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Research Article

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Autoreactive T Cells to Platelet GPIIb-IIIa in Immune Thrombocytopenic Purpura

Role in Production of Anti–Platelet Autoantibody

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Abstract

T cell proliferative responses to platelet membrane GPIIb-IIIa were examined in 14 patients with chronic immune thrombocytopenic purpura (ITP), 7 systemic lupus erythematosus (SLE) patients with or without thrombocytopenia, and 10 healthy donors. Although peripheral blood T cells from all subjects failed to respond to the protein complex in its native state, reduced GPIIb-IIIa stimulated T cells from three ITP patients and one SLE patient with thrombocytopenia, and tryptic peptides of GPIIb-IIIa stimulated T cells from nearly all subjects. The specificity of the responses for GPIIb-IIIa was confirmed by activation of GPIIb-IIIa–primed T cells by a recombinant GPIIbα fragment in secondary cultures. Characterization of T cell response induced by modified GPIIb-IIIa showed that the response was restricted by HLA-DR, the responding T cells had a $CD4^+$ phenotype, and the proliferation was accelerated only in ITP patients, suggesting in vivo activation of these T cells. In vitro IgG anti–GPIIb-IIIa synthesis in PBMC cultures was induced by modified GPIIb-IIIa specifically in ITP patients with platelet-associated anti–GPIIb-IIIa antibody. Anti–GPIIb-IIIa antibody produced in supernatants was absorbed by incubation with normal platelets. In summary, $CD4^+$ and HLA-DR–restricted T cells to GPIIb-IIIa are involved in production of anti–platelet autoantibody in ITP patients and are related to the pathogenic process in chronic ITP. (*J. Clin. Invest.* 1998. 102:1393–1402.)

Key words: autoantibody • autoimmunity • helper T cells • HLA • SLE

Introduction

Chronic immune thrombocytopenic purpura (ITP)[1] is an autoimmune disease characterized by increased platelet clearance caused by anti-platelet autoantibodies, which bind to circulating platelets, resulting in destruction by the reticuloendothelial system (1, 2). Major targets of anti-platelet antibodies are platelet membrane GPs, including GPIIb-IIIa, GPIb-IX, GPIa-IIa, and GPIV (3). Although results vary among studies, the most common target recognized by anti-platelet antibodies in ITP patients is GPIIb-IIIa (3–5), also designated $\alpha_{IIb}\beta_3$ integrin or CD41/CD61, which is a calcium-dependent heterodimeric membrane receptor for fibrinogen (6).

It is now evident that autoantibody production by B cells requires autoantigen-specific T cell help (7). T cells reactive with autoantigens targeted by autoantibodies were identified in patients with various systemic or organ-specific autoimmune diseases (8–10). These autoantigen-specific T cells almost always had a $CD4^+$ phenotype, known as helper T cells. Previous studies using lupus-prone mice and patients with lupus nephritis showed that autoreactive T cells selectively provided help to autoantibody-producing B cells (11). Furthermore, one of us (M. Kuwana) recently showed that autoreactive $CD4^+$ T cells can provide cognate help to B cells in an antigen-specific manner, resulting in the production of autoantibody in scleroderma patients (12).

It has been shown that circulating or platelet-associated anti–GPIIb-IIIa antibodies in ITP patients are mainly of the IgG isotype (3) and recognize multiple epitopes on the complex, including linear and conformational determinants (13–15). These features strongly suggest that anti–GPIIb-IIIa antibody production in ITP patients requires antigen-specific T cell help. However, only a limited number of studies analyzing T cell responses to platelet antigens in ITP patients have been performed to date. Earlier studies using whole human platelets as an antigen source found the presence of platelet-reactive T cells in ITP patients (16, 17). Recently, Filion et al. reported that autoreactive T cells to GPIIb-IIIa, which escape thymic deletion but are tolerized in the periphery, were identified in healthy individuals (18). However, the role of GPIIb-IIIa–reactive T cells in anti-platelet autoantibody production as well as the pathogenesis of ITP is unknown. In the present study, affinity-purified human GPIIb-IIIa was used to examine antigen-specific T cell responses in ITP patients.

Methods

Patients and controls. Peripheral blood T cells from 14 patients with chronic ITP were analyzed in this study. Chronic ITP was defined as thrombocytopenia (platelet count $< 150 \times 10^9$/liter) persisting for longer than 6 mo, normal or increased bone marrow megakaryocytes without morphological evidence for dysplasia, and no secondary immune or nonimmune diseases that account for the thrombocytopenic state (1, 2). Three patients took low-dose corticosteroids (< 10 mg of prednisolone/d), and one patient was on prednisolone (5 mg/d) and danazol at the time of blood examination. Previous medical treatment was with corticosteroids in three patients, splenectomy in six, intravenous IgG in nine, azathioprine in one, danazol in one, and platelet transfusion in one. Three patients had never received medical treatment. Control blood was obtained from 7 systemic lupus erythematosus (SLE) patients and 10 healthy volunteers. All SLE patients

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; GST, glutathione S-transferase; ITP, immune thrombocytopenic purpura; SLE, systemic lupus erythematosus; TBS, Tris-buffered saline; TT, tetanus toxoid.

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satisfied the American Rheumatism Association revised criteria for classification of SLE (19). Two SLE patients had chronic thrombocytopenia that had required medical treatment. All blood samples were obtained in accordance with the Keio University Institutional Review Board guidelines. Peripheral blood T cells from seven ITP patients and six healthy donors were examined on multiple occasions.

HLA-DRB1 allele genotyping. Genomic DNA was amplified by PCR using primers specific for the DRB1 gene. DRB1 alleles were determined based on the RFLPs of PCR-amplified products (20).

Preparation of human GPIIb-IIIa. GPIIb-IIIa was purified from outdated platelet concentrates using affinity chromatography. Briefly, human platelets ($3–5 \times 10^9$) suspended in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 1 mM benzamidine (GP buffer) were sonicated and ultracentrifuged at 100,000 *g* for 60 min at 4°C. The pellet was then resuspended in GP buffer containing 1% Triton X-100 (wt/vol) and allowed to stand on ice for 1 h. The solubilized platelet membrane preparation was ultracentrifuged again as indicated above. The platelet membrane lysate was applied to the affinity column, in which the IgG fraction of mAb to human GPIIb-IIIa (21) was coupled to cyanogen bromide-activated Sepharose CL-4B (Amersham Pharmacia Biotech Inc., Piscataway, NJ). After extensive washing with 0.1% Triton X-100/GP buffer containing 0.5 M LiCl, the bound GPIIb-IIIa was eluted with 0.05 M diethylamine in 0.05% Triton X-100/GP buffer (pH 11.5). Fractions with a peak in the OD_{280} reading were collected as GPIIb-IIIa antigen. After GPIIb-IIIa was eluted, fractions in which the OD_{280} reading was below 0.1 were collected as a control preparation. GPIIb-IIIa and control preparations were immediately neutralized with solid glycine, dialyzed against TBS containing 0.05% Triton X-100, sterilized by passage through 0.45-μm pore-size syringe filters, and stored at −80°C. The relative amounts of purified GPIIb-IIIa determined by Coomassie blue staining revealed that GPIIb and IIIa represented > 95% of the total stained protein. GPIIb-IIIa was also purified from platelets of two healthy donors on a small scale.

