INVESTIGATION OF THE GENETIC BACKGROUND OF INFLAMMATORY RHEUMATOLOGICAL DISEASES

ZOLTAN SZABO M.D.

Medical and Health Science Center, University of Debrecen
3rd Department of Internal Medicine
Division of Rheumatology

DEBRECEN
2007.
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ZOLTAN SZABO M.D.

Head of immunological programme:
Prof. Margit Zeher M.D., D.Sc.

Supervisors:
Prof. Tibor Glant M.D. D.Sc., Zoltan Szekanecz M.D., D.Sc.

Medical and Health Science Center, University of Debrecen
3rd Department of Internal Medicine
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INTRODUCTION

Ankylosing spondylitis (AS) is the prototype of spondylarthropathies. Despite of the well known pathogenetic factors (genetic background, gender, environmental factors, inflammation of sacroiliac and other joints, increased bone formation etc.), the cause of the disease is still unknown. According to genetic studies of human ankylosing spondylitis major histocompatibility complex (MHC) is the most important factor of disease susceptibility. Whereas, effect of non-MHC genes is might as important as MHC on susceptibility of any autoimmun disease. Family studies of AS (genome scans) implicated several chromosome regions: MHC: 6p, non-MHC: 2q, 10q, 11q, 16q, 17q, 19q.

To date, spontaneous or experimentally induced spondylarthropathy has been reported in only a few animal models, and autoimmune mechanisms are thought to be involved in only HLA–B27–transgenic rodents and mice with proteoglycan (PG)–induced arthritis (PGIA) and spondylitis (PGIS). Because HLA–B27–transgenic animals, when maintained in germ-free conditions, do not develop spondylitis, the molecular mimicry of bacterial antigens is believed to be a contributing factor to the pathomechanisms of spondylarthropathy in transgenic rodents and, perhaps, in humans as well. Although neither the HLA–B27–transgenic model nor the PGIA model is identical to human AS, both models mimic genetic and/or pathologic abnormalities present in the human disease.

Immunization of BALB/c or C3H/HeJCr (C3H) mice with cartilage PG induces progressive polyarthritis (PGIA), which is frequently accompanied by spondylitis. Since PGIA reaches 100% incidence in these 2 susceptible murine strains, and because the
onset of spondylitis cannot be precisely determined in vivo in mice, involvement of the
spine has been considered a concomitant symptom of PGIA. However, our recent study
suggested that PGIA and PGIS could be 2 independent diseases, even though both are
induced by immunization with cartilage PG. The major supporting argument for this
notion is that, in a mixed genetic background, PGIA occurs without spondylitis, and a
number of intercrossed mice with PGIS do not have peripheral arthritis.

Rheumatoid arthritis (RA) is an autoimmune disease leading to chronic synovitis
and eventually bone destruction. The etiology of the disease is still unknown, however,
both genetic and environmental factors play important roles in the onset of RA. The
major histocompatibility complex (MHC) is encoded in human leukocyte antigen (HLA)
gene clusters on chromosome 6. Among MHC class II molecules, various HLA-DR
alleles have been associated with susceptibility to RA in several racial groups. In
addition, some HLA-DRB1 alleles have also been related to the severity and outcome of
RA.

These disease associated HLA molecules share a common amino acid sequence in
the third hypervariable region of the DRB chain, the so-called "shared epitope" (SE). SE
is a sequence of five amino acids. Among the SE variants, the QKRAA motif is found in
DRB1*0401 and DRB1*0409; the QRRAA sequence has been described in DRB1*0101,
DRB1*0102, DRB1*0404, DRB1*0405, DRB1*0408, DRB1*0410, DRB1*1402 and
DRB1*1406 variants, while the RRRAA motif is specific for the DRB1*1001 subtype.

There are also significant ethnical and geographical differences. For example, in
RA patients from Northern Europe ong ethnic groups. In the Northern European
population HLA-DRB1*04 is strongly associated with RA, while Mediterranean RA patients rather carry the DRB1*01 and DRB1*10 alleles. Furthermore, in North American natives, DRB1*14 is associated with susceptibility to RA. There has been one recent report on Hungarian RA patients showing strong association of the HLA-DRB1*0404 subtype with RA.

