M.K.) and the Japanese Ministry of Health, Labor, and Welfare (to M.K.).

We thank Y. Tanaka and Y. Okazaki for their excellent technical assistance, and Drs. Y. Fukuuchi, A. Koto, T. Amano, N. Tanahashi, J. Hamada, S. Nogawa, and H. Sato for coordinating the blood specimen collection and providing clinical information.

References

Aarli, J.A., Stefansson, K., Marton, L.S., Wollmann, R.L., 1990. Patients with myasthenia gravis and thymoma have in their sera IgG autoantibodies against titin. Clin. Exp. Immunol. 82, 284–288.

Bohan, A., Peter, J.B., 1975. Polymyositis and dermatomyositis (second of two parts). N. Engl. J. Med. 292, 403–407.

Braunwald, E., Fauci, A.S., Kasper, L.K., Hauser, S.L., Longo, D.L., Jameson, J.L., Isselbacher, K.J. (Eds.), Harrison's Principles of Internal Medicine, (15th edition). McGraw-Hill, New York.

Buckley, C., Newsom-Davis, J., Willcox, N., Vincent, A., 2001. Do titin and cytokine antibodies in MG patients predict thymoma or thymoma recurrence? Neurology 57, 1579–1582.

Drachman, D.B., 1994. Myasthenia gravis. N. Engl. J. Med. 330, 1797–1810.

Evoli, A., Minisci, C., Di Schino, C., Marsili, F., Punzi, C., Batocchi, A.P., Tonali, P.A., Doglietto, G.B., Granone, P., Trodella, L., Cassano, A., Lauriola, L., 2002. Thymoma in patients with MG: characteristics and long-term outcome. Neurology 59, 1844–1850.

Hart, I.K., Waters, C., Vincent, A., Newland, C., Beeson, D., Pongo, O., Christine, M., Newsom-Davis, J., 1997. Autoantibodies detected to expressed K^+ channels are implicated in neuromyotonia. Ann. Neurol. 41, 238–246.

Hoch, W., McConville, J., Helms, S., Newsom-Davis, J., Melms, A., Vincent, A., 2001. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. Nat. Med. 7, 365–368.

Hofstad, H., Ohm, O.J., Mork, S.J., Aarli, J.A., 1984. Heart disease in myasthenia gravis. Acta Neurol. Scand. 70, 176–184.

Jaretzki III, A., Barohn, R.J., Ernstoff, R.M., Kaminski, H.J., Keesey, J.C., Penn, A.S., Sanders, D.B., 2000. Myasthenia gravis: recommendations for clinical research standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. Neurology 55, 16–23.

Kuwana, M., Kaburaki, J., Mimori, T., Tojo, T., Homma, M., 1993. Autoantibody reactive with three classes of RNA polymerases in sera from patients with systemic sclerosis. J. Clin. Invest. 91, 1399–1404.

Kuwana, M., Okano, Y., Kaburaki, J., Medsger Jr., T.A., Wright, T.M., 1999. Autoantibodies to RNA polymerases recognize multiple subunits and demonstrate cross-reactivity with RNA polymerase complexes. Arthritis Rheum. 42, 275–284.

Kuwana, M., Kimura, K., Kawakami, Y., 2002. Identification of an immunodominant epitope on RNA polymerase III recognized by systemic sclerosis sera: application to enzyme-linked immunosorbent assay. Arthritis Rheum. 46, 2742–2747.

Liyanage, Y., Teo, M., MacLennan, C., Buckel, A., Beeson, D., Willcox, N., Newsom-Davis, J., Vincent, A., 1998. Expression of muscle proteins in thymomas of patients with myasthenia gravis. Ann. N. Y. Acad. Sci. 841, 411–413.

Masiarz, F.R., Malcolm, B.A., 1994. Rapid determination of endoprotease specificity using peptide mixture and Edman degradation analysis. Methods Enzymol. 241, 302–310.

Mimori, T., Hinterberger, M., Pettersson, I., Steitz, J.A., 1984. Autoantibodies to the U2 small nuclear ribonucleoprotein in a patient with scleroderma-polymyositis overlap syndrome. J. Biol. Chem. 259, 560–565.