Affinity-purified GPIIb-IIIa was chemically modified by treatment with porcine trypsin (0.1 μg/ml) or DTT (2 mM). Trypsin activity was neutralized by prompt addition of an equal volume of fresh FBS. A control preparation was also treated with trypsin and DTT in the same manner. Migration of DTT-treated and untreated GPIIb-IIIa was apparently different under native condition, indicating sufficient reduction of the GPIIb-IIIa complex. In contrast, many small fragments (< 50 kD) corresponding to tryptic peptides of GPIIb-IIIa were seen when GPIIb-IIIa treated with trypsin was fractionated.

Preparation of GPIIbα fusion proteins. Recombinant fusion proteins consisting of three different portions of GPIIbα were generated. These included IIbα18-259, IIbα244-575, and IIbα566-841, which encompass amino acid residues 18–259, 244–575, and 566–841, respectively, of a total of 871 amino acids of GPIIbα. Briefly, the fusion proteins were derived by subcloning of PCR products of GPIIb into the 3′ end of the *Schistosoma japonicum* glutathione S-transferase (GST) gene in the bacterial expression vector, pGEX-6P-1 (Amersham Pharmacia Biotech Inc.). Recombinant proteins were expressed in *Escherichia coli* strain DH5α and purified by glutathione-agarose affinity chromatography (22). The fusion proteins and GST were dialyzed against TBS, concentrated by ultrafiltration, and filter sterilized. The relative amounts of GPIIbα fusion proteins and GST in purified preparations represented > 95% of the total stained protein by Coomassie blue staining.

Cell preparations. PBMCs were isolated from heparinized blood using Histopaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. In some experiments, erythrocytes were recovered and washed twice with TBS. PBMCs were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C, 5% CO_2. A T cell-enriched fraction used as T cells was separated from PBMCs by passage through a nylon wool column and non-T cells were removed from the nylon wool by compression against the sides of the tubes (8). T cells that were depleted of $CD3^+$, $CD4^+$, or $CD8^+$ cells were prepared by incubating the T cells with anti-CD3, anti-CD4, or anti-CD8 mAb-coupled magnetic beads (PerSeptive Diagnostics, Cambridge, MA), respectively, at 4°C for 1 h, followed by magnetic removal of bead-bound cells (8). Platelets were isolated from platelet-rich plasma and stored at −80°C until use.

Detection of IgG anti–GPIIb-IIIa antibody. IgG anti–GPIIb-IIIa antibody levels in sera and platelet eluates were measured by ELISA using affinity-purified GPIIb-IIIa as an antigen source (5). Antibodies bound to the platelet surface (platelet eluates) were eluted from platelet aliquots (10^8) using 0.1 M HCl, followed by prompt neutralization with 0.2 M NaOH. The 96-well microtitration plates were coated with GPIIb-IIIa at a concentration of 0.25 μg/ml. After being blocked with 2% BSA, wells were incubated with sera (1:100) or platelet eluates (1:1) and subsequently with peroxidase-conjugated goat anti–human IgG (1:10,000; Cappel, Aurora, OH). Finally, wells were incubated with a substrate (tetramethylbenzidine/sodium perborate) for 10 min. The reaction was stopped by addition of an equal volume of 1 M H_2SO_4, and the OD_{450} was read with an automatic microplate reader. All samples were tested in duplicate, and results were calculated as the duplicate mean. Cutoff values were considered to be the mean+3 × SD of 20 samples from healthy individuals (0.108 for serum samples and 0.155 for platelet eluates). SD are < 20% of the mean or < 0.010 (OD_{450}) unless indicated otherwise.

T cell proliferation assays. Non–T cells were cultured in complete medium in the presence or absence of antigen for 1 h. Antigen-pulsed non–T cells (2×10^4/well) were mixed with autologous T cells (10^5/well) and cultured in 96-well flat-bottomed culture plates. In cultures with reduced antigen preparations, antigen-pulsed non-T cells were washed twice with complete medium before being mixed with T cells. Cells were cultured for 7 d (unless indicated otherwise) and then incubated with 1 μCi/well of [^{3}H]thymidine during the final 16 h of culture. The cells were harvested, and [^{3}H]thymidine incorporation was determined in a TopCount microplate scintillation counter (Packard Instruments, Meriden, CT). All cultures were carried out in triplicate, and all values represent the mean of triplicate determinations. SDs are < 20% of the mean or < 100 cpm unless indicated otherwise. Native and chemically modified GPIIb-IIIa (10 μg/ml, unless indicated otherwise) were added to the cultures as antigens, and the same volumes of untreated or treated control preparations were also used as controls. Antigen-specific T cell proliferation was expressed as the stimulation index, which was calculated as cpm incorporated in antigen-stimulated cultures/cpm incorporated in control cultures. Purified tetanus toxoid (TT; 5 μg/ml) and PHA (1 μg/ml) were used to stimulate T cells to exclude possible nonspecific depression of responsiveness. A positive response was defined as having both stimulation index > 3 and the increase in cpm associated with antigenic stimulation > 500. In a smaller number of samples, more detailed studies of the time course of the antigen-specific response were carried out, with proliferation being examined at 3, 5, 6, and 7 d.

Secondary stimulation of PBMCs was also performed. After the priming stimulation of PBMCs with trypsin-digested GPIIb-IIIa for 7 d, the viable cells were then cultured for 3 d in the presence of 50 U/ml rIL-2 (GIBCO BRL, Grand Island, NY) and autologous irradiated (3,000 rad) PBMCs as antigen-presenting cells (APCs) in medium alone, or supplemented with trypsin-digested GPIIb-IIIa, GPIIbα fusion proteins, or GST. Proliferation was measured by incorporation of [^{3}H]thymidine as described above.

In vitro anti–GPIIb-IIIa antibody production in PBMC cultures. In vitro assay to analyze antigen-induced anti–GPIIb-IIIa antibody synthesis by PBMCs was carried out as described (12). Briefly, PBMCs (2×10^6/well) were cultured in 2 ml of complete medium in 24-well tissue culture plates with or without antigen in the presence of pokeweed mitogen (1 μg/ml) for 10 d. Native GPIIb-IIIa, reduced GPIIb-IIIa, and trypsin-digested GPIIb-IIIa, as well as trypsin and TT were used as antigens. $CD4^+$ or $CD8^+$ cell-depleted PBMCs were also tested. IgG anti–GPIIb-IIIa antibody levels in undiluted culture supernatants were measured by anti–GPIIb-IIIa ELISA described above. All experiments were done in duplicate, and anti–GPIIb-IIIa antibody results represent the mean of duplicate values.

