APS proposed by the International Workshop.[11] Primary APS was diagnosed in 3 of the patients, whereas the remaining 2 had secondary APS accompanied by systemic lupus erythematosus. At the time of blood examination, all the patients were on low-dose corticosteroids (< 10 mg/d) and aspirin. Samples from 6 healthy individuals possessing DRB4*0103 confirmed by polymerase chain reaction–based genotyping[12] were used in experiments on priming the p276-290–specific T-cell response. All samples were obtained after the patients and control subjects gave their written informed consent in accordance with the Declaration of Helsinki, as approved by the Keio University Institutional Review Board (Tokyo, Japan).

Antigen preparations

Native β_2GPI was purified from normal pooled human plasma as described elsewhere.[13] Nicked and reduced β_2GPI was prepared by treating β_2GPI with plasmin[14] and dithiothreitol,[5] respectively. Fusion recombinant maltose-binding proteins (MalBPs) expressed in *Escherichia coli* included GP-F and GP3, which encoded the entire amino acid sequence (amino acids 1-326) and domains IV and V (amino acids 182-326), respectively, of human β_2GPI.[5] MalBP, a fusion partner, was also prepared as a control antigen. GP-F and GP3 lacking MalBP, GP-F/MalBP(−), and GP3/MalBP(−) were prepared by incubating MalBP fusion proteins with factor Xa followed by the removal of MalBP and factor Xa by passing the mixture through amylose resin and benzamidine-Sepharose columns, respectively. A recombinant polypeptide encoding domain V of β_2GPI (amino acids 242-326; rDomain V) was expressed by a *Pichia pastoris* expression system.[15] Peptides encompassing amino acids 276-290 and 306-320 of β_2GPI (p276-290 and p306-320) were synthesized using a solid-phase multiple synthesizer (Advanced ChemTech, Louisville, KY) and purified by high-performance liquid chromatography. These peptides had potential DR53-binding anchor residues,[16] but the binding capacity to the DR53 molecule was not examined. Capacity of individual antigen preparations to bind anionic PL was evaluated by competitive inhibition of an interaction between native β_2GPI and immobilized cardiolipin.[13] Briefly, cardiolipin-coated plates were incubated with native β_2GPI in the presence of an excess amount of individual antigen preparations. The β_2GPI-cardiolipin complex was detected by incubation with a monospecific APS serum positive for a high titer of anti-β_2GPI antibodies.

Preparation of PL liposomes

Dipalmitoylphosphatidylserine (DPPS), phosphatidylserine from bovine brain (BBPS), and cardiolipin from bovine heart were purchased from Sigma Chemical (St Louis, MO); dilauroylphosphatidylserine (DLPS), dimyristoylphosphatidylserine (DMPS), dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylcholine (DOPC), and monooleoylphosphatidylserine (MOPS) were from Avanti Polar Lipids (Alabaster, AL); and a lyso form of BBPS (lyso-BBPS) was from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). All chemicals were of reagent-grade quality. The fatty acid chains of bovine tissue–derived BBPS and cardiolipin were not well characterized, but all other PLs were chemically synthesized. Liposomes were prepared principally as described previously,[17] with a lipid composition of DOPC at a molar ratio of 7:3 with the following PLs: DOPS, DPPS, DLPS, DMPS, MOPS, BBPS, lyso-BBPS, and cardiolipin. A mixture of the desired lipids in chloroform-methanol (1:1) was placed in a pear-shaped flask and solvent was removed in a rotary evaporator under reduced pressure. The dried lipids were dispersed with a vortex mixture in sterilized 0.3 M glucose solution. The liposome solutions were then sonicated for 1 minute at 70°C with a bath-type sonicator and adjusted to 1 μmol lipid/mL. These PL liposomes were preincubated with or without native β_2GPI (100 μg/mL) for 30 minutes at room temperature before addition to the cultures. The capacity of PLs to bind β_2GPI was evaluated by an assay as described[13] with some modifications. Briefly, individual PLs were coated on microtiter plates and subsequently incubated with β_2GPI. The PL-β_2GPI complex was detected by anti-β_2GPI monoclonal antibody (mAb) Cof-23.[18]

Culture media

All cultures were incubated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Before use in culture, the fetal bovine serum was depleted of bovine β_2GPI using heparin-Sepharose as described previously[5] to avoid its potential influence on the generation of the epitope peptide.

The p276-290–specific CD4+ T-cell lines

T-cell lines reactive with p276-290 were generated from peripheral blood T cells by the repeated stimulation with GP-F followed by limiting dilution as described previously.[6] A total of 7 $CD4^+$ T-cell lines established from patients with APS were used in this study. Four of them (KS3, OM2, OM7, and EY3) were confirmed to be clones based on the single functional T-cell receptor β-chain and were reported in detail previously.[6,7] The clonality of the remaining 3 lines (OM-b, KY-a, KM-b) was not determined. All the T-cell lines recognized p276-290 in the context of HLA-DRB4*0103, had the T-helper 0 (Th0)/Th1 phenotype expressing interferon γ (IFN-γ), and had the capacity to induce anti-β_2GPI antibody production from autologous B cells. T-cell lines were maintained by repetitive stimulation with GP-F, recombinant human interleukin 2 (IL-2; 100 U/mL), and irradiated autologous APCs at 7- to 10-day intervals.

Preparation of APCs

Epstein-Barr virus–transformed lymphoblastoid B-cell line cells (LBLs) were generated from all patients with APS. Circulating monocytes and B cells were isolated from peripheral blood mononuclear cells using anti-CD14 or anti-CD19 mAb-coupled magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) followed by magnetic-activated cell separation (MACS) column separation according to the manufacturer's protocol. Flow cytometric analysis revealed that purity of monocyte and B-cell fractions was greater than 98%. Macrophages and immature monocyte-derived dendritic cells (DCs) were obtained from plastic adherent peripheral blood mononuclear cells in the presence of macrophage-colony stimulating factor (R&D Systems, Minneapolis, MN) or granulocyte-macrophage-colony stimulating factor and IL-4 (PeproTech, Rocky Hill, NJ), respectively, according to previously published methods.[19] Macrophages and immature DCs were pulsed with antigen and subsequently incubated with 50 ng/mL tumor necrosis factor α (TNF-α; PeproTech) for 24 hours to induce activation/maturation before being used in the assays. Flow cytometric analysis revealed that the activated macrophage fraction contained greater than 98% $CD14^+CD80^+$ cells, and the mature DC fraction contained greater than 95% $CD83^+HLA\text{-}DR^+$ cells. Allogeneic splenocytes were obtained from DR53-carrying patients with gastric cancer who required splenectomy as part of the dissection of tumor tissues.[20] In some experiments, endosomal processing inhibitor chloroquine (0.1 μM; Sigma Chemical) or brefeldin A (1 μg/mL; Sigma Chemical) was added to the APC cultures 1 hour before the addition of antigen.

Assays for antigen-specific T-cell response

Antigen-specific proliferation of T-cell lines was determined principally as described previously.[6] T-cell lines (2×10^4/well) were cultured with various combinations of irradiated APCs (2×10^4/well) and antigen. The APCs were autologous LBLs, monocytes, B cells, monocyte-derived DCs, macrophages, and allogeneic CD53-carrying splenocytes. Native β_2GPI, nicked β_2GPI, reduced β_2GPI, GP-F, GP3, GP-F/MalBP(−), GP3/MalBP(−), rDomain V (10 μg/mL), and p276-290 (5 μg/mL) were used as antigens containing amino acids 276-290 of human β_2GPI. Control antigens were MalBP (10 μg/mL) and p306-320 (5 μg/mL). PL liposomes that were preincubated with native β_2GPI, GP3/MalBP(−), or rDomain V were used at a final concentration of 0.1 μmol lipid and 10 μg protein per 1 mL. A combination of immobilized anti-CD3 mAb (30 ng/mL) and phytohemagglutinin (1 μg/mL) was also used to exclude nonspecific unresponsiveness. LBLs were irradiated at 100 Gy and all other APCs at 40 Gy before being mixed with T-cell lines. After 60 hours of incubation with antigen, 0.5

μCi/well (0.0185 MBq) [^{3}H]-thymidine was added to the cultures for 16 hours. The cells were then harvested and [^{3}H]-thymidine incorporation was determined in a Top-Count microplate scintillation counter (Packard, Meriden, CT). The antigen-induced T-cell response was also evaluated from the production of IFN-γ as described previously.[21] In some experiments, anti–HLA-DR (L243; immunoglobulin G2a [IgG2a]), anti–HLA-DQ (1a3; IgG2a), or an isotype-matched control mAb (1 μg/mL; Leinco Technologies, Ballwin, MO) were added at the initiation of the cultures. All experiments were carried out in duplicate or triplicate, and the values are the mean of multiple determinations.

In vitro priming of T cells responsive to p276-290 with β_2GPI-PL liposome complex

Peripheral blood T cells (2×10^6) isolated from peripheral blood mononuclear cells using anti-CD3 mAb-coupled magnetic beads (Miltenyi Biotech) were cultured with autologous mature DCs or TNF-α–stimulated macrophages that were previously pulsed with a mixture of BBPS liposomes and β_2GPI, BBPS liposomes, or β_2GPI alone. On day 3, IL-2 (30 units/mL) was added to the cultures. On day 10, viable T cells were harvested and we examined the capacity to produce IFN-γ in response to antigenic stimulation with autologous LBLs pulsed with MalBP, GP-F, native β_2GPI, p276-290, or p306-320. All culture experiments were carried out in duplicate, and all values represent the mean of duplicate determinations. Results were expressed after the background IFN-γ production was deducted.

Results

Conditions that induce the expression of p276-290 as a consequence of antigen processing

Various combinations of antigens and APCs were tested for their ability to stimulate p276-290–reactive CD4$^+$ T-cell lines generated from patients with APS. In this in vitro assay system, a response of p276-290–reactive T-cell line can be used as an indicator for the efficient presentation of p276-290 by APCs as a consequence of antigen processing. First, various types of DR53-carrying APCs pulsed with native β_2GPI were examined for their capacity to stimulate p276-290–reactive T-cell lines. Autologous LBLs, monocytes, B cells, mature DCs, activated macrophages, or allogeneic splenocytes bearing native β_2GPI failed to induce a proliferative response of the T-cell lines OM7 and KS3 (Figure 1A). Identical results were obtained using all 7 T-cell lines. Next, autologous LBLs were pulsed with various forms of β_2GPI and cultured with p276-290–reactive T-cell lines OM7 and KS3 (Figure 1B). A significant response was detected in the cultures with reduced β_2GPI and recombinant fusion proteins expressed in a bacterial expression system (GP-F and GP3), as shown in our previous study.[6] Interestingly, the capacity of GP-F and GP3 to stimulate p276-290–reactive T-cell lines was largely reduced when the fusion partner MalBP was removed from these recombinant fusion proteins. The addition of MalBP to these cultures did not reverse the response (data not shown), indicating that MalBP expressed as a fusion protein played a role in the T-cell response. In contrast, LBLs pulsed with native and nicked forms of β_2GPI or with rDomain V expressed in the eukaryotic expression system did not induce the T-cell response. Analogous findings were obtained from all 7 T-cell lines examined.

