by their strong association with the active disease status of BD. Moreover, recognition of the endogenously generated MICA peptide by autoreactive CTLs in the context of HLA–B51 may explain why HLA–B51 is a marker of susceptibility and severity in BD (1,2). Thus, MICA is the first "behçetogenic" CD8 autoantigen identified in association with HLA–B51. In BD patients without HLA–B51, it is possible that appropriate MICA-derived peptides are presented in the context of HLA class I molecules other than HLA–B51 and recognized by autoreactive CTLs.

Many extrinsic pathogenic candidates, including bacterial and viral antigens, have been reported in BD (2). An excessive inflammatory response induced by these extrinsic agents is one of the fundamental disease processes of BD (2). Neutrophil hyperfunction and a cross-reactive autoimmune response between microbial and human HSPs are proposed to be responsible for this hyperreactivity (1,14). MICA is a stress-inducible protein that acts as a ligand for a subset of γ/δ T cell receptors (6) and a killer cell–activating receptor, NKG2D, expressed by most cytotoxic cells, including CTLs and natural killer cells (15). Cytotoxic activity is augmented via the engagement of NKG2D to MICA, whose expression is induced on epithelial and endothelial cells under stress conditions. The physiologic role of MICA remains unclear, but it is thought to function as a marker for damaged or infected epithelial and endothelial cells and to facilitate clearance of those injured cells by an immune surveillance system. This process would not normally induce harmful inflammation but may cause tissue injuries in the presence of MICA-reactive CTLs in patients with BD. Namely, after nonspecific minor injuries and microbial infection, MICA-reactive CTLs are activated upon recognition of the MICA-derived peptide presented on the epithelium and endothelium and subsequently lead to excessive and prolonged inflammatory responses at the site of stress by enhancing the MICA-mediated cytotoxicity. This response would be further augmented in the predominant Th1 cytokine environment observed in patients with BD (1,2).

In summary, this report provides the first direct evidence showing the potential involvement of HLA–B51–restricted autoreactive CTLs in the pathogenesis of BD. Moreover, our findings will stimulate further research on MICA as a key molecule in the development of BD. Because this is a preliminary study preformed in a small number of patients with BD, additional studies involving a larger population are necessary to confirm our hypothesis.

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Concise Report

Lack of circulating autoantibodies to bone morphogenetic protein receptor-II or activin receptor-like kinase 1 in mixed connective tissue disease patients with pulmonary arterial hypertension

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Objectives. To examine whether autoantibodies against bone morphogenetic protein receptor-II (BMPR-II) or activin receptor-like kinase 1 (ALK-1) are associated with pulmonary arterial hypertension (PAH) in patients with mixed connective tissue disease (MCTD).

Methods. We studied sera from 37 MCTD patients with or without PAH, six patients with idiopathic PAH, and 30 healthy controls. Circulating anti-BMPR-II and anti-ALK-1 antibodies were detected using immunoprecipitation of recombinant antigens generated by *in vitro* transcription/translation and indirect immunofluorescence of cultured cells that were induced to express these antigens by gene transfer. Anti-BMPR-II antibodies were further examined by immunoprecipitation and immunoblotting using a recombinant fragment of the extracellular domain of BMPR-II.

Results. Serum anti-BMPR-II and anti-ALK-1 autoantibodies were not detected in MCTD patients irrespective of the presence or absence of PAH, or in patients with idiopathic PAH.

Conclusions. Our finding does not support the hypothesis that autoantibody-mediated dysregulation of signals through BMPR-II or ALK-1 contributes to the development of PAH in patients with connective tissue diseases.

KEY WORDS: Pulmonary arterial hypertension, Autoantibody, Mixed connective tissue disease, Bone morphogenetic protein receptor-II, Activin receptor-like kinase 1.

Pulmonary arterial hypertension (PAH) is a life-threatening organ involvement in patients with various connective tissue diseases, including scleroderma [1]. PAH also occurs without an underlying disease, and is then termed idiopathic pulmonary arterial hypertension (IPAH; formerly primary pulmonary hypertension) [2]. Recent genetic analyses in IPAH patients have identified germ-line mutations in the gene for bone morphogenetic protein receptor-II (BMPR-II), a type II receptor for transforming growth factor β (TGF-β) superfamilies, in both familial and sporadic cases [3–5]. In addition, families with hereditary haemorrhagic telangiectasia are known to develop PAH frequently, and mutations in the gene for activin receptor-like kinase 1 (ALK-1), a type I receptor for TGF-β, have been identified as a genetic factor responsible for this hereditary disease [6]. Both BMPR-II and ALK-1 are receptors for TGF-β family proteins, with intracellular serine/threonine kinase domains that are preferentially expressed on vascular endothelial cells [7, 8]. Upon ligand binding, these receptors participate in the phosphorylation of a series of Smad proteins that regulate the growth and functional properties of vascular endothelial and smooth muscle cells. It is currently believed that dysregulated Smad signals through BMPR-II and ALK-1 are involved in the pathogenesis of IPAH [9, 10].

