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KRN/I-A^{g7} Mouse Arthritis Is Independent of Complement C3

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Abstract

Background—KRN/I-A^{g7} (KxB/N) is a mouse model of inflammatory arthritis, which resembles human rheumatoid arthritis. Arthritis in these animals is caused by autoreactivity to a ubiquitously expressed autoantigen, glucose-6 phosphate isomerase. Tolerance is broken at both the T cell and B cell level. The sera from KRN/I-A^{g7} mice can induce mouse arthritis in healthy mice. Complement components of the alternative complement pathway, including C3, have been shown to be required in induction of mouse arthritis by serum transfer.

Methods—We have bred KRN/I-A^{g7} mice onto a C3-deficient background and followed cohorts for the spontaneous appearance of arthritis. We have also transferred KxB/N serum to B6.I-A^{g7} recipients.

Results—C3-deficient KRN/I-A^{g7} mice spontaneously developed severe, destructive arthritis, comparable to that seen in C3-intact KRN/I-A^{g7} mice. However, serum transfer experiments confirmed the strong requirement for C3 in the passive model.

Conclusion—The pathogenesis of spontaneous KRN/I-A^{g7} arthritis can largely proceed by complement-independent pathways and must have pathology effector mechanisms in addition to those seen in the passive serum transfer model.

Keywords

Arthritis; autoantibody; KRN; GPI; complement C3

Introduction

Rheumatoid arthritis (RA) is the most common inflammatory polyarthritis, which affects about two million Americans. Many of the important aspects of its pathophysiology have been elucidated and are being targeted with novel therapeutic agents. However, the fundamental etiology of RA remains mysterious [1]. For some time, T cell autoimmunity was thought to be the key dysfunction, particularly given the clear genetic association with a T cell recognition molecule (HLA-DR4 and the shared epitope), but failure to find reproducible evidence of autoreactive T cells and failure of some anti-T cell therapy dissuaded investigators from this path [2, 3]. Several autoantibody species are strongly associated with RA, in particular rheumatoid factor and anti-cyclic citrullinated peptide (CCP). Although it has not yet been fully demonstrated that these autoantibodies are pathogenic in RA, circumstantial evidence for their direct role has been increasing [4]. The recent demonstration that B cell depletion therapy with rituximab is highly effective in many patients with RA appears to establish an incontrovertible central role for B cells [5, 6].

A pathogenic role for autoantibodies in inflammatory arthritis has been clearly demonstrated in animal models [7]. Since 1996, the KRN/I-A^{g7} (or K/BxN) model has gained a great deal of attention, given that it occurs spontaneously as a result of a well-defined T cell and B cell autoreactivity to a single antigen [8]. The KRN T cell receptor (TCR) recognizes a foreign antigen, bovine pancreas ribonuclease (RNase), in the context of major histocompatibility complex class II (MHC II) I-A^k [9]. Serendipitously, it also recognizes a ubiquitously expressed self protein, glucose-6-phosphate isomerase (GPI), in the context of MHC II I-A^{g7} [10, 11]. The KRN/I-A^{g7} mouse develops an inflammatory joint disease with many characteristics of RA. A key feature of KRN/I-A^{g7} model is that the arthritis can be transferred by serum or with monoclonal antibodies to a wide range of mouse strains [12, 13]. The serum transfer system has allowed the rapid assessment of the role of many inflammatory pathways in the pathogenesis of arthritis. Specifically, it has been found that mice that are genetically deficient in mast cells, neutrophils, Fcγ receptors, and complement component factor B, C3, C5, or C5a receptor lacked the responses to transferred serum [4, 14–20]. Notably, the early components of the classical pathway, e.g., C1q and C4, did not appear to be necessary. However, the alternative pathway has been thought to be essential [7].

We have tested further the role of complement by producing KRN/I-A^{g7} mice that were C3-deficient. Surprisingly, these mice developed severe destructive arthritis not dissimilar to that seen in C3-intact KRN/I-A^{g7} mice. Nevertheless, C3-deficient mice were confirmed to be highly resistant to passive arthritis induced by serum transfer. Since C3 is essential to the function of all three known complement pathways, these results indicate important complement-independent mechanisms in the spontaneous induction of KRN/I-A^{g7} arthritis.

