Table 1 Demographic and laboratory findings at first visit of 46 patients later diagnosed as having ITP and 16 later diagnosed as having a non-ITP disorder

Demographic and laboratory findings	Number (%), Mean ± SD		
	ITP (n = 46)	Non-ITP (n = 16)	*P* value
Women	33 (72)	8 (50)	0.2
Age at first visit (years)	50.2 ± 18.2	59.0 ± 17.4	0.09
Anemia	11 (24)	14 (88)	<0.001
Leukocyte count (x 10^9/L)	5.3 ± 1.5	4.0 ± 0.9	<0.001
Platelet count (x 10^9/L)	46 ± 24	59 ± 20	0.04
Anti-GPIIb/IIIa antibody-producing B cells (/10^5 peripheral blood mononuclear cells)	7.6 ± 4.2	2.9 ± 4.1	<0.001
Platelet-associated anti-GPIIb/IIIa antibodies (units)	16.3 ± 22.3	4.2 ± 8.3	0.01
Plasma anti-GPIIb/IIIa antibodies (units)	2.9 ± 2.5	4.1 ± 5.9	0.8
Reticulated platelets (%)	2.8 ± 1.9	1.4 ± 0.9	0.001
Plasma thrombopoietin (pg/mL)	126 ± 85	525 ± 429	<0.001

GP = glycoprotein; ITP = idiopathic thrombocytopenic purpura.

and by McMillan et al,[7] who suggested that the guideline's recommendations are not rigorous enough to make an accurate diagnosis of ITP. In addition, this diagnostic process largely relies on the experience of hematologists who specialize in this field, whereas many less experienced physicians see patients with decreased platelet counts. Therefore, other practical criteria are needed for diagnostic accuracy in clinical settings.

The presence of anti-platelet antibodies is a hallmark of the autoimmune nature of ITP.[8] Anti-platelet antibodies in patients with ITP preferentially recognize platelet surface glycoproteins (GP), and the most common target is GPIIb/IIIa.[8] Several antigen-specific assays are reported to be useful in identifying patients with ITP.[7,9,10] We have also reported that an enzyme-linked immunospot assay for the detection of circulating B cells secreting anti-GPIIb/IIIa antibodies is a sensitive, specific, and convenient method for evaluating the presence or absence of autoantibody-mediated thrombocytopenia.[11] In addition, the percentage of reticulated platelets and the circulating thrombopoietin level are reported to be useful in discriminating a state of accelerated platelet destruction from that of decreased platelet production.[12-15] To evaluate the potential usefulness of these laboratory tests for the diagnosis of ITP, we conducted a prospective study of patients who had thrombocytopenia without any other morphologic abnormalities in their peripheral blood film at first visit.

Subjects and methods

Subjects

We prospectively investigated all 62 adult patients who had thrombocytopenia, who first visited the outpatient clinic at Keio University Hospital during a 3-year period (from January 2000 to December 2002), and who met the inclusion criteria: thrombocytopenia $<100 \times 10^9$/L; the absence of any other morphologic abnormalities in the peripheral blood film; exclusion of pseudothrombocytopenia; no clinical or serologic evidence of associated conditions or factors that can cause thrombocytopenia, such as systemic lupus erythematosus, infection with the human immunodeficiency virus, lymphoproliferative disorders, liver cirrhosis, or therapy with drugs such as heparin or quinidine; and no previous treatment with corticosteroids or splenectomy. In our outpatient clinic, antinuclear antibody testing was routinely performed on patients with thrombocytopenia, and serologic tests for human immunodeficiency virus and hepatitis C virus were performed on patients who were judged to be at clinical risk. At the first visit, a detailed history and physical examination and routine laboratory tests, including complete blood count and peripheral blood film, were performed on all patients. At the same time, 20 mL of peripheral blood was obtained for the evaluation of the anti-GPIIb/IIIa antibody response and platelet turnover. A total of 10 demographic and laboratory findings were recorded for each patient at study entry. These included sex, age at first visit, erythrocyte count, leukocyte count, platelet count, anti-GPIIb/IIIa antibody-producing B cell frequency, platelet-associated and plasma anti-GPIIb/IIIa antibodies, percentage of reticulated platelets, and plasma thrombopoietin level. An erythrocyte count $<4.3 \times 10^{12}$/L (men) or $<3.7 \times 10^{12}$/L (women) was regarded as anemia. All blood samples were obtained after the patients gave written informed consent, as approved by the Keio University Institutional Review Board.

All patients underwent a bone marrow examination and were followed by one of the investigators for 22.5 ± 9.8 months (range, 8 to 41 months). The investigator was blinded to the results of the 5 specialized tests for evaluating

Table 2 Sensitivity, specificity, positive predictive value, and negative predictive value of initial laboratory findings associated with a later diagnosis of ITP

Laboratory findings	Number				*P* value	Percentage (95% confidence interval)			
	ITP (n = 46)		Non-ITP (n = 16)			Sensitivity	Specificity	Positive predictive value	Negative predictive value
	Positive	Negative	Positive	Negative					
Erythrocyte count ≥ 4.3 × 10^{12}/L (male) ≥3.7 × 10^{12}/L (female)	35	11	2	14	<0.001	76 (61-87)	88 (62-98)	95 (82-99)	56 (35-76)
Leukocyte count ≥ 4 × 109/L	38	8	5	11	<0.001	83 (69-92)	69 (41-89)	88 (75-96)	58 (33-80)
Anti-GPIIb/IIIa antibody-producing B cells ≥ 2/105 peripheral blood mononuclear cells	43	3	3	13	<0.001	93 (82-99)	81 (54-96)	93 (82-99)	81 (54-96)
Platelet-associated anti-GPIIb/IIIa antibodies ≥ 3.3 units	28	18	2	14	0.002	61 (45-75)	88 (62-98)	93 (78-99)	44 (26-62)
Percentage of reticulated platelets ≥2%	27	19	1	15	<0.001	59 (43-73)	94 (70-100)	96 (82-100)	44 (27-62)
Plasma thrombopoietin <300 pg/mL	43	3	5	11	<0.001	93 (82-99)	69 (41-89)	90 (77-97)	79 (49-95)

GP = glycoprotein; ITP = idiopathic thrombocytopenic purpura.

the anti-GPIIb/IIIa antibody response and platelet turnover, which were done for this study.

Diagnosis

A diagnosis of ITP was made on the basis of clinical history, physical examination, complete blood count, bone marrow findings, and the clinical course for at least 8 months; the diagnosis was not based on the results of any of the 5 specialized studies done as part of this study. All patients with ITP fulfilled the criteria advised by the American Society of Hematology Practice Guidelines[4] and had chronic ITP based on thrombocytopenia persisting longer than 6 months. Diagnoses of myelodysplastic syndrome, aplastic anemia, and amegakaryocytic thrombocytopenia were based principally on bone marrow findings and cytogenetic analysis.[16-18]

Sample preparation

Platelet-rich plasma was prepared from heparinized venous blood by centrifugation at 120 g for 10 minutes, and the remaining cell component was subjected to a Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation to isolate the peripheral blood mononuclear cells. After 10^6 platelets were used in the assay for reticulated platelets, the remaining platelet-rich plasma was spun to separate the platelets from platelet-poor plasma.

Evaluation of anti-GPIIb/IIIa antibody responses

B cells producing immunoglobulin G (IgG) anti-GPIIb/IIIa antibodies were detected using the enzyme-linked immunospot assay.[11,19] Briefly, affinity-purified human GPIIb/IIIa was coated onto a polyvinylidene difluoride-bottomed 96-well microplate. Peripheral blood mononuclear cells were pipetted into the wells (10^5/well) and cultured at 37°C for 4 hours. During this incubation period, secreted anti-GPIIb/IIIa antibodies were bound to the immobilized GPIIb/IIIa in the immediate vicinity of the secreting cells. After washing away the cells, the membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG, and anti-GPIIb/IIIa antibodies bound to the membrane were visualized as spots by incubation with a substrate. Blue-purple spots that were round and had a dark center with slightly fuzzy edges were regarded as the sites of anti-GPIIb/IIIa antibody-secreting B cells. Each assay was conducted in 5 independent wells, and the results represent the mean of the 5 values. The frequency of circulating anti-GPIIb/IIIa antibody-producing B cells was calculated as the number per 10^5 peripheral blood mononuclear cells, and an abnormal value was defined as ≥2.0 based on 5 standard deviations above the mean obtained from 52 healthy individuals.[11]

IgG anti-GPIIb/IIIa antibodies in platelet eluates (from 5 × 10^7 platelets) and plasma were measured by enzyme-linked immunosorbent assay using purified human GPIIb/IIIa as the

Table 3 Sensitivity, specificity, positive predictive value, and negative predictive value of 3 simple ITP-associated laboratory tests* in combination with other tests for the diagnosis of ITP

Combinations of ITP-associated laboratory tests	Number of findings required	Number					Percentage (95% confidence interval)			
		ITP (n = 46)		Non-ITP (n = 16)						
		Positive	Negative	Positive	Negative	*P* value	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Three simple ITP-associated laboratory tests*	2 or more	38	8	2	14	<0.001	83 (69-92)	88 (62-98)	95 (83-99)	64 (41-83)
±Anti-GPIIb/IIIa antibody-producing B cells	2 or more	43	3	2	14	<0.001	93 (82-99)	88 (62-98)	96 (85-99)	82 (57-96)
±Platelet-associated anti-GPIIb/IIIa antibodies	2 or more	41	5	2	14	<0.001	89 (76-96)	88 (62-98)	95 (84-99)	74 (49-91)
±Plasma thrombopoietin	2 or more	42	4	3	13	<0.001	91 (79-98)	81 (54-96)	93 (82-99)	77 (50-93)
±Anti-GPIIb/IIIa antibody-producing B cells and platelet-associated anti-GPIIb/IIIa antibodies	2 or more	45	1	5	11	<0.001	98 (88-100)	69 (41-89)	90 (78-97)	92 (62-100)
±Anti-GPIIb/IIIa antibody-producing B cells and plasma thrombopoietin	2 or more	44	2	4	12	<0.001	96 (85-99)	75 (48-93)	92 (80-98)	86 (57-98)
±Platelet-associated anti-GPIIb/IIIa antibodies and plasma thrombopoietin	2 or more	45	1	3	13	<0.001	98 (88-100)	81 (54-96)	94 (83-99)	92 (66-100)
±Anti-GPIIb/IIIa antibody-producing B cells, platelet-associated anti-GPIIb/IIIa antibodies, and plasma thrombopoietin	3 or more	44	2	1	15	<0.001	96 (85-99)	94 (70-100)	98 (88-100)	88 (64-99)

GP = glycoprotein; ITP = idiopathic thrombocytopenic purpura.