None of the individual β_2GPI preparations that stimulated p276-290–reactive T-cell lines were able to bind anionic PL (Figure 1B). Since β_2GPI binds anionic PLs mainly through the major PL-binding site located on a surface-exposed turn,[22] we hypothesized that the loss of the binding capacity in the β_2GPI preparations was due to internalization of the major PL-binding site by a

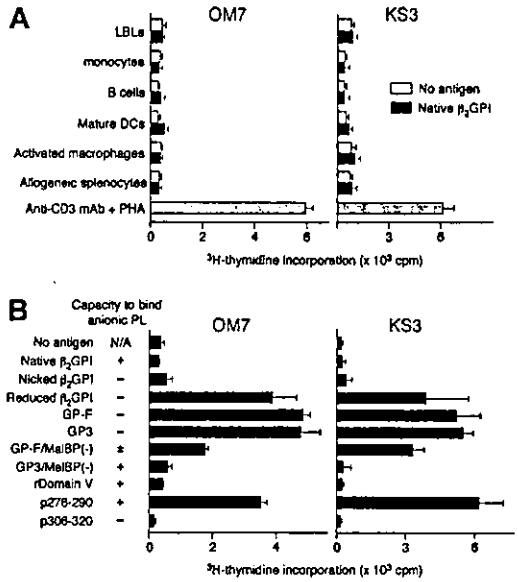

Figure 1. Response of p276-290–reactive T-cell lines to various combinations of DR53-positive APCs and β_2GPI preparations. (A) Various DR53-positive APCs were pulsed with or without native β_2GPI (■ and □, respectively) and subsequently cocultured with the p276-290–reactive T-cell lines OM7 and KS3. All APCs except allogeneic splenocytes were autologous cells. The antigen-induced T-cell response was measured by [^{3}H]thymidine incorporation. A □ indicates a control T-cell line stimulated by a combination of anti-CD3 mAb and phytohemagglutinin (PHA). A representative result from at least 2 independent experiments is shown. (B) Autologous LBLs were pulsed with various β_2GPI preparations and subsequently cocultured with the p276-290–reactive T-cell lines OM7 and KS3. The antigen-induced T-cell response was measured by [^{3}H]thymidine incorporation. The capacity to bind β_2GPI in individual preparations was assessed by inhibition of the interaction between native β_2GPI and immobilized cardiolipin. Results are shown as mean and standard deviation. A representative result from 3 independent experiments is shown.

structural modification and that this feature is important for the subsequent presentation of p276-290 that occurs as a result of antigen processing. If this were the case, the β_2GPI-PL complex, in which the major PL-binding site is covered by anionic PL, should stimulate p276-290–reactive T-cell lines. To test this possibility, immature DCs and macrophages were pulsed with DOPS liposomes that were preincubated with or without native β_2GPI, induced to mature/activate by TNF-α treatment, and used to stimulate the p276-290–reactive T-cell lines KS3 and EY3 (Figure 2). DCs bearing DOPS-bound β_2GPI induced a T-cell response, as was observed in those pulsed with GP-F or p276-290, but those bearing native β_2GPI or DOPS liposomes alone did not. In contrast, macrophages pulsed with DOPS-bound β_2GPI were less efficient in stimulating p276-290–reactive T-cell lines. Analogous findings were obtained from all 7 T-cell lines used in this study.

We tested various PL liposomes for their ability to stimulate the p276-290–reactive T-cell lines KS3, EY3, OM-b, and KM-b in the presence of β_2GPI using DCs as APCs. All p276-290–reactive T-cell lines proliferated upon recognition of DCs preincubated with β_2GPI and liposomes containing DOPS or BBPS (Figure 3A). There was a borderline response to a mixture of β_2GPI and cardiolipin-containing liposomes, but all other PLs failed to induce a proliferation irrespective of the presence or absence of β_2GPI. When T-cell response was evaluated by IFN-γ release in response to antigenic stimulation, the response of T-cell lines was specifically induced by liposomes containing DOPS or BBPS and to a lesser extent by liposomes containing cardiolipin (Figure 3B). All 4 T-cell lines represented similar findings, and the IFN-γ release assay appeared to be more sensitive than the proliferation assay. The assay to evaluate capacity of individual PLs to bind β_2GPI

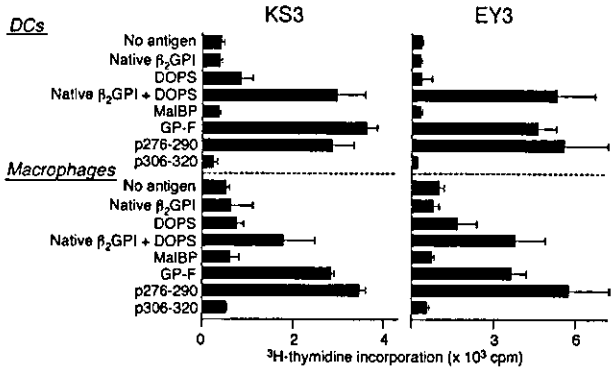

Figure 2. Response of p276-290–reactive T-cell lines in cultures of β_2GPI and anionic PL. Autologous immature DCs and macrophages were pulsed with native β_2GPI alone, DOPS liposomes, a mixture of native β_2GPI and DOPS liposomes, MalBP, or GP-F; induced to maturate or activate; and used to stimulate the p276-290–reactive T-cell lines KS3 and EY3. Synthetic peptides were pulsed with APCs before their coculture with T cells. The antigen-induced T-cell response was measured by [^{3}H]thymidine incorporation. Results are shown as mean and standard deviation. A representative result from at least 2 independent experiments is shown.

revealed that DOPS, BBPS, and cardiolipin were able to bind β_2GPI but the others were not (Figure 3C). These findings indicate that the capacity of individual PLs to induce a T-cell response was correlated with their β_2GPI-binding capacity. We further tested whether β_2GPI preparations with capacity to bind anionic PL (GP3/MalBP(−) and rDomain V) that were preincubated with PL liposomes induced a response of p276-290–reactive T-cell lines KS3 and EY3. DCs bearing DOPS liposomes preincubated with GP3/MalBP(−) or rDomain V induced a T-cell response, although these 2 β_2GPI preparations alone failed to induce a response.

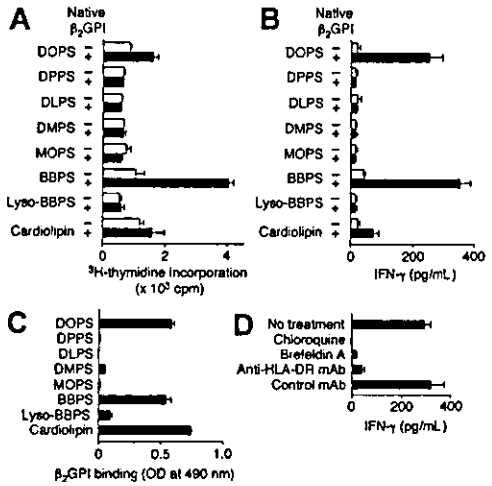

Figure 3. Binding of β_2GPI to anionic PL facilitates the processing and presentation of p276-290, which activates p276-290–reactive T-cell lines. (A) Autologous immature DCs were pulsed with or without a mixture of native β_2GPI and various PL liposomes (■ and □, respectively), induced to mature, and used to stimulate the p276-290–reactive T-cell line KS3. The antigen-induced T-cell response was evaluated by [^{3}H]thymidine incorporation. A representative result from 2 independent experiments is shown. (B) Autologous immature DCs were pulsed with or without a mixture of native β_2GPI and various PL liposomes (■ and □, respectively), induced to mature, and used to stimulate the p276-290–reactive T-cell line KS3. The antigen-induced T-cell response was evaluated by IFN-γ production. A representative result from 3 independent experiments is shown. (C) The capacity of individual PLs to bind β_2GPI was evaluated by a solid-phase assay. A representative result from 2 independent experiments is shown. OD indicates optical density. (D) The p276-290–reactive T-cell line OM-b was cultured with autologous DCs bearing DOPS-bound β_2GPI in the presence or absence of chloroquine, brefeldin A, anti–HLA-DR mAb, or control mAb. The antigen-induced T-cell response was evaluated by IFN-γ production. Results are shown as the mean and standard deviation. A representative result from 2 independent experiments is shown.

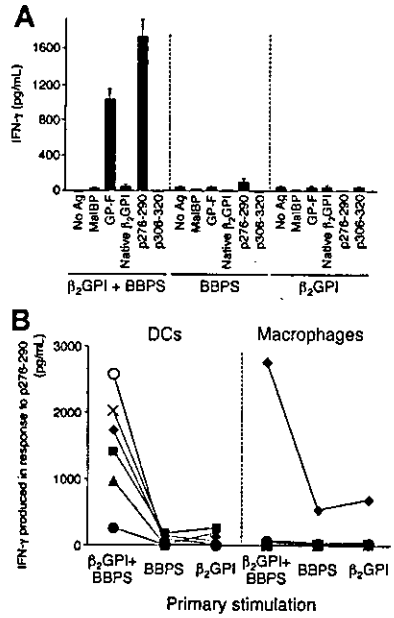

Figure 4. Induction of a T-cell response to p276-290 by BBPS-bound β_2GPI in peripheral blood T cells from DR53$^+$ healthy individuals in vitro. (A) Peripheral blood T cells from a representative DR53$^+$ healthy individual were stimulated with DCs pulsed with BBPS-bound β_2GPI, BBPS, or β_2GPI alone, and the T-cell response to MalBP, GP-F, native β_2GPI, p276-290, or p306-320 was measured by IFN-γ production. A representative result from 2 independent experiments is shown. (B) T-cell response to p276-290 after priming with autologous DCs or macrophages pulsed with BBPS-bound β_2GPI, BBPS, or β_2GPI alone in 6 DR53$^+$ healthy individuals. The p276-290–specific T-cell response was evaluated by IFN-γ production. The results from each of 6 individuals are indicated by a different symbol. Results are shown as mean and standard deviation.

In addition, the T-cell response induced by BBPS-bound β_2GPI was completely abolished by the pretreatment of antigen-captured DCs with chloroquine or brefeldin A, which impair the antigen-processing pathway (Figure 3D). Moreover, the T-cell response induced by BBPS-bound β_2GPI was completely blocked by an anti–HLA-DR antibody during the DC–T-cell interaction. These findings indicate that the response of p276-290–reactive T-cell lines induced by β_2GPI-PL complex–pulsed DCs is endosomal antigen-processing dependent and HLA class II dependent.

Induction of T-cell response to p276-290 by PL-bound β_2GPI in peripheral blood T cells from healthy individuals

We further tested whether β_2GPI bound to anionic PLs primes a T-cell response to p276-290 in healthy individuals in vitro. In this experiment, BBPS was used since liposomes containing this PL constantly induced a strong response of p276-290–reactive T-cell lines in the presence of β_2GPI. Peripheral blood T cells from 6 healthy individuals carrying DR53 were stimulated once with autologous DCs or macrophages pulsed with BBPS-bound β_2GPI, BBPS liposomes, or β_2GPI alone, and the antigen-specific T-cell response was evaluated by IFN-γ production in response to various antigens, including p276-290. Figure 4A shows representative results obtained using DCs as APCs. T cells stimulated with BBPS-bound β_2GPI showed profound responses to GP-F and p276-290, whereas those stimulated with BBPS liposomes or β_2GPI alone did not. Successful priming of p276-290–reactive T cells was obtained in 5 of 6 individuals when DCs were used as APCs but detected in only one when macrophages were used instead (Figure 4B).