Methods

Mice

C57BL/6 (B6), B6.CgH-2^{g7}-Tg (Ins2-CD80)₃B7Flv/LwnJ (B6.I-A^{g7}), and B6.129S4-C3^{tm1Crr}/J (B6.C3KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). KRN TCR transgenic mice were originally obtained from Diane Mathis (IGMBC, Strasbourg, France). The strain was maintained on the B6 background (B6.KRN). Arthritis mice that express both KRN and I-A^{g7} were produced by crossing B6.I-A^{g7} females and B6.KRN males (KRN/I-A^{g7} F1).

B6.C3KO mice were crossed with B6.KRN to establish a C3-deficient and KRN heterozygous strain (B6.C3KO-KRN) and with B6.I-A^{g7} to produce a C3-deficient and I-

A^{g7} homozygous strain (B6.C3KO-I-A^{g7}). C3-deficient mice expressing both KRN and I-A^{g7} were produced by crossing B6.C3KO-I-A^{g7} females and B6.C3KO-KRN males (C3KO KRN/I-A^{g7} F1).

All mice were bred and maintained in the animal facility of the School of Medicine, University of Pennsylvania. The animal breeding, maintenance, and experimental procedures were carried out by the protocols approved by the Institutional Animal Care and Use Committee of the University.

Mouse Genotyping

KRN TCR expression, C3 wild-type, and disrupted C3 alleles were determined by polymerase chain reaction (PCR) amplification of genomic DNA from mouse tail DNA [21].

MHC II I-A^{g7} and MHC II I-A^b alleles were identified by tail DNA PCR also, following the protocol developed in Diane Mathis' laboratory. The conditions of the PCR, resulting in a 180-bp DNA fragment for both genotypes, were: 1 cycle of 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; followed by 5 min at 72°C. The 5' primer sequence for I-A^{g7} is 5'-TTC AAG GGC GAG TGC TAC TT, and the 3' primer sequence is 5'-GTT CGC TCC AGG TAC TGC TT. The 5' primer sequence for I-A^b is 5'-TTC ATG GGC GAG TGC TAC TT, and the 3' primer sequence is 5'-CGT TCG CTC CAG GAT CTC. Each PCR was carried out in the volume of 25 µl with 3.0 mM MgCl₂, 1 M betaine, 0.5 mM dNTP, 400 nM 5' primer, 400 nM 3' primer, and 0.25 units of Platinum Taq polymerase (Invitrogen, San Diego, CA, USA) and 1 µl of mouse tail digestion.

Double Immunodiffusion Assay

Serum C3 was detected by double immunodiffusion (Ouchterlony) test, using goat anti-mouse C3 antiserum (Bethyl Laboratories, Inc., Montgomery, TX, USA).

Micro-CT

Micro-computed tomographic (micro-CT) imaging of formalin-fixed mouse hind limbs in air was performed with an eXplore Locus SP micro-CT specimen scanner (GE Healthcare Technologies, London, Ontario, Canada), using the following parameters: 80 kVp, 80 µA, 250-µm Al filter, and 2 frame averages. Image data were acquired using 400 views in 0.5° steps with 1.6 s exposure time, 2×2 detector bin mode, and 40 min scan time. Raw data were processed by a filtered back-projection Feldkamp algorithm and reconstructed at an isotropic resolution of 25 µm. The 3D image data were volume-rendered using OsiriX software (www.osirix-viewer.com) with a color palette representative of bones and muscles together with a logarithmic inverse opacity function to enhance subtle differences in intensity. Using the same window level and width for all data sets allowed for qualitative visual comparison of bone damage severity, with lighter colors representing greater apparent bone density.

Serum Transfer

Eight- to 12-week-old male B6 and B6.C3KO mice received intraperitoneal injections (i.p.) of KRN/I-A^{g7} F1 serum on day 1 and day 3. Ankle thickness and clinical index were evaluated as described below.

Mouse Arthritis Evaluation

Ankle thickness was measured at the malleoli with a caliper (World Precision Instrument, Sarasota, FL, USA). The clinical index of arthritis was scored on the scale from 0 to 4. Level 0, no redness and swelling of ankles or wrists; level 1, mild, but definite redness and

swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits regardless of the number of affected digits; level 2, moderate redness and swelling of ankles or wrists; level 3, severe redness and swelling of the entire paw including digits; levels 4, maximally inflamed limb with involvement of multiple joints. The scores of all four limbs were summed.

Mouse arthritis was evaluated daily until the joint swelling reached plateau, then every other day until swelling was no longer present.