*Anemia, leukocyte count, and percentage of reticulated platelets.

antigen.[19,20] Antibody units were calculated from the OD_{450} results, based on a standard curve obtained from serial concentrations of pooled plasma with a high titer of IgG anti-GPIIb/IIIa antibodies at dilutions ranging from 1:50 to 1:6400. One unit of anti-GPIIb/IIIa antibody was defined as the amount present in the pooled plasma diluted 1:3200. All samples were examined in duplicate, and the results were calculated as the mean of two values. An abnormal value for platelet-associated antibody was defined as ≥ 3.3 units and an abnormal value for plasma anti-GPIIb-IIIa antibody was defined as ≥ 5.0 units, based on 5 standard deviations above the mean obtained from 40 healthy persons.

Evaluation of platelet turnover

Reticulated platelets were detected by staining platelets with thiazole orange (Retic-COUNT, Becton Dickinson, San Jose, Calif) followed by the flow cytometric analysis described previously.[21,22] The fluorescence histogram was analyzed using a linear gate set to capture 1% of the reticulated platelet counts. This standard gate was used to analyze data from all samples and measure the percentage of thiazole orange-positive platelets in this gate. When a single such marker was used to analyze a series of 40 healthy control samples to determine the variability of the technique, the average percentage of reticulated platelets was 0.85% ± 0.23% (range, 0.2% to 1.5%). An abnormal value for the percentage of reticulated platelets was defined as greater than the mean plus 5 standard deviations (2.0%) in our assay system. Possible day-to-day variability was assessed in 2 healthy control subjects; the inter-assay coefficient of variation was 9.6%, and the intra-assay coefficient of variation was 5.4%. The plasma thrombopoietin level was measured using a commercially available enzyme-linked immunosorbent assay kit (Quantikine; R&D Systems, Minneapolis, Minn) according to the manufacturer's protocol. The mean plasma thrombopoietin level in 30 healthy persons was 83.9 ± 11.7 pg/mL (range, 50 to 126 pg/mL). A thrombopoietin level of ≥ 142 pg/mL was defined as abnormal based on a normal mean plus 5 standard deviations.

Statistical analysis

Confidence intervals were calculated for each sensitivity, specificity, positive predictive value, and negative predictive value. Comparisons between 2 patient groups were tested for statistical significance using the nonparametric Mann-Whitney test or the chi-squared test, as appropriate.

Results

Clinical diagnosis of patients with thrombocytopenia

For the 62 patients, the clinical diagnosis was ITP in 46 patients (74%), myelodysplastic syndrome in 8 (13%), aplastic anemia in 2 (3%), and amegakaryocytic thrombocytopenia in 1 (2%). Five patients (8%) did not have a definitive final diagnosis at the last observation (observation period ranged from 11 to 23 months), because they continued to have reduced megakaryocytes in the bone marrow, alone or in combination with other lineages, without morphologic evidence for dysplasia. These patients were tentatively diagnosed as having reduced platelet production, with or without other cytopenias, and without dysplasia or evidence for platelet destruction. Among the 46 patients with a diagnosis of ITP, 32 received corticosteroids and showed a subsequent partial or complete response. Three patients who were refractory to corticosteroid therapy underwent splenectomy, which successfully increased the platelet count. All 6 patients who were treated with intravenous immunoglobulin experienced an increase in platelet count. Five patients with ITP were treated for *Helicobacter pylori*, and 3 showed a significant increase in platelet count after the successful eradication.

Clinical and laboratory findings in ITP versus non-ITP

The initial findings for evaluation of anti-GPIIb/IIIa antibody responses and platelet turnover were compared according to final clinical diagnosis (Figure). High levels of anti-GPIIb/IIIa antibody-producing B cell frequency, platelet-associated anti-GPIIb/IIIa antibodies, and the percentage of reticulated platelets were detected almost exclusively in patients who later received a final diagnosis of ITP. The plasma thrombopoietin level was normal or slightly increased in patients with ITP, but more than half the patients with myelodysplastic syndrome, aplastic anemia, amegakaryocytic thrombocytopenia, or reduced platelet production, with or without other cytopenias, without dysplasia or evidence for platelet destruction, had an extremely high plasma thrombopoietin level. An increased level of plasma anti-GPIIb/IIIa antibodies was found in only a small number of patients, irrespective of the final diagnosis.

The 46 patients with a final diagnosis of ITP tended to be younger than the 16 patients with other diagnoses (Table 1). Six laboratory findings, including the presence or absence of anemia, the leukocyte count, the frequency of anti-GPIIb/IIIa antibody-producing B cells, the platelet-associated anti-GPIIb/IIIa antibody level, the percentage of reticulated platelets, and the plasma thrombopoietin level, were different at presentation in patients later diagnosed as having ITP than in other patients. The initial platelet count was marginally lower in ITP compared with other diagnoses. The sensitivity, specificity, positive predictive value, and negative predictive value for each of these laboratory findings (Table 2) showed that no single test was sufficient to diagnose ITP in patients with thrombocytopenia. The combination of 3 simple and inexpensive tests (anemia, leukocyte count, and reticulated platelets) also had a low sensitivity and negative predictive value, but the addition of any one of

the substantially more costly tests improved sensitivity and negative predictive value (Table 3). When the presence of 3 or more of the 6 initial ITP-associated laboratory findings was regarded as possible ITP, a positive result was detected in 44 of 46 patients ultimately diagnosed as ITP (96%) but in only 1 (6%) of 16 patients without ITP, indicating a sensitivity of 96%, specificity of 94%, positive predictive value of 98%, and negative predictive value of 88%.

Discussion

This prospective study demonstrates that 6 laboratory tests (erythrocyte count, leukocyte count, anti-GPIIb/IIIa antibody-producing B cells, platelet-associated anti-GPIIb/IIIa antibodies, reticulated platelets, and plasma thrombopoietin level) performed at the first visit are useful to predict a future diagnosis of ITP. These laboratory findings associated with the diagnosis of ITP are categorized into 3 groups that individually reflect different aspects of the disease: a lack of abnormality in myeloid and erythroid lineages, the presence of autoantibody response to platelet-specific GPIIb/IIIa, and preserved platelet production. Three simple and inexpensive tests plus at least 1 of the other more costly ITP-associated laboratory tests measured together at first visit were a relatively sensitive and specific way to discriminate between the future diagnosis of ITP as compared with other conditions. However, it was difficult to determine which combination was most reasonable, probably because the number of patients examined in this study was relatively small. Further multicenter studies involving larger patient cohorts are necessary to make a decision regarding the least expensive and more efficient combination of laboratory tests to detect ITP.

To evaluate the autoimmune nature of the disease, the autoantibody response to GPIIb/IIIa, a major target of antiplatelet autoantibodies, was examined using 2 different methods that have complementary advantages and disadvantages. The enzyme-linked immunospot assay is very sensitive, but this assay system detects B cells that produce antibodies reacting with immobilized GPIIb/IIIa irrespective of their capacity to bind platelet surfaces. This assay would therefore give a false-positive result in patients who have antibodies that react with GPIIb/IIIa but fail to bind platelet surfaces, ie, antibodies that recognize a cytoplasmic domain of GPIIIa.[23] This result may have occurred in 1 of the patients with myelodysplastic syndrome, who showed highly positive results with anti-GPIIb/IIIa antibody-producing B cells and plasma anti-GPIIb/IIIa antibodies but who did not have platelet-associated anti-GPIIb/IIIa antibodies. Conversely, immunologic assays for platelet-associated anti-GPIIb/IIIa antibodies specifically detect antibodies capable of binding platelet surfaces, although the sensitivity is somewhat low.[7,9,10,24] The plasma anti-GPIIb/IIIa antibody level was not useful in the diagnosis of ITP, as reported previously,[20,24] because the majority of pathogenic anti-GPIIb/IIIa antibodies capable of binding platelets are present on platelet surfaces, rather than in the circulation. An increase in sensitivity might be achieved by simultaneously measuring the autoantibody response to other platelet-specific GP, such as GPIb/IX.[8]

Reticulated platelets are young platelets that have been recently released into the circulation and contain elevated levels of nucleic acid components.[25] The proportion of reticulated platelets among circulating platelets reflects platelet turnover.[21,22] Thrombopoietin is a principal regulator of megakaryo-thrombopoiesis, and its circulating level is inversely correlated with the absolute number of bone-marrow megakaryocytes and platelets.[26] These parameters are rapid, noninvasive tests that provide information about activity of the megakaryocytes in the bone marrow and platelet life span. Our prospective study showed that these convenient tests are useful for discriminating between ITP and hypoplastic thrombocytopenia, consistent with previous reports.[12-15]

In summary, initial laboratory findings that predicted future diagnosis of ITP identified in this study could potentially be included in future diagnostic criteria for adult ITP. Because the various combinations of diagnostic tests were retrospectively classified in this study, prospective evaluation of the same diagnostic criteria on another, independent set of patients is necessary.