In general, the HLA-DR4 allele is found in 75% of Caucasian RA patients, as well as in 30% of the healthy Caucasian population. Among the DRB1*04 alleles, the DRB1*0401 and DRB1*0404 alleles have been associated with increased susceptibility to RA in Caucasian patients with RA, whereas the frequency of the DRB1*0405 allele is increased among Japanese and Chinese patients. Among HLA-DR1 subtypes, the association of the DRB1*0101 allele with RA has been reported in Ashkenazy Jews and in various Caucasians patient populations.
OBJECTIVES OF THE STUDY

1.) We analyzed the occurrence and incidence of PGIS in arthritis-susceptible and arthritis-resistant murine strains, to gain information about the aethiology and pathomechanism of this autoimmune spondylitis. Determination of genetic susceptibility has clinical and prognostical implications as well. We hope, that our findings may contribute to our knowledge about the pathogenesis of certain human diseases (e.g.: AS) and also might help identifying new potential biomarkers and therapeutic targets of spondyloarthropathies.

Our specific aims were:

• to characterize the incidence and severity of PGIS using F1 and F2 hybrids of PGIS-susceptible and PGIS-resistant mouse strains;
• to determine the incidence and severity of PGIS in the context of disease-related parameters of inflammation and the immune response;
• to investigate the role of genetic background in disease susceptibility and severity.

2.) In our second study, we determined the frequency of various HLA-DRB1*01 and DRB1*04 alleles in a North-Eastern Hungarian population of RA patients.
MATERIALS, PATIENTS AND METHODS

STUDY I.

PGIS was induced in susceptible BALB/c and C3H strains of mice, and in their F1 and F2 generations derived from intercrosses with arthritis- and/or spondylitis-resistant DBA/2 and DBA/1 parent strains, by systemic immunization with cartilage PG aggrecan. Incidence and severity of arthritis and spondylitis was determined and correlated with serum antibody and cytokine levels, and in vitro T-cell responses to cartilage PG aggrecan.

Antigens and immunization of animals.

Total cartilage extracts were obtained by 4M guanidinium chloride extraction from adult human articular cartilage. These cartilage extracts were further purified by repeated CsCl gradient ultracentrifugation under dissociative conditions (in 4M guanidinium chloride). Purified PG extracts were treated with chondroitinase ABC (Seikagaku America/Associates of Cape Cod, Falmouth, MA, USA), 5 units/100 mg of PG in 0.1M Tris–acetate buffer, pH 7.6, for 24 hours at 37°C, to deplete chondroitin sulfate side chains. PG samples were subsequently digested with endo-β-galactosidase (Seikagaku America), 0.1 unit/100 mg of PG in sodium acetate buffer, pH 5.8, to remove residual keratan sulfate side chains present in adult cartilage. Since the keratan sulfate and chondroitin sulfate side chains may mask a number of dominant/arthritogenic epitopes, the depletion of these glycosaminoglycan side chains is critical in order to “retrieve” dominant arthritogenic T cell epitopes of the core proteins of PG. PG extract
was obtained by diethylaminoethyl (DEAE; Whatman, Clifton, NJ, USA) ion-exchange chromatography. The unbound fraction was retrieved at 0.15\(M\) \(NaCl\) and was further absorbed with hyaluronan–Sepharose to remove glycosaminoglycan-free G1-domain fragments of PG and cartilage link protein. Samples were dialyzed against water and lyophilized.

All animal experiments were approved by the Institutional Animal Care and Use Committee, of Rush University, Chicago, IL. Different experimental groups were maintained under the same conditions.

Female and male mice (12–16 weeks of age) of different inbred strains (BALB/c, C3H, DBA/1, DBA/2) as well as their F1 and F2 hybrids were used for immunization according to standard protocols. F1 and F2 hybrids of these inbred mouse strains were generated in our animal facility, maintained in a pathogen-free, but not germ-free, environment, and immunized exactly the same way as their parent strains. Some inbred mice and their F1 and F2 offspring were also studied retrospectively, by using available laboratory data and by examining new histologic sections of formalin-fixed archived spine tissues. To induce arthritis and spondylitis, 100 \(\mu\)g PG protein in 100 \(\mu\)l phosphate buffered saline (PBS) (sterile; pH 7.4) was injected intraperitoneally (IP) with a synthetic adjuvant, dimethyldioctadecyl–ammonium bromide (DDA) (Sigma-Aldrich, St. Louis, Mo, USA), or with 100 \(\mu\)g PG protein in 100 \(\mu\)l PBS (sterile; pH 7.4) and 100 \(\mu\)l Freund’s complete adjuvant (CFA) (2 mg/100 \(\mu\)l) in three weeks intervals. For immunization with human type II collagen (CII), the CII was dissolved in 0.1\(M\) acetic acid, diluted in PBS. One hundred micrograms of CII in 100 \(\mu\)l was then emulsified with an equal volume of CFA or DDA and injected intradermally into the base of the tail on
days 0 and 21. Animals immunized with cartilage PG received a total of 3 injections (days 0, 21 and 42) with adjuvant CFA or DDA. Animals were killed at 98–126 days after the first injection.

