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Induction of a B-cell-dependent chronic arthritis with glucose-6-phosphate isomerase

Robert Bockermann¹, David Schubert², Thomas Kamradt³ and Rikard Holmdahl⁴

¹Section for Medical Inflammation Research, University of Lund, Lund, Sweden
²Deutsches Rheumaforschungszentrum Berlin, Berlin, Germany
³Deutsches Rheumaforschungszentrum Berlin, and Institut für Immunologie, Klinikum der FSU, Jena, Germany
⁴Section for Medical Inflammation Research, University of Lund, Lund, Sweden

Corresponding author: Rikard Holmdahl, Rikard.Holmdahl@med.lu.se

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Abstract

Antibodies specific for glucose-6-phosphate isomerase (G6PI) from T-cell receptor transgenic K/BxN mice are known to induce arthritis in mice, and immunization of DBA/1 mice with G6PI led to acute arthritis without permanent deformation of their joints. Because rheumatoid arthritis is a chronic disease, we set out to identify the capacity of G6PI to induce chronic arthritis in mice. Immunization with recombinant human G6PI induced a chronically active arthritis in mice with a C3H genomic background, whereas the DBA/1 background allowed only acute arthritis and the C57BL/10 background permitted no or very mild arthritis. The disease was associated with the major histocompatibility region sharing an allelic association similar to that of collagen-induced arthritis (i.e. q > p > r). All strains developed a strong antibody response to G6PI that correlated only in the C3H.NB strain with arthritis severity. Similarly, a weak response to type II collagen in a few mice was observed, which was associated with arthritis in C3H.NB mice. Mice on the C3H background also developed ankylosing spondylitis in the vertebrae of the tail. Both C3H.Q and B10.Q mice deficient for B cells were resistant to arthritis. We conclude that G6PI has the ability to induce a chronic arthritis, which is MHC associated and B-cell dependent. Thus, there are striking similarities between this and the collagen-induced arthritis model.

Introduction

Glucose-6-phosphate isomerase (G6PI) is a widely expressed protein with multiple functions. It is an essential cytosolic enzyme in the energy cycle and has glycolytic activity, but it also has additional functions as an extracellular signalling molecule. Thus, G6PI is also known as AMF (autocrine motility factor) and neuroleukine, and may play roles in both cancer and autoimmunity [1,2].

Coincidentally, it was found that G6PI plays an essential role in the development of arthritis in mice. This originally stemmed from the observation that a bovine pancreas ribonuclease specific T-cell receptor transgenic mouse crossed with NOD mice (the so-called K/BxN mouse) spontaneously developed arthritis. Through a series of elegant experiments it was demonstrated that this transgenic T-cell receptor recognized G6PI within the context of major histocompatibility complex (MHC) class II molecule A07 [3,4]. The transgenic autoreactive T cells triggered autoreactive B cells to produce arthritogenic antibodies specific to G6PI [4-6]. After transferring G6PI-reactive serum from arthritic K/BxN mice, these antibodies bound to peripheral joints and induced arthritis in a manner strikingly similar to that shown previously for antibodies to the cartilage-specific antigen collagen type II (CII) [7,8]. B-cell activation in response to G6PI appeared to occur primarily in lymph nodes draining the joints [9], indicating that recognition of G6PI is joint specific. The reason for this specificity is not apparent because the G6PI protein is a ubiquitous protein.

Even though there are inconsistent data regarding the role of G6PI in rheumatoid arthritis (RA) [10-13], it appears that antibodies to G6PI occur predominantly in patients with Felty's syndrome – a variant of RA [14]. It is still unclear whether this is a unique phenomenon of Felty's syndrome or whether it...
reflects a generalized higher autoreactivity in these patients. Support for the latter comes from a study using affinity purified sera from arthritis patients that compared different kinds of arthritides and suggested a role for G6PI antibodies [15]. G6PI is nevertheless an interesting autoantigen and may represent a unique pathway leading to aggressive subtypes of RA. It is thus interesting to investigate this model further and to compare it with other models, such as the classic collagen-induced arthritis (CIA) model [16]. Here we show that immunization with G6PI leads to a chronically active arthritis in mice with genes from the C3H background, and that susceptibility is both controlled by the MHC region and dependent on B cells.