To absorb anti–GPIIb-IIIa antibody in culture supernatants with normal platelets, culture supernatants were incubated with serial numbers of platelets (10^5–10^8) from two independent healthy donors for 15 min at room temperature. Erythrocytes (10^8) and PBMCs (10^7) obtained from the same donors were used as controls. After centrifugation, the pretreated supernatants were applied to anti–GPIIb-IIIa ELISA. To block nonspecific Ig binding sites on the cell surface, each cell fraction was preincubated with purified human γ-globulin (50 mg/ml; Sigma Chemical Co.) for 30 min and washed twice with TBS.

Anti-HLA class II mAb blocking. To examine the effects of anti-HLA class II mAbs on antigen-specific T cell proliferation and in vitro anti–GPIIb-IIIa antibody synthesis, mAbs were added to the cultures at the start of the culture (8, 12). Anti-DR (L243), anti-DQ (1a3), and anti-DP (B7/21) as well as isotype control mouse IgG_2a and IgG_3 mAb (Leinco Technologies, St. Louis, MO) were dialyzed against TBS and used at a final concentration of 0.2 μg/ml unless indicated otherwise.

Results

T cell proliferative response to native GPIIb-IIIa. The proliferative responses of peripheral blood T cells with native GPIIb-IIIa and TT were examined in 14 ITP patients, 7 SLE patients, and 10 healthy donors. All subjects showed significant T cell proliferation upon stimulation with PHA (data not shown). As shown in Fig. 1, none of the subjects showed a significant T cell response to native GPIIb-IIIa. Moreover, native GPIIb-IIIa failed to induce a T cell response over a wide range of antigen concentrations (1–100 μg/ml). In contrast, a TT-induced T cell response was detected in 11 ITP patients, 5 SLE patients, and 9 healthy donors.

T cell proliferative response to modified GPIIb-IIIa. T cell responses to reduced or trypsin-digested GPIIb-IIIa were examined (Table I). Three ITP patients (P1, P2, and P3) showed

Table I. Proliferative Responses of Peripheral Blood T Cells to Reduced or Trypsin-digested GPIIb-IIIa in 14 ITP Patients, 7 SLE Patients, and 10 Healthy Donors

				IgG anti–GPIIb-IIIa antibody			Antigen-specific T cell proliferative responses			
							Reduced GPIIb-IIIa		Trypsin-digested GPIIb-IIIa	
#	Age/gender	Current therapy*	Platelet ($\times 10^9$/liter)	Serum	Platelet eluate	DRB1	cpm	SI	cpm	SI
ITP patients										
P1	68/F	None	13	+	+	0405/0901	7246	10.6	5364	6.9
P2	56/F	None	41	+	+	0403/0901	8827	9.8	14542	8.8
P3	54/M	None	32	−	+	0405/0901	8401	8.2	10842	12.5
P4	66/M	None	46	−	+	0405/0901	1003	2.6	7996	10.5
P5	53/F	None	35	−	−	1502/0406	1522	2.3	5390	5.8
P6	69/M	PSL	21	+	+	0410/0802	720	2.3	5050	7.2
P7	27/F	PSL	67	−	+	0405/0901	808	2.1	8881	9.6
P8	59/M	None	59	−	+	1502/1401	2097	1.8	5192	5.1
P9	53/F	PSL	34	−	−	1302/0901	1940	1.0	10205	3.3
P10	75/F	None	65	−	+	1501/1502	545	1.0	1203	1.3
P11	69/F	PSL, DZ	24	+	+	1302/0802	460	1.0	9910	7.1
P12	59/M	None	39	−	−	1502/1101	1004	1.0	2629	3.3
P13	39/F	None	63	−	+	1502/1302	301	0.9	1734	3.9
P14	49/F	None	38	−	−	1501/0404	536	0.8	3503	5.9
SLE patients										
L1	40/F	PSL, AZ	32	+	+	0101/0406	1094	6.0	2192	10.5
L2	43/F	PSL, CY	46	+	+	1401	704	2.4	1640	4.4
L3	54/M	PSL	278	−	−	1501/0405	222	1.9	3343	7.8
L4	55/F	PSL	254	−	−	1401/0802	124	1.5	621	3.4
L5	20/F	PSL, MZ	226	+	−	0403/0901	102	1.2	798	5.1
L6	26/M	PSL, AZ	250	−	−	1502/0901	99	1.2	282	1.0
L7	34/F	PSL	218	−	−	1501/0901	382	1.0	201	1.0
Healthy donors										
D1	39/M			−	−	1502/1302	2410	2.4	10943	11.5
D2	44/F			−	−	1302/0803	708	2.0	2659	4.6
D3	29/F			−	ND	1502/0405	1986	1.8	8827	8.0
D4	34/M			−	−	1202/1403	541	1.0	13021	16.9
D5	29/F			−	ND	0405/1403	1320	1.0	6451	5.0
D6	38/F			−	ND	1502/0901	440	1.0	2605	3.1
D7	27/M			−	−	1501/0901	986	0.8	10400	7.9
D8	34/M			−	−	0803/0901	798	0.8	6349	3.1
D9	32/M			−	−	0101/0901	274	0.8	4508	12.1
D10	58/F			−	ND	0101/1302	769	0.8	3075	3.2

ND, not determined; SI, stimulation index; *PSL, prednisolone; DZ, danazol; AZ, azathioprine; CY, cyclophosphamide; MZ, mizoribine.

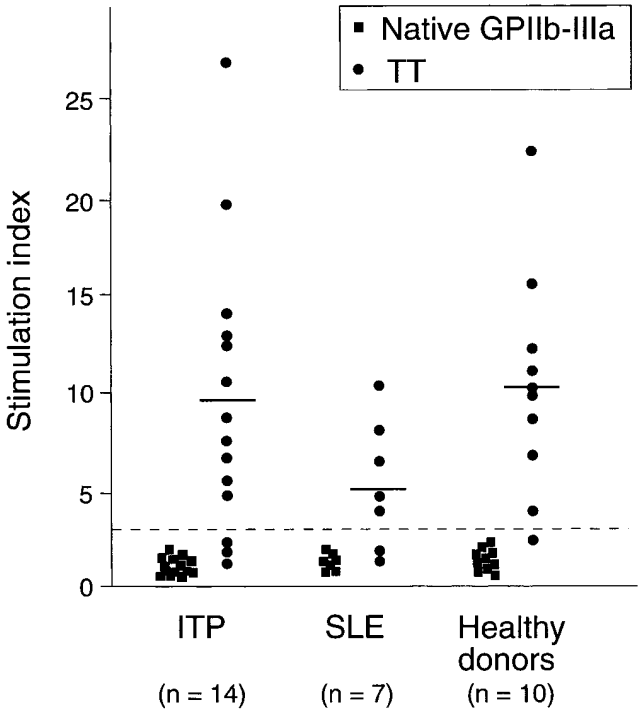

Figure 1. T cell proliferative responses to native GPIIb-IIIa and TT in 14 ITP patients, 7 SLE patients, and 10 healthy donors. Peripheral blood T cells were stimulated for 7 d with native GPIIb-IIIa (10 μg/ml) or TT (5 μg/ml). The short horizontal line indicates the mean of stimulation indices, and the broken line indicates the cut-off level.

