How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease

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Arthritis in the K/BxN mouse model results from pathogenic immunoglobulins (Igs) that recognize the ubiquitous cytoplasmic enzyme glucose-6-phosphate isomerase (GPI). But how is a joint-specific disease of autoimmune and inflammatory nature induced by systemic self-reactivity? No unusual amounts or sequence, splice or modification variants of GPI expression were found in joints. Instead, immunohistological examination revealed the accumulation of extracellular GPI on the lining of the normal articular cavity, most visibly along the cartilage surface. In arthritic mice, these GPI deposits were amplified and localized with IgG and C3 complement. Similar deposits were found in human arthritic joints. We propose that GPI–anti-GPI complexes on articular surfaces initiate an inflammatory cascade *via* the alternative complement pathway, which is unbridled because the cartilage surface lacks the usual cellular inhibitors. This may constitute a generic scenario of arthritogenesis, in which extra-articular proteins coat the cartilage or joint extracellular matrix.

A hallmark of rheumatoid arthritis (RA) is the specific destruction of the synovial joints, but the role played by joint-specific autoreactivity remains controversial[1]. It is not known, for example, whether T cells are dominant players in local inflammation or whether they act upstream during the process by helping pathogenic B cells. Data obtained with the K/BxN mouse, a recently described arthritis model, have further agitated debate on these issues. These T cell receptor–transgenic (TCR-Tg) mice have a T cell repertoire that is highly skewed for a specificity directed against the major histocompatibility complex (MHC) class II A^{g7} molecule loaded with self-peptide derived from the ubiquitous glycolytic enzyme glucose-6-phosphate isomerase (GPI)[2–5]. These animals develop an aggressive form of arthritis that begins at 3–4 weeks of age. As for human RA, this spontaneous disease requires a particular MHC class II allele, is chronic, progressive, symmetrical and results in the severe destruction of cartilage and bone. A classical histological ensemble of synovitis, leukocyte invasion of the articular cavity and pannus formation causes this destruction. In this model, progression of arthritis is also typically dependent on the inflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α) (our unpublished data). The articular manifestations result from arthritogenic Igs that are also directed against GPI[3,4]. High titers of these Igs develop in Tg K/BxN mice because of the preferential help that B cells expressing GPI-reactive Igs receive from the Tg T cells[6]. In the absence of lymphocytes in the host, affinity-purified anti-GPI IgG from these mice can transfer destructive arthritis and, alone, can provoke the full range of inflammatory functions that underlie the disease. Activation of the alternative pathway of complement and signaling through Fc receptors are both essential to the downstream effector phase of disease[7].

A major paradox lies in the exquisite specificity of the joint attack provoked by these Igs, which react against a ubiquitously expressed antigen. GPI is an enzyme of the glycolytic pathway; it is essential for basic carbohydrate metabolism. Consequently, it is expressed in all cells, and GPI-deficient mouse embryos die at the two-cell stage[8]. It is normally sequestered in the cytoplasm and is only released into the circulation in minute amounts during pathological states such as liver metabolic injury or tumor growth, which correlate with cell damage or apoptosis. The presence of circulating GPI enzymatic activity in serum had been investigated as a potential diagnostic tumor marker, but was not followed up because of inconsistent results[4]. Thus, GPI makes an unlikely candidate target for arthritogenic Igs, particularly because disease manifestations in K/BxN mice appear to be limited exclusively to the joints. We explore here the potential reasons for this paradoxical joint specificity.

Results

No evident joint specificity to GPI expression

That the pathology provoked by anti-GPI Igs is confined to the joint raises the question of whether there might be something special about how GPI is expressed in the articulations. As a first approach, we looked for