Materials and methods

Mice
The mouse strains were bred and used in the animal facility of the Section for Medical Inflammation Research (University of Lund, Lund, Sweden). Mice of DBA/1J and C3H.NB origins were from Jackson Laboratories (Bar Harbor, Maine, USA), and those of B10.Q, B10.P and B10.RIII origins were from Dr Jan Klein (Max-Planck-Institut für Biologie, Abteilung Immunogenetik, Tübingen, Germany). C3H.Q mice were established through backcrossing (4n) of the H2q fragment derived from an original C3H.Q mouse into C3H.NB [17]. The human DR4 transgenic and the backcrossed B-cell deficient mouse strains were described previously [18-20].

Experimental mice were matched for sex and age in all experiments. The founder µMT mouse was kindly provided by Dr Werner Müller (Institute of Genetics, Cologne, Germany), which we backcrossed to B10.Q for 13 generations before they were intercrossed for the experiment. C3H.Q µMT mice were backcrossed for 10 generations and finally intercrossed.

The experiments were conducted in accordance with guidelines from the Swedish Ethical Committee.

Antigens
Recombinant human G6PI was produced as previously described [21]. G6PI cDNA fragments were introduced into a modified pQE100 expression vector for expression of Histagged proteins in Escherichia coli strain BI21. Supernatants of bacterial lysates were subjected to purification over a NTA column (Qiagen, Hilden, Germany), in accordance with the manufacturer’s instructions. Using the same strategy, human creatine kinase (hCK) and mouse G6PI (mG6PI) was produced. The purity of proteins was checked using standard SDS gels. Rat collagen type II (rcII) was prepared from SWARM chondrosarcoma by pepsin digestion [22] and further purified as described previously [23].

Immunization and arthritis scoring
An emulsion for immunization was made by sonication, using an aliquot of human G6PI and complete Freund’s adjuvant (Difco, Detroit, MI, USA), resulting in an emulsion of 2 mg/ml human G6PI. A total of 100 µl of the emulsion (200 µg G6PI) was injected at the base of the tail in each mouse. For the titration experiments G6PI was diluted with phosphate-buffered saline to adjust to the required concentration. In some experiments mice were given an intradermal boost with 50 µg G6PI in incomplete Freund’s adjuvant. Mice were visually scored for arthritis using an extended scoring protocol ranging from 1 to 15 for each paw, allowing a maximum score of 60 per mouse. Each arthritic (red and swollen) toe and knuckle was scored as 1, whereas an affected ankle was scored as 5 (total: 15/paw) [24].

Antibody analysis
Serum for analysis of antibody levels was taken at indicated time points and at the end of all experiments. Serum was diluted 1:1,000 for G6PI, mG6PI, hCK and 1:100 for rcII antibody analysis. ELISA Maxisorp plates (Nunc, Roskilde Denmark) were coated with 50 µl of 10 µg/ml of the recombinant proteins or rat CII. The amounts of total specific IgG was determined through quantitative ELISA using peroxidase-conjugated goat anti-mouse IgG (H+L; 115-035-062; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies [25]. ABTS (2,2’-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid), #11204521001; Roche Diagnostics GmbH, Penzberg, Germany) was used as substrate. Values were measured at 405 nm and are expressed as optical density values.

Histology
At the end of the experiments paws, knees, and tails were fixed in 4% paraformaldehyde for 24 hours and decalcified with EDTA. The paraffin sections were stained with haematoxylin and erythrosine [26].

Statistical analysis
Frequency of arthritis was analyzed using the χ² test, and antibody levels and arthritis severity were analyzed using the Mann-Whitney U-test. Disease score and antibody correlations were analyzed using the Spearman rho correlation test from the StatView software package (Version 5.0.1, SAS Institute Inc., Cary, NC, USA).