IPAH and PAH accompanied by connective tissue diseases have similar characteristics [1], including medial hypertrophy and plexogenic arteriopathy in the pulmonary artery histology; progressive clinical course and poor prognosis; and therapeutic responses to continuous infusion of the prostacyclin analogue and endothelin-1 receptor antagonist. Therefore, we hypothesized that dysfunction in BMPR-II or ALK-1 also contributes to the pathogenesis of PAH in patients with connective tissue diseases. However, it seemed unlikely that genetic mutations would be found as the underlying cause because two independent research groups failed to detect mutations in the BMPR-II gene in patients with scleroderma spectrum disorders and PAH [11, 12]. Another potential mechanism includes the presence of autoantibodies that functionally interfere with ligand binding to BMPR-II or ALK-1, which is reasonably likely, because patients with connective tissue diseases, especially those with systemic lupus erythematosus (SLE), are intrinsically prone to producing autoantibodies. To test this hypothesis, we developed assay systems to detect autoantibodies that react with BMPR-II and ALK-1. Since the majority of Japanese patients with connective tissue disease and PAH are positive for antibodies to U1 small nuclear ribonucleoprotein (U1RNP) and have a diagnosis of mixed connective tissue disease

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(MCTD) [13, 14], we examined MCTD patients with or without PAH in this study.

Materials and methods

Patients and samples

We examined 37 patients who were positive for anti-U1RNP antibodies and fulfilled the diagnostic criteria for MCTD proposed by Porter *et al.* [15]. MCTD patients represented heterogeneous clinical characteristics: 21 patients met the American College of Rheumatology (ACR) classification criteria for SLE [16], 24 met the ACR preliminary classification criteria for scleroderma [17], and six met the diagnostic criteria for definite polymyositis or dermatomyositis as proposed by Bohan and Peter [18]. Fourteen patients satisfied two of these three sets of criteria (SLE and scleroderma in nine, and scleroderma and polymyositis/dermatomyositis in five). Echocardiogram was performed on all patients, and those with a mean pulmonary artery pressure ≤25 mmHg at rest were regarded as non-PAH [9]. The remaining patients, with a mean pulmonary artery pressure >25 mmHg, were further divided into two groups: patients with definite PAH in whom the presence of PAH was confirmed by right ventricle catheterization, and those with questionable PAH in whom right ventricle catheterization was not carried out. Serum samples from six patients with IPAH [2] and 30 healthy individuals were used as controls. Written consent was obtained according to the Declaration of Helsinki, and the study design was approved by Keio University Institutional Review Boards.

IgG fraction

The immunoglobulin G (IgG) fraction was isolated from sera by affinity chromatography using a HiTrap™ Protein G HP Column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. The protein concentration was measured with the Bradford assay using serial concentrations of bovine serum albumin (BSA) as a standard.

cDNA constructs

Full-length cDNAs encoding human BMPR-II and ALK-1 were kindly provided by Dr Kohei Miyazono (University of Tokyo Graduate School of Medicine, Tokyo, Japan). These cDNAs were subcloned into pcDEF3-FLAG (BMPR-II) or pcDNA3-HA (ALK-1), which add FLAG- and HA-epitope tags, respectively, at the carboxyl terminal end [19, 20].

Immunoprecipitation of recombinant polypeptides generated by in vitro *transcription and translation*

Recombinant BMPR-II-FLAG and ALK-1-HA were produced by *in vitro* transcription and translation of full-length cDNAs using the Single Tube Protein™ System 3 (Novagen, Darmstadt, Germany) in the presence of ^{35}S-labelled methionine (ICN Biomedicals, Irvine, CA, USA). ^{35}S-labelled BMPR-II-FLAG and ALK-1-HA were applied to immunoprecipitation assays as described previously [21]. Mouse monoclonal antibodies (mAbs) to BMPR-II (clone 18; Becton Dickinson, San Jose, CA, USA) and FLAG (clone M2; Sigma-Aldrich, St Louis, MO, USA) were used as positive controls for BMPR-II-FLAG, and rat anti-HA mAb (clone 3F10; Roche Applied Science, Mannheim, Germany) was used as a positive control for ALK-1-HA. Mouse anti-His mAb (Amersham Biosciences) was used as a negative control. The immunoprecipitated materials were separated on sodium dodecyl sulphate (SDS)-6% (for BMPR-II-FLAG) or 8.5% (for ALK-1-HA) polyacrylamide gels, and subjected to autoradiography using a Fuji BAS 5000 Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation and immunoblotting using recombinant BMPR-II as an antigen

