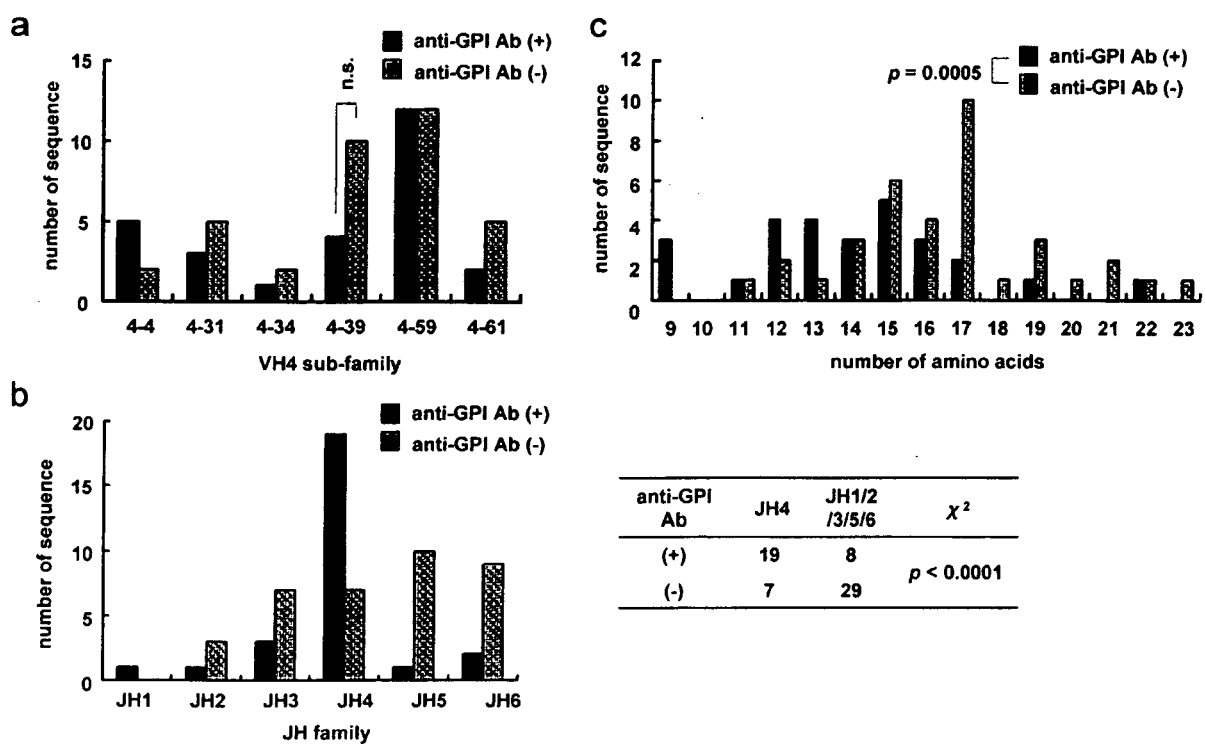
Table III. Synovial immunoglobulin VH4 repertoire of anti-GPI Ab (+) and (-) RA patients.

	VH	DH	H-CDR3	bp	JH	R/S ratio within CDR
Anti-GPI Ab (+) RA patients						
RA1	4-04	2-21	NMAGDVIGFFDY	14	4	2/0
	4-04	4-23	SRNPIDYPLGYFDY	16	4	2/1
	4-04	5-05	GYSYGLFDV	11	4	12/2
	4-04	6-25	QRHRRGDFDI	12	3	2/0
	4-31	3-10	EELRRIRGPFFDY	15	4	9/3
	4-34	6-06R	GEQDEHQVSSRFFFYYYIDV	22	6	9/2
	4-39	2-15	QGYCSGGTCQDFDY	16	4	3/0
	4-39	3-10	QGARQWFGEFGAFDY	17	4	6/0
	4-59	3-09	LSPGGNFDFDL	13	4	6/1
	4-59	3-10	DGEGGSYYFDY	13	4	9/1
	4-59	3-10R	HNNTWHPFDY	12	4	8/1
	4-59	3-16R	LPPRGNYRLDS	13	4	5/1
	4-59	6-13	VPGFSSTWFEVDY	15	4	6/2
	4-59	6-13	FSGSFYGWFDP	13	5	7/0
	4-59	IR	VSTQTDY	9	4	5/1
	4-59	1-07	APPPWLRRVSTGTWL	17	2	5/3
	4-61	2-02R	GRQPDYYYAMDV	14	6	8/4
RA2	4-04	5-12R	SPDNRNTLDI	12	3	7/2
	4-31	3-10	GYYYGPGSYHPFET	16	4	3/1
	4-31	6-13	DRDAAAGRWVDY	14	4	3/0
	4-39	1-26	PVVGARDPAPFDL	15	3	4/2
	4-59	3-03	RGGPTEH	9	1	4/1
	4-59	3-09	DRGQEYGIDS	12	4	4/2
	4-59	IR1R	LGQLGDH	9	4	12/2
	4-61	1-20	VSLLGYKRNDGKYHFDY	19	4	6/2
RA3	4-39	3-10	YIRGVRSGGYFDY	15	4	4/2
	4-59	1-26	HGVDSGSFYAFDY	15	4	3/0
Anti-GPI Ab (-) RA patients						
RA4	4-31	3-10	DHGSGSSYFFSPNYGMDV	20	6	3/1
	4-34	5-12	GNSGNGYYFYNYMDV	17	6	11/3
	4-39	2/OR15-2R	FTITLFRGKEGNY	15	4	5/0
	4-39	3/OR15-3	QNGLQSRVDYFDF	15	4	7/1
	4-39	IRR	GGGVNLGSGAFYDE	16	4	18/7
	4-59	1-01	GGGFSSNWSLAPFAFDI	19	3	3/2
	4-59	2-15	DVDCVGGSCYSSDWFDP	19	5	5/1
	4-59	3-22	LWGSSGLYGENWFDP	17	5	5/1
	4-59	4/OR15-4	DVTSVQTTMVPAFDY	17	4	9/2
	4-59	5-05	DIRGYGYGYFDL	14	2	13/1
	4-59	6-19	DTHTAVPGDDYFES	16	4	7/3
	4-61	1-26	ESLKVGSTCFDP	14	5	9/3
	4-61	3-10	ARPDGSESFYRYLDL	17	2	4/1
	4-61	3-10	EQTGLRGQNM	12	3	7/2
	4-61	4-23	EGDYGGSYYYYYMDL	17	6	11/0
RA5	4-04	2-15	AGGGDCSGATCYSYYYGMDV	22	6	5/0
	4-31	2-21	GFGSSVIAMAYYFDY	17	4	3/1
	4-31	4-04	LHAERALGFWFDP	15	5	17/3
	4-31	4/OR15-4	VAPGAMPDDASEI	15	3	8/1
	4-34	3-09	MANLTGTPGLGI	14	3	7/2
	4-39	2/OR15-2R	DYITIFGVAPFDP	15	5	4/1

Table III. Continued.

	VH	DH	H-CDR3	bp	JH	R/S ratio within CDR
	4-39	3-03	**H**VNFEVVIGRWFD**H**	16	5	13/3
	V39	3-03	LGALFGADSYYGMDV	17	6	6/1
	V39	4-23	**K**DYADYEGFAY	13	5	6/0
	V39	5-12	YISATMEDF	11	3	11/2
	V39	6-13	DAGYSSS**RH**PVGFDP	17	5	8/3
	V39	6-19R/3-16	**HAR**IGA**H**YTYGSF**R**LFDAFDV	23	3	5/1
	V59	3-03	D**K**SGYYTPGGYYYYYGMDV	21	6	3/2
	V59	3-03	APYWSGYVYGLDV	15	6	7/1
	V59	3-10	ETYYSASGSYYSGQYYFEY	21	4	6/1
	V59	4/OR15-4	**H**GGLYPYYYFAMDV	16	6	5/0
	V59	6-19	**R**TDDYS**R**GWYWYFDP	17	2	6/1
	V59	6-19R	**HAIHR**FSTAFPNWFDP	18	5	3/2
	V61	4-17	DASLLYGDYVSWFDP	17	5	8/5
RA6	V04	1-14R	DP**R**TV**K**TMDV	12	6	6/4
	V59	3-22	GP**H**DTMTNYYGLNAFDI	19	3	7/4

The characters of immunoglobulin sequences using VH4 family genes are shown. Bold characters represent based-ionized amino acids (R, arginine; H, histidine; K, lysine). Underline indicates R/S ratio >3.