Mygland, A., Tysnes, O.B., Matre, R., Volpe, P., Aarli, J.A., Gilhus, N.E., 1992. Ryanodine receptor autoantibodies in myasthenia gravis patients with a thymoma. Ann. Neurol. 32, 589–591.

Namba, T., Brunner, N.G., Grob, D., 1974. Idiopathic giant cell polymyositis. Report of a case and review of the syndrome. Arch. Neurol. 31, 27–30.

Newsom-Davis, J., Mills, K.R., 1993. Immunological associations of acquired neuromyotonia (Isaacs' syndrome). Report of five cases and literature review. Brain 116, 453–469.

Philipson, L.H., Schaefer, K., LaMendola, J., Bell, G.I., Steiner, D.F., 1990. Sequence of a human fetal skeletal muscle potassium channel cDNA related to RCK4. Nucleic Acids Res. 18, 7160.

Scheele, G.A., 1975. Two-dimensional gel analysis of soluble proteins. Characterization of guinea pig exocrine pancreatic proteins. J. Biol. Chem. 250, 5375–5385.

Shillito, P., Molenaar, P.C., Vincent, A., Leys, K., Zheng, W., van der Berg, R.J., Plomp, J.J., Kempen, G.H.V., Chauplannaz, G., Wintzen, A.R., van Dijk, J.G., Newsom-Davis, J., 1995. Acquired neuromyotonia: evidence for autoantibodies directed against K^+ channels of peripheral nerves. Ann. Neurol. 38, 714–722.

Tamkun, M.M., Knoth, K.M., Walbridge, J.A., Kroemer, H., Roden, D.M., Glover, D.M., 1991. Molecular cloning and characterization of two voltage-gated K^+ channel cDNAs from human ventricle. FASEB J. 5, 331–337.

Veh, R.W., Lichtinghagen, R., Sewing, S., Wunder, F., Grumbach, I.M., Pongo, O., 1995. Immunohistochemical localization of five members of the Kv1 channel subunits: contrasting subcellular locations and neuron-specific co-localizations in rat brain. Eur. J. Neurosci. 7, 2189–2205.

Wakkach, A., Guyon, T., Bruand, C., Tzartos, S., Cohen-Kaminsky, S., Berrin-Aknin, S., 1996. Expression of acetylcholine receptor genes in human thymic epithelial cells: implications for myasthenia gravis. J. Immunol. 157, 3752–3760.

Wickenden, A.D., Jegla, T.J., Kaprielian, R., Backx, P.H., 1999. Regional contributions of $K_V1.4$, $K_V4.2$, and $K_V4.3$ to transient outward K^+ current in rat ventricle. Am. J. Physiol. 276, H1599–H1607.

Williams, C.L., Lennon, V.A., 1986. Thymic B lymphocyte clones from patients with myasthenia gravis secrete monoclonal striational autoantibodies reacting with myosin, alpha actinin, or actin. J. Exp. Med. 164, 1043–1056.

Yellen, G., 2002. The voltage-gated potassium channels and their relatives. Nature 419, 35–42.

Rheumatology 2006;45:150–156 doi:10.1093/rheumatology/kei118
Advance Access publication 27 September 2005

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

M. Nakamura, Y. Tanaka, T. Satoh, M. Kawai, M. Hirakata[1], J. Kaburaki[2], Y. Kawakami, Y. Ikeda[1] and M. Kuwana

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.

Institute for Advanced Medical Research and [1]Department of Internal Medicine, Keio University School of Medicine and [2]Department of Internal Medicine, Tokyo Electric Power Company Hospital, Tokyo, Japan.

Submitted 24 May 2005; revised version accepted 12 August 2005.