Anti-BMPR-II antibodies were detected using a recombinant chimeric protein consisting of the extracellular domain of human BMPR-II and the histidine-tagged Fc region of human IgG1 (BMPR-II/Fc chimera; R & D Systems, Minneapolis, MN, USA). Serum samples (10 μl) were incubated with protein A-Sepharose™ CL-4B beads (Amersham Biosciences) for 2 h at room temperature. Goat anti-BMPR-II polyclonal antibodies (R & D systems) and anti-His mAb were used as positive controls, and anti-HA mAb was used as a negative control. The beads were incubated with BMPR-II/Fc chimera (50 ng) for 2 h at 4°C in 10 mM Tris–HCl, 500 mM NaCl, 0.1% Nonidet P-40, pH 8.0, containing 60% fetal bovine serum to block non-specific protein A binding to the Fc region of the BMPR-II/Fc chimera. The immunoprecipitated materials were subsequently separated on SDS–8.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk, and incubated with anti-BMPR-II polyclonal antibodies and then with alkaline phosphatase-conjugated anti-goat IgG (Sigma-Aldrich). Positive signals were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Indirect immunofluorescence of cultured cells expressing exogenous genes

COS-7 cells grown on fibronectin-coated eight-well chamber slides (Becton Dickinson Labware, Bedford, MA, USA) were transfected with plasmids harbouring BMPRII-FLAG or ALK-1-HA, using the Effectene transfection reagent (Qiagen, Hilden, Germany). COS-7 cells transfected with the empty vector were used as a control. After a 24-h culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, the cells were fixed with cold acetone/methanol for 5 min. Each slide was blocked with 5% BSA, and incubated with purified IgG (40 μg/ml) and then with Alexa Fluor 488-conjugated goat anti-human IgG antibodies (Molecular Probes, Eugene, OR, USA). The cells were further incubated with biotinylated anti-FLAG mAb and streptavidin-conjugated Alexa Fluor 568 (for BMPR-II transfectants) or anti-HA mAb and Alexa Fluor 568-conjugated goat anti-rat IgG antibody (for ALK-1 transfectants). Anti-BMPRII mAb with Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies were used as a positive control for BMPR-II transfectants. Nuclei were counterstained with TO-PRO-3 (Molecular Probes). The cells were observed under a confocal laser fluorescent microscope (LSM5 PASCAL; Carl-Zeiss, Göttingen, Germany).

Results

Of 37 patients with MCTD, 15 and two had definite and questionable PAH, respectively, while 20 did not have PAH. There was no difference in the distribution of clinical diagnosis between patients with PAH and those without. Nine of 15 patients with definite PAH died within 3 yr after the serum sample collection. Autopsy in seven patients revealed a typical medial hypertrophy with or without plexiform lesions in pulmonary arteries. Of the remaining six patients, five are being treated with epoprostenol and one is on bosentan.

Sera from 37 MCTD patients with and without PAH, six patients with IPAH and 30 healthy controls were screened for antibodies to BMPR-II and ALK-1 by immunoprecipitation

