β2GPI peptide-reactive T cell lines derived from two healthy controls were mapped in three β2GPI fragments, one of which was shared between patients and controls (p244–264). Flow-cytometric analysis of the seven T cell lines revealed a CD4^+ phenotype (data not shown).

Restriction Molecules for β2GPI-Reactive T Cells

To identify HLA molecules that present β2GPI-derived peptides to established T cell lines, blocking experiments using anti-HLA class II mAbs were done. The proliferative response of AK2 was completely blocked by HU4 (specific for DRB1 and DRB5) and L243 (specific for DRB1 and DRB4, reactivity to DRB3 and DRB5 is yet to be determined), whereas HU11 (specific for DQ4, 5 and 6), HU18 (specific for DQ7, 8 and 9) and B7/21 (specific for DP) were incapable of blocking the response (Fig. 2A). Because HLA of the donor patient was typed as DRB1*0403, DRB1*0901, and DRB4*0103, it seems likely that the T cell line was restricted by either one of DRB1*0403 or DRB1*0901. Indeed, an L-cell transfectant expressing DRA + DRB1*0403 molecules (L-B19D7) presented the peptide to AK2, whereas L

RESULTS

Establishment of T Cell Lines Autoreactive to β2GPI Peptides and Identification of T Cell Epitopes

PBMC isolated from 18 unrelated APS or SLE (lupus-like disease were included) patients with high anti-β2GPI antibody titer and 10 healthy controls were stimulated with the mixture of human β2GPI peptides, as shown in Table 1. We found proliferative responses of peptide-reactive T cells in 4 patients and 2 controls, from which 7 T cell lines (4 from patients and 3 from controls) were established. Autologous PBMC pre-pulsed with the mixture of β2GPI-derived peptides were used as feeder cells for these T cell lines and some T cell lines were cloned for further studies. AK2, NT5, and KM27 were derived from patients, as well as SM21 derived from a control subject responded to a β2GPI peptide p244–264. OAA derived from a patient responded to a β2GPI peptide p154–174, IT090 and SM2 derived from controls responded to β2GPI peptide p226–246 and p64–83, respectively (Fig. 1). SM2 and SM21 responded to p64–83 and p244–264, respectively; the response pattern, when combined, is shown in Fig. 1. Thus, epitopes for the four independent β2GPI peptide-reactive T cell lines derived from patients were mapped in two β2GPI fragments. However the epitopes for three β2GPI peptide-reactive T cell lines derived from two healthy controls were mapped in three β2GPI fragments, one of which was shared between patients and controls (p244–264). Flow-cytometric analysis of the seven T cell lines revealed a CD4^+ phenotype (data not shown).
cells expressing DRA + DRB1*0901 (L/KR1-3-A10) or DR53 (DRA + DRB4*0103; L-DRw53-Dw15) and L cells transfected with the Neo gene alone (L-Neo) did not. These observations indicate that the T cell line AK2 recognized β2GPI peptide p244–264 in the context of DR53 (DRA + DRB4*0103); and (b) SM2 (Fig. 2E) recognized β2GPI peptide p64–83 in the context of DR52 (DRA + DRB3*0101). Response of KM27 (Fig. 2C) was completely blocked by B7/21, whereas HU4, L243, HU11, and HU18 were incapable of blocking the response. The patient KM was typed for DPB1*0901 and DPB1*0501 and allogeneic PBMC sharing DPB1*0901 and presented peptide p244-264 to KM27, whereas those sharing DPB1*0501 failed to do so. These observations indicate that KM27 recognizes β2GPI peptide p244–264 in the context of DP9 (DPA1*0201-DPB1*0901). Using the same methods, IT090 (Fig. 2D) recognized β2GPI peptide p226–246 in the context of DRA + DRB1*1502. We could not identify restriction HLA class II molecule of T cell line OAA because cell growth gradually slowed and numbers of T cells were too scarce. Each T cell clone (AK2.2.9, NT5.3.1, and IT090.3.1) was confirmed to carry the same restriction characteristics as the mother T cell lines (data not shown).
Identification of a Core Peptide Fragment Recognized by T Cells