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Autoantibody to CD40 ligand in systemic lupus erythematosus: association with thrombocytopenia but not thromboembolism

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Objectives. **To examine the prevalence, clinical associations and pathogenic roles of autoantibodies to CD40 ligand (CD40L) in patients with systemic lupus erythematosus (SLE).**

Methods. **Plasma anti-CD40L antibodies from 125 patients with SLE, 24 with primary antiphospholipid syndrome (APS) and 90 with idiopathic thrombocytopenic purpura (ITP) and from 62 healthy individuals were measured with an enzyme-linked immunosorbent assay (ELISA). HeLa cells transfected with human CD40L cDNA (HeLa/CD40L) were used to confirm the presence of anti-CD40L autoantibodies. The effect of anti-CD40L antibodies on the CD40L-CD40 interaction was evaluated by observing CD40L-induced IκB activation in CD40-expressing fibroblasts.**

Results. **Anti-CD40L autoantibody was detected in seven (6%) SLE, three (13%) primary APS and 11 (12%) ITP patients, but in no healthy controls. Antibody binding in an ELISA was competitively inhibited by membrane components of HeLa/CD40L. Anti-CD40L antibody-positive IgG specifically bound the surface of living HeLa/CD40L, as shown by flow cytometry. The frequency of thrombocytopenia was significantly higher in SLE patients with the anti-CD40L antibody than in those without (100 *vs* 14%; $P < 0.00001$), whereas there was no association between the anti-CD40L antibody and thrombosis. Binding of the anti-CD40L antibodies in patients' plasma to CD40L was competitively inhibited by a series of mouse anti-CD40L monoclonal antibodies. Anti-CD40L antibody-positive IgG failed to inhibit CD40L-induced IκB activation.**

Conclusions. **Anti-CD40L autoantibody is associated with thrombocytopenia but not thromboembolism. Our findings are potentially useful in understanding the complex roles of CD40L in the pathophysiology of thrombosis and haemostasis as well as the thromboembolic complications that occur during treatment with anti-CD40L humanized antibody.**

KEY WORDS: Autoantibody, CD40 ligand, Costimulatory molecule, Humanized antibody, Platelet, Systemic lupus erythematosus, Thrombocytopenia, Thrombosis.

CD40 ligand (CD40L), also known as CD154, is a transmembrane protein expressed mainly on $CD4^+$ T cells and platelets in an activation-dependent manner [1]. The interaction of CD40L on activated $CD4^+$ T cells with CD40 on antigen-presenting cells is essential for the T-cell-dependent humoral immune response [1, 2]. The therapeutic efficacy of blocking this interaction with an anti-CD40L monoclonal antibody (mAb) has been shown in animal models of various autoimmune diseases, including rheumatoid arthritis [3] and systemic lupus erythematosus (SLE) [4]. In these models, the anti-CD40L mAb both prevented disease development and interfered with ongoing disease. Thus, the disruption of CD40L-CD40 signalling has been proposed as a novel strategy for treating human T-cell-mediated diseases. Recently, several clones of anti-human CD40L humanized mAbs that block antigen-specific immunoglobulin G (IgG) responses *in vivo* in non-human primates have been manufactured, and two of them [hu5c8, BG-9588, ruplizumab, Antova™ (Biogen, Cambridge, MA, USA) and E6040/IDEC-131 (IDEC Pharmaceuticals, San Diego, CA, USA)] were used in clinical trials in patients with various autoimmune diseases, including SLE and idiopathic thrombocytopenic purpura (ITP) [5]. In an open-label study in SLE patients with active nephritis, patients receiving anti-CD40L humanized mAb showed reductions in disease activity indices and anti-double-stranded DNA (dsDNA) antibody titres [6, 7]. Another phase I, dose-escalating trial of a humanized mAb to CD40L in patients with refractory ITP showed an increase in platelet count in parallel with a transient suppression of platelet-specific autoantibody responses in patients who received the highest dosage [8]. These findings indicate that CD40L–CD40 signal blockade is a promising strategy for treating human autoimmune diseases.

However, clinical studies of anti-CD40L humanized mAbs have raised serious concerns that thromboembolitic events could be a complication of this treatment [9, 10], although the precise mechanism of this adverse effect is not understood currently. This clinical observation led us to hypothesize that autoantibodies reactive with CD40L could be a risk factor for acquired thrombophilia, if they were present. To test this hypothesis, we developed assay systems to detect anti-CD40L autoantibodies and used them to screen patients with SLE, one of the acquired prothrombotic autoimmune diseases. We also examined the clinical characteristics associated with anti-CD40L autoantibodies and their pathogenic roles in patients with SLE.

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Materials and methods

Patients and controls

We studied 125 consecutive patients with SLE, 24 with primary antiphospholipid syndrome (APS) and 90 with ITP, who were followed up at Keio University Hospital. All SLE patients satisfied the American College of Rheumatology (ACR) preliminary criteria [11]. Patients with primary APS satisfied the Sapporo criteria [12] but did not meet the preliminary criteria for SLE. Eighteen SLE patients satisfied the Sapporo criteria as well, and thus had secondary APS. The criteria for the diagnosis of ITP were: (i) thrombocytopenia of 100×10^9/l or less; (ii) normal or increased bone marrow megakaryocytes without morphological evidence of dysplasia; (iii) no other primary diseases or conditions that account for the thrombocytopenic state; and (iv) disease duration of more than 6 months [13]. Sixty-two healthy subjects were used as the control. Both serum and heparinized platelet-poor plasma samples were obtained from all subjects. Blood samples and clinical information were obtained after the patients and controls had given their written informed consent, in accordance with the declaration of Helsinki. The design of the work was approved by the Keio University Institutional Review Board.

Clinical features of SLE patients

The demographic and clinical features were evaluated for each SLE patient at the time of blood collection. Thirty-seven clinical and laboratory findings were recorded; these were individual items included in the ACR preliminary classification criteria [11] and the SLE disease activity index (SLEDAI) [14] as well as histories of thromboembolism and fetal loss. Thrombocytopenia was defined as a platelet count below 100×10^9/l. The SLEDAI was calculated and used to evaluate the disease activity in SLE patients. The number of bone marrow megakaryocytes was semiquantitatively assessed for some SLE patients with thrombocytopenia, from whom bone marrow films were available [15].

Autoantibody analysis

Anti-dsDNA antibody was measured quantitatively with the Farr assay, and anti-Sm, anti-U1RNP, anti-SSA/Ro and anti-SSB/La antibodies were identified using an RNA immunoprecipitation assay with unlabelled HeLa cell extracts [16]. IgG anti-cardiolipin antibodies were measured with an enzyme-linked immunosorbent assay (ELISA) kit (MBL, Nagano, Japan). Lupus anticoagulant was determined by a cross-mixing test using a commercially available kit based on the diluted Russell's viper venom test (Gradipore, Sydney, Australia). The antibody response to GPIIb/IIIa, a major platelet autoantigen recognized by anti-platelet antibodies [13], was evaluated by detecting circulating B cells producing IgG anti-GPIIb/IIIa antibodies using an enzyme-linked immunospot assay [17].

Quantification of circulating soluble CD40L

Soluble CD40L in plasma was measured in 35 SLE patients by ELISA (R & D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

Purification of IgG from plasma

IgG was purified from patients' plasma by affinity chromatography using a HiTrap Protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden). IgG fractions were dialysed against phosphate-buffered saline (PBS) and sterilized by passage through 0.22 μm pore syringe filters.

HeLa cells transfected with human CD40L

HeLa cells transfected with full-length human CD40L cDNA (HeLa/CD40L), a kind gift from Dr Kazunori Kato (Sapporo Medical College, Japan), were maintained in RPMI1640 containing 10% fetal bovine serum and 500 μg/ml Geneticin (Invitrogen, Carlsbad, CA, USA). Untransfected wild-type HeLa cells were used as a control.

ELISA for the detection of IgG anti-CD40L autoantibody

CD40L is rapidly expressed on the surface of platelets and is released in a soluble form after platelet activation and thrombus formation [18]. To prevent the potential effect of CD40L up-regulated during *in vitro* clot formation on the anti-CD40L antibody reactivity, platelet-poor plasma, instead of serum, was used in all assays measuring the anti-CD40L antibody. An ELISA system for detecting anti-CD40L autoantibodies was developed as described [19] with some modifications. Briefly, polyvinyl 96-well plates were coated with a recombinant soluble CD40L (PeproTech, London, UK) diluted in PBS to 0.5 μg/ml, at 4°C for 12 h. The recombinant CD40L contains amino acids 113–261 of human CD40L, comprising the extracellular receptor-binding domain, and has been shown to form a bioactive homotrimer. The remaining free binding sites were blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 2 h. Plasma samples diluted 1:100 with ELISA buffer (PBS containing 0.1% BSA and 0.1% Tween 20) were then added to the wells, and incubated at room temperature for 2 h. Peroxidase-conjugated goat anti-human IgG (ICN/Cappel, Aurora, OH, USA) diluted 1:5000 in ELISA buffer was then added, and the samples were incubated for one additional hour. All incubations were followed by three washes with ELISA buffer. The bound antibodies were visualized by adding tetramethylbenzidine (1 mg/ml) in phosphate–citrate buffer containing dimethylsulphoxide. After the reaction had been stopped by adding 1 M sulphuric acid, the optimal density at 450 nm (OD_{450}) was read with an automatic plate reader (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested in duplicate, and the antibody units were calculated from the OD_{450}, using a standard curve obtained from serial concentrations (0.125–2.5 μg/ml) of E6040, a humanized mAb to human CD40L. One unit of anti-CD40L antibody was defined as 0.125 μg/ml of E6040. The cutoff value was the mean plus five times the standard deviation of 62 healthy control plasma (3.75 U).

ELISA competition assay

We set up two different competition assays using the anti-CD40L antibody ELISA. In one assay, soluble membrane fractions prepared from HeLa/CD40L and wild-type HeLa cells were used as competitors for the antigen. Briefly, the cells were sonicated and spun in an ultracentrifuge at 100 000 *g* for 1 h, and the pellet was resuspended in PBS containing 1% Triton X-100. After a second ultracentrifugation, as above, the solubilized cell membrane preparation was obtained as the supernatant. Diluted plasma samples positive for anti-CD40L antibody and E6040 were preincubated with the soluble membrane fraction (1 mg/ml) of HeLa/CD40L or wild-type HeLa cells before being subjected to the ELISA.