Mouse Serum Anti-GPI Antibody Detection

Serum anti-GPI antibodies were detected by solid-phase enzyme-linked immunosorbent assays (ELISA). GPI was expressed from a his-tagged mouse GPI construct obtained from Diane Mathis in Protein Expression and Libraries Facility (Wistar Institute, Philadelphia, PA, USA). Polyvinylchloride flat bottom microtiter plates (Thermo Electron Corp., Milford, MA, USA) were coated with 5 µg/ml GPI in BBS (0.2 M borate buffered solution, pH 8.2) at 4°C overnight. Coated plates were blocked with BBT (0.5% bovine serum albumin and 0.4% Tween-80 in BBS) at room temperature for 1 h. The plates were washed three times with BBS after each incubation. Serum samples and controls were diluted in BBT, added to plates, and incubated at 4°C overnight. Mouse serum anti-GPI κ was detected with 25 ng/ml biotinylated rat anti-mouse κ (BD Bioscience, San Jose, CA, USA), followed by avidin-alkaline phosphatase in 1:10,000 dilution (Sigma-Aldrich, St. Louis, MO, USA). Each reagent was added and incubated at room temperature for 1 h following three washes with BBST (0.5% Tween-80 in BBS). Phosphatase substrate 4-nitrophenyl phosphate (Sigma-Aldrich) at the concentration of 1 mg/ml in 10 mM diethanolamine was added and incubated at room temperature for 4 h. Optical density at 405 nm of each well was measured using a microplate reader (E-Max, Molecular Devices Corp., Sunnyvale, CA, USA). All reagents used in this ELISA were added to plate at the volume of 100 µl per well. A laboratory-pooled KRN/I-A^{g7} F1 anti-GPI serum was used as experimental reference and positive control [22].

Statistical Analysis

The levels of serum anti-GPI were analyzed with Soft-Max v4.5. and reported as EDF (equivalent dilution factor to standards). The data were presented as geometric means from each group of samples. Error bars represent SE of each group of data. Statistical significance (*p* value) was determined by Student's *t* test.

Results

Mouse Arthritis in C3-Deficient KRN/I-A^{g7} Mice

In contrast to the reported results with the transfer of anti-GPI-positive serum, we were surprised to observe that KRN/I-A^{g7} F1 that were totally C3-deficient exhibited pronounced joint inflammation of all distal joints of paws at 4 weeks, similar to that seen in KRN/I-A^{g7} that had normal C3 genes (Fig. 1a). In order to verify C3 deficiency in these mice, disrupted C3 allele was reconfirmed by PCR, and the absence of serum C3 was indicated by double immunodiffusion assay (data not shown.)

To check whether the inflammation only occurred in soft tissue or whether bone structure was involved also, the bone damage of affected joints was evaluated by micro-CT. Post-mortem limbs from B6, C3KO KRN/I-A^{g7} F1, and KRN/I-A^{g7} F1 mice at the age of 12 weeks were scanned by micro-CT. Figure 1b shows the plantar surface of the left foot from B6, C3KO KRN/I-A^{g7} F1, and KRN/I-A^{g7} F1 mice. Strikingly, massive bone destruction was obvious in the paws of both C3KO KRN/I-A^{g7} F1 and KRN/I-A^{g7} F1 mice. All

interphalangeal, metatarsal, and tarsal joints (and their fore limb equivalents, not shown) were damaged; the calcaneum was also damaged, depicting a typical image of bone erosion together with anarchic reconstruction. Consistent results were seen in an additional four mice of each genotype that were imaged (data not shown).

In a longitudinal experiment, matched cohorts of C3-deficient KRN/I-A^{g7} mice and C3-intact KRN/I-A^{g7} were followed from weaning for clinical signs of arthritis and serum anti-GPI antibody production. All mice developed severe clinical arthritis over the following 4 weeks, accompanied by increasing titers of anti-GPI antibody (Fig. 2). The C3KO mice had an earlier onset of arthritis, but had slightly less severe disease after age 6 weeks. Interestingly, the clinical disease was apparent in both groups at 4 weeks, at which time serum anti-GPI antibodies were not detectable, and two C3KO mice already had joint inflammation at 3 weeks. Measurement of ankle thickness was also consistent (Fig. 2b).

