A Longitudinal Study on an Autoimmune Murine Model of Ankylosing Spondylitis

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Running title: A Murine Model of Ankylosing Spondylitis

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Abbreviations: AS, ankylosing spondylitis; CFA, complete Freund’s adjuvant; CIA, collagen-induced arthritis; ELISA, enzyme-linked immunosorbent assay; IVD, intervertebral disk; JAS, juvenile ankylosing spondylitis; PG, proteoglycan aggrecan; PGIA, PG-induced arthritis; PGISp, PG-induced spondyloarthropathy; RA., rheumatoid arthritis; SpA, spondyloarthropathy; TNF-α, tumor necrosis factor-alpha.
Abstract

Background. Proteoglycan (PG) aggrecan-induced arthritis (PGIA) is the only systemic autoimmune murine model which affects the axial skeleton, but no studies have been performed characterizing the progression of spine involvement.

Objectives. To follow pathologic events in experimental spondylitis, and underscore its clinical, radiographic, and histological similarities to human ankylosing spondylitis (AS); and determine whether the spondyloarthropathy is a shared phenomenon with PGIA, or an “independent” disease.

Methods. Arthritis/spondylitis-susceptible BALB/c and resistant DBA/2 mice, and their F1 and F2 hybrids were immunized with cartilage proteoglycan aggrecan, and radiographic and histological studies were performed prior to onset and during the progression of spondylitis at weekly intervals.

Results. Approximately 70% of the PG-aggrecan immunized BALB/c mice develop spondyloarthropathy (PGISp), and the progression of the disease is very similar to human AS. It begins with inflammation in the sacroiliac joints and with enthesitis, and then progresses upwards involving multiple intervertebral disks. In F2 hybrids of arthritis/spondylitis-susceptible BALB/c and resistant DBA/2 mice the arthritis incidence was 43.5%, whereas the frequency of spondylitis was over 60%. Some arthritic F2 hybrid mice had no spondylitis, others developed spondylitis in the absence of peripheral arthritis.

Conclusions. The PGISp model provides a valuable tool for studying autoimmune reactions in spondylitis, and identifying genetic loci associated with spondyloarthropathy.

[Key words: animal model, spondylitis, autoimmune, proteoglycan aggrecan, mouse]
(Introduction)

Ankylosing spondylitis (AS) is a chronic inflammatory autoimmune disease of the axial skeleton, and considered to be a “prototype” of spondyloarthopathies (SpA). AS frequently shows familial aggregation with male preponderance, and the association of AS with the human leukocyte antigen (HLA)-B27 as strong evidence of autoimmune etiology, was first described more than 30 years ago.1 The combination of HLA-B27 with other HLA alleles (HLA-B60 and HLA-B35) was found to increase the genetic predisposition up to six-fold,2 3 and genome-wide screening studies suggested the polygenic character of AS.4 5 Despite intensive research, the pathological mechanism of AS is unknown. Studies on putative autoantigens implicated the role of molecular mimicry represented by *Klebsiella* antigens,6-9 *Yersinia* antigens,10-12 self-recognized HLA-B27,11 13 14 or epitopes in the cartilage proteoglycan (PG) aggrecan15-19 in AS.

Animal models are invaluable aids in the research of human autoimmune diseases, and there are only a few systemic animal models, mostly in genetically altered rodents, which involve intervertebral disk (IVD) pathology.20-27 Among the rodent models of SpAs, immunization of susceptible mice (BALB/c and some C3H sub-strains) with human cartilage proteoglycan (PG) aggrecan,28-30 or with the G1 domain of aggrecan or versican,31 32 induces progressive polyarthritis which is frequently associated with spondylitis resembling human AS. While the spine involvement in PG aggrecan-induced arthritis (PGIA) has been known since the murine model was first described,28 33 no systemic study has been performed to determine whether it is only a concomitant phenomenon of peripheral arthritis, or whether arthritis and spondylitis represent two different diseases; although both are induced by immunization with cartilage PG. Here we describe a longitudinal study on the spine during the progression of PGIA in BALB/c mice. We also show the clear divergence of the two models (arthritis and spondylitis) in F2 hybrids of arthritis-susceptible (BALB/c) and arthritis-resistant (DBA/2) strains of mice.
MATERIALS AND METHODS