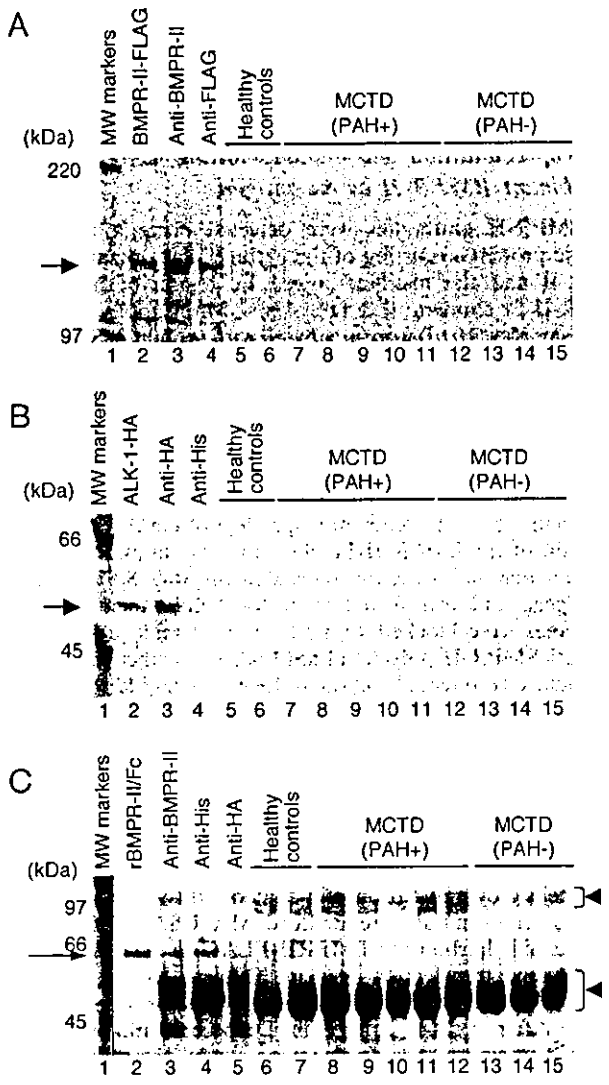

FIG. 1. Screening of anti-BMPR-II and anti-ALK-1 autoantibodies in representative sera from healthy controls and MCTD patients with or without PAH. (A) Detection of anti-BMPR-II antibodies using immunoprecipitation of ^{35}S-labelled BMPR-II-FLAG generated by *in vitro* transcription/translation. Lane 1, ^{14}C-labelled molecular weight markers; lane 2, recombinant BMPR-II-FLAG without immunoprecipitation; lane 3, anti-BMPR-II mAb; lane 4, anti-FLAG mAb; lanes 5 and 6, healthy control sera; lanes 7–11, sera from MCTD patients with definite PAH; lanes 12–15, sera from MCTD patients without PAH. Arrow denotes intact recombinant BMPR-II-FLAG. (B) Detection of anti-ALK-1 antibodies using immunoprecipitation of ^{35}S-labelled ALK-1-HA generated by *in vitro* transcription/translation. Lane 1, ^{14}C-labelled molecular weight markers; lane 2, recombinant ALK-1-HA without immunoprecipitation; lane 3, anti-HA mAb; lane 4, anti-His mAb; lanes 5 and 6, healthy control sera; lanes 7–11, sera from MCTD patients with definite PAH; lanes 12–15, sera from MCTD patients without PAH. Arrow denotes intact recombinant ALK-1-HA. (C) Detection of anti-BMPR-II antibodies by immunoprecipitation and immunoblotting using a recombinant BMPR-II/Fc chimera. Lane 1, molecular weight markers; lane 2, BMPR-II/Fc chimera without immunoprecipitation; lane 3, anti-BMPR-II polyclonal antibodies; lane 4, anti-His mAb; lane 5, anti-HA mAb; lanes 6 and 7, healthy control sera; lanes 8–12, sera from MCTD patients with definite PAH; lanes 13–15, sera from MCTD patients without PAH. Arrow denotes recombinant BMPR-II/Fc chimera and arrowheads denote non-specific IgG bands.

of ^{35}S-labelled BMPR-II-FLAG and ALK-1-HA, respectively. As shown in Fig. 1A, BMPR-II-FLAG produced by *in vitro* transcription and translation represented a 135-kDa intact protein with several degradation products. These proteins were immunoprecipitated by anti-BMPR-II and anti-FLAG mAbs, but recognized by none of the MCTD sera, IPAH sera or by 30 healthy control sera. A 55-kDa ALK-1-HA protein generated by *in vitro* transcription/translation was specifically precipitated by the anti-HA mAb (Fig. 1B). Like the BMPR-II-FLAG, ALK-1-HA was not recognized by any of the MCTD, IPAH or healthy control sera. Anti-BMPR-II or anti-ALK-1 antibodies were not detected when the amount of serum or antigens applied was increased to two-fold in all the MCTD samples.

Anti-BMPR-II autoantibodies were screened in an additional assay using BMPR-II/Fc chimera as an antigen. As shown in Fig. 1C, immunoprecipitation in the presence of an excess of bovine IgG successfully suppressed non-specific protein A binding to the Fc region of the chimeric protein. The BMPR-II/Fc chimera was recognized by anti-BMPR-II polyclonal antibodies and anti-His mAb, but not by an irrelevant anti-HA mAb. None of the sera from the 37 MCTD patients or 10 healthy controls reacted with the BMPR-II/Fc chimera in this assay system.

Because BMPR-II and ALK-1 are transmembrane proteins, recombinant proteins produced in the cell-free systems may have a conformation different from that of the membrane-bound native form. To evaluate antibody binding to membrane-bound BMPR-II and ALK-1, COS-7 cells were induced to transiently express BMPR-II-FLAG or ALK-1-HA by gene transfer, and subjected to an indirect immunofluorescence assay (Fig. 2). At least 10% of the treated cells constantly expressed BMPR-II-FLAG, as assessed by positive staining with anti-BMPR-II (green) and anti-FLAG (red) mAbs. Both mAbs stained principally the same portions of the cell membrane and cytoplasm (yellow). Anti-BMPR-II and anti-FLAG mAbs did not stain cells transfected with a control vector without the cDNA insert. To detect anti-BMPR-II antibodies in MCTD sera, IgG purified from serum samples was used instead of anti-BMPR-II mAb. Figure 2A shows representative results obtained from MCTD sera and a healthy control serum. Both MCTD sera stained nuclei, and one serum additionally stained the cytoplasm of all the cultured cells, but the staining pattern was clearly different from that produced by the anti-FLAG mAb. No staining pattern comparable to that produced by the anti-FLAG mAb was detected in additional IgG samples from four MCTD patients, including three with definite PAH, or from two healthy controls. Similarly, anti-HA mAb specifically stained the cellular membrane and cytoplasm of cells that were induced to express ALK-1-HA (red), but none of the IgG samples purified from the MCTD patients or the healthy controls produced the staining pattern we observed with the anti-HA mAb (Fig. 2B).