Results
Titreration of the arthritogenic dose of G6PI
DBA/1 mice were used to confirm induction of arthritis using human recombinant G6PI in our animal house and to titrate the dose. Almost 100% of the mice developed arthritis upon immunization with all of the doses used, although the severity was dose dependent. With the lowest dose (100 µg) the arthritis started as early as day 9 and subsequently progressed to a severe arthritis peaking 2 weeks after immunization.

Thereafter the disease gradually resolved and no macroscopic signs of arthritis were apparent at day 40 (Fig. 1a), enabling
mice to climb under the lids of their cages. The intermediate dose of 200 µg per mouse, which resulted in arthritis in 100%, was selected for further study.

**Pronounced genetic control of chronic arthritis involving both MHC and non-MHC genes**

G6PI immunization of different mouse strains resulted in marked differences in arthritis susceptibility and severity. The C3H.NB strain developed arthritis approximately 2 days later than did DBA/1 mice. However, the arthritis in C3H.NB mice was more severe and, most importantly, these mice developed a chronically active inflammation that lasted throughout the observation period of 90 days (Fig. 1b).

Histological analysis of joints at 90 days after immunization showed active erosive inflammation (Fig. 2e,j). It should be pointed out that only active inflammatory arthritis with redness and oedema was evaluated for clinical scoring. Even though the oedema declined over time in C3H mice, these animals still had tissue depositions that rendered the joints dysfunctional. There was also massive cell infiltration of the joint space causing erosion and destruction of bone and cartilage, as demonstrated by histology (Fig. 2e,j). The destructive character of this process was striking; for instance, it was even able to dissolve joint cartilage. Simultaneously, new bone formation could be observed, creating large osteophytes. In contrast, DBA/1 mice regained function of many finger joints without
The inflammatory process, in contrast to CIA, was not limited to synovial joints but also eroded vertebra and the annulus fibrosus, together with the nucleus pulposus in mice of the C3H background (Fig. 2g). Healthy vertebrae are shown in Fig. 2f. B10.P mice, which share the MHC region with the highly susceptible C3H.NB mice, were resistant to arthritis, showing the strong influence of non-MHC genes. The MHC congenic B10.Q strain, on the other hand, developed significant but mild acute arthritis, whereas another MHC congenic strain – B10.RIII – was also totally resistant to joint destruction. It should be noted that the B10.Q strain used in this study is different from the B10.Q mouse available from Jackson Laboratories, which has an arthritis-protective mutation of the Tyk2 gene [28], which explains the earlier reported resistance in these animals to G6PI-induced arthritis [21]. The (B10.Q × DBA/1)F1 mice developed arthritis almost as severe as that in DBA/1 mice, showing that part of the genetic contribution from DBA/1 dominates the suppressive B10.Q background genes. Mice expressing the human DR4 (0401) molecule on the B10 background were resistant to arthritis. These observations indicate that the most susceptible MHC haplotype is H2q, which is similar to earlier observations in the CIA model [29,30].

Because the highly susceptible C3H.NB strain harboured the less susceptible MHC haplotype (H2s), we tested it in comparison with the C3H.Q strain, which is a MHC congenic strain that carries the H2q haplotype. The C3H.Q strain developed slightly more chronic arthritis than the C3H.NB mice, although the difference between the strains in single experiments did not always achieve statistical significance because of the high severity of arthritis in both strains (Fig. 1c).

A booster immunization after resolution of arthritis induced a relapse in most strains (Fig. 1b), but this was milder than the first arthritic episode and started at the exact same time after immunization, suggesting that there is no memory effect from the primary immunization.