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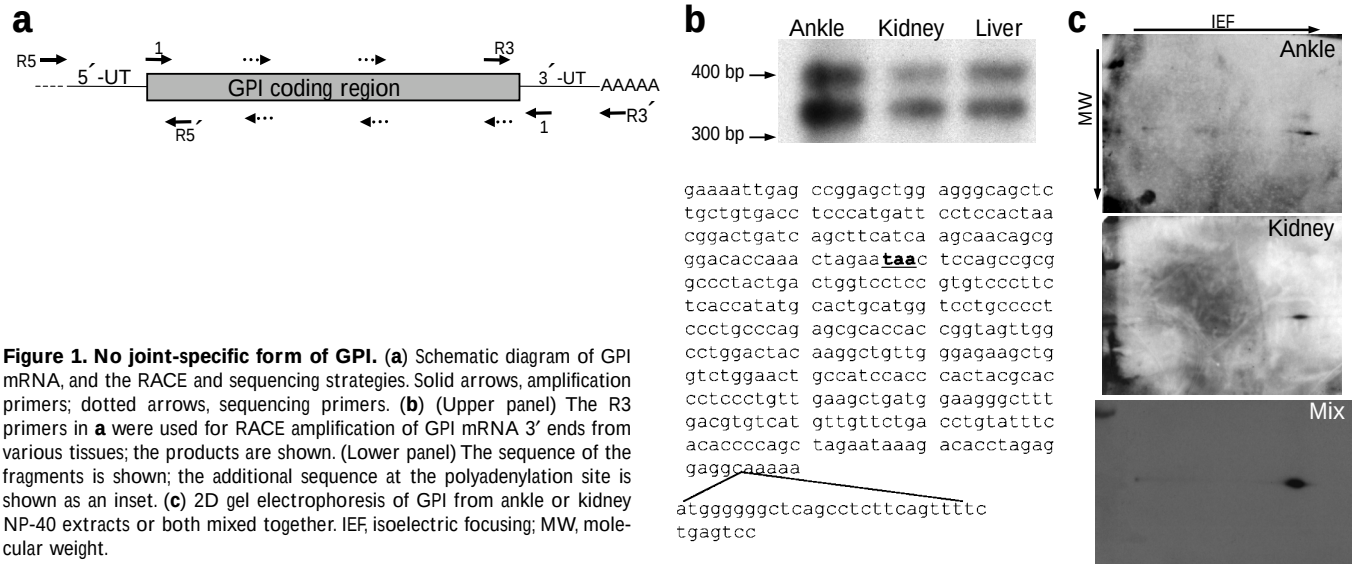

Figure 1. No joint-specific form of GPI. (**a**) Schematic diagram of GPI mRNA, and the RACE and sequencing strategies. Solid arrows, amplification primers; dotted arrows, sequencing primers. (**b**) (Upper panel) The R3 primers in **a** were used for RACE amplification of GPI mRNA 3′ ends from various tissues; the products are shown. (Lower panel) The sequence of the fragments is shown; the additional sequence at the polyadenylation site is shown as an inset. (**c**) 2D gel electrophoresis of GPI from ankle or kidney NP-40 extracts or both mixed together. IEF, isoelectric focusing; MW, molecular weight.

particularities in GPI gene transcripts. One possibility was that there are differences in the coding sequences of GPI mRNA in joint tissue. To address this issue, RNA was prepared from dissected ankle material and various segments of the coding region were amplified by polymerase chain reaction (PCR) using overlapping primers (**Fig. 1a**). The resulting fragments were then sequenced. No differences from the GenBank reference sequence for GPI mRNA were found (data not shown), except for the known strain-specific polymorphism at amino acid position 95 (D or N), which forms the basis for the classical genetic mapping test that relies on GPI electrophoretic polymorphism. Another possibility was that alternate coding region termini characterize GPI mRNA in the joint, resulting in a leader peptide and/or transmembrane region and a secreted or membrane-bound form of GPI. This possibility was tested by rapid amplification of cDNA ends (RACE) PCR[9] of both mRNA extremities (**Fig. 1a**). A single band was obtained by RACE at the 5′ end, which indicated a unique 5′ terminus; this was confirmed by sequencing the fragment (data not shown). At the 3′ end, two bands were detected, signifying two different transcripts (**Fig. 1b**). However, they proved to be irrelevant, as they were observed in equivalent proportions in RNA from joint and control tissues and merely corresponded to an alternative polyadenylation site that was of no consequence to the protein structure.

As a second approach, we searched for translational or post-translational modifications that could result in a different form of GPI in the joint. Extracts of various tissues were separated by two-dimensional (2D) isoelectric focusing–polyacrylamide gel electrophoresis (IEF-PAGE), and GPI was revealed by electroblotting and probing with affinity-purified anti-GPI. The mobilities of articular and extra-articular GPI were indistinguishable, as confirmed by mixing the two samples (**Fig. 1c**).

As a third approach, we compared GPI expression. Semiquantitative PCR showed that there were essentially no differences in GPI transcript levels in joint *versus* kidney (**Fig. 2a**). This result was confirmed by quantitative real-time PCR (**Fig. 2b**). To test whether GPI protein translation efficiency or post-translational stability varied, cytoplasmic extracts from ankle and other tissues were tested by immunoblotting with anti-GPI (**Fig. 2c**). Again, when normalized relative to actin as an internal standard, no difference was observed (**Fig. 2d**).

Thus, we found nothing unusual about either the amounts or forms of GPI mRNA or protein in joint tissue, although a particular form of GPI produced by a minor cell population cannot be formally ruled out.