**Assessment of onset and severity of arthritis.**

The onset and severity of arthritis were determined using a visual scoring system that was based on the extent of swelling and redness of the paws. Animals were inspected weekly during the first 3 weeks and then (after the second injection) 2 times per week. The degree of joint swelling for each paw (scores ranging from 0 to 4) was expressed as a cumulative arthritis score, with a possible maximum severity index of 16 per animal. For scoring of arthritis onset, the first clinical appearance of swelling was defined as the onset, and an empirical onset score ranging from 6 (the earliest date) to 0 (end of experiment) was established over a 60-day period from day 10 after the second PG injection (i.e., from day 31 of the experiment).

**Radiological appearance of PGIS**

PGIS was confirmed by radiography of the spines and sacroiliac joints of PG-immunized mice using a Hewlett Packard Faxitron instrument (18 seconds, 65 kV; Buffalo Grove, IL) and high-resolution film (Kodak X-omat TL, Rochester, NY).

Characteristic signs of axial involvement during the development of PGIS could be detected 4 to 8 weeks after the onset of arthritis: irregular joint surface in the sacroiliac joint (*sacroileitis*) and narrowing of the intervertebral space. Later ankylosis occurs due to degeneration of intervertebral discs (IVD), which is similar that of human AS.
**Histological evaluation of PGIS**

The severity of PGIS was characterized by the histological abnormalities observed. After acquisition of radiographic images, spines were decalcified and embedded in paraffin, and light microscopy scoring of hematoxylin and eosin–stained sections was performed. In brief, spondyldiscitis, i.e., inflammatory cell infiltration around the IVD, was recorded as a severity score of 1, while <50% resorption of the IVD received a score of 2, advanced 50–100% resorption of the IVD was recorded as a score of 3, and cartilaginous/bony ankylosis of neighboring vertebral bodies was given a score of 4. An average of 18.7 IVDs per mouse was scored histologically, which included the distal cervical (from C3/C4) segment and all thoracic and proximal lumbar (to L3/L4) IVDs. Finally, a spondylitis index (SPI) was calculated for each animal, by dividing the cumulative score by the number of IVDs examined.

**Measurement antigen specific T and B cell responses**

Sera were collected at the end of experiments, and spleen cells were used for testing PG specific T cell responses. Antigen specific T cell proliferation was assessed on day 5 by measuring the incorporation of 3H-thymidine. Spleen cells (3x10^5 cells/well, 96 well plate) were stimulated in the presence of 50 µg of PG protein/ml. Antigen specific T cell responses were measured in quadruplicate samples. Antigen-specific IL-2 production (the response of CTLL-2 cells to IL-2 in the supernatants) was measured in 48-hour supernatants by the CTLL-2 bioassay. The antigen-specific T cell response was expressed as a stimulation index (SI), which represents the ratio of the counts per minute of incorporated 3H-thymidine in antigen-stimulated cultures to the cpm in nonstimulated cultures.
Antigen-specific production of interferon-γ (IFN-γ), interleukin-4 (IL-4), and tumor necrosis factor-α (TNF-α) was measured in cell culture supernatants (3x10^6 cells/ml) on day 4. Cytokine production of spleen cells, serum cytokine levels (TNF-α, interleukin-1β [IL-1β], interleukin-6 [IL-6], IL-4 and serum amyloid A (SAA, an acute-phase protein in mice) were measured using enzyme-linked immunosorbent assays (ELISAs; BD Biosciences, San Diego, CA, or R&D Systems, Minneapolis, MN).

Serum levels of PG-specific antibodies were also measured by ELISA. Maxisorp immunoplates (96 well, Nunc, Roskilde, Denmark) were coated with human or mouse cartilage PGs (0.1 µg of protein/well), and the free binding sites were blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions, and levels of both total anti-PG antibodies and isotypes of PG-specific antibodies were determined using peroxidase-conjugated goat anti-mouse IgG (Accurate, Westbury, NY) or rat mAb to mouse IgG1 or IgG2a (Zymed, South San Francisco, CA) as secondary antibodies. Serum antibody levels were calculated relative to the corresponding mouse IgG isotype standards (all from Zymed) or mouse serum immunoglobulin fractions (Sigma-Aldrich).