**Development of G6PI arthritis is associated with a strong antibody response to G6PI and a weak response to type II collagen**

All arthritis susceptible mouse strains developed a strong antibody response to human G6PI (hG6PI; Fig. 3a). However, the hG6PI specific antibody response did not always correlate with arthritis severity; strong correlation was found only in the C3H.NB strain (Table 1). The anti-G6PI antibody response made use of all IgG isotypes (data not shown). ELISA plates were also coated with recombinant mouse G6PI because human G6PI was used for immunization. The antibody responses were very similar using the two proteins (Fig. 3a,b). To exclude the His-tag as an allogeneic B-cell epitope, we also investigated the antibody response against a His-tag fusion protein of hCK (Fig. 3d) and His-tag labelled recombinant Aα as negative controls (data not shown). No significant response...
Antibody analysis. Indicated mouse strains were immunized with 200 µg human glucose-6-phosphate isomerase (hG6PI) in complete Freund’s adjuvant and bled at day 40 for antibody analysis. ELISA plates were coated with (a) 10 µg/ml hG6PI, (b) mouse G6PI (mG6PI), (c) collagen type II (CII), or (d) human creatine kinase (hCK). Sera from nonimmunized mice (n = 5) of different genetic backgrounds were used as negative controls. The figures show the optical density (OD) value for total IgG responses at a serum dilution of 1:1,000 for hG6PI, mG6PI and hCK (panels a, b and d) and 1:100 for CII (panel c). The results are represented as box plots, indicating the median, the 25th and 75th centiles as boxes, and the 10th and 90th centiles as whiskers. Outliers are indicated as circles.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Anti-hG6PI versus score</th>
<th>Anti-CII versus score</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Rho</td>
<td>P value</td>
<td>Rho</td>
</tr>
<tr>
<td>C3H.NB</td>
<td>0.917*</td>
<td>0.009*</td>
<td>0.793*</td>
</tr>
<tr>
<td>B10.Q</td>
<td>0.402*</td>
<td>0.046*</td>
<td>-0.094</td>
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<td>(B10.Q × DBA1)F₁</td>
<td>0.018</td>
<td>0.956</td>
<td>0.139</td>
</tr>
<tr>
<td>DBA/1</td>
<td>0.317</td>
<td>0.370</td>
<td>0.033</td>
</tr>
</tbody>
</table>

To investigate correlations between arthritis severity and antibody production the Spearman correlation test was applied. Mice of indicated strains were immunized with 200 µg glucose-6-phosphate isomerase (G6PI) in complete Freund’s adjuvant. Blood was drawn at day 40 and analyzed by ELISA for anti-hG6PI and anti-CII total IgG responses, as shown in Fig. 1. The accumulative arthritis score until day 40 was tested for correlation with antibody production using the Spearman rank correlation test. A rho value close to 1 indicates correlation of high ranks for IgG with high ranks for arthritis scores; 0 indicates that there is no correlation between values; and a number close to -1 indicates that high ranks for one variable correlate with low ranks for the other. *Significant positive correlations.
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Development of G6PI-induced arthritis is B-cell dependent

To determine conclusively whether B cells play a critical role in the pathology of G6PI-induced arthritis we used mice with a disrupted IgM gene, which are therefore deficient in mature B cells. No arthritis developed in B-cell-deficient B10.Q mice (Fig. 4a). C3H.Q µMT mice developed only very mild oedema for no longer than 2 days (Fig. 4b), leaving no histological changes (data not shown).

Discussion

Immunization with G6PI induces arthritis of various degrees of severity and chronicity, depending on the mouse strain. Interestingly, the C3H genetic background permits a chronically active disease course that leads to loss of joint function. This is an important feature of an animal model of RA because the human disease is already chronic when it becomes diagnosed. RA is most likely often preceded by many years of subclinical inflammatory activity. This is not only reflected by raised C-reactive protein levels but also by the production of autoantibodies such as rheumatoid factors and antibodies to citrullinated proteins [31-33]. The chronic disease course of arthritis in C3H.Q and C3H.NB mice, induced with G6PI, will be useful in the analysis of mechanisms of chronicity and as a model to develop new therapeutic protocols.