To identify a core sequence of the \( \beta_2 \)GPI peptide p244–264 (\(^{244}\)SCKLPVKKATVVYQGERVKIQ\(^{264}\)) for the DRB1*0403-restricted antigen presentation to AK2.2.9 clone, a series of peptides truncated either from the C- or N-terminus was synthesized and reactivities of AK2.2.9 were determined. As shown in Fig. 3, truncation from the N-terminus to p248 or from the C-terminus to p261 abrogated the proliferative response of AK2.2.9. These

FIGURE 2 Identification of restriction HLA class II molecules for \( \beta_2 \)GPI peptide-reactive T cell lines, (A) AK2, (B) NT 5, (C) KM 27, (D) IT090, (E) SM2 and (F) SM21. Proliferative responses of T cells to specific peptides were investigated in the presence of autologous PBMC and anti-HLA class II mAbs, allogeneic PBMC and/or L cells transfected with HLA genes. To block the peptide presentation of APC, irradiated autologous PBMC (1.5 \( \times \) 10\(^5\)) and T cells (3 \( \times \) 10\(^4\)) were incubated with anti-HLA class II mAbs at 37°C for 1–2 h before the addition of peptide (2 \( \mu \)M). HU4 is specific to DRB1 + DRB5, L243 is specific to DRB1 + DRB4, and reactivity of L243 to DRB3/DRB5 is not determined. HU11 is specific to DQ7 + DQ8 + DQ9, and B7/21 is specific to DP. In the allogeneic combinations, peptide (10 \( \mu \)M)-pulsed (for 2 h) allogeneic adherent cells were irradiated at 6000 cGy and used as APC. In an experiment with L cells, mitomycin C-treated L cells (3.5 \( \times \) 10\(^4\)) were prepulsed with (or without) 10 \( \mu \)M peptide for 2 h and used as APC. Mean cpm of triplicate responses ± SD is indicated.
data indicate that the 14-mer peptide (248PVKKATV
VYQGERV261) is a minimum sequence required for
activation of AK2.2.9. Based on use of the same meth-
ods, the core sequences required for activation of three
other T cell lines were as follows: NT5.3.1; p247–
262(261), KM27; p246 –260 and SM21; p247(248)–
261.

**Cytokine Production of T Cells in Response to β2GPI Peptide**

All T cell lines produced both IFN-γ and IL-4 in culture
supernatants, when stimulated by 10 μM of β2GPI
peptides (Fig. 4A), where cytokine production reached
the plateau phase (data not shown). However, the IFN-
γ/IL-4 ratio was significantly different between T cell
lines derived from patients and those from healthy con-
trols, as shown in Fig. 4B (p = 0.028, by Mann-Whit-
ney’s U-test); IFN-γ/IL-4 ratios were lower in T cell lines
derived from patients. Such a difference was mainly due
to low levels of IFN-γ produced by patients’ T cells (Fig.
4A). It is also important to note that although NT5.3.1
from a patient and SM21 from a healthy individual
recognized the same peptide in the context of the same
HLA class II molecule, the IFN-γ/IL-4 ratio was distinct;
being 1.39 and 336, respectively.

**DISCUSSION**

The patients we studied were treated with corticosteroids
and some were treated with immune suppressive agents
such as azathioprine. Therefore, efficiency with regard to
establishing T cell lines cannot be readily compared
between patients and healthy controls. Because three of
four epitopes for patient-derived CD4+ T cell lines were
p244 –264, it seems likely that the major T cell epitope
for patients is p244 –264. However, it would not be
specific to patients, because one CD4+ T cell line derived
from a healthy control subject recognized p244 –264
(summarized in Table 2).

Peptide presentation to T cell lines was restricted by
HLA-DRB1*0403, DRB4*0103 (DR53), DPB1*0901
(complexed with DPA1*0201, speculated by linkage
disequilibrium; 33), DRB3*0101 and DRB1*1502.
Thus, there was no tendency for particular HLA class II
molecules, except for predominant usage of DR mole-
cules as restriction molecules. In this concern, various
HLA class II alleles were found to be associated with
patients carrying anti-β2GPI antibodies, and some re-
striction molecules identified in the present study have
been reported to be associated with patients carrying
anti-β2GPI antibodies and/or aCL. For instance, DR53
and DR4 were associated with anti-β2GPI antibodies
[18] and the high titer of aCL was associated with DR5
(possibly DRB1*1201) [20], which is in linkage disequi-
librium with DRB3*0101. These observations col-
lectively indicate that association of certain HLA class II
alleles with APS might be explained, at least in part, as