Correspondence to: M. Kuwana, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kuwanam@sc.itc.keio.ac.jp

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 $5 \times$ 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

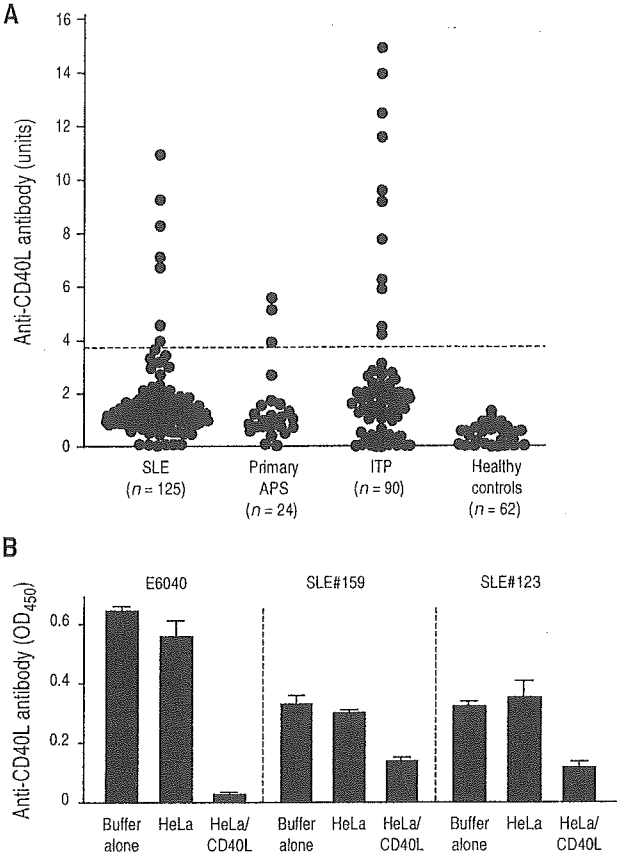

FIG. 1. (A) Plasma anti-CD40L antibody levels measured by ELISA in 125 patients with SLE, 24 with primary APS and 90 with ITP and in 62 healthy controls. The broken line denotes the cut-off set at the mean plus $5 \times$ S.D. of 62 healthy controls (3.75 units). (B) Competitive inhibition in an anti-CD40L 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.

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).

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).

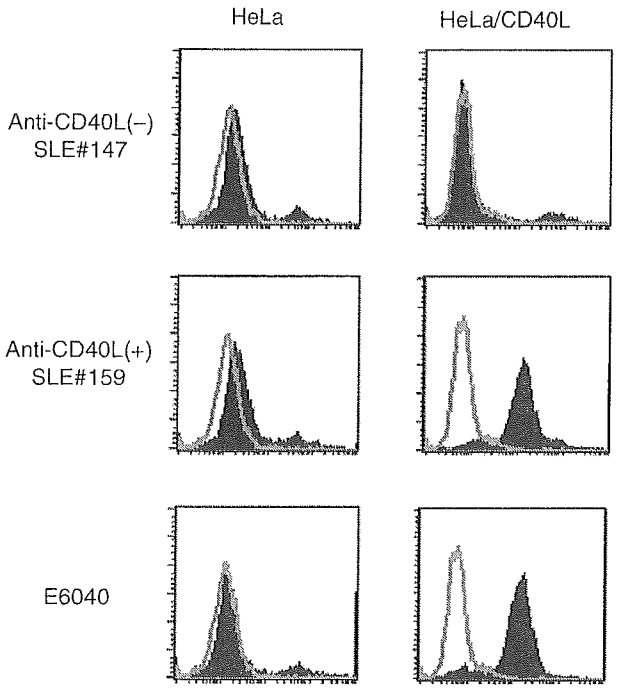

FIG. 2. Binding of anti-CD40L autoantibody in patients' plasma to CD40L expressed on the living cell surface. Unfixed wild-type HeLa and HeLa/CD40L cells were preincubated with anti-CD40L antibody-positive or -negative IgG from SLE patients (250 μg/ml), or anti-CD40L humanized mAb E6040 (10 μg/ml), followed 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.