anti-GPI Ab	JH4	JH1/2 /3/5/6	χ^2
(+)	19	8	$p < 0.0001$
(-)	7	29	

Figure 2. Comparison of synovial VH4 B cells from rheumatoid arthritis (RA) patients with or without anti-glucose-6-phosphate isomerase (GPI) Ab. (a) Usage of VH4 subfamily frequencies of VH4 gene usage in synovial B cells from RA patients are shown. In anti-GPI Ab (+) patients, VH4-59 (12 products), VH4-4 (5 products), VH4-39 (4 products), VH4-31 (3 products), VH4-61 (2 products), and VH4-34 (1 products) were identified. In anti-GPI Ab (-) patients, VH4-59 (12 products), VH4-39 (10 products), VH4-31 and VH4-61 (5 products each), and VH4-4 and VH4-34 (2 products each) were identified. VH4-39 showed a relatively low frequency in synovial B cells of anti-GPI Ab (+) RA patients, although the comparison is statistically not significant. (b) The usage of immunoglobulin heavy chain joining segment (JH) family. The frequencies of JH gene usage of synovial VH4 B cells from RA patients are shown. JH4 (19 products) was the most frequent gene used in anti-GPI Ab (+) RA patients, although this gene was not predominant in anti-GPI Ab (-) individuals ($p<0.0001$ by two-tailed Fisher exact test between JH4 and others). (c) The number of amino acids in the immunoglobulin heavy chain complementarity-determining region 3 (IgH-CDR3). The IgH-CDR3 lengths of VH4 B cells are shown. Lengths varied between 9 and 23 (mean, 15.46 ± 3.09) amino acids. In anti-GPI Ab (+) RA patients, the IgH-CDR3 lengths (14.00±2.96 amino acids) of synovial VH4 B cells were significantly shorter than those of anti-GPI Ab (-) individuals (16.56±2.75 amino acids) ($p=0.0005$ by Mann-Whitney's U test).

between the groups in terms of amino acids usage of IgH-CDR3, although this region in anti-GPI Ab (+) RA patients was rich in basic-ionized amino acids (arginine, histidine, and lysine) in their near central position, compared to the composition in anti-GPI Ab (-) individuals (Table III).

The IgH-CDR3 amino acid lengths varied from 9 to 23 amino acids (mean, 15.46±3.09 amino acids). In anti-GPI Ab (+) RA patients, the IgH-CDR3 length of synovial immunoglobulins using VH4 was significantly shorter than the length of those in anti-GPI Ab (-) individuals (p=0.0005, Fig. 2c). These findings suggest the prevalence of affinity-matured VH4 B cells in the synovium of anti-GPI Ab (+) RA patients.

Discussion

Anti-GPI Ab is frequently detected in patients with aggressive forms of RA (12,13), and its level correlates significantly with extra-articular manifestations such as rheumatoid nodules, rheumatoid vasculitis, and Felty's syndrome (10). We reported previously that serum IgG from anti-GPI Ab (+) RA patients preferentially attached to the articular surface of the meta-carpophalangeal joints of the monkey, inducing recruitment of granulocytes and mononuclear cells into the synovium (29). These results indicated that human serum immunoglobulins from RA patients include autoantibodies to specific protein(s) expressed in the joint cavity. Furthermore, human GPI protein is expressed on the cartilage and synovial surface in RA (7) and anti-GPI Ab is present in the synovial fluid, suggesting that the local production of such autoantibodies might be associated with arthritis. To address this hypothesis, we focused on the synovial B cells of anti-GPI Ab (+) patients.

In the present study, VH4 genes were detected in the synovium of all patients with RA. In some autoimmune diseases such as systemic lupus erythematosus (SLE) (30,31), VH4 genes are overrepresented in peripheral B cells (18), although negative selection of VH4 genes occurs in healthy individuals (19,20). These observations implicate VH4 genes as a self-reactive gene family. The frequency of VH4 genes in peripheral B cells from RA patients was not different from that of healthy individuals (32), however VH4 genes were highly expressed in the rheumatoid synovial B cells (21,22). In addition, antigen-driven immune maturation of B cells is characterized by an R/S ratio >3 within the CDR (33). Our study demonstrated that VH4 (+) synovial B cells in patients with RA are affinity-matured, because immunoglobulins with a high R/S ratio were dominant.

A skewed VH4 subfamily in RA synovium was not identified in this study, but when we compared anti-GPI Ab (+) with (-) patients, VH4 subfamily usages of synovial B cells from anti-GPI Ab (+) RA patients were less frequent for VH4-39. In peripheral blood of SLE, VH4-34 (V4.21) was overexpressed and correlated with some autoantibodies (30,31), but no specific subfamily repertoire has been identified in the synovium of RA patients. We do not know whether these skewed VH4 subfamilies are related to arthritogenicity, however, there are reports that some autoantibodies with VH4-34 (V4.21) segments are related to the pathogenicity of SLE (34,35). Since anti-GPI Ab is a candidate arthritogenic antibody, it would be interesting to identify the skewed VH4 subfamilies by increasing numbers of the sequence.

Our sequence analysis noted that synovial IgH-CDR3 from anti-GPI Ab (+) RA patients was enriched in basic-ionized amino acids. The ionized side-chains of arginine in the CDRs contribute to higher binding affinity for some antigens such as DNA, cardiolipin (36,37), and TAG72 (38). A previous study found that arginine in IgH-CDR3 of human and murine anti-dsDNA was most likely to be generated during V-D-J rearrangement in B cells, and the higher frequency of arginine in the IgH-CDR might similarly be due to the clonal expansion of B cells (38). In addition, the precise location of arginine is important for the binding (37).

IgH-CDR3 length and amino acid composition is the major contributor to antigen specificity and affinity (39-41). Matured immunoglobulins have shorter CDR3s than non-matured ones in both mice and humans (42,43). In the present anti-GPI Ab (+) RA patients, the CDR3 length of synovial immunoglobulins using VH4 was significantly shorter and the JH4 usage was significantly higher than those of anti-GPI Ab (-) individuals. These data support the notion that synovial B cells of anti-GPI Ab (+) patients are affinity-matured with higher affinity to a particular antigen.

In conclusion, our findings on synovial B cells in RA patients positive for anti-GPI Ab clearly demonstrated a high frequency of VH4-JH4 subfamily genes rich in basic amino acids and shorter CDR3 length, indicating affinity-matured B cells, reactive to autoantigens such as GPI. Future studies using anti-GPI Ab-producing B cell hybridomas should shed light on the functional role of anti-GPI Ab in the pathogenesis of RA.

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The exploration of joint-specific immunoreactions on immunoglobulins G of anti-glucose-6-phosphate isomerase antibody-positive patients with rheumatoid arthritis

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Abstract. The pathogenic role of autoantibodies in rheumatoid arthritis (RA) remains elusive. Anti-glucose-6-phsosphate isomerase (GPI) antibodies (Abs) are candidates for arthritogenic Abs because they directly induce arthritis in mice. High titers of anti-GPI Abs are found in some RA patients with severe forms. The aim of this study was to analyze the role of IgG, including anti-GPI Abs, in the joints of RA patients. Synovial tissue was obtained from 6 patients with RA (3 anti-GPI Abs- positive and 3 anti-GPI Abs-negative) and compared histologically and immunohistochemically for IgG and C3 deposition. IgG fractions were separated from the sera of anti-GPI Abs-positive RA patients and healthy subjects, and injected into the metacarpophalangeal joints of 4 cynomolgus monkeys. On day 16, the joints were harvested and examined histologically and immunohistochemically. The expression of the C5a receptor (C5aR) molecule in the synovium was quantified by real-time PCR using cDNA from the monkeys' joints. The synovia of anti-GPI Abs-positive RA patients showed diffuse infiltration of cells, including mast cells, and strong deposition of IgG and C3. In monkeys, IgG from RA patients, including anti-GPI Abs, resulted in recruitment of granulocytes and mononuclear cells, strong deposition of IgG on the articular surface, and overexpression of C5aR, but no joint swelling. No infiltrated cells or IgG deposition were observed in monkeys injected with IgGs from healthy subjects. Our results suggest that IgG fraction from RA patients, including that of anti-GPI Abs, may play a role in the synovitis of RA, although the pathogenesis of human anti-GPI Abs is still uncertain.

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Introduction

Recently, the K/BxN T cell receptor transgenic mouse model was described as a model of inflammatory arthritis, characterized by arthritic manifestations similar to those of rheumatoid arthritis (RA) (1). Matsumoto *et al* (2) reported that arthritis could be almost entirely sustained by autoantibodies to the self-antigen glucose-6-phosphate isomerase (GPI). However, once the pathogenic antibodies (Abs) have been produced by the autoimmune reaction, there is no further requirement for lymphoid cells (3). The effector mechanisms of anti-GPI Abs have been confirmed by the requirement of innate immune system players (e.g. complement cascade, FcγR, neutrophils and mast cells) (4-7). More recently, immunization with human GPI was reported to provoke arthritis in DBA/1 mice, supporting the notion that autoimmunity to GPI directly plays some role in arthritis in genetically unaltered mice (8). These results also indicated that ubiquitous antigens might be the targets of arthritogenic Abs.