Discussion

This is the first report examining the autoantibody response to BMPR-II or ALK-1 in patients with connective tissue disease complicated by PAH and in IPAH patients. Contrary to our expectations, we failed to detect IgG autoantibodies that reacted with BMPR-II or ALK-1 in sera from MCTD patients, irrespective of the presence or absence of PAH. Since the recombinant antigens used in our assays are predicted to have a native conformation, our findings indicate a lack of circulating IgG antibodies capable of binding to BMPR-II or ALK-1 expressed on the endothelium. However, there is no human control serum positive for anti-BMPR-II or anti-ALK-1 antibody, although non-human antibodies to human BMPR-II or tags expressed on the recombinant proteins were used as positive controls in the assays. The possibility that our assays were less sensitive in

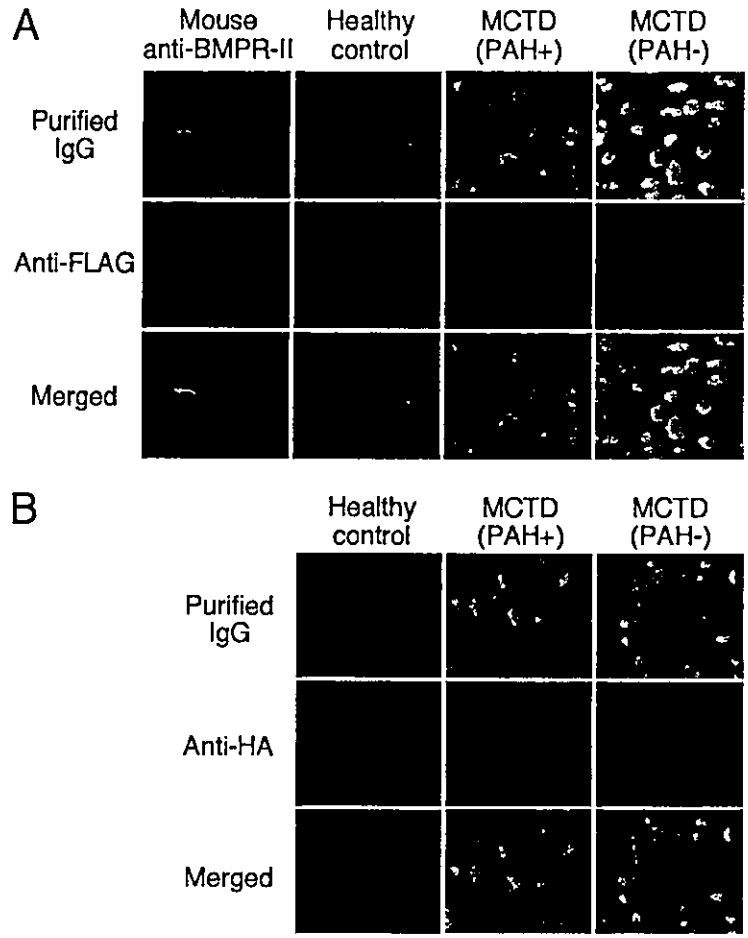

FIG 2. Screening of anti-BMPR-II and anti-ALK-1 autoantibodies using indirect immunofluorescence to observed COS-7 cells induced to express recombinant BMPR-II-FLAG and ALK-1-HA by gene transfer in representative samples from a healthy control, an MCTD patient with definite PAH, and an MCTD patient without PAH. (A) Fixed BMPR-II-FLAG-transfected cells were incubated with anti-BMPR-II mAb or IgG purified from serum samples (green) in combination with anti-FLAG mAb (red). (B) Fixed ALK-1-HA-transfected cells were incubated with IgG purified from serum samples (green) in combination with rat anti-HA mAb (red). The cells were examined with a confocal laser fluorescence microscope. Nuclei were counterstained with TO-PRO-3 (blue). Original magnification, ×40.

detecting human autoantibodies cannot be excluded. In addition, because the endothelium consists of approximately 10^{13} endothelial cells, occupying almost $7\,m^2$ in adults [22], it is still possible that patients have pathogenic autoantibodies to BMPR-II or ALK-1 that are present on endothelial cell surfaces but not in the circulation.

