Raised levels of anti-glucose-6-phosphate isomerase IgG in serum and synovial fluid from patients with inflammatory arthritis

M Schaller, W Stohl, S M Tan, V M Benoit, D M Hilbert, H J Ditzel

Background: In K/BxN mice, anti-glucose-6-phosphate isomerase (GPI) antibodies (Abs) are arthritogenic, and their transfer into naive mice induces arthritis. Anti-GPI Abs develop in many human patients with RA and are associated with more severe forms of the disease.

Objective: To elucidate the serum and synovial fluid (SF) anti-GPI IgG profiles among different patient groups with a variety of arthritides.

Methods: Blood and SF obtained concomitantly from 91 patients with clinically well defined arthritis were tested for concentrations of total anti-GPI IgG, anti-GPI IgG subclasses, B lymphocyte stimulator (BLyS), and APRIL by ELISA.

Results: Anti-GPI IgG was detected in sera and SF of patients with many arthritic diseases, but was preferentially associated with inflammatory arthritis, in general, and RA, in particular. The anti-GPI IgG subclass usage was skewed and varied among the different arthritic disease groups. Inverse correlations between serum levels of BLyS and anti-GPI IgG and positive correlations between serum levels of APRIL and anti-GPI IgG were seen among immune based arthritic patients and patients with RA but not among non-immune based patients. No correlations were found in SF from any group of arthritic patients.

Conclusion: Raised circulating anti-GPI Abs are not unique to patients with RA but are present in many patients with inflammatory arthritides. The difference in anti-GPI IgG subclass usage among disease groups may influence effector function and disease outcome. The inverse correlation between serum BLyS and anti-GPI IgG levels suggests that anti-GPI B cells may be regulated differently from other autoantibody producing B cells. Anti-GPI Abs may serve a pathogenic function in humans by promoting the maintenance of existing disease.

PATIENTS AND METHODS

Subjects were recruited for this study from the Los Angeles County and University of Southern California Medical Center.

Abbreviations: Abs, antibodies; AP, alkaline phosphatase; BLyS, B lymphocyte stimulator; Crys, crytal induced arthritis; ELISA, enzyme linked immunosorbent assay; GPI, glucose-6-phosphate isomerase; OA, osteoarthritis; PBS, phosphate buffered saline; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SpA, spondyloarthropathies; Tr, traumatic arthritis
concentration of total IgG (Accurate Chemical, M1590, m serial dilutions (0.02–5 \(100\) mM citric acid and dialysed against phosphate buffered research Institute and USC. concentrations by enzyme linked immunosorbent assay these patients have recently been reported. 34 Coded serum treatment data of the study patients. Purification of anti-GPI Abs from serum and SF of patients with RA I gG from this standard curve. After blocking for 2 hours at 37°C with 3% bovine serum albumin, sera and SF samples diluted 1:50–1:800 were incubated for 3 hours at 37°C. For the standard curve, the wells coated with standard serum were incubated with PBS in this step. After washing with PBS-0.05% Tween 20, the bound IgG from the serum or SF samples and the standard serum were detected with an alkaline phosphatase (AP) labelled F(ab')2 fragment of a goat antihuman IgG Fe-specific Ab (Jackson, West Grove, PA, 1:1000 in PBS), visualised with p-nitrophenyl phosphate substrate and read at 405 nm. As an internal standard, two RA sera were included, one with high and one with a moderate anti-GPI titre, as well as the affinity purified anti-GPI Ab preparation obtained from patients with RA. The exact concentration of anti-GPI IgG (in \(\mu g/ml\)) in the patient sera or SF was calculated from the standard curve in the linear range. Interassay variation was <15%. For the anti-GPI IgG subclass distribution analysis the following subclass-specific Abs: murine antihuman IgG1 at 1 \(\mu g/ml\), murine antihuman IgG2 and murine antihuman IgG4 at 5 \(\mu g/ml\) (all BD Pharmingen, San Diego, CA), murine antihuman IgG3 at 5 \(\mu g/ml\) (Zymed, San Francisco, CA), were incubated for 1 hour at 37°C. Bound anti-IgG subclass specific Abs were detected with an AP labelled goat antimurine F(ab')2 fragment specific antibody (Jackson, West Grove, PA), visualised with p-nitrophenyl phosphate substrate, and read at 405 nm. As standards, serial dilutions (0.02–20.0 \(\mu g/ml\)) of a serum with known concentration of each of the four IgG subclasses as well as a serum for specific quantification of IgG2 (both from Accurate Chemical, M1564 Westbury, NY) were coated on the same ELISA plate, allowing calculation of the exact concentration of anti-GPI IgG from this standard curve. BlyS and APRIL levels were determined by ELISA as described previously. 29 32 36

