Mod Rheumatol (2005) 15:258–263
DOI 10.1007/s10165-005-0405-y

ORIGINAL ARTICLE

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

Received: January 11, 2005 / Accepted: May 12, 2005

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

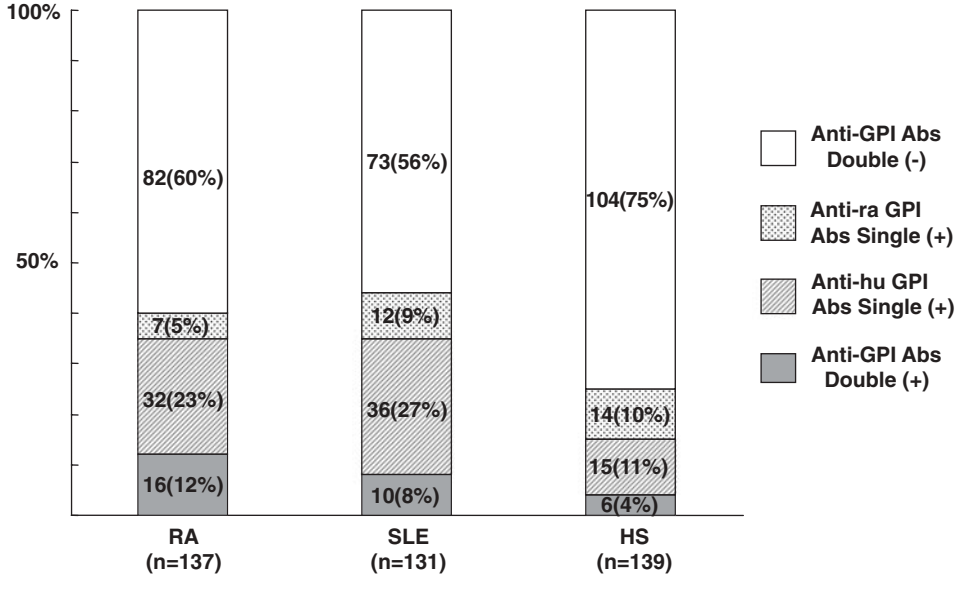

Fig. 1. Positivity against recombinant human (hu) and native rabbit (ra) glucose-6-phosphate isomerase (*GPI*) in rheumatoid arthritis (*RA*) and systemic lupus erythematosus (*SLE*) patients and healthy subjects (*HS*). This figure shows the distribution of the double-negative, single-positive (hu or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