Discussion

The present study demonstrates that the binding of β_2GPI to anionic PL surfaces renders the molecule highly immunogenic by an enhanced generation of the cryptic T-cell determinant as a direct consequence of antigen processing in functional APCs. To date, little information has been obtained about the mechanisms that induce the expression of cryptic self-peptides and elicit autoimmunity in human autoimmune diseases. Potential mechanisms that reveal cryptic self-determinants in APCs include modulation of antigen processing and/or increased antigen delivery to the processing compartment.[10] One possible explanation is that the PL binding physically shields the p276-290 determinant from proteolytic attack in endocytic compartments. In this regard, Simitsek et al[23] reported that antibody binding to the antigen suppresses the generation of some epitopes and boosts that of others. Remarkably, both suppressed and boosted epitopes were present within a protein domain that was "fingerprinted" by the antibody, whereas epitopes that lay outside this domain were not affected. Based on these findings, they speculated that the antibody, by binding and stabilizing a protein domain, might influence the accessibility of the site to proteases during antigen processing. In addition, processing of the thyroglobulin-autoantibody complex has been described to promote generation of cryptic pathogenic peptides in APCs in murine models for autoimmune thyroid disease.[24] A similar mechanism can be proposed for the generation of p276-290 from the processing of PL-bound β_2GPI. The major PL-binding site located on the surface of a β_2GPI molecule[22] should be easily accessed by proteases in endocytic compartments during antigen processing, and therefore, the peptides containing the intact major PL-binding site would not be generated from native β_2GPI. In contrast, the binding of β_2GPI to anionic surfaces may protect the major PL-binding site from protease attack by masking the site, resulting in the appearance of the previously cryptic peptide containing the entire major PL-binding site. Anionic PLs on apoptotic bodies and platelet microparticles were potentially present in our cultures, but p276-290–reactive T-cell lines did not respond to native β_2GPI in the absence of PL liposomes. The precise reason for this phenomenon is unclear, but quantity and quality of the lipid vesicles may be important in inducing the presentation of the previously cryptic p276-290 in APCs.

The capacity of individual PLs to induce a response of the p276-290–reactive T-cell line was principally correlated with their β_2GPI-binding capacity. However, β_2GPI had a strong binding affinity to solid-phase cardiolipin, but the cardiolipin-β_2GPI complex demonstrated only a weak T-cell stimulatory capacity. β_2GPI interacts with lipid vesicles containing anionic PLs via the PL-binding patch (ie, a cluster of basic amino acid residues and a hydrophobic flexible loop in the domain V).[22] In contrast to the β_2GPI interaction with solid-phase cardiolipin, binding capacity of β_2GPI to lipid vesicles depends not only on the strength of the negative charge but also on fluidity of the lipid vesicles. In general, the membrane fluidity is influenced by length of fatty acid chains, number of unsaturated double bonds, and/or phase transition temperature of each lipid composed in liposomes. Therefore, it is likely that such structural differences also contribute to the efficiency to reveal the cryptic epitope containing the intact major PL-binding site in APCs.

The in vitro priming experiment strongly suggested that an event inducing the presentation of p276-290 by DCs would activate β_2GPI-reactive CD4$^+$ T cells in the normal T-cell repertoire in genetically susceptible individuals. Therefore, exposure of p276-290 to the immune system might be a critical step for inducing APS by triggering the activation of disease-relevant β_2GPI-reactive CD4$^+$ T cells. Activated β_2GPI-reactive T cells would subsequently stimulate B cells to produce pathogenic anti-β_2GPI antibody through the expression of CD40 ligand and IL-6, as reported previously.[6] Since β_2GPI is a plasma protein abundant in the circulation (~ 200 μg/mL), excessive exposure to anionic surfaces, such as microorganisms and apoptotic cells, may induce the formation of a large quantity of β_2GPI bound to anionic surfaces in vivo. In this regard, associations between various types of infections and the production of antiphospholipid antibodies with or without APS manifestations have been reported,[25] and infection is one of the major precipitating factors contributing to the development of catastrophic APS.[26] Furthermore, an enhanced yield of cryptic determinants and T-cell stimulatory capacity can be achieved by highly potent DCs that have specialized mechanisms for antigen capture and increased expression of HLA class II and costimulatory and adhesion molecules. These 2 mechanisms may act synergistically to elicit the β_2GPI-specific T-cell response, but additional factors, such as impaired regulatory function and nonspecific inflammation mediated by cytokines and toll-like receptor ligands, are apparently required to initiate the pathogenic autoimmune response. Once the T-cell response to p276-290 is primed, the specific T-cell response could be sustained and amplified by professional APCs that have taken up the β_2GPI complexed with anionic surfaces that are normally present in a small quantity in vivo, such as apoptotic cells, platelet microparticles, and oxidized low-density lipoprotein.[27] In addition, this response can be further boosted by the formation of immune complexes consisting of anti-β_2GPI antibodies and β_2GPI bound to anionic surfaces.

In summary, our finding is the first demonstration of a mechanism that elicits pathogenic autoreactive T-cell responses in APS. Further studies examining anionic surfaces that bind to β_2GPI and induce the presentation of the cryptic peptide of β_2GPI in vivo would be useful in clarifying the pathogenesis of APS. In addition, it is likely that modulation of antigen processing is an inevitable consequence of the high-affinity binding and influence processing of autoantigens that are bound by high-affinity ligands. This theory encourages further research examining the possibility that the unveiling of cryptic self-determinants by the altered processing of autoantigens complexed with certain ligands is a major mechanism of the initiation of the autoimmune spiral in other autoimmune diseases.

Acknowledgments

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Is Eradication Therapy Useful as the First Line of Treatment in *Helicobacter pylori*–Positive Idiopathic Thrombocytopenic Purpura? Analysis of 207 Eradicated Chronic ITP Cases in Japan

Kingo Fujimura,[a] Masataka Kuwana,[b] Yoshiyuki Kurata,[c] Masahiro Imamura,[d] Hiroshi Harada,[e] Hisashi Sakamaki,[f] Masanao Teramura,[g] Kyuhei Koda,[h] Shosaku Nomura,[i] Sayaka Sugihara,[j] Takeshi Shimomura,[k] Tetsuro-Takahiro Fujimoto,[a] Kazuma Oyashiki,[l] Yasuo Ikeda[m]

[a]Department of Hematology and Oncology, Division of Clinical Pharmacotherapeutics, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima; [b]Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo; [c]Department of Blood Transfusion, Osaka University Hospital, Suita; [d]Department of Hematology, Hokkaido University School of Medicine, Sapporo; [e]Department of Hematology, Showa University Fujigaoka Hospital, Yokohama; [f]Department of Internal Medicine, Tokyo Komagome Metropolitan Hospital, Tokyo; [g]Department of Hematology, Tokyo Women's Medical University School, Tokyo; [h]Department of Internal Medicine, Asahikawa Red Cross Hospital Asahikawa; [i]Department of Hematology, Kansai Medical School, Osaka; [j]Department of Hematology, Hiroshima Red Cross Hospital Hiroshima; [k]Department of Hematology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima; [l]Department of Hematology, Tokyo Medical School, Tokyo; [m]Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

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Abstract

A retrospective study was performed to determine the prevalence of *Helicobacter pylori* (*H pylori*) infection, the effect of *H pylori* eradication on platelet counts, and the characteristic clinical features of chronic immune or idiopathic thrombocytopenic purpura (ITP) with *H pylori* infection. *H pylori* infection was found in 300 patients, a group that was significantly older ($P < .005$) and had more cases of hyperplastic megakaryocytes in the bone marrow ($P = .01$) than patients without *H pylori* infection. *H pylori* eradication therapy was performed in 207 *H pylori*–positive ITP cases, and the platelet count response was observed in 63% of the successful eradication group and in 33% of the unsuccessful eradication group ($P < .005$). In the successful group, the complete remission and partial remission rates were 23% and 42%, respectively, 12 months after eradication. In the majority of responders, the platelet count response occurred 1 month after eradication therapy, and the increased platelet count continued without ITP treatment for more than 12 months. *H pylori* eradication therapy was effective even in refractory cases, which were unresponsive to splenectomy. In conclusion, *H pylori* infection was involved in most ITP patients older than 40 years in Japan, and eradication therapy should be the first line of treatment in *H pylori*–positive ITP patients.
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Key words: ITP; *Helicobacter pylori*; Eradication; Older ITP patients

1. Introduction

The pathological mechanisms of idiopathic thrombocytopenic purpura (ITP) have not been clearly determined; however, the immunological platelet destruction mechanism is accepted in most cases as immune thrombocytopenic purpura [1-3]. The etiology and mechanism of induction of immune mechanisms against platelets are unknown.

In 1998, Gasbarrini et al reported that in ITP cases with *Helicobacter pylori* (*H pylori*) infection, the platelet count was elevated after eradication of this bacterium [4]. That report presented some interesting problems to us. One was whether *H pylori* infection is involved in the pathological mechanisms of some cases of immune thrombocytopenia, and the other was whether eradication therapy is effective for treatment of ITP cases with *H pylori* infection.

Correspondence and reprint requests: Kingo Fujimura, MD, PhD, Department of Hematology and Oncology, Division of Clinical Pharmacotherapeutics, Program for Applied Biomedicine, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan; 81-82-257-5295; fax: 81-82-257-5299 (e-mail: fujimura@hiroshima-u.ac.jp).

H pylori is a gram-negative bacterium that causes gastritis and is an important risk factor for gastric ulcers, duodenal ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [5]. However, recent reports have suggested that *H pylori* infection can cause immune diseases not related to the gastric mucosa, such as chronic thyroiditis, rheumatoid arthritis, and Sjögren syndrome [6-8]. Several reports from Italy and Japan appear to confirm the effect of *H pylori* eradication on platelet count in *H pylori*–positive ITP cases [9-13]. Therefore *H pylori* eradication in *H pylori*–positive ITP is likely to be an effective ITP treatment strategy. On the other hand, reports from Spain, northern Europe, and the United States did not support the effectiveness of *H pylori* eradication in *H pylori*–positive ITP [14-16]. The cause of this discrepancy and the mechanisms by which eradication is effective in ITP patients are not clear. The questions then are whether specific characteristic backgrounds are present in *H pylori*–positive ITP and eradication-effective ITP cases and whether eradication therapy is useful for thrombocytopenia.

We designed a nationwide retrospective study in Japan to answer these questions, to evaluate the incidence of *H pylori*–positive ITP cases and the effect of eradication on platelet count, and to determine whether the eradication procedure can be included in the standard regimen of ITP treatment.

2. Materials and Methods

2.1. Patients

ITP patients older than 18 years who were examined for *H pylori* infection in 11 hospitals in Japan from July 2002 to December 2003 were enrolled in this study. The diagnosis was made and management provided by a hematologist in each hospital.

Questionnaires were used to gather the following data: age, sex, laboratory data for ITP (white blood cell count, hemoglobin concentration, red blood cell count, platelet count, bone marrow findings such as megakaryocyte poiesis expressed as decrease, normal, or increase as found with marrow smears), bleeding symptoms including all forms and grades, ITP disease duration, previous and recent medication for ITP, whether splenectomy had been performed, laboratory data for diagnosis of *H pylori* infection, whether eradication therapy was used, the protocol used for eradication, the effect of eradication on *H pylori* infection, the effect of eradication on platelet count, and side effects of the eradication procedure. Platelet count was measured with an automatic blood cell count analyzer in each hospital.

From the enrolled patients the following groups were selected for analysis of the involvement of *H pylori* in ITP: (1) Platelet counts were monitored for at least 3 months after eradication therapy, counts being measured every 4 or 8 weeks. (2) To evaluate the eradication effects, the dose of the ongoing medication was maintained for at least 4 weeks after eradication therapy. (3) After informed consent was obtained, the eradication therapy was selected by *H pylori*–positive ITP patients themselves. Approval for the eradication treatment of ITP was obtained at each hospital before the start of the study, and informed consent was provided by all patients in each hospital according to the Declaration of Helsinki.

2.2. Diagnosis and Evaluation of Effectiveness

Diagnosis of ITP was performed according to the diagnostic criteria for ITP determined by the Research Committee for ITP supported by the Ministry of Health and Welfare of Japan (1990). The main points were as follows: thrombocytopenia was less than $10 \times 10^4/\mu L$; no other hematological abnormalities were present in peripheral blood; whenever other diseases were suspected bone marrow was examined to exclude them; no underlying diseases were causing thrombocytopenia; and thrombocytopenia had been present for at least 6 months.