**Statistical analysis**

Statistical analysis was performed using SPSS software, version 10.0 (SPSS, Chicago, IL). Student’s t-test was used to compare the results of 2 groups. Spearman’s rho test was used to determine correlation coefficients. P values of less than 0.05 were considered significant.
STUDY II.

The frequency of various HLA-DRB1*01 and DRB1*04 alleles was determined enrolling eighty-three Hungarian RA patients (70 females and 13 males, all Caucasian) into the study.

RA patients and controls

Eighty-three Hungarian RA patients (70 females and 13 males, all Caucasian) were included in our study. All patients fulfilled the 1987 revised classification criteria of the American College of Rheumatology (ACR) (21). The mean age of the patients was 50 ± 15 years (range: 17-82). The mean disease duration at the time of the study was 6 ± 4 years (range: 0.5-22 years). Peripheral blood was drawn from each patient for DNA isolation.

Peripheral blood was also obtained from 55 healthy Caucasian controls (47 females and 8 males) with similar mean age (46 ± 13 years). These individuals were either hospital employees or visitors who were unrelated to the patients. Informed consent was obtained from each RA patient and control subject. For this study we also obtained ethical committee approval.

Polymerase chain reaction with sequence specific primers (PCR-SSP)

Genomic DNA was isolated from buffy coats of EDTA-anticoagulated blood using QIAamp Blood Mini Kit (QIAGEN) according to the instructions of the manufacturer.
Polymerase chain reaction (PCR)-based HLA-DRB genotyping (DRB1*01-DRB1*16) was performed with the help of sequence specific primers (Olerup SSP, Genovision, Norway). All samples were processed according to the instructions of the manufacturer using recombinant Taq DNA polymerase (Invitrogen). PCR amplification of DNA was performed using Hybaid PCR express thermal cycler. HLA genotypes were determined on the basis of the PCR product pattern obtained using 2% agarose gel electrophoresis. DNA bands were detected using Alpha Imager MultiImage Light Cabinet (Alpha Innotech Corporation, San Leandro, CA, USA).

**Statistical analysis of the second study**

HLA-DR allele frequencies in patients with RA were compared to those obtained from healthy subjects. Frequency comparisons for different antigens were performed by chi-square analysis with Yartes’ correlation or Fisher’s exact test using SPSS software, version 10.0 (SPSS, Chicago, IL). Differences between any two data groups were considered to be significant if p value was <0.05.

**RESULTS**

Incidence and severity of PGIA and PGIS in inbred mice and their F1 and F2 hybrids.

Incidence of arthritis and spondylitis in different inbred strains of mice and their F1 and F2 generations shows remarkable variance according to immunization protocols [cartilage PG (PGIA, PGIS) and CII (collagen induced arthritis, CIA)] and adjuvants used
As has been reported previously, 97–100% of PG-immunized BALB/c and C3H mice developed peripheral arthritis at 2 weeks after the third antigen injection. Massive inflammatory cell infiltration, pannus formation, and cartilage and bone erosion characterized the histopathologic picture of the affected joints. None of the DBA/1 or DBA/2 parents nor the (BALB/c x DBA/2)F1 hybrids developed arthritis until the end of the 14–18-week experimental period.

Whole-body radiographs revealed axial involvement (initial narrowing of the sacroiliac joint or intervertebral space) at ∼8 weeks after the third PG + CFA injection and as early as 3–4 weeks after the third PG + DDA injection. Thus, DDA accelerated the onset of PGIS, but the spondylitis still developed several weeks later than the peripheral arthritis. Importantly, PGIS was detected in only PG-immunized, but not in CII-immunized, mice, and abnormalities could not be detected in any of the control (arthritis-resistant) animals/strains. Cartilage surface erosions were first observed in the sacroiliac joints. PGIS was progressive and very similar in all affected animals/strains, and radiographic abnormalities of the spine and sacroiliac joints were supported by the histologic observations. During the course of the spine disease, the lumbar and then the proximal thoracic and distal cervical segments became involved, but not all IVDs were equally affected at any given time point. Some IVDs seemed to be intact or mildly damaged even when the vast majority of the disks were resorbed and the vertebral bodies underwent ossification or fusion.

