human C3 (ICN Biomedicals, Costa Mesa, CA) and 1/500 diluted Texas-red conjugated anti-IgG (Jackson Immunoresearch) using an FW4000 fluorescent microscope (Leica Microsystems, Tokyo). Nuclei were weakly counterstained with 4'-6'-diamidine-2-phenylindole dihydrochloride (DAPI) (50 ng/section, Molecular Probes, Eugene, OR).

Intra-articular injection of human IgGs into cynomolgus monkeys. In the next step, experiments were conducted on four 5-year-old male cynomolgus monkeys (two received RA IgG while the other two received IgG of healthy subjects). Sera containing anti-GPI Abs from RA1 patients and sera from healthy subjects were separated by protein G column (Pharmacia, Piscataway, NJ), and after purification, the same quantity of IgG fractions (0.15 mg x4 times) were injected into the metacarpophalangeal (MP) joints of cynomolgus monkeys on days 0, 3, 6, and 9. Joint swelling and blood tests were monitored, and H&E and immunohistochemical studies of joints were performed on day 16. Human IgG in the joints were detected by 1/500 diluted Texas-red conjugated anti-IgG (Jackson Immunoresearch) using an FW4000 fluorescent microscope (Leica). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Tsukuba University.

Sequencing and quantification of monkey C5aR in the synovium. Total RNA from the MP joint synovium of the monkeys (on day 16) was prepared with Isogen (Nippon gene, Co., Tokyo). Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) were carried out using the methods described by Matsumoto *et al* (19). Briefly, first strand cDNAs were synthesized in a 20-μl reaction mixture containing oligo(dT) primer by reverse transcriptase from 1 μg of total RNA. Using a set of primers (Table II), the fragment was amplified and cloned into pCR2.1 TM vector (Invitrogen, San Diego, CA), and nucleotides were analyzed with an ABI377 sequencer (PE Applied Biosystems, Foster city, CA). According to the sequence, we set the probe for Taq man PCR. For quantitation, PCR was performed on a TaqMan PRISM 7700 instrument (PE Applied Biosystems), and the data were analyzed using the instrument software. Amplification was performed in triplicate in 96-well plates, using specific primers and probes (see Figure 5A). As a positive control for the Taq Man system, we used neutrophils from the cynomolgus monkeys. Differences in C5aR expression between RA-GPI positive vs. HS were compared and analyzed with the non-parametric Mann-Whitney U test. P values < 0.05, with 95% confidence interval, were considered significant.

Results

Studies in RA patients

Massive cell infiltration and lack of germinal center in synovia of anti-GPI Abs-positive patients with RA. Table I summarizes the clinical data including the anti-GPI titer of the three anti-GPI Abs-positive and three anti-GPI Abs-negative RA patients. Interestingly, two patients in the anti-GPI Abs-positive group had rheumatoid nodules, although they had neither rheumatoid vasculitis nor Felty's syndrome. Histopathological examination of H&E-stained synovial specimens showed three distinct patterns; diffuse, aggregate, and germinal center (GC)-like infiltrates (20,21). In anti-GPI Abs-positive specimens, massive cell infiltration was observed (Fig. 1A-C). However, neither GC-like formation, nor aggregation of cells, was noted in the synovia. In contrast, all anti-GPI Abs-negative RA specimens showed GC-like structures or aggregation of cells (Fig. 1D-F) that were not infrequently seen in RA. These findings suggest that diffuse infiltrate, without GC-like structures, is one of the features of synovia with anti-GPI Abs-positive specimens.

Mast cell involvement in synovia of anti-GPI Abs-positive RA. Previous studies have indicated the involvement of mast cells in anti-GPI Abs-induced arthritis in mice, and suggest that they may function as a cellular link between autoantibodies and effector populations (7). Toluidine blue staining showed the presence of mast cells in all three anti-GPI-Abs-positive synovia (Fig. 2A-C). In anti-GPI Abs-negative patients, all three specimens also showed comparable mast cells (Fig. 2D). These findings suggest the possible involvement of mast cells in anti-GPI Abs-positive RA patients, although mast cell infiltration is a common phenomenon in all RA synovia.

IgG and C3 deposition in anti-GPI Abs-positive RA synovium. Previous studies have shown the expression of GPI on articular and synovial surfaces of normal mice and humans (22). Immunohistochemical examination of the synovia of three anti-GPI Abs-positive patients showed clear deposition of IgG and C3 on the synovial surface (Fig. 3A-C, IgG, C3, and merge in RA1 patients; and Fig. 3D and E, RA2 and 3), suggesting an immunocomplex formation with complement fixation in the synovium of these patients. In contrast, two of the three synovia from the anti-GPI Abs-negative RA group lack this formation (Fig. 3G and H). This finding suggests that immunocomplex formation and complement activation on the synovial surface may be one of the specific features of synovia with anti-GPI Abs-positive RA.

Studies in cynomolgus monkeys

Deposition of injected human IgG anti-GPI Abs on the articular surface. To examine the arthritogenic effects of serum IgG including anti-GPI Abs from RA, human IgGs containing anti-GPI Abs purified from the serum of RA1 patients, or serum IgGs from healthy subjects, were injected four times directly into MP joints of cynomolgus monkeys (Fig. 4A). On day 16, the joints were harvested, stained with H&E and examined histologically and immunohistochemically. Recruitment of infiltrated cells into the joints, and strong deposition of human IgG onto the articular surface, were clearly evident in monkeys that received the intra-articular injection of RA IgG (Fig. 4B and D), although no finger-joint swelling was noted. In contrast, no infiltrated cells or IgG deposition were observed in monkeys that received IgGs from healthy subjects (Fig. 4C and E). These results indicate that serum IgG including anti-GPI Abs from RA patients, deposit preferentially on the articular surface and might recruit effector cells via C5aR or FcγR.

Overexpression of C5aR in synovia of monkeys treated with IgG anti-GPI Abs. Previous studies show that an injection of anti-GPI Abs did not induce arthritis in C5aR-deficient

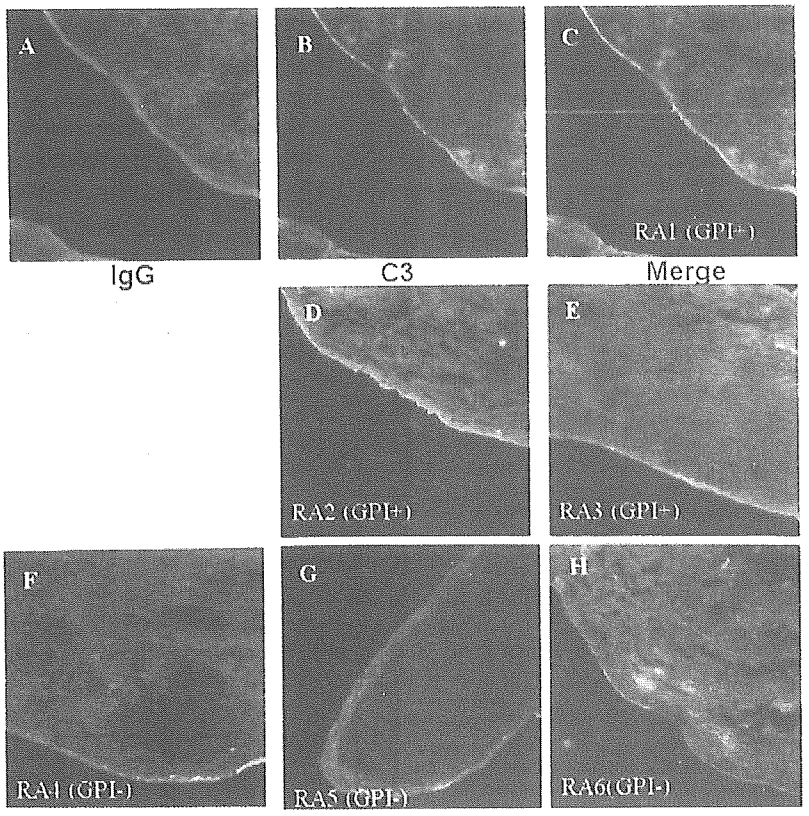


Figure 3. Immunocomplexes with C3 involvement in the synovial tissue of RA patients with anti-GPI Abs. Immunohistochemical study with IgG (red, A), C3 (green, B), and merge (yellow, C) were performed. RA1 (anti-GPI Abs-positive RA) (A-C), RA2 (anti-GPI Abs-positive RA) (D), RA3 (anti-GPI Abs-positive RA) (E), RA4 (anti-GPI Abs-negative RA) (F), RA5 (anti-GPI Abs-negative RA) (G), RA6 (anti-GPI Abs-negative RA) (H). Superimposed images positive for IgG and C3 indicate an immunocomplex formation on the synovial surface in all three anti-GPI Abs-positive RA patient samples (C-E), but not in two of the anti-GPI Abs negative RA patient samples (G,H). Magnification, x200 A-H.

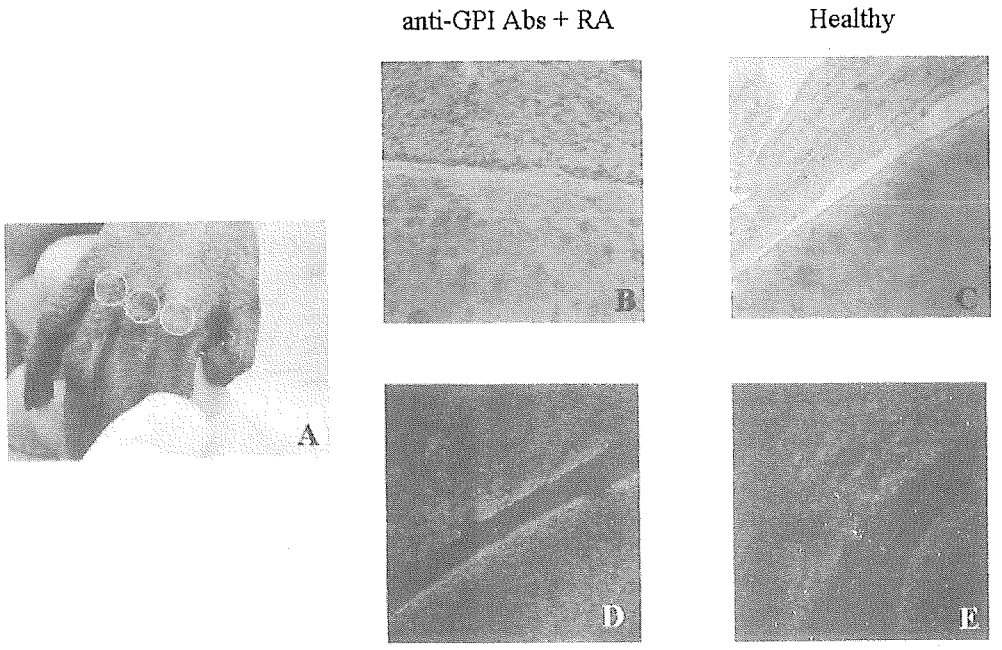


Figure 4. Serum IgG anti-GPI Abs from RA patients formed immunocomplexes on the articular surface and provoked infiltration of effector cells. Injection of human serum IgG into MP joints of cynomolgus monkeys (yellow circles in A represent the site of injection). Cryostat sections of MP joints of cynomolgus monkeys (B, C, D, E) injected 4 times with IgGs from anti-GPI Abs-positive RA patients or IgGs from healthy subjects. H&E staining of joints injected with IgGs containing anti-GPI Abs from RA subjects, (B) and IgGs from healthy subjects (C). Note that infiltration of the effector cell occurred only in response to anti-GPI Abs. Immunohistochemical studies using anti-human IgG (red) with anti-GPI Abs from RA patients (D), or IgG from healthy subjects (E). Note the strong deposition of human IgG on the articular surface by IgG anti-GPI Abs. Magnification x200 B-E.

