Glucose-6-phosphate isomerase is not a specific autoantigen in rheumatoid arthritis

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Objective. To test the hypothesis that glucose-6-phosphate isomerase (GPI) is a novel autoantigen in RA.

Methods. Eighty-eight serum samples from 23 patients with rheumatoid arthritis (RA), 25 with Sjögren’s syndrome, 20 with systemic lupus erythematosus and 20 healthy controls were tested by enzyme-linked immunosorbent assay (ELISA) using a commercially available, partially purified rabbit GPI as antigen. Beside each duplicate well containing antigen (10 µg/ml), uncoated blocked duplicate wells (phosphate-buffered saline only) were included as controls for non-specific binding for every serum tested. We also examined antibodies binding to various polypeptides in the GPI preparation by immunoblotting in 73 of the sera.

Results. By ELISA, binding levels were low and there was no difference between serum from patients with RA, other rheumatic diseases and normal controls. By immunoblotting, antibodies binding to the GPI polypeptide were present in 70–80% of all groups tested. In addition, we showed that another polypeptide identified as phosphoglucomutase was also present in the preparation and reacted with human immunoglobulins.

Conclusion. Our findings suggest that GPI is not a specific autoantigen in RA.

Key words: Glucose-6-phosphate isomerase, Rheumatoid arthritis, Specific autoantigen.

Amongst the autoimmune rheumatic diseases, the autoantigens in rheumatoid arthritis (RA) have been particularly elusive. At least three have stood the test of time and reproducibility in independent laboratories: the antiperinuclear factor, anti-rat oesophageal keratin and the Sa antigen [1, 2]. It was recently proposed that the three antigens are structural proteins (fillagrin, keratin and vimentin) whose arginines are deiminated to form citrulline. This would account for the reactivity of such apparently diverse proteins with autoantibodies in RA [3, 4]. It was therefore a surprise to us that a completely unrelated protein, glucose-6-phosphate isomerase (GPI) was recently proposed as a novel autoantigen in RA [5]. Using a commercially available semi-purified enzyme, 64% of humans with RA had high levels of antibodies, compared with healthy controls and those with Sjögren’s syndrome (SS) [5]. Antibodies to GPI were also detected by immunoblotting in 12 RA serum samples but not in an unspecified number of healthy controls [5]. The observation was cited as providing a linkage with a mouse model of RA transgenic for a T-cell receptor [6] which recognizes peptides from GPI. Taken together, these studies suggest that GPI could be a novel and perhaps unexpected autoantigen in RA.

More recently, two studies [7, 8] challenged this finding by suggesting that antibodies to GPI were rare, and not specific for RA. One study [7] also showed that the antigen preparation contained polypeptides other than GPI, including creatine kinase which was contaminating the preparation. The original authors then challenged these findings by showing that recombinant GPI gave similar results to the commercial preparation and that contaminants were therefore not contributing to false-positive results [9]. It was therefore concluded that the question as to whether GPI was a specific autoantigen in RA was unresolved.

In this study we use a similar enzyme-linked immunosorbent assay (ELISA) to that originally reported, but corrected for background in every serum tested. We also analysed the components of the antigen by...
mass spectrometry and examined antibodies to various polypeptides in the GPI preparation by immunoblotting in a much larger number of sera than previously reported.

**Methods**

Serum samples from 88 patients and controls, taken with informed consent and approval by the Riverside Regional Ethics Committee (RREC 102), were tested by ELISA using the same antigen as that used by Schaller et al. (Sigma, cat. number P-9544) [5] and with an almost identical method. Sera diluted 1:50 in phosphate-buffered saline (PBS) were from 23 patients with RA fulfilling revised American College of Rheumatology criteria, 25 patients with SS, 20 with systemic lupus erythematosus (SLE) and 20 healthy controls. Beside each duplicate well containing antigen (coated overnight at room temperature in 50 µl at 10 µg/ml) uncoated blocked duplicate wells (PBS only) were included as controls for non-specific binding for every serum tested. The plates were blocked with PBS containing 2% casein (BDH, Poole, UK) and 0.05% Tween-20 for 2 h at room temperature. Incubation with the serum for 2 h at 37 °C, as reported [5], resulted in such a high background that we used the more conventional incubation of 2 h at room temperature. Bound antibody was detected with peroxidase-conjugated goat anti-human IgG (whole molecule) (Bio-Rad, Hertfordshire, UK) incubated for 1 h. After seven washes with 0.05% Tween-20 PBS, substrate solution [3,3′,5,5′-tetramethylbenzidine (TMB), KPL Laboratories, Insight Biotechnology Ltd, Middlesex, UK] was added for a few minutes and stopped with H₂SO₄ (1:16). The absorbance at 450 nm was measured with a Victor 1420 Multilabel Counter (Wallac and Berthold, Crownhill, Milton Keynes MK8 0AB, UK).