Table 1 Subject demographic and drug treatment

<table>
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<th>Age (years), mean (range)</th>
<th>RA (n = 37)</th>
<th>OA (n = 13)</th>
<th>Tr (n = 6)</th>
<th>Cry (n = 14)</th>
<th>SpA (n = 11)</th>
<th>Other (n = 10)</th>
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<td>3/3</td>
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Ar, Arabic; As, Asian; B, Black; H, Hispanic; W, White.

Westbury, NY) were coated on the same ELISA plate, allowing calculation of the exact concentration of anti-GPI IgG from this standard curve. After blocking for 2 hours at 37°C with 3% bovine serum albumin, sera and SF samples diluted 1:50–1:800 were incubated for 3 hours at 37°C. For the standard curve, the wells coated with standard serum were incubated with PBS in this step. After washing with PBS-0.05% Tween 20, the bound IgG from the serum or SF samples and the standard serum were detected with an alkaline phosphatase (AP) labelled F(ab')2 fragment of a goat antihuman IgG Fe-specific Ab (Jackson, West Grove, PA, 1:1000 in PBS), visualised with p-nitrophenyl phosphate substrate and read at 405 nm. As an internal standard, two RA sera were included, one with high and one with a moderate anti-GPI titre, as well as the affinity purified anti-GPI Ab preparation obtained from patients with RA. The exact concentration of anti-GPI IgG (in \(\mu g/ml\)) in the patient sera or SF was calculated from the standard curve in the linear range. Interassay variation was <15%.

For the anti-GPI IgG subclass distribution analysis the following subclass-specific Abs: murine antihuman IgG1 at 1 \(\mu g/ml\), murine antihuman IgG2 and murine antihuman IgG4 at 5 \(\mu g/ml\) (all BD Pharmingen, San Diego, CA), murine antihuman IgG3 at 5 \(\mu g/ml\) (Zymed, San Francisco, CA), were incubated for 1 hour at 37°C. Bound anti-IgG subclass specific Abs were detected with an AP labelled goat antimurine F(ab')2 fragment specific antibody (Jackson, West Grove, PA), visualised with p-nitrophenyl phosphate substrate, and read at 405 nm. As standards, serial dilutions (0.02–20.0 \(\mu g/ml\)) of a serum with known concentration of each of the four IgG subclasses as well as a serum for specific quantification of IgG2 (both from Accurate Chemical, M1564 Westbury, NY) were used. The exact concentration of anti-GPI Abs (in \(\mu g/ml\)) in the patient sera and SF was calculated from the linear range of the standard curves, as described above. Interassay variation was <20%.

The total anti-GPI IgG concentration as measured by adding the individual anti-GPI subclass concentrations was shown to correlate (r = 0.768) with the total anti-GPI IgG concentration measured using a polyclonal anti-Fc Ab, such as represented in figs 1A, 1B, and fig 2A, indicating that the anti-Fc Ab detected the different subclasses equally well.

BlyS and APRIL levels in serum and SF samples BlyS and APRIL levels were determined by ELISA as described previously. 29 32 36

Statistical analysis All analyses were performed using either Statview 4.1 (Abacus Concepts Inc, Cary, NC) or SigmaStat (SPSS, Chicago, IL) software. Because neither sera nor SF samples were distributed normally, non-parametric testing using the
Mann-Whitney rank sum test between two groups, or Kruskal-Wallis one way analysis of variance on ranks among three or more groups was performed. Correlations were determined by the Spearman rank order correlation.

RESULTS

Detection of anti-GPI Abs in serum and SF

Several previous studies of circulating anti-GPI Ab levels have reported results as arbitrary units. To quantify anti-GPI Ab concentrations as \( \mu g/ml \) of IgG, a human IgG anti-GPI Ab standard was prepared by affinity purification of sera from patients with RA with high titre anti-GPI Abs. Analysis of the purified human anti-GPI IgG showed that the antibody preparation bound specifically to hGPI (but minimally to a panel of control antigens, fig 1A) and had a functional affinity comparable to that of serum from 60 day old arthritic K/BxN mice (fig 1B). In contrast, little GPI binding was seen in total IgG from sera of healthy donors purified by the same procedure as that used for the human RA sera and the flowthrough from the GPI column of the RA IgG fraction.