A

β-actin

forward 5'-cactggcattgtcatggactct

reverse 5'-gctcggtgaggatcttcatgag

probe 5'-tcacccacactgtgcccatctacgag

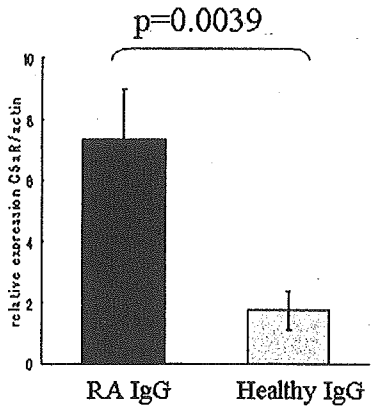
C5aR

forward 5'-ggtccaccaagagacactcaaggt

reverse 5'-ggagtccagcttcttcagtagca

probe 5'-tgccctaccaggtgac

B



C

sample	Relative expression (mean) C5aR / β-actin
RA IgG	7.33
Healthy IgG	1.76
Positive control	353

Figure 5. Sequences of primers and probes used and enhanced expression of monkey C5aR by human IgG anti-GPI Abs (A). Primer and probe sequences used for quantitation of cynomolgus monkey C5aR. ß-actin was used as a reference. The expression of C5aR was reported relative to that of ß-actin. Six MP joints' synovium (three for IgG-anti GPI Abs from RA patients and three for IgG from healthy subjects) were used in this study. Data are mean +SD of relative expression of the gene in each joint (B). The mean and positive control value of the relative expression in each joints are shown in (C).

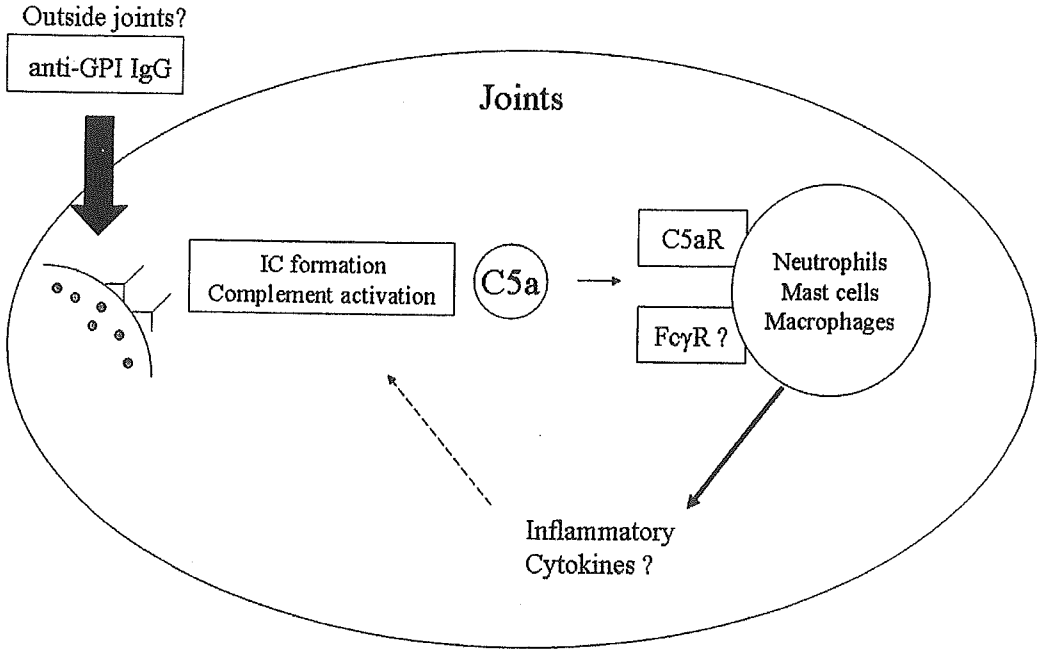


Figure 6. Schematic diagram of possible mechanisms of anti-GPI Abs-induced synovitis in RA. Anti-GPI Abs from RA patients attach directly to the surface of the joint cavity, recruit complement- and C5aR-positive cells, and ultimately result in synovitis. In addition to GPI, other ubiquitous antigens could introduce arthritogenic Abs in RA.

Table II. Alignment of nucleotide sequence of C5a receptor of rhesus and cynomolgus monkeys.

```
Rhesus      688 ggt ccaccaagac actcaaggtg gtggtggcag tggtggccag tttctttatc
Cynomolgus  688 --- ---------- --------- ---------- ---------- ----------

ttctggttgc cctaccaggt gacggggatg atgatgtcct tcctggagcc atcgtcaccc
---------- ---------- --------ca ----------- -----c-c-- g---------

acattccTGC TACTGAAGAA GCTGGACTCC 830
----a---TGC ---------- ---------- 830
```

Boxes represent 5' and 3' primers used in the experiment.

K/BxN mice (4), implying that C5a/C5aR interaction in the joint is important for the development of arthritis. To determine the role of C5aR in our study, we quantified the expression level of C5aR in the MP synovia using Taq Man real-time PCR. Since the C5aR of cynomolgus monkeys has not yet been cloned, we prepared several sets of primers encoding the C5aR sequence of rhesus monkeys. We selected primers that amplify the C5aR genes of cynomolgus monkeys (Fig. 5A and Table II), and sequencing was performed (alignment between rhesus and cynomolgus monkeys is shown in Table II). Homology between rhesus and cynomolgus monkeys was 95.8% at nucleotide level. As a positive control, we used neutrophils from a cynomolgus monkey. C5aR mRNA was highly expressed in the synovia of monkeys injected with IgG from anti-GPI Abs-positive RA, but not in those injected with IgG from healthy subjects (p=0.0039) (Fig. 5B and C). These findings suggest that immunocomplex deposition by human RA IgG including anti-GPI Abs might induce complement cascade activation via C5aR-bearing cells. A possible explanation for the incomplete perturbation might be due to differences in innate immune systems between humans and monkeys.

Discussion

The effector mechanisms of arthritogenic autoantibodies, which recognize GPI, have been clarified in detail using several knockout mice. Previous studies indicated that the key players involved in the development of arthritis, after anti-GPI Abs transfer, included Fcγ receptor (especially FcγRIII) (4); alternative complement pathway, including factors B, C3, C5, C5aR (4); subsets of Fcγ receptor or C5a-receptor bearing cells (6,7); and some inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α (5). Schaller *et al* (9) and our report (10) clearly demonstrated the presence of anti-GPI in the serum of RA patients. However, the pathogenic role of anti-GPI Abs in RA is still elusive. The present study challenged to translate the mechanisms of anti-GPI Abs in the K/BxN mouse model to anti-GPI Abs-positive RA.

Our study is the first to show diffuse cell infiltration without GC-like structures in the synovia of three anti-GPI Abs-positive RA patients, and infiltration of mast cells and immunocomplex deposition with complement C3 on the synovial surface in anti-GPI Abs-positive RA patients. Several groups have reported the accumulation of synovial mast cells in RA (23,24). Furthermore, in the anti-GPI induced arthritis model, mast cell-deficient mice were completely resistant to the induction of arthritis, while reconstitution of these mice with mast cell precursors restored sensitivity to the disease (7). Other studies showed that the formation of immunocomplexes presumably triggered mast cell activation through the production of complement-derived anaphylatoxins and FcγR crosslinking (25,26). Unfortunately, it is impossible to stain anti-GPI Abs in the rheumatoid synovium. This does not exclude, however, the involvement of rheumatoid factor (RF) or other IgGs, such as anti-cyclic citrullinated peptide (CCP) Abs, which were frequently detected and evaluated as predictors of arthritis in RA (27), in the synovium. However, we could detect neither IgG RF nor anti-CCP Abs in RA1 patients (data not shown).

What is the pathogenic role of serum IgG including anti-GPI Abs in RA? Two monoclonal anti-GPI Abs could cause arthritis in different strains of mice (28). In addition to mice, human GPI accumulates on the synovium and joint articular surfaces (22), and anti-GPI Abs in humans probably attach to the articular surface in the affected joints. However, this phenomenon has never been analyzed *in vivo*. To analyze the arthritogenic role of serum IgG including anti-GPI Abs of RA patients, we injected these antibodies directly into the joints of cynomolgus monkeys, whose complement system mimics that of humans. The present study demonstrated that serum IgG from anti-GPI Abs-positive RA patients preferentially attached to the articular surface of the MP joints of the monkey, and resulted in the recruitment of granulocytes and mononuclear cells in the synovium. These findings indicate that human serum Igs from RA patients include autoantibodies to specific protein(s) expressed in the joint cavity. However, no joint swelling was noted, probably because these Abs from RA patients are not enough to induce arthritis in cynomolgus monkeys, which still has some differences in innate immune systems compared to humans. We waited until day 16 to harvest in order to monitor arthritis occurrences for a week after the final injection on day 9.

Quantitative PCR analysis of the C5aR gene showed augmentation of monkey C5aR mRNA expression in the

synovium following the injection of IgG anti-GPI Abs from RA patients. C5aR is a critical molecule in arthritis, based on the high expression level of C5aR in the RA synovium (29), and deletion of the C5aR completely protects against experimental arthritis induced by anti-GPI-Abs (4) or anti-collagen Abs (30). In this study, we provided evidence showing that serum IgGs from anti-GPI positive RA patients recruited C5aR-bearing cells through complement activation *in vivo*. While it is necessary to use affinity-purified human anti-GPI Abs, the amount of affinity-purified anti-GPI Abs obtained in the study was very small, probably reflecting the lower affinity of human anti-GPI Abs than K/BxN mice (12). Alternatively, the small amount, similar to that described for anti-proteinase-3 Abs in Wegener granulomatosis (31), might be due to the presence of an idiotypic network that includes Abs that recognize Abs in GPI, because anti-GPI Abs have also been related to vasculitis (15). These idiotypic Abs may block the association between anti-GPI Abs and GPI column. Future studies using hybridoma cells that produce anti-GPI monoclonal Abs from peripheral blood mononuclear cells and synovium of RA patients, should shed some light on the pathogenic role of anti-GPI Abs.

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References

1. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C and Mathis D: Organ-specific disease provoked by systemic autoreactivity. Cell 87: 811-822, 1996.
2. Matsumoto I, Staub A, Benoist C and Mathis D: Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. Science 286: 1732-1735, 1999.
3. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, Degott C, Kikutani H, Rajewsky K, Pasquali JL, Benoist C and Mathis D: From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 10: 451-461, 1999.
4. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, Takahashi K, Holers VM, Walport M, Gerard C, Ezekowitz A, Carroll MC, Brenner M, Weissleder R, Verbeek JS, Duchatelle V, Degott C, Benoist C and Mathis D: Arthritis critically dependent on innate immune system players. Immunity 16: 157-168, 2001.
5. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, Gravallese E, Mathis D and Benoist C: Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. J Exp Med 196: 77-85, 2002.
6. Wipke BT and Allen PM: Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol 167: 1601-1608, 2001.
7. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D and Brenner M: Mast cells: a cellular link between auto-antibodies and inflammatory arthritis. Science 297: 1689-1692, 2002.
8. Schubert D, Maier B, Morawietz L, Krenn V and Kamradt T: Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in genetically unaltered mice. J Immunol 172: 4503-4509, 2004.
9. Schaller M, Burton DR and Ditzel H: Autoantibodies to GPI in rheumatoid arthritis: linkage between an animal model and human disease. Nat Immunol 2: 746-753, 2001.
10. Matsumoto I, Lee DM, Goldbach-Mansky R, Sumida T, Hitchon CA, Schur PH, Anderson RJ, Coblyn JS, Weinblatt ME, Brenner M, Duclos B, Pasquali JL, El-gabalawy H, Mathis D and Benoist C: Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and spectrum of other chronic autoimmune disorders. Arthritis Rheum 48: 944-954, 2003.
11. Schaller M, Benoit VM and Ditzel HJ: Correspondence: Response. Nat Immunol 3: 412-413, 2002.
12. Kassahn D, Kolb C, Solomon S, Bochtler P and Illges H: Few human autoimmune sera detect GPI. Nat Immunol 3: 411-412, 2002.
13. Schubert D, Schmidt M, Zaiss D, Jungblut PR and Kamradt T: Autoantibodies to GPI and creatine kinase in RA. Nat Immunol 3: 411, 2002.
14. Herve CA, Wait R and Venables PJ: Glucose-6-phosphate isomerase is not a specific autoantigen in rheumatoid arthritis. Rheumatology 42: 645-651, 2003.
15. Van Gaalen FA, Toes RE, Ditzel HJ, Schaller M, Breedveld FC, Verweij CL and Huizinga TW: Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis. Arthritis Rheum 50: 395-399, 2004.
16. Mestas J and Hughes CW: Of mice and not men: differences between mouse and human immunology. J Immunol 172: 2731-2738, 2004.
17. Sumichika H, Sakata K, Sato N, Takeshita S, Ishibuchi S, Nakamura M, Kamahori T, Ehara S, Itoh K, Ohtsuka T, Ohbora T, Mishina T, Komatsu H and Naka Y: Identification of a potent and orally active non-peptide C5a receptor antagonist. J Biol Chem 277: 49403-49407, 2002.
18. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH and Luthra HS: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315-324, 1988.
19. Matsumoto I, Tsubota K, Satake Y, Kita Y, Matsumura R, Murata H, Namekawa T, Nishioka K, Iwamoto I, Saitoh Y and Sumida T: Common T cell receptor clonotype in lacrimal glands and labial salivary glands from patients with Sjögren's syndrome. J Clin Invest 97: 1969-1977, 1996.
20. Schoroder AE, Greiner A, Seyfert C and Berek C: Differentiation of B cells in nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. Proc Natl Acad Sci USA 93: 221-225, 1996.
21. Takemura S, Braun A, Crowson C, Kurtin PJ, Cofield RH, O'Fallon WM, Goronzy JJ and Weyand CM: Lymphoid neogenesis in rheumatoid synovitis. J Immunol 167: 1072-1080, 2001.
22. Matsumoto I, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, Mathis D and Benoist C: How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. Nat Immunol 3: 360-365, 2002.
23. Itoh K, Meffre E, Albesiano E, Farber A, Dines D, Stein P, Asnis SE, Furie RA, Jain RI and Chiorazzi N: Immunoglobulin heavy chain variable region gene re-placement as a mechanism for receptor revision on rheumatoid arthritis synovial tissue B lymphocytes. J Exp Med 192: 1151-1164, 2000.
24. Kim HJ, Krenn V, Steinhauser G and Berek C: Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis. J Immunol 162: 3053-3062, 1999.
25. Mandik-Nayak L, Wipke BT, Shih FF, Unanue ER and Allen PM: Despite ubiquitous autoantigen expression, arthritogenic autoantibody response initiates in the local lymph node. Proc Natl Acad Sci USA 99: 14368-14373, 2002.
26. Crisp AJ, Chapman CM, Kirkham SE, Schiller AL and Krane SM: Articular mastocytosis in rheumatoid arthritis. Arthritis Rheum 27: 845-851, 1984.
27. Wooley DE and Tetlow LC: Mast cell activation and its relation to proinflammatory cytokine production in the rheumatoid lesion. Arthritis Res 2: 65-74, 2000.
28. Okayama Y, Hagaman DD and Metcalfe DD: A comparison of mediators released or generated by IFN-γ treated human mast cells following aggregation of Fc gamma RI or Fc epsilon RI. J Immunol 166: 4705-4712, 2001.
29. Prodeus AP, Zhou X, Maurer M, Galli SJ and Carrol MC: Impaired mast cell-dependent natural immunity in complement C3 deficient mice. Nature 90: 172-175, 1997.