GPI deposits in the joints

As GPI expression in the joint appeared to have no particularities, we turned to immunohistology to examine its distribution in joints. Cryostat sections of several tissues from normal C57BL/6 (B6) mice were prepared without fixation or decalcification of the tissues. These sections were probed with fluorescein isothiocyanate (FITC)-conjugated anti-GPI—a polyclonal reagent prepared by affinity purification from the sera of arthritic K/BxN mice—and were examined by conventional and confocal fluorescence microscopy. As expected, GPI was homogenously distributed in the cytoplasm of all cells in all organs (data not shown). This finding extended to the joints where intracytoplasmic GPI was present in chondrocytes and synoviocytes, with staining intensities comparable to

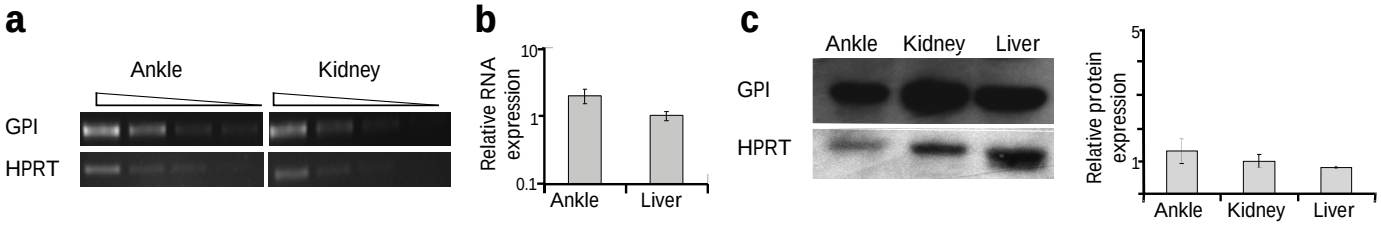

Figure 2. No overexpression of GPI in joints. (**a**) Semiquantitative RT-PCR analysis of GPI mRNA in RNA samples from ankle or kidney. Amplification with HPRT-specific primers was done as a control. (**b**) Analysis of ankle and kidney RNA by real-time quantitative PCR. (**c**) Expression of GPI protein analyzed by immunoblotting ankle, kidney and liver NP-40 extracts. GPI amounts, normalized on the basis of HPRT expression, are shown in the graphs.