Using both the affinity purified anti-GPI Ab preparation and a standard serum with known total IgG and IgG subclass concentrations as reference standards, we tested sera from 37 patients with RA, 13 with OA, 6 with Tr, 14 with Crys, 11 2.5 2.0 1.5 1.0 0.5 0

Figure 1 Anti-GPI-specific IgG purified from the serum of a patient with RA with high anti-GPI titre. (A) Samples from different purification steps, including the RA serum before purification, the purified total IgG fraction, and the purified anti-GPI IgG fraction, were tested for their binding to rabbit GPI (rGPI), human GPI (hGPI), bovine serum albumin, and glutathione-S-transferase as control antigens in an ELISA. Mouse K/BxN serum and normal serum from a healthy subject (NS), were included as positive and negative controls. The fraction containing the purified anti-GPI IgG preparation was highly specific for hGPI and did not react with the other control antigens. (B) Titration of the human anti-GPI IgG fraction showed positive GPI reactivity down to 0.06 \( \mu g/ml \) of IgG, comparable to the mouse K/BxN serum, whereas no reactivity was found in the remaining RA IgG fraction after affinity purification.

Figure 2 Anti-GPI IgG concentration in sera and SF of patients with various arthritic diseases. Sera (A) and SF (B) obtained concomitantly from 37 patients with RA, 13 with OA, 6 with Tr, 14 with crystal induced arthritis (Crys), 11 with seronegative SpA, 10 with other forms of inflammatory arthritis (Other) and in 21 normal healthy control sera (NS) were tested for binding to recombinant hGPI by ELISA. Bound IgG was detected with an AP conjugated F(ab)\(_2\) goat antihuman IgG-Fc and the concentration calculated according to a standard serum with known concentration of IgG. The cut off points for positivity were calculated as the 95% centile of the concentrations in healthy control subjects (>35.4 \( \mu g/ml \)). Immune based arthritis (Imm) includes patients with RA, SpA, SLE, undifferentiated inflammatory polyarthritis, and polymyalgia rheumatica. Non-immune based arthritis (Non) includes patients with OA, Tr, Crys, infectious arthritis, and adenocarcinomatous arthritis. The lines inside the boxes indicate the medians, the outer boxes indicate the 25th and 75th centiles; the bars extending from the boxes indicate the 10th and 90th centiles.
with SpA, and 10 with other diagnoses. Measurable anti-GPI IgG Abs were routinely detected in all but the OA patient groups (fig 2A), although the anti-GPI IgG concentrations and frequencies of anti-GPI IgG positive sera varied significantly among the patient groups (p < 0.001). Positivity for serum anti-GPI IgG was defined as a concentration greater than the 95th centile of the anti-GPI IgG concentration of 21 sera from normal healthy controls (≥35.4 μg/ml). Eighteen of 37 (49%) RA sera, 3/6 (50%) Tr sera, 5/14 (36%) Crys sera, 4/11 (36%) SpA sera, 3/10 (30%) Other sera (1 SLE, 1 tuberculosis arthritis, and 1 undifferentiated inflammatory polyarthritis), and 0/13 OA sera were positive for anti-GPI IgG Abs. The median concentration of anti-GPI IgG Abs (32.8 μg/ml, range 0.8–72 μg/ml) in serum from patients with RA was significantly higher than that in serum from patients with OA (0.5 μg/ml, p < 0.0001) or in normal healthy control sera (7.6 μg/ml, p = 0.028). However, no statistically significant differences in the median concentration of anti-GPI IgG Abs were found between the RA and the Crys (29.7 μg/ml), Tr (31.0 μg/ml), SpA (27.7 μg/ml) or Other (32.0 μg/ml) groups.