Research article

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A functional variant of Fcγ receptor IIIA is associated with rheumatoid arthritis in individuals who are positive for anti-glucose-6-phosphate isomerase antibodies

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Abstract

Anti-glucose-6-phosphate isomerase (GPI) antibodies are known to be arthritogenic autoantibodies in K/B×N mice, although some groups have reported that few healthy humans retain these antibodies. The expression of Fcγ receptors (FcγRs) is genetically regulated and has strong implications for the development of experimental arthritis. The interaction between immune complexes and FcγRs might therefore be involved in the pathogenesis of some arthritic conditions. To explore the relationship between functional polymorphisms in FcγRs (*FCGR3A*-158V/F and *FCGR2A*-131H/R) and arthritis in individuals positive for anti-GPI antibodies, we evaluated these individuals with respect to *FCGR* genotype. Genotyping for *FCGR3A*-158V/F and *FCGR2A*-131H/R was performed by PCR amplification of the polymorphic site, followed by site specific restriction digestion using the genome of 187 Japanese patients with rheumatoid arthritis (including 23 who were anti-GPI antibody positive) and 158 Japanese healthy individuals (including nine who were anti-GPI antibody positive). We report here on the association of *FCGR3A*-158V/F functional polymorphism with anti-GPI antibody positive status. Eight out of nine healthy individuals who were positive for anti-GPI antibodies possessed the homozygous, low affinity genotype *FCGR3A*-158F (odds ratio = 0.09, 95% confidence interval 0.01–0.89; $P = 0.0199$), and probably were 'protected' from arthritogenic antibodies. Moreover, among those who were homozygous for the high affinity genotype FCGR3A-158V/V, there were clear differences in anti-human and anti-rabbit GPI titres between patients with rheumatoid arthritis and healthy subjects ($P = 0.0027$ and $P = 0.0015$, respectively). Our findings provide a molecular model of the genetic regulation of autoantibody-induced arthritis by allele-specific affinity of the FcγRs.

Introduction

Rheumatoid arthritis (RA) is a heterogeneous autoimmune disease that is characterized by chronic inflammatory polyarthritis [1]. One of the characteristic features of RA is the expression of several autoantibodies. The presence of such autoantibodies (e.g. rheumatoid factor [RF]), identified by screening, is commonly used as a diagnostic marker, although the pathogenic role played by autoantibodies in RA remains a mystery.

Fcγ receptors (FcγRs) play a pivotal role in the reaction between immune complex and myeloid cells. Three FcγR types have been identified in mice and humans (FcγRI, FcγRII and FcγRIII). In mouse arthritis models, FcγRIII deficient hosts exhibit resistance to collagen type II induced arthritis and anti-glucose-6-phosphate isomerase (GPI) antibody induced arthritis [2,3], suggesting that FcγRIII is indispensible in autoantibody dependent arthritis. In humans FcγRs are encoded by eight genes, and the genes encoding the low affinity FcγRs (*FCGR2A*, *FCGR3A*, *FCGR2C*, *FCGR3B* and

AP = alkaline phosphatase; bp = base pairs; ELISA = enzyme-linked immunosorbent assay; FcγR = Fcγ receptor; GPI = glucose-6-phosphate isomerase; GST = gluthathione-S-transferase; OD = optical density; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RF = rheumatoid factor.

FCGR2B) are located within a gene cluster on chromosome 1q22-23. Of these FcγRs, FcγRIIIa and FcγRIIa are known to be stimulatory receptors. Various genetic polymorphisms of these receptors were reported to be associated with several autoimmune diseases [4,5], one of which is a polymorphism in *FCGR3A*, with either a phenylalanine (F) or a valine (V) at amino acid position 158 [6,7]. Moreover, based on findings from a co-crystalization study with IgG_1 and FcγRIIIa [8], this residue directly interacts with the lower hinge region of IgG_1, suggesting strong binding between IgG_1 and FcγRIIIa-158V on both natural killer cells and macrophages. For *FCGR2A* genes, a polymorphism at position 131 (with either histidine [H] or arginine [R]) alters the ability of the receptor to bind to certain IgG subclasses [9,10].

In RA patients, *FCG3A*-158V/F polymorphisms were reported to be frequent in UK Caucasian, North Indian and Pakistani individuals [11,12], but not in Japanese, Spanish and French individuals [13-15]. The reason for these differences between populations is unknown, although it is possible that they might depend on the prevalence in these populations of patients with autoantibody related forms of RA, in particular the prevalence of those who have pathogenic autoantibodies that directly interact with FcγRs (especially FcγRIIIa).

Anti-GPI antibodies are candidate arthritogenic antibodies. In K/B×N mice, polyclonal or two monoclonal anti-GPI antibodies induced arthritis in several strains of mice [16]. Moreover, FcγRIII deficient mice were resistant to anti-GPI antibody induced arthritis [3]. Another recent report [17] also confirmed that immune complex and FcγRIII are essential initiators of arthritis through sequential activation of effector cells, thus giving antibodies access into the joint. In human RA, anti-GPI antibodies have frequently been detected in patients with aggressive forms of arthritis [18,19], and their levels correlated significantly with extra-articular manifestations such as rheumatoid nodules, rheumatoid vasculitis and Felty's syndrome [20]. Moreover, a modest association of homozygosity for the *FCGR3A*-158V allele with RA in the nodular phenotype was suggested by Morgan and coworkers [11], suggesting the presence of a link between anti-GPI antibodies and *FCGR3A* allele. However, whether anti-GPI antibody positive status correlates with RA is a matter of controversy [18-22]. In our assay few healthy individuals retained anti-GPI antibodies; however, we do not know whether these protective phenotypes are associated with certain human gene polymorphisms.

In order to determine the relationship between functional polymorphisms of *FCGR* and possible arthritogenic anti-GPI antibodies in human conditions, we examined the correlation of these polymorphisms with anti-GPI positivity.

Materials and methods

Patients

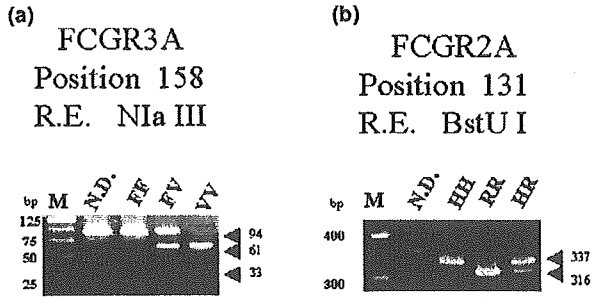
The study was approved by the local ethics review committee and written informed consent was obtained from all participants. Blood samples were collected from 187 Japanese patients with RA (mean age 46 ± 17 years; 33 females; mean disease duration 12.9 years [range 1–46 years]) including four with vasculitis and three with Felty's syndrome. These patients, randomly selected from among patients visiting the clinic, were followed at University of Tsukuba Hospital. The diagnosis of RA was based on the criteria presented by the American College of Rheumatology [23]. In addition, 158 Japanese volunteers (mean age 30 ± 9 years; 105 females) were recruited from our institute to serve as a healthy comparison group. All healthy individuals were free of rheumatic disease symptoms, and derived from the same geographic locations.

Enzyme-linked immunosorbent assay for GPI

In order to select anti-GPI antibody positive patients, we used recombinant human GPI (described in detail previously [18]) or rabbit muscle GPI (Sigma, St Louis, MO, USA). Both antigens were used at 5 μg/ml (diluted in phosphate-buffered saline [PBS]) to coat microtitre plates (12 hours, 4°C). After washing twice with washing buffer (0.05% Tween 20 in PBS), Block Ace (diluted 1/4 in 1 × PBS; Dainippon Pharmaceuticals, Osaka, Japan) was used for saturation (30 min at 37°C). After two washes, sera (diluted 1/50) were added and the plates were incubated for 12 hours at 4°C. After washing, alkaline phosphatase (AP)-conjugated anti-human IgG (Fc fragment specific; Jackson Immuno Research, West Grove, PA, USA) was added to the plate (dilution 1/1000, for 1 hour at room temperature). After three washes, colour was developed with AP reaction solution (containing 9.6% diethanol amine, 0.25 mmol/l $MgCl_2$; pH 9.8) with AP substrate tablets (Sigma; one AP tablet per 5 ml AP reaction solution). Plates were incubated for 1 hour at room temperature, and the optical density (OD) was measured by plate spectrophotometry at 405 nm. Determinations were performed in triplicate and standardized between experiments by reference to a highly positive human anti-GPI serum. The primary reading was processed by subtracting OD readings of control wells (coated with gluthathione-S-transferase (GST) and Block Ace for recombinant GPI–GST and rabbit GPI, respectively). The cut-off OD was calculated from the ELISA reactions of 158 healthy Japanese donors. Those who were double positive to both antigens were considered anti-GPI antibody positive. Because we used two antigens for the discrimination, the cut-off OD (mean value + 1 standard deviation) was 0.98 for human recombinant GPI and 0.64 for rabbit native GPI.

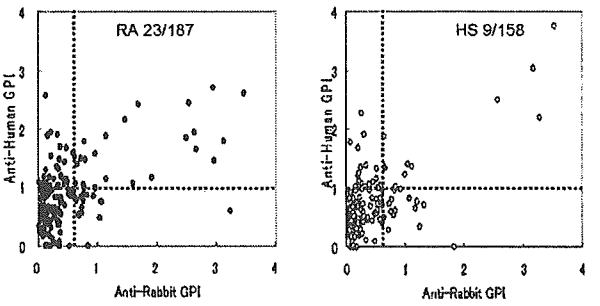
Genomic DNA was isolated from 0.5 ml anticoagulated peripheral blood, from 187 RA patients and 158 healthy individuals, by using DNA QuickII DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan). FcγR polymorphisms (*FCGR3A*-158V/F) were identified, as described by Koene

Figure 1



PCR-RFLP analysis of the *FCGR3A* and *FCGR2A* genes. cDNA was amplified with primers and restriction digested using appropriate enzymes. Digested PCR products were visualized with ethidium bromide. (a) *FCGR3A* gene and (b) *FCGR2A* gene. ND, nondigested PCR product; RE, restriction enzyme.

