Ph.D. Thesis

THE ROLE OF HUMAN CARTILAGE PROTEOGLYCAN AGGREGAN IN INDUCTION OF ARTHRITIS IN HLA-HUMANIZED MICE

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Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease that usually affects diarthrodial joints and leads to the destruction of cartilage and erosion of bone. While the primary target organ is the synovial joint, there is no clear evidence that any macromolecule of the cartilaginous tissues, bone, or synovium would serve as a preferential autoantigen. Thus, the etiology of RA is unknown, and both environmental and genetic factors are thought to be involved in its pathogenesis. Among the genetic components, the major histocompatibility complex (MHC) is probably the most important predisposing factor for RA, but the MHC alone is insufficient for disease induction. Recently, several non-MHC loci have been identified that contain genes that are likely to be involved in the disease mechanism.

The association of RA with certain HLA alleles has been known for more than 2 decades, and more recently, relatively large numbers of subtypes of MHC alleles have been identified by polymerase chain reaction. Although there are considerable variations among ethnic groups, it is clear that individuals carrying the HLA-DRB1 allele *0101, *0401, *0404, or *0405 are at increased risk of developing RA. The different MHC alleles, however, are inherited together as a complex, and because the DRB1 alleles show a strong linkage disequilibrium with DQB1 alleles, such as DQB1*0301 (HLA-DQ7) and DQB1*0302 (HLA-DQ8), it is uncertain whether HLA-DRB1 alone or in combination with HLA-DQB1 alleles is the true predisposing factor for RA.

Although the MHC has the strongest effect on RA susceptibility, several non-MHC loci show linkage with the disease in studies of multicare families. A general problem with genome-wide mapping studies, however, is the relatively weak linkage between the non-MHC loci and disease susceptibility because of the extremely high genetic variation within the
human population. In support of studies in humans, a useful experimental approach to the identification of non-MHC loci is the use of genetically susceptible rodents in which the disease simulates certain characteristics of RA.

The most appropriate animal models for RA seem to be those that are induced by systemic immunization of genetically susceptible mice or rats with cartilage matrix components, such as type II collagen, proteoglycan (PG) aggrecan, link protein, or human cartilage glycoprotein-39. Proteoglycan-induced arthritis (PGIA) shows many similarities to RA, as demonstrated by the results of clinical assessments, immunologic tests, radiographic analyses, and histopathologic studies of diarthrodial joints. As with RA, PGIA is polygenic, and both MHC and non-MHC components are critical to the development of arthritis. Many of the non-MHC susceptibility loci identified in PGIA correspond to human chromosome regions where quantitative trait loci have also been found in family studies of RA.

PG is a complex macromolecule. Its core protein consists of 2,400 amino acids to which glycosaminoglycan (GAG) side chains of chondroitin sulfate and keratan sulfate are covalently attached. The presence of the large number of GAG side chains (>120/PG molecule) may mask the antigenic sites of the core protein; thus, an intact PG is a relatively weak antigen. Enzymatic depletion of the negatively charged GAG side chains significantly enhances the immunogenicity of the molecule, a feature that is exploited in the induction of PGIA. Some structural changes, such as partial deglycosylation or cleavage of the core protein of PG by various metalloproteinases, occur in vivo during the normal turnover of cartilage PG, but these processes are more extensive in inflammatory conditions. Fragments released during PG degradation may trigger and/or maintain local immune reactions in the synovial joints in arthritis-susceptible animals and perhaps in human individuals as well. Indeed, immune responses to human cartilage PG have been detected in patients with RA,
supporting the hypothesis that, among other candidate autoantigens, cartilage PG might be a target of the autoimmune inflammatory attack in RA joint lesions.

**Objectives**

1. To apply in PGIA and analyze the effect of dimethyldioctadecyl ammonium-bromide (DDA), a powerful adjuvant that does not have the side effects of the conventionally used Freund’s adjuvant

2. To address whether human cartilage PG can be presented to mouse T cells in the context of the RA-predisposing MHC class II alleles.

3. To determine whether any of the core protein epitopes of the human PG can induce arthritis in the presence of either a resistant or susceptible genetic background.