significant T cell responses to reduced GPIIb-IIIa, whereas none of the healthy donors showed a T cell response to reduced GPIIb-IIIa. An SLE patient with thrombocytopenia (L1) also showed a reduced GPIIb-IIIa–induced T cell proliferation. There were no differences in age, gender, current or previous therapy, or platelet count between the 3 ITP responders and 11 ITP nonresponders. All four responders, including three ITP patients and one SLE patient, had platelet-associated anti–GPIIb-IIIa antibody. It is worthy to note that the DR4-associated alleles were detected in all four responders. Surprisingly, a T cell response to trypsin-digested GPIIb-IIIa was detected in 13 ITP patients, 5 SLE patients, and 10 healthy donors. The experiments using reduced and trypsin-digested GPIIb-IIIa were repeated in seven ITP patients and six healthy donors, and the results were consistently reproducible. Furthermore, peripheral blood T cells from two healthy donors (D4 and D8) responded to purified autologous GPIIb-IIIa which was digested with trypsin.

Specific induction of a secondary proliferative response by recombinant GPIIbα fusion proteins. To confirm the specificity of the T cell response for GPIIb-IIIa, peripheral blood T cells primed by trypsin-digested GPIIb-IIIa were stimulated with recombinant IIbα fusion proteins in secondary cultures in three ITP patients and one heathy donor. As shown in Fig. 2, when [^{3}H]thymidine incorporation was measured after 3 d of the secondary culture, trypsin-digested GPIIb-IIIa, as well as one recombinant GPIIbα fragment (IIbα18-259), but not GST, induced T cell proliferation.

Effect of anti-HLA class II mAbs on T cell proliferation induced by modified GPIIb-IIIa. The effect of anti-DR, anti-DQ, and anti-DP mAbs on the T cell proliferative response to reduced or trypsin-digested GPIIb-IIIa was examined in six ITP patients, two SLE patients, and four healthy donors, and the representative results are shown in Fig. 3. Anti-DR mAb was effective in inhibiting T cell proliferation induced by modified GPIIb-IIIa, compared with anti-DQ and anti-DP mAbs. Inhibition of T cell proliferation by anti-DR mAb was dose dependent when various concentrations (0.01–1 μg/ml) of anti-DR mAb were tested.

Determination of T cell subsets responsive to modified GPIIb-IIIa. To identify T cell subsets reactive with modified GPIIb-IIIa, the effect of depletion of CD3$^+$ cells, CD4$^+$ cells, and CD8$^+$ cells on T cell proliferation induced by reduced or trypsin-digested GPIIb-IIIa was examined in two ITP patients and one healthy donor. As shown in Fig. 4, T cell proliferation was almost completely lost when CD3$^+$ or CD4$^+$ cells, but not

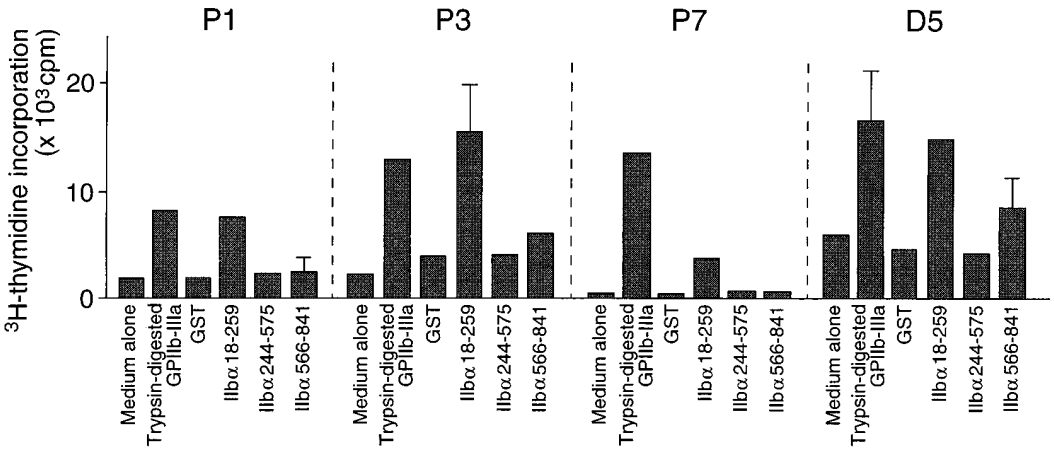

Figure 2. Proliferative responses to GPIIbα fusion proteins in trypsin-digested GPIIb-IIIa–primed T cells of ITP patients P1, P3, P7, and in healthy donor D5. Peripheral blood T cells were stimulated with trypsin-digested GPIIb-IIIa (10 μg/ml) for 7 d. The viable cells were stimulated with trypsin-digested GPIIb-IIIa (10 μg/ml), GST (10 μg/ml), or GPIIbα fusion proteins (IIbα18-259, IIbα244-575, or IIbα566-841; 10 μg/ml) for 3 d in the presence of rIL-2 (50 U/ml). SDs are not shown in case of $< 20\%$ of the mean or < 100 cpm.