Figure 2



Population of anti-GPI antibody positive individuals, and *FCGR3A* and *FCGR2A* genotypes. The study included 187 patients with rheumatoid arthritis and 158 healthy Japanese individuals. The horizontal and vertical dotted lines represent the cutoff optical density values calculated from ELISA reactions of 158 healthy individuals for human recombinant GPI and rabbit native GPI, respectively. Individuals positive for both antibodies were considered anti-GPI antibody positive. Numbers in each graph represent the proportions of individuals positive for anti-GPI antibodies relative to the total number of individuals in that group. GPI, glucose-6-phosphate isomerase; HS, healthy subjects; RA, rheumatoid arthritis.

and coworkers [6], using a nested PCR followed by allele specific restriction enzyme digestion. For homozygous FcγRIIIA-158F patients only one undigested band (94 bp) was visible. Three bands (94 bp, 61 bp and 33 bp) were seen in heterozygous individuals, whereas for homozygous FcγRIIIA-158V patients only two digested bands (61 bp and 33 bp) were detected (Fig. 1a). These genotyping findings were confirmed by direct sequencing in some individuals.

FcγRIIA-131H/R genotyping

Genotyping of FcγRIIA-131H/R also consisted of PCR followed by an allele specific restriction enzyme digestion, in accordance with the method reported by Jiang and coworkers [24]. The *FCGR2A*-131H and *FCGR2A*-131R alleles were visualized as 337 bp and 316 bp DNA fragments, respectively (Fig. 1b). These genotyping findings were confirmed by direct sequencing in some individuals.

Statistical analysis

The data were analyzed using the Student's *t*-test and the χ^2 test, and Fisher's exact test was used when expected frequencies were lower than 5. We used Mann–Whitney U-test to evaluate the distribution of anti-GPI antibodies in FcγRIIIA-158V/V RA patients and healthy individuals. $P < 0.05$ was considered statistically significant.

Results

Our ELISA assay is highly specific because we used recombinant bacterial human GPI and native rabbit GPI, and double positivity for the two antibodies correlated significantly with the results of western blotting to GPI [18]. Because two GPI antigens were used for discrimination, the cutoff value of the OD was the mean value + one standard deviation from 158 healthy individuals, estimated using ELISA. Those who were positive for both antibodies were considered to be anti-GPI antibody positive. Using these definitions, 23 (12.3%) RA patients were anti-GPI antibody positive, and nine (5.7%) healthy individuals were anti-GPI antibody positive (Fig. 2). Statistical analysis revealed a significant difference in anti-GPI antibody positivity between RA patients and healthy individuals ($\chi^2 = 4.438$, with one degree of freedom; $P = 0.0352$).

To analyze whether functional *FCGR* polymorphisms were correlated with anti-GPI antibody positive and negative individuals, we performed *FCGR* genotyping. *FCGR3A* and *FCGR2A* genotypes in the control group were in Hardy–Weinberg equilibrium. The *FCGR3A*-158V allele (high affinity genotype) was more frequently identified in patients with RA than in healthy individuals within the anti-GPI antibody positive population ($\chi^2 = 0.012$, with one degree of freedom; $P = 0.012$; Tables 1 and 2). In addition, these differences were evident when individuals were categorized according to the presence or absence of these genotypes: 56.5% of patients with RA were homozygous or heterozygous with respect to *FCGR3A*-158V, as compared with 11.1% of healthy individuals; and 43.5% of patients with RA were homozygous with respect to *FCGR3A*-158F, as compared with 88.9% of healthy individuals ($\chi^2 = 5.42$ with one degree of freedom; $P < 0.02$; Tables 1 and 2). Comparison of *FCGR3A*-158V allele frequency between RA patients and healthy individuals revealed no statistically significant difference: 48.7% of patients with RA were homozygous or heterozygous with respect to *FCGR3A*-158V, as compared with 42.4% of healthy individuals; and 51.3% of patients with RA were homozygous with respect to *FCGR3A*-158F, as compared with 57.6% of healthy individuals ($\chi^2 = 1.04$ with one degree of freedom; $P = 0.245$; Table 1).

Table 1

Frequencies of *FCGR3A* and *FCGR2A* genotypes in patients with RA and positive and negative for anti-GPI antibodies

	FCGR3A-158			*FCGR2A*-131		
	FF low	F/V	VV high	HH high	H/R	RR low
GPI+ RA ($n = 23$)	10 (43.5)	9 (39.1)	4 (17.4)	16 (69.6)	6 (26.1)	1 (4.3)
GPI- RA ($n = 164$)	86 (52.4)	68 (41.5)	10 (6.1)	128 (78)	29 (17.7)	7 (4.3)
GPI+ Control ($n = 9$)	8(88.9)	1 (11.1)	0 (0)	4 (44.4)	5 (55.6)	0 (0)
GPI- Control ($n = 149$)	83 (55.7)	58 (38.9)	8 (5.4)	109 (73.2)	40 (26.8)	0 (0)

Data are expressed as number (percentage) of individuals. GPI, glucose-6-phosphate isomerase; high, high affinity genotype; low, low affinity genotype; RA, rheumatoid arthritis.

Table 2

Alleic skewing of *FCGR3A* and *FCGR2A* in anti-GPI antibody positive healthy individuals

Polymorphism	Allele	RA GPI+ ($n = 46$)	Healthy GPI+ ($n = 18$)	P (χ^2)	P (Fisher's)	OR (95% CI)
FCGR3A-158	F	29	17	0.012	0.013	0.10 (0.01–0.82)
	V	17	1			
FCGR2A-131	H	38	13	0.35	0.4902	1.83 (0.51–6.59)
	R	8	5			

P values are given for RA versus healthy individuals using a 2×2 contingency table. CI, confidence interval; Fisher's, Fisher's probability test; OR, odds ratio; RA, rheumatoid arthritis.

Table 3

Genotype skewing of *FCGR3A* and *FCGR2A* gene polymorphisms in anti-GPI antibody positive healthy individuals

Polymorphism	Genotype	RA GPI+ ($n = 23$)	Healthy GPI+ ($n = 9$)	P (χ^2)	P (Fisher's)	OR (95% CI)
FCGR3A-158	FF	10 (43.5%)	8 (88.9%)	0.019	0.044	0.09 (0.01–0.89)
	FV/VV	13(56.5%)	1 (11.1%)			
FCGR2A-131	HH	16 (69.6%)	4(44.4%)	0.19	0.24	2.86 (0.58–13.96)
	HR/RR	7 (30.4%)	5 (55.6%)			

P values are given for RA versus healthy individuals using a 2×2 contingency table. CI, confidence interval; Fisher's, Fisher's probability test; OR, odds ratio; RA, rheumatoid arthritis.

Next, *FCGR2A* genotyping was conducted in the same cohort (Table 1). In contrast to *FCGR3A*, the frequency of the *FCGR2A*-131H allele (high affinity genotype) was not significantly different between the two groups within the anti-GPI antibody positive population ($\chi^2 = 0.862$ with one degree of freedom; $P = 0.35$; Tables 1 and 2). These differences were also not evident when individuals were categorized according to the presence or absence of these genotypes ($P = 0.19$; Tables 1 and 3).

We also analyzed the association between FcγR and other related autoantibodies such as RF. There was no difference between RF positive and RF negative populations of RA patients ($P = 0.82$ and $P = 0.4$ for *FCGR3A* and *FCGR2A*, respectively; Table 4).

Finally, in order to identify the relationship between *FCGR3A*-158V allele and anti-GPI antibodies more clearly, we focused on individuals who were homozygous for the high affinity *FCGR3A*-158V/V genotype (14 RA patients and eight healthy individuals) and compared their anti-GPI antibody titres. Surprisingly, both anti-human GPI antibodies and anti-rabbit GPI antibodies were significantly elevated in the RA group ($P = 0.0027$ and $P = 0.0015$ for anti-human GPI antibodies and anti-rabbit GPI antibodies, respectively, by Mann–Whitney U-test; Fig. 3). This suggests that anti-GPI antibody positivity

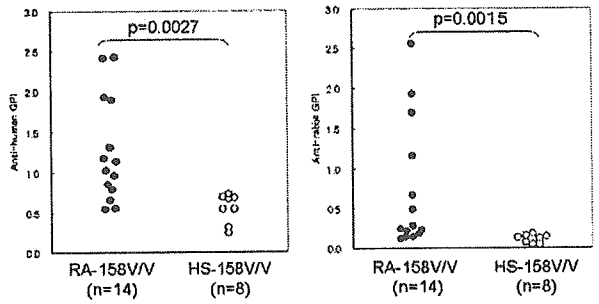
Table 4

***FCGR3A* and *FCGR2A* genotypes in rheumatoid factor positive and negative RA patients**

Polymorphism	Genotype	RA RF+ (n = 130)	RA RF- (*n* = 57)	*P* (χ^2)	OR (95% CI)
FCGR3A-158	FF	66 (50.8%)	30(52.6%)	0.82	0.93 (0.50–1.73)
	FV/VV	64(49.2%)	27 (47.4%)		
FCGR2A-131	HH	103 (79.2%)	42(73.7%)	0.4	1.36 (0.66–2.82)
	HR/RR	27 (20.8%)	15 (26.3%)		

P values are given for RA RF+ versus RA RF- using a 2×2 contingency table. CI, 95% confidence interval; OR, odds ratio; RA, rheumatoid arthritis; RF, rheumatoid factor.

Figure 3



Higher titres of anti-human and anti-rabbit GPI antibodies in *FCGR3A*-158V/V RA patients versus healthy individuals. In individuals homozygous for the *FCGR3A* high affinity V/V genotype (14 RA patients and 8 healthy individuals), both anti-human GPI antibodies and anti-rabbit GPI antibodies were significantly elevated in the RA group ($P = 0.0027$ and $P = 0.0015$ for anti-human GPI antibodies and anti-rabbit GPI antibodies, respectively, by Mann–Whitney U-test). GPI, glucose-6-phosphate isomerase; RA, rheumatoid arthritis.

might predispose individuals with the *FCGR3A*-158V/V genotype to arthritis.

Discussion

Several studies have indicated that anti-GPI antibodies are potential arthritogenic antibodies [18-20] because they were frequently detected in patients with severe forms of RA. Because high titres of these antibodies (IgG, not IgM) were also detected in healthy individuals, the arthritogenicity of these antibodies should be due to modulation – by the low affinity genotype of FcγRs – of the bypass between immune complex and FcγR bearing cells. In a GPI immunized mouse model severe arthritis occurred only in DBA/1 mice, although the production of anti-GPI antibodies was almost equal in arthritis susceptible and resistant mouse strains [25]. Thus, the incidence of arthritis might depend on certain genetic factors such as FcγR. Anti-GPI antibody positive individuals express several GPI variant mRNAs in peripheral blood monocytes [26]. This observation supports the notion that the presence of GPI variants is necessary to produce anti-GPI autoantibodies, and that genetic factors such as FcγRIIIA are important in the development of arthritis. Based on this conclusion, it is conceivable that the production of anti-GPI antibodies does not occur as a 'result' of joint destruction.

Our results do not indicate that individual polymorphisms in the *FCGR3A* and *FCGR2A* genes play roles in susceptibility to RA. Despite the lack of association with individual *FCGR* polymorphisms in the whole cohort, our studies suggest that *FCGR3A*-158V/F polymorphisms play a crucial role in RA among those individuals who are positive for anti-GPI antibodies (Tables 2 and 3). Moreover, focusing on *FCGR3A*-158V/V homozygous individuals, anti-GPI antibodies were clearly evident in patients with RA. These findings suggest that anti-GPI antibodies might have arthritogenic potential in individuals homozygous for *FCGR3A*-158V/V.

Conclusion

Our findings show that *FCGR3A*-158V/F functional polymorphisms were associated with RA among anti-GPI antibody positive individuals. This is the first report on possible mechanisms of arthritic diseases; they are tightly regulated by some genes, especially by FcγR genotype, as well as by production of arthritogenic autoantibodies.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

IM wrote the manuscript and conceived the study. HZ performed FcγR genotyping and coordinated the statistical analysis. YM, TY and YK performed GPI ELISA. TH participated in clinical assessment. TS participated in the full design and coordination of the study, and DG, SI and AT participated in writing the discussion.