The hematological response after eradication therapy was assessed during the final examination in each case, and the effectiveness was determined by the following criteria: If the platelet count before eradication therapy was less than $1 \times 10^4/\mu L$ and the platelet count had increased to more than 3 times the pre-eradication level after eradication therapy, the case was considered a responder case. If the initial platelet count was $1 \times 10^4/\mu L$ or greater and less than $3 \times 10^4/\mu L$ and the platelet count had increased to more than $5 \times 10^4/\mu L$ after eradication or if the initial platelet count was $3 \times 10^4/\mu L$ or greater but less than $10 \times 10^4/\mu L$ and the platelet count had increased to greater than $3 \times 10^4/\mu L$ of the pre-eradication level, the case also was considered to be a responder case. Responder cases with platelet counts less than $15 \times 10^4/\mu L$ were referred to as partial remission (PR). Complete remission (CR) was defined as platelet counts greater than $15 \times 10^4/\mu L$ after eradication without maintenance therapy. Nonresponder (NR) cases were defined as cases in which the platelet count did not meet the above criteria after eradication therapy.

H pylori infection was diagnosed according to the *H pylori* diagnostic guidelines established in 2003 by the Japan *Helicobacter pylori* Research Association [17]. That is, *H pylori* infection was diagnosed when the result of at least 1 or more of the following 3 tests was positive: serum *H pylori* antibody test, ^{13}C urea breath test (UBT), and detection of *H pylori* in gastric mucosa biopsy specimens. Four or more weeks after eradication treatment, the effect of eradication on *H pylori* infection was assessed with the ^{13}C UBT.

2.3. Analysis

We used a multiple logistic regression model and extended Poisson regression model on the *H pylori* data to examine the effect of background factors on the following binary response variables: whether the patient had positive or negative results for *H pylori*, whether platelet count increased, and whether eradication was successful. The background factors considered were sex, age, duration of ITP, tendency to bleed, platelet count, megakaryocyte count, treatment of ITP, and whether splenectomy had been performed. For category variables, we used dummy values.

3. Results

3.1. Patient Numbers and Prevalence of H pylori Infection

We enrolled 467 patients over 18 months. However, the following 32 cases were excluded: 6 cases in the CR state of ITP, 2 cases of acute ITP, 17 cases diagnosed as myelodysplastic syndrome or systemic lupus erythematosus, 4 cases of Evans syndrome, and 3 cases in which results of *H pylori* examinations were not adequate. Thus the final total was 435 cases. Previous ITP management of the enrolled cases was as follows: 70 cases were refractory cases to steroids and splenectomy. A total of 181 cases were treated with steroids; 48 cases were treated with ascorbate or cepharanthine (plant alkaloid agent) without steroids; 133 cases were fresh ITP cases or had not been treated, and 3 cases had unknown previous management. The age distribution in 435 ITP cases showed 2 peaks. One peak was small in the 31- to 40-year age group, and the other was large in the 51- to 60-year age group (Figure 1A). The average age among the patients was relatively high, and approximately 70% of the patients in this study were older than 50 years. The male to female ratio was 1/2.63.

UBTs in conjunction with *H pylori* antibody tests were performed in 152 cases. The remaining cases were diagnosed with either a UBT (154 cases) or a *H pylori* antibody test (92 cases), or a biopsy was performed in addition to 1 of the other tests (37 cases).

Three hundred cases were diagnosed as *H pylori* positive (positive rate, 69%), including the cases of 217 female patients (68.8% positive in female patients) and those of 83 male patients (69.2% positive in male patients), and 135 cases were found to be *H pylori* negative, including those of 98 female and 37 male patients. The *H pylori*-positive rate did not differ between female and male patients (Table 1).

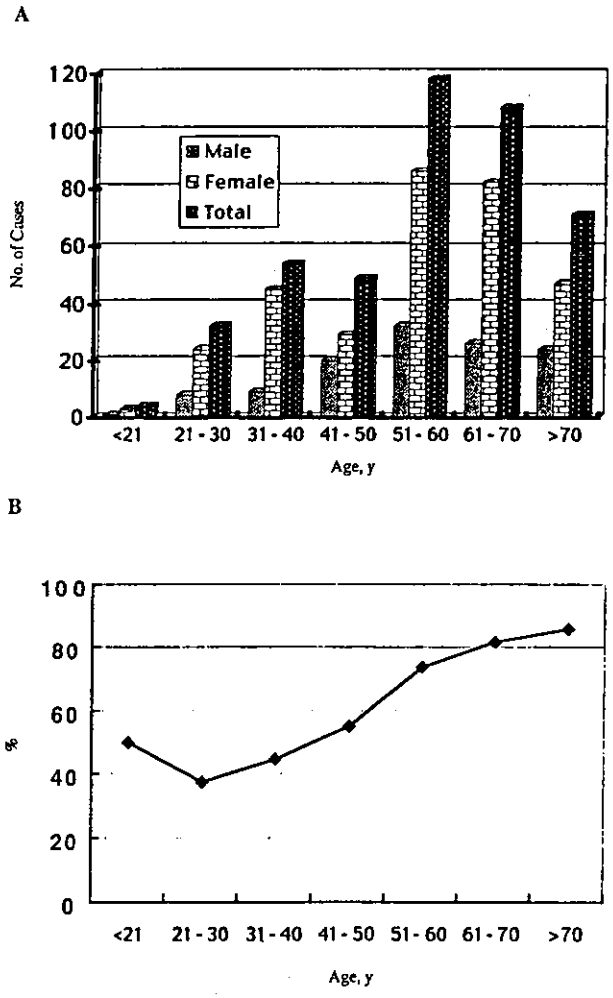

Figure 1. A, Age distribution of 435 patients with idiopathic thrombocytopenic purpura (ITP). There are 2 waves in the total number of patients. The first is patients 21 to 50 years old, and the second is patients 41 years and older. More than 70% of patients are in the second wave. B, *Helicobacter pylori* infection–positive rates in each age population of ITP patients. The *H pylori*–positive rate increased gradually between the ages of 41 and more than 71 years.

3.2. Comparison of the Backgrounds between H pylori–Positive and –Negative Groups

As shown in Table 1, there was no difference in sex ratio between the groups. The mean age of the *H pylori*–positive patient group (58.92 ± 13.76 years) was significantly higher than that of the *H pylori*–negative patient group (47.36 ±

Table 1.
Initial Background Factors of *Helicobacter pylori*–Positive and –Negative Groups

	H pylori Positive	*H pylori* Negative
No. of cases	300	135
Male/female ratio	1/2.6	1/2.65
Mean age, y*	58.92 ± 13.76	47.36 ± 15.87
Disease period, y	8.18 ± 6.80	8.74 ± 7.23
Bleeding tendency		
+	161 (54.8%)	85 (64.9%)
–	133 (45.2%)	46 (35.1%)
Unknown	6	4
Initial platelet count		
5-10 × 10^4	61 (21.1%)	24 (18.6%)
3-5 × 10^4	76 (26.3%)	25 (19.4%)
1-3 × 10^4	115 (39.8%)	53 (41.1%)
<1	37 (12.8%)	27 (20.9%)
Unknown	11	6
Bone marrow megakaryocytes		
Increased†	140 (54.9%)	51 (44.7%)
Normal	110 (43.1%)	59 (51.8%)
Decreased	5 (2.0%)	4 (3.5%)
Unknown	5	21
Treatment before eradication		
No treatment	91	42
Steroid treatment	122	59
Other treatment	40	8
Splenectomy	45	25
Unknown	2	1

*P < .005 (odds ratio, 1.72/10 y).
†P = .011 (odds ratio, 2.03).

Table 2.
Eradication Effects on Platelet Response*

Helicobacter pylori–negative ITP cases, n	135 (31%)
H pylori–positive ITP cases, n	300 (69%)
Eradication cases, n	228
Eradication successful group, n	161 (78%)
Platelet count: Response cases, n	101 (63%) †
Platelet count: Nonresponse cases, n	60 †
Eradication unsuccessful group, n	46 (22%)
Platelet count: Response cases, n	15 (33%) †
Platelet count: Nonresponse cases, n	31 †
Unknown cases, n	19
Previous treatment unknown cases, n	2
Noneradication cases, n	72

*ITP indicates idiopathic thrombocytopenic purpura.
†$P < .005$ (odds ratio, 4.45).

15.87 years). The estimated regression coefficients were 0.054 ($P < .005$), and the odds ratio was 1.72 (per 10 years). The durations of ITP in the *H pylori*–positive and –negative patient groups were 8.18 ± 6.80 and 8.74 ± 7.23 years, respectively, and were not significantly different. The clinical features and laboratory findings at diagnosis of ITP showed that there were trends among the patients with fewer bleeding events (55% versus 65%) and that there were fewer platelet counts less than $1 \times 10^4/\mu L$ (13% versus 21%) in *H pylori*–positive ITP cases than in *H pylori*–negative cases, but the estimated regression coefficient and odds ratio were not significant. However, in the *H pylori*–positive group there were significantly more cases of hyperplastic megakaryocytes in the bone marrow, an estimated regression coefficient of $P = .01$ and odds ratio of 2.03. The cases of ITP that had not been treated, had been treated with steroids, or had been treated with splenectomy before enrollment did not differ significantly, but more patients in the *H pylori*–positive group received other treatment during ITP (Table 1).

3.3. Age Distribution in H pylori–Positive and –Negative ITP Cases

The rate of positive results for *H pylori* infection in this study increased with age (Figure 1B). *H pylori*–positive ITP was found more frequently in patients older than 41 years; that is, 55% of the patients in the 41-year age group had positive results. This rate increased to 73% in 51-year-old patients and to more than 80% in the group older than 61 years.

Most *H pylori*–negative ITP cases were found in patients younger than 50 years. The peak of negative cases was found in the 21- to 40-year age group. No difference between male and female patients in *H pylori*–positive rate was found in any age group.

3.4. Use of Eradication Treatment in H pylori–Positive ITP Cases

As shown in Table 2, 228 (76%) of 300 *H pylori*–positive ITP patients consented to eradication treatment, and treatment was performed. The standard eradication regimen in Japan (lansoprazole 60 mg/d, clarithromycin 400 mg/d, and amoxicillin 1500 mg/d, taken together twice a day for 7 days) was selected in most cases. Twenty-one cases were excluded after analysis because the eradication effect was not evaluated or because previous ITP treatments were unclear. Eradication of *H pylori* infection succeeded in 161 (78%) of 207 cases. The success rate did not differ between male (success rate, 78.8%) and female (success rate, 77.2%) patients or between age groups.

The initial ITP treatment regimen or treatment immediately before eradication by steroids or other immunosuppressive agents had no significant influence on the success rate of the eradication treatment.

3.5. Eradication Effects of H pylori Infection on Platelet Count

A significant increase in platelet count at the final observation was found in 101 (63%) of the 161 cases in which eradication of *H pylori* was successful and in 15 (33%) of the 46 cases in which eradication was not successful. These findings showed that the increase in platelet count correlated with successful eradication treatment of *H pylori*–positive ITP cases with an estimated regression coefficient of $P < .005$ and an odds ratio of 4.45 (Table 2).

The background characteristics of the increased platelet and NR groups with successful eradication treatment were compared (Table 3). The shorter duration of ITP (6.52 ± 4.67 years versus 9.85 ± 7.77 years) in the increased platelet group was significant with an estimated regression coefficient of $P < .005$ and an odds ratio of 0.34 (per 10 years), but age did not influence the platelet count response after eradication. The results of platelet count recovery were not affected by platelet count immediately prior to eradication or by ITP treatment before eradication whether or not eradication was successful.

3.6. Follow-up Study

Overall, 155 (75%) of all eradicated ITP cases were followed up for 12 months after eradication, 42 (20%) of the cases for 6 months, and 10 (5%) of the cases for 3 months.