Passive Arthritis in C3-Deficient KRN/I-A^{g7} Mice

Several previous studies have shown that KRN/I-A^{g7} serum transfer induced little or no arthritis in C3-deficient mice. Since C3KO KRN/I-A^{g7} F1 mice had remarkable amount of serum anti-GPI antibodies and pronounced joint inflammation, we speculated that higher levels of anti-GPI might be able to bypass the complement system to induce arthritis. To test this hypothesis, we transferred increasing quantities of anti-GPI serum to B6.C3KO-I-A^{g7} and B6 mice (Fig. 3). In the C3-sufficient B6 mice, the inflammation was visible 24 h after first serum injection, and it rapidly attained maximal clinical index value, particularly in the groups that received 400 and 800 μ l serum injection. The B6.C3KO-I-A^{g7} mice showed minimal inflammation in the two higher dose groups, comparable to what has been reported in the past.

Sera of all groups of mice were collected at 24 h after the second anti-GPI serum injection (day 4) and when ankle redness and swelling generally started to decrease (day 15). Figure 4 shows that in the highest dose group (800 μ l $\times$ 2), the serum levels of anti-GPI were apparently comparable to those seen in sera for 12-week-old KRN/I-A^{g7} F1 mice with or without C3, although the numbers of mice that could be tested were limited. In all groups the anti-GPI antibody levels decreased substantially 11 days later.

Discussion

Previous findings have clearly implicated the alternative complement pathway in the pathogenesis of inflammatory arthritis in the KRN model. Mice receiving KRN serum, containing anti-GPI autoantibodies, failed to develop clinical arthritis, if they were deficient in C3 or factor B. In addition, C5 appeared to be critical, since deficiency in C5 or its receptor also provided protection from joint inflammation. In contrast, early complement components that were essential only for the classical complement pathway or the lectin (MBP) pathway were found to be irrelevant [17, 23]. This body of information fit nicely with the parallel work that suggested that the main isotype of the pathogenic anti-GPI autoantibodies was IgG1, which is known to be incapable of fixing complement by the classical pathway [24].

Our present findings indicate that this conception of the pathogenesis of the genetic KRN model needs to be revised [16]. We found that KRN/I-A^{g7} F1 mice that produce endogenous anti-GPI autoantibodies do not require C3 to develop severe, rapidly progressive inflammatory arthritis, including extensive bone destruction as seen on micro-CT. Thus, C3KO KRN/I-A^{g7} F1 mice begin to show clinical arthritis at weaning or soon after, and then express increasing levels of anti-GPI autoantibodies in the serum. Over the next 4 weeks, the prevalence grows to 100%, and the severity reaches the maximum. Although our current

clinical study suggested that the chronic disease in the C3KO mice was slightly milder, we have not performed repeated studies of large cohorts to confirm this point. It would perhaps be unlikely that C3 would be totally irrelevant, given its strong influence on disease in the serum transfer model. It would certainly be expected that all three complement pathways, classical, alternative, and MBP, would be blocked in the absence of C3. The essential point is that the destructive process of the KRN/I-A^{g7} F1 model can proceed in the presumed complete absence of participation of the complement systems.

Why are the conclusions regarding the role of complement so different in the intact genetic and serum transfer models of KRN arthritis? Clearly this implies that certain pathogenic mechanisms must operate differently in these two variants. It is possible that the levels of autoantibody in the intact model are so high that they can compensate for the lack of C3 and still activate the other known players in this system, such as mast cells, neutrophils, macrophages, and follicular dendritic cells [14, 25–27]. However, we do not believe that serum antibody levels alone are responsible, since large doses of passive antibody (i.e., 800 μ L $\times$ 2) induced little joint inflammation in C3KO recipients, even though the levels of antibody reached in the passive recipient of these doses were at least transiently comparable to those seen in the intact genetic model. In addition, severe clinical arthritis appeared in the KRN/I-A^{g7} F1 mice several weeks before maximum antibody levels were measurable in the serum, and even began before autoantibody was detectable (Fig. 2). In individual KRN/I-A^{g7} F1 mice, we saw no overall correlation between anti-GPI levels and clinical arthritis, in either the C3KO or C3 WT mice. However, the pattern of earlier onset arthritis in the C3KO mice was reflected in the earlier appearance of anti-GPI antibodies in the group. The *persistence* of autoantibodies in the intact genetic model may be more critical than peak levels (see Fig. 2). Unfortunately, this is very difficult to test, as it would require impractical amounts of KRN serum for transfer. We have not directly tested the effectiveness of the anti-GPI autoantibodies produced in C3-deficient KRN/I-A^{g7} F1 mice to transfer arthritis to either C3KO or C3 WT recipients. Alternatively, the presence of autoantibodies at an earlier age in the intact genetic model may allow for complement-independent inflammation to develop. Finally, the pathogenesis of arthritis in the intact genetic model may depend on the presence of anti-GPI T cells or B cells by mechanisms that are independent of circulating autoantibody. We would, in fact, propose that all the relevant mechanisms that have been demonstrated in the serum transfer model should in principle be revisited in the spontaneous genetic model in order to understand fully the implications of anti-GPI autoimmunity. To this point, recent data have implicated a role for CD8 T cells in the genetic model [28].