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References

1. Firestein GS: **Evolving concepts of rheumatoid arthritis.** *Nature* 2003, **423**:356-361.

2. Diaz de Stahl T, Andren M, Martinsson P, Verbeek JS, Kleinau S: **Expression of FcgammaRIII is required for development of collagen-induced arthritis.** *Eur J Immunol* 2002, **32**:2915-2922.
3. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, Takahashi K, Holers VM, Walport M, Gerard C, *et al.*: **Arthritis critically dependent on innate immune system players.** *Immunity* 2002, **16**:157-168.
4. Dijstelbloem HM, Scheepers RH, Oost WW, Stegeman CA, van der Pol WL, Sluiter WJ, Kallenberg CG, van de Winkel JG, Tervaert JW: **Fcgamma receptor polymorphisms in Wegener's granulomatosis: risk factors for disease relapse.** *Arthritis Rheum* 1999, **42**:1823-1827.
5. Myhr KM, Raknes G, Nyland H, Vedeler C: **Immunoglobulin G Fc-receptor (FcgammaR) IIA and IIIB polymorphisms related to disability in MS.** *Neurology* 1999, **52**:1771-1776.
6. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M: **Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype.** *Blood* 1997, **90**:1109-1114.
7. Wu J, Edberg JC, Redecha PB, Bansal V, Guyre PM, Coleman K, Salmon JE, Kimberly RP: **A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease.** *J Clin Invest* 1997, **100**:1059-1070.
8. Sondermann P, Huber R, Oosthuizen V, Jacob U: **The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex.** *Nature* 2000, **406**:267-273.
9. Warmerdam PA, van de Winkel JG, Vlug A, Westerdaal NA, Capel PJ: **A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding.** *J Immunol* 1991, **147**:1338-1343.
10. Parren PW, Warmerdam PA, Boeije LC, Arts J, Westerdaal NA, Vlug A, Capel PJ, Aarden LA, van de Winkel JG: **On the interaction of IgG subclasses with the low affinity Fc gamma RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2.** *J Clin Invest* 1992, **90**:1537-1546.
11. Morgan AW, Griffiths B, Ponchel F, Montague BM, Ali M, Gardner PP, Gooi HC, Situnayake RD, Markham AF, Emery P, Isaacs JD: **Fcgamma receptor type IIIA is associated with rheumatoid arthritis in two distinct ethnic groups.** *Arthritis Rheum* 2000, **43**:2328-2334.
12. Morgan AW, Keyte VH, Babbage SJ, Robinson JI, Ponchel F, Barrett JH, Bhakta BB, Bingham SJ, Buch MH, Conaghan PG, *et al.*: **FcgammaRIIIA-158V and rheumatoid arthritis: a confirmation study.** *Rheumatology (Oxford)* 2003, **42**:528-533.
13. Kyogoku C, Tsuchiya N, Matsuta K, Tokunaga K: **Studies on the association of Fc gamma receptor IIA, IIB, IIIA and IIIB polymorphisms with rheumatoid arthritis in the Japanese: evidence for a genetic interaction between HLA-DRB1 and FCGR3A.** *Genes Immun* 2002, **3**:488-493.
14. Radstake TR, Petit E, Pierlot C, van de Putte LB, Cornelis F, Barrera P: **Role of Fcgamma receptors IIA, IIIA, and IIIB in susceptibility to rheumatoid arthritis.** *J Rheumatol* 2003, **30**:926-933.
15. Nieto A, Caliz R, Pascual M, Mataran L, Garcia S, Martin J: **Involvement of Fcgamma receptor IIIA genotypes in susceptibility to rheumatoid arthritis.** *Arthritis Rheum* 2000, **43**:735-739.
16. Matsumoto I, Staub A, Benoist C, Mathis D: **Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme.** *Science* 1999, **286**:1732-1735.
17. Wipke BT, Wang Z, Kim J, McCarthy TJ, Allen PM: **Dynamic visualization of a joint-specific autoimmune response through positron emission tomography.** *Nat Immunol* 2002, **3**:366-372.
18. Matsumoto I, Lee DM, Goldbach-Mansky R, Sumida T, Hitchon CA, Schur PH, Anderson RJ, Coblyn JS, Weinblatt ME, Brenner M, *et al.*: **Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and a spectrum of other chronic autoimmune disorders.** *Arthritis Rheum* 2003, **48**:944-954.
19. Schaller M, Burton DR, Ditzel HJ: **Autoantibodies to GPI in rheumatoid arthritis: linkage between an animal model and human disease.** *Nat Immunol* 2001, **2**:746-753.
20. van Gaalen FA, Toes RE, Ditzel HJ, Schaller M, Breedveld FC, Verweij CL, Huizinga TW: **Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis.** *Arthritis Rheum* 2004, **50**:395-399.
21. Kassahn D, Kolb C, Solomon S, Bochtler P, Illges H: **Few human autoimmune sera detect GPI.** *Nat Immunol* 2002, **3**:411-412.
22. Schubert D, Schmidt M, Zaiss D, Jungblut PR, Kamradt T: **Autoantibodies to GPI and creatine kinase in RA.** *Nat Immunol* 2002, **3**:411.
23. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, *et al.*: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.** *Arthritis Rheum* 1988, **31**:315-324.
24. Jiang XM, Arepally G, Poncz M, McKenzie SE: **Rapid detection of the Fc gamma RIIA-H/R 131 ligand-binding polymorphism using an allele-specific restriction enzyme digestion (ASRED).** *J Immunol Methods* 1996, **199**:55-59.
25. Schubert D, Maier B, Morawietz L, Krenn V, Kamradt T: **Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in genetically unaltered mice.** *J Immunol* 2004, **172**:4503-4509.
26. Muraki Y, Matsumoto I, Chino Y, Hayashi T, Suzuki E, Goto D, Ito S, Murata H, Tsutsumi A, Sumida T: **Glucose-6-phosphate isomerase variants play a key role in the generation of anti-GPI antibodies: possible mechanism of autoantibody production.** *Biochem Biophys Res Commun* 2004, **323**:518-522.

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ORIGINAL ARTICLE

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Identification of three novel peptides that inhibit CD40–CD154 interaction

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Abstract The CD40–CD154 interaction is an attractive target for therapeutic intervention in various autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis, and myasthenia gravis. In this study, to develop a new disruption strategy of the CD40–CD154 interaction, we screened for peptides with inhibitory effects on such ligation. 2×10^{11} phage display libraries displaying liner peptides of 12-mer amino acids were screened by CD40-Ig binding assay and eight phages which expressed a different respective peptide (40BP-1 to -8) were able to specifically bind to CD40. Competitive inhibition analyses showed that 3 of the 8 peptides (40BP-N1-1 – APELPNMTPSWT; 40BP-N1-2 – APRPHTSYSPLP; and 40BP-N1-3 – GMTAPPPPRLTQ) blocked CD40–CD154 interaction when used at high concentrations. A consensus sequence (APxPPxxT) was conserved in these three peptides. These peptides may constitute a useful and novel strategy for the inhibition of the interaction between CD40 and CD154 molecules.

Key words Autoimmune disease · CD40 · CD154 · Peptide · Phage display

Introduction

CD40 is a 48 kDa membrane glycoprotein expressed on B cells, monocytes, dendritic cells, and thymic epithelium, as well as on certain carcinomas. It belongs to the tumor necrosis factor receptor superfamily, a group of type I transmembrane molecules. The ligand for CD40 (CD154) belongs to the tumor necrosis factor family and is predominantly expressed on activated T cells, although variable expression has been reported on mast cells, B cells, monocytes, and basophils. The CD40–CD154 interaction plays a central role in the control of both humoral and cellular immunity.[1,2]

Blockade of the CD40–CD154 interaction with a monoclonal antibody directed against CD154 has been shown to inhibit autoimmune responses in vivo in various murine models of autoimmune disease, including rheumatoid arthritis,[3] systemic lupus erythematosus (SLE),[4] and multiple sclerosis.[5,6] It has been proposed that disruption of CD40–CD154 could be a useful strategy in the treatment of autoimmune diseases.

In the present study, we screened the phage libraries to find peptides that could inhibit CD154–CD40 interaction. We found eight phage peptides that could specifically bind to CD40, and three of them inhibited the binding of CD40 and CD154 molecules. A consensus sequence (APxPPxxT) was identified in the three phages that showed inhibitory activity. Based on the inhibitory action of these regulatory peptides on CD40–CD154 interaction, we discuss the possible use of such strategy for the treatment of autoimmune diseases.

Materials and methods

Fusion proteins

CD40-Ig, which is fused to the extracellular domain of human CD40 and the human IgG1 Fc region, were prepared by constitutive transfection of HEK293 cells and purified with Hitrap-rProtien A affinity chromatography (Pharmacia Biotech, Uppsala, Sweden).[7,8] Soluble CD154 (sCD154), consisting of the extracellular domain of murine CD8α tagged with FLAG and the extracellular domain of human CD154, were also obtained using HEK293 cells and were purified with anti-FLAG M2-agarose affinity resin (Sigma, St. Louis, MO, USA) as described previously.[9] Bioactivity of sCD154 was checked by the induction of CD95

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on Daudi lymphoma cells, which constitutively express functional CD40.

Selection of CD40-binding phages

M13 phage display libraries displaying linear peptides of 12-mer amino acids (Ph.D. System, New England Biolabs, Beverly, MA, USA) were used to select CD40-Ig binding phages (40BP). Panning was performed as described in the product manual with the following modifications. CD40-Ig was diluted to 50μg/ml in 0.1M $NaHCO_3$ (pH 8.6) and adsorbed onto 96-well microtiter plates (Falcon 1172, non-tissue culture treated) for 12h at 4°C. After blocking for 3h with 0.5% bovine serum albumin in 0.1M $NaHCO_3$ (pH 8.6), aliquots of the phage display libraries (2×10^{11} plaque-forming units) were diluted in Tris-buffered saline (TBS) (150mM NaCl, 50mM Tris–HCl, pH 7.5) containing 0.1% Tween-20, and phages were allowed to bind for 1h at room temperature. Unbound phages were removed by washing with TBS containing 0.5% Tween-20, and bound phages were eluted with 250μg/ml sCD154 in TBS containing 0.5% Tween-20, and amplified in *Escherichia coli* ER2537. Phages were prepared from the culture supernatant by standard polyethylene glycol/NaCl precipitation and used for the next round of panning. After the third round of panning randomly selected phage plaques were amplified, and single-strand DNA was sequenced using the Amplitaq FS sequencing kit (Applied Biosystems, Foster City, CA, USA) with a primer (5′-CCCTCATAGTT AGCGTAACG-3′).

Phage-binding assay

CD40-Ig or human IgG_1 (Sigma, I-8640) was coated on 96-well plates as described above. Phages amplified from single plaques and purified by polyethylene glycol/NaCl precipitation were serially diluted in TBS containing 0.5% Tween-20 and allowed to bind to CD40-Ig for 2h at room temperature. After washing, bound phages were detected with a horseradish peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Tetramethylbenzidine peroxidase substrate mixture (Sumitomo Bakelite, Akita, Japan) was added and the absorbance was measured at 450nm by a microplate reader (SPECTRAmax190; Molecular Devices, Sunnyvale, CA, USA).

Recombinant phage protein

Recombinant g3p N1 fragments displaying distinct 12-mer peptides of 40BP (40BP-N1) were constructed using pET-20b(+) His-tagging expression vector. The polymerase chain reaction (PCR) 5′-primer was GATCCCATGGGTA TGAAAAAATTATTATTCGCAATTCC and the 3′-primer was GAATCTCGAGTTCAGGGATAGCAAGC CCA. The PCR products were cleaved with *Nco*I and *Xho*I and inserted into pET-20b(+). 40BP-N1 were obtained using *Escherichia coli*. BL21(DE3) transformed with the constructed vector and 40BP-N1 production was induced by 0.5mM isopropyl-β-D-thiogalactopyranoside. The produced 40BP-N1 in periplasmic and cytoplasmic fraction was purified with Ni affinity chromatography. The amino acid sequences of each 40BP-N1 were confirmed by a peptide sequencer. 12-mer peptide was identified on the N-end of each 40BP-N1.