The platelet count responses at 12 months after successful eradication (122 cases) were as follows (Table 4): 28 (23%) of

Table 3.
Backgrounds of the Platelet Count Response Group and the Nonresponse Group in Eradication-Effective Cases*

	Platelet Count	
	Response	Nonresponse
No. of cases	101	60
Male/female ratio	37/64 (0.58)	15/45 (0.33)
ITP duration, y†	6.52 ± 4.67	9.85 ± 7.77
Mean age, y	58.70 ± 12.6	56.73 ± 14.31
Mean platelet count immediately before eradication, $\times 10^4$	4.01 ± 2.22	5.18 ± 5.68
Treated ITP cases immediately before eradication, n	52 (50.5%)	27 (45.0%)

*ITP indicates idiopathic thrombocytopenic purpura.
†$P < .005$ (odds ratio, 0.34/10 years).

Table 4.
Platelet Count Response 12 Months after Eradication Therapy*

Platelet Count Immediately before Eradication, $\times 10^4$	Complete Remission	Partial Remission	No Response	Total
Eradication successful group, n = 122 cases				
<1	0	2 (67)	1 (33)	3
1-3	8 (20)	23 (58)	9 (23)	40
3-5	8 (21)	16 (42)	14 (37)	38
5-10	12 (29)	10 (24)	19 (46)	41
Total	28 (23)	51 (42)	43 (35)	122
Eradication unsuccessful group, n = 33 cases				
<1	0	0	2 (100)	2
1-3	1 (11)	1 (11)	7 (78)	9
3-5	1 (7)	3 (21)	10 (71)	14
5-10	3 (38)	0	5 (63)	8
Total	5 (15)	4 (12)	24 (73)	33

*Values in parentheses are percentages.

the cases were in CR, 51 (42%) of the cases were in PR, and 43 of the cases were NR. In all, 79 (65%) of the cases showed a response in platelet count (Table 4). In most of the PR cases in which platelet count increased to more than $5 \times 10^4/\mu L$, ITP treatment was discontinued after eradication. The cases in which platelet counts were less than $1 \times 10^4/\mu L$ just before eradication did not show an increase in platelet count above $5 \times 10^4/\mu L$ at 12 months after eradication, although the platelet count had increased to at least 3 times the initial count and the tendency toward bleeding had disappeared in 2 cases (Figure 2A).

We used the extended Poisson regression model to analyze the relation between the previous ITP treatment or platelet count immediately prior to eradication and the platelet count response after eradication. At 12 months the relation between platelet count before eradication and the effect of platelet count response on CR and PR rate after eradication was not significant (Table 4). The PR and CR rates tended to be higher in the nontreatment (40 cases) and the steroid treatment groups (52 cases) before eradication than in the splenectomy (12 cases) or other treatment groups (18 cases); however, there were no significant differences between these groups (Table 5).

In the group in which eradication was not successful (33 cases), 5 cases were in CR (15%), 4 cases were in the increased platelet response group (PR, 12%), and 24 cases were NR (Tables 4 and 5). The 9 responder cases were previously diagnosed as *H pylori* infection by UBT or UBT plus stomach biopsy. In all, the response rate in this group was 27% at 12 months after eradication. The clinical course of platelet count in these 9 platelet count response cases is shown in Figure 2B.

Most platelet count response cases showed that the increase in platelet count occurred 1 month after eradication and then gradually increased further for 12 months (Figure 2A). To examine the increase in platelet count for success status data, we used the paired t test for each neighbor time point. The increase in platelet count was significant ($P < .01$) in every span.

In the NR group, there were 6 cases in which platelet counts increased transitionally and returned to the baseline level within 6 months, even though eradication had been successful. There were no relapse cases after 6 months regardless of the success of the eradication treatment.

3.7. Adverse Effects of Eradication Treatment

Adverse effects of eradication treatment were found in 39 (17.6%) of 222 cases. These adverse effects were gastrointestinal trouble, such as discomfort at the epigastrium and diarrhea. Soft stool and rash occurred in 64% and 23%,

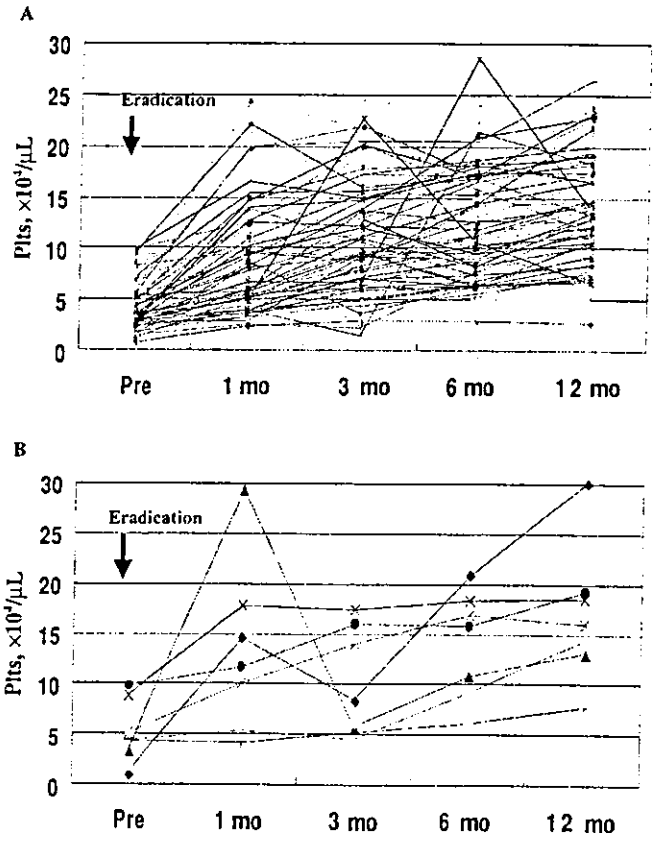

Figure 2. Laboratory course of platelet count responders in successful eradication cases (79 cases) (A) and unsuccessful eradication cases (9 cases) (B) followed for more than 12 months. The platelet count in most responder cases began to increase 1 month after eradication and continued to increase gradually.

Table 5.
Previous Idiopathic Thrombocytopenic Purpura Treatment and Eradication Effect on Platelet Response 12 Months after Eradication

Treatment	Eradication Effect	No. of Cases	Platelet Response, n (%)		
			Complete Remission	Partial Remission	No Response
No treatment	Yes	40	11 (28)	16 (40)	13 (33)
	No	7	3 (43)	1 (14)	3 (43)
Steroid treatment	Yes	52	12 (23)	24 (46)	16 (31)
	No	18	2 (11)	3 (17)	13 (72)
Other treatment	Yes	18	3 (17)	6 (33)	9 (50)
	No	5	0	0	5 (100)
Splenectomy	Yes	12	2 (17)	5 (42)	5 (42)
	No	3	0	0	3 (100)
Total	Yes	122	28 (23)	51 (42)	43 (35)
	No	33	5 (15)	4 (12)	24 (73)

respectively, of cases showing adverse effects. These problems were the main adverse reactions, and except in 1 case in which the tendency to bleed became more serious, the patients were able to overcome these events and complete the eradication schedule.

4. Discussion

The incidence of *H pylori* infection in the general population of Japan is approximately 10% to 40% until 40 years of age, after which it increases to approximately 80% [18]. Because the incidence of *H pylori* infection in patients with ITP does not seem to differ from that in the general population of Japan, the incidence of *H pylori* infection in ITP is not considered a specific feature of the disease.

H pylori infection did not influence the severity of the clinical features of ITP. This retrospective study revealed that the *H pylori*–positive group had a higher mean age and incidence of hyperplastic bone marrow megakaryocytes, but there was no evidence to explain the bone marrow findings.

The success rate of *H pylori* eradication in *H pylori*–positive ITP cases was slightly lower than that found in gastric diseases without ITP in Japan. The former was 78%, and the latter was 82% to 91% [19]. ITP treatment just before *H pylori* eradication did not influence this rate. Eradication in *H pylori*–positive ITP cases induced a significant increase in platelet count in the successful eradication group compared with that of the unsuccessful eradication group.

These findings suggest that *H pylori* infection is involved in the mechanisms of thrombocytopenia in most ITP patients in Japan who are older than 41 years. However, in the younger generation, other, unknown factors must be involved in the development of ITP.

Approximately 33% of the eradication failure group had an increase in platelet count. Although it is difficult to explain this response, a possibility is the occurrence of false-positive results of the ^{13}C UBT, because an insufficient period of time had passed to allow for an accurate result, or from inadequacy in the ^{13}C UBT itself. The other possibility is that reduction of *H pylori* organisms shuts down the circuit of platelet immunity or that another, unknown mechanism is at work [20,21].

The management of ITP initially or immediately prior to eradication treatment did not influence the increase in platelet count. That is, the platelet response rate in nontreatment groups was higher than in any treatment group, but there was no significant difference between them. This evidence suggests that refractory ITP cases with *H pylori* infection should be eradicated prior to other treatment procedures.

Examination of the clinical course after *H pylori* eradication clearly showed that a significant increase in platelets began 1 month after eradication and gradually continued to increase to a plateau. This finding suggests that the effect of eradication therapy on platelet counts can be predicted at an early stage. Several cases relapsed within 6 months, but in the remaining cases, platelet counts continued to increase for 12 months, even in the eradication failure group. The low incidence of relapse is one of the benefits of eradication therapy in *H pylori*–positive ITP cases.

In some cases, although eradication was successful, NR cases occurred. Several possibilities may account for this result. One possibility is that the UBT gave false-negative results in some cases or that small numbers of organisms remained after treatment and continued to stimulate an immune reaction against the platelets, even though the UBT gave negative results. An alternative is that *H pylori*–false-positive cases were present. Eleven cases of *H pylori* infection had been detected initially by serum antibody test. The other possibility is that this organism is not involved in the thrombocytopenia in some *H pylori*–positive ITP cases and another mechanism is taking place in these thrombocytopenia cases, because many other persons with *H pylori* infection do not develop thrombocytopenia.

This study confirmed previous reports with large-scale retrospective analyses. That is, eradication of *H pylori* is effective in significantly increasing platelet count, and this organism is involved in the pathogenesis of some ITP cases [4,12]. However, the mechanisms of thrombocytopenia that affect some persons with *H pylori* infection are unknown.

Several specific proteins derived from *H pylori*, such as blood group Ag–binding adhesin, *H pylori* neutrophil activation factor, lipopolysaccharide (LPS), and CagA (cytotoxin-associated gene A) protein, which is produced by the Cag pathogenicity island gene, induce colonization of *H pylori*, neutrophil infiltration, and cellular immunity. These agents may be involved in the development of gastritis, gastric ulcer, and MALT lymphoma [5,22]. However, it is not understood why localized *H pylori* infection at the gastric mucosa develops into immune thrombocytopenia in some cases.

The products derived from microorganisms such as LPS, bacterial DNA, and viruses are able to act as adjuvants and induce immune reactions to unrelated antigens [23]. Whether this phenomenon is due to the ability of the organisms to activate innate immunity and induce lymphocyte response in a nonspecific way, by molecular mimicking, or both has not been resolved. It recently was reported that platelet eluates from *H pylori*–positive ITP cases reacted with CagA protein [24]. This evidence seems to support the molecular mimicking mechanism in ITP development with *H pylori* infection. However, further investigations are required to confirm this hypothesis.

Although many persons in the world have *H pylori* infection, only a very small proportion develop thrombocytopenia. This discrepancy can be explained, as a working hypothesis, by the multiplicity of infective *H pylori* strains, diversity of host immune responses, differences in the age of infected persons, and environmental factors. In addition, genetic effects are likely to play a significant role in the development of thrombocytopenia [25,26].