The mutations in the BMPR-II and ALK-1 genes that were detected in patients with IPAH included frameshifts, deletions, nonsense and missense mutations, and splice-site variations, most of which impaired the proteins' functional properties or transcriptional activities. This is consistent with a model of haploinsufficiency [23] or a dominant-negative effect [24]. The expression level of the BMPR-II protein is in fact reduced in the lungs of patients with severe IPAH, most of whom have an underlying mutation in the BMPR-II gene [25]. Based on these findings, it is widely accepted that a mutation in the BMPR-II and ALK-1 genes presents a major risk of the loss of TGF-β family-mediated growth inhibition of pulmonary arterial endothelial and smooth muscle cells, which leads to the development of the typical IPAH plexiform lesions in the pulmonary arteries [9, 10]. Taken together with the lack of germ-line mutations in the BMPR-II genes of patients with scleroderma spectrum disorders and PAH [11, 12], our findings strongly suggest that the underlying mechanisms for developing PAH are different between patients with connective tissue diseases and those without. This is supported by a recent paper reporting that the endothelial cell proliferation in the plexiform lesions is monoclonal in IPAH and polyclonal in scleroderma [26]. In this regard, the maintenance of the endothelial vascular network is regulated by several factors other than TGF-β superfamily proteins and their signalling pathways, including the angiopoietin-Tie system [27]. It has been reported recently that the expression of angiopoietin-1 and the phosphorylation of Tie-2 are higher than normal in the lungs of patients with non-familial PAH, including IPAH and scleroderma [28]. Further studies are necessary to identify the mechanisms contributing to the development of PAH in patients with connective tissue diseases.

Rheumatology	Key messages
	• Circulating autoantibodies to BMPR-II or ALK-1 were undetectable in connective tissue disease patients irrespective of the presence or absence of PAH.

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The authors have declared no conflicts of interest.

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Binding of β_2–glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells

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Antiphospholipid syndrome (APS) is an autoimmune prothrombotic disorder in association with autoantibodies to phospholipid (PL)–binding plasma proteins, such as β_2-glycoprotein I (β_2GPI). We have recently found that CD4+ T cells autoreactive to β_2GPI in patients with APS preferentially recognize a cryptic peptide encompassing amino acid residues 276-290 (p276-290), which contains the major PL-binding site, in the context of DR53. However, it is not clear how previously cryptic p276-290 becomes visible to the immune system and elicits a pathogenics autoimmune response to β_2GPI. Here we show that presentation of a disease-relevant cryptic T-cell determinant in β_2GPI is induced as a direct consequence of antigen processing from β_2GPI bound to anionic PL. Dendritic cells or macrophages pulsed with PL-bound β_2GPI induced a response of p276-290–specific CD4+ T-cell lines generated from the patients in an HLA-DR–restricted and antigen-processing–dependent manner but those with β_2GPI or PL alone did not. In addition, the p276-290–reactive T-cell response was primed by stimulating peripheral blood T cells from DR53-carrying healthy individuals with dendritic cells bearing PL-bound β_2GPI in vitro. Our finding is the first demonstration of an in vitro mechanism eliciting pathogenic autoreactive T-cell responses to β_2GPI and should be useful in clarifying the pathogenesis of APS. (Blood. 2005;105:1552-1557)

Introduction

Antiphospholipid syndrome (APS) is characterized by arterial and venous thrombosis as well as recurrent intrauterine fetal loss in association with antiphospholipid antibodies.[1] β_2–glycoprotein I (β_2GPI), a plasma protein that binds negatively charged substances including phospholipids (PLs), is the most common target for the antiphospholipid antibody associated with the clinical features of APS.[2] Pathogenicity of the anti-β_2GPI antibody has been demonstrated in animal models, including normal mice immunized with human β_2GPI[3] and severe combined immunodeficiency mice into which peripheral blood lymphocytes from patients with APS were transferred.[4] We recently identified autoreactive CD4+ T cells to β_2GPI that promote anti-β_2GPI antibody production in patients with APS.[5-7] β_2GPI–specific CD4+ T cells recognize amino acid residues 276-290 (p276-290), which define the immunodominant β_2GPI peptide, in the context of DRB4*0103 (DR53). This epitope is located on domain V and contains the major PL-binding site at amino acids 280-288.[8] The p276-290–reactive T-cell clones did not respond to functional antigen-presenting cells (APCs) bearing native β_2GPI but did to those bearing chemically reduced β_2GPI or recombinant β_2GPI fragments produced in bacteria.[6] Given that β_2GPI-reactive T cells are also detected in some healthy individuals,[5] the p276-290 epitope defined by β_2GPI-specific T cells is "cryptic," since it is generated at a subthreshold level by the processing of native β_2GPI under normal circumstances.[9]

There is increasing interest in the possibility that crypticity is an important characteristic of epitopes recognized by the autoreactive T cells and thus is relevant to autoimmune pathogenesis.[10] T cells recognizing self-determinants generated in sufficient amounts in APCs undergo deletion in the thymus or anergy 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. This concept represents the major hypothesis for the pathogenesis of autoimmune diseases, but the fundamental question is how epitopes that are normally cryptic become visible to the immune system and elicit a sustained pathogenic response. In this study, p276-290–specific T-cell lines generated from patients with APS were used to investigate the mechanisms that induce the efficient processing and presentation of cryptic p276-290 as a consequence of antigen processing.