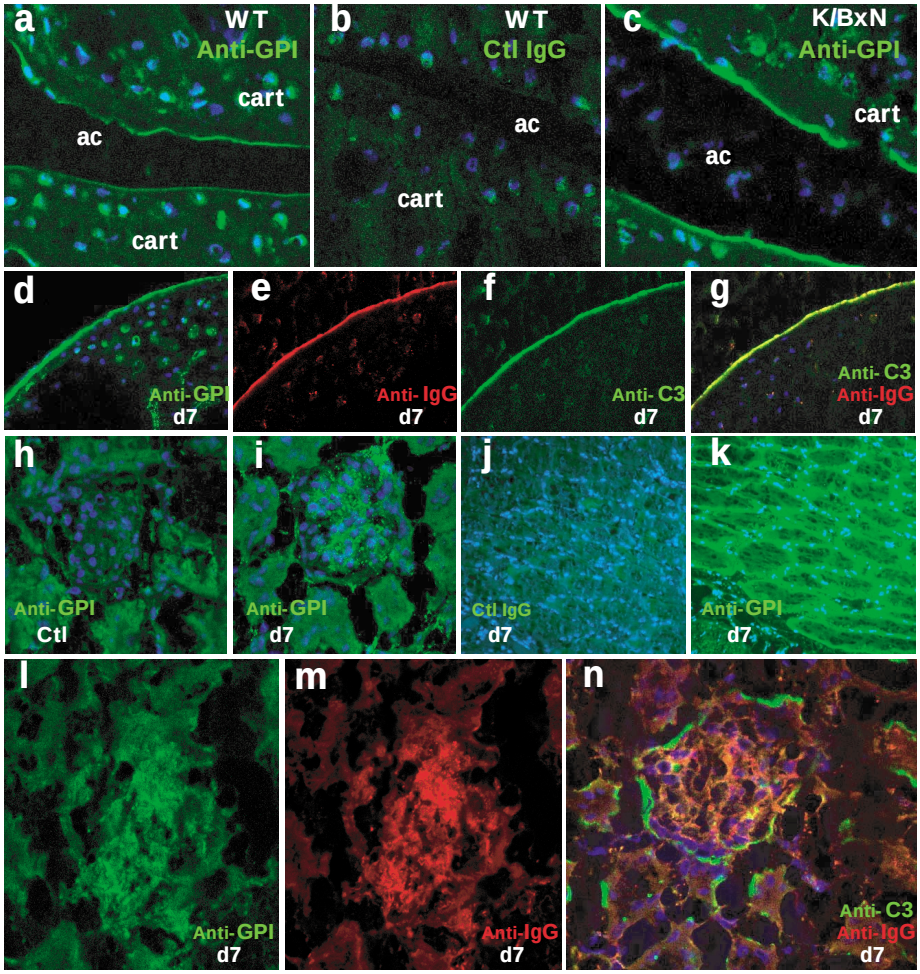

Figure 3. Presence of GPI on joint surfaces. (**a,b**) Serial cryostat sections from normal (WT) ankle joint stained with anti-GPI or control Ig (green); nuclei were counterstained with DAPI (blue) and examined with a confocal microscope and ×63 objective (images representative of three experiments, one mouse each). Ac, articular cavity; cart, cartilage. (**c**) Similar staining of an ankle section from an arthritic K/BxN mouse. Representative of four experiments with either transgenic K/BxN or serum-transferred mice. (**d–g**) Ankle cartilage surface of a B6 mouse 7 days after injection of arthritogenic K/BxN serum, stained as indicated. (**g**) Overlay image of **e** and **f**. (**h,i**) Kidney glomeruli from normal or K/BxN serum-injected mice stained with anti-GPI and a DAPI counterstain (images representative of three experiments, one mouse each). Images were obtained by confocal imaging with a ×40 objective. (**j,k**) Serial muscle sections from a B6 mouse taken 7 days after injection of arthritogenic K/BxN serum. Sections were stained with anti-GPI or control Ig and a DAPI counterstain was used. Images were obtained by classical microscopy with a ×20 objective. (**l–n**) Kidney glomeruli from a B6 mouse taken 7 days after injection of arthritogenic K/BxN serum, stained as indicated. (**l,m**) Images are from the same costained section. (**n**) Overlay image of anti-C3 and anti-IgG stains with a DAPI counterstain.

those of other tissues. However, there was also evidence of GPI in extracellular structures along the articular cavity; this was particularly clear for the cartilage surface, where a thin and very sharp crescent of GPI lined the cartilage surface (**Fig. 3a**). No GPI was detectable within the body of the cartilage nor at the bone-cartilage junctions. Staining was specific as it was not detectable with control FITC-conjugated IgG, which was prepared and used in parallel (**Fig. 3b**).

GPI deposited at the articular surface could target pathogenic Igs, which prompted the question of its fate in arthritic K/BxN Tg mice or the recipients of K/BxN serum. The amount of GPI deposited along the joint surfaces increased markedly in both types of arthritic mice (**Fig. 3c**). This GPI was most likely in the form of immune complexes, as deposits of IgG and C3 complement fragments were clearly visible in doubly stained serial sections (**Fig. 3d–g**). This localization contrasted with that observed in the joints of normal mice: in normal mice, the GPI deposits were not localized with IgG (data not shown). In arthritic mice, the GPI–anti-GPI immune complexes extended beyond the cartilage surface, along the thickened synovium and the pannus.

An explanation of joint specificity

These data suggested that GPI on the articular surface is the target for pathogenic antibodies, which results in complex formation and complement activation. Circulating complexes of GPI–anti-GPI formed extra-articulately may also participate in these deposits, eventually leading to lattice formation. But, still, why the joint specificity? Initial analyses of K/BxN mice showed large amounts of Ig deposits in a number of tissues[2], but it was not known whether these were GPI-IgG complexes. Cryostat sections of various tissues were prepared and stained as above. The cytoplasmic GPI present in the kidney made it difficult to reliably detect GPI bound to exposed extracellular surfaces in normal mice, as had been readily detected on cartilage. However, there was a marked increase in the amount of GPI that could be detected in the kidney mesangium 7 days after transfer of K/BxN serum (**Fig. 3h,i**). Similarly, GPI could be detected in the muscle perimesium and/or endomesium (**Fig. 3j,k**). As in the joint, GPI localized with IgG in the kidney glomeruli (**Fig. 3l,m**), but there was a marked difference: the complement component C3 did not localize with these complexes (**Fig. 3n**). Some C3 deposits were observed, but in the peripheral membranous region of the glomeruli, and there was essentially no overlap with the mesangial GPI-IgG complexes (these C3 deposits are commonly seen in control unmanipulated mice). This may be an important clue to the joint specificity: GPI-IgG complexes do form in extra-articular organs, but they do not initiate a complement cascade.

The source of GPI and anti-GPI

Next we identified the source of the GPI deposited on the surface of articular cartilage. A sandwich ELISA was developed to assess the presence of free GPI in serum and showed circulating GPI in normal mouse serum (**Fig. 4a**). Concentrations were high in mice at 1 week of age, dropped to 400 ng/ml by 3 weeks of age and remained low thereafter. In K/BxN mice, free GPI was present in a low concentrations at week 1 and became