CD40–CD154 binding assay

sCD154 was iodinated with ^{125}I-labeled Bolton-Hunter reagent (NEN Life Science Products, Boston, MA, USA) as described in the product manual. The binding of [^{125}I]sCD154 to CD40-Ig was performed using protein-A scintillation proximity assay (SPA) regent, type I (Amersham Life Science, Buckinghamshire, UK) in 96-well Opti-plate. CD40-Ig (0.2μg/ml) diluted in TBS containing 10% fetal bovine serum was incubated with 10nM [^{125}I]sCD154 for 30min. Then, SPA reagent (4mg/ml) was added and the plate was incubated for 1h with agitation. The binding of [^{125}I]sCD154 was counted with scintillation counter (Packard, Meriden, CT, USA). All procedures were carried out at room temperature. The competition assay was performed using the purified 40BP-N1. The serially diluted 40BP-N1 was incubated with CD40-Ig for 10min before the addition of [^{125}I]sCD154. The subsequent procedures were the same as described above.

Results

CD40 binding phage peptides

By CD40 binding activity using CD40-Ig-coated plates, 2×10^{11} phage display libraries displaying linear peptides of 12-mer amino acids were screened. After the third round of panning, eight phage plaques were randomly selected. These CD40 binding phages (40BP-1, -2, -3, -4, -5, -6, -7, and -8) had distinct 12-mer amino acid on their N-terminal of g3p (Fig. 1). The sequences of all 40BPs did not show homology to CD40 and CD154 at the amino acid level. To confirm the specificity of their CD40 binding, these phages were applied to CD40-Ig-coated plates or control human IgG-coated plates. As control, wild-type phages (WT) and the phages randomly selected from the plaques before the first panning (NP-1, -2, and -3) were also examined. As shown in Fig. 1, 40BPs bound to CD40-Ig but not to human control IgG. On the other hand, WT, NP-1, -2, and -3 did not bind to both CD40-Ig and human IgG. 40BP-1, -2, -3, and -6 bound to CD40-Ig more preferably than the other 40BPs.

Inhibitory effects of 40BP peptides for CD40–CD154 interaction

To examine whether the peptides expressed on 40BPs could inhibit the binding between CD40 and CD154 molecules,

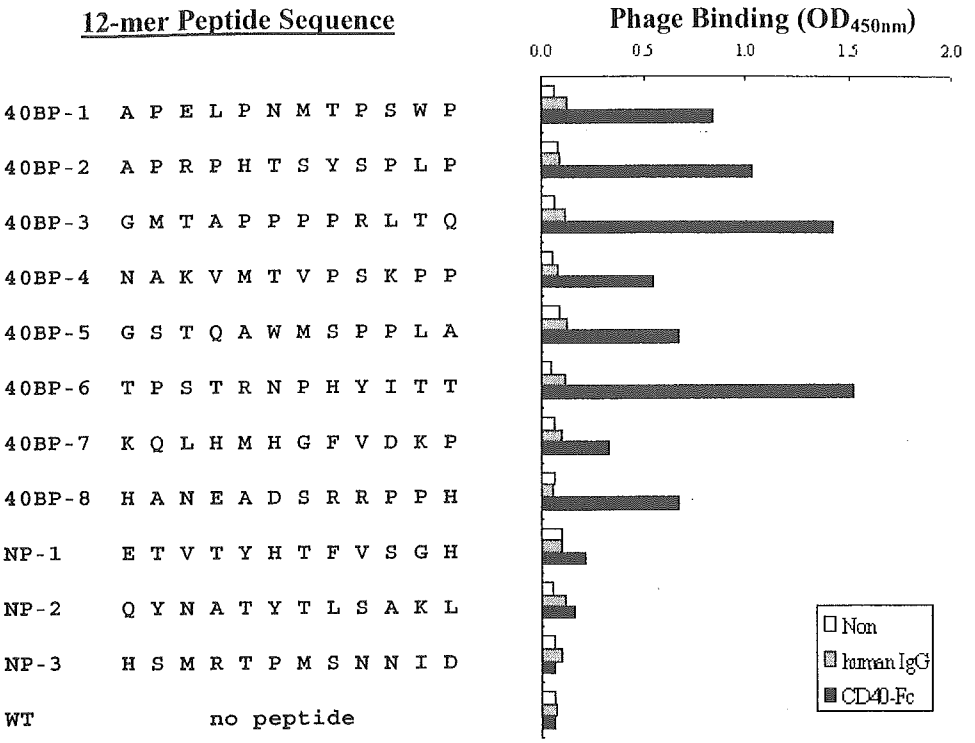

Fig. 1. Specificity of phage peptides. Eight CD40 binding phages (40BP-1 to -8) selected from the plaques after the third panning were able to bind to CD40-Ig fusion protein but not to human IgG. The three other phages randomly selected from the plaques before the first panning (NP-1 to -3) are control peptides that do not have binding activity to CD40-Ig. *Wt*, wild-type phages with no peptides

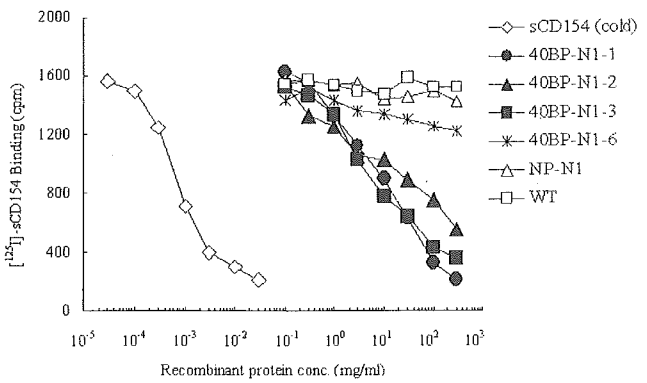

Fig. 2. Competitive inhibition of CD40–sCD154 binding. CD40-Ig and [^{125}I]sCD154 binding was competitively inhibited by cold sCD154 (positive control), recombinant phage proteins (40BP-N1-1, -2, -3, -6, NP-N1), and WT phage g3p N1 fragment (WT, negative control). Three peptides (40BP-N1-1, -2, and -3) with a consensus sequence interfered with CD40–sCD154 interaction, whereas 40BP-N1-6 with no consensus sequence, NP-N1, and WT with no binding activity to CD40-Ig did not inhibit CD40–CD154 binding

the recombinant fusion proteins consisting of 12-mer peptide and N1 fragment of phage g3p from 40BP-1, -2, -3, and -6 were prepared (40BP-N1-1, -2, -3, and -6, respectively), and the soluble CD154 (sCD154) and CD40-Ig binding inhibition assay was carried out. The purity of recombinant proteins was analyzed by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis method (data not shown). As shown in Fig. 2, the binding of CD40-Ig and [^{125}I]sCD154 was competitively inhibited by using cold sCD154 at concentrations of 0.001–0.01 mg/ml. The binding of [^{125}I]sCD154 to CD40-Ig was inhibited by 40BP-N1–1, -2, and -3, when they were used at high concentrations of 1–300 mg/ml. In contrast, 40BP-N1-6, NP-N1 (the recombinant fusion proteins consisted of 12-mer peptide and N1 fragment of phage g3p from NP-1), and WT (wild-type phage g3p N1 fragment) did not show such an inhibitory effect even at a higher concentration (up to 300 mg/ml). A consensus sequence (APxPPxxT) was identified among the three inhibitory peptides, whereas no motif was identified in the other peptides that had no inhibitory effect.

Discussion

We have screened eight phages that could specifically bind to CD40. These phages have expressed a different 12-mer peptide on their N-terminal of g3p fragment protein, respectively. Four phages (40BP-1, -2, -3, and -6) bound to CD40-Ig more preferably than the other 40BPs. In the present study we provided evidence for the inhibitory effects of three novel peptides (40BP-N1-1 – APELPNMTPSWT; 40BP-N1-2 – APRPHTSYSPLP; and 40BP-N1-3 – GMTAPPPPRLTQ) on CD40–CD154 interaction when used at high concentrations, and that a consensus sequence (APxPPxxT) was conserved in these three peptides. These peptides did not show any homology with CD40 and its ligands CD154 by computer analyses at the amino acid level. Thus, we conclude that these peptides with the APxPPxxT motif could be considered as novel inhibitory molecules for the CD40–CD154 interaction.

Interestingly, 40BP-N1-6 constructed from 40BP-6 phage, which preferably bound to CD40, did not inhibit CD40–CD154 interaction. A multidimensional protein structure of 12-mer peptide and full-length g3p protein (phage minor capsid protein) may contribute to the binding of the 40BP-6 phage to CD40. The structure could not retain in 40BP-N1-6 recombinant protein, which is composed of only 12-mer peptide and g3p N1 fragment, a part of g3p protein.

The efficacy of blocking the CD40–CD154 interaction therapeutically with an anti-CD154 monoclonal antibody has been shown in animal models for many autoimmune diseases, including rheumatoid arthritis, SLE, multiple sclerosis, and myasthenia gravis.[3-6,10] Therefore, disruption of the CD40–CD154 pathway has been proposed as a strategy for treating various human autoimmune diseases mediated by T cells and autoantibodies. At present, antibody against CD154 or CD40 is the only way that has been shown to block the CD40–CD154 interaction. In this study, we have devised a new strategy to inhibit CD40–CD154 interaction by peptides. What is the difference between anti-CD154 antibodies and inhibitory peptides identified in the present study? First, there are major differences in the molecular weight. IgG antibody is a molecule of about 150 kDa, while the inhibitory peptides are only 12-mer amino acids and are estimated to be 1–2 kDa molecules. Thus, it is very easy to produce these peptides and it is difficult to generate antibodies against these molecules in vivo. Secondly, the affinity of the antibody to CD154 may be higher than that of the peptides to CD40 molecule. The K_d is 10^{-3} M in the case of antigen–antibody interaction, whereas the peptide–CD40 interaction might be less than the K_d of T-cell receptors and peptides in the context of the major histocompatibility complex (10^{-4}–10^{-5} M).[11] Thus, several peptides are required to regulate the CD40–CD154 interaction. Further studies are also necessary to clarify the inhibitory activity of these peptides on the CD40–CD154 interaction in vitro and in vivo.

In conclusion, we have described three novel peptides that could specifically inhibit the CD40–CD154 interaction, suggesting that these might constitute a novel therapeutic method in autoimmune disease mediated by CD40–CD154.

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References

1. Foy TM, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ. Immune regulation by CD40 and its ligand GP39. Annu Rev Immunol 1996;14:591–617.
2. Hollenbaugh D, Ochs HD, Noelle RJ, Ledbetter JA, Aruffo A. The role of CD40 and its ligand in the regulation of the immune response. Immunol Rev 1994;138: 23–37.
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(5126):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(3):1470–80.
5. Howard LM, Miga AJ, Vanderlugt CL, Dal Canto MC, Laman JD, Noelle RJ, et al. Mechanisms of immunotherapeutic intervention by anti-CD40L (CD154) antibody in an animal model of multiple sclerosis. J Clin Invest 1999;103(2):281–90.
6. Howard LM, Dal Canto MC, Miller SD. Transient anti-CD154-mediated immunotherapy of ongoing relapsing experimental autoimmune encephalomyelitis induces long-term inhibition of disease relapses. J Neuroimmunol 2002;129(1–2): 58–65.
7. Fanslow WC, Anderson DM, Grabstein KH, Clark EA, Cosman D, Armitage RJ. Soluble forms of CD40 inhibit biologic responses of human B cells. J Immunol 1992;149(2):655–60.
8. Hollenbaugh D, Douthwright J, McDonald V, Aruffo A. Cleavable CD40Ig fusion proteins and the binding to sgp39. J Immunol Methods 1995;188(1):1–7.
9. Hollenbaugh D, Grosmaire LS, Kullas CD, Chalupny NJ, Braesch-Andersen S, Noelle RJ, et al. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. EMBO J 1992;11(12):4313–21.
10. Im SH, Barchan D, Maiti PK, Fuchs S, Souroujon MC. Blockade of CD40 ligand suppresses chronic experimental myasthenia gravis by down-regulation of Th1 differentiation and up-regulation of CTLA-4. J Immunol 2001;166(11):6893–8.
11. Matsui K, Boniface JJ, Reay PA, Schild H, Fazekas de St Groth B, Davis MM. Low affinity interaction of peptide-MHC complexes with T cell receptors. Science 1991;254:1788–91.