The first report on anti-GPI Abs in humans showed a high frequency of Abs in the sera of RA patients (9), though their frequency is still debated (10-14). Our anti-GPI Abs assay (10) seemed to be highly specific because it employs two different GPIs: a recombinant human GPI and a rabbit native GPI. Our results showed that only 15% of RA patients had high titers of anti-GPI Abs, although the severity of arthritis correlated with serum levels of anti-GPI Abs (10). Other authors have shown that extra-articular complications in RA are associated with serum levels of anti-GPI Abs (15). However, the arthritogenic role of these Abs remains unclear. To explore the role of human IgG including anti-GPI Abs in the joints, we compared the synovia of anti-GPI Abs-positive or -negative RA patients by histology and immunohistochemistry. We demonstrated the diffuse infiltration of inflammatory cells, including mast cells, in the synovium, lack of germinal-center

Table I. Clinical data and anti-GPI titer in six patients with RA.

	Age/Sex	Disease duration (years)	RF	CRP (mg/dl)	Rheumatoid nodule	Hu-GPI Abs OD405	Ra-GPI Abs OD405	Anti GPI-Abs
RA1	68/F	23	+	4.01	-	2.43	2.55	+
RA2	66/F	20	+	4.47	+	1.78	3.13	+
RA3	71/F	25	+	0.92	+	2.6	3.47	+
RA4	72/F	24	+	1.1	-	0.62	0.05	-
RA5	67/F	20	-	3.78	-	0.59	0.34	-
RA6	60/F	15	+	1.61	-	1.07	0.02	-

Cutoff values; Hu-GPI, 1.32 OD, Ra-GPI, 0.94 OD. RF, rheumatoid factor; +, positive; -, negative.

like structures, and the formation of immuno-complexes with C3 on the synovial surface.

There are significant differences between mice and humans with regard to the immune system, especially the innate immune system, including complement and immuno-complex-Fc receptor cascades (16). To investigate whether human anti-GPI Abs present in the serum have arthritogenic properties, we injected serum anti-GPI Abs, obtained from patients with RA, into several strains of mice. However, this did not produce swelling of the joints in mice (unpublished data). The results of these preliminary experiments suggested that human GPI Abs did not solely induce arthritis, although we could not confirm the differences in the innate immune system between humans and mice. In such experiments, it is better to use monkeys because they are genetically closer to humans than mice. Moreover, recent studies reported that orally-administered C5aR antagonist is only effective in humans and cynomolgus monkeys (17). Based on these properties, we used cynomolgus monkeys in this study. Intra-articular injection of human serum IgGs containing anti-GPI Abs in cynomolgus monkeys resulted in immunocomplex formation on the articular surface and intra-articular accumulation of granulocytes and mononuclear cells. These findings suggest that RA IgG including anti-GPI Abs, present in serum, preferentially form on the articular surface of immunocomplexes that might induce complement activation via C5aR-bearing cells, resulting in minor synovitis. However, the direct pathogenic role of IgG from human RA is still uncertain.

Patients and methods

Patients and tissue samples. Patients with RA were recruited from the Autoimmune Disease Clinic at Tsukuba University Hospital. Samples of synovial tissue were obtained from six RA patients including three anti-GPI Abs-positive, and three anti-GPI Abs-negative, who underwent knee replacement surgery. The study was approved by the local ethics com-mittee, and written informed consent was obtained from all participants. The diagnosis of RA was based on the criteria of the American College of Rheumatology (18). Table I summarizes the clinical data of participating patients.

Enzyme-linked immunosorbent assay for GPI. To select anti-GPI-positive patients, we used recombinant human GPI (huGPI), or rabbit muscle GPI (raGPI) (Sigma, St Louis, MO), which has been described in detail previously (10). Briefly, both antigens were used at 5 μg/ml (diluted in phosphate-buffered saline, PBS) to coat microtiter plates (12 h at 4°C). After washing, Block Ace (diluted 1/4 in 1xPBS, Dainippon Pharmaceuticals, Osaka, Japan) was used for saturation (30 min at 37°C). After washing, sera (diluted 1/50) were added and the plates were incubated for 12 h at 4°C. After washing again, alkaline phosphatase (AP)-conjugated anti-human IgG (Fc-fragment specific, Jackson Immuno Research, West Grove, PA) was added to the plate (dilution: 1/1000, 1 h, room temperature). After three washes, color was developed with AP reaction solution (containing 9.6% diethanol amine, 0.25 mM $MgCl_2$, pH 9.8) with AP substrate tablets (Sigma; one AP tablet per 5 ml of AP reaction solution). Plates were incubated for 1 h at room temperature and the OD was measured by plate spectrophotometry at 405 nm. Determinations were performed in triplicate, and standardized by reference to a highly positive human anti-GPI serum. The primary reading was processed by subtracting OD readings of control wells (coated with GST and Block Ace for huGPI-GST and raGPI, respectively). The cutoff OD was calculated from the ELISA reaction of 137 healthy control Japanese donors, the mean value ± two standard deviation was 1.32 to human recombinant GPI, and 0.94 to rabbit native GPI. Double-positive populations were considered anti-GPI Abs-positive.

Histopathological and immunohistochemical examinations. After knee replacement surgery (written informed consent was also obtained), the synovium from patients with RA were embedded in optimal cutting temperature (OCT) compound, frozen in dry ice isopentane, and 5-μm thick sections were mounted at -25°C. Slides were stored at -80°C until use, then acetone fixed for 30 sec and dried for 30 min. Tissue sections were lightly counterstained with hematoxylin-eosin (H&E) or standard toluidine blue. Stained sections were examined at 1000 diameters and at least 100 fields were evaluated in each specimen. Total mast cells / randomized 10fields were counted in three anti-GPI Abs-positive and -negative RA. The deposition of C3 and IgG was detected by 1/100 diluted fluorescein iso-thiocyanate (FITC)-conjugated sheep anti-

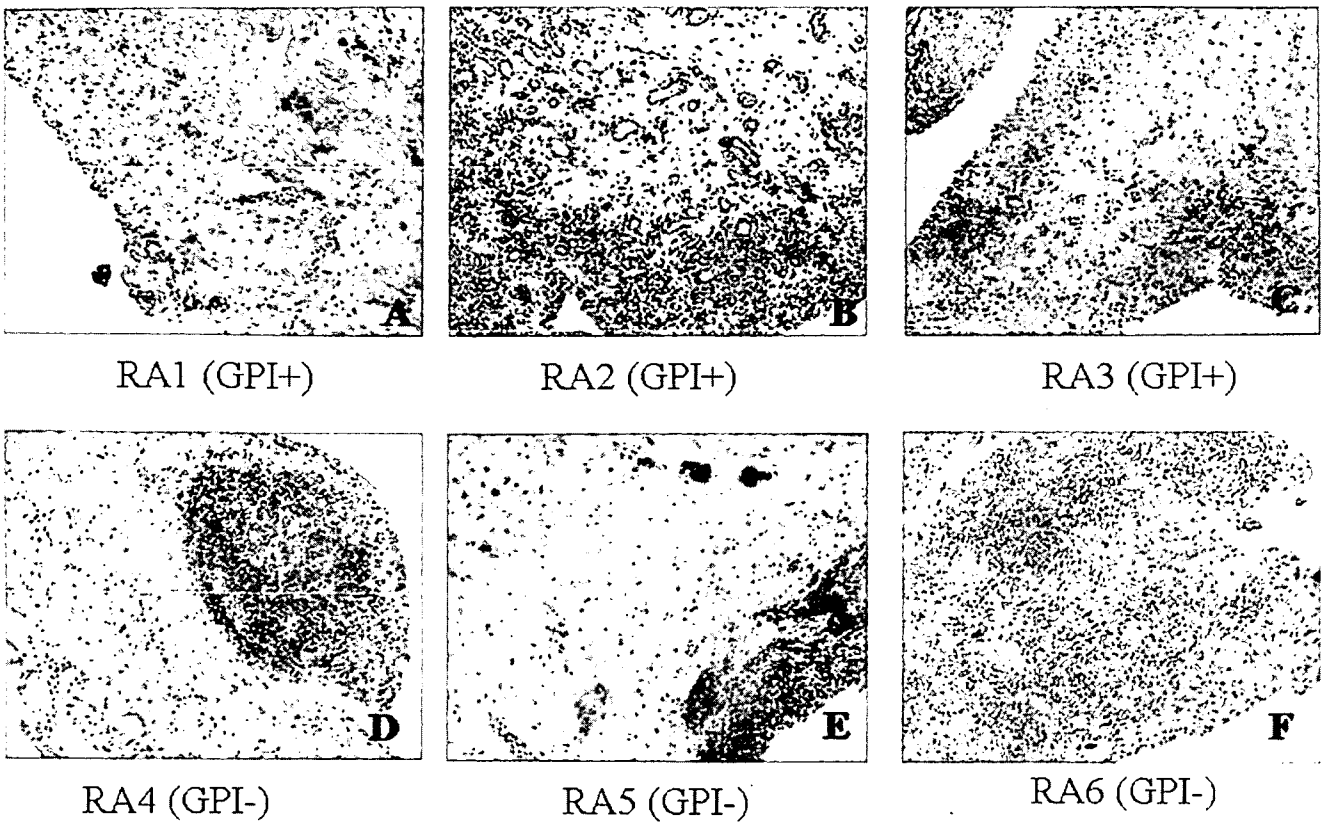


Figure 1. H&E staining of the synovium of RA patients with and without anti-GPI Abs. H&E staining of RA synovium. A, RA1 (anti-GPI Abs-positive RA) x100; B, RA2 (anti-GPI Abs-positive RA) x100; C, RA3 (anti-GPI Abs-positive RA) x100; D, RA4 (anti-GPI Abs-negative RA) x100; E, RA5 (anti-GPI Abs-negative RA) x100; F, RA6 (anti-GPI Abs-negative RA) x100. Note the diffuse distribution of infiltrated cells throughout the tissue samples in anti-GPI-positive patients (A, B, C). In contrast, note the germinal center-like structure (D, F) or cell aggregation (E) in three anti-GPI negative samples.