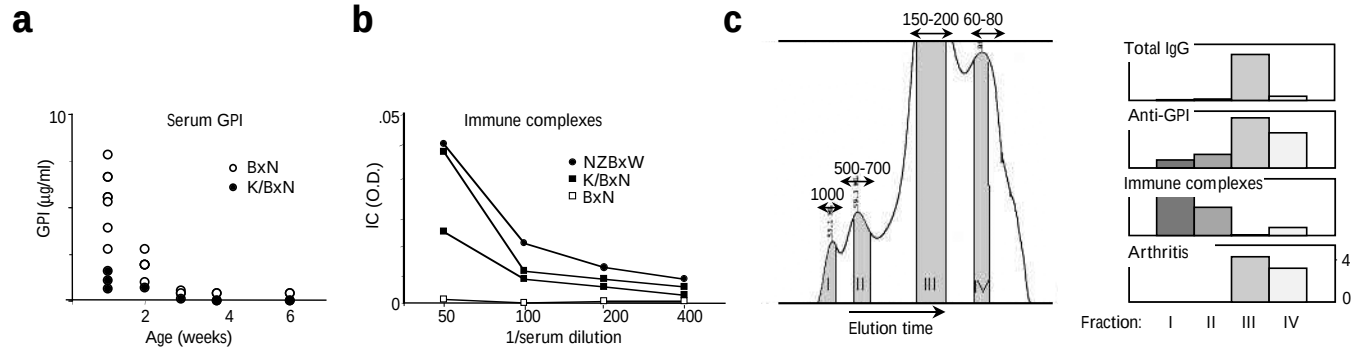

Figure 4. Circulating GPI or GPI–anti-GPI immune complexes. (**a**) GPI was quantified by sandwich ELISA analysis of sera from normal (B6×NOD)F_1 mice (BxN) or their arthritic Tg littermates (K/BxN). (**b**) Immune complexes were detected by ELISA; the target C1q was used to coat plates. Sera was taken from 5-week-old K/BxN mice, and serum from an (NZB×NZW)F_1 mouse (NZBxW) was used as a positive control for circulating immune complexes. Data are representative of two independent experiments; there were two recipient mice for each fraction. (**c**) K/BxN serum was fractionated by size-exclusion chromatography. (Left) Total protein elution profile; the fractions that were collected are shaded and their average molecular weights (in kD) shown. The total IgG, anti-GPI and C1q binding complex contents and arthritogenic potential of the four fractions were examined by ELISA. Data are given as arbitrary units; arthritogenic potential was assessed by injecting the fraction into naïve mice and assigning a clinical index (max clinical index determined as in[31]).

undetectable, at least in an immunoreactive form, by week 3, which coincided with the time at which detectable anti-GPI titers initially appeared[4]. To determine whether GPI might, at that point, circulate as immune complexes, we did ELISA assays with plate-bound C1q, an indicator of immune complex formation. Complexes were indeed found in the serum of arthritic K/BxN mice at titers comparable to those found in (NZB×NZW)F_1 mice, which we used as positive controls (**Fig. 4b**). As predicted, these C1q binding complexes were only detected after 30 days of age, coincident with arthritis onset (data not shown).

Given the presence of these complexes in mice early in the arthritic process, we then asked whether these were the pathogenic agents, that is, whether complexes or free IgG enter the joint and cause disease. Stable immune complexes in K/BxN serum were separated from free anti-GPI IgG by high-performance gel filtration, and mice were injected with an equivalent proportion of each fraction (**Fig. 4c**). Total IgG fractionated in peak III, as expected, but ~20% of the anti-GPI was found in fractions of heavier molecular weight that contained the C1q-reactive immune complexes. Yet, when tested by injection into naïve mice, all disease-conferring activity was found in the low molecular weight fractions. This experiment must be interpreted with a degree of caution, as some complex formation will occur after transfer of free IgG (although the amount of serum GPI in an adult mouse, ~1 µg, is dwarfed by the amount of anti-GPI that was injected). However, the results indicate that the pathogenic potential is not preferentially found in the immune complex fraction.

Applicability to human patients

A key question is whether arthritis development in K/BxN mice represents a generic mechanism that is applicable to RA or other human arthritides. In particular, is GPI also a target in humans, as has been suggested[10]? We examined, therefore, whether the pattern of GPI deposition observed in the joints of mice might be present in joints from RA patients. Two specimens of joint tissue from RA patients were prepared for immunohistological examination. In one sample, the staining intensity was modest; in the other, clear deposits of GPI were detected along the surface of the erosive pannus, although the bone surface itself was free of GPI (**Fig. 5**, no cartilage was present in the section). Igs were also present at the same location (data not shown); these probably corresponded to IgG deposits that have been described on the articular cavities of RA patients[11–13]. However, we did not observe the juxta-endothelial deposits described by another group[10]. Thus, patterns of anti-GPI deposits similar to those found in K/BxN mice can be observed in human RA patients.