The incidence of PGIS was 62–70% in inbred BALB/c and C3H mice, whereas only weak and sporadic discitis (involvement of only 1–2 IVDs) occurred in 2 (4%) of 50 PG + DDA–immunized DBA/2 mice. Moreover, no spondylitis was found in PG- or
human CII–immunized animals of the DBA/1 strain. The incidence and severity of spondylitis were highly comparable in both PGIS-susceptible inbred strains (BALB/c and C3H), regardless of the adjuvant used (CFA or DDA); nevertheless, as described above, PG immunization with DDA as adjuvant resulted in an earlier onset of PGIS.

Although F1 hybrids of the BALB/c x DBA/2 intercross were fully resistant to PGIA, unexpectedly, more than 30% of them developed PGIS, whereas none of the F1 hybrids of BALB/c x DBA/1 developed PGIS. Even more surprising was that F2 hybrids of BALB/c x DBA/2 (PGIS-susceptible versus PGIS-resistant parent strains, respectively) and (BALB/c x C3H)F2 mice (both of which have PGIS-susceptible parent strains) showed a similarly high incidence (63–70%) and severity of spondylitis, but none of the F2 hybrids of the BALB/c x DBA/1 intercross developed spine involvement. This finding was unexpected, because the F2 hybrids of both genetic intercrosses (BALB/c x DBA/2 and BALB/c x DBA/1) exhibited a similar incidence and severity of PGIA when immunized using the same protocol. Since the F2 hybrids of major histocompatibility complex (MHC)–matched mice (BALB/c x DBA/2; both having the H-2d haplotype) and MHC-unmatched mice (BALB/c H-2d versus C3H H-2k) exhibited a similarly high incidence and severity of PGIS, we focused, in subsequent studies, on the PGIS in these 2 intercrosses.

**Characterization of PGIS in F2 hybrids of BALB/c x DBA/2 and BALB/c x C3H mice.**

Although the onset of arthritis (PGIA) was earlier and the severity was higher in (BALB/c x C3H)F2 hybrid mice than in (BALB/c x DBA/2)F2 mice, the incidence of
PGIA and severity of spondylitis were comparable in the 2 hybrid groups. We found that 137 of 223 (BALB/c x DBA/2)F2 mice (61.4%) and 148 of 212 (BALB/c x C3H)F2 mice (69.8%) developed PGIS, with a mean ± SD spondylitis (histologic) severity score of 0.69 ± 0.63 and 0.66 ± 0.57, respectively. However, the number of mice considered negative (33.2%) (developing neither spine nor joint involvement) and the number that developed only PGIS without arthritis (23.3%) was significantly higher in the (BALB/c x DBA/2)F2 group than in the (BALB/c x C3H)F2 hybrids (9.9% negative and 2.8% with PGIS only). Nevertheless, when PGIA and PGIS occurred together, more progressive arthritis was usually associated with more severe spondylitis.

Immunological features of PGIS in F2 hybrids of BALB/c x DBA/2 and BALB/c x C3H mice.

The next evident question was whether any of the 4 clinical groups (i.e., negative, affected with arthritis only, affected with spondylitis only, or affected by both diseases) could be distinguished based on differences in T or B cell responses and/or disease-related markers measured in the serum. Although the power of statistical significance was reduced because of the high individual differences in F2 hybrid mice and because of the relatively low number of arthritic (BALB/c x DBA/2)F2 and negative or spondylitis only–affected (BALB/c x C3H)F2 hybrids, we could still observe significant differences in the serum IL-6 levels when the 4 groups in each of the 2 genetic combinations were compared. IL-6 was very high in the sera of animals having both arthritis and spondylitis, and the levels of both serum IL-6 and SAA positively correlated with the severity of PGIS.
We found very strong T and B cell responses to both human (immunizing) and mouse (self) PGs and high cytokine (TNF-α, IL-1β, IL-4, IL-6) concentrations in all PG-immunized mice, and compared all possible combinations. Although the anti-PG antibody (both auto- and heteroantibodies), IL-6, and serum TNF-α levels showed a significant correlation with the presence of PGIS in (BALB/c x C3H)F2 mice, only the serum IL-6 and SAA levels were significantly higher in (BALB/c x DBA/2)F2 mice with PGIS. However, this difference could be due to the earlier onset and increased severity of arthritis, but was not necessarily related to the spondylitis, in (BALB/c x C3H)F2 mice.