Antigens, animals, experimental groups, and immunization

The use of human cartilage from joint replacement surgeries for antigen isolation was approved by the Institutional Review Board, and all animal experiments were approved by the Institutional Animal Care and Use Committee. Human cartilage PG was used for immunization of 24-26-week-old female BALB/c mice purchased from the National Cancer Institute (NCI, Kingston colony, NY). Preparation of cartilage PG aggrecan (henceforth PG) was performed as described earlier.\textsuperscript{34, 35} As a standard method,\textsuperscript{30, 34, 35} the first antigen injection (100 µg PG protein) was given in complete Freund’s adjuvant (CFA; Difco, Detroit, MI), and the same doses of antigen were injected as second and third boosts in incomplete Freund’s adjuvant on week 3 and 6 (n=210). Age-matched control female BALB/c mice were either not immunized (n=75) or immunized with ovalbumin, a non-cartilage-related antigen in adjuvant (n=75). A small group of PG-immunized males (n=12) was used as a positive, and ovalbumin plus adjuvant-injected BALB/c males (n=8) as a negative control group, and all were sacrificed at the end of experiments (on week 36; figure 1).

In a second set of experiments, arthritis- and spondylitis-susceptible BALB/c female mice\textsuperscript{28, 30, 33, 34} were mated with arthritis-resistant DBA/2 males and the resulting F1 offspring were mated to generate F2 hybrids. These F1 (36 females and 12 males) and all F2 hybrids (106 females and 117 males) of BALB/c x DBA/2 intercrosses were also immunized with cartilage PG aggrecan as described above.\textsuperscript{29, 34, 36}

Clinical and histological assessments of arthritis and spondylitis

Immunized BALB/c mice were examined twice a week for clinical symptoms of arthritis. A standard scoring system, based upon swelling and redness of each paw (a maximum score of 4 per paw) was used for the assessment of arthritis.\textsuperscript{30, 34, 35} During the first 6 weeks of
immunization (until the third injection), we sacrificed five immunized mice every week. During the next 5 weeks, as arthritic mice became available (figure 1; inductive phase of arthritis), we sacrificed five arthritic and five yet non-arthritic mice each week (figure 1). From week 12 (i.e., 6 weeks after the third injection), when eventually all PG aggrecan-immunized BALB/c mice were arthritic, except for three females (Table 1), we sacrificed five mice weekly until week 26, and then 20 arthritic mice were sacrificed on weeks 30. The remaining 32 arthritic and three non-arthritic PG-immunized females, and all males, were sacrificed on the last day (week 36) of the experimental period (figure 1).

In the second set of experiments, PG-immunized BALB/c (n=81), DBA/2 (n=48), and their F1 (n=48) and F2 (n=223) hybrid offspring were scored weekly, and onset and severity of arthritis were recorded as described above for BALB/c mice. Animals were sacrificed on week 30 and disease onset and severity were correlated with the final histological score of spine.

Serial sections were stained with hematoxylin-eosin, safranin O-fast green, and picrosirius red.37 38 Spines of control, and age-matched PG-immunized arthritic BALB/c mice were stained with alizarin red and alcian blue for gross pathologic examination as described.39

A histology scoring system was established for spine involvement. Enthesitis, inflammatory cell accumulation around the IVD and/or infiltration of the annulus fibrosus was recorded as severity score 1, less than 50% absorption/erosion of the IVD received a score of 2, essentially complete resorption (>50%) of the IVD was recorded as score 3, and cartilaginous/bony ankylosis as score 4. A minimum of 18-22 IVDs (from the distal cervical to lumbar regions) of each control and immunized mouse was scored. Finally, a spondylitis score of each animal was calculated by dividing the cumulative score (all IVD scores) with the number of IVDs examined histologically. Autoimmune reactions (antibody production and T-cell
responses) to immunizing human and mouse (self) PGs were determined as described previously.  