In another competitive assay, a series of mouse anti-CD40L mAbs, clone 24–31 (Ancell, Bayport, MN, USA), TRAP-1 (Immunotech, Marseille, France) and 5c8 (American Type Culture Collection, Manassas, VA, USA), was used to compete with the anti-CD40L autoantibody in patients' plasma. Clone 24–31 is the parent line of the humanized mAb E6040. The antigen-coated ELISA wells were incubated with serial concentrations (0.05–50 μg/ml) of mouse anti-CD40L mAbs at room temperature

for 30 min, and subsequently with E6040 (2.5 μg/ml) or patients' plasma diluted 1:100, followed by incubation with peroxidase-conjugated goat human-specific IgG (ICN/Cappel). Significant inhibition was defined as less than 60% of the OD_{450} results obtained from mock-treated wells.

Immunoblots

Reactivity to recombinant CD40L was examined by immunoblotting as described previously [19]. A 1:50 dilution of patients' plasma, E6040 (2.5 μg/ml) or goat anti-CD40L polyclonal antibodies (0.2 μg/ml; R & D Systems) was used as a primary antibody.

Flow cytometric analysis

Unfixed HeLa/CD40L and wild-type HeLa cells were incubated with IgG (250 μg/ml) purified from patients' plasma or E6040 (10 μg/ml), then with fluorescein-5-isothiocyanate-conjugated goat anti-human IgG $(Fab')_2$ fragment. Cell staining was analysed on a FACSCalibur® flow cytometer (Becton Dickinson, San Diego, CA, USA).

Effects of IgG on IκB phosphorylation

An adenovirus vector harbouring a full-length human CD40 cDNA was prepared using the AdEasy™ Adenoviral Vector System (Stratagene, La Jolla, CA, USA). Cultured human dermal fibroblasts were induced to express CD40 by adenoviral gene transfer. After the cell-surface expression of CD40 had been confirmed by flow cytometry on day 3, the fibroblasts were cultured in serum-free medium for 10 min with a recombinant soluble CD40L (0.5 μg/ml), which was preincubated with or without E6040 (0.01–1 μg/ml) or IgG (10 or 250 μg/ml) derived from SLE patients with or without anti-CD40L antibody or healthy controls, for 30 min at room temperature. The cells were lysed, and the equivalent of 1.25×10^4 cells was subjected to immunoblotting using anti-phospho-IκB or anti-IκB antibody (Cell Signaling Technology, Beverly, MA, USA) as a probe. The signal was visualized with a LumiGLO® chemiluminescence detection system (Cell Signaling Technology).

Statistical analysis

All comparisons for statistical significance between two patient groups were performed using the χ^2 test or Student's *t*-test.

Results

Detection of IgG anti-CD40L autoantibody by ELISA

IgG anti-CD40L antibody was measured in plasma samples from 125 patients with SLE, 24 with primary APS, 90 with ITP and 62 healthy individuals by ELISA using a recombinant CD40L as the antigen source (Fig. 1A). When the cut-off value was set as the mean plus 5 × S.D. of 62 healthy control sera, anti-CD40L antibody was positive in seven SLE patients (6%), three primary APS patients (13%) and 11 ITP patients (12%), but in none of the healthy controls.

To examine the specificity of the anti-CD40L antibody reactivity in the ELISA, we conducted a competitive ELISA in which plasma samples were preincubated with the soluble membrane fraction from HeLa/CD40L or wild-type HeLa cells. Representative results obtained from E6040 and SLE plasma determined to be positive by ELISA are shown in Fig. 1B. The anti-CD40L antibody reactivity in E6040 and SLE plasma was inhibited by preincubation with the soluble membrane fraction from HeLa/CD40L, but not with the fraction from wild-type HeLa cells. We analysed 12 additional plasma samples from SLE, primary APS, or ITP patients that showed an anti-CD40L antibody level above the cut-off, and obtained concordant results in all samples. In contrast, no apparent inhibition of the anti-CD40L antibody reactivity by preincubation with the soluble membrane fraction of HeLa/CD40L was observed in the plasma from two SLE patients who showed an antibody level just below the cut-off (3.4 and 3.2 U).

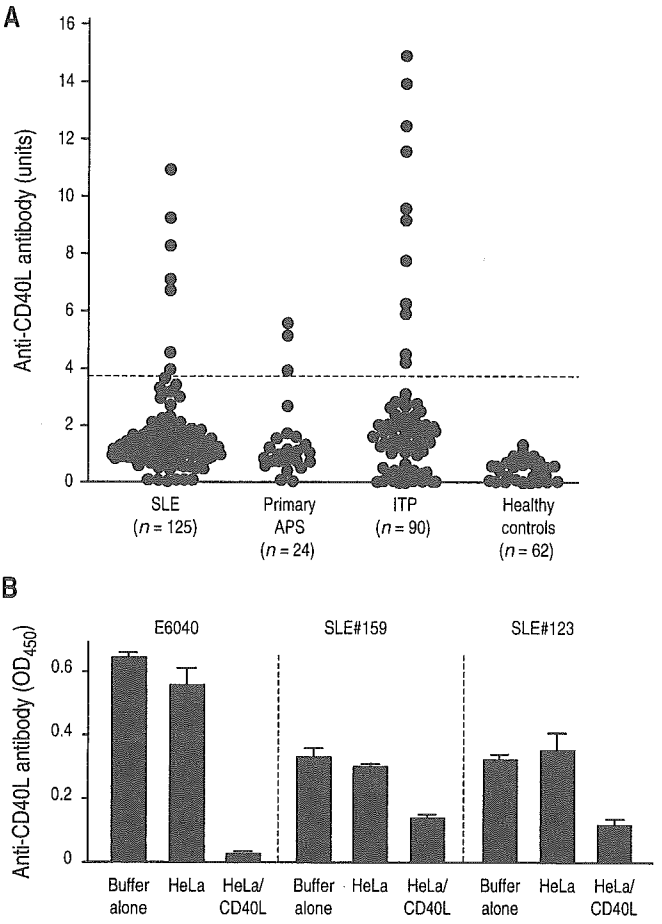

FIG. 1. (A) Plasma anti-CD40L antibody levels measured by ELISA in 125 patients with SLE, 24 with primary APS and 90 with ITP and in 62 healthy controls. The broken line denotes the cut-off set at the mean plus 5 × S.D. of 62 healthy controls (3.75 units). (B) Competitive inhibition in an anti-CD40L antibody ELISA using the soluble membrane fraction from HeLa/CD40L cells as a competitor. Anti-CD40L humanized mAb E6040 and plasma samples from representative SLE patients were pre-incubated with buffer alone or with the soluble membrane fraction from HeLa/CD40L or wild-type HeLa cells before applying them to the anti-CD40L antibody ELISA. Results shown are representative of two experiments.

Antigen recognition profiles of anti-CD40L autoantibodies

All 21 plasma samples from SLE, primary APS and ITP patients that were positive for anti-CD40L antibody in the ELISA were further examined by immunoblotting using the same antigen used in the ELISA. The recombinant CD40L trimer was separated into monomers (17 kDa) under a denaturing condition. The denatured CD40L monomer was recognized by goat anti-CD40L polyclonal antibodies, but not by E6040 or any of the patients' plasma that was positive for anti-CD40L antibody in the ELISA (data not shown).

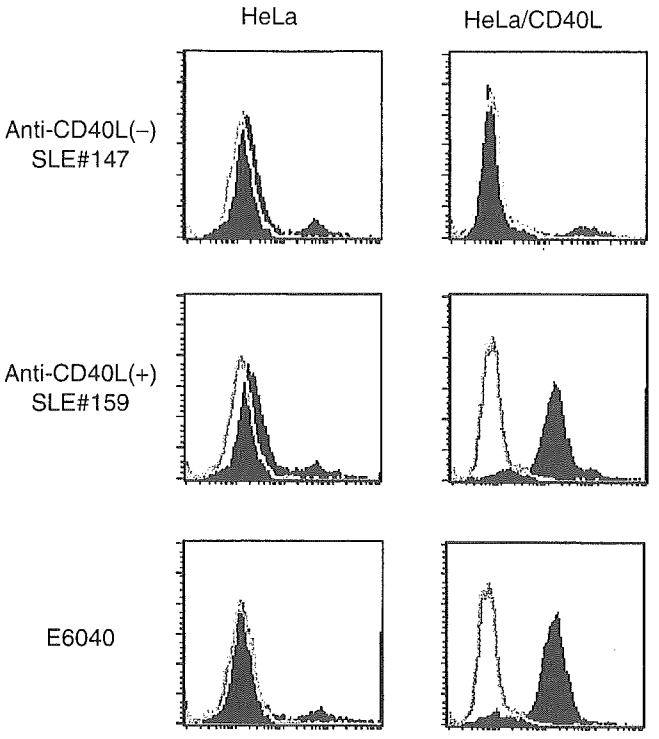

FIG. 2. Binding of anti-CD40L autoantibody in patients' plasma to CD40L expressed on the living cell surface. Unfixed wild-type HeLa and HeLa/CD40L cells were preincubated with anti-CD40L antibody-positive or -negative IgG from SLE patients (250 μg/ml), or anti-CD40L humanized mAb E6040 (10 μg/ml), followed by incubation with fluorescein-5-isothiocyanate-conjugated goat anti-human IgG $(Fab')_2$ fragment. Cell staining was analysed by flow cytometry and is shown as shaded histograms. Open histograms represent controls stained with secondary antibody alone. Results shown are representative of three experiments.

Binding of the IgG anti-CD40L antibody in patients' plasma to CD40L molecules expressed on the living cell surface was examined by flow cytometry using HeLa/CD40L. IgG fractions purified from four anti-CD40L antibody-positive patients (three SLE and one primary APS) and four anti-CD40L antibody-negative patients (three SLE and one healthy control) were used in this analysis. As shown in Fig. 2, no specific binding was detected when HeLa/CD40L cells were incubated with the IgG from an anti-CD40L antibody-negative SLE patient. IgG from a representative anti-CD40L antibody-positive SLE patient bound to HeLa/CD40L, but not to wild-type HeLa cells, as observed with anti-CD40L humanized mAb E6040. Specific binding to HeLa/CD40L was detected for all the anti-CD40L antibody-positive IgG, but not for anti-CD40L antibody-negative IgG.