Discussion

We found that GPI deposits lined the surface of the articular cavity, particularly the cartilage surface, in normal mice. As a result of these findings, we suggest a new model of arthritis pathogenesis, one that applies to disease in K/BxN mice but perhaps also to disease in humans. Diffusible molecules of extra-articular origin bind to cartilage surfaces and serve as targets for autoantibodies, which are generated either through immune responses or loss of self-tolerance. These immobilized Igs activate the

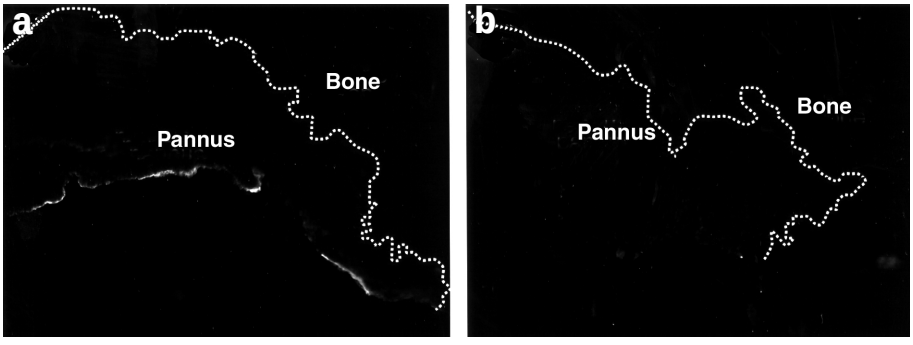

Figure 5. GPI deposits on a human RA pannus. Cryostat sections from a surgically removed digit joint of an RA patient stained with (**a**) anti-GPI or (**b**) control Ig. Conventional immunofluorescence was examined with a ×20 objective.

alternative complement pathway as a result of the noncellular surface of cartilage, which therefore lacks several complement inhibitors.

There are several tenets to this model. First, the target need not be of articular origin. None of the experiments done here identified a joint-specific form of GPI. Although it may originate in part from local release into the synovial fluid, the GPI deposited on the joint surfaces can also be passively transferred from the serum. In this respect, GPI may be only one example of molecules that can diffuse into the joint from the circulation, facilitated by the absence of a basal membrane under the joint vasculature. This origin may account for the arthritis found as secondary manifestations in a large variety of infections or autoimmune disorders, in which immune reactions develop against self or microbial components, such as hepatitis-associated arthritis or the reactive arthritis that follows gut infection.

Second, the key to joint specificity may be that the development of K/BxN arthritis is dependent on activation of the alternative complement pathway[7]. With an operational logic parallel to that of natural killer cells, the alternative pathway can be activated on all surfaces, but is normally kept in check on self-surfaces by regulatory enzymes that destroy the spontaneously activated C3b[14]. These include soluble proteins (factors H and I) and cell surface proteins such as DAF (decay-accelerating factor) and MCP (membrane cofactor protein). Igs bound to surfaces enhance the alternative pathway by binding C3b covalently[15,16], which protects it by restricting its accessibility to factor H[17]; this protection is further stabilized by properdin[18,19]. Cartilage is an unusual body surface. It is devoid of cell membrane–bound C3 inactivators and is only protected from alternative pathway activation by the soluble inhibitors and by the dampening effect of surface sialic acid[20,21]. Ig deposition could interfere by preventing factor H access and GPI–anti-GPI complexes may also mask sialic acid residues; both these processes may contribute to turning cartilage into an activating surface. In contrast, surfaces in the kidney or muscle are more efficient at controlling the alternative pathway. Thus, the joint is a special location at which the cartilage surface maintains an uneasy alternative complement pathway equilibrium that is readily perturbed. The dominant IgG isotype in K/BxN arthritis is IgG1[2,6], whereas IgG3 and IgG2a are the dominant isotypes for nephritis[22,23]. This is perhaps related to the fact that the classical complement pathway does play a role in kidney pathology[24]. Thus, the joint specificity of K/BxN arthritis, and perhaps more generally of arthritis in contexts of systemic immune dysregulation, may merely reflect a differential sensitivity to innate effector mechanisms rather than a response to some particular joint antigen.

Third, there is no *a priori* link between the cartilage and the inciting molecule. It has been proposed that cell-surface receptors, perhaps similar to the high-affinity receptors cloned from tumor cells[25], might "present" GPI on vascular endothelial cells[10]. We have not been able to confirm endothelial surface staining on mouse sections or in the few human RA samples we analyzed. It is unlikely that cell-surface transmembrane molecules would be present on articular cartilage. We do not know which cartilage surface molecule(s) bind GPI, but hypothesize that binding may occur *via* multiple low-affinity interactions with repeated carbohydrate moieties of cartilage proteoglycans. The cartilage would then act as a low-affinity but high-avidity "molecular sponge". That GPI is usually found as a dimer[26,27] and may be important in stabilizing such interactions. Also, it may not be mere coincidence that its natural substrates are phosphorylated sugars. It will be important to determine how GPI binds to joint surfaces and how widespread this binding may be.