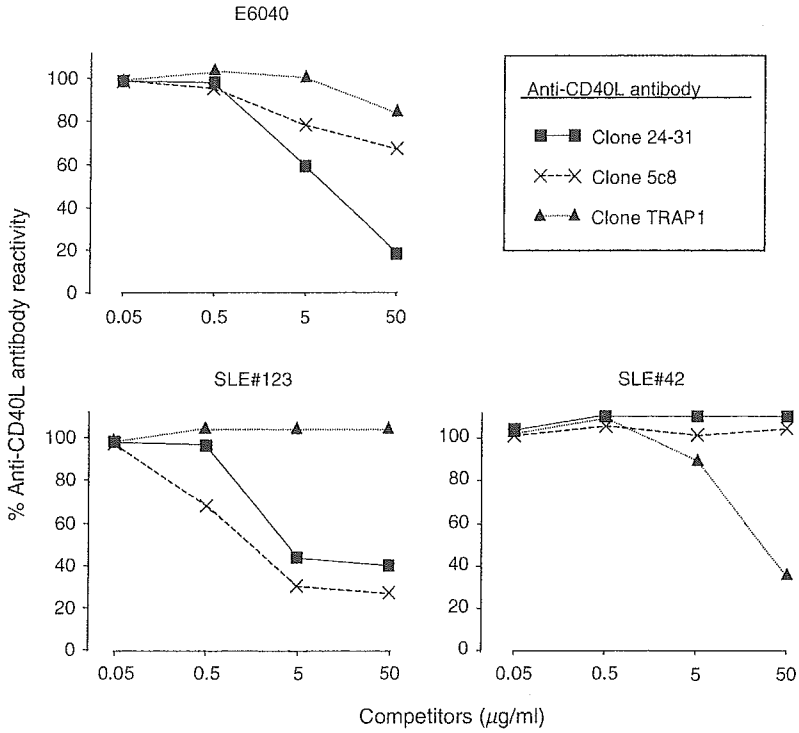

FIG. 3. Competitive inhibition in an anti-CD40L antibody ELISA using a series of mouse anti-CD40L mAbs. The antigen-coated ELISA wells were incubated with serial concentrations (0.05–50 μl/ml) of mouse anti-CD40L mAb (24–31, TRAP-1 or 5c8), and subsequently with anti-CD40L humanized mAb E6040 or SLE 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

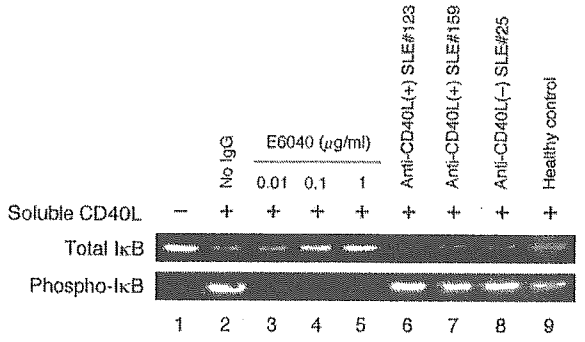

FIG. 4. Effects of patients' plasma-derived IgG on soluble CD40L-induced IκB activation in CD40-expressing fibroblasts. Human dermal fibroblasts induced to express CD40 by adenoviral gene transfer were incubated with or without recombinant soluble CD40L (0.5 μg/ml), preincubated with E6040 (0.01–1 μg/ml) 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.

Acknowledgements

We thank Yuka Okazaki, Mutsuko Ishida and Masaaki Kubota for their expert technical assistance and Dr Kazunori Kato for providing the HeLa/CD40L cells. This work was supported by grants from the Japanese Ministry of Health, Labour and Welfare, and Eisai Company Ltd.

The authors have declared no conflicts of interest.