**Distribution of HLA-DRB1 alleles**

The distribution of HLA-DR antigens in our population is shown in Table 2. HLA-DR1 (HLA-DRB1*01) alleles were detected in 32.5% of RA patients compared to 18% of controls. However, this difference was statistically not significant (p=0.06). HLA-DR4 (HLA-DRB1*04) alleles were expressed in significantly more RA patients (31%) than controls (11%) (p<0.05). The frequency of HLA-DR12 (HLA-DRB1*12) was significantly lower in RA patients compared to controls (0% vs 18%, respectively) (p<0.05). No differences were found between RA patients and controls with regards to HLA-DR3, DR7-11 and DR13-16 allele frequencies.
DISCUSSION

In the present study, we analyzed the relationship between arthritis and spondylitis, both of which can be induced in PGIA-susceptible inbred mouse strains and their F1 and F2 hybrids. Although spondylitis, as a concomitant phenomenon of PGIA, has been known since the first description of the model, this is the first study in which the effects of antigens, adjuvants, and genetic backgrounds have been systematically investigated. We compared not only known spondylitis-susceptible BALB/c and C3H strains, but also their MHC-matched and MHC-unmatched F1 and F2 generations, which were derived from intercrosses with arthritis (CIA)-susceptible (DBA/1) and arthritis-resistant (DBA/2) strains.

The development of PGIS, like PGIA, requires cross-reactive immune (both T and B cell) responses between human cartilage PG used for immunization and mouse (self) PG. IVDs, both nucleus pulposus and annulus fibrosus, contain large amounts of the PG aggrecan, which is similar to that in articular cartilage. Since human PG shows very high homology with mouse PG, it is not surprising that in mice immunized with human cartilage PG, an immune attack is mounted against cartilaginous tissues in the mouse joints and IVDs. However, this occurs only in special genetic backgrounds, indicating that arthritis susceptibility per se is insufficient for the development of spondylitis. As described previously, DBA/1 mice develop arthritis (CIA), but not spondylitis, in any experimental condition, i.e., using either males or females, immunization with either cartilage PG or human CII, in either CFA or DDA. Although 30–40% of the F1 and F2 hybrids of the BALB/c x DBA/1 intercross, immunized with
either PG or human CII, developed arthritis, we could not detect inflammatory cells around or adjacent to the IVDs in these arthritic or nonarthritic animals when more than 3,500 IVDs of more than 230 spine sections were examined. This observation suggests that the DBA/1 strain carries very strong protective genes against PGIS.

Within the DBA/2 strain, which was completely resistant to PGIA and mostly to PGIS as well (i.e., only a small percentage of animals had very mild inflammation adjacent to 1 or 2 IVDs), ∼30% of their F1 hybrids with the PGIS-susceptible BALB/c strain developed spondylitis, with a relatively high severity score (mean ± SD 0.13 ± 0.06). In their F2 hybrids, the incidence (61%) and severity (0.69 ± 0.63) of spondylitis increased further, eventually reaching the values found in the parent BALB/c strain, when both the parental and hybrid mice were immunized with PG in DDA adjuvant. Thus, the DBA/2 genome should contain spondylitis susceptibility and protective genes that might be silent in the original background. These observations in the genetic combination of BALB/c and DBA/2, however, are the first proof that PGIA and PGIS possibly represent 2 different diseases. BALB/c and DBA/2 strains carry the same H-2d allele. This indicates that the MHC alone (e.g., in DBA/2 mice) is insufficient to control PGIS susceptibility. This notion was also supported by the results in the (BALB/c x DBA/1)F2 generation, in which ∼25% of the immunized mice were homozygous for the H-2d allele but none of the F2 hybrids developed spondylitis.

In contrast, when 2 spondylitis-susceptible strains (BALB/c and C3H) were intercrossed, the incidence of PGIS in the F2 hybrids was essentially the same as in any of the 2 parent strains. Thus, it is likely that neither the BALB/c nor the C3H strain has additional genetic loci that could either reduce, as in (BALB/c x DBA/1)F2 hybrids, or
increase, as in F2 hybrids of BALB/c x DBA/2 strains, the susceptibility to PGIS. This is not surprising, since the common ancestor of the C3H and BALB/c strains is a female Bagg albino. Thus, spondylitis-susceptibility genes could have been present in the Bagg albino before the BALB/c and C3H lines were separated as inbred strains. Because no other inbred strains have, as yet, been found to be susceptible to PGIS, we believe that only a very few genes control spondylitis susceptibility, and that these genes are most likely the same in the BALB/c and C3H strains. The first genome-wide screening studies of 223 (BALB/c x DBA/2)F2 hybrid mice have been completed recently, and indeed, only 2 definitive loci (on chromosomes 2 and 18) and 4 suggestive loci (on chromosomes 11, 12, 15, and 19) seem to be linked to PGIS in this MHC-matched generation.