4. To map all predicted T cell epitopes of PG that can be presented by the most frequent predisposing alleles in RA.

**Materials and methods**

**HLA class II-transgenic mice**

These mice do not carry functional endogenous class II MHC genes because of a spontaneous mutation in the promoter region of the H-2Eα gene (in H-2b haplotypes), a defect which was then combined with a targeted disruption of the H-2Aβ gene (Aβ0 knockout). The original 4 transgenic lines used for the studies were HLA-DR2.Ab0 (DRB1*1502), HLA-DR3.Ab0 (DRB1*0301), HLA-DR4.Ab0 (DRB1*0401), and HLA-DQ8.Ab0 (DQA1*0301 coexpressed with DQB1*0302).
Antigens, immunization, and assessment of arthritis

PG was isolated from human cartilage, and the GAG side chains were depleted by digestion with chondroitinase ABC. Peptide sequence selection was based on the presence of MHC-binding motifs, \( \alpha \)-helix amphipathic sequence motifs that are common in T cell epitopes, and the hydrophobic strip-of-helix algorithm. As a result of this selection, a total of 143 aggrecan core protein-specific peptides were synthesized. These peptides were used for initial screening of T cell responses (T cell proliferation and interleukin-2 [IL-2] production) in PG-immunized HLA-transgenic and wild-type BALB/c mice. Peptides that elicited T cell responses were used for both the peptide-immunization experiments and the epitope-screening studies.

For epitope mapping, 6-8-week-old mice were injected intraperitoneally or into the footpad of the hind paws with PG antigen or synthetic peptide in Freund's complete adjuvant (CFA). Mice were killed 9 days after the priming injection or 9-10 days after the second or third intraperitoneal injection of antigen. Spleens were harvested from the intraperitoneally injected mice, and popliteal and inguinal lymph nodes were harvested from the footpad-primed mice. T cell responses of spleen or lymph node cells were measured in the presence of PG or each synthetic peptide.

For arthritis induction, 8-12-week-old mice expressing different HLA class II transgenes and lacking endogenous class II MHC molecules, along with their wild-type littermates, were immunized intraperitoneally with PG at 3-week intervals. Since immunization with PG and DDA/CFA caused lower mortality and more severe arthritis than that with PG and CFA/IFA, mice were injected alternately (every second injection) with either 1) PG antigen dissolved in PBS and mixed with 1 mg of the adjuvant dimethyldioctadecylammonium bromide (DDA) in PBS or 2) PG antigen emulsified with CFA. The appearance of joint swelling was recorded as the time of onset of arthritis. A
standard scoring system based upon swelling and redness (range 0-4 for each paw) was used for the assessment of disease severity. The limbs of arthritic and nonarthritic mice were dissected, fixed, decalcified, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin for histopathologic examination.

**Measurements of antigen-specific T cell responses, antibodies, and cytokines**

PG-immunized mice were killed 4-5 weeks after the fifth antigen injection. Sera were collected, and spleen and lymph node cells were used for testing PG/peptide-specific T cell responses. Antigen-specific T cell responses were measured in the presence of PG protein or synthetic peptide. Antigen-specific IL-2 production was measured in 48-hour supernatants by the CTLL-2 bioassay, and T cell proliferation was assessed on day 5 by measuring the incorporation of $^3$H-thymidine. The antigen-specific T cell response was expressed as a stimulation index. Antigen-specific production of interferon-γ (IFNγ) and IL-4 was measured in cell culture supernatants using enzyme-linked immunosorbent assays (ELISAs).

PG-specific antibodies were also measured by ELISA. Immunoplates were coated with human or mouse cartilage PG, and free binding sites were blocked with fat-free milk in PBS. Sera were applied at increasing dilutions, and both total anti-PG antibodies and isotypes of PG-specific antibodies were determined using peroxidase-conjugated goat anti-mouse IgG or rat anti-mouse IgG1 or IgG2a.

**Flow cytometry**

To determine the expression of mouse class II MHC and HLA molecules on the surface of antigen-presenting cells, leukocytes from heparinized peripheral blood or spleen cells were incubated with fluorescein isothiocyanate- or phycoerythrin-labeled monoclonal antibodies to mouse I-Ad or to human HLA-DR or HLA-DQ. Fluorescence was measured and analyzed with a FACSscan flow cytometer and CellQuest software.
Statistical analysis

Statistical analysis was performed using the SPSS software package. Mann-Whitney and Wilcoxon tests were used for intergroup comparisons. P values less than 0.05 were considered significant.

Results

Incidence and severity of PGIA in BALB/c mice immunized with PG and DDA/CFA or CFA/IFA

The major clinical parameters (arthritis onset, severity and incidence) were compared in commercially available BALB/c mice immunized with the two different method. The incidence was 100% and arthritis score was significantly higher at week 9-10 among the DDA/CFA immunized mice compared with CFA/IFA immunized group. The mortality caused by DDA was much lower than that of caused by Freund’s adjuvant.