We also measured anti-GPI IgG Abs in SF samples using the cut off value described for the serum samples (>35.4 μg/ml) to determine positivity (fig 2B). Twenty of 37 (54%) RA SF, 5/13 (38%) OA SF, 5/14 (36%) Crys SF, 2/6 (33%) Tr, 5/11 (45%) SpA SF, and 6/10 (60%) Other SF were positive. The median concentration of anti-GPI IgG Abs in the SF samples was significantly higher than that of the serum samples (p < 0.0001). The median SF anti-GPI IgG Concentration in the RA (42.1 μg/ml, range 1–266 μg/ml, p = 0.041), SpA (86.9 μg/ml, p = 0.03), and the Other groups (87.0 μg/ml, p = 0.0257) was significantly higher than that in the OA group (13.5 μg/ml). Grouping the SF samples according to the 95th centile of the mean anti-GPI concentration of 21 sera from healthy controls (for IgG1 >29.0 μg/ml, for IgG2 >12.5 μg/ml, for IgG3 >6.1 μg/ml and for IgG4 >2.0 μg/ml). Only one RA serum sample, and none from the other arthritis groups, exhibited an IgG1 subclass anti-GPI response, the subclass generally constituting the major part of the Ab response to proteins (table 2). The RA sera predominantly contained IgG3 and IgG4 anti-GPI Abs; OA and Crys sera predominantly contained IgG2 anti-GPI Abs; and Reiter’s syndrome sera predominantly contained IgG4 anti-GPI Abs (fig 3, table 2). The median concentration of anti-GPI IgG3 in RA sera was significantly higher than in Crys (p = 0.003), Reiter’s syndrome (p = 0.016), and OA sera (p = 0.003). The sum of the anti-IgG1–4 concentrations correlated well (r = 0.768) with the directly measured total IgG anti-GPI concentrations, indicating that the secondary antibody used in the ELISA was able to detect anti-GPI IgG of different subclasses.

### Table 2 Percentage of patient serum samples positive for anti-GPI IgG subclasses

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<tr>
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<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
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<tr>
<td>RA (n = 37)</td>
<td>2.7</td>
<td>2.7</td>
<td>21.6</td>
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<tr>
<td>Crys (n = 12)</td>
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<tr>
<td>Reiter’s syndrome (n = 6)</td>
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<td>–</td>
<td>66.6</td>
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<tr>
<td>OA (n = 12)</td>
<td>–</td>
<td>33.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NS (n = 21)</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
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</tr>
</tbody>
</table>

RA, rheumatoid arthritis; Crys, crystal induced arthritis; OA, osteoarthritis; NS, normal serum.

### Correlations between anti-GPI IgG concentration and BlyS or APRIL protein levels in serum and SF samples of arthritic patients

Previous studies have demonstrated positive correlations between serum BlyS and specific autoantibody levels. To assess whether similar correlations exist between BlyS or APRIL and anti-GPI IgG levels, we measured anti-GPI levels in paired sets of sera and SF in which BlyS and APRIL levels had already been determined. Surprisingly, serum anti-GPI concentrations correlated inversely, rather than positively, with serum BlyS levels among all arthritic patients regardless of their diagnoses (fig 4A, p = 0.008), but did correlate positively with serum APRIL levels (fig 4B, p = 0.028). These correlation patterns were mimicked when only patients with immune based arthritis (figs 4E and F, p = 0.010, p = 0.089) or only patients with RA (figs 4I and J, p = 0.052, p = 0.014) were evaluated but not when patients with non-immune based arthritis were evaluated (data not shown). In contrast with the serum findings, no significant correlations between the concentrations of anti-GPI IgG and BlyS or APRIL in SF were found in any of these patient cohorts (figs 4C and D, G and H, K and L).
The inverse correlation among patients with RA between serum levels of BLyS and anti-GPI IgG was especially surprising, because serum levels of BLyS and RF have previously been shown to correlate positively with each other. When we focused on the 18 patients with RA for whom we had concurrent serum BLyS and RF levels, we again found a positive correlation between the levels of serum RF and BLyS (fig 5A) with no correlation seen between serum RF levels and serum APRIL levels (fig 5B). Among this subset of patients with RA, we again found an inverse correlation between the levels of serum anti-GPI IgG and serum RF levels (fig 5C).

DISCUSSION
To assess the potential human relevance of anti-GPI mediated arthritis in K/BxN mice, we measured anti-GPI levels in sera and SF from human patients with a wide variety of arthritic conditions. We detected anti-GPI Abs in a large proportion of patients with RA (fig 2), although at a frequency somewhat lower than that previously reported (49% vs 64%), and also in sera and SF from patients with other forms of arthritis. Serum anti-GPI IgG Ab levels were significantly higher in patients with immune based arthritis compared with non-immune based arthritis, demonstrating that anti-GPI Abs are not disease-specific for RA but, rather,
Further studies are needed, therefore, to determine whether the differences in IgG subclass distribution of the anti-GPI Abs found in different arthritic diseases are indeed pathogenically important.