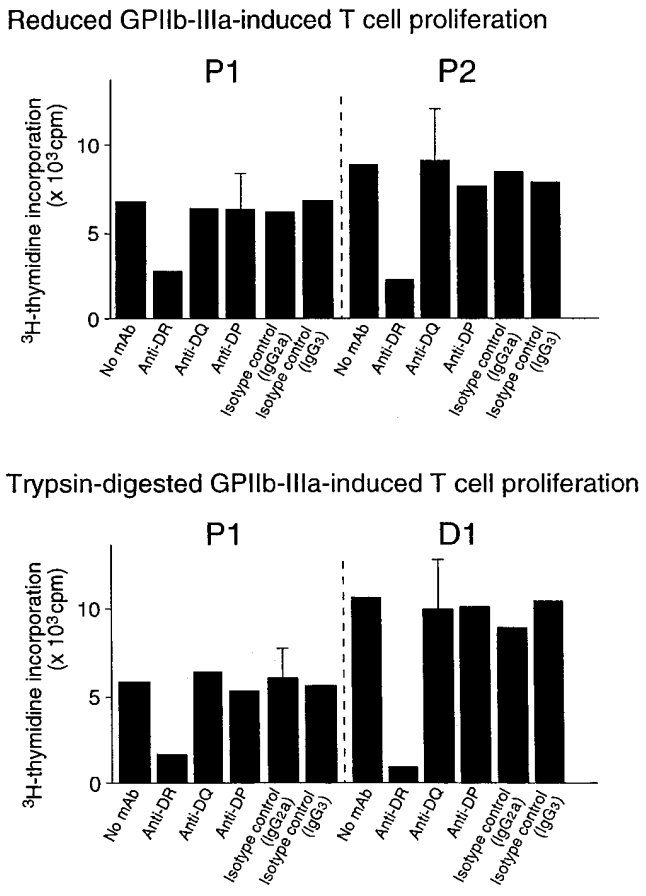

Figure 3. Effect of HLA class II mAbs on T cell proliferation induced by modified GPIIb-IIIa in ITP patients P1 and P2, and in healthy donor D1. Peripheral blood T cells were stimulated with reduced or trypsin-digested GPIIb-IIIa (10 μg/ml) for 7 d. Anti-DR, anti-DQ, anti-DP, and mouse isotype control (IgG_2a or IgG_3) mAbs were added at the initiation of cultures. SDs are not shown in case of $< 20\%$ of the mean or < 100 cpm.

$CD8^+$ cells, were depleted. Similar results were obtained in additional six ITP patients and four healthy donors.

Kinetics of T cell response to modified GPIIb-IIIa. T cell proliferative responses to reduced GPIIb-IIIa, trypsin-digested GPIIb-IIIa, and TT were measured on days 3, 5, 6, and 7 of cultures in responders, including five ITP patients and four healthy donors. Representative results are shown in Fig. 5. T cell responses to reduced or trypsin-digested GPIIb-IIIa in ITP patients were detected at day 5 and peaked at day 6, whereas T cells from healthy donors did not show a T cell response to trypsin-digested GPIIb-IIIa at day 5, but responded at day 7. Three additional ITP patients and two additional healthy donors showed a similar time course of T cell proliferation. The mean stimulation index of T cell proliferation induced by trypsin-digested GPIIb-III at day 5 in five ITP patients was significantly greater than that in four healthy donors (6.7 ± 1.0 versus 1.1 ± 0.2, $P = 0.001$ by Student's t test). The accelerated T cell response to modified GPIIb-IIIa observed in ITP patients but not in healthy donors is consistent with in vivo activation of these T cells.

In vitro antigen-induced anti–GPIIb-IIIa antibody production in PBMC cultures. IgG anti–GPIIb-IIIa antibody levels were measured in the supernatants of PBMCs cultured with native or modified GPIIb-IIIa in five ITP patients, two SLE patients, and three healthy donors (Table II). Three ITP patients, P1, P2, and P3, all of whom showed T cell proliferation to reduced GPIIb-IIIa, produced anti–GPIIb-IIIa antibody when PBMCs were stimulated with reduced GPIIb-IIIa. Anti–GPIIb-IIIa antibody was also detected in PBMC cultures stimulated with trypsin-digested GPIIb-IIIa in all five ITP patients. None of the PBMC culture supernatants from healthy donors contained a significant amount of IgG anti–GPIIb-IIIa antibody, whereas all of them showed trypsin-digested GPIIb-IIIa–induced T cell proliferation. PBMC cultures without antigenic stimulation did not produce anti–GPIIb-IIIa antibody in ITP patients. In contrast, anti–GPIIb-IIIa antibody was produced without antigenic stimulation in PBMC culture of SLE patient L1, although enhancement of anti–GPIIb-IIIa antibody synthesis was observed upon stimulation with modified GPIIb-IIIa.

We further examined whether anti–GPIIb-IIIa antibody synthesized in vitro by PBMCs bound to normal platelets. As shown in Fig. 6, anti–GPIIb-IIIa antibody reactivity was absorbed by preincubation of culture supernatants of ITP patient P2 with platelets from two healthy donors, but not by preincubation with erythrocytes or PBMCs. Similar results were obtained when culture supernatants from another ITP patient (P1) were tested.

The effect of $CD4^+$ T cell or $CD8^+$ T cell depletion and anti-HLA class II mAbs on in vitro anti–GPIIb-IIIa antibody production was examined in four ITP patients, and the representative results are shown in Fig. 7. Anti–GPIIb-IIIa antibody synthesis induced by reduced or trypsin-digested GPIIb-IIIa was almost completely blocked by the depletion of $CD4^+$ T cells or the addition of anti-DR mAb, indicating that $CD4^+$ and HLA-DR–restricted T cells responsive to modified GPIIb-IIIa are necessary for in vitro IgG anti–GPIIb-IIIa antibody synthesis.

Longitudinal analysis of T cell response to modified GPIIb-IIIa. Antigen-specific T cell proliferative response and in vitro anti–GPIIb-IIIa antibody production induced by trypsin-

Table II. IgG Anti–GPIIb-IIIa Antibody Levels in Supernatants of PBMC Cultures in ITP Patients, SLE Patients, and Healthy Donors

	Antigens added to PBMC cultures					
	No antigen	Native GPIIb-IIIa	Reduced GPIIb-IIIa	Trypsin-digested GPIIb-IIIa	Trypsin	TT
ITP patients						
P1	0.013	0.021	0.324	0.269	0.009	0.019
P2	0.018	0.012	0.378	1.160	0.012	0.005
P3	0.019	0.012	0.109	0.381	0.000	0.009
P7	0.009	0.024	0.041	0.992	0.018	0.011
P13	0.011	0.030	ND	0.417	0.002	0.010
SLE patients						
L1	0.106	0.092	0.182	0.243	0.123	0.140
L4	0.002	0.004	0.000	0.006	0.002	0.000
Healthy donors						
D1	0.007	0.017	0.011	0.017	0.010	0.008
D4	0.000	0.001	0.007	0.009	0.004	0.000
D9	0.006	ND	ND	0.019	ND	0.004

ND, not determined. Results are expressed as OD_{450}.