PG immunization of HLA-transgenic mice on the original mixed (PGIA-resistant) genetic background

Mice of the 4 HLA-transgenic lines on the original mixed (likely PGIA-resistant) genetic background and PGIA-susceptible wild-type BALB/c mice were immunized and screened for arthritis. Approximately 70% of the PGIA-susceptible BALB/c mice developed arthritis 7-10 days after the third PG injection, and 100% developed arthritis within 3 weeks after the fourth PG injection. In contrast, no clinical or histologic signs of inflammation in peripheral joints were detected up to 4 weeks after the fifth PG antigen injection.

To assess whether the lack of arthritis in the transgenic mice could be attributed to impaired immune responses, we measured both cellular and humoral immune responses to
PG. The antigen-specific T cell responses, measured either as proliferation or as IL-2 production, in the different transgenic lines and in the arthritic wild-type BALB/c mice were highly comparable. Similar to the T cell response, each transgenic mouse line exhibited antibody responses to the immunizing human PG, although serum levels of both PG-specific IgG1 and IgG2a were lower in HLA-transgenic mice (except for the HLA-DQ8.Ab\(^0\) group) than in the wild-type BALB/c mice. These results indicated that, while none of the HLA-transgenic lines developed arthritis, all responded well to immunization with human cartilage PG, i.e., the human PG was presented effectively to mouse T cells in the context of HLA class II transgenes.

**T cell epitope repertoire in PG-immunized HLA-transgenic mice**

The next evident step was to determine which T cell epitopes within the PG core protein were presented by the different MHC alleles. For these experiments, HLA-transgenic mice were immunized intraperitoneally with human PG, and spleen cell responses to each of the 143 peptides that represented the predicted T cell epitopes were determined in vitro. All transgenic mice exhibited immune responses to the whole PG molecule, and a number of peptide epitopes of the PG core protein could be identified by T cell proliferation.

Mice expressing different HLA transgenes recognized distinct sets of PG epitopes. However, 2 sites within the G1 domain of the core protein (represented by peptides p25-42 and p268-285) contained sequences that were highly immunogenic in all HLA-transgenic mice. Moreover, 3 of the 4 dominant/arthritogenic epitopes of the human PG core protein, which were previously identified in BALB/c mice, also induced T cell responses in mice transgenic for HLA-DR4.Ab\(^0\) and/or HLA-DQ8.Ab\(^0\).

In addition to the 2 dominant regions of the G1 domain, a number of partially overlapping or nonoverlapping peptides also induced T cell responses, particularly in DR4.Ab\(^0\) and DQ8.Ab\(^0\)-transgenic mice, although they were usually of lower intensity.
Comparison of the epitope profile recognized by all 4 groups of HLA-transgenic mice indicated that 50% of the positive peptides induced strong T cell responses in DR4.Ab\(^0\) and DQ8.Ab\(^0\) mice (11 out of 20 in DR 4 and 8 out of 15 in DQ8 group, respectively), while this ratio was only 20% in DR2.Ab\(^0\) and DR3.Ab\(^0\) mice (2 out of 11 in DR2 and 3 out of 15 in DR3 group, respectively). Moreover, 1 of the dominant/arthritogenic epitopes identified in wild-type BALB/c mice (amino acids 2379-2394 in the G3 domain), containing the shared epitope sequence (QKRAA) with 1 or 2 conservative amino acid replacements, was also positive in DR4- and DQ8-transgenic mice.

**Effect of backcrossing HLA-DR4- and HLA-DQ8-transgenic mice onto the PGIA-susceptible BALB/c background and induction of arthritis**

To address whether HLA class II alleles in the absence of the mouse’s own class II MHC, but in the presence of an arthritis-susceptible genetic background, are capable of mediating autoimmune arthritis upon immunization with human cartilage PG, DR4- and DQ8-transgenic mice were backcrossed onto the arthritis-susceptible BALB/c background. These 2 transgenic lines were chosen, because of the strong linkage of DR4 and DQ8 to RA, because a number of peptide epitopes of the PG core protein induced strong T cell responses in these transgenic mice, and because DR4- and DQ8-transgenic lines were responsive to at least 2 of the 4 epitopes that were dominant/arthritogenic in wild-type BALB/c mice. The expression of DR4 and DQ8 alleles and the lack of I-Ad on splenocytes and peripheral blood mononuclear cells in transgenic mice were confirmed by flow cytometry.