The particular IgG subclass profile found for each type of arthritide seem to be specific for the anti-GPI response and not a general pattern of autoantibody responses to common autoantigens. In contrast with the subclass profiles we observed for the anti-GPI response, Cook et al found that the type II collagen response in patients with RA was predominantly IgG1 and IgG3, and in patients with OA and SLE predominantly IgG4.41 Further, Cambridge et al found that the anti-myeloperoxidase response in patients with RA was predominantly of the IgG1 and IgG3 subclasses,42 whereas Todome et al found that the anti-streptococcal cell wall peptidoglycan response in patients with RA was predominantly IgG2.43

Recently, increased circulating levels of BlyS have been detected in several B cell dependent autoimmune diseases, and a positive correlation between the circulating BlyS levels and the magnitude of systemic autoimmune antibody responses such as anti-dsDNA, anti-Sm, anti-SSA/SSB, antiphospholipid, and RF has been observed.27–29 44 These correlations and results from animal models suggest that BlyS may preferentially affect autoreactive B cells, leading to autoantibody production. It was, thus, highly surprising that instead of observing a positive correlation between serum BlyS and anti-GPI IgG levels, we routinely observed among our arthritic patients positive correlations between serum APRIL and anti-GPI IgG levels but inverse correlations between serum BlyS and anti-GPI IgG (fig 4). The inverse correlations between serum BlyS and anti-GPI IgG levels were found despite a positive correlation between serum BlyS and RF levels in patients with RA (fig 5).

A possible explanation for this surprising result is based on the highly complex relation between BlyS and its receptors. The three known receptors for BlyS (BAFF-R, TACI, and BCMA) are all present on B cells to various degrees.45 The last two receptors can also bind APRIL.46 Whereas BlyS binding to BAFF-R leads to maturation of B cells at several differentiation stages, BlyS binding to TACI inhibits B cell activation,47 thereby maintaining B cell homeostasis. Promotion of autoantibody production by BlyS may be mediated through BAFF-R, which has been shown to be the major BlyS receptor on human primary B cells.48 However, TACI is up regulated in activated B cells, and incubation with agonistic anti-TACI Abs inhibits the ability of BlyS to costimulate B cell activation.49 Possibly, anti-GPI producing B cells express high surface levels of TACI, which, upon engagement with BlyS, down modulate activation of these B cells. Thus, high circulating levels of BlyS might be associated with low levels of circulating anti-GPI Abs.

An important role for APRIL may emerge from the recent findings that plasmablasts (CD38+) up regulate surface expression of BCMA,7 rendering them sensitive to survival signals delivered by both BlyS and APRIL. Findings that APRIL circulates at higher concentrations in patients with RA,45 and that APRIL levels are positively correlated with anti-GPI Abs concentrations (figs 4B, F, J) may indicate that plasmablasts could be the anti-GPI producing cells, and are especially sensitive to APRIL. Our findings raise the intriguing question of whether BlyS or APRIL antagonistic treatment would actually lower autoantibody levels of all specificities. Current clinical trials in patients with SLE with a
neutralising anti-BlyS Ab may help to answer this question for SLE associated autoantibodies.

In conclusion, our results indicate that anti-GPI Abs are frequently found not just in patients with RA but also in patients with other forms of inflammatory arthritis. Assaying for anti-GPI IgG is not straightforward and may account for some of the conflicting results reported. Our data suggest that anti-GPI IgG is not the primary disease causing event, but may perpetuate disease, in agreement with results from van Gaalen et al., who found high titres of anti-GPI Abs particularly associated in patients with severe forms of RA—that is, Felty’s syndrome, vasculitis, or rheumatoid nodules. Future studies will examine whether neutralisation of anti-GPI IgG will alleviate the clinical and/or pathological manifestation of inflammatory arthritis.

ACKNOWLEDGEMENTS

We thank Charysh Randolph for her outstanding technical assistance and M. Kat Occhipinti for editorial assistance. Supported in part by grants from the NIH (HL63651) and the Danish Medical Research Council and a fellowship to Dr. Schaller. M. Schaller, V. M. Benoit, H. J. Ditzel, and T. Hirano are employed by the National Arthritis Foundation (Sontag Foundation fellowship) to Dr. Schaller.

REFERENCES