Clinical characteristics of SLE patients with the anti-CD40L antibody

Of the 21 total patients positive for the anti-CD40L antibody, including seven with SLE, three with primary APS and 11 with ITP, thromboembolism was detected in only two (10%); one each with SLE (cerebral infarction and deep venous thrombosis of the leg) and primary APS (deep venous thrombosis of the leg). A history of fetal loss in such patients was also infrequent (14%); one with SLE who also had thrombosis and two with primary APS had a history of spontaneous abortion or intrauterine fetal death. All of these patients were diagnosed as having primary or secondary APS. In contrast, 20 (95%) of 21 anti-CD40L antibody-positive patients had thrombocytopenia and 11 of them had ITP.

TABLE 1. Clinical and laboratory findings in SLE patients with and without plasma anti-CD40L autoantibody

Clinical and laboratory findings	Anti-CD40L-positive ($n=7$)	Anti-CD40L-negative ($n=118$)	P
Sex (% female)	100	90	NS
Age at examination (yr)	37.9 ± 12.7	41.6 ± 13.2	NS
History of thromboembolism (%)	14	22	NS
History of fetal loss (%)	20 (1/5)	8 (5/64)	NS
Malar rash (%)	71	59	NS
Discoid rash (%)	14	9	NS
Photosensitivity (%)	43	34	NS
Oral ulcers (%)	14	27	NS
Arthritis (%)	71	66	NS
Serositis (%)	14	20	NS
Renal disorder (%)	14	36	NS
Neurological disorder (%)	29	6	NS
Haemolytic anaemia (%)	29	3	0.02
Leucopenia (%)	57	60	NS
Thrombocytopenia (%)	100	14	<0.00001
Anti-Sm antibody (%)	29	12	NS
Anti-SSA/Ro antibody (%)	86	31	0.01
Anti-dsDNA antibody (U)	93 ± 99	55 ± 81	NS
SLEDAI	14.8 ± 9.2	4.6 ± 4.8	0.02

NS, not significant ($P \geq 0.05$); dsDNA, double-stranded DNA; SLEDAI, SLE disease activity index.

TABLE 2. Platelet count, anti-GPIIb/IIIa antibody response and bone marrow megakaryocytes in SLE patients with anti-CD40L autoantibody

Patient	Sex/age (yr)	Anti-CD40L antibody (U)	Platelet count ($\times 10^9$/l)	Anti-GPIIb/IIIa antibody response[a]	Bone marrow megakaryocytes
123	F/32	10.9	34	NT	NT
251	F/50	9.2	18	+	Increased
284	F/19	8.3	25	+	Normal
159	F/46	7.1	47	+	Decreased
22	F/38	6.7	43	+	NT
109	F/24	4.6	8	+	Normal
29	F/56	4.0	38	NT	Normal

[a]Anti-GPIIb/IIIa antibody response was evaluated by detecting circulating anti-GPIIb/IIIa antibody-producing B cells. NT, not tested.

To further characterize the clinical associations with the anti-CD40L autoantibody in SLE patients, demographic and clinical findings as well as coexisting autoantibodies were compared between SLE patients with and without the anti-CD40L antibody (Table 1). Haemolytic anaemia and thrombocytopenia were more frequently detected in patients with the anti-CD40L antibody than in those without ($P=0.02$ and $P<0.00001$, respectively). It was notable that all SLE patients with the anti-CD40L antibody had thrombocytopenia. Anti-SSA antibody was more frequently detected in SLE patients with the anti-CD40L antibody than in those without ($P=0.01$). The frequencies of other clinical and serological features, including thromboembolism and fetal loss, were similar in these two patient groups, but SLEDAI was significantly higher in the anti-CD40L-positive than in the negative group ($P=0.02$). There was no difference in the soluble CD40L level in plasma between five SLE patients with the anti-CD40L antibody and 30 patients without it (106 ± 61 *vs* 102 ± 76 pg/ml).

In addition, the platelet count, anti-GPIIb/IIIa antibody response and cellularity of bone marrow megakaryocytes were assessed in seven SLE patients with the anti-CD40L antibody (Table 2). All the patients had a platelet count below 50×10^9/l,

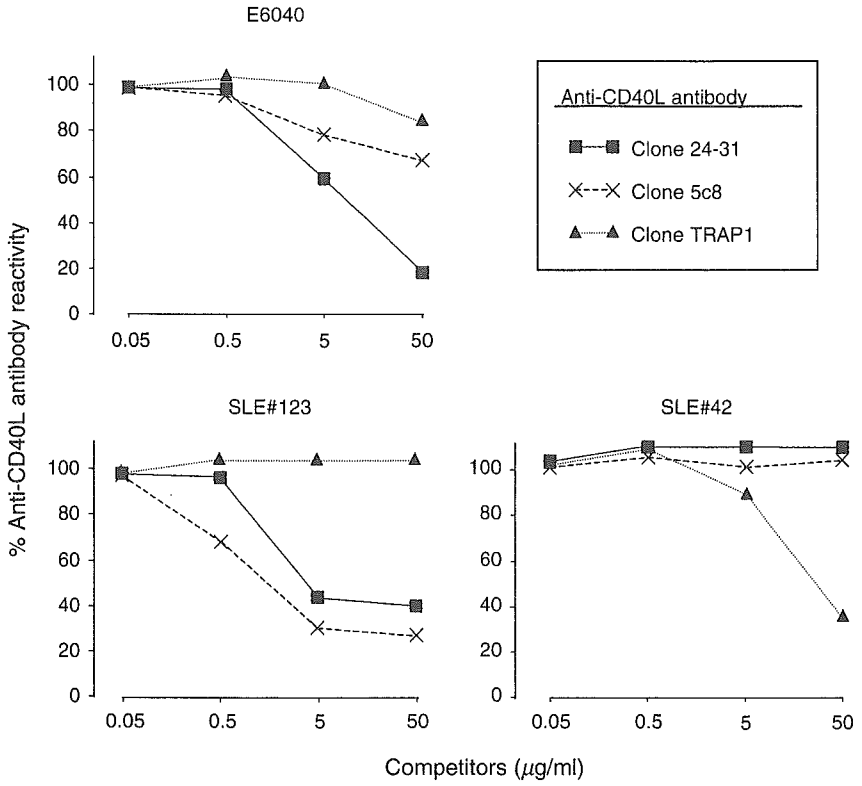

FIG. 3. Competitive inhibition in an anti-CD40L antibody ELISA using a series of mouse anti-CD40L mAbs. The antigen-coated ELISA wells were incubated with serial concentrations (0.05–50 μl/ml) of mouse anti-CD40L mAb (24–31, TRAP-1 or 5c8), and subsequently with anti-CD40L humanized mAb E6040 or SLE patients' plasma diluted 1:100. Clone 24–31 is the parent line of E6040. Results shown are representative of three experiments.

and five required corticosteroid therapy to control bleeding, which successfully increased the platelet count. An anti-GPIIb/IIIa antibody response was detected in all five patients examined, and all 11 ITP patients with the anti-CD40L antibody also had elevated anti-GPIIb/IIIa antibody-producing B cells. Of five anti-CD40L antibody-positive patients for whom bone marrow films were available, all but one had normal or increased megakaryocytes.

Autoantigenic epitopes on CD40L

To further examine the specificity of the anti-CD40L autoantibody and autoantigenic epitopes on the CD40L molecule, we performed competitive ELISAs in which a series of mouse anti-CD40L mAbs was used to compete. A total of nine anti-CD40L antibody-positive plasma samples from seven SLE patients and two ITP patients were analysed in this assay. We first confirmed that our procedure was reliable by examining E6040, a humanized version of mouse clone 24–31. As shown in Fig. 3, the binding of E6040 to immobilized CD40L was specifically inhibited by 24–31, but not by other mouse anti-CD40L mAbs. Anti-CD40L antibody reactivity in a representative SLE patient (patient 123) was suppressed by anti-CD40L mAbs 24–31 and 5c8, while the reactivity in SLE patient 42 was inhibited by another mAb, TRAP1. The antibody binding in all nine anti-CD40L antibody-positive patients was inhibited by at least one of the anti-CD40L mAbs, indicating the specific binding of the autoantibodies to CD40L. There were two patterns of inhibition among the patients: inhibition by both 24–31 and 5c8 in four, and inhibition by TRAP1 alone in five. Interestingly, 24–31 and 5c8 have been shown to functionally inhibit the CD40L-CD40 interaction [20, 21], whereas TRAP1 binds to CD40L independently of the CD40-binding site [22].

Effects of the anti-CD40L autoantibody on CD40L-induced IκB phosphorylation in CD40-expressing fibroblasts

To investigate whether the anti-CD40L autoantibody inhibits the functional interaction between CD40L and CD40, we examined IκB activation, a downstream signal induced by the CD40L–CD40 engagement. That is, the binding of CD40L to CD40 induces the rapid degradation and phosphorylation of IκB in CD40-expressing cells [2]. Consistent with this, human dermal fibroblasts induced to express CD40 by adenoviral gene transfer exhibited a decrease in total IκB and the appearance of phospho-IκB upon ligation to soluble CD40L (Fig. 4). When serial concentrations of E6040 were preincubated with the soluble CD40L, the IκB degradation was suppressed in a dose-dependent manner, while the phosphorylation of IκB was nearly completely inhibited at all antibody concentrations. In contrast, this inhibitory effect was not observed when soluble CD40L was preincubated with the IgG from two anti-CD40L antibody-positive SLE patients, one anti-CD40L antibody-negative SLE patient or a healthy control. IgG from SLE patient 123 competed with the mouse anti-CD40L mAbs 24–31 and 5c8 for the binding site, whereas IgG from SLE patient 159 competed with TRAP1. IgG from three additional anti-CD40L antibody-positive SLE patients also lacked the inhibitory effect, independently of the epitope profiles determined by the patterns of competitive inhibition with mouse anti-CD40L mAbs. A higher concentration of anti-CD40L antibody-positive IgG (250 μg/ml) also failed to inhibit the CD40L-induced IκB degradation and phosphorylation.