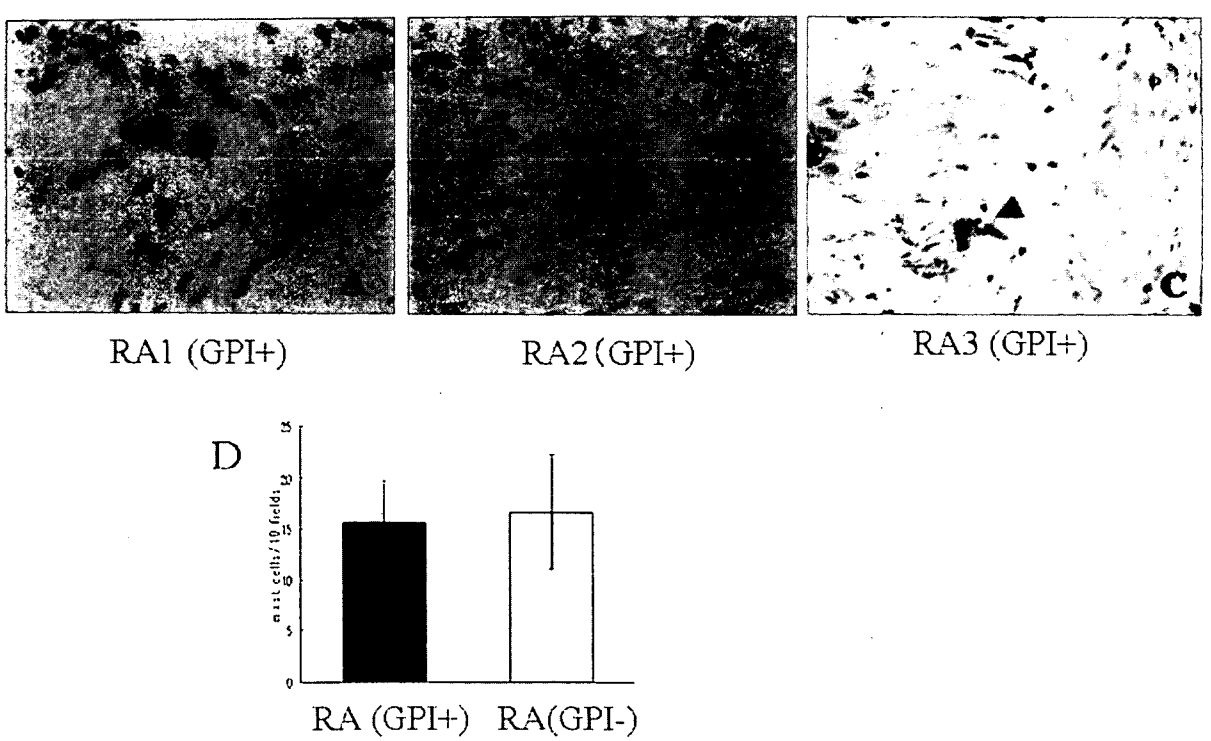


Figure 2. Mast cell involvement in the synovial tissue of RA patients with anti-GPI Abs. Toluidine blue staining (A, B, C) was performed. RA1 (anti-GPI Abs-positive RA) (A, D), RA2 (anti-GPI Abs-positive RA), RA3 (anti-GPI Abs-positive RA). Arrowheads indicate mast cells (A, B, C). Anti-GPI Abs-negative RA patients also had comparable mast cells in the synovium (D). Magnification, x200 A-C.

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

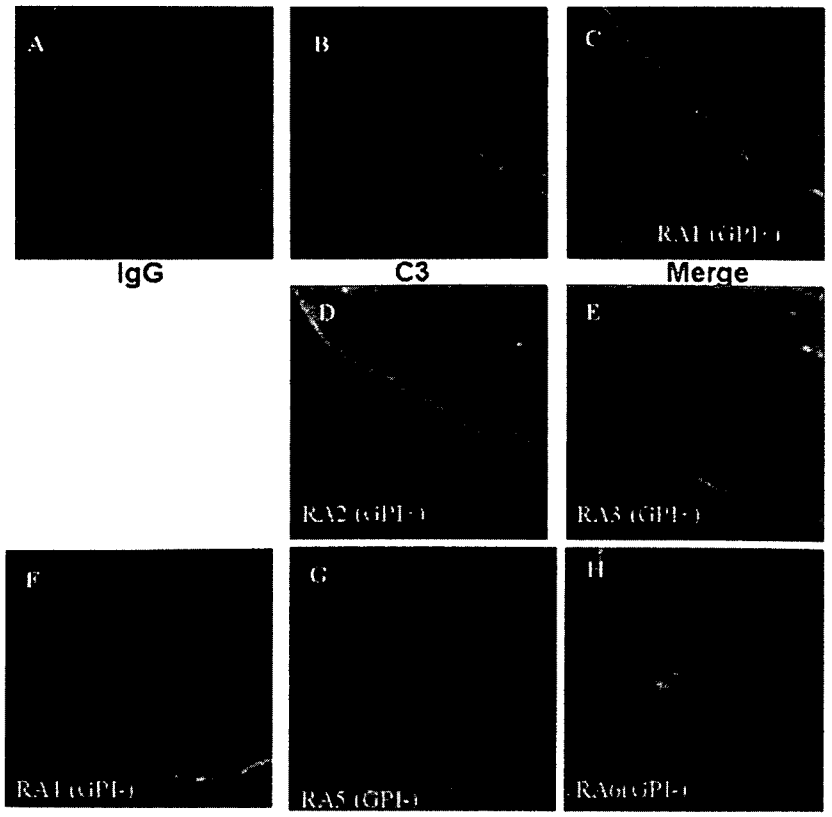


Figure 3. Immunocomplexes with C3 involvement in the synovial tissue of RA patients with anti-GPI Abs. Immunohistochemical study with IgG (red, A), C3 (green, B), and merge (yellow, C) were performed. RA1 (anti-GPI Abs-positive RA) (A-C), RA2 (anti-GPI Abs-positive RA) (D), RA3 (anti-GPI Abs-positive 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.

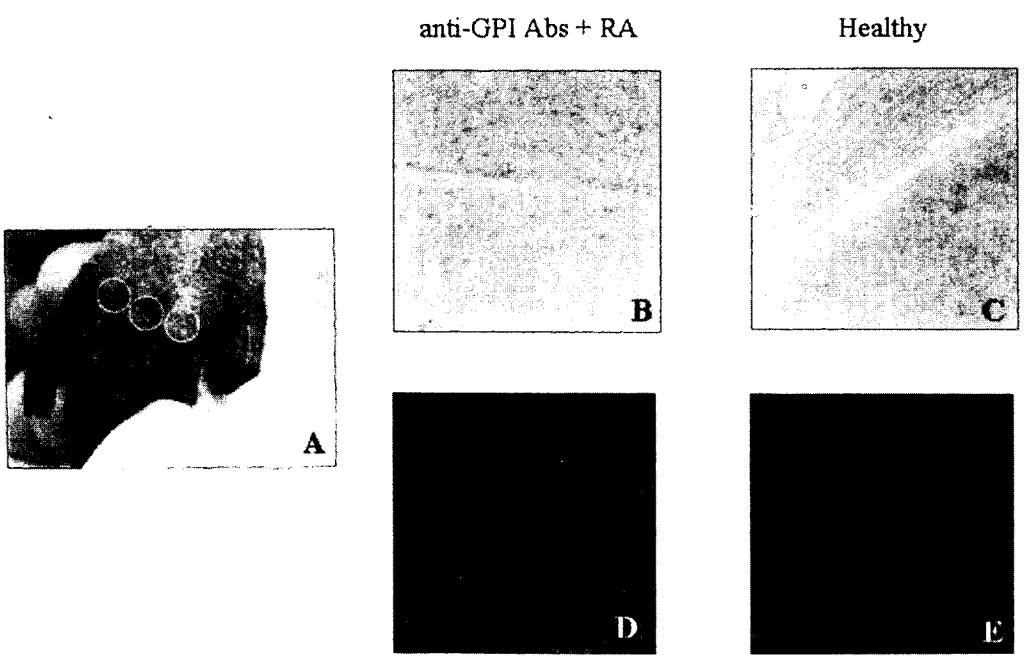


Figure 4. Serum IgG anti-GPI Abs from RA patients formed immunocomplexes on the articular surface and provoked infiltration of effector cells. Injection of human serum IgG into MP joints of cynomolgus monkeys (yellow circles in A represent the site of injection). Cryostat sections of MP joints of cynomolgus 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

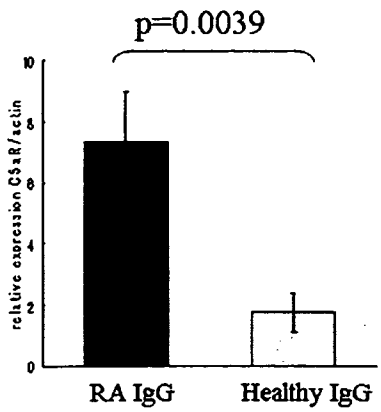
C5aR

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).