Interestingly, the C3H background also allows a more severe CIA [16,34]. In addition, in both models, the DBA/1 mouse develops a severe but acute and self-limited type of arthritis. Another striking similarity between the G6PI model and CIA is the association with MHC. In both models the H2 q haplotype confers a more severe form of arthritis than does H2 p. In the CIA model this difference has been shown to be due to the Aq molecule, which binds the immunodominant CII 260–270 glycopeptide with greater affinity than the corresponding A p molecule [35]. It would therefore be of interest to identify the G6PI peptide that binds to Aq and to investigate its affinity to the different MHC molecules, in analogy to the CII peptide. However, a difference from CIA is that the H2r haplotype, despite a strong anti-G6PI antibody response, does not confer susceptibility to G6PI-induced arthritis, although in the CIA model the association with H2r is dependent on binding and recognition of peptides other than 260–270 [36]. Another apparent difference is that DR4 (DRB1*0401/DRA) expressing mice are susceptible to CIA [19,37] but not to G6PI-induced arthritis. This is possibly due to a threshold effect, in which the mice developed a strong autoimmune response to G6PI but which, combined with the relative nonpermissive B10 background, did not lead to arthritis. It may not be unexpected that G6PI-induced arthritis is critically dependent on functional B cells, as shown by our findings in B-cell deficient µMT mice on the B10 and the C3H backgrounds. However, it is interesting that a mild transient oedema was observed in B-cell-deficient mice on the highly arthritis susceptible C3H background.

Role of B cells in the pathology of G6PI-induced arthritis

The role of B cells in the pathology of glucose-6-phosphate isomerase (G6PI)-induced arthritis was addressed using B-cell deficient µMT mice on B10.Q and C3H.Q backgrounds. Mice aged between 6 and 7 weeks were immunized with 200 µg G6PI in complete Freund’s adjuvant and scored for arthritis. (a) Five out of seven B10.Q mice developed arthritis, whereas none of the six B10.Q µMT mice exhibited signs of inflammation. (b) Four out of 12 C3H.Q µMT mice exhibited mild oedema for no longer than 48 hours, whereas all nine C3H.Q littermate control mice demonstrated a destructive inflammatory disease course. Error bars indicate the standard error of the mean.

for these antigens could be detected. That the response was truly directed against conserved G6PI epitopes was also confirmed by using tissue purified commercial (Sigma-Aldrich Sweden AB, Stockholm, Sweden) rabbit G6PI (data not shown).

Nevertheless, many of the strains used, such as B10.RIII and B10.DR4, did not develop arthritis even though they exhibited strong antibody responses against G6PI, indicating the presence of other protective factors. Interestingly, arthritis susceptible strains developed significant titres to CII (Fig 3c), but again only the C3H.NB mice exhibited a positive correlation between anti-CII antibody response and arthritis severity (Table 1).
It has been shown in the K/BxN transgenic model that anti-G6PI serum antibodies readily transfer arthritis [4,6]. In the protein-induced G6PI model, this has so far not been demonstrated [21], and the antibody titres in the different strains do not exhibit a convincing correlation with arthritis susceptibility. Thus, high levels of antibodies to G6PI do not always lead to arthritis, indicating that other pathogenic factors play a role. Interestingly, a few mice with severe arthritis developed detectable amounts of antibodies to CII. There is no evidence for a cross-reactivity between G6PI and CII, and the most likely explanation is an activation of an autoimmune response to cartilage-derived CII, as has been seen in pristane-induced arthritis and in various spontaneous arthritides in mice [38-41].

On the C3H background the G6PI model is also useful in investigating ankylosing spondylitis because it generates inflammation of vertebral joints followed by ankylosis after a single round of immunization. Careful serum analysis of patients with different forms of arthritides revealed that G6PI-specific antibodies may be identified not only in severe forms of RA but also in ankylosing spondylitis and Reiter’s syndrome [15]. The G6PI-induced arthritis model on the C3H background demonstrates that an initial G6PI immune response is sufficient to induce destructive activity in the spine. Which C3H genetic factors actually contribute to this arthritis pathway remain to be determined, and this needs a careful and cautious analysis of the precise genetic background of any mice used.