Discussion

This study demonstrates that a subset of SLE patients, primary APS patients and ITP patients have IgG anti-CD40L

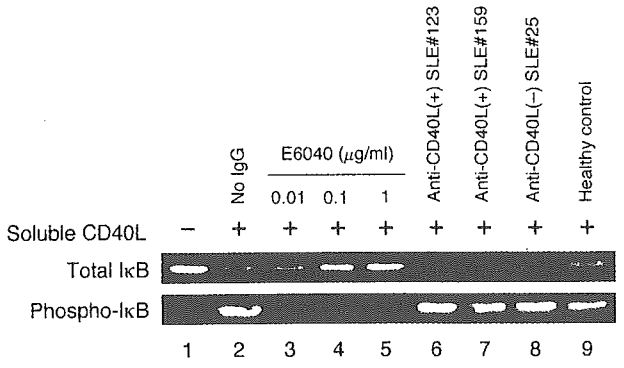

FIG. 4. Effects of patients' plasma-derived IgG on soluble CD40L-induced IκB activation in CD40-expressing fibroblasts. Human dermal fibroblasts induced to express CD40 by adenoviral gene transfer were incubated with or without recombinant soluble CD40L (0.5 μg/ml), preincubated with E6040 (0.01–1 μg/ml) or IgG (10 μg/ml) from SLE patients with or without anti-CD40L antibodies or healthy controls. Total cellular lysates were fractionated on SDS–polyacrylamide gels and transferred onto nitrocellulose membranes, which were probed with an anti-IκB (upper panel) or anti-phospho-IκB (lower panel) antibody. Lane 1, no stimulation; lane 2, stimulation with soluble CD40L preincubated with PBS; lanes 3–5, stimulation with soluble CD40L preincubated with serial concentrations of E6040 (0.01–1 μg/ml); lanes 6 and 7, stimulation with soluble CD40L preincubated with anti-CD40L antibody-positive SLE IgG (10 μg/ml); lane 8, stimulation with soluble CD40L preincubated with anti-CD40L antibody-negative SLE IgG (10 μg/ml); lane 9, stimulation with soluble CD40L preincubated with healthy control IgG (10 μg/ml). IgG from SLE patient 123 competed with mouse anti-CD40L mAbs 24–31 and 5c8 for the antibody-binding site, whereas IgG from SLE patient 159 competed with TRAP1. One of three experiments with similar results is shown.

autoantibodies in their circulation. Anti-CD40L antibody was detected by ELISA using a recombinant CD40L as an antigen source, and the specificity of the antibody binding was confirmed by competitive inhibition assays using the soluble membrane fraction of CD40L-expressing cells and mouse anti-CD40L mAbs as competitors. In addition, the anti-CD40L autoantibodies could bind the surface of living CD40L-expressing cells. Contrary to our initial hypothesis, the anti-CD40L autoantibody was not clinically associated with thromboembolism, but was strongly associated with thrombocytopenia, although the number of anti-CD40L antibody-positive patients was rather small.

All plasma samples that reacted with immobilized CD40L in the ELISA exhibited poor reactivity to the same antigen in its denatured form in immunoblots. This discordant result could be explained simply by the recognition of conformational epitope(s) expressed on the CD40L homotrimer by the autoantibodies, because the anti-CD40L antibodies in patients' plasma bound to the surface of living CD40L-expressing cells, as assessed by flow cytometry. Based on the competition patterns of the anti-CD40L autoantibody with mouse anti-CD40L mAbs, there are at least two distinct autoantigenic epitopes on CD40L, and the epitope reactivity is heterogeneous among patients.

The anti-CD40L autoantibodies were not disease-specific; rather they were associated with thrombocytopenia. The majority of the anti-CD40L antibody-positive SLE patients exhibited normal or elevated bone marrow megakaryocytes, and the thrombocytopenia in these patients responded to corticosteroid therapy; these clinical features were compatible with immune thrombocytopenia, including ITP [13]. Since CD40L pre-exists within the intracellular stores of circulating platelets and is expressed on their surface after activation [18], anti-CD40L autoantibodies potentially work as antiplatelet antibodies *in vivo*, by binding to the surface of activated platelets and enhancing platelet clearance by phagocytes. However, all the anti-CD40L antibody-positive SLE and ITP patients examined had concomitant anti-GPIIb/IIIa antibodies, pathogenic antiplatelet antibodies found in patients with ITP [13]. Therefore, it is still possible that the production of anti-CD40L autoantibodies is just a consequence of excessive platelet destruction. This hypothesis could be tested by examining patients with non-immune thrombocytopenia, although our preliminary survey showed that none of 11 non-SLE patients with thrombotic thrombocytopenic purpura or disseminated intravascular coagulation was positive for anti-CD40L antibody.

Our *in vitro* assay examining CD40L-induced IκB activation in CD40-expressing fibroblasts strongly suggests that the anti-CD40L autoantibodies in patients' plasma lack the capacity to block the CD40L-CD40 interaction *in vivo*. In this regard, the pathogenic process of SLE would be suppressed if the autoantibody blocked the functional CD40L-CD40 interaction, as observed in clinical trials of anti-CD40L humanized mAb in SLE patients. However, SLE patients with the anti-CD40L autoantibodies had higher disease activity than those without. Alternatively, the anti-CD40L autoantibody may contribute to the formation of immune complexes, because SLE patients are known to have upregulated CD40L expression on T and B cells [23] and an increased level of circulating soluble CD40L [24].

The anti-CD40L autoantibodies in patients' plasma recognized conformational epitopes on CD40L expressed on the cell surface, but failed to functionally block the CD40L-CD40 interaction. One explanation for this phenomenon is that the binding of autoantibody to CD40L may not interfere with the CD40-binding site. This would be expected for anti-CD40L autoantibodies that competed with mouse anti-CD40L mAb TRAP1, which lacks the ability to interfere with the CD40L-CD40 interaction [22]. However, the anti-CD40L autoantibody in nearly half the patients competed for the binding site with anti-CD40L mAbs 24–31 and 5c8. These two mouse mAbs are known to functionally block the CD40L-CD40 interaction [20, 21], suggesting that epitopes recognized by the anti-CD40L autoantibodies in these samples are located adjacent to the receptor-binding site on the molecule. Another possibility is that the anti-CD40L autoantibody in patients' plasma has an intrinsic low affinity for CD40L. However, there was not much difference in the binding affinity for CD40L between anti-CD40L autoantibodies and E6040, because both antibody specificities failed to bind denatured CD40L monomers in immunoblots, and inhibition of the CD40L binding of these antibodies was achieved by similar concentrations of mouse anti-CD40L mAbs in the competitive ELISA.

Thromboembolic complications during anti-CD40L humanized mAb treatment led to a temporary halt in all clinical trials. CD40L–CD40 blockade is a potentially effective therapy for various T-cell-mediated diseases, including SLE and other autoimmune diseases [25], and transplant rejection [26], but the potential risk of thromboembolic complications haunts its future development. Our results showed that the presence of anti-CD40L autoantibody is not a risk factor for thromboembolism in SLE patients. The precise mechanism of thrombophilia during anti-CD40L mAb treatment is not clear at present, but several have been proposed. It is intriguing that CD40L is rapidly expressed on the surface of platelets during thrombus formation [18]. An interaction between the CD40L on activated platelets and CD40 on platelets, endothelial cells and monocytes facilitates their inflammatory and prothrombotic properties [27]. It is conceivable that the binding of the anti-CD40L antibody to activated platelets might enhance their aggregation, through the additional interaction of the anti-CD40L antibody with Fcγ receptors on platelets and endothelial cells. The observation that anti-CD40L autoantibodies in SLE patients without thromboembolism could bind

the cell surface of living CD40L-expressing cells disfavours the hypothesis of an Fcγ receptor-mediated mechanism during anti-CD40L mAb treatment [7, 8]. On the other hand, CD40L has a lysine–arginine–glutamic acid motif that allows it to bind platelet surface GPIIb/IIIa, and this interaction is involved in stabilizing the thrombus [28]. Disruption of this interaction by an anti-CD40L antibody might render the platelet plugs unstable and thus ready to embolize. Nearly all the SLE and ITP patients with anti-CD40L autoantibody had a concomitant anti-GPIIb/IIIa antibody, which may alter the clot-stabilizing properties of CD40L and mask the potential prothrombotic effect of the anti-CD40L autoantibodies. In this regard, an increased thrombotic risk is reported in patients with acute coronary syndromes and elevated soluble CD40L, and this risk is significantly reduced by treatment with abciximab, an anti-GPIIb/IIIa chimeric mAb [29]. In addition, thromboembolic complication has not been reported in clinical trials of anti-CD40L humanized mAb in ITP patients [8].

In summary, anti-CD40L autoantibody is associated with thrombocytopenia but not with thromboembolism in SLE patients. Our findings are potentially useful for better understanding the mechanisms underlying the thromboembolic complications associated with anti-CD40L humanized mAb treatment. Further studies are necessary to elucidate the complex roles of CD40L in the pathophysiology of thrombosis and haemostasis.

Rheumatology	Key messages
	• Autoantibody to CD40L is associated with thrombocytopenia in SLE patients. • Anti-CD40L autoantibody is not a risk factor for thromboembolism, which was observed in clinical trials of anti-CD40L humanized antibody.

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The authors have declared no conflicts of interest.