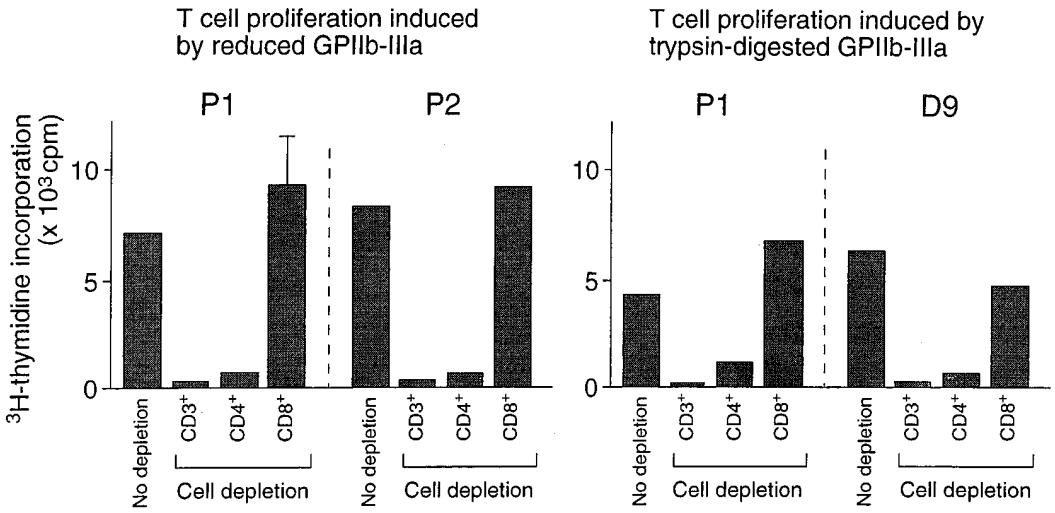

Figure 4. Effect of depletion of $CD3^+$ cells, $CD4^+$ cells, and $CD8^+$ cells on T cell proliferation induced by modified GPIIb-IIIa in ITP patients P1 and P2, and in healthy donor D9. $CD3^+$, $CD4^+$, and $CD8^+$ cells were removed from peripheral blood T cells using anti-CD3, anti-CD4, and anti-CD8 mAb-coupled magnetic beads, respectively. T cell fractions were stimulated with reduced or trypsin-digested GPIIb-IIIa (10 μg/ml) for 7 d. SD are not shown in case of < 20% of the mean or < 100 cpm.

digested GPIIb-IIIa were serially analyzed in a representative ITP patient (P7). This patient maintained safe platelet count (25–94 $\times$ 10^9/liter) without any treatment until May of 1997, when she was given 60 mg/d of oral prednisolone because of significant mucous membrane bleeding with a platelet count of 12 $\times$ 10^9/liter. An effective response occurred initially, but platelet count decreased gradually as prednisolone was tapered. Splenectomy was performed in November, 1997, and thereafter her platelet count remains > 150 $\times$ 10^9/liter. As shown in Table III, amounts of IgG anti–GPIIb-IIIa antibody produced in vitro in response to trypsin-digested GPIIb-IIIa were negatively correlated with platelet count during the course of the disease.

Discussion

We have identified autoreactive T cells to platelet membrane GPIIb-IIIa, a major target of anti–platelet autoantibodies in chronic ITP. Peripheral blood T cells from ITP patients and healthy donors were virtually unresponsive to native GPIIb-IIIa complex but responded upon stimulation with reduced or trypsin-digested GPIIb-IIIa. The specificity of the T cell proliferative response for GPIIb-IIIa was confirmed by the specific activation of modified GPIIb-IIIa–primed T cells by one of recombinant GPIIbα fragments in secondary cultures. A major proportion of T cells reactive with GPIIb-IIIa had a $CD4^+$ phenotype and were restricted by HLA-DR. These features are consistent with those of previously reported T cells to antigens targeted by autoantibodies, such as topoisomerase I (23), insulin (10), and acetylcholine receptor α (24). We believe that these T cells reactive with GPIIb-IIIa are involved in the production of pathogenic anti–GPIIb-IIIa antibody in ITP patients, since T cell responses to GPIIb-IIIa were accelerated in ITP patients, suggesting that these T cells were activated in vivo in ITP patients; IgG anti–GPIIb-IIIa antibody capable of binding to normal platelets could be induced in vitro by HLA-DR–restricted and $CD4^+$ T cells from ITP patients in the presence of GPIIb-IIIa; and amounts of IgG anti–GPIIb-IIIa antibody produced in vitro were negatively correlated with platelet count in parallel with treatment response in a representative ITP patient.

Native GPIIb-IIIa induced no proliferative response of peripheral blood T cells from all subjects examined. T cells recognizing peptides generated from native GPIIb-IIIa by normal processing pathways are hypothesized to be deleted in the thymus ("negative selection") since GPIIb-IIIa is shown to be expressed abundantly as early as the 16th week of intrauterine life on epithelial cells of thymic stroma (25). However, Filion et al. recently reported that $CD4^+$ T cells reactive with dioleoylphosphatidyl choline/dioleoylphosphatidyl serine liposome-encapsulated GPIIb-IIIa were detected in peripheral

Table III. Serial Analysis of T Cell Proliferative Response to GPIIb-IIIa and In Vitro Anti–GPIIb-IIIa Antibody Production in ITP Patient P7

	October, 1996	July, 1997	October, 1997	March, 1998
Prednisolone dosage (mg/d)	None	12.5	8	4
Platelet count ($\times$ 10^9/liter)	67	144	21	220
IgG anti–GPIIb-IIIa antibody in platelet eluate (OD_{450})	0.528	0.446	0.506	0.258
T cell proliferative response to trypsin-digested GPIIIb-IIIa (SI)	9.6	5.9	6.1	10.6
T cell proliferative response to TT (SI)	10.8	7.8	ND	8.4
IgG anti–GPIIb-IIIa antibody produced in vitro in response to trypsin-digested GPIIb-IIIa (OD_{450})	0.992	0.183	0.867	0.314

ND, not determined. P7 received oral prednisolone (60 mg/d) in May of 1997, and was splenectomized in November of 1997.

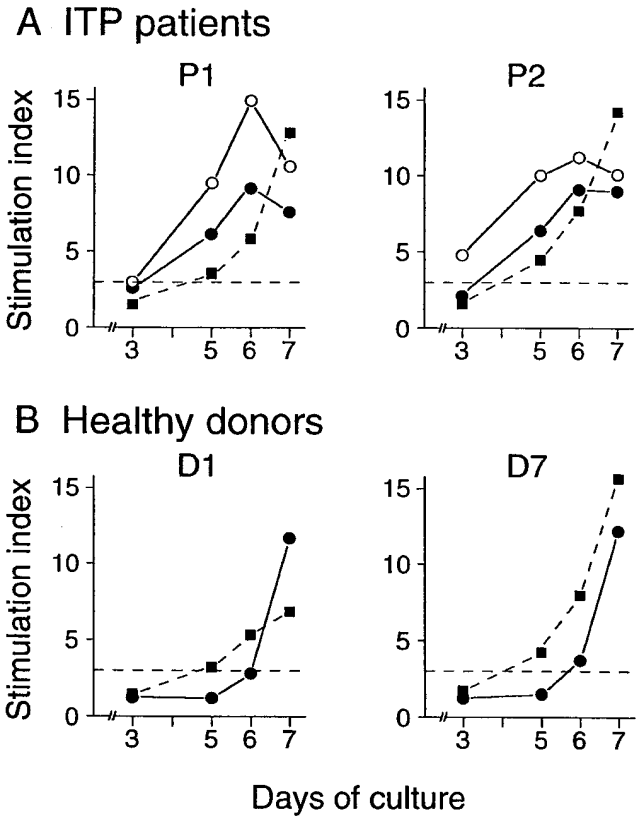

Figure 5. Kinetics of T cell proliferation induced by modified GPIIb-IIIa and TT in (*A*) ITP patients P1 and P2 and in (*B*) healthy donors D1 and D7. Peripheral blood T cells were stimulated with reduced GPIIb-IIIa (10 μg/ml; *open circles*), trypsin-digested GPIIb-IIIa (10 μg/ml; *closed circles*), or TT (5 μg/ml; *closed squares*) for 3, 5, 6, and 7 d.