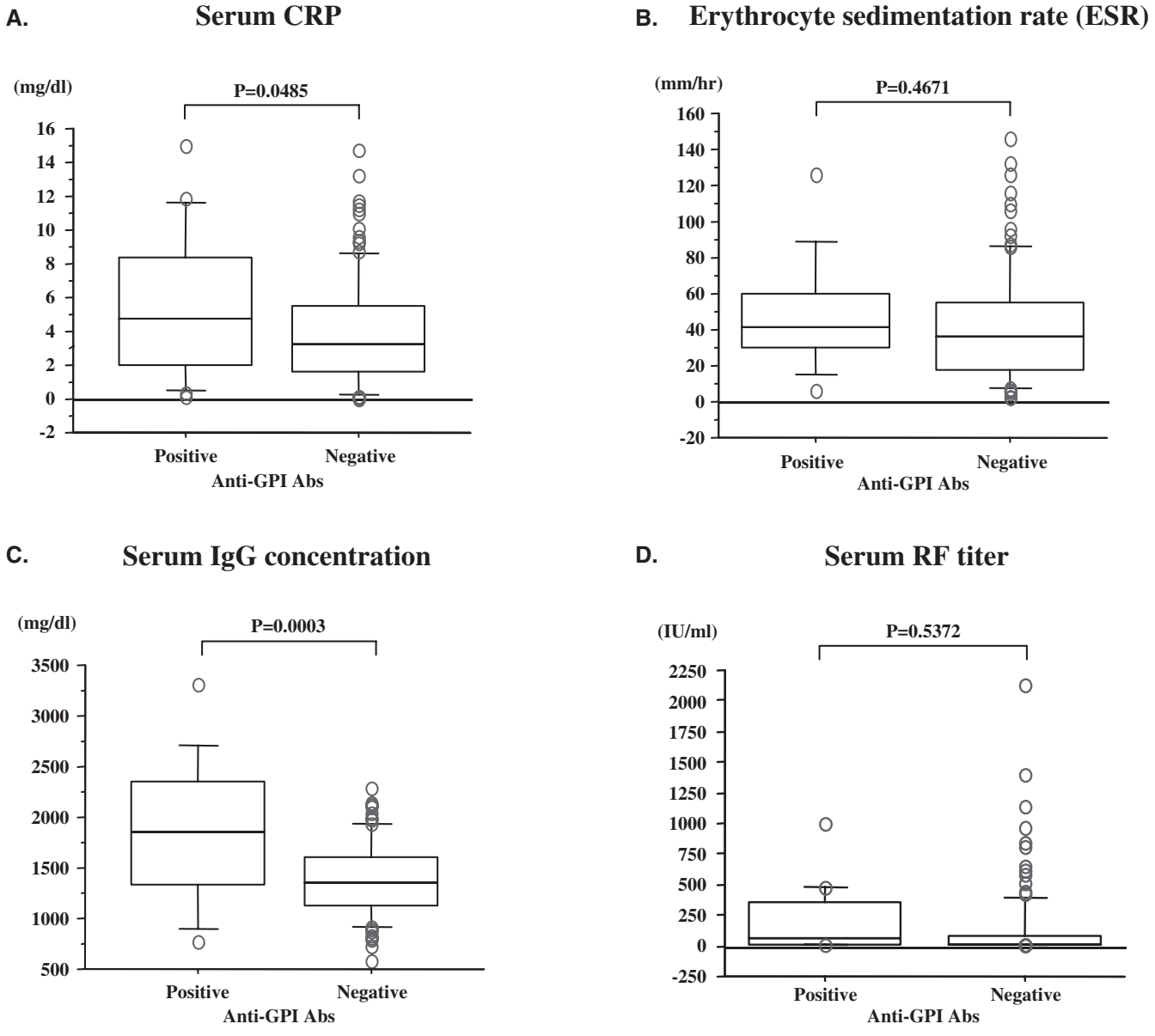

Fig. 2A–D. Comparison of laboratory data for anti-GPI-positive and -negative RA patients. **A** C-reactive protein (*CRP*); **B** erythrocyte sedimentation rate (*ESR*); **C** serum immunoglobulin G (*IgG*) concentration; and **D** the rheumatoid factor (*RF*) titer are compared. Each box represents 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.

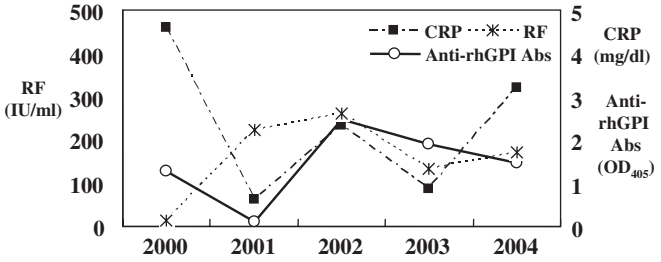

Fig. 3. Consecutive follow-up of hematological parameters in an anti-GPI Ab-positive patient. The figure shows changes in the hematological parameters (anti-rh GPI Abs, CRP concentration, RF titer) in the disease course of an anti-GPI Ab-positive RA patient. The anti-GPI Ab titer was more highly correlated 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]

Acknowledgments This work was supported in part by the Japanese Ministry of Science and Culture (I.M., T.S.) and PRESTO, Japan Science and Technology Agency (I.M.). I.M. is also a recipient of a fellowship from the Japan Intractable Diseases Research Foundation, Uehara Memorial Foundation, and Japan Rheumatoid Foundation. We thank Mrs. Chitose Okabe and Miss Hitomi Ikeda for their excellent technical assistance and Dr. Marianne Kimura for her critical reading of the manuscript.

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