Our results in the KRN model recall parallel findings in murine lupus models. Although complement consumption and complement deposition are characteristic of both murine and human lupus, MRL/lpr.CD3^{-/-} and C57BL/6. C4^{-/-}, C3^{-/-} mice had disease a little different from that seen in the C3 wild-type strains [29, 30]. Curiously, factor D-deficient or factor B-deficient MRL/lpr mice had decreased disease [31, 32].

The implications for human disease are not clear at this time. Is complement consumption an epiphenomenon in RA? In this condition, serum complement levels are not decreased, although joint fluid may have evidence for complement depletion [33]. We would speculate that complement is a facultative mechanism that may play more of a role in some patients than others. Similarly, serum antibody in RA (most likely anti-CCP) may be pathogenic, but other mechanisms of the adaptive immune system may also have the potential to contribute.

Conclusion

The pathogenesis of spontaneous KRN/I-A^{g7} arthritis can largely proceed by complement-independent pathways and must have pathology effector mechanisms in addition to those seen in the passive serum transfer model.

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Fig. 1. Destructive arthritis in complement deficient mice. **a** Ankle arthritis of KRN/I-A^{g7} mice with or without complement C3. Representative images of right hind paws of 2-month-old C3-deficient B6.I-A^{g7} (left), B6.KRN/I-A^{g7} (middle), and C3-deficient B6.KRN/I-A^{g7} (right) mice. Both ankles of KRN/I-A^{g7} mice, with or without functional C3, showed severe redness and swelling. **b** Bone destruction of KRN/I-A^{g7} mice with or without complement C3. Representative micro-computed tomography (micro-CT) of left hind paws of 3-month-old C3-deficient B6.I-A^{g7} (left), B6.KRN/I-A^{g7} (middle), and C3-deficient B6.KRN/I-A^{g7} (right) mice. Lighter colors indicate higher bone density

Fig. 2. Development of arthritis in matched cohorts of C3-sufficient and C3-deficient KRN/I-A^{g7} mice. The clinical index (CI) and ankle thickness of mice were recorded weekly from age 3 to 12 weeks. Anti-GPI antibodies levels were tested biweekly. Shown are arithmetic means and SE ($N=6$ in each group). In these figures, *solid diamonds* represent C3-sufficient mice while *open squares* represent C3-deficient mice. Although the arthritis onset was earlier in C3-deficient mice (week 3) than C3-sufficient mice (week 4), the arthritis was more severe in C3-sufficient mice than that of C3-deficient mice after 6 weeks. $*p<0.05$ for comparison of C3KO and C3-sufficient groups

Fig. 3. KRN/I-A^{g7} serum-induced arthritis. Two hundred, 400, or 800 μl of KRN/I-A^{g7} serum was administrated IP on day 1 and day 3 to C3-intact mice (*black diamonds*: 200 μl; *black squares*: 400 μl; *black circles*: 800 μl.) or to C3-deficient mice (*open diamonds*: 200 μl; *open squares*: 400 μl; *open circles*: 800 μl). *N*=3 mice per group

Fig. 4. Ant-GPI levels in KRN/I-A^{g7} serum transferred mice. Blood samples were collected 1 day and 12 days after second serum injection (day 4 and day 15, respectively). Each mouse is represented by a *vertically paired solid* (day 4) and *open* (day 15) symbol: C3-intact B6.I-A^{g7} mice (*solid squares*: day 4; *open squares*: day 15); C3-deficient B6.I-A^{g7} mice (*solid circles*: day 4; *open circles*: day 15). For comparison, titers are shown from 12-week-old KRN/I-A^{g7} F1 mice with or without C3 (*solid and dotted lines*, respectively). Values shown are EDF in reference to a pooled KRN/I-A^{g7} F1 anti-GPI serum