While 70-80% of PG-immunized wild-type BALB/c mice developed arthritis after the third injection and 100% were arthritic after the fourth PG injection, both transgenic/congenic lines required a fifth antigen injection to develop arthritis. Although the HLA-transgenic mice received at least 1 additional antigen injection, the incidence and severity of PGIA were lower.
in the HLA-transgenic/congenic BALB/c than in the wild-type BALB/c mice. This was particularly evident in the DR4.Ab\(^0\)-transgenic/congenic line.

**Clinical and histopathologic features of PGIA in HLA-transgenic BALB/c mice**

While the clinical scores, the early histologic abnormalities, and the progression of the disease seemed to be slightly milder in the HLA-transgenic/congenic mice than in the wild-type BALB/c mice, there were essentially no differences in the histologic appearance of inflamed joints from wild-type and transgenic animals.

**Immunogenicity of PG-specific peptides in HLA-DR4.Ab\(^0\)- and HLA-DQ8.Ab\(^0\)-transgenic BALB/c mice**

The overall immune responses (assessed by T cell proliferation, antibody production, and epitope mapping) in the BALB/c DR4.Ab\(^0\) and DQ8.Ab\(^0\) PG-immunized transgenic/congenic mice were highly comparable with those described for mice harboring these HLA alleles in the mixed genetic backgrounds. To further characterize the immunogenicity of PG epitopes, spleen cells from PG-immunized DR4.Ab\(^0\) and DQ8.Ab\(^0\) BALB/c mice were cultured in the presence of selected core protein peptides, and T cell proliferation and cytokine production were measured. While some of the peptides in PG-immunized mice provoked stronger T cell proliferation than IL-2 production, others elicited the opposite response. The 11 strong/dominant DR4-associated peptides and the 8 DQ8-associated peptides also induced positive responses in the DR4-Ab\(^0\) and DQ8.Ab\(^0\) BALB/c mice. Cells stimulated with some of these peptides produced significant amounts of both IL-2, but very low levels of IL-4. In general, a higher IFN:IL-4 ratio was noted in arthritic mice, which was usually more pronounced in DQ8.Ab\(^0\)- than in DR4.Ab\(^0\)-transgenic BALB/c mice. However, no significant correlation was found between the presence of arthritis and T cell recognition of any particular peptide.
To distinguish between dominant and cryptic PG epitopes, DR4.Ab\(^0\)- and DQ8.Ab\(^0\)-transgenic/congenic BALB/c animals were primed with individual peptides that induced strong T cell responses after immunization with whole PG, and assays were performed 9 days after peptide injection. Among these selected peptides, again, peptides p28-42 and p271-285 provoked the highest T cell proliferation and predominantly Th1-type cytokine production in both transgenic lines. In DQ8.Ab\(^0\)-transgenic/congenic BALB/c mice, stimulation of lymph node cells with peptides p250-264 and p1989-2004 resulted in particularly strong IL-2 secretion, whereas these peptides induced only relatively weak IFN production and essentially no IL-4 production. Three other peptides (p76-90, p265-79, and p277-291) in DR4.Ab\(^0\), and peptide p2379-2394 in DQ8.Ab\(^0\) BALB/c mice, although recognized by T cells from DR4- and DQ8-transgenic mice after PG immunization, induced only very weak T cell proliferation and IL-2, IFN, and IL-4 production upon priming. Lymph node and spleen cells from mice primed with any of these synthetic peptides did not respond to stimulation with human PG. Thus, the peptides selected on the basis of strong T cell responses upon PG immunization represent cryptic epitopes.

**Discussion**

In this study, our aim was to determine whether the RA-prediposing class II molecules of the MHC can present cartilage PG aggrecan, and if so, to determine the epitope repertoire of the human cartilage PG in HLA-transgenic mice and determine whether HLA transgenic mice develop arthritis in response to immunization with human cartilage PG.