Fourth, the progression of arthritis appears to entail a positive feedback loop that recruits more of the target GPI molecules and/or GPI–anti-GPI immune complexes to the surfaces of the articular cavity. The amount of GPI on the cartilage surface was far higher in arthritic transgenic or serum-transferred mice than in normal animals. In imaging experiments in which radioactively labeled anti-GPI was transferred into mice, increased binding of anti-GPI in the joint was found 48–72 h after the onset of disease, indicating that this accumulation of joint GPI is an early event (U. Mahmood *et al.*, unpublished data). One possibility is that early cartilage alterations, which expose proteoglycan structures that are normally shielded, result in increased GPI binding. It is also possible that deposited, bivalent anti-GPI would recruit more GPI to the site—as free molecules or as GPI–anti-GPI complexes—resulting in lattice formation. Consistent with this idea, analysis of the pathogenic potential of monoclonal antibodies (mAbs) has shown that arthritogenesis requires coordinate binding to multiple epitopes and the simultaneous injection of several mAbs is required for efficacy[6].

Fifth, which form of anti-GPI is responsible for disease? One should distinguish between two phases. During the first phase, several arguments suggest that upon injection of GPI antibodies, free Igs enter the joint and then bind GPI onto the cavity surface, rather than immune complexes being preformed in the serum and then filtering into the cavity. This is because the very existence of a GPI lining along the articular cavity now provides a direct target for the binding of anti-GPI, so there is no need to invoke pre-existing immune complexes. Also, in gel filtration experiments, the pathogenic activity fractionates with free IgG, not with complexes, and several attempts at enhancing the pathogenicity of mAbs or serum IgG by supplementing with titrated amounts of recombinant GPI have not worked[6]. In addition, antibodies to GPI accumulate in the joint within a matter of minutes[28], which is more consistent with the immediate entry of Igs than with the accumulation of complexes. In the second phase, the accumulation of GPI and anti-GPI may reflect deposits of the complexes circulating in serum: the increased vascular permeability now allows better entry of complexes and, by then, all circulating GPI is complexed by the large excess of antibodies present.

RA is a multifaceted disease and the model described here certainly represents a simplification of the mechanisms that could contribute to the variety of arthritic conditions in humans. Additional pathways and mechanisms, such as secondary immune responses to joint structures or indirect consequences of the synovial hyperproliferation, must contribute to the complex events of established RA. The model proposed above also has many conceptual precedents, in particular in the ideas of immune complex pathology that were prevalent in the early 1970s[29,30]. In essence, this view of arthritis is a form of type III hypersensitivity (Arthus) reaction, which is localized to the joint. In focusing attention on specific molecules and effector pathways, the K/BxN mouse does, however, provide a focal point around which to synthesize this view of the early stages of arthritogenesis.

Finally, the evolution of serum GPI concentrations follows an interesting pattern, with a precipitous drop during the first weeks of age. This may reflect a fetal or early neonatal cellular release of the enzyme, perhaps related to the extensive tissue proliferation and growth that occurs during this period or to feto-maternal interactions. In any case, the pattern likely explains the development of T cell tolerance observed in K/BxN mice[2]. It is complete during the first weeks of age, shows strong clonal deletion in the thymus and is less stringent later on, so that clonotype-positive reactive cells mature in the peripheral organs and are only partially anergized. It will be critical to determine whether this pattern is of importance in establishing imperfect tolerance and is a general feature of self-antigens that are involved in certain forms of arthritis.

The KRN model of arthritis, although very different in its immunological phase, dovetails with the classical arthritis model induced by collagen II immunization: immunoglobulin binding to the cartilage surface. This

shows how different pathogenic mechanisms and autoimmune targets may converge to a common effector pathway in human arthritic diseases.

Methods

Mice and arthritis. K/BxN TCR–Tg mice were as described[2]. They were maintained by serial crossing onto the B10.BR genetic background and, as such, were asymptomatic. Arthritic offspring were produced by crossing to the NOD/Lt strain. Recombination-activating gene 1–deficient mice were a gift of F. Alt (Children's Hospital, Boston). B6 mice were from the Jackson Laboratory (Bar Harbor, ME). Arthritis was spontaneous in K/BxN mice or was induced in normal inbred mice by injection of 2×150 μl of K/BxN serum. For fractionation, K/BxN and control BxN sera (2–3 ml) were separated by size-exclusion chromatography with a Superdex 200 column (Amersham Pharmacia Biotech, Piscataway, NJ). Fractions (4 ml) were collected, pooled and concentrated before ELISA analysis of injection into mice.

RNA analysis. RNA was prepared from kidney tissue by a modified LiCl-Urea technique[31]. After dissection of ankles (sectioned at the long bones of the lower leg and in the metatarsal area), the skin and superficial tendons were removed and the remaining tissue was immersed in 1 ml of RNA solubilization solution (without LiCl). Articular cavities were opened with a scalpel and were dissected in the medium to release the cellular contents. Concentrated LiCl solution (6 M LiCl, 6 M urea and 10 mM Na acetate at pH 5) was then added to precipitate the RNA. cDNA was synthesized from these RNAs by MuLV reverse transcriptase (Gibco-BRL, Gettysburg, PA).