blood from the majority of healthy individuals (18). In Filion's report, the GPIIb-IIIa–reactive T cells, which proliferated only in the presence of exogenous IL-2, did not produce their own IL-2 but expressed a high-affinity IL-2 receptor α on their surface. These features are consistent with characteristics of anergic T cells, which is a functionally inactivated state of T cells after antigenic stimulation in the periphery (26). Therefore, T cell tolerance to GPIIb-IIIa is tightly maintained under normal circumstances; some T cells to native GPIIb-IIIa escape thymic deletion and exist in the periphery, but are inactivated by a post-thymic mechanism of peripheral tolerance (27).

GPIIb-IIIa is known to contain several polymorphisms, known as human platelet antigen systems, which are targets of alloantibodies leading to neonatal thrombocytopenia or post-transfusion purpura (28). In case of neonatal alloimmune thrombocytopenia, an HLA-DR–restricted T cell response to GPIIIa peptides containing the polymorphic region was detected in a susceptible mother (29). However, it is unlikely that GPIIb-IIIa–reactive T cells detected in our study were alloreactive T cells for the following reasons: T cell responses to modified GPIIb-IIIa were accelerated in ITP patients, indicating that these T cells were already primed in vivo, although only one of the ITP patients had received a platelet transfusion; T cells from healthy donors responded to purified autologous trypsin-digested GPIIb-IIIa; and GPIIb-IIIa–primed T cells from ITP patients and a healthy donor responded to a recombinant protein expressing amino acids 18–259 of GPIIbα, in which no polymorphism has been described (28).

It is now apparent that T cells recognizing "cryptic" self-peptides which, under normal circumstances, are not processed and presented with resultant induction of central or peripheral self tolerance, exist in the normal T cell repertoire (30). It has been shown that autoreactive T cells are not activated by pep-

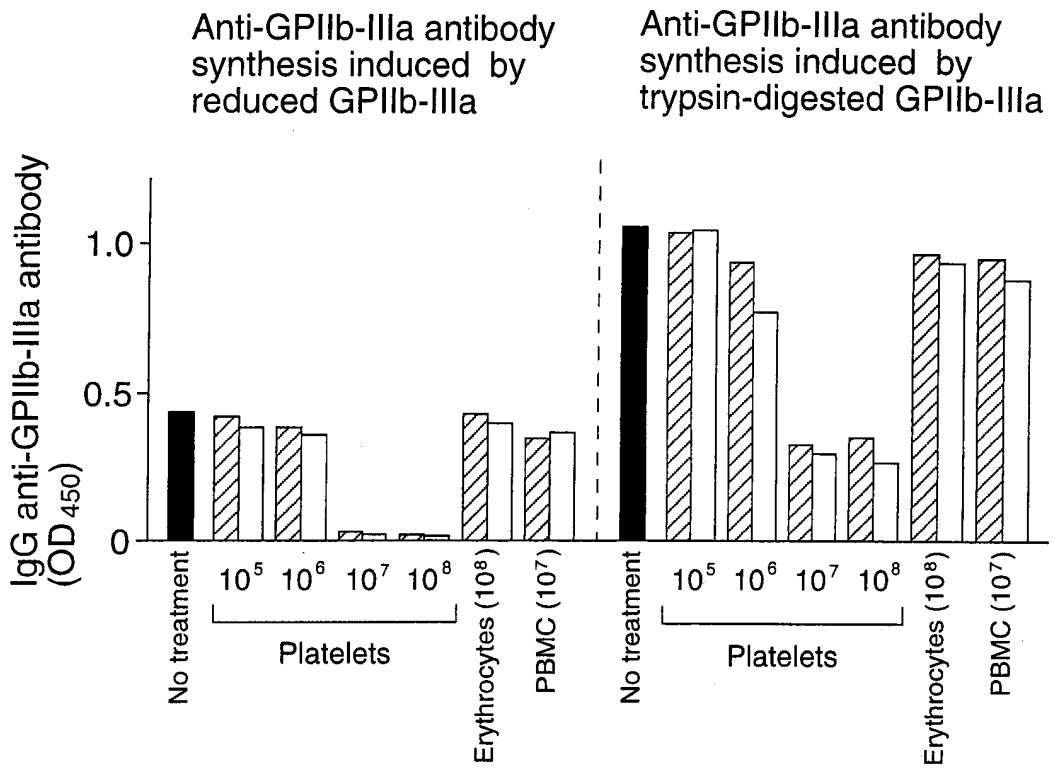

Figure 6. Absorption of IgG anti–GPIIb-IIIa antibody in PBMC culture supernatants by incubation with normal platelets. PBMCs from ITP patient P2 were stimulated with reduced or trypsin-digested GPIIb-IIIa (10 μg/ml) for 10 d in the presence of PWM (1 μg/ml). Culture supernatants were then incubated with serial numbers of platelets (10^5–10^8), erythrocytes (10^8), or PBMCs (10^7) obtained from two independent healthy donors (*hatched* and *open bars*). IgG anti–GPIIb-IIIa antibody levels in untreated (*closed bars*) or treated supernatants were measured by anti–GPIIb-IIIa ELISA. SD are not shown in case of < 20% of the mean or < 0.010.