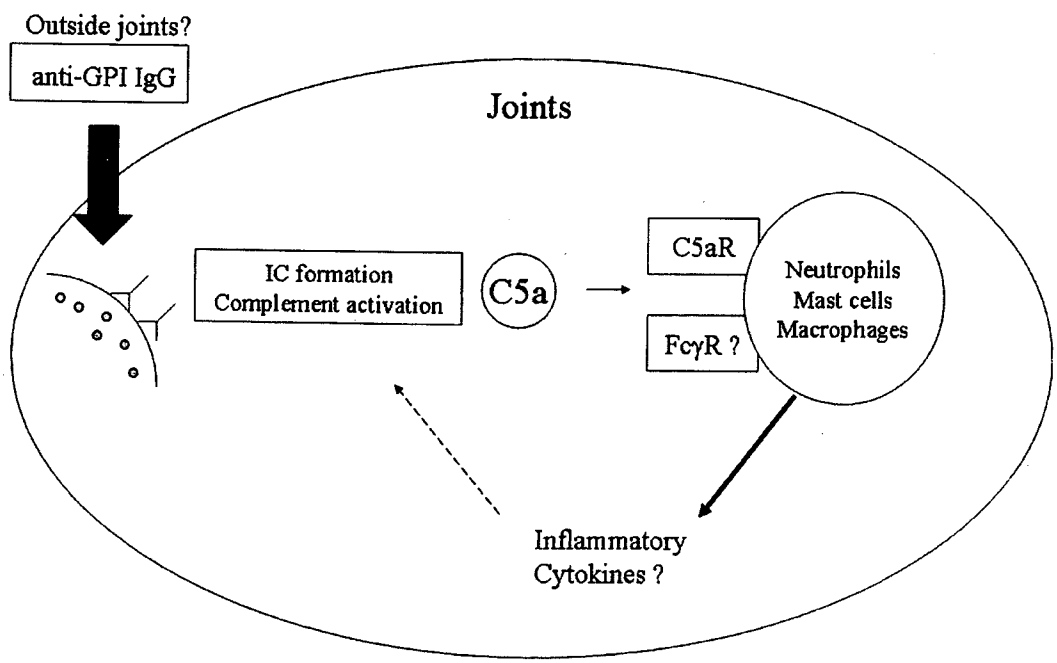


Figure 6. Schematic diagram of possible mechanisms of anti-GPI Abs-induced synovitis in RA. Anti-GPI Abs from RA patients attach directly to the surface of the joint cavity, recruit complement- and C5aR-positive cells, and ultimately result in synovitis. In addition to GPI, other ubiquitous antigens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

acattcctgc tactgaagaa gctggactcc 830
----a---a- ---------- ---------- 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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ORIGINAL ARTICLE

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Clinical characteristics of anti-glucose-6-phosphate isomerase antibody-positive Japanese patients with rheumatoid arthritis

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Abstract Anti-glucose-6-phosphate isomerase (GPI) antibodies (Abs) are known to be arthritogenic in mice. These Abs are elevated in several forms of arthritic condition in humans, although their prevalence in rheumatoid arthritis (RA) patients is still in debate. Some RA patients have increased levels of anti-GPI Abs, but their clinical manifestation and relevance to other Abs are not clearly elucidated. The aims of this study were to explore the clinical and hematological characteristics of RA with anti-GPI Abs, and to compare their prevalence in RA patients, systemic lupus erythematosus (SLE) patients, and healthy subjects (HS) in a Japanese population. Anti-GPI Abs were positive in 16 patients with RA (12%, $n = 137$), in 10 patients with SLE (8%, $n = 131$), and in 6 HS (4%, $n = 139$). C-reactive protein (CRP), immunoglobulin G, and the antinuclear antibody titer were higher in anti-GPI-positive patients than in those who were negative ($P = 0.049$, $P = 0.0003$, and $P = 0.002$, respectively). Moreover, the positivity of anti-GPI Abs was correlated with CRP more than with rheumatoid factor in RA patients. It is unclear whether anti-GPI Abs can predict the progress of disease, but the prevalence of these Abs was higher in active RA patients with severe arthritis, suggesting that anti-GPI Abs may be related to the pathogenesis of severe forms of arthritis.

Key words Auto-antibody · Clinical parameters · Glucose-6-phosphate isomerase (GPI) · Rheumatoid arthritis (RA) · Systemic lupus erythematosus (SLE)

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Introduction

Rheumatoid arthritis (RA) is the most common type of inflammatory polyarthritis. This disease is considered to be mainly an autoimmune disorder, although the detailed etiology is still unknown.[1] Several autoantibodies have been identified [e.g., rheumatoid factor, anti-cyclic citrullinated peptide (CCP) antibodies (Abs), and anti-fillagrin Abs] in the serum of RA patients, and many of them are useful markers for diagnosis, but they have not been proven to be pathogenic.

Anti-glucose-6-phosphate isomerase (GPI) Abs detected in the serum of K/BxN T-cell receptor transgenic mice[2,3] have been confirmed to have arthritogenic potential.[4] One research group reported a high prevalence (64%) of these Abs in RA patients,[5] although this observation could not be reproduced.[6–8] Because anti-GPI Abs are one of the major candidates for arthritogenic antibodies,[9] their relationship with certain clinical parameters should be elucidated in detail. The aims of this study were to find the characteristic hematological and clinical features of RA patients with anti-GPI Abs, and to clarify their prevalence in human RA patients and in a control group of Japanese subjects.

In anti-GPI Ab-positive RA patients, CRP was higher than in RA patients negative for these Abs ($P = 0.049$), suggesting that anti-GPI Abs are correlated with disease activity. Moreover, immunoglobulin G and antinuclear antibody titers were also higher in anti-GPI Ab-positive than in -negative patients ($P = 0.0003$ and $P = 0.002$, respectively), so it is possible that anti-GPI Ab production is relevant to production of some types of autoantibody. These findings suggest that anti-GPI Abs are correlated with severe arthritis in RA patients and might be a useful arthritic marker in some RA patients.

Materials and methods

Patients

Serum samples were obtained from 137 patients with RA (23 men, 114 women) in Tsukuba University Hospital. All RA patients satisfied the classification criteria of the American College of Rheumatology (1987).[10] Their mean age was 56.8 years (range, 15–85), and mean disease duration was 12.6 years (range, 1–46 years). At the time of this study, 133 patients were receiving medication (50 were receiving methotrexate, and 99 were receiving oral prednisolone).

Serum samples were also obtained from 131 patients with systemic lupus erythematosus (SLE) in Tsukuba University Hospital, and from 139 healthy subjects (HS). Their mean ages were 42.0 years (range, 18–75) and 32 years (range, 20–63), respectively. All SLE patients satisfied the 1997 revised American College of Rheumatology criteria for SLE.[11]

At the time of routine venepuncture, informed consent for drawing blood was obtained from all patients and participating HS.

Enzyme-linked immunosorbent assay for detecting anti-GPI antibody

Blood samples were centrifuged, and the serum obtained was divided into aliquots. To select anti-GPI-positive patients, we used recombinant human GPI (huGPI), which has been described in detail previously,[6] or rabbit muscle GPI (raGPI) (Sigma, St Louis, MO, USA). Both antigens were used at 5 μg/ml (diluted in phosphate-buffered saline, PBS) to coat microtiter plates (Sumilon S, Sumitomo Bakelite, Tokyo, Japan) (12 h, 4°C). After the plates were washed three times 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 incubated for 12 h at 4°C. After washing, alkaline phosphatase (AP)-conjugated anti-human IgG (Fc-fragment specific, American Qualex, San Clemente, CA, USA) was added to the plate (dilution: 1/500, 1 h, room temperature). After three washes, color was developed with AP reaction solution (containing 9.6% diethanol amine and 0.25 mM $MgCl_2$, pH 9.8) with AP substrate tablets (Sigma; one AP tablet per 5 ml of AP reaction solution). Plates were incubated for 1 h 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 huGPI-GST and raGPI, respectively). The cutoff OD was calculated from the enzyme-linked immunosorbent assay (ELISA) reaction of 137 healthy control Japanese donors. The mean value plus one standard deviation was 0.98 to human recombinant GPI, and 0.63 to rabbit native GPI. Double-positive populations were considered anti-GPI Abs-positive.