RESULTS

Development of peripheral arthritis, and PG-specific antibody and T-cell responses

As described earlier and above (experimental groups), most of the PG-immunized BALB/c mice developed peripheral arthritis between days 7 and 14 after the third antigen injection (table 1). All arthritic mice showed strong humoral and cellular immune responses to PG as described in detail in our earlier papers. In this respect, we found no differences between mice with and without spine involvement, and no correlation between the severity of spondylitis and PG-specific antibody levels. Similar to that described above for antibody responses, T-cell reactions in PG-immunized BALB/c mice with and without spondylitis were highly comparable, and neither PG-specific antibodies nor T-cell responses were detected in non-immunized or ovalbumin/adjuvant-immunized mice.

Histological assessment of spine involvement in PG-immunized BALB/c mice

Histological analysis revealed axial involvement as early as week 8 after the third PG injection (data not shown). The disease affected only the PG-immunized mice and abnormalities could not be detected in any of the control animals. Cartilage surface erosions were found first in the sacroiliac joints (figure 2B); IVDs of the proximal tail became affected simultaneously with, or shortly after the development of inflammation in the sacroiliac joint. During the course of the disease, first the lumbar, and later the proximal thoracic and distal cervical segments became involved, but not all IVDs were equally affected at a certain time point. The fibro-cartilaginous articular surface of sacroiliac joints became eroded (figure 3B), which was followed by a focal (figure 2C) and then more extensive (figure 2D) chondrocyte proliferation. This reactive cell
proliferation gave rise to a chondrophyte-like tissue, completely ankylotizing the sacroiliac joint within 1-2 months after the first X-ray (not shown) or histological abnormalities could be observed (figure 2D).

In the acute phase of spondylitis, strong mononuclear infiltration appeared, leading to destabilization of the IVD. The damaged or resorbed annulus fibrosus allowed the nucleus pulposus to protrude in a ventral direction. The protruded IVD or the growing osteophytes frequently caused compression of the spinal cord, particularly at the lower cervical regions (figure 3D).

In order to study the changes in the extracellular matrix and its components, sections were stained with safranin O to assess the PG content (figures 3B and 3E), and picrosirius red staining was used to determine the orientation of the collagen fibers by polarization microscopy (figures 3C and 3F). Safranin O staining revealed that, in addition to the growth plate and end plate of the vertebra body, the nucleus pulposus and the annulus fibrosus also contained large amounts of PG (figure 3B). While PG was lost from endplates, large chondrophytes, and later osteophytes, grew at connecting adjacent vertebra bodies (figure 3E). A polarization microscopic study revealed the normal lamellar orientation of the collagen fibers in the annulus fibrosus and end plate in normal IVDs (figure 3C); an orientation which was lost in affected IVDs (figure 3F).

**Macroscopic analysis of spondylitic spines**

For macroscopic examination, spines were stained with alizarin red and alcian blue (figure 6). This staining reveals more details of the cartilaginous and bony components of the deformed spine regions. In contrast to the normal disk where the growth plates and end plates of the vertebral bodies appeared as sharp solid lines (figure 4A), the contours of these cartilaginous tissues became diffuse due to chondrophyte formation (figure 4B). Progression of spondylitis, was observed in each animal that developed spondylitis: the disk space disappeared and a
chondrophyte/osteophyte, fusing the neighboring vertebral bodies, replaced the original IVD (figure 4C).