Taken together, *H pylori* infections were involved in most thrombocytopenic patients older than 40 years in Japan. In addition, eradication therapy proved to be significantly effective in increasing platelet count in cases of short duration of ITP, and this effect was found even in cases in which splenectomy was not effective. The increase occurred soon after eradication and continued for a long period without ITP treatment in many cases, preventing bleeding events and improving the quality of life in more than 60% of *H pylori*–positive ITP patients. From these results, it is highly recommended that eradication therapy be used as a first line of treatment in *H pylori*–positive ITP cases because the treatment schedule is economical and patients are not subject to adverse effects such as those of steroid and other immunosuppressive therapies.

Acknowledgments

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The Role of Autoreactive T-Cells in the Pathogenesis of Idiopathic Thrombocytopenic Purpura

Masataka Kuwana, Yasuo Ikeda

Institute for Advanced Medical Research and Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

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Abstract

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease mediated by antiplatelet autoantibodies. The major target of these autoantibodies is a platelet membrane glycoprotein, GPIIb-IIIa, which is a receptor for fibrinogen and other ligands. We recently identified $CD4^+$ T-cells autoreactive to GPIIb-IIIa in ITP patients. These T-cells are considered pathogenic because they help B-cells produce antibodies that bind to normal platelet surfaces. GPIIb-IIIa–reactive T-cells respond to chemically reduced and tryptic peptides of GPIIb-IIIa but not to native GPIIb-IIIa, indicating that the epitopes they recognize are "cryptic" determinants generated at a subthreshold level by the processing of native GPIIb-IIIa under normal circumstances. Although GPIIb-IIIa–reactive T-cells are also detected in healthy individuals, they are activated in vivo only in ITP patients. Activation of GPIIb-IIIa–specific T-cells and the subsequent production of pathogenic anti–GPIIb-IIIa antibodies can be induced by functional antigen-presenting cells in the spleen that present cryptic GPIIb-IIIa peptides to these T-cells. The pathogenic process of ITP can be explained as a continuous loop in which B-cells produce antiplatelet autoantibodies, splenic macrophages phagocytose antibody-coated platelets and present GPIIb-IIIa–derived cryptic peptides, and GPIIb-IIIa–reactive $CD4^+$ T-cells exert their helper activity. Further studies examining the mechanisms that induce the processing and presentation of cryptic peptides derived from the platelet antigen at disease onset will clarify how the pathogenic autoantibody response in ITP is initiated.
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Key words: Idiopathic thrombocytopenic purpura; Autoreactive T-cell; GPIIb-IIIa; Cryptic epitope

1. Introduction

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

It is now evident that autoantibody production by B-cells requires autoantigen-specific T-cell help. T-cells that react with autoantigens targeted by autoantibodies have been identified in patients with various systemic or organ-specific autoimmune diseases. These autoantigen-specific T-cells almost always have a $CD4^+$ helper phenotype. Previous studies in patients with lupus nephritis and antiphospholipid syndrome (APS) showed that autoreactive T-cells selectively provide help to autoantibody-producing B-cells [6,7]. Circulating or platelet-associated anti–GPIIb-IIIa antibodies in ITP patients have been shown to be mainly of the immunoglobulin G (IgG) isotype [3] and to recognize multiple epitopes on the complex, including linear and conformational determinants [8,9]. The presence of anti–GPIIb-IIIa antibodies is associated with certain HLA class II alleles in ITP patients [10]. Furthermore, a recent study by Roark et al demonstrated that platelet-specific antibodies derived from

Correspondence and reprint requests: Masataka Kuwana, MD, PhD, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; 81-3-5363-3778; fax: 81-3-5362-9259 (e-mail: kuwanam@sc.itc.keio.ac.jp).

splenic B-cells undergo somatic mutation, a genetic process strictly dependent on T-cell help [11]. Taken together, these features of ITP patients strongly suggest that antiplatelet autoantibody production is under the control of platelet-specific helper T-cells. Therefore, a detailed analysis of the T-cells autoreactive to platelet antigens is essential to understanding the pathogenic process in patients with ITP. This article focuses on the role of GPIIb-IIIa–reactive T-cells in the pathogenesis of ITP and particularly on how these autoreactive T-cells initiate and maintain pathogenic antiplatelet autoantibody production.

2. Characterization of GPIIb-IIIa–Reactive CD4+ T-Cells

The presence of T-cells autoreactive to platelets was first described by Semple and Freedman in 1991 [12]. In that report, they demonstrated that peripheral blood T-cells from ITP patients secreted interleukin 2 (IL-2) upon stimulation with autologous platelets. The production of IL-2 from circulating T-cells in response to platelets was further confirmed by Ware and Howard [13]. A subsequent study by Filion et al showed that this autoreactive T-cell response observed in ITP patients is the result of a breakdown in T-cell tolerance to platelet autoantigens in association with uncontrolled IL-2 expression [14]. In addition, Shimomura et al demonstrated an oligoclonal accumulation of CD4+ T-cells that frequently used the Vβ3, 6, 13.1, and 14 genes for their T-cell receptors in the peripheral blood of ITP patients [15]. These earlier studies together indicated that platelet antigen–reactive T-cells are expanded during and are associated with the pathogenic processes of ITP. However, the antigenic specificity of these platelet-reactive T-cells and whether they stimulated antiplatelet autoantibody production remained unclear.

In 1998, we found that GPIIb-IIIa is one of the major target antigens recognized by platelet-reactive CD4+ T-cells in ITP patients [16]. In that study, human GPIIb-IIIa affinity-purified from outdated platelet concentrates was used to examine antigen-specific T-cell responses, but peripheral blood T-cells from ITP patients and healthy individuals were virtually unresponsive to GPIIb-IIIa in its native form. Previous studies in our laboratory had indicated that structural modification of autoantigens could induce the expression of antigenic self-determinants that were recognized by pathogenic autoreactive T-cells in other autoimmune diseases [17,18]. With this result in mind, we prepared disulfide-reduced GPIIb-IIIa and tryptic peptides of GPIIb-IIIa and used them to stimulate T-cells. Using this system, we reproducibly detected T-cell proliferative responses induced by modified GPIIb-IIIa from the majority of ITP patients. We confirmed the specificity of the T-cell response by specifically activating T-cells primed with modified GPIIb-IIIa by means of recombinant GPIIb-IIIa fragments expressed in *Escherichia coli* in secondary cultures. A major proportion of the GPIIb-IIIa–reactive T-cells had a CD4+ helper phenotype and were restricted by HLA-DR. However, an in vitro T-cell proliferative response to chemically modified GPIIb-IIIa was also detected in many healthy individuals.

To evaluate the ability of GPIIb-IIIa–reactive CD4+ T-cells to stimulate antiplatelet autoantibody production, we set up an in vitro culture system in which peripheral blood T-and B-cells were cultured in the presence or absence of modified GPIIb-IIIa and the IgG anti–GPIIb-IIIa antibody levels were subsequently measured in the culture supernatants. IgG anti-GPIIb-IIIa antibodies were detected in the supernatants of ITP patients in an antigen-dependent manner but not in the supernatants of healthy individuals who showed T-cell proliferation induced by modified GPIIb-IIIa. This result is analogous to the in vitro production of antiphospholipid antibodies in peripheral blood mononuclear cell cultures from APS patients versus healthy individuals in response to a major autoantigen, β_2-glycoprotein I [18]. The difference between ITP patients and healthy individuals in the autoantibody response can be explained by a mechanism in which circulating memory B-cells capable of producing IgG autoantibodies are present in the patients but absent in healthy individuals [19]. This antibody production was inhibited specifically by a monoclonal antibody (MoAb) to HLA-DR, indicating a requirement for T-cell recognition of the antigen during the T-cell–B-cell interaction [16]. The IgG anti–GPIIb-IIIa antibodies produced in the in vitro cultures potentially act as antiplatelet antibodies in vivo in ITP patients because they are capable of binding to normal platelet surfaces (Figure 1). Therefore, it is likely that the GPIIb-IIIa–reactive T-cells detected in our assay are involved in the production of pathogenic antiplatelet antibodies in ITP patients.

To map the T-cell epitopes on GPIIb-IIIa, we examined the T-cell responses to 6 recombinant fragments covering different portions of GPIIbα and GPIIIa [20]. Peripheral blood T-cells from ITP patients and healthy individuals proliferated in response to the recombinant fragments in various combinations but frequently recognized the amino-terminal portions of both GPIIbα (amino acid residues 18-259) and GPIIIa (amino acid residues 22-262). More importantly, these amino-terminal fragments also stimulated the production of antiplatelet antibodies from autologous B-cells. When GPIIb-IIIa–reactive T-cell lines were generated from ITP patients by the repeated stimulation of peripheral blood T-cells with tryptic peptides of GPIIb-IIIa, most of the specific T-cell lines recognized the amino-terminal portion of GPIIbα or GPIIIa. From these findings, the amino-terminal portion of GPIIb-IIIa was deemed to be primarily responsible for the stimulation of autoreactive T-cells and subsequent autoantibody production. Notably, the antiplatelet autoantibodies in ITP patients mainly recognize the conformational epitope consisting of the amino-terminal portion of GPIIbα as well [9]. A study to finely map T-cell epitopes has been underway in our laboratory, but many epitopes appear to be associated with different HLA class II alleles, even in the amino-terminal portion of GPIIb-IIIa. On the other hand, Semple and coworkers showed that a synthetic peptide corresponding to amino acid residues 496 to 510 of GPIIIa stimulated IL-2 production in T-cell lines generated from the ITP spleen [21]. Moreover, another Japanese group reported that CD4+ T-cell lines from an ITP patient produced interferon γ (IFN-γ) in response to stimulation with a synthetic peptide consisting of GPIbα amino acid residues 429 to 443; this region contains anchor motifs for HLA-DRB1*0405 [22]. Interestingly, these T-cell lines also responded to autologous

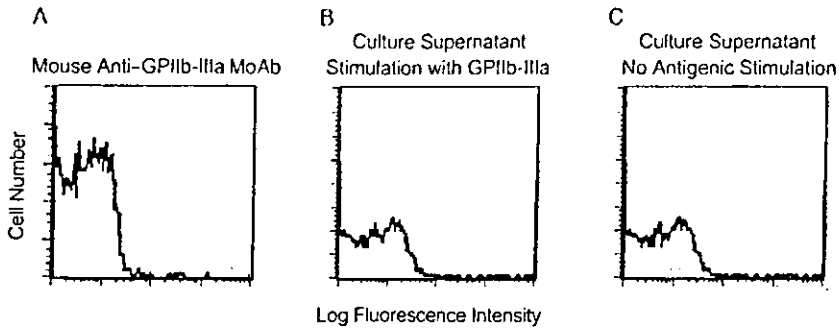

Figure 1. Binding of immunoglobulins in peripheral blood mononuclear cell (PBMC) culture supernatants to platelet surfaces. Platelets derived from healthy individuals were incubated with fluorescein-5-isothiocyanate (FITC)-conjugated mouse anti-glycoprotein IIb-IIIa (GPIIb-IIIa) monoclonal antibody (MoAb) (A). Platelets were also incubated with ITP patient-derived PBMC culture supernatants with (B) or without (C) antigenic stimulation with modified GPIIb-IIIa and then incubated with FITC-conjugated goat antihuman immunoglobulin G $F(ab')_2$ fragment. Cell staining analyzed by flow cytometry is shown as shaded histograms. Open histograms represent controls stained with FITC-conjugated isotype-matched MoAb to an irrelevant antigen (A) or with secondary antibody alone (B and C). Results shown are representative of 5 experiments using 4 different PBMC samples.

dendritic cells preincubated with platelet lysate. However, it is not known whether these T-cells, which are responsive to epitopes located in the center of GPIIbα and GPIIIa, can induce antiplatelet autoantibody production. These findings taken together indicated that T-cell epitopes are present throughout the GPIIbα and GPIIIa molecules, suggesting that the entire GPIIb-IIIa molecule is a target of the autoimmune response. In addition, these different results may reflect the heterogeneity of the T-cell response to GPIIb-IIIa in ITP patients.