Rheumatoid factor, antinuclear antibodies, and immunoglobulin G (IgG) concentrations

Rheumatoid factor (RF) was determined by a nephelometric commercial test (RFII: Tina-quant, Nissui, Tokyo, Japan). Antinuclear antibodies (ANAs) were determined by a standard indirect immunofluorescence technique on HEp-2 cells. Serum ANA titer at >1/40 was considered positive.

The immunoglobulin G (IgG) concentration was evaluated by a sandwich ELISA.

Statistical analysis

Patient groups were compared using the χ-squared test for proportions. Differences were considered significant whenever $P < 0.05$. Statistical analysis was performed using StatView for Macintosh statistical software (StatView Software; SAS Institute, Cary, NC, USA).

Results

ELISA for detecting anti-GPI antibody

As discussed in length previously,[6] we utilized two different sources of GPI, mainly to avoid cross-reactivity or contamination during preparation; in particular, contaminated protein in native rabbit GPI has been reported previously.[7] The recombinant form of human GPI is 100% identical to human GPI protein (confirmed by sequencing), but it may not have the same conformation or posttranslational modifications as the natural enzyme. Thus, we also used native GPI from rabbit liver, which is commercially available. While native rabbit GPI is not strictly identical to the human GPI protein (93% identity), it provides the native conformation of the enzyme with normal posttranslational modifications. Double positivity for human and rabbit GPI Abs was clearly correlated with the positive Western blot results.[6] When discriminated in this way, positivity of anti-GPI Abs in the serum was 12% (16/137) in patients with RA, 8% (10/131) in those with SLE, and 4% (6/139) in HS. The distribution of anti-GPI Abs, especially those showing independent positivity to huGPI or raGPI in RA and SLE patients and HS, are summarized in Fig. 1. Statistical analysis showed no significant difference in the anti-GPI Ab positivity between RA and SLE patients ($\chi^2 = 1.251$ with 2 degrees of freedom, $P = 0.3618$), although a significant difference was found between RA patients and HS ($\chi^2 = 5.098$ with 2 degrees of freedom, $P = 0.0418$).

Comparison of clinical features of anti-GPI-positive and -negative RA patients

To analyze the differences in clinical manifestations between anti-GPI Ab-positive and -negative RA patients, we compared mean age, disease duration, sex, and X-ray stage. Mean age was 56.0 (range, 40–70) versus 56.8 (range, 15–85) years, and mean disease duration was 9.8 (range, 1–25) versus 12.6 (range, 1–46) years for positive and negative patients, respectively. All 16 (100%) anti-GPI Ab-positive RA patients were women, but 23 (19%) anti-GPI Ab-negative RA patients were men ($P = 0.0559$) (Table 1). In terms of X-ray stage, we divided the patients into two groups, those in stage I or II and those in stage III or IV. Nine (56%) patients were stage III or IV and 7 (44%) were stage I or II among anti-GPI Ab-positive RA patients. Among anti-GPI Ab-negative RA patients, 71 (61%) were stage III or IV and 45 (39%) were stage I or II ($P = 0.705$) (Table 1).

Table 1. Clinical features and antinuclear antibodies (ANAs) of anti-glucose-6-phosphate isomerase (GPI) antibody (Ab)-positive and -negative rheumatoid arthritis patients

	Anti-GPI Abs		*P*
	Positive	Negative	
Mean age ($n = 137$)	56	56.8	*0.9000*
Disease duration (years) ($n = 137$)	9.8	12.6	*0.2336*
Sex (no. of patients) ($n = 137$)			
Male	0	23	*0.0559*
Female	16	98	
Disease stage ($n = 132$)			
I or II	7	45	*0.7050*
III or IV	9	71	
ANAs (no. of patients) ($n = 94$)			
Positive	8	7	*0.0003**
Negative	8	71	

*Statistically significant

This analysis showed that anti-GPI Abs were expressed predominantly in female patients, but the disease progression was not clearly different between anti-GPI Ab-positive and -negative RA patients.

Comparison of laboratory data for anti-GPI-positive and -negative RA patients

To investigate whether anti-GPI Abs were relevant with respect to inflammation markers, we compared C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) between anti-GPI Ab-positive and -negative RA patients. The CRP concentration in anti-GPI Ab-positive RA patients (mean, 2.56 mg/dl; range, 0.11–10.30) was higher ($P = 0.049$) than that in anti-GPI Ab-negative RA patients (mean, 1.47 mg/dl; range, 0.02–8.71) (Fig. 2A). In contrast, ESR was comparable; mean ESR was 48.1 mm/h (range, 6–126) versus 42.3 mm/h (range, 2–146) ($P = 0.467$) in positive and negative patients, respectively (Fig. 2B). We also analyzed the maximum CRP concentration throughout the disease course of RA; the mean maximum CRP was 5.71 (range, 0.82–11.83) in positive patients, versus 3.91 (range, 0.03–14.74) in negative patients ($P = 0.106$). These findings suggest that RA patients with anti-GPI Abs had an active form of arthritis.

We also investigated the relationship between the Abs IgG, RF, and ANA between anti-GPI Ab-positive and -negative RA patients. The mean IgG concentration was 1819 mg/dl (range, 763–3308) in positive patients versus 1440 mg/dl (range, 576–2095) in negative patients ($P = 0.0003$) (Fig. 2C). IgA and IgM titers, by contrast, were comparable between positive and negative patients (data not shown). The mean RF titer at the time of collection was 189 U/ml (range, 6–992) in positive patients versus 138 U/ml (range, 2–2120) in negative patients ($P = 0.5372$) (Fig. 2D). To discriminate whether RF positivity had any connection to anti-GPI Abs positivity, we screened RF positivity

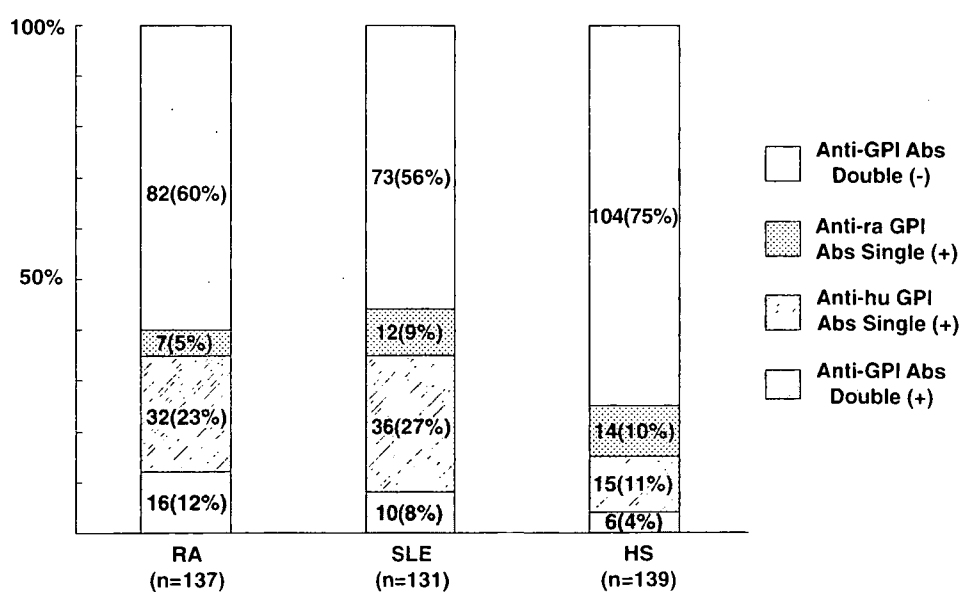

Fig. 1. Positivity against recombinant human (hu) and native rabbit (ra) glucose-6-phosphate isomerase (*GPI*) in rheumatoid arthritis (*RA*) and systemic lupus erythematosus (*SLE*) patients and healthy subjects (*HS*). This figure shows the distribution of the double-negative, single-positive (hu or ra), and double-positive population in patients with RA or SLE and in HS. *Ab*, antibody