To search for alternative forms within the coding region, PCR primers were used to amplify the coding sequence (Fig. 1a, pair 1; all primer sequences were derived by the use of the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), the products were cloned into PCR2.1 vector and three independent plasmids were sequenced on an Applied Biosystems ABI 373A with internal primers (Fig. 1a). For RACE analysis of mRNA termini, the protocol was as described[9]. For the 3′ end, the mRNA was reverse-transcribed with a dT-adaptor primer (R3′). A 10 μl aliquot was used for 40 cycles of PCR with the adaptor primer and sense primer R3. The reaction products were separated by agarose gel electrophoresis, blotted and detected with a labeled PCR product that covered the 3′ end of the GPI coding region. These RACE products were also sequenced. For quantitative real-time analysis, PCR was done on a TaqMan PRISM 7700 instrument (PE Applied Biosystems, Foster City, CA), and data was analyzed with the instrument software. Amplification was done in triplicate in 96-well plates using intron-spanning specific primers and the antisense fluorogenic probe labeled with a 5′ 6-carboxy-fluoroscein (FAM) reporter dye and a 3′ 6-carboxy-tetramethyl-rhodamine (TAMRA) quencher (sense primer 5′–AGATCAACTACACCGAGGATCG–3′; antisense primer 5′–ACATCTTTGCCGTCCACCTT–3′; probe 5′–FAM-TGGCCCTTCG GAACCGGTCC-TAMRA–3′). A cyclophilin primer combination was used as a standard.

Immunoblot and protein analysis. For immunoblotting, ankles and kidney (two of each) were minced and the contents extracted in 500 μl of lysis solution (50 mM Tris-HCl at pH 7.4, 5 mM $MgCl_2$, 0.5% NP-40 and 2 mM PMSF). Whole lysate (15 μg) was separated by PAGE and blotted into PVDF membranes (Bio-Rad, Hercules, CA). After blocking with 5% milk in PBS, K/BxN serum (1/1000) or mouse anti-actin (Sigma, St. Louis, MO) was used as a probe. Bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti–mouse IgG (Jackson ImmunoResearch, West Grove, PA) using a Super-Signal kit (Pierce, Rockford, IL) and chemiluminescence was imaged quantitatively. For 2D immunoblotting, kidney or ankle extracts (10 μg) or a 1/1 mixture were diluted in sample buffer (40 μl, 9.5 M urea, 2% NP-40, 2% ampholines at pH 3.5–10 and 5% β-mercaptethanol) and were loaded onto a first-dimension tube gel (9.5 M urea, 3.35% acrylamide, 2% NP40 and 2% ampholines at pH 3.5–10). After electrophoresis, the tube gels were loaded onto a 10% acrylamide slab gel and GPI was detected as above.

Immunohistology. For immunohistology, cryostat sections from ankle joints were prepared with the tape-capture technique as described[7]. Anti-GPI polyclonal Igs were affinity-purified from K/BxN serum as described[4]; protein G–purified IgG from normal BxN mice was used as a control. Igs were conjugated to FITC (Fluorescein-EX, Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Other reagents were Texas red–conjugated anti–mouse IgG (Jackson), and FITC-conjugated goat anti–mouse C3 (ICN Biomedicals, Costa Mesa, CA). After blocking with 2% bovine serum albumin and 0.04% Tween in PBS, the sections were stained with anti-GPI or control Ig (500 ng/section). Nuclei were counterstained with DAPI (50 ng/section, Molecular Probes). Fluorescence was detected on a conventional Zeiss Axioplan2 microscope or by confocal microscopy (LSM 410 confocal microscope, with 364, 488 or 568 nm excitation, Zeiss, Thornwood, NY). Images were acquired and processed digitally (Photoshop 6.0).

ELISA assays. Antibodies to GPI were detected as described[4]. To quantify GPI in serum, a sandwich ELISA assay was developed with a recently isolated GPI mAb (M. M., unpublished data). Anti-GPI (mAb 6.121, 10 μg/ml) was used to coat a microtiter plate, and test serum (1/100) was added and incubated for 2 h at room temperature. After washing, biotinylated (Fluoreporter biotin–dinitrophenol, Molecular Probes) polyclonal anti–GPI Ig, which was affinity-purified from K/BxN serum, was added to the plate (1/1000). The plate was incubated for 1 h and was detected with allophycocyanin-streptavidin (Jackson ImmunoResearch); all incubations were done at room temperature. Recombinant mouse GPI[4] was used as a standard.

To detect immune complexes, ELISA plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with 20 μg/ml of C1q (Sigma) in PBS at 4 °C overnight, blocked with bovine serum albumin before incubation with serum dilutions and bound complexes were detected with phosphatase-coupled anti–mouse IgG (Jackson Immunoresearch).

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Competing interests statement

The authors declare that they have no competing financial interests.

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