Induction of an autoimmune arthritis in genetically susceptible rodents using CFA–based protocols requires at least 1 injection of antigen (either PG or CII) in CFA. This indicates that the activation of the innate immune system with mycobacterial components in oil is as important as the antigen-induced activation of the adaptive immune system. Mycobacterial compounds, such as heat-shock proteins, are known as potent nonspecific cellular stimulators, and muramyl-dipeptide (a peptidoglycan) and trehalosedimycolate (a glycolipid equivalent to lipopolysaccharide of \textit{Escherichia coli}) could play a role in the enhancement of immune reactions to self antigens in autoimmune models. Recently, we found that a hydrophilic/lipophilic quaterner ammonium base, DDA, which incorporates antigens into a liposomal micelle, is as effective an adjuvant as CFA (without the side effects of CFA) in the induction of either PGIA or CIA. Both CFA and DDA stimulate the innate immune system equally well and also activate antigen-specific effector and regulatory T cells of the Th1 arm of adaptive immunity.
We have thus compared the adjuvant effects of CFA and DDA on PGIS. As described earlier, DDA together with PG could induce arthritis and spondylitis in BALB/c and C3H mice. In these 2 parent (inbred) strains, we found no difference using either CFA or DDA with PG, but DDA appeared to be a more potent adjuvant than CFA in (BALB/c x DBA/2)F2 hybrid mice. We found a ~2-fold increase in the incidence of both PGIS and PGIA in F2 hybrids of the BALB/c x DBA/2 intercross, when these mice were immunized with PG in DDA instead of PG in CFA. However, if a strain was resistant to either PGIA, CIA, or PGIS, the antigen in DDA adjuvant was not sufficient to induce tissue-specific inflammation. In conclusion, although DDA, as a more potent adjuvant than CFA, may influence the incidence of spondylitis in susceptible mice, the (auto)immune responses to tissue/organ-specific antigen (in this case, PG) and the genetic background (including the appropriate MHC) are the most critical factors in the development of PGIS.

The MHC, in general, is the strongest genetic component in autoimmune disorders. The association of HLA–B27 with AS, as evidence of the autoimmune etiology of AS, was first described more than 30 years ago. The combination of HLA–B27 with other HLA alleles (HLA–B60 and HLA–B35) was found to increase the genetic predisposition to AS, and genomewide screening studies suggested a polygenic character of the disease. Despite intensive research in this area, the pathologic mechanism of AS is unknown. Studies on putative autoantigens implicated the role of molecular mimicry, represented by *Klebsiella* antigens, *Yersinia* antigens, self-recognized HLA–B27, or epitopes in cartilage PG or versican. The present study could not reveal which subdomains within the murine H-2 locus are responsible and/or involved in the genetic
predisposition to PGIS in either of the F2 hybrids of BALB/c x C3H or the BALB/c x DBA/2 intercross.

PGIS has been detected in the same inbred strains that are susceptible to PGIA. Nevertheless, the genetic, clinical, and laboratory findings are different in PG-induced arthritis and spondylitis. These differences may reflect only quantitative differences in animals affected with both diseases, however, the complete resistance to either arthritis or spondylitis, or both, in different genetic combinations suggests that PGIA and PGIS are 2 distinct diseases. Although many immune system traits and laboratory parameters (including antigen-specific T cell responses, PG-specific IgG isotype ratios, and levels of interferon-γ, IL-1β, or TNF-α) could distinguish between arthritic and nonarthritic mice, only the high serum IL-6 level seemed to be consistently associated with the presence of SpA in PG-immunized mice. This observation may indicate that PGIS is a more uniform disease than PGIA.