Patients, materials, and methods

Study subjects

Peripheral blood T cells from 5 Japanese patients with APS were analyzed in this study. All patients fulfilled the preliminary classification criteria for

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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-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

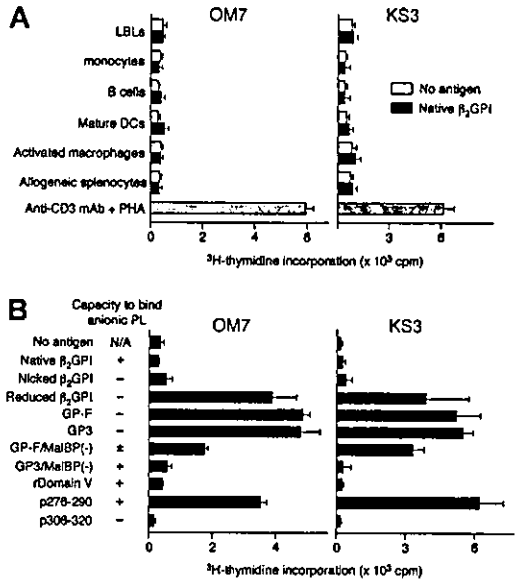

Figure 1. Response of p276-290–reactive T-cell lines to various combinations of DR53-positive APCs and β_2GPI preparations. (A) Various DR53-positive APCs were pulsed with or without native β_2GPI (■ and □, respectively) and subsequently cocultured with the p276-290–reactive T-cell lines OM7 and KS3. 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

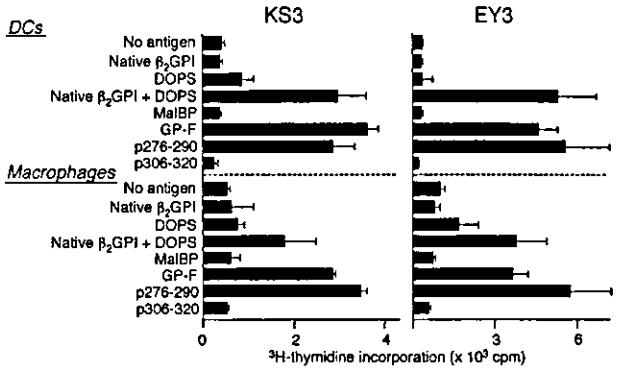

Figure 2. Response of p276-290–reactive T-cell lines in cultures of β_2GPI and anionic PL. Autologous immature DCs and macrophages were pulsed with native β_2GPI alone, DOPS liposomes, a mixture of native β_2GPI and DOPS liposomes, MalBP, or GP-F; induced to maturate or activate; and used to stimulate 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.

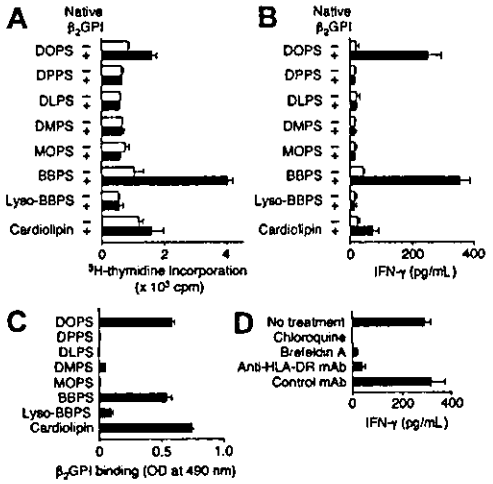

Figure 3. Binding of β_2GPI to anionic PL facilitates the processing and presentation of p276-290, which activates p276-290–reactive T-cell lines. (A) Autologous immature DCs were pulsed with or without a mixture of native β_2GPI and various PL liposomes (■ and □, respectively), induced to mature, and 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.

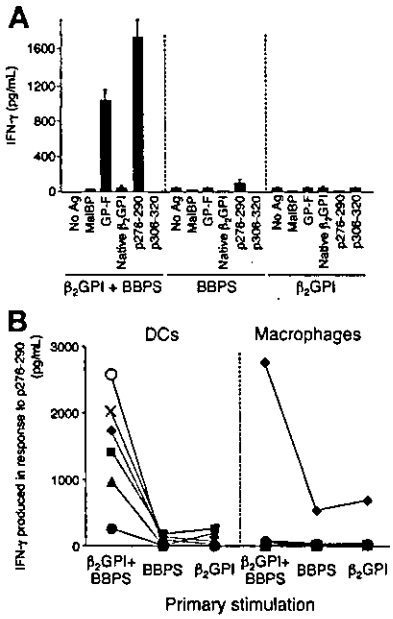

Figure 4. Induction of a T-cell response to p276-290 by BBPS-bound β_2GPI in peripheral blood T cells from DR53$^+$ healthy individuals in vitro. (A) Peripheral blood T cells from a representative DR53$^+$ healthy individual were stimulated with DCs pulsed with BBPS-bound β_2GPI, BBPS, or β_2GPI alone, 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.