Our work over many years with congenic and transgenic mice has made us aware of pitfalls relating to the purity of genetic backgrounds. One should be careful in extrapolating data without having full control over the genetic backgrounds. It is not too surprising that, for instance, the C3H.He mice used by Ji and coworkers [42] did not exhibit high sensitivity for their serum transfer model, as might be suggested by our results. Not only does the C3H.He mouse from Jackson, used by those investigators, has a defect in the Toll-like receptor 4 that renders it unresponsive to lipopolysaccharide stimulation, but also it carries another MHC haplotype (H2-K) compared with our congenic mice. Furthermore, it is likely that the different MHC congenic inbred strains have accumulated mutations over the years, as well as carrying several contaminating fragments due to incomplete backcrossing. Bearing these problems in mind, we backcrossed our C3H.Q mice for several generations to C3H.NB to be sure that we compared the MHC effect only. Therefore, it will be of great interest in future investigations to use a panel of highly controlled congenic mice to identify chronicity factors in C3H mice.

In an examination of the IgG isotypes active in human Reiter’s syndrome, they appeared to be predominantly of the T-helper-2-like isotype IgG4, equivalent to IgG1, in K/BxN mice [15]. It will be interesting to investigate whether a T-helper-2 driven immune response is responsible for the chronic severity and spine involvement with the C3H background.

Taken together, there are several similarities but also differences between G6PI-induced arthritis and CIA. Most strikingly, the genetic control in the two models allows only acute arthritis in DBA/1 mice but a more chronic relapsing form in mice of the C3H background. In the CIA model the B10 background allows chronic development of arthritis [43]. Interestingly, both models appear to follow a central pathogenic pathway that involves B-cell autoreactivity and arthritogenic antibodies. Experiments using antibodies to CII and G6PI over the years have shown extensive similarities [6,8,42,44-49].

The most obvious difference between the two models is the tissue distribution of the autoantigen. G6PI is systemically distributed in the body because it is expressed intracellular in all cells as an enzyme of glycolysis and can furthermore be secreted. CII is also widely expressed during foetal development (for review see Holmdahl and coworkers [50]). In the adult CII expression is more restricted, mainly to cartilage (e.g. in diarthroial joints, larynx, spine and sternum). It is also expressed in the vitreous body of the eye [50]. G6PI-induced arthritis developed much earlier after immunization than did CIA and with a stronger oedematous appearance, which could extend into the knees, although no prominent histological changes were observed in DBA/1 mice. Thus, in both CIA and G6PI-induced arthritis the tissue distribution of the autoantigen could not account for the specificity of the inflammatory disease. In fact, models with unknown autoantigens, like induction of arthritis with the alkane pristane, are also specific in that the resulting inflammation only affects joints [51,52].

One important question to address is where the immune system detects the autoantigen. The antigen might be differently expressed in various tissues and be processed differently depending on the kind of antigen-presenting cell. Another issue to address is the role played by synovial tissue in diarthroial joints because these joints are predominantly affected. The cartilage surface may be of importance for triggering antibody-mediated inflammation, as shown for both anti-CII and anti-G6PI antibodies. Taken together, we believe that G6PI-induced arthritis is a very useful model for studies of RA and it may represent a unique pathway, in particular with respect to its autoantigen specificity and chronicity.

**Conclusion**

This study showed that G6PI-induced arthritis can be converted into a chronic inflammatory arthritis model by using the C3H genetic background. Mice of the C3H background also develop arthritis in their vertebra, supporting a role for G6PI reactivity in ankylosing spondylitis.

We conclude that G6PI has the ability to induce a chronic form of arthritis, which is MHC associated and B-cell depend-
ent. Thus, there is a striking similarity between G6P-induced arthritis and the CIA model. Genetic factors determining chronicity – a hallmark of RA – will be addressed using this model in future experiments.

Competing interests

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

Authors’ contributions

RB performed the experiments, and was involved in designing the study and in writing the manuscript under the guidance of RH. DS and TK produced the recombinant proteins (hG6PI, mG6Pi and hCK) and were involved in designing the study and critically read the manuscript.

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