The GPI was examined by 10% polyacrylamide electrophoresis and Coomassie staining. To identify the bands, they were excised from the Coomassie-stained gel, digested with trypsin according to published methods [10] and characterized by matrix-assisted laser desorption ionization mass spectrometry (MS) and by tandem electrospray MS.

Seventy-three serum samples were tested by immunoblotting. After being reduced by boiling for 5 min with dithiothreitol, GPI (7 µg/well) was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Immobilon PVDF membranes (Millipore, Hertfordshire, UK). Blots were blocked for 2 h at room temperature with 5% milk–PBS–0.05% Tween, incubated with human sera at a dilution of 1:100 for 1 h at room temperature and developed with the same peroxidase conjugate used in the ELISA for 1 h at room temperature. Reactive bands were visualized by chemiluminescence using ECL (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK).

**Results**

Using the ELISA method reported [5] we found that most of the binding was due to background, i.e. binding to the plate regardless of the presence of antigen. Incubation of the conjugate in the absence of serum indicated that the background was not due to its binding to the GPI preparation. By substituting casein for milk powder as a blocking agent and by performing incubations at room temperature rather than 37 °C, background binding was reduced to more acceptable levels. Under these conditions, there was no difference in the level of antibodies in RA compared with SS, SLE or healthy controls, whether the background was subtracted (Fig. 1a) or not (not shown). By SDS-PAGE, the preparation of GPI contained a dominant band at 55 kDa and a fainter one at 62 kDa, as well as a few lighter bands around 30 kDa (Fig. 1b). Mass spectrometry and tandem electrospray MS indicated that the major band at 55 kDa was GPI. The bands at 62 kDa and 30 kDa were identified as rabbit phosphoglucomutase and fructose-1,6-bisphosphatase, respectively. Traces of rabbit creatine kinase M chain were also present.

We then examined 73 of the sera by immunoblotting. Sera from patients with RA, SS, SLE and healthy controls (Fig. 1c and Table 1) reacted with rabbit GPI at 55 kDa and a fainter one at 62 kDa, as well as a few lighter bands around 30 kDa (Fig. 1b). Mass spectrometry and tandem electrospray MS indicated that the major band at 55 kDa was GPI. The bands at 62 kDa and 30 kDa were identified as rabbit phosphoglucomutase and fructose-1,6-bisphosphatase, respectively. Traces of rabbit creatine kinase M chain were also present.

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It has been argued [9] that differences in patient cohorts or reagents used in ELISA can contribute to different results in serological analyses. The RA group studied by Schaller et al. contained a high proportion of patients with Felty’s syndrome. Our own cohort were all patients with severe, active disease but none of them had this rare complication. However, when other established autoantibody systems have been examined in other diseases, it is highly unusual for such gross discrepancies to arise between laboratories even in the face of diverse patient populations or different reagents.

In conclusion, antibodies to GPI are not readily detectable by ELISA in our hands and immunoblotting of rabbit GPI detects a variety of immunoreactive bands, none of which are restricted to RA sera. Based on these and other results [7, 8], we conclude that GPI is unlikely to be a specific autoantigen in rheumatoid arthritis.

**Table 1. Frequency of anti-GPI and anti-phosphoglucomutase antibodies detected by immunoblotting in the different populations tested**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Anti-GPI antibodies</th>
<th>Anti-phosphoglucomutase antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Strong*</td>
</tr>
<tr>
<td>Systemic lupus erythematosus (n = 19)</td>
<td>16 (84.2%)</td>
<td>9 (47.4%)</td>
</tr>
<tr>
<td>Rheumatoid arthritis (n = 22)</td>
<td>16 (72.7%)</td>
<td>5 (22.7%)</td>
</tr>
<tr>
<td>Sjögren’s syndrome (n = 16)</td>
<td>12 (75%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>Healthy controls (n = 16)</td>
<td>13 (81.25%)</td>
<td>5 (31.2%)</td>
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</tbody>
</table>

* + * indicates a strong reaction observed on immunoblotting.

**Discussion**

By ELISA we were unable to detect any convincing antibodies to GPI in any of the sera. Under all conditions that we examined, the binding was barely above background so that we considered it essential to include duplicate background wells for every serum sample. Interestingly, the optical density values that we obtained were very similar to those of Kassahn et al. [8], who also subtracted background from their ELISA. It is possible that using a larger number of sera we might find the occasional serum in which genuine antibodies were present. However, we felt that 23 RA samples should be more than enough to demonstrate specific antibody if it really were present in over 60% of serum samples as originally claimed [5].

By immunoblotting, immunoglobulins binding to the GPI polypeptide were identified in approximately 80% of serum samples examined, but again there was no difference between the sera from patients with RA and controls. Why such a high proportion of samples reacted by immunoblotting but not by ELISA is unexplained. GPI is an essential enzyme involved in glycolysis and gluconeogenesis and its sequence is conserved throughout evolution, including bacteria. It is possible that antibodies to bacterial GPI may cross-react with linear epitopes in rabbit GPI. Given that we found no difference in binding of serum antibodies to GPI in RA compared with normal serum, we have not investigated this further.

**References**