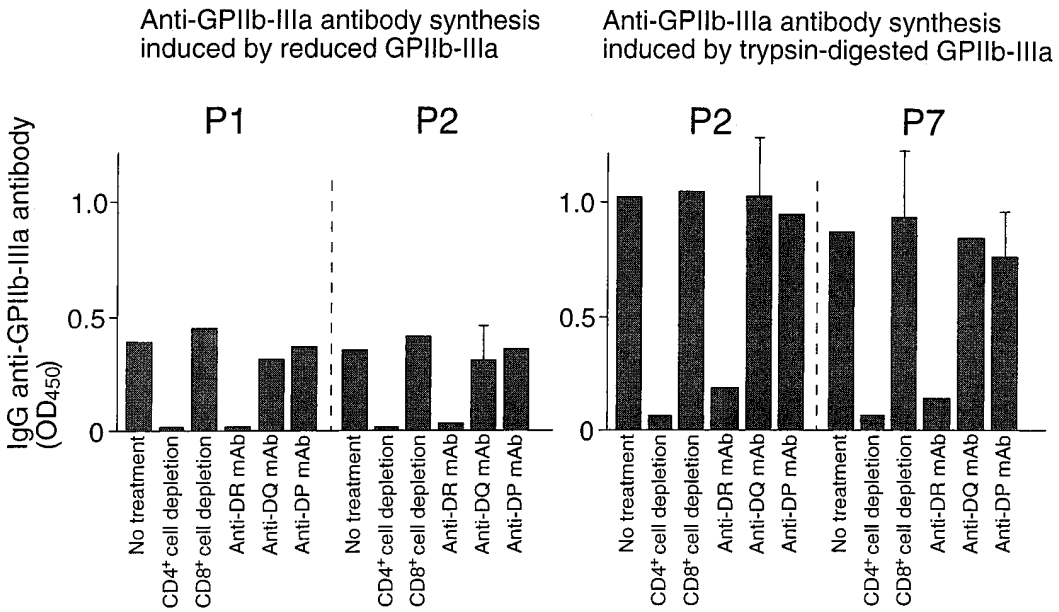

Figure 7. Effect of $CD4^+$ cell depletion, $CD8^+$ cell depletion, and anti-HLA class II mAbs on modified GPIIb-IIIa–induced IgG anti–GPIIb-IIIa antibody production by PBMCs from ITP patients P1, P2, and P7. $CD4^+$ and $CD8^+$ cells were removed from PBMCs using anti-CD4 and anti-CD8 mAb-coupled magnetic beads, respectively. Cells were stimulated with reduced or trypsin-digested GPIIb-IIIa (10 μg/ml) for 10 d in the presence of PWM (1 μg/ml). Anti-DR, anti-DQ, and anti-DP mAbs (0.2 μg/ml) were added at the initiation of cultures. IgG anti–GPIIb-IIIa antibody levels in culture supernatants were measured by anti–GPIIb-IIIa ELISA. SD are not shown in case of $< 20\%$ of the mean or < 0.010.

tides generated by processing from a native self-protein, but can be activated by cryptic determinants (31). Because determinant dominance is influenced by protein structure, conditions that change the molecular context of autoantigens may permit presentation of previously cryptic determinants, thereby breaking T cell tolerance. T cells directed against cryptic self-peptides are presumed to remain naive in that they rarely encounter the appropriate self-peptides. These features of T cells recognizing cryptic epitopes are consistent with those of T cells to GPIIb-IIIa based on the following findings. First, trypsin-digested GPIIb-IIIa–reactive T cells were detected in healthy donors, indicating that these T cells were a component of the normal T cell repertoire. Second, accelerated T cell responses to GPIIb-IIIa in ITP patients indicate recent antigen exposure or in vivo activation of T cells, whereas kinetics of modified GPIIb-IIIa–induced T cell responses in healthy donors are more typical of those of naive T cell responses (32). Similar differences in kinetics of autoantigen-specific T cell proliferation in patients versus healthy individuals were shown in T cell responses to type II collagen in rheumatoid arthritis (33) and to topoisomerase I in scleroderma (8).

The mechanism of antigen exposure that resulted in the activation of GPIIb-IIIa–reactive T cells in ITP patients is unknown. It has been shown that cryptic epitopes can be revealed due to factors that affect normal processing of self-proteins, such as artificial cleavage of self-proteins or complex formation of self-proteins with other proteins (31, 34). Similar mechanisms that affect normal processing of GPIIb-IIIa might occur in ITP patients. For example, the complex of GPIIb-IIIa and its ligand may induce the expression of cryptic epitopes, since fibrinogen and other GPIIb-IIIa-binding proteins have the Arg-Gly-Asp (RGD) sequence with the proper conformation and it has been shown that other unrelated proteins with the RGD sequence can bind to GPIIb-IIIa (35). In this case, platelets themselves may be effective APCs that activate GPIIb-IIIa–reactive T cells, in view of the finding of Semple et al. that an enhanced expression of HLA-DR and CD80 has been observed on circulating and splenic platelets from ITP patients (36). Another possibility is that ITP patients have been exposed to proteins that cross-react with GPIIb-IIIa. It is reported that autoreactive T cells and autoantibodies can be generated in normal mice by coimmunization with a mixture of self- and foreign autoantigen (37). The mechanism is thought to be the generation of cross-reactive B cells, initially primed by foreign protein serving as a molecular mimic, that then bind, process, and present self-protein. Since B cells that acquire antigen via surface Ig require approximately 1,000- to 10,000-fold less antigen to induce responses, B cells would have the ability to efficiently concentrate and present to T cells small quantities of determinants that are typically sequestered (7, 38). Cross-reactive B cells can subsequently prime naive autoreactive T cells if they express the appropriate costimulatory molecules (39). In this regard, it has been reported that anti-GPIIIa antibodies in patients with HIV-related ITP cross-react with HIV-associated gp120 (40).

It is interesting to note that antigen-induced IgG anti–GPIIb-IIIa antibody production in PBMC cultures was not observed in healthy individuals, although T cells reactive with trypsin-digested GPIIb-IIIa were present in peripheral blood. This is analogous to in vitro production of anti–topoisomerase I autoantibody in PBMC cultures from scleroderma patients versus healthy individuals (12). This finding could be explained by the mechanism that circulating B cells capable of producing IgG anti–GPIIb-IIIa antibody were present in ITP

patients but were absent in healthy donors, since circulating B cells producing IgG anti–GPIIb-IIIa antibody are presumed to be "memory" B cells, which have already undergone isotype-switching and affinity maturation. Another possibility is a difference in the functional property of GPIIb-IIIa–reactive T cells, such as cytokine profiles (Th1 versus Th2 phenotype) (41).

IgG anti–GPIIb-IIIa antibody synthesis was observed in PBMC cultures without antigenic stimulation in a SLE patient with thrombocytopenia and platelet-associated anti–GPIIb-IIIa antibody. This is consistent with spontaneous production of anti-DNA antibodies in PBMC cultures of SLE patients (42). However, antigenic stimulation with GPIIb-IIIa enhanced anti–GPIIb-IIIa antibody production, suggesting that GPIIb-IIIa–reactive T cells play some roles in activating B cells in SLE. This might reflect an intrinsic difference in anti–platelet autoantibody production between ITP and SLE: antigen-specific response in ITP versus polyclonal response in SLE. Further studies analyzing a large number of patients are necessary to test this hypothesis.

In summary, our results indicate that possible pathogenic anti–GPIIb-IIIa autoantibodies in ITP patients are produced by activation of autoreactive T cells to GPIIb-IIIa. Possible mechanisms that result in the activation of GPIIb-IIIa–reactive T cells include expression of cryptic determinants of GPIIb-IIIa and generation of cross-reactive B cells. Further investigation of autoreactive T cells to platelet membrane GPs may provide a clue to the pathogenesis of chronic ITP and may be of therapeutic use in treating refractory patients.

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