**Analysis of peripheral arthritis and spondylitis in F2(BALB/c x DBA/2) hybrid mice**

As described above, three PG-immunized BALB/c female mice did not develop PGIA, but two of the three showed spondyloarthritis at the end of the experiment (table 1). Therefore, we assume that spondyloarthritis develops independently of arthritis in PG aggrecan-immunized BALB/c mice. To confirm this hypothesis and separate overlapping clinical, histopathological and laboratory parameters of the two diseases, we immunized 223 F2(BALB/c x DBA/2) hybrids, and their parents. All mice (BALB/c, DBA/2, F1 and F2 hybrids) were sacrificed on week 30 of immunization, as the development of PGISp achieved a constant (~70%) level by week 26 of immunization (table 1). Figure 5A shows the major characteristics and distribution of peripheral arthritis and spondylitis in these experimental groups. Approximately 40% of the PG aggrecan-immunized F2(BALB/c x DBA/2) mice developed PGIA, but unexpectedly, over 60% had spondylitis. Most of the arthritic mice had spondyloarthritis (85 of 97), and 23.3% of the PG aggrecan-immunized F2 hybrids had spondyloarthritis without joint inflammation (figure 5A; right-side panel).

In this second set of experiments, 81 (63 female and 18 male) BALB/c mice developed PGIA (mean arthritis score: 11.6 ± 2.4), which was associated with PGISp (mean spondylitis score of affected animals: 1.45 ± 0.62) in 57 animals (70.4%).
DISCUSSION

The normal IVD is a complex fibrocartilaginous structure containing poorly-defined chondrocyte- and fibroblast-like cells that can survive in a relatively avascular environment. The extracellular matrix of the IVD contains two major groups of macromolecules: collagens and PGs. While the nucleus pulposus contains almost uniformly aggrecan and type II collagen, the annulus fibrosus is formed mostly by type I collagen fibrils embedded into aggrecan and versican.41 The insertion site of the ligaments into the cortical bone, i.e., the enthesis, also contains PG, mostly versican,42 which might be degraded in IVDs undergoing degenerative processes.43 44

The core protein of human cartilage (and nucleus pulposus) PG aggrecan is over 2,300 amino acid-long, and contains 20-27 T cell epitopes30 presented to T cells by various major histocompatibility complexes (MHC) of antigen presenting cells.45 Most of these epitopes, however, are in “cryptic” position, i.e., masked by glucosaminoglycan side chains. A few T cell epitopes of human PG aggrecan have been characterized as dominant/arthritogenic, and due to the high sequence homology between human and mouse PGs,46 47 immunization of susceptible BALB/c and C3H mice with human PG provokes autoimmune responses to mouse (self) PGs, which then induce arthritis and spondylitis.28-30 34

Spondylodiscitis (a robust form of inflammation involving the entire IVD) occurs in 15% of patients with AS,48 but the incidence is greater (up to 60%) when diagnosed by magnetic resonance imaging.49 The primary site of the inflammation in AS, however, is still debated; it is proposed to be subchondrium49 or in the synovium (sacroiliac joint) close to the entheses.50

To date, spontaneous or experimentally induced disk degeneration have been reported only in a few animal models.20-22 24 26 27 51 Autoimmune mechanisms are thought to be involved only in HLA-B27 transgenic rodents23 25 52 and in PGIA.28-30 34 As HLA-B27 transgenic animals, when
maintained in germ free conditions, do not develop spondylitis,\textsuperscript{25, 53} the molecular mimicry of bacterial antigens may be a strong contributing factor to the pathomechanisms of SpA either in these transgenic rodents or in human individuals. Nevertheless, none of these animal models is identical with human AS, but may mimic genetic and/or pathological abnormalities present in the human disease. One of the most exclusive examples is that, in addition to the MHC, the major genetic loci of the PGISp in F2 hybrids of BALB/c and DBA/2, and BALB/c and C3H intercrosses was localized on chromosome 2 (authors yet unpublished observation), which correspond to that identified in patients with AS.\textsuperscript{5, 54} PGIA is characterized by a fine cooperation between antibody-producing and antigen presenting B cells and T helper cells,\textsuperscript{30, 55} and affects both the axial and peripheral skeleton. The chronology of pathological events in this model suggests that the involvement of the sacroiliac joint and the spine is similar to AS. PG-immunized mice first exhibit strong mononuclear influx and develop pannus-like tissue in the sacroiliac joint. The infiltrated tissue gradually destroys cartilage, which is followed by complete ankylosis of the joint. Simultaneously with, or shortly after, the initial signs of sacroiliitis, spondylitis/spondylodiscitis flares up with mononuclear infiltration around the annulus, followed by destabilization of the annulus and protrusion of the nucleus. Matrix components from the nucleus could be additional local sources of autoantigens. The abundance of antigens in this environment can initiate self-sustaining (auto)immune reactions that ultimately lead to the complete loss of IVD, and reactive chondrophyte/osteophyte formation and ankylosis fusing together vertebra bodies.