References

1. Laman JD, Claassen E, Noelle RJ. Functions of CD40 and its ligand, gp39 (CD40L). Crit Rev Immunol 1996;16:59–108.
2. van Kooten C, Banchereau J. CD40-CD40 ligand. J Leukoc Biol 2000;67:2–17.
3. Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA, Noelle RJ. Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. Science 1993;261:1328–30.
4. Mohan C, Shi Y, Laman JD, Datta SK. Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. J Immunol 1995;154:1470–80.
5. Dumont FJ. IDEC-131 IDEC/Eisai. Curr Opinion Invest Drugs 2002;3:725–34.
6. Huang W, Sinha J, Newman J *et al.* The effect of anti-CD40 ligand antibody on B cells in human systemic lupus erythematosus. Arthritis Rheum 2002;46:1554–62.
7. Boumpas DT, Furie R, Manzi S *et al.* A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis. Arthritis Rheum 2003;48:719–27.
8. Kuwana M, Nomura S, Fujimura K *et al.* The effect of a single injection of humanized anti-CD154 monoclonal antibody on the platelet-specific autoimmune response in patients with immune thrombocytopenic purpura. Blood 2004;103:1229–36.
9. Biogen says it has stopped ongoing trials of anti-CD40 ligand monoclonal antibody. Biogen Inc. Press Release, 2 January 1999.
10. IDEC Pharmaceuticals announces a clinical hold on ongoing clinical trials of its IDEC-131 antibody. IDEC Pharmaceuticals. Press Release, 10 June 2002.
11. Tan EM, Cohan AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
12. Wilson WA, Gharavi AE, Koike T *et al.* International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome. Arthritis Rheum 1999;42:1309–11.
13. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. N Engl J Med 2002;346:995–1008.
14. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH; Committee on Prognosis Studies in SLE. Derivation of the SLEDAI: a disease activity index for lupus patients. Arthritis Rheum 1992; 35:630–40.
15. Kuwana M, Okazaki Y, Kajihara M *et al.* Autoantibody to c-Mpl (thrombopoietin receptor) in systemic lupus erythematosus: relationship to thrombocytopenia with megakaryocytic hypoplasia. Arthritis Rheum 2002;46:2148–59.
16. Forman MS, Nakamura M, Mimori T, Gelpi C, Hardin JA. Detection of antibodies to small nuclear ribonucleoproteins and small cytoplasmic ribonucleoproteins using unlabelled cell extracts. Arthritis Rheum 1985;28:1356–61.
17. Kuwana M, Okazaki Y, Kaburaki J, Ikeda Y. Detection of circulating B cells secreting platelet-specific autoantibody is useful in the diagnosis of autoimmune thrombocytopenia. Am J Med 2003;114:322–5.
18. Henn V, Slupsky JR, Grafe M *et al.* CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 1998;391:591–4.
19. Kuwana M, Medsger TA Jr, Wright TM. Detection of anti-DNA topoisomerase I antibody by an enzyme-linked immunosorbent assay using overlapping recombinant polypeptides. Clin Immunol Immunopathol 1995;76:266–78.
20. Noelle RJ, Ledbetter JA, Aruffo A. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. Immunol Today 1992;13:431–3.
21. Lederman S, Yellin MJ, Cleary AM *et al.* T-BAM/CD40-L on helper T lymphocytes augments lymphokine-induced B cell Ig isotype switch recombination and rescues B cells from programmed cell death. J Immunol 1994;152:2163–71.
22. Kroczek RA, Graf D, Brugnoni D *et al.* Defective expression of CD40 ligand on T cells causes 'X-linked immunodeficiency with hyper-IgM (HIGM1)'. Immunol Rev 1994;138:39–59.
23. Crow MK, Kirou KA. Regulation of CD40 ligand expression in systemic lupus erythematosus. Curr Opin Rheumatol 2001;13:361–9.
24. Kato K, Santana-Sahagun E, Rassenti LZ *et al.* The soluble CD40 ligand sCD154 in systemic lupus erythematosus. J Clin Invest 1999; 104:947–55.
25. Kelsoe G. Therapeutic CD154 antibody for lupus: promise for the future? J Clin Invest 2003;112:1480–2.
26. Graca L, Le Moine A, Cobbold SP, Waldmann H. Antibody-induced transplantation tolerance: the role of dominant regulation. Immunol Res 2003;28:181–91.
27. Henn V, Steinbach S, Büchner K, Presek P, Kroczek RA. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. Blood 2001;98:1047–54.
28. André P, Prasad KS, Denis CV *et al.* CD40L stabilizes arterial thrombi by a β_3 integrin-dependent mechanism. Nature Med 2002; 8:247–52.
29. Heeschen C, Dimmeler S, Hamm CW *et al.* Soluble CD40 ligand in acute coronary syndromes. N Engl J Med 2003;348:1104–11.