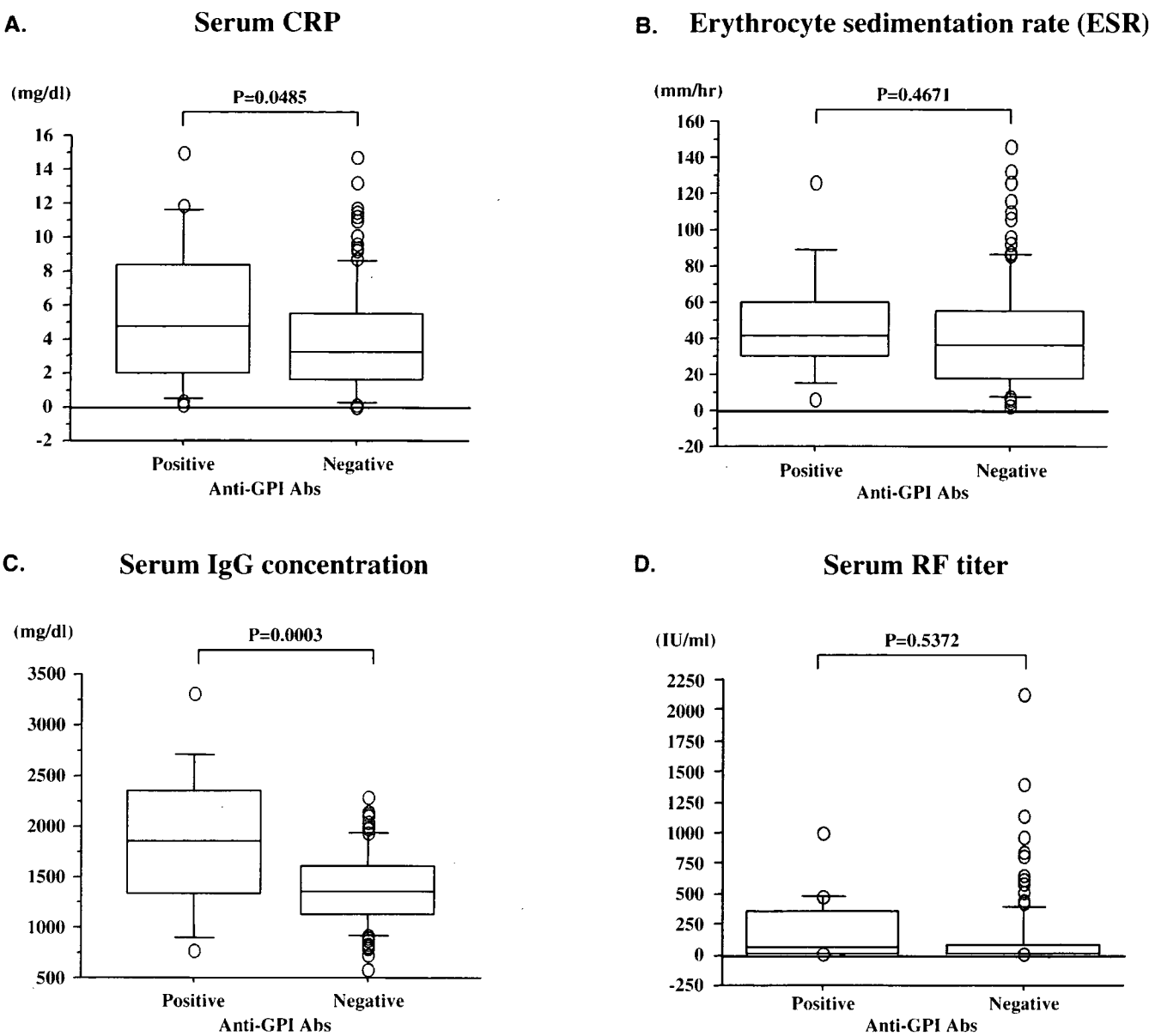

Fig. 2A–D. Comparison of laboratory data for anti-GPI-positive and -negative RA patients. **A** C-reactive protein (*CRP*); **B** erythrocyte sedimentation rate (*ESR*); **C** serum immunoglobulin G (*IgG*) concentration; and **D** the rheumatoid factor (*RF*) titer are compared. Each box represents statistical values. The intermediate line within the box marks the median, and the upper boundary of the box indicates the 75th percentile. The whiskers above and below the box indicate the 90th and 10th percentiles, respectively. The significance is expressed by the *P* values

throughout the disease course. Among anti-GPI Ab-positive RA patients, 13 (81%) were RF positive and 3 (19%) were negative. Among anti-GPI Ab-negative RA patients, 79 (66%) were RF positive and 40 (44%) were RF negative (P = 0.360). Because ANAs were not checked in all RA patients, we compared ANA positivity only in some of the RA patients. Among anti-GPI Ab-positive RA patients, ANAs were positive in eight (50%) and negative in eight (50%). In contrast, among anti-GPI Ab-negative RA patients, ANAs were positive in 7 (9%) and negative in 71 (91%) (P = 0.0003) (Table 1). The pattern of distribution of ANAs was not distinctive, but the prevalence was different between the two groups. In summary, anti-GPI Ab-positive RA patients had higher IgG and ANA positivity than negative patient, but the relationship between anti-GPI Ab positivity and RF was statistically unclear.

Consecutive follow-up study of anti-GPI Abs, RF, and CRP in an anti-GPI Ab-positive patient

To investigate the changes in hematological parameters in an anti-GPI Ab-positive patient, we checked anti-GPI Abs, RF, and CRP from the onset of arthritis. In 2000, at the disease onset, the patient was already positive for anti-GPI Abs, although completely negative for RF. In 2001, when

the disease was almost controlled, anti-GPI Abs and CRP had decreased, but RF had increased. In this patient, fluctuations of anti-GPI Abs were similar to those of CRP, suggesting that anti-GPI Abs might be a good marker for arthritis in some RA patients.

Discussion

The etiology of RA is multifactorial. To understand RA, autoAbs have been used as diagnostic tools and indicators of disease activity. Some autoAbs are elevated in RA patients, but a specific marker is not available.[12] Only few autoAbs have been identified as disease-specific Abs, and anti-GPI Abs are one of these candidate autoAbs identified has having arthritogenicity.[9] Here, we identified some anti-GPI Ab-positive RA patients in a Japanese cohort. Our ELISA assay was highly specific because we used both recombinant bacterial human GPI and native rabbit GPI; double positivity for the two antigens correlated significantly with the results of Western blotting for GPI.[6] However, the prevalence of these Abs in RA patients was only 12%; thus, their sensitivity is very low. In previous studies, when only native rabbit GPI was used, the prevalence of anti-GPI Abs in RA patients was 64%,[5] 45%,[13] or 23%.[14] Several research groups also stated that contaminant proteins introduced during rabbit GPI purification enhanced the reactivity.[6–8,14] On the other hand, can anti-GPI Ab-positive patients be accurately identified if we use only human recombinant GPI? In our previous study,[6] some cross-reactivity also occurred with human recombinant GPI, as confirmed by visualization of a band that was not identical to that of human recombinant GPI. This artifact was probably due to the lack of conformational structure and the glycosylation status of bacterial human recombinant GPI; the latter role was substituted for by native rabbit GPI in this study. Therefore, we utilized two different sources of GPI antigen to discriminate true-positive patients.

The prevalence of anti-GPI Abs was not high compared with that of anti-CCP Abs or RF. As long ago as several decades, RF was utilized as a useful diagnostic marker for RA. RF is elevated in 70%–80% of RA patients, but its pathogenic role is still uncertain.[15] As shown by our consecutive follow-up study of an RA patient, anti-GPI Abs were more highly correlated with disease activity than with RF, suggesting that anti-GPI Ab positivity is a good marker for discriminating the activity of RA in some patients (Fig. 3). Recently, anti-CCP Abs have been recognized as a better marker for the diagnosis of RA,[16] because of their early appearance[17] and their ability to predict the course of the disease.[18,19] We could not identify any difference in radiographic progression between anti-GPI Ab-positive and -negative RA patients, but almost all of the anti-GPI Ab-positive RA patients had elevated levels of anti-CCP Abs (unpublished observation, Yasukochi et al.). It is possible that GPI is also citrullinated and cross-reacts with CCP to some extent, so further analysis needs to be done.

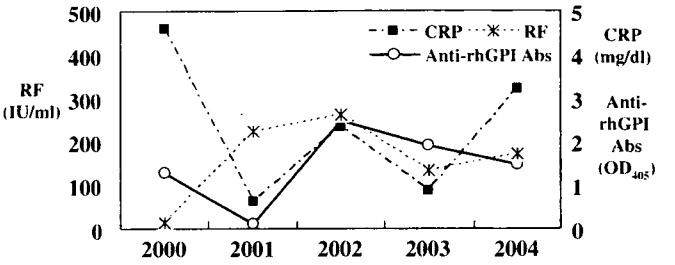

Fig. 3. Consecutive follow-up of hematological parameters in an anti-GPI Ab-positive patient. The figure shows changes in the hematological parameters (anti-rh GPI Abs, CRP concentration, RF titer) in the disease course of an anti-GPI Ab-positive RA patient. The anti-GPI Ab titer was more highly correlated with the CRP concentration than with the RF titer. This tendency was also detected in some other anti-GPI Ab-positive RA patients (data not shown). *OD*, optical density

Anti-GPI Ab-positive patients tend to have a high CRP concentration. Regarding the relationship between disease activity and anti-GPI Abs, several groups have reported that anti-GPI Ab positivity is correlated with disease activity and severity, in particular, extraarticular manifestations;[5,6,20] Felty's syndrome patients were highly represented in the first report.[5] Therefore, we also checked for the occurrence of vasculitis with RA (in Japanese, we termed this type of malignant RA "MRA"); only two MRA patients were part of the study, and one was anti-GPI Ab-positive and the other was negative. Unfortunately, there were no Felty's syndrome patients in our study. From these data, it is unclear whether anti-GPI Abs positivity is correlated with the occurrence of extraarticular manifestations in Japanese patients. However, some patients with C1q immune complexes were followed. Two anti-GPI Ab-positive patients among four RA patients (50%) had elevated levels of C1q immune complexes.