PG aggrecan represents one of the major extracellular matrix proteins located in immunoprivileged sites of the body (such as in the articular cartilage or nucleus pulposus of the IVD), therefore, it might become a target antigen in autoimmune diseases.\textsuperscript{15-17, 19, 56-58} Here, we demonstrate that spondylitis develops in arthritis-susceptible mice immunized with PG, and the
progression of the spine disease resembles that seen in human AS. Therefore, we postulate that the PG aggrecan plays an important role in PG aggrecan-induced spondyloarthropathy (PGISp) in this model, and perhaps also has a role in humans with autoimmune SpA.\textsuperscript{15 16 18 19} This is the first time that a systematic and longitudinal study has been performed on spine disease in mice with systemic immunization with and autoantigen (cartilage PG aggrecan). We found that spondylitis developed without arthritis, especially in PG aggrecan-immunized F2 hybrids of PGIA-susceptible BALB/c and resistant DBA/2 strains. Vice versa, approximately 30\% of arthritic mice did not show evidence of spondylitis. These observations strongly suggest that PGIA and PGISp are independent diseases, although immunization with cartilage PG is an absolute requirement for the induction of either PGIA or PGISp.

In conclusion, it is very likely that there are both overlapping/shared and distinct genetic components at the level of (auto)immune regulation in these two models. While the autoimmune pathomechanisms of PGIA and PGISp seem to be similar, the incidence, severity, and particularly the genetic control of joint and spine involvement are likely distinct. Using F2 hybrids of arthritis- and spondylitis-resistant DBA/2 and susceptible BALB/c mice we could show a maximum of 57\% (85 of 149 affected animals) overlap between PGIA and PGISp, whereas 40\% of the F2 hybrid mice with spondylitis did not have arthritis. Therefore, this report supports the relevance of performing genome-wide screening studies for identification of susceptibility loci controlling AS in this experimental murine model, which seems to mimic the human disease.
ACKNOWLEDGEMENT

This work was supported in part by grants (AR40310, AR45652, AR51163) from the National Institute of Health (USA).

COMPETING INTEREST STATEMENT

Authors state that there is nothing to declare
**Table 1. Incidence of arthritis and spondylitis in cartilage PG aggreca-immunized mice**

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<td>10</td>
<td>14</td>
<td>20</td>
<td>24</td>
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| Incidence of peripheral arthritis | 0/210 (0%) | 8/180 (4.4%) | 127/130 (97.7%) | 107/110 (97.3%) | 82/85 (97.1%) | 52/55 (94.5%) | 20/20 (100%) | 44/47 (93.9%) |

| Incidence of spondylitis | N.d. | 0/30 (0%) | 0/50 (0%) | 13/25 (52%) | 16/25 (64%) | 8/25 (72%) | 14/20 (70%) | 34/47 (71.4%) |

†Incidence is expressed as the number of affected/total BALB/c mice (results are also shown as % in parentheses). The last column includes the results of the 12 positive control males (PGISp incidence: 75%), whereas all other animals were female BALB/c mice.

‡By week 5 after the third PG injection, only three PG-immunized mice remained non-arthritic, and these animals were followed until the end of the experiment. Two of these three non-arthritic BALB/c mice developed spondylitis.

‡Calculation is based upon histological analysis of the spine, and positive/total number of sacrificed animals (percent) is shown. N.d. - not determined.
REFERENCES


Figure legend

**Figure 1.** Time course of immunization, number of animals sacrificed and analyzed weekly, and the time of onset of arthritis in peripheral joints (PGIA; thick black arrow) and spondylitis (PGISp; thick white arrow). A total of 210 female BALB/c mice were immunized with human cartilage PG aggrecan in the first experimental group, and 5 sacrificed weekly.