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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

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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.

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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 3×10^4 /μL or greater but less than 10×10^4 /μL 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 15×10^4 /μL were referred to as partial remission (PR). Complete remission (CR) was defined as platelet counts greater than 15×10^4 /μL 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.

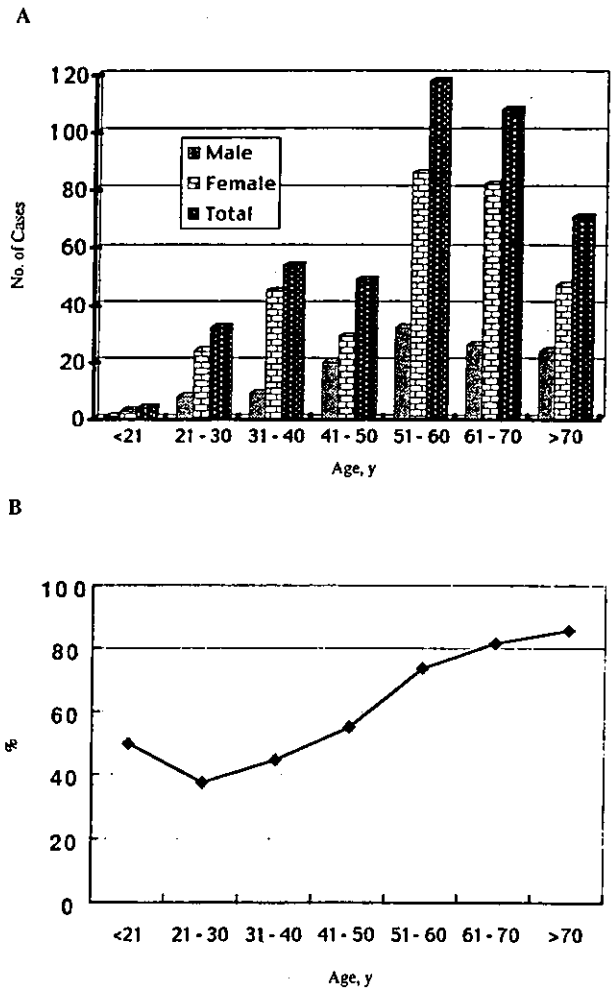

Figure 1. A, Age distribution of 435 patients with idiopathic thrombocytopenic purpura (ITP). There are 2 waves in the total number of patients. The first is patients 21 to 50 years old, and the second is patients 41 years and older. More than 70% of patients are in the second wave. B, *Helicobacter 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.

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).

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%)
	Nonresponse cases, n	60
Eradication unsuccessful group, n		46 (22%)
Platelet count	Response cases, n	15 (33%)
	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%,

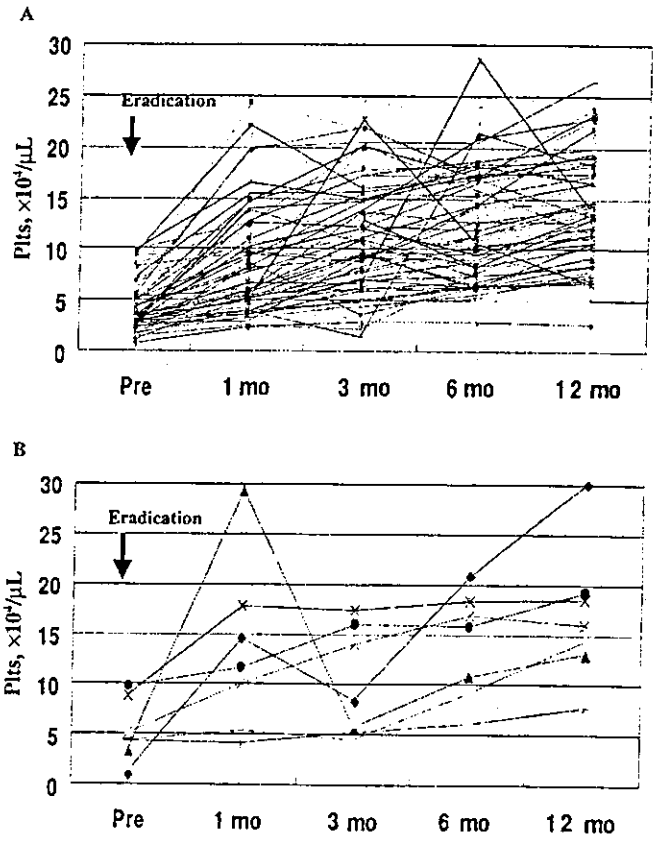

Figure 2. Laboratory course of platelet count responders in successful eradication cases (79 cases) (A) and unsuccessful eradication cases (9 cases) (B) followed for more than 12 months. The platelet count in most responder cases began to increase 1 month after eradication and continued to increase gradually.

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.

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

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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

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