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特集

ITP

Idiopathic thrombocytopenic purpura
: Analysis of the pathogenic process and recent progresses in therapeutic strategies

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臨床血液学 最近の進歩

Key words 抗血小板抗体 自己反応性T細胞 *Helicobacter pylori* 分子標的療法 副刺激

特発性血小板減少性紫斑病(ITP)は血小板破壊が亢進して血小板減少をきたす後天性疾患のうち，明らかな原因や基礎疾患がないものと定義される．発症様式と自然経過から急性と慢性型に分類されるが，共通する病態は免疫学的機序による血小板破壊である．抗血小板抗体が結合してオプソニン化した血小板が網内系でFcγ受容体を介してマクロファージに捕捉・貪食されることが主たる血小板破壊の機序である．本稿では血小板に対する自己免疫を中心にITPの病態について概説し，さらに新しい治療戦略についてまとめた．

Ⅰ．抗血小板抗体の役割

ITP患者に出現する抗血小板抗体の標的は血小板凝集にかかわる各種血小板膜糖蛋白で，最も頻度が高い標的抗原はGPⅡb/Ⅲaである[1]．これら自己抗体は血小板に結合して血小板の網内系での破壊を誘導するだけでなく，骨髄巨核球に結合して血小板産生を阻害したり[2]，血小板凝集を阻害することで出血傾向を助長する作用も有する．

B細胞からの高親和性IgG産生には$CD4^+$ T細胞からのヘルパー活性が必須である．私たちは抗血小板抗体の主要な標的抗原GPⅡb/Ⅲaを認識する$CD4^+$ T細胞がITP患者末梢血中に検出され，それらは自己B細胞からの抗血小板抗体産生を誘導するヘルパー活性を持つことを報告した[3)4)]．GPⅡb/Ⅲa反応性T細胞は抗原を認識することで活性化されると，CD40リガンド(CD40L)やIL-6の発現を介したヘルパー活性によりB細胞からの抗GPⅡb/Ⅲa抗体産生を誘導する．

近年，胸腺でのnegative selectionによる自己反応性T細胞の除去は不完全で，末梢における自己反応性T細胞の不活化がトレランス維持に重要なことが示された．実際にGPⅡb/Ⅲa反応性T細胞は多くの健常人末梢血中に検出されることから，ITPの病態解明にはその活性化機構の解析が必要である．GPⅡb/Ⅲa反応性T細胞は人為的に構造を修飾したGPⅡb/Ⅲaを取り込んだ抗原提示細胞に対して反応性を示すが，血小板膜上の本来の構造を有するGPⅡb/Ⅲaを取り込んだ抗原提示細胞に対して反応しない[3)4)]．そのため，GPⅡb/Ⅲa反応性T細胞は本来の分子構

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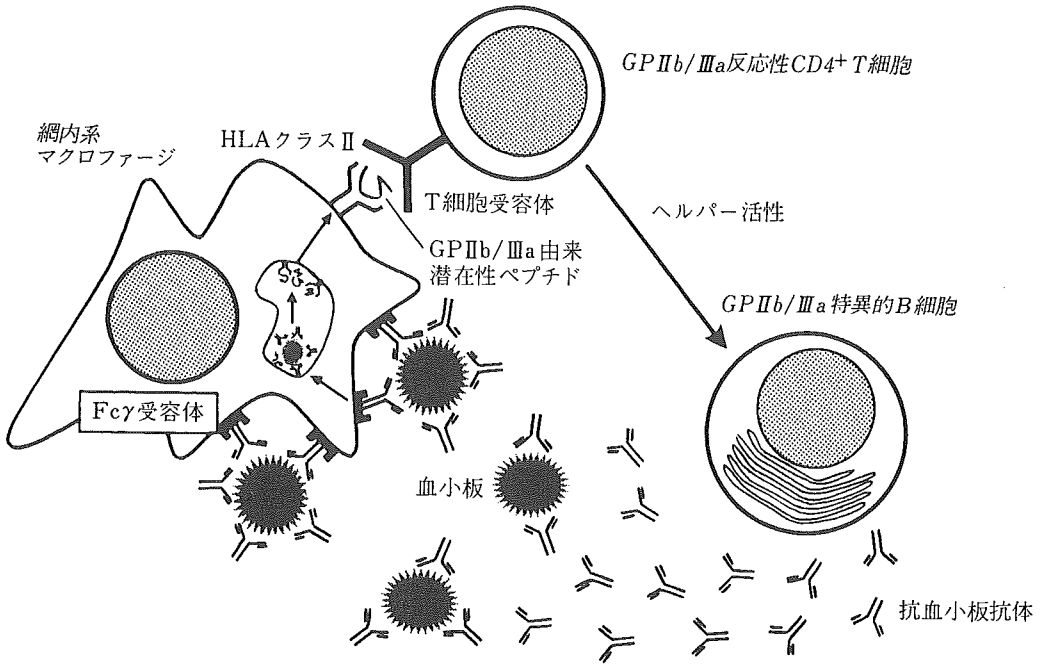

図1 ITPにおける抗血小板抗体の産生メカニズム

造を持つGPⅡb/Ⅲaからのプロセッシングでは作られない潜在性(cryptic)ペプチドを認識すると考えられる．潜在性ペプチドとは，通常は抗原プロセッシングにより免疫応答を誘導するのに十分な量が提示されないペプチドのことで，その詳細は他を参照していただきたい[5]．したがって，ITP患者ではGPⅡb/Ⅲaの潜在性ペプチドが末梢の抗原提示細胞により提示されることがGPⅡb/Ⅲa反応性T細胞活性化の誘因と推測され，その発現細胞は脾臓など網内系のマクロファージであることが示されている[6]．網内系マクロファージはFcγ受容体を介してオプソニン化血小板を大量に貪食するため，大量に取り込んだGPⅡb/Ⅲaからのプロセッシングにより従来は潜在性のペプチドが免疫応答を誘導するのに十分量発現される可能性がある．以上の結果から想定されるITP患者における抗血小板抗体の産生メカニズムを図1に示す．GPⅡb/Ⅲa反応性$CD4^+$ T細胞が活性化されて抗血小板抗体が産生されると，オプソニン化血小板を貪食した網内系マクロファージがGPⅡb/Ⅲa由来の潜在性ペプチドを発現することで特異的T細胞を活性化し，抗血小板抗体産生を維持・増強する．このようなGPⅡb/Ⅲa反応性T細胞，B細胞，網内系マクロファージのサイクルが成立すると抗血小板抗体産生は持続する[7]．

Ⅱ．細胞傷害性T細胞の役割

ITP患者における血小板破壊機序に関して古くから細胞傷害性T細胞の関与が想定されてきた．最近，ITP患者末梢血T細胞で細胞傷害性T細胞に高発現されるperforinやgranzymeの遺伝子発現亢進が報告され，細胞傷害性T細胞の活性化が示された[8]．ただし，この細胞傷害活性がGPⅡb/Ⅲaなどの血小板特異抗原の認識を介した反応か，非特異的な傷害活性によるかは明らかでない．

Ⅲ．ITPに対する治療戦略

ITP治療の基本方針は15年以上変わっていない．すなわち，血小板数を正常に戻すのでなく，出血症状の改善，重篤な出血の予防を目標とし，

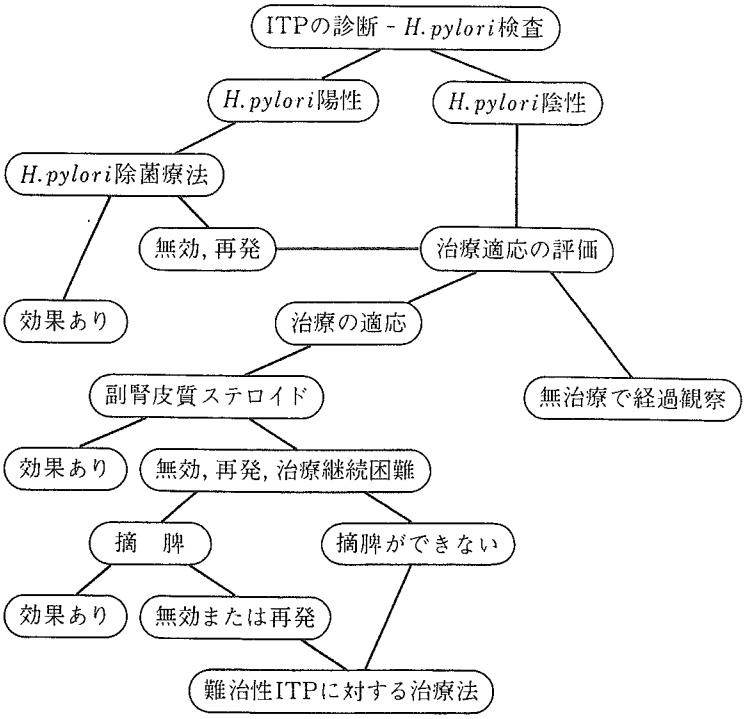

図2　ITPの新しい治療指針案(試案)
*H. pylori*を保菌するITP患者に対する除菌療法を優先し，除菌無効例および*H. pylori*陰性例に対して従来の副腎皮質ステロイド療法，摘脾を行う．

血小板数と出血症状の程度に基づいて治療適応を決定する．適応症例では，まず副腎皮質ステロイド，次いで摘脾を行う．一方，これら一次治療で血小板数を出血の危険の少ないレベルに維持できない例を難治性とよび，ITP症例の10%程度を占める．難治例は一般人口に比べて死亡率が約4倍高く，特に60歳を越えた高齢者では重篤な出血のリスクが非常に高まる．また，副腎皮質ステロイドで血小板数のコントロール可能な症例でも非特異的な免疫抑制効果による感染症や骨粗鬆症などの副作用が問題となる．そのため，有効性と選択性の高い，病態に基づいた治療法が求められており，近年ようやく従来の治療指針の変更につながる知見が集積されてきた．

1. *Helicobacter pylori*(*H. pylori*)除菌療法

*H. pylori*はらせん型小桿菌で，慢性萎縮性胃炎，消化性潰瘍，胃癌などの病因として注目されている．イタリアのGasbarriniらは*H. pylori*を保菌するITP患者に除菌療法を行うと，血小板数が増加することを初めて報告した[9]．この成績は追試され，わが国では*H. pylori*を保菌するITP患者の半数以上で除菌療法の有効性が確認された[10]．血小板増加は一過性でなく，長期寛解例も少なくない．*H. pylori*除菌療法はプロトンポンプ阻害薬と抗菌薬2種類を1週間服用するだけで，副腎皮質ステロイド療法に比べて寛解導入率は高く，副作用も胃腸障害や皮疹など軽度である．そのため，ITPと診断されればまず*H. pylori*を検索し，保菌例では副腎皮質ステロイドの前に除菌療法を行うこと，さらに*H. pylori*保菌例に対しては血小板数や出血症状にかかわらず全例で除菌療法を行うことが提案されている(図2)．*H. pylori*除菌が血小板数を増やす機序として抗血小板抗体と*H. pylori*構成蛋白の交差反応などが推測されているが，現時点で詳細は明らかでない．