**Figure 2.** Inflammation of the sacroiliac joints and progression of ankylosis in PG-immunized BALB/c mice with spondylitis. The normal sacroiliac joint (A), which is first eroded by a synovial pannus-like hyperproliferative tissue (black arrows) at the articular surfaces indicated with dotted lines in panel B, as early as 4-6 weeks after the onset of arthritis in peripheral joints. This was followed shortly by massive proliferation of chondrocyte-like cells (white arrowheads on panel C), which formed a junction of chondrophyles resulting in complete ankylosis of the sacroiliac joint (D). The tissue sections were stained with hematoxylin and eosin.

**Figure 3.** Cervical spine segments in normal mice (A, B, C) and in mice afflicted with spondylitis (D, E, F). The affected disks are shown with the ankylosing osteophytes (black arrows and dotted white lines: IVD C4/C5) which protrude and compress the spinal cord (SpC) (D). Massive enthesitis develops with a pannus-like tissue eroding the rest of the IVD at the ventral side of the spine. Safranin O/fast green staining (B, E) demonstrates the PG content (red) in the growth plate (GP), in the cartilaginous end plate (EP), and, less intensively, in the annulus fibrosus (AF) and nucleus pulposus (NP) of the normal (B) or spondylitic (E) spine. In the affected spine (E) the annulus fibrosus and the nucleus pulposus have been completely resorbed and the end plate (black arrowheads) is essentially absent (E). The neighboring vertebra bodies
are connected by chondrocytes (black arrows), which ankylotize the spine. Polarization microscopy of picrosirius red-stained sections (panels C and F) indicates that the regularity of collagen fibers, due to parallel orientation in the normal annulus fibrosus (C)(white arrows) is completely lost in the annulus fibrosus of the disk affected by inflammation (F).

**Figure 4.** Progression of spondylitis in the lumbar spine (L3-L5) in PG-immunized BALB/c mice. Alizarin red stains bone red, while alcian blue stains the cartilaginous structures blue. Black arrowheads indicate the cartilaginous growth plates of the vertebra bodies (A; control), which became moderately deformed by 12 weeks (B) and grossly altered by 24 weeks (C) after the onset of peripheral joint inflammation. White arrows show chondrocyte formation at the marginal regions of the vertebra bodies.

**Figure 5.** Analysis and comparison of PG-induced arthritis (PGIA: A) and PG-induced spondylitis (PGISp: Sp) in 81 BALB/c (B/c), 48 DBA/2 (D/2) mice and their F1 and F2 hybrids (BALB/c x DBA/2) immunized with human cartilage PG (A). All mice were injected four times with PG and sacrificed on week 30 of the experiment, i.e., 21 weeks after the last immunization. Panel B summarizes significant differences when males and females with arthritis and/or spondylitis were compared. Significant differences are indicated with asterisks (*p<0.05; **p<0.01).
**IMMUNIZATION**

- 1st
- 2nd
- PGIA
- 3rd

- 0 weeks:
  - 5 non-arthritis PG-immunized mice/week (n=30)

**INDUCTIVE PHASE OF ARTHRITIS**

- 6 weeks:
  - 5 non-arthritis PG-immunized mice/week (n=25)
  - 5 arthritic PG-immunized mice/week (n=25)

**PROGRESSIVE PHASE OF ARTHRITIS**

- 11 weeks:
  - 5 PG-immunized arthritic mice/week (n=75)

- 36 weeks:
  - 20 PG-immunized arthritic mice
  - 35 female and 12 male PG-immunized arthritic BALB/c mice
### Figure 5

#### A

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Arthritis 43.50%  Spondylitis 61.43%

#### B

Onset, severity or spondylitis scores

**F2 (BALB/c x DBA/2)**

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Ann Rheum Dis published online January 7, 2005

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