Indeed, although several genetic loci have been implicated in different forms of autoimmune/experimental arthritis and in rheumatoid arthritis, only a very few, probably only 2 dominant, non-MHC loci (on chromosomes 2 and 18) have been identified that could control susceptibility to spondylitis in (BALB/c x DBA/2)F2 hybrid mice. Importantly, both of these 2 genetic loci correspond to those identified in human patients with AS. Therefore, experimentally induced spondylarthropathy in mice can serve as a tool for elucidating the genetic, immunomodulatory, and other components that control the development of spondylitis.

In the second study, we assessed HLA-DRB1 genotype frequencies in a North-Eastern Hungarian population of RA patients in comparison to healthy controls. HLA-
DR4 was significantly more common in RA patients compared to controls. In addition, HLA-DR1 showed a tendency of being more frequent in RA. There are great geographical differences regarding this issue. Similarly to our results, HLA-DR4 is strongly associated with RA in Northern Europe, the United States, Germany and Argentina. In a single recent Hungarian study, HLA-DR4, as well as HLA-DR1, similarly to our present findings, was associated with RA. However, Mediterranean RA patients tend to carry HLA-DR1, as well as HLA-DR10, rather than HLA-DR4. Native North-American indians tend to carry HLA-DR14. Thus, with regards to HLA-DRB1 association of RA, our Hungarian RA patient population seems to be similar to Northern and Western, rather than Southern European RA patients.

Here we found diminished frequency of HLA-DR12 among our RA patients in comparison to controls. A study from Funland also suggests the protective role of HLA-DR12 in RA.

**SUMMARY OF RESULTS**

1. Spondylarthropathy was induced in susceptible BALB/c and C3H strains of mice, and in their F1 and F2 generations derived from intercrosses with arthritis- and/or spondylitis-resistant DBA/2 and DBA/1 parent strains, by systemic immunization with cartilage proteoglycan (PG) aggrecan.

2. This is the first study in which the autoimmune spondylitis (PGIS) has been characterized according to effects of antigens, adjuvants, and different genetic backgrounds.
3. The incidence and severity of PGIS were scored histologically. Our newly developed histological scoring method was used to compare the severity of spondylitis in different groups of mice examined.

4. These scores for spine involvement were correlated with serum antibody and cytokine levels and with in vitro T cell responses to cartilage PG. Serum IL-6 and SAA levels showed significant correlation with severity of PGIS.

5. New observations of the study are the following:
   - 60–70% of susceptible mouse strains and their F2 hybrids developed spondylitis either with or without arthritis.
   - Adjuvants, particularly those activating the innate immune system and enforcing the Th1 dominance, had significant effects on the outcome and progression of PGIS.
   - The DBA/1 strain appeared to carry genes protecting this strain and its F1 and F2 hybrids from spondylitis.
   - The DBA/2 strain, although resistant to PGIS, harbored genes permitting PGIS in its hybrid generations.
   - Arthritis- and/or spondylitis-susceptible BALB/c and C3H parent strains and their F2 hybrids exhibited the highest incidence and severity of spondylitis.

These are new informations regarding genetic predisposition to spondylitis. On the basis of our findings we think, that presumably only a very few non-MHC genes control susceptibility to PGIS, and that these genes are most likely the same in the BALB/c and C3H strains. The results of our investigations can serve as the base of an extensive research project about the genetic background of autoimmune spondylitis.
Assessing HLA-DRB1 genotype frequencies in our North-Eastern Hungarian population of RA patients we found, that HLA-DR4 was significantly more common in RA patients compared to controls. In addition, HLA-DR1 showed a tendency of being more frequent in RA.
PUBLICATIONS

Papers on the topic of the thesis:


Other papers related to the topic of the thesis:

1) Szanto S, Bardos T, **Szabo Z**, David CS, Buzas EI, Mikecz K, Glant TT.: Induction of arthritis in HLA-DR4-humanized and HLA-DQ8-humanized mice by human cartilage proteoglycan aggrecan but only in the presence of an appropriate (non-MHC) genetic background. Arthritis Rheum 2004; 50:1984-95. **IF: 7.19**


List of other publications:


Posters and presentations on the topic of the thesis:


Other posters and presentations:


16. Szabó, Z., Szanto, S., Bardos, T., Hanyecz, A., David, CS., Glant, TT.: Human MHC class II transgenic mice (HLA-DR4 and HLA-DQ8) recognize peptide epitopes of human cartilage proteoglycan (PG), but develop arthritis only in a genetically susceptible background. Rush University Forum for Research, Chicago, IL 2004.


Publications: 12

Impact factor of papers: 35,1