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<th>Peptide</th>
<th>3H-thymidine incorporation (cpm x 10^-3)</th>
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<td>None</td>
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<tr>
<td>244SCKLPKKATVYQG258</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 3** Identification of core peptide fragments for
stimulation of two T cell clones AK2.2.9 and NT5.3.1, and
two T cell lines KM27 and SM21. Various peptides truncated
either from the N-terminus or from C-terminus of β2GPI
p244–264 were investigated for their capacity to stimulate T
cells. Irradiated autologous PBMC (1.5 × 10⁵) or allogeneic
PBMC carrying restriction HLA molecules were used as APC,
and T cells (3 × 10⁴) were cultured for 72 h in the presence
of 10 μM of each truncated soluble peptide. Cultures were
pulsed with ³H-thymidine and incubated during the final
18-h period, and the incorporated radioactivity was measured.
Values shown are the mean cpm of triplicate cultures ± SD.
a classical immune response gene phenomenon related to β₂GPI. The shortest epitope for AK2.2.9 restricted by DRB1*0403 was p248–261. The amino acid sequence of DRB1*0403 is the same as that of DRB1*0406 except for one residue; residue 37 is Tyr and Ser in DRB1*0403 and DRB1*0406, respectively. We suggested in our previous study that the DRB1*0406-specific peptide-binding motif is composed of hydrophobic residues (F, I, L and M) at position 1, hydrophobic residues (L, I, and M) at position 4 and neutral hydrophilic residues (N, S, and Q) at position 6 [34]. Because residue 37 has a lesser influence on HLA-DR-peptide binding, it is reasonable to speculate that 252A, 255V, and 257Q may be the first, fourth, and sixth DR-anchor.

FIGURE 4 Cytokine production by T cell lines in response to specific β₂GPI peptides. T cell lines were cultured with irradiated autologous PBMC or allogeneic PBMC carrying restriction HLA molecules in the presence of soluble peptides (10 μM). After 48-h incubation, 80 from 150 μl of triplicate culture supernatants were collected. (A) IFN-γ (open bar) and IL-4 (closed bar) concentrations were assessed by ELISA. Results are expressed as the geometric means ± SD of duplicate measurement. (B) IFN-γ/IL-4 ratio are calculated based on (A) and plotted to be compared between patients and controls.

TABLE 2 Characteristics of the β₂GPI peptide reactive T cell lines and data on subjects

<table>
<thead>
<tr>
<th>T cell line (clone)</th>
<th>Disease</th>
<th>Age and gender</th>
<th>Serum anti-β₂GPI IgG* (U/ml)</th>
<th>Epitope (core)</th>
<th>Restriction HLA class II molecule</th>
<th>IFN-γ/IL-4b</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK2 (AK2.2.9)</td>
<td>APS secondary to SLE</td>
<td>41 F</td>
<td>&gt;125</td>
<td>p244–264</td>
<td>DRB1*0403</td>
<td>1.54</td>
</tr>
<tr>
<td>NT5 (NT5.3.1)</td>
<td>SLE</td>
<td>39 F</td>
<td>11.9</td>
<td>p244–264</td>
<td>DRB4*0103</td>
<td>1.39</td>
</tr>
<tr>
<td>KM27</td>
<td>SLE</td>
<td>52 F</td>
<td>28.8</td>
<td>p244–264</td>
<td>DPA1<em>0201 + DPB1</em>0901[a]</td>
<td>12.4</td>
</tr>
<tr>
<td>OAA</td>
<td>ARS secondary to SLE</td>
<td>36 F</td>
<td>67.7</td>
<td>p246–260</td>
<td>ND[f]</td>
<td>2.96</td>
</tr>
<tr>
<td>IT090 (IT090.3.1)</td>
<td>Healthy</td>
<td>32 M</td>
<td>&lt;1.2</td>
<td>p226–246</td>
<td>DRB1*1502</td>
<td>77.8</td>
</tr>
<tr>
<td>SM2</td>
<td>Healthy</td>
<td>23 F</td>
<td>&lt;1.2</td>
<td>p64–83</td>
<td>DRB3*0101</td>
<td>36.1</td>
</tr>
<tr>
<td>SM21</td>
<td>Healthy</td>
<td>23 F</td>
<td>&lt;1.2</td>
<td>p244–264</td>
<td>DRB4*0103</td>
<td>336</td>
</tr>
</tbody>
</table>