2. 分子標的療法

近年，病態にかかわる分子や細胞を標的とした治療法(分子標的療法)が次々に臨床の場に導入さ

表1 ITPに対する新しい分子標的療法とその作用標的

治療法	商品名	投与量	作用標的	主な副作用
Cyclosporin A	サンディミュン ネオーラル	2.5～3 mg/kg/day	T細胞機能抑制	感染症 悪性腫瘍
Myophenolate mofetil	セルセプト	1.5～2 g/day	T，B細胞機能抑制	感染症，悪性腫瘍
Anti-CD20 chimeric antibody (rituximab)	リツキサン	375mg/m^2 weekly を4週間	B細胞除去	感染症
TNF receptor p75-Ig fusion (etanercept)	エンブレル	25mg/day を週2回	TNFα，TNFβ活性阻害	感染症(特に結核)
Anti-CD52 humanized antibody (alemtuzumab/Campath-1H)	日本で未承認	10mg/day を10日間	T，B細胞除去	感染症 投与時反応
Anti-CD40 ligand humanized antibody (IDEC-131/E6040)	日本で未承認	10mg/kg を反復	副刺激阻害	感染症 血栓症？
CTLA4-Ig	日本で未承認	10mg/kg を2週毎に3回，以後は4週に1回	副刺激阻害	投与時反応

れた．ITP患者では網内系マクロファージ，GPⅡb/Ⅲa反応性T，B細胞のサイクルが成立しており(図1)，いずれかのステップを標的とすることで抗血小板抗体産生の抑制が可能である．**表1**にITPでの有効性が報告されている分子標的療法およびその作用標的をまとめた．

1)新規免疫抑制薬

作用機序が明確な免疫抑制薬cyclosporin Aとmycophenolate mofetil(MMF)がITPに対し用いられ，良好な成績が報告されている．cyclosporin AはT細胞の増殖とサイトカイン産生を選択的に抑制するが，MMFはT，B細胞両者の増殖を抑制する．cyclosporin Aを5 mg/kg以上用いると高頻度に腎機能障害，高血圧がみられるが，2.5～3 mg/kgと低容量でも多くの症例で効果を認める[11]．これら薬剤はリンパ球に選択性が高いが，過度の免疫抑制は感染症や二次性悪性腫瘍を誘発する可能性がある．

2)抗CD20キメラ抗体(rituximab)

元来悪性リンパ腫の治療薬として開発されたrituximabは抗血小板抗体産生B細胞を含む成熟B細胞をすべて除去することで効果を発揮する．rituximabを投与された難治例の約半数で血小板数が増加し，そのうち半数で長期に渡る寛解が得られた報告がある[12]．rituximabに反応しやすい要因として，短い罹病期間，摘脾後があげられている．投与後は末梢血B細胞が一過性にほとんど消失するが，重篤な感染症の頻度は予測していたほど高くない．

3)抗CD52ヒト化抗体(alemtuzumab)

alemtuzumabは元来慢性リンパ性白血病などのリンパ系腫瘍に対する治療薬として開発された生物製剤で，リンパ球に広く発現するCD52を標的とすることでリンパ球すべてを一過性に除去する．少数例ながらITPでの使用が報告され，半数以上で効果を認めた．ただし，悪寒，発熱などの非常に強い投与時反応を伴い，リンパ球著減による感染症のリスクも高い．

4)TNF阻害薬

関節リウマチ(RA)治療薬として欧米で承認されているetanerceptが有効であった難治性ITPが報告されている[13]．etanerceptはTNF受容体TNFR-p75をヒトIgGのFc領域との融合蛋白として発現させた生物製剤でTNFαとTNFβ両者の活性を阻害するが，ITPで血小板数を増やす機序は明らかでない．

5)副刺激遮断薬

T細胞と抗原提示細胞の相互作用に必須な副刺激を標的とした手法もITPに有効な治療の候補である．T細胞活性化にはT細胞受容体による抗原認識のみでは不十分で，同時に副刺激が必要である．T細胞と抗原提示細胞膜上にはさまざまな副刺激分子が存在するが，特に重要なシグナルとしてCD40L-CD40，CD28-CD80/CD86の2つ

がある．副刺激なしにT細胞受容体から主シグナルが伝わるとT細胞は活性化されないだけでなく，その後の適正な刺激に対しても反応できない不応答状態に陥る．この性質を利用して，副刺激を遮断することで抗原に曝露しているT細胞の不活化が可能である．

このような副刺激阻害薬として臨床試験中の製剤としてヒト化抗CD40L抗体とCTLA4-Igがある．難治性ITP患者を対象としたヒト化抗CD40L抗体の単回投与により，GPⅡb/Ⅲaに対するT細胞，B細胞の抑制とともに一過性の血小板数の増加が報告されている[14]．ただし，抗CD40L抗体療法と血栓症との関連が疑われ，現在臨床試験が中断されている．CTLA4は活性化T細胞に一過性に発現される分子で，CD80/CD86に対してCD28より高い親和性で結合することで副刺激シグナルを抑制する．CTLA4-IgはCTLA4とヒトIgG-Fcとの融合蛋白である．各種自己免疫疾患を対象とした第Ⅱ相試験が進行中で，RAでの試験では投与群でコントロール群に比べて明らかな改善がみられている．ITP患者を対象とした報告はないが，CTLA4-Ig存在下でITP患者由来の血小板反応性T細胞のトレランス誘導が *in vitro* の実験系で示されている[15]．

おわりに

抗血小板抗体産生を誘導する免疫動態が明らかにされ，分子標的療法の標的となる細胞あるいは分子が同定されてきた．今後，薬剤開発と臨床試験の両面からの検討が進み，難治性ITPに有効な治療法が確立されることが期待される．

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● 自己免疫疾患の概念と知見

特発性血小板減少性紫斑病

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要旨

ITP は血小板膜糖タンパク質に対する自己抗体により誘導される後天性の血小板減少症である．抗血小板抗体が結合した血小板が網内系で Fcγ 受容体を介してマクロファージなどの貪食細胞に捕捉され，貪食・破壊されることが主たる病態である．最近の研究成果により，自己反応性 $CD4^+$ T 細胞による抗血小板抗体の産生機構が明らかにされた．また，診断，治療に関する新しい知見が集積され，15 年以上も用いられてきた従来の診断基準や治療ガイドラインの見直しが検討されている．

はじめに

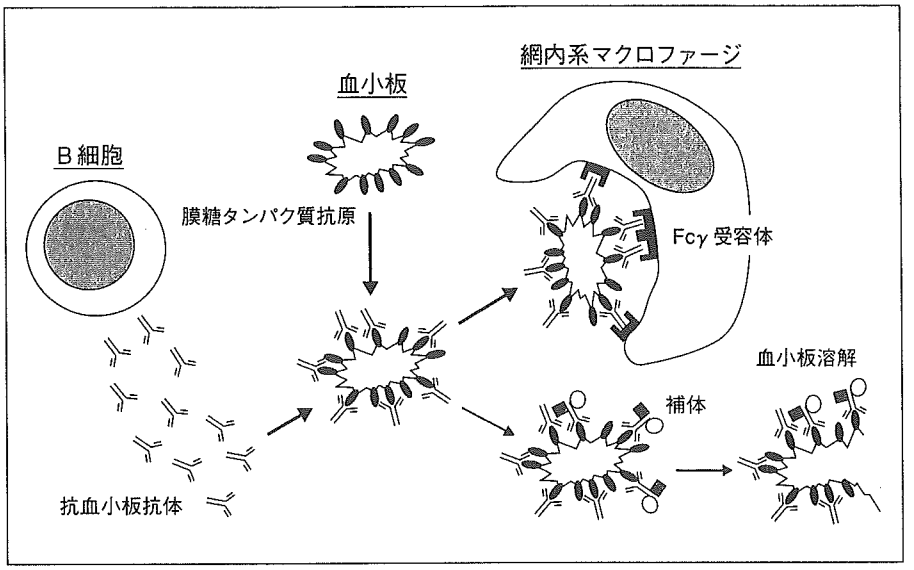
特発性血小板減少性紫斑病（ITP）は薬剤などの原因や基礎疾患が明らかでないにもかかわらず，血小板の破壊が亢進し，血小板減少をきたす後天性疾患である．主体となる血小板破壊の機序は，主に血小板に対する自己抗体を介した免疫反応による．本稿では，ITP の病態，診断，治療の関する最近の知見を概説する．

病　態

ITP における血小板破壊の機序は，血小板に対する自己抗体が結

キーワード：抗血小板抗体，自己反応性T細胞，網状血小板，
Helicobacter pylori，分子標的療法

図1 ITPにおける血小板破壊の機序



B細胞から産生された抗血小板抗体は血小板膜糖タンパク質抗原に結合し，Fcγ 受容体を介して網内系マクロファージにより貪食，破壊される．IgM または大量の IgG 抗血小板抗体が血小板に結合すれば，補体活性化を介して血管内での血小板溶解がみられる可能性もある．

合してオプソニン化された血小板が網内系で Fcγ 受容体を介してマクロファージなどの貪食細胞に捕捉され，貪食・破壊される病態である（図1）[1]．IgM 型あるいは大量の IgG 型抗血小板抗体が血小板に結合すれば補体活性化を介して血小板の血管内破壊が起こる可能性もあるが，病態におけるその貢献度は低い．多くの ITP 患者で脾臓がおもな血小板破壊場所であるとともに抗血小板抗体産生部位であり[2]，その摘出が ITP に対して有効率の高い治療であることが実証されている．一方，血小板を直接傷害する $CD8^+$ 細胞傷害性T細胞の存在も報告されているが[3]，その認識が血小板に特異的かどうかは明らかではない．

抗血小板抗体の標的抗原として GPⅡb／Ⅲa，GPⅠb／Ⅸ，GPⅠa／Ⅱa など各種血小板膜糖タンパク質が同定されている．特に抗 GPⅡb／Ⅲa 抗体の頻度が高く，高感度な検出法を用いると 90％ 以上で陽性となる．これら対応抗原はいずれも血小板凝集にかかわるリガンドの受容体のため，自己抗体による血小板機能障害も出血を助長する[4]．また，抗血小板抗体の対応抗原は血小板のみならず骨髄巨核球にも発現することから，これら自己抗体は巨核球の成熟障害や細胞傷害を誘