$CD4^+$ helper T-cells are functionally classified according to their cytokine expression profiles. In this regard, several reports on serum T-cell–derived cytokines in ITP patients describe a helper T-cell subtype 1 (Th1) response or a mixed Th0/Th1 response [23,24]. We have generated more than 50 T-cell lines responsive to GPIIb-IIIa from the peripheral blood or spleen of ITP patients and have found that all of these T-cell lines produced IFN-γ and had a Th1- or Th0-like cytokine expression profile. In addition, IL-6 expression by these T-cell lines correlated with their ability to provide help to B-cells. It is now known that the Th1/Th2 antagonism in humans is not as strict as it is reported to be in mice, and T-cell function is associated with individual cytokine profiles rather than with the Th1/Th2 phenotype. Therefore, we further examined the roles of individual cytokines in regulating the production of anti–GPIIb-IIIa antibodies by using an in vitro assay system consisting of GPIIb-IIIa–reactive $CD4^+$ T-cell lines and autologous peripheral blood B-cells [20]. In this assay system, an anti–IL-6 MoAb blocked the T-cell–dependent production of anti–GPIIb-IIIa antibodies, but anti–IFN-γ and anti–IL-4 MoAbs did not, supporting the idea that IL-6 is the major B-cell–activating factor produced by GPIIb-IIIa–reactive $CD4^+$ T-cells. On the other hand, an anti-CD154 MoAb almost completely inhibited the in vitro production of anti–GPIIb-IIIa antibody induced by GPIIb-IIIa–reactive $CD4^+$ T-cell lines as well, indicating that CD40-CD154 engagement is also essential for the T-cell helper activity [25]. Taken together, these findings indicate that $CD4^+$ T-cell–dependent B-cell activation depends on 2 types of stimuli: T-cell–derived IL-6 and CD40-CD154 engagement.

$CD4^+$ T-cells that preferentially secrete transforming growth factor β1 (TGF-β1) are known to exert an immunosuppressive function through a bystander effect and are now called the *Th3 subset* [26]. In this regard, Andersson et al recently reported that ITP patients in remission had significantly elevated plasma levels of TGF-β1 [27], a finding later confirmed by Mouzaki et al [28]. Moreover, peripheral blood mononuclear cell cultures from ITP patients with active disease showed a reduced production of TGF-β1 [29]. Therefore, the T-cell cytokine profile is skewed toward a Th1/Th0 pattern in patients with active ITP and toward a Th3 response in patients in remission. This finding is potentially useful because the induction of platelet-reactive Th3 cells might be a therapeutic strategy for ITP. It is possible that the oral administration of autologous platelets induces platelet-specific tolerance through the induction of platelet antigen–reactive Th3 cells [30].

3. The Role of Cytotoxic T-Cells in ITP Pathogenesis

Earlier studies of the cell-mediated immune response in ITP patients considered cytotoxic platelet destruction as a possible pathogenic mechanism because a certain percentage of ITP patients do not have detectable antiplatelet antibodies [31]. Olsson et al recently demonstrated that the cell-mediated cytotoxic lysis of platelets by cytotoxic T-cells might contribute to thrombocytopenia in ITP [32]. In this study, a microarray analysis of peripheral blood T-cells revealed the increased expression of genes for ApoI/Fas, granzyme A, granzyme B, and perforin, together with Th1-related genes such as those for IFN-γ and IL-2 receptor, in ITP patients compared with healthy controls. An in vitro cytotoxicity assay showed the lysis of autologous platelets in patients with active ITP but not in patients in remission. In addition, ITP patients in remission showed increased gene and protein expression for several members of the killer cell immunoglobulin-like receptor family, which inhibit cytotoxic T-cells and natural killer cells. Currently, it is not known whether this phenomenon is mediated by the specific recognition of platelet antigens such as GPIIb-IIIa, by cytotoxic T-cells, or by the nonspecific activation of cytotoxic T-cells.

4. GPIIb-IIIa–Reactive T-Cells Recognize Cryptic Determinants

Circulating GPIIb-IIIa–reactive T-cells are detectable not only in ITP patients but also in many healthy individuals who do not have anti–GPIIb-IIIa antibodies [16,20], indicating that GPIIb-IIIa–reactive T-cells are a component of the normal T-cell repertoire. T-cells that recognize peptides generated from native GPIIb-IIIa by normal processing pathways are hypothesized to be deleted in the thymus through the process of "negative selection" because GPIIb-IIIa is expressed abundantly on thymic epithelial cells as early as the 16th week of intrauterine life [33]. However, Filion et al reported that $CD4^+$ T-cells reactive with liposome-encapsulated GPIIb-IIIa, which should have a native conformation, were detected in the peripheral blood from a majority of healthy individuals [34]. According to the report of Filion et al, 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 surfaces. These features are consistent with the characteristics of anergic T-cells, which are T-cells in a functionally inactivated state after their antigenic stimulation in the periphery. Therefore, T-cell tolerance to GPIIb-IIIa is tightly maintained under normal circumstances. Some T-cells reactive to native GPIIb-IIIa escape thymic deletion and exist in the periphery; however, they are inactivated by a postthymic mechanism of peripheral tolerance [35].

To evaluate whether the GPIIb-IIIa–reactive T-cells in ITP patients are activated in vivo, we performed the following assays. First, the precursor frequency of GPIIb-IIIa–reactive T-cells in circulation was directly determined with limiting-dilution analysis [19]. The frequency of GPIIb-IIIa–reactive circulating T-cells was significantly increased in ITP patients, compared with healthy individuals. Second, the time course of the T-cell response to modified GPIIb-IIIa was evaluated by measuring the T-cell proliferative response on days 3, 5, 6, and 7 [16]. The T-cell response to modified GPIIb-IIIa in ITP patients was detectable on day 5 and peaked on day 6, whereas T-cells from healthy responders did not show the response on day 5 but did respond on day 7. These accelerated T-cell responses to modified GPIIb-IIIa in ITP patients indicate recent antigen exposure or the in vivo activation of such T-cells, whereas the kinetics of the T-cell responses induced by modified GPIIb-IIIa in healthy individuals were more typical of a naive T-cell response. Similar differences in the kinetics of autoantigen-specific T-cell proliferation in patients versus healthy individuals were shown for the T-cell responses to type II collagen in rheumatoid arthritis [36] and to topoisomerase I in scleroderma [37,38]. Taken together, these findings indicate that GPIIb-IIIa–reactive T-cells are activated in vivo in ITP patients but not in healthy individuals.

Because GPIIb-IIIa–reactive $CD4^+$ T-cells responded to the reduced GPIIb-IIIa or to tryptic peptides of GPIIb-IIIa but not to native GPIIb-IIIa complex [16], it is likely that the epitopes recognized by the GPIIb-IIIa–reactive T-cells are "cryptic" determinants. The concept of the cryptic self-determinant was initially proposed by Sercarz and colleagues [39,40] and has been extended by other investigators [41]. In this model, extracellular antigens are taken up by antigen-presenting cells (APCs) and degraded into peptide fragments by endosomal proteases (antigen-processing). Only the peptides with a capacity to bind HLA class II molecules are expressed on the cell surface and presented to T-cells. Of the numerous peptides predicted by the amino acid sequences of the autoantigen, a small proportion are presented to T-cells as a consequence of antigen processing and presentation. These peptides are representative of the autoantigen and termed *dominant self-determinants*, whereas the remaining peptides, which are not efficiently presented and are normally hidden from the immune system, are termed *cryptic self-determinants*. There is increasing interest in the possibility that crypticity is an important characteristic of the epitopes recognized by autoreactive T-cells and thus is relevant to autoimmune pathogenesis [41-43]. T-cells recognizing the dominant self-determinants in APCs undergo deletion in the thymus or inactivation in the periphery. On the other hand, T-cells specific for cryptic self-determinants are a component of the normal T-cell repertoire but normally do not encounter antigenic peptides in the periphery. These T-cells might become activated and autoaggressive if the previously cryptic self-determinants were presented at a higher concentration. Our findings regarding the T-cell response to GPIIb-IIIa in ITP patients are consistent with this cryptic epitope model because the GPIIb-IIIa–reactive T-cells recognize epitope peptides generated from structurally modified GPIIb-IIIa but not from native GPIIb-IIIa, exist in the normal T-cell repertoire, and are activated in vivo in ITP patients but not in healthy individuals.

5. Mechanisms for the Activation of GPIIb-IIIa–Reactive T-Cells

A fundamental question is how normally cryptic epitopes of platelet antigens such as GPIIb-IIIa become visible to the immune system and elicit a sustained pathogenic response in ITP patients. Because T-cells that are responsive to cryptic self-determinants would not encounter antigenic peptides in the periphery under normal circumstances, it is likely that a pathogenic autoreactive T-cell response is induced by the de novo presentation of a previously cryptic epitope under special conditions (Figure 2). To examine the mechanisms for activating GPIIb-IIIa–reactive T-cells in ITP patients, we sought to identify the site where GPIIb-IIIa–derived cryptic peptides are efficiently presented by APCs and subsequently induce the activation of GPIIb-IIIa–reactive T- and B-cells. The reticuloendothelial system, including the spleen, was a likely place because approximately 60% of ITP patients achieve a stable increased platelet count after surgical splenectomy [44] and because the spleen is considered the primary site of both platelet destruction and antiplatelet autoantibody production [45]. To test our hypothesis, we evaluated the frequencies and activation status of GPIIb-IIIa–reactive T- and B-cells in the peripheral blood and spleen obtained from ITP patients who had undergone splenectomy [19]. There were no differences in GPIIb-IIIa–reactive T-cell frequencies between the peripheral blood and spleen as determined by limiting-dilution analysis, but activated T-cells responsive to GPIIb-IIIa with accelerated proliferation rates and activated T-cells expressing CD154 were more frequent in the spleen than in the peripheral

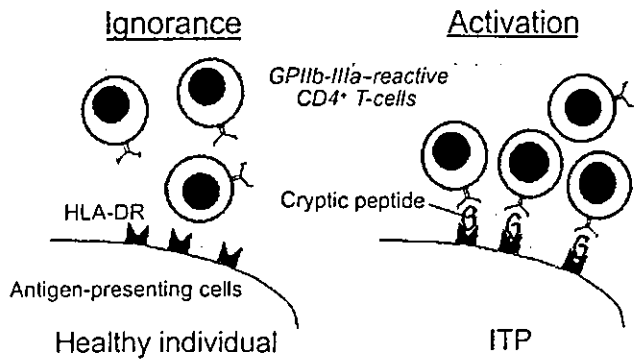

Figure 2. Activation of glycoprotein IIb-IIIa (GPIIb-IIIa)-reactive CD4+ T-cells in the normal T-cell repertoire by de novo presentation of a previously cryptic peptide of GPIIb-IIIa. T-cells recognizing a GPIIb-IIIa–derived cryptic peptide would not encounter the antigenic peptide in the periphery in healthy individuals (immunologic "ignorance"), whereas GPIIb-IIIa–reactive CD4+ T-cells are activated on recognition of the previously cryptic peptide expressed on functional antigen-presenting cells in patients with idiopathic thrombocytopenic purpura (ITP).

blood. The frequencies of B-cells producing anti-GPIIb-IIIa antibodies in the peripheral blood and spleen, as determined by enzyme-linked immunospot assay, were also similar; however, anti-GPIIb-IIIa antibodies were spontaneously produced by splenocytes in vitro but were scarcely secreted by peripheral blood mononuclear cells. $CD19^-$/surface immunoglobulin–negative/$CD38^+$/$CD138^+$ plasma cells secreting anti-GPIIb-IIIa antibodies were detected exclusively in the spleen. Serial analysis showed that the frequencies of circulating GPIIb-IIIa–reactive T- and B-cells were markedly decreased after splenectomy in patients with a complete response but were unchanged in nonresponders. These findings indicated that the T-cell–B-cell interaction through recognition of the cryptic peptides of GPIIb-IIIa in ITP patients occurs primarily in the spleen. An increased delivery of antigen to the processing compartment is one of the potential mechanisms for revealing cryptic self-determinants in APCs [41], and splenic macrophages are likely to be primarily involved in this process. Splenic macrophages that phagocytose opsonized platelets via Fcγ receptors may have the ability to efficiently concentrate small quantities of platelet antigens that were previously cryptic. Taking these data into consideration leads us to propose a pathogenic loop that consists of antiplatelet autoantibody production by B-cells, phagocytosis of opsonized platelets and presentation of GPIIb-IIIa–derived cryptic peptides by splenic macrophages, and the exertion of helper activity by the GPIIb-IIIa–reactive CD4+ T-cells in ITP patients (Figure 3). Once this pathogenic loop is established, the antiplatelet autoantibody production is maintained endlessly. Therefore, therapeutic strategies for ITP should be aimed at interrupting this pathogenic cycle.