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Significance of Valine/Leucine^{247} Polymorphism of β_2-Glycoprotein I in Antiphospholipid Syndrome

Increased Reactivity of Anti–β_2-Glycoprotein I Autoantibodies to the Valine^{247} β_2-Glycoprotein I Variant

Shinsuke Yasuda,[1] Tatsuya Atsumi,[1] Eiji Matsuura,[2] Keiko Kaihara,[2] Daisuke Yamamoto,[3] Kenji Ichikawa,[1] and Takao Koike[1]

Objective. **To clarify the consequences of the valine/leucine polymorphism at position 247 of the β_2-glycoprotein I (β_2GPI) gene in patients with antiphospholipid syndrome (APS), by investigating the correlation between genotypes and the presence of anti-β_2GPI antibody. The reactivity of anti-β_2GPI antibodies was characterized using recombinant Val^{247} and Leu^{247} β_2GPI.**

Methods. **Sixty-five Japanese patients with APS and/or systemic lupus erythematosus who were positive for antiphospholipid antibodies and 61 controls were analyzed for the presence of the Val/Leu^{247} polymorphism of β_2GPI. Polymorphism assignment was determined by polymerase chain reaction followed by restriction enzyme digestion. Recombinant Val^{247} and Leu^{247} β_2GPI were established to compare the reactivity of anti-β_2GPI antibodies to β_2GPI between these variants. The variants were prepared on polyoxygenated plates or cardiolipin-coated plates, and the reactivity of a series of anti-β_2GPI antibodies (immunized anti-human β_2GPI monoclonal antibodies [Cof-19–21] and autoimmune anti-β_2GPI monoclonal antibodies [EY1C8, EY2C9, and TM1G2]) and IgGs purified from patient sera was investigated.**

Results. **A positive correlation between the Val^{247} allele and the presence of anti-β_2GPI antibodies was observed in the patient group. Human monoclonal/polyclonal anti-β_2GPI autoantibodies showed higher binding to recombinant Val^{247} β_2GPI than to Leu^{247} β_2GPI, although no difference in the reactivity of the immunized anti-β_2GPI between these variants was observed. Conformational optimization showed that the replacement of Leu^{247} by Val^{247} led to a significant alteration in the tertiary structure of domain V and/or the domain IV–V interaction.**

Conclusion. **The Val^{247} β_2GPI allele was associated with both a high frequency of anti-β_2GPI antibodies and stronger reactivity with anti-β_2GPI antibodies compared with the Leu^{247} β_2GPI allele, suggesting that the Val^{247} β_2GPI allele may be one of the genetic risk factors for development of APS.**

The antiphospholipid syndrome (APS) is characterized by arterial/venous thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL) (1–3). Among the targets of aPL, β_2-glycoprotein I (β_2GPI), which bears epitopes for anticardiolipin antibodies (aCL), has been extensively studied (4–6). APS-related aCL do not recognize free β_2GPI, but do recognize β_2GPI when it is complexed with phospholipids or negatively charged surfaces, by exposure of cryptic epitopes (7) or increment of antigen density (8).

The significance of antigen polymorphism in the production of autoantibodies or the development of autoimmune diseases is now being widely discussed. It is speculated that amino acid substitution in antigens can lead to differences in antigenic epitopes of a given protein. In particular, β_2GPI undergoes conformational

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alteration upon interaction with phospholipids (9). β_2GPI polymorphism on or near the phospholipid binding site can affect the binding or production of aCL (anti-β_2GPI autoantibodies), the result being altered development of APS. Polymorphism near the antigenic site, or which leads to alteration of the tertiary structure of the whole molecule, may affect the binding of autoantibodies. Five different gene polymorphisms of β_2GPI attributable to a single-nucleotide mutation have been described: 4 are a single amino acid substitution at positions 88, 247, 306, and 316 (10), and the other is a frameshift mutation associated with β_2GPI deficiency found in the Japanese population (11). In particular, the Val/Leu^{247} polymorphism locates in domain V of β_2GPI, between the phospholipid binding site in domain V and the potential epitopes of anti-β_2GPI antibodies in domain IV, as we reported previously (12). Although anti-β_2GPI antibodies are reported to direct to domain I (13) or domain V (14) as well, it should be considered that a certain polymorphism alters the conformation of the molecule, affecting function or antibody binding at a distant site.

We previously reported that, in a group of British Caucasian subjects, the Val^{247} allele was significantly more frequent in primary APS patients with anti-β_2GPI antibodies than in controls or in primary APS patients without anti-β_2GPI antibodies (15), but the importance of the Val^{247} allele in patients with APS is still controversial. In this study, we analyzed the correlation between the β_2GPI Val^{247} allele and anti-β_2GPI antibodies in the Japanese population. We also investigated the reactivity of anti-β_2GPI antibodies to recombinant Val^{247} β_2GPI and Leu^{247} β_2GPI, using a series of monoclonal anti-β_2GPI antibodies and IgGs purified from sera of patients with APS. Finally, to investigate the difference in anti-β_2GPI binding to those variants, we conformationally optimized to domain V and the domain IV–V complex of β_2GPI variants at position 247, referring the crystal structure of β_2GPI.

PATIENTS AND METHODS

Patients and controls. The study group comprised 65 patients (median age 38 years [range 18–74 years]; 57 women and 8 men) who attended the Hokkaido University Hospital, all of whom were positive for aPL (IgG, IgA, or IgM class aCL, and/or lupus anticoagulant). Thirty-four patients had APS (16 had primary APS, and 18 had secondary APS), and 31 patients did not have APS (24 had systemic lupus erythematosus [SLE], and 7 had other rheumatic diseases). Among all subjects, 19 had a history of arterial thrombosis, and 6 had venous thrombosis. Of the 31 patients with a history of pregnancy, 8 experienced pregnancy complications (some patients had more than 1 manifestation of pregnancy morbidity). Anti-β_2GPI antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as β_2GPI-dependent aCL (16). IgG, IgA, or IgM class β_2GPI-dependent aCL were found in 30, 14, and 21 patients, respectively (some patients had >1 isotype), and 34 patients had at least 1 of those isotypes. Lupus anticoagulant, detected by 3 standard methods described previously (17), was found in 51 patients. The diagnoses of APS and SLE, respectively, were based on the preliminary classification criteria for definite APS (18) and the American College of Rheumatology criteria for the classification of SLE (19). Informed consent was obtained from each patient or control subject. The control group comprised 61 healthy individuals with no history of autoimmune, thrombotic, or notable infectious disease.

Determination of β_2GPI gene polymorphism. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a standard phenol–chloroform extraction procedure or the DnaQuick kit (Dainippon, Osaka, Japan). Polymorphism assignment was determined by polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion (PCR–restriction fragment length polymorphism) using *Rsa* I (Promega, Southampton, UK) as described previously (15).

Purification of patient IgG. Sera from 11 patients positive for IgG class β_2GPI-dependent aCL were collected. The mean (±SD) titer of aCL IgG from these patients was 29.0 ± 21.5 IgG phospholipid (GPL) units (range 12.4 to >98 GPL units). IgG was purified from these sera using a protein G column and the MAbTrap GII IgG purification kit (Pharmacia Biotech, Freiburg, Germany), as recommended by the manufacturer.

Monoclonal anti-β_2GPI antibodies. Two types of anti-β_2GPI monoclonal antibodies were used. Cof-19, Cof-20, and Cof-21 are mouse monoclonal anti-human β_2GPI antibodies obtained from immunized BALB/c mice, directed to domains V, III, and IV of β_2GPI, respectively. These monoclonal antibodies recognize the native structure of human β_2GPI (12).

EY1C8, EY2C9, and TM1G2 are IgM class autoimmune monoclonal antibodies established from patients with APS (20). These antibodies bind to domain IV of β_2GPI, but only after interaction with solid-phase phospholipids or with a polyoxygenated polystyrene surface. EY1C8 and EY2C9 were established from a patient whose genotype of β_2GPI was heterozygous for Val/Leu^{247}. The genotype of the patient with TM1G2 was not determined.

Preparation of recombinant β_2GPI. As previously reported, genes were expressed in *Spodoptera frugiperda* Sf9 insect cells infected with recombinant baculoviruses (12). A full-length complementary DNA of human β_2GPI coding Val^{247} was originally obtained from Hep-G2 cells (21), and the valine residue was replaced by leucine, using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI). The sequence of the primers for a mutant Val^{247}→Leu (GTA→TTA) is as follows: 5′-GCATCTTGTAAATTACCTGTGAAAAAAG-3′. A DNA sequence of the mutant was verified by analysis using ABI Prism model 310 (PE Applied Biosystems, Foster City, CA).

Binding assays of monoclonal anti-β_2GPI antibodies and purified IgGs to the recombinant β_2GPI (cardiolipin-coated plate). The reactivity of a series of monoclonal anti-β_2GPI antibodies and IgG fractions (purified from the sera of APS patients positive for IgG class anti-β_2GPI) against 2 β_2GPI variants was investigated using an ELISA. ELISAs were performed using a cardiolipin-coated plate as previously reported (16) but with a slight modification. Briefly, the wells of Sumilon Type S microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were filled with 30 μl of 50 μg/ml cardiolipin (Sigma, St. Louis, MO) and dried overnight at 4°C. After blocking with 2% gelatin in phosphate buffered saline (PBS) for 2 hours and washing 3 times with 0.05% PBS–Tween, 50 μl of 10 μg/ml recombinant β_2GPI and controls were distributed and incubated for 30 minutes at room temperature. Wells were filled with 50 μl of serial dilutions of monoclonal antibodies (Cof-19–21, EY1C8 and EY2C9, and TM1G2) or purified patient IgG (100 μg/ml), followed by incubation for 30 minutes at room temperature. After washing 3 times, 50 μl of alkaline phosphatase–conjugated anti-mouse IgG (1:3,000), anti-human IgM (1:1,000), or anti-human IgG (1:6,000) was distributed and incubated for 1 hour at room temperature. The plates were washed 4 times, and 100 μl of 1 mg/ml *p*-nitrophenyl phosphate disodium (Sigma) in 1*M* diethanolamine buffer (pH 9.8) was distributed. Optical density (OD) was read at 405 nm, with reference at 620 nm. One percent fatty acid–free bovine serum albumin (BSA) (A-6003; Sigma)–PBS was used as sample diluent and control.

Binding assays of monoclonal anti-β_2GPI antibodies to recombinant β_2GPI (polyoxygenated plate). Anti-β_2GPI antibody detection assay using polyoxygenated plates was performed as previously reported (22), with minor modifications. Briefly, the wells of polyoxygenated MaxiSorp microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated with 50 μl of 1 μg/ml recombinant β_2GPI in PBS and incubated overnight at 4°C. After blocking with 3% gelatin–PBS at 37°C for 1 hour and washing 3 times with PBS–Tween, 50 μl of monoclonal antibodies, diluted with 1% BSA–PBS, were distributed and incubated for 1 hour at room temperature. The following steps were taken, in a similar manner.

Conformational optimization of domain V and the domain IV–V complex in human β_2GPI variants at position 247. A conformation of domain V in the valine variant at position 247 was first constructed from the crystal structure of the leucine variant (implemented in Protein Data Bank: 1C1Z) (23). Replacement of leucine by valine at position 247 was performed using the Quanta system (Molecular Simulations, San Diego, CA), and the model was optimized by 500 cycles of energy minimization by the CHARMm program (24), with hydrophilic hydrogen atoms and TIP3 water molecules (25). Molecular dynamics simulation (5 psec) of the model was then performed with 0.002 psec time steps. The cutoff distance for nonbonded interactions was set to 15Å, and the dielectric constant was 1.0. A nonbonded pair list was updated every 10 steps. The most stable structure of each domain in the dynamics iterations was then optimized by 500 cycles of energy minimization. The final structures of domain V consisted of 2,616 atoms, including 603 TIP3 water molecules, and had a total energy of -1.63×10^4 kcal/mole with a root-mean-square force of 0.869 kcal/mole.

Molecular models of a domain IV–V complex (leucine and valine variants at position 247) were further constructed by considering the location of the oligosaccharide attachment site in domain IV, the location of epitopic regions of the Cof-8 and Cof-20 monoclonal antibodies, the junction between domains IV and V, and molecular surface charges of both domains. These models were again optimized by molecular dynamics simulation and by energy minimization as described above. The final structures of the complex in the leucine and valine variants consisted of 3,773 and 3,778 atoms, respectively, including hydrophilic hydrogen atoms and 806 and 808 TIP3 water molecules, respectively, and had total energy of -2.07×104 and -2.03×104 kcal/mole with a root-mean-square force of 0.985 and 0.979 kcal/mole, respectively.