Anti-GPI Ab-positive patients had elevated levels of several Abs, including IgG and ANA ($P = 0.0003$ and $P = 0.002$, respectively). In the anti-GPI Ab-positive population, 70% of SLE patients had arthritis. It could be argued that among anti-GPI Ab-positive patients with RA or SLE, anti-GPI Abs are correlated with the occurrence of arthritis. Another possibility is that anti-GPI Abs are expressed as a result of polyclonal activation.

In summary, these findings suggest that anti-GPI Abs are correlated with severe arthritis in Japanese patients with RA, and so might be a useful arthritic marker in some RA patients. The role of this Ab in disease progression remains to be elucidated, and it is possible that there is an autoAb-dependent pathway in the development of human arthritis.[21]

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Multiple Autoimmune Diseases after Autologous Stem-Cell Transplantation

TO THE EDITOR: Hematopoietic stem-cell transplantation can be an effective treatment in patients with refractory systemic sclerosis.[1] We report on a 19-year-old woman with systemic sclerosis who underwent CD34+-selected autologous hematopoietic stem-cell transplantation in March 2001. Before the transplantation, the physical and laboratory findings showed no evidence of any other autoimmune diseases. After written consent was obtained from the patient, CD34+ hematopoietic stem cells were transplanted according to a method used for systemic sclerosis.[1] The dermal sclerosis improved immediately after transplantation, but thrombocytopenia and Graves' disease developed.

In June 2005, the patient was admitted to the hospital because of fever and edema. Blood tests revealed proteinuria (11.4 g per day) and new autoantibodies in the serum (Fig. 1A). On the sixth hospital day, paralysis developed on the left side as the result of a right cerebral infarction. Systemic lupus erythematosus with membranous-type lupus nephritis (Fig. 2) and the antiphospholipid-antibody syndrome were diagnosed; the patient was treated with prednisolone, warfarin, and cyclosporine. She is currently in clinical remission and is back at work.

During the early phases of immune reconstitution, residual lymphocytes undergo proliferation and expansion, a process controlled by regulatory T cells.[2,3] These cells, defined by the phenotype CD4+CD25+FOXP3+, are important in the prevention of autoimmunity. Interleukin-17–producing helper T (Th17) cells may play a role in the induction of autoimmunity.[4,5] In our patient, the level of serum interleukin-17, released mainly by Th17 cells, was elevated at the onset of the systemic lupus (Fig. 1B). Levels of *FOXP3* messenger RNA, a marker of regulatory T cells, were reduced, suggesting a deficiency of such cells (Fig. 1C). The findings in our patient suggest a role of both regulatory T cells and Th17 in the development of systemic lupus.

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1. Farge D, Passweg J, van Laar JM, et al. Autologous stem cell

Figure 1 (facing page). Clinical and Laboratory Findings after CD34+-Selected Autologous Hematopoietic Stem-Cell Transplantation.
Panel A shows the association between clinical events (including the onset of autoimmune thrombocytopenia [AITP], Graves' disease, systemic edema, and cerebral infarction) and changes in titers of each autoantibody. At the onset of edema, a serum sample from the patient contained anti-Sm, anti-Scl70, and anticardiolipin IgG antibodies (IgG-aCL), in addition to anti-DNA autoantibodies and lupus anticoagulant. The solid line indicates the modified Rodnan total skin thickness score (ranging from 0 to 51, with higher values indicating more thickness). Normal ranges for these levels are as follows: anti-Sm, 0 to 5.9 U per milliliter, anti-Scl70, 0 to 18.9 U per milliliter; and IgG-aCL, <1.3 U per milliliter. Panel B shows serum levels of interleukin-17, transforming growth factor β1 (TGF-β1), and interleukin-6. Normal ranges for these levels are as follows: TGF-β1, 30.95 to 38.65 ng per milliliter; interleukin-6, 0.54 to 1.10 ng per milliliter; and interleukin-17, not detected. Panel C shows changes in T cells, including the ratio of interferon-γ–producing CD4+ T cells (Th1) and interleukin-4–producing CD4+ T cells (Th2) and FOXP3 messenger RNA (mRNA) on peripheral-blood mononuclear cells. The solid squares indicate levels of CD19+ cells, and the circles indicate levels of CD4+CD25+ cells. Normal ranges are as follows: ratio of Th1 to Th2, 7.22 to 47.52; FOXP3 mRNA, 57.10 to 175.19 copies per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard; CD19+, 9.24 to 17.01%; and CD4+CD25+, 5.66 to 10.24%. Calculations were made with the JMP statistical software package, version 5.0 (SAS Institute).

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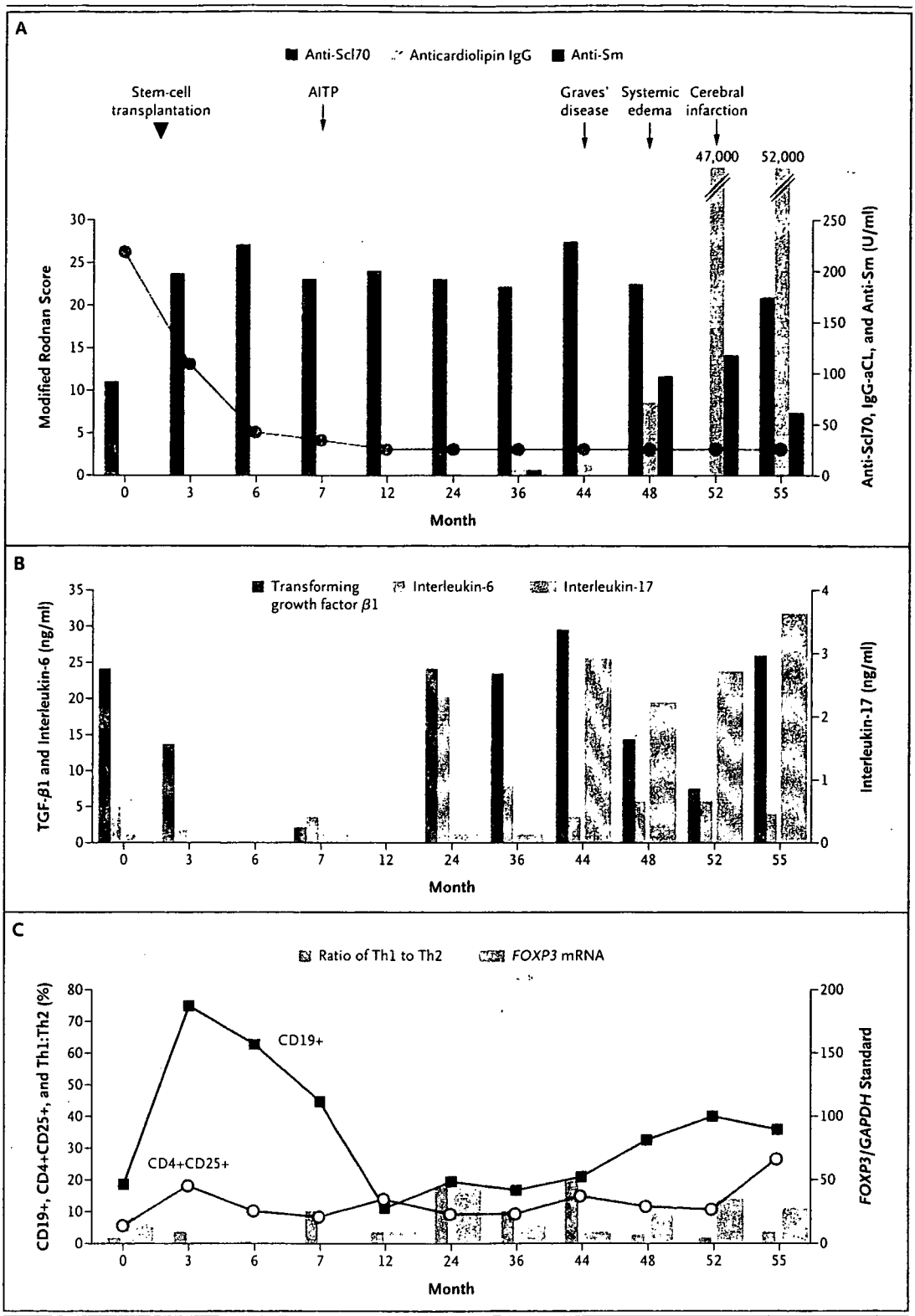
A
Anti-Scl70
Anticardiolipin IgG
Anti-Sm
Stem-cell transplantation
AITP
Graves' disease
Systemic edema
Cerebral infarction
47,000
52,000
Modified Rodnan Score
Anti-Scl70, IgG-aCL, and Anti-Sm (U/ml)
Month
B
Transforming growth factor β1
Interleukin-6
Interleukin-17
TGF-β1 and Interleukin-6 (ng/ml)
Interleukin-17 (ng/ml)
Month
C
Ratio of Th1 to Th2
FOXP3 mRNA
CD19+
CD4+CD25+
CD19+, CD4+CD25+, and Th1:Th2 (%)
FOXP3/GAPDH Standard
Month