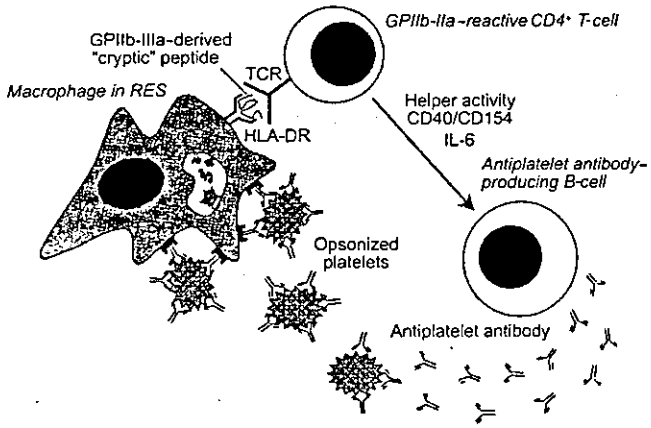

Figure 3. Schematic representation of a continuous pathogenic loop carried out by macrophages in the reticuloendothelial system (RES), glycoprotein IIb-IIIa (GPIIb-IIIa)-reactive CD4+ T-cells, and antiplatelet antibody-producing B-cells that maintain antiplatelet antibody production in patients with idiopathic thrombocytopenic purpura. A macrophage in the RES takes up opsonized platelets via the Fcγ receptor (FcγR) and presents a GPIIb-IIIa–derived cryptic peptide in the context of HLA-DR as a result of antigen processing. The GPIIb-IIIa–reactive CD4+ T-cell is activated by recognition of the HLA/cryptic peptide complex via the T-cell receptor (TCR). The activated GPIIb-IIIa–reactive CD4+ T-cell subsequently stimulates a specific B-cell to produce immunoglobulin G antiplatelet antibodies through the expression of CD154 and interleukin 6 (IL-6).

6. Potential Pathogenesis for ITP

In patients with ITP, macrophages in the reticuloendothelial system are a source of cryptic peptides that activate GPIIb-IIIa–reactive T- and B-cells. However, other mechanisms should contribute to the expression of cryptic peptides at the onset of the disease. It is difficult to identify those mechanisms by using samples from ITP patients because the triggering event may no longer be present in the patients (ie, a hit-and-run event). Figure 4 summarizes potential events that could induce the expression of cryptic epitopes derived from platelet antigens and the APCs that present the cryptic epitopes in the induction and chronic phases of ITP. Because determinant dominance during antigen processing in APCs is influenced by protein structure, conditions that change the molecular context of GPIIb-IIIa may permit the presentation of previously cryptic determinants, thereby breaking the

	Induction phase	Chronic phase
Magnitute of cryptic epitope expression	*Genetic disposition* *Nonspecific inflammation* *Suppressed regulatory function*	
Possible events	Infection of microorganism Drug/chemical exposure Massive destruction of platelets	Massive destruction of opsonized platelets
APC	Cross-reactive B-cell Dendritic cell/macrophage	Splenic macrophage

Figure 4. Potential events that induce the expression of cryptic epitopes of platelet antigens and the antigen-presenting cells (APCs) that present the cryptic epitopes in the induction and chronic phases of idiopathic thrombocytopenic purpura.

T-cell tolerance. Potential mechanisms for revealing cryptic self-determinants in APCs include the modulation of antigen processing, such as structural modification of self-antigens due to an unusual cleavage event or the formation of a complex with ligands that masks or unmasks cleavage sites for proteases in endosomes [41]. The factors that induce the expression of GPIIb-IIIa–derived cryptic peptides as a consequence of antigen processing in ITP patients are unknown, but several potential mechanisms can be proposed. For example, because chemical treatment of GPIIb-IIIa induces the expression of cryptic peptides that are recognized by T-cells, cryptic peptides may be revealed by chemical or drug exposure and a resultant structural modification. In this regard, patients taking D-penicillamine or gold salt sometimes develop a thrombocytopenia that is indistinguishable from ITP [46,47]. Alternatively, the complex of GPIIb-IIIa and its ligand may induce the expression of cryptic epitopes because fibrinogen and other GPIIb-IIIa–binding proteins have the Arg-Gly-Asp (RGD) sequence, and other unrelated proteins with the RGD sequence have been shown to bind GPIIb-IIIa [48]. In this regard, autoreactive T-cells and autoantibodies reportedly can be generated in normal mice by coimmunization with a mixture of self- and foreign antigens [49]. We recently found that β_2-glycoprotein I complexed with anionic phospholipids induces the expression of a cryptic epitope that is recognized by pathogenic autoreactive CD4$^+$ T-cells in APS patients [50].

Another potential mechanism is that ITP patients may have been exposed to foreign proteins that cross-react with GPIIb-IIIa. This mechanism is thought to involve the generation of cross-reactive B-cells that are initially primed by a foreign protein; they then bind a self-antigen and process and present self-peptides to T-cells [51]. Because B-cells that acquire antigen via surface immunoglobulin require approximately 1000 to 10,000 times less antigen for a subsequent T-cell response, B-cells may be able to efficiently concentrate small quantities of determinants that were previously cryptic [52]. In this regard, anti-GPIIIa antibodies in patients with human immunodeficiency virus (HIV)-related immune thrombocytopenia cross-react with HIV-associated gp120 [53]. The eradication of *Helicobacter pylori* from ITP patients was recently reported to induce a complete remission in nearly half of the patients [54]. The precise mechanism for this phenomenon remains unclear, but cross-reactivity between GPIIb-IIIa and the *H pylori* CagA protein has been proposed as a potential mechanism [55].

The efficient presentation of cryptic peptides derived from GPIIb-IIIa or other platelet antigens in APCs through one of the mechanisms described above would induce the activation of specific CD4$^+$ T-cells and the production of antiplatelet autoantibodies. However, to initiate the harmful autoimmune response requires additional factors, such as genetic disposition, nonspecific inflammation mediated by cytokines and toll-like receptor ligands, and/or suppressed regulatory function.

7. Summary and Future Perspectives

Accumulating evidence in the field of cellular immunity in ITP patients indicates an important role for platelet antigen–specific CD4$^+$ T-cells in antiplatelet antibody production and the pathogenesis of ITP. Further studies examining the mechanisms that induce the efficient presentation of cryptic T-cell determinants of platelet antigens as a consequence of antigen processing would clarify the etiology of ITP. In addition, the specific elimination or inactivation of pathogenic GPIIb-IIIa–reactive CD4$^+$ T-cells is a potential selective therapy for inhibiting the pathogenic antiplatelet antibody production in patients with ITP.

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Genetic Defects Leading to Hereditary Thrombotic Thrombocytopenic Purpura

Koichi Kokame and Toshiyuki Miyata

In patients with thrombotic thrombocytopenic purpura (TTP), unusually large multimers of von Willebrand factor (VWF) circulate in the plasma. This is caused by a functional deficiency of VWF-cleaving protease, ADAMTS-13. Although TTP usually occurs as an acquired form due to autoantibodies against ADAMTS-13, the condition may be inherited in an autosomal recessive fashion. Thus far, genomic DNA from 23 patients with hereditary TTP and their families has been analyzed and 33 causative mutations identified in the *ADAMTS13* gene: 19 missense, five nonsense, five frameshift, and four splice mutations. Common missense polymorphisms have been also found, one of which significantly reduces ADAMTS-13 activity. No cases have been found without mutations in the *ADAMTS13* gene, suggesting that genetic defects in *ADAMTS13* are the dominant cause of hereditary TTP. Further analysis may reveal the genetic background associated with acquired TTP and other thrombotic diseases.
Semin Hematol 41:34-40.

THROMBOTIC thrombocytopenic purpura (TTP) is a highly lethal disease characterized by five features: thrombocytopenia, microangiopathic hemolytic anemia, renal failure, fever, and neurological dysfunction.[1] In TTP, platelet thrombi form within the microvasculature, which is thought to be caused by accumulation of unusually large multimers of von Willebrand factor (VWF). The majority of TTP is acquired, often affecting adolescents and adults. In a smaller number of cases, TTP is inherited in an autosomal recessive fashion, and hereditary TTP with neonatal onset and frequent relapses is often diagnosed as Upshaw-Schulman syndrome.[2]

VWF is synthesized primarily in vascular endothelial cells and secreted into the plasma as large multimeric forms.[3-6] Normally, the large multimers are cleaved into smaller forms by a plasma metalloprotease, ADAMTS-13,[7-11] which is a member of the ADAMTS (a disintegrin-like and metalloprotease [reprolysin type] with thrombospondin type 1 motif) gene family. In acquired TTP, autoantibodies against ADAMTS-13 are produced that inhibit the VWF-cleaving activity of ADAMTS-13, leading to the accumulation of highly active large VWF multimers.[12,13] In contrast, patients with hereditary TTP innately have no plasma ADAMTS-13 activity. Thus far, 33 causative mutations have been identified in the *ADAMTS13* gene and here we summarize the genetic defects leading to hereditary TTP. We describe the gene symbol of ADAMTS-13 as *ADAMTS13*, according to gene nomenclature provided by the Human Gene Nomenclature Committee. We refer to the protein as ADAMTS-13 according to recommendation of Dr Suneel S. Apte (http://www.lerner.ccf.org/bme/apte/adamts/nomenclature.php).

Structural Properties of ADAMTS-13

Human ADAMTS-13 was purified from plasma[14-16] and its cDNA was cloned.[16-18] At the same time, *ADAMTS13* was also identified as the responsible gene for hereditary TTP by linkage analysis.[19] ADAMTS-13 consists of 1,427 amino acid residues, containing an N-terminal signal peptide, a propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type-1 motif (TSP1), a cysteine-rich domain, a spacer domain, seven additional TSP1 repeats, and two CUB domains (Fig 1). Its mRNA is predominantly expressed in liver.[16,17,19,20]

Functional Properties of ADAMTS-13

The only known physiological substrate of ADAMTS-13 is VWF multimers, which play a pivotal role in platelet aggregation. ADAMTS-13 specifically cleaves the peptidyl bond between Y1605 and M1606 in the VWF A2 domain.[21-24] Systematic studies on the structure and function of ADAMTS-13, using recombinant ADAMTS-13 with progressive C-terminal truncations, determined the domains required for

From the National Cardiovascular Center Research Institute, Osaka, Japan.

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Address correspondence to Toshiyuki Miyata, PhD, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan.

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