Statistical analysis. Correlations between the allele frequencies and clinical features such as the positiveness of β_2GPI-dependent aCL were expressed as odds ratios (ORs) and 95% confidence intervals (95% CIs). *P* values were determined by chi-square test with Yates' correction. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Val/Leu^{247} polymorphism of β_2GPI and the presence of β_2GPI-dependent aCL. As shown in Table 1, the Leu^{247} allele was dominant in the population of healthy Japanese individuals, compared with Caucasians, which is consistent with a previous report (26). Japanese patients with anti-β_2GPI had a significantly increased frequency of the Val^{247} allele, compared with Japanese patients without anti-β_2GPI ($P = 0.0107$) or Japanese controls ($P = 0.0209$).

The binding of autoimmune anti-β_2GPI to recombinant Val^{247} and Leu^{247} β_2GPI. Representative binding curves using cardiolipin-coated plates and polyoxygenated plates are shown in Figure 1. Regardless of the type of plates, Cof-20 bound equally to valine and leucine variants of β_2GPI (Figures 1a and c), in any concentration of Cof-20. The binding curves of Cof-19 and Cof-21 were similar to that of Cof-20 (results not

Table 1. Frequency of the Val^{247} allele of β_2GPI in patients with APS*

Group	Japanese	British Caucasians
Patients with anti-β_2GPI	23/68 (33.8)†	48/56 (85.7)‡
Patients without anti-β_2GPI	9/62 (14.5)	39/58 (67.2)
Controls	23/122 (18.9)	55/78 (70.5)

* Values are the number (%). β_2GPI = β_2-glycoprotein I; APS = antiphospholipid syndrome.
† $P = 0.0107$ versus patients without anti-β_2GPI (odds ratio [OR] 3.01, 95% confidence interval [95% CI] 1.26–7.16), and $P = 0.0209$ versus controls, by chi-square test (OR 2.15, 95% CI 1.09–4.23).
‡ $P = 0.204$ versus patients without anti-β_2GPI (OR 2.92, 95% CI 1.16–7.39), and $P = 0.0396$ versus controls, by chi-square test (OR 2.51, 95% CI 1.03–6.13).

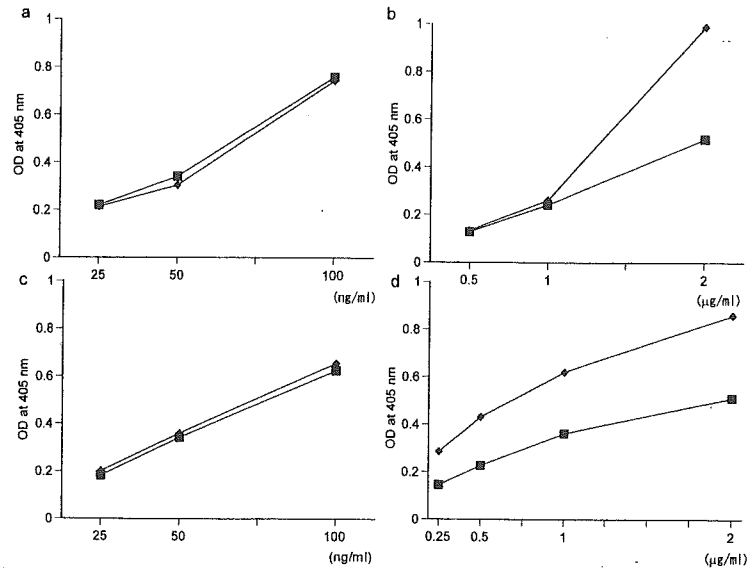

Figure 1. Representative binding curves of monoclonal anti-β_2-glycoprotein I (anti-β_2GPI) antibodies to recombinant valine/leucine^{247} β_2GPI. **a,** Binding curve of Cof-20 using cardiolipin-coated plate. **b,** Binding curve of EY2C9 using cardiolipin-coated plate. **c,** Binding curve of Cof-20 using polyoxygenated plate. **d,** Binding curve of EY2C9 using polyoxygenated plate. Binding to Val^{247} β_2GPI and Leu^{247} β_2GPI are indicated with diamonds and squares, respectively. OD = optical density.

shown). In contrast, EY2C9 showed stronger binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI (Figures 1b and d). EY1C8 and TM1G2 also showed stronger binding to Val^{247} β_2GPI. Figure 2a shows the binding of the monoclonal antibodies, on cardiolipin-coated plates, in the following concentrations: for Cof-19–21, 100 ng/ml;

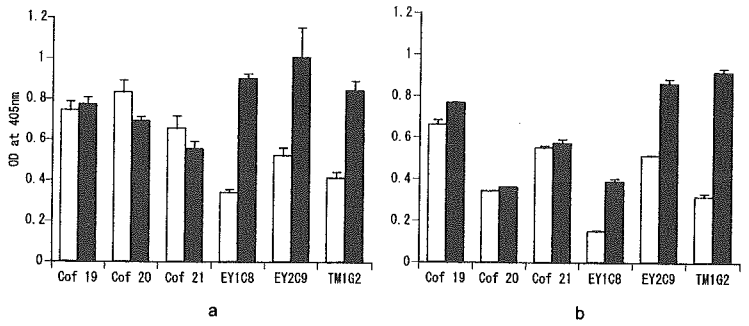

Figure 2. Reactivity of anti-β_2-glycoprotein I (anti-β_2GPI) antibodies to β_2GPI variants. **a,** The binding of monoclonal anti-β_2GPI antibodies to the recombinant valine/leucine^{247} β_2GPI was investigated using enzyme-linked immunosorbent assay (ELISA) on cardiolipin-coated plates. Concentrations of antigens and antibodies were as follows: for recombinant β_2GPI, 10 μg/ml; for Cof-19–21, 100 ng/ml; for EY1C8 and EY2C9, 2 μg/ml; for TM1G2, 5 μg/ml. **b,** The binding of monoclonal anti-β_2GPI antibodies to the recombinant Val/Leu^{247} β_2GPI was investigated using ELISA on polyoxygenated plates. Concentrations of antigens and antibodies were as follows: for recombinant β_2GPI, 1 μg/ml; for Cof-19–21, 50 ng/ml; for EY1C8 and EY2C9, 2 μg/ml; for TM1G2, 5 μg/ml. Results were presented as the optical density (OD) at 405 nm. Open columns indicate binding activity to Leu^{247} β_2GPI, and solid columns indicate binding activity to Val^{247} β_2GPI. Bars show the mean and SD.

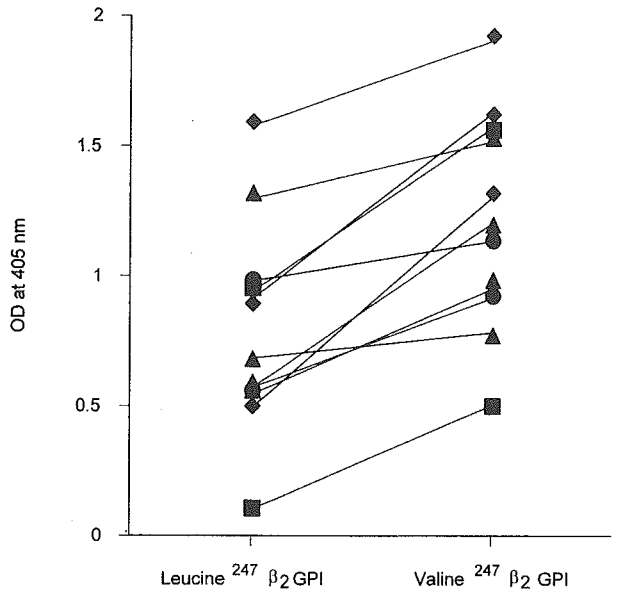

Figure 3. Reactivity of purified IgG from patients (100 μg/ml) to recombinant Val/Leu^{247} β_2-glycoprotein I (β_2GPI) (10 μg/ml), presented as the optical density (OD) at 405 nm. Squares, circles, and triangles indicate patients homozygous for the Leu^{247} allele, homozygous for the Val^{247} allele, and heterozygous for the Val/Leu^{247} allele, respectively. Diamonds indicate patients whose genotypes were not available.

for EY1C8 and EY2C9, 1 μg/ml; and for TM1G2, 2.5μg/ml. In contrast with the close reactivity of Cof-19, Cof-20, and Cof-21 between Val^{247} β_2GPI and Leu^{247} β_2GPI, autoimmune monoclonal antibodies (EY1C8, EY2C9, and TM1G2) showed higher binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI. The autoimmune monoclonal antibodies also showed a higher binding to Val^{247} β_2GPI directly coated on polyoxygenated plates (Figure 2b). IgG in sera collected from 11 patients (100 μg/ml) also showed higher binding to Val^{247} β_2GPI than to Leu^{247} β_2GPI on cardiolipin-coated plates, regardless of the patients' genotypes (Figure 3).

Conformational alteration by leucine replacement by valine at position 247. Each domain V conformation in 2 variants at position 247 is shown in Figure 4a. The root-mean-square deviations for matching backbone atoms and equivalent atoms in the leucine and valine variants were 0.76 and 1.11 Å, respectively. The largest shift was observed at Val^{303}, one of the residues located on the backbone neighboring position 247. The shift seemed to be caused by weak flexibility of side chains consisting of Val^{247}, Pro^{248}, and Val^{249} and the electrostatic interactions between Lys^{250}, Lys^{251}, Glu^{307}, and Lys^{308}.

The molecular models of the IV–V complex in leucine and valine variants are shown in Figure 4b. The root-mean-square deviations for matching these backbone atoms and equivalent atoms were 1.72 and 2.03 Å, respectively. Electrostatic interactions and hydrogen bonds between Asp^{193} and Lys^{246}/Lys^{250}, Asp^{222} and Lys^{305}, and Glu^{228} and Lys^{308} appeared in the IV–V complex, but the interaction between Glu^{228} and Lys^{308} was disrupted by the leucine replacement by valine, because direction of the Lys^{308} side chain was significantly changed in the complex. As a result, Trp^{235} of domain IV, located on the contact surface with domain V, was slightly shifted.

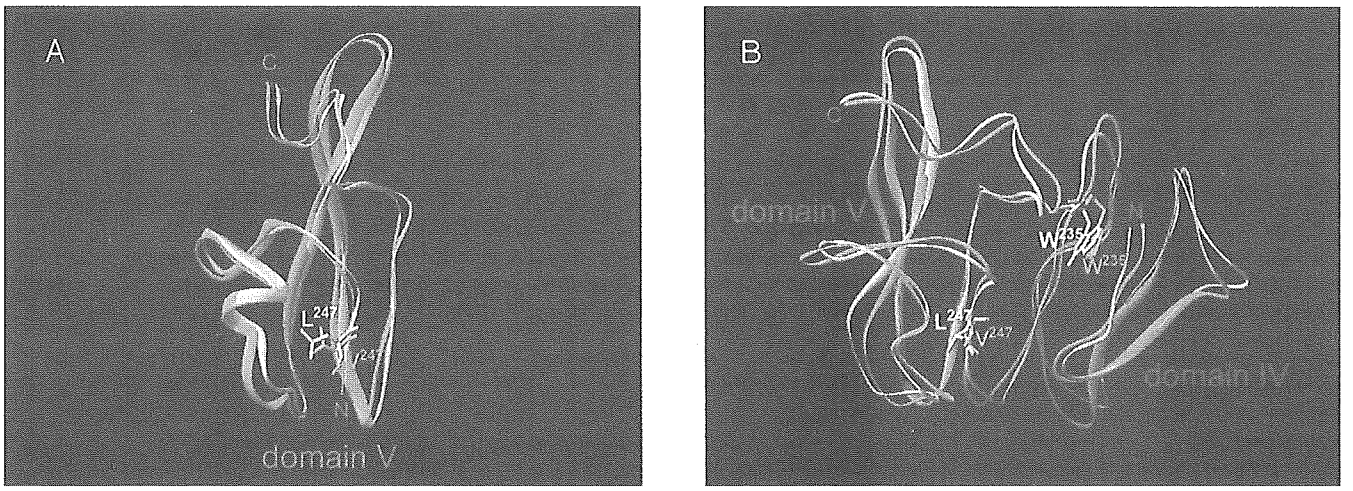

Figure 4. Conformational alterations in domain V (**A**) and in the domain IV–V complex (**B**), replacing leucine by valine at position 247. Structure of the valine (light blue) and leucine (white) variants was shown by a ribbon representation with the secondary structure.