[a]The normal value of serum anti-β₂GPI IgG is less than 3.5 U/ml.
[b]Ratio of produced cytokines in response to specific peptide.
[c]AK has a history of cerebral infarction.
[d]DPA1*0201 + DPB1*0901 complex was speculated based on their linkage disequilibrium and T cell proliferation assay using allogeneic PBMC as APC.
[e]OA has a history of recurrent spontaneous abortion.
[f]Restriction molecule of OA could not be identified.
[g]SM2 and SM21 were established from the same subject.
respectively, for peptide binding to the DRA + DRB1*0403 complex. The shortest epitope for KM27 restricted by DPB1*0901 was p246–260. It has been suggested that the DP9-specific peptide binding motif is composed of positively charged residues (R or K) at position 1 and hydrophobic residues (A, G, or L) at position 6 [35]. Accordingly, $^{247}$Leu and $^{251}$V of p248–260 are likely to be the first and sixth DP-anchor, respectively. The shortest epitopes for NT5.3.1 and SM21 restricted by DRB4*0103 were p247–262(261) and p247(248)–261, respectively. The shortest epitopes for NT5.3.1 and 260 are likely to be the first and sixth DP-anchor, respectively. The shortest epitopes for NT5.3.1 and SM21 restricted by DRB4*0103 were p247–262(261) and p247(248)–261, respectively. The DR53-specific peptide binding motif has been reported [36]. However, p247–262 does not carry such motifs.

All CD4$^+$ T cell lines produced both IFN-γ and IL-4 in response to antigenic peptides. However, it is noteworthy that T cells from patients are the Th0–Th2 phenotype, whereas T cells from healthy subjects are Th0–Th1 phenotype. It is likely that $\beta_2$GPI-reactive effector T cells of patients (Th0–Th2) may induce increased antibody production as well as immunoglobulin class switching, including IgG and IgA [37]. Indeed, BALB/c, a typical Th2-prone strain [38], affords a model for APS [21, 22, 39]. In contrast to these observations, a recent study by Visvanathan et al. [23] revealed that $\beta_2$GPI-reactive human T cells produced high levels of IFN-γ. These points need further investigation.

The complete amino acid sequence of human $\beta_2$GPI proved to be 326 residues in length [5–7]. Steinacker et al. [6, 40] reported valine/leucine dimorphism at position 247; gene frequency for $^{247}$Val and $^{247}$Leu were 0.765 and 0.235, respectively, in European populations. In contrast, $^{247}$Leu is of a higher frequency in Japanese individuals, as we investigated using a documented method [40] (data not shown). Of four subjects whose T cell lines recognize p244–264, three were positive for $^{247}$Leu, one patient NT was not positive for $^{247}$Leu. We then synthesized variant p244–264 of which $^{247}$Leu was replaced by Val and confirmed that NT5.3.1 cells respond to this peptide as well (data not shown). It is thus conceivable that T cell epitopes demonstrated in the present study reacted to purified $\beta_2$GPI added in excess amounts to cultures (data not shown). It is thus conceivable that T cell epitopes demonstrated in the present study cannot be presented physiologically, but might be presented under special conditions, such as changes in cytokine concentrations, pH, activation of some proteases, exposure of phospholipids of endothelial cell membrane by injury and expression of adhesion molecules/HLA molecules/co-stimulatory molecules, in a local milieu in vivo.

Identification of conditions in which cryptic epitopes are presented to $\beta_2$GPI peptide-reactive T cell lines, will unravel mechanisms involved in the development of APS. In this respect, AK2.2.9 did not react to purified soluble $\beta_2$GPI presented by APC, the antigen presenting capacity of which is augmented by treatment with IFN-γ or granulocyte macrophage-colony stimulating factor (GM-CSF). We also tested the following treatment on $\beta_2$GPI, but antigen was not presented: (a) boiling; (b) reduction by 2-mercaptoethanol; and (c) incubation of soluble or cardiolipin-binding $\beta_2$GPI with coagulation proteases, such as thrombin, VIIa, IXa, Xa, XIa and XIIa, plasmin and inflammatory proteases, such as cathepsin G and elastase. Conditions under which cryptic epitopes are presented to T cell lines are currently under investigation.
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