Although PGIA-resistant and PGIA-susceptible strains expressed highly comparable immune responses to cartilage PG upon immunization, arthritis susceptibility was clearly dependent on the presence of both the appropriate MHC allele (either H-2d, DRB1*0401, or DQB1*0302) and an appropriate (BALB/c) genetic background. The critical role of MHC and
non-MHC genes in PGIA was highlighted by previous findings. One of the best earlier examples was that wild-type BALB/c and congenic BALB/K mice (carrying H-2d and H-2k alleles, respectively) were susceptible to PGIA, whereas the congenic BALB/B line (carrying the resistant H-2b allele on the BALB/c background) was completely resistant. Similarly, while BALB/c mice (H-2d) exhibited 100% susceptibility to PGIA, neither DBA/2 nor NZB mice (both carrying H-2d) developed arthritis upon PG immunization. Therefore, it was not surprising that the HLA-humanized mice with RA-predisposing MHC alleles in mixed genetic backgrounds (CBA/J, B10.M, 129/Sv, C57BL/6) were uniformly resistant to PGIA.

By replacing the mouse H-2d (PGIA-susceptibility) allele with DR4 or DQ8 alleles in mice of the arthritis-susceptible (BALB/c) background, PGIA could be induced, indicating that disease development requires an optimal combination of MHC and non-MHC alleles. Although the RA-predisposing DRB1 (DR4) alleles have often been detected in linkage with DQB1 (DQ8) genes in humans, in the humanized mice, the presence of DRB1*0401 or DQB1*0302 alone was sufficient to reveal the arthritogenicity of human cartilage PG on an appropriate genetic (BALB/c) background.

An important observation of this study was a discrepancy between the predicted and the actual recognition of T cell epitopes of PG in immunized mice. We used various prediction algorithms, based upon known matches between MHC/peptide and peptide/T cell receptor (TCR) structures in BALB/c (I-Ad) mice, as well as between human DR4 and DQ8 alleles and TCR. Collectively, these results suggested that, except for a few gaps, the entire G1 domain, the highly homologous B and B loops of the G2 domain, and a part of the G3 domain C-terminal of the epidermal growth factor-like module, were saturated with frequently overlapping T cell epitopes. We synthesized peptides covering the entire G1, G2, and G3 domains with a 3-mer amino acid offset, as well as sequences that represented predicted epitopes in the keratan sulfate- or chondroitin sulfate-attachment regions. Of 143
synthetic peptide sequences tested in wild-type BALB/c mice immunized with human cartilage PG, 27 were identified as T cell epitopes. All 143 synthetic peptides were also tested for T cell response in DR4.Ab\(^0\)- and DQ8.Ab\(^0\)-transgenic mice.

Based upon earlier epitope-mapping studies in arthritic wild-type BALB/c mice, it was not surprising to find only a few peptides that were recognized in context with HLA in PG-immunized transgenic mice. Also, we were not surprised to identify a few, originally nonpredicted, T cell epitopes. However, we found it interesting that peptides spanning 2 regions of the PG core protein (p22-51 and p253-285) proved to be highly immunogenic in both DR4- and DQ8-transgenic mice, since these sequences were not predicted to be potential T cell epitopes for either DRB1*0401 or DQB1*0302 alleles in studies using multiple prediction methods. By contrast, a few peptides in the G1 domain and a peptide sequence (1785GAYGSGTPSSFP) in the chondroitin sulfate-attachment region with predicted high-affinity binding to DRB1*0401, which were selected as strong T cell epitopes, were found not to be recognized in the humanized mice.

We therefore subsequently used additional algorithms and epitope-prediction methods and selected those core protein peptide sequences that could elicit T cell responses in DR4- and DQ8-transgenic mice. As a result, while these p22-51 and p250-291 sequences originally were not selected as being efficient MHC-binding peptides, multiple T cell epitopes and MHC-binding sites were identified in both core protein regions. Thus, while epitope prediction can facilitate mapping studies, in vivo tests are necessary to confirm the correctness of the predictions. It is also possible that we might have missed a number of unpredicted T cell epitopes in the GAG-attachment regions, where only predicted T cell epitopes with high probability scores were synthesized and tested.

Despite the fact that the DR4 allele apparently presents a larger repertoire of PG core peptides than does the DQ8 allele, the incidence and severity of arthritis in the DR4.Ab\(^0\)-
transgenic/congenic BALB/c mice were significantly lower than the incidence and severity in the DQ8.Ab\(^0\)-transgenic/congenic BALB/c line. In both DR4.Ab\(^0\)- and DQ8.Ab\(^0\)-transgenic/congenic BALB/c mice, the overall severity and especially the incidence of arthritis were far lower than those in wild-type BALB/c mice, and HLA-transgenic BALB/c mice required at least 1 additional PG injection to provoke arthritis. The difference in disease incidence and severity between wild-type and HLA-transgenic BALB/c mice could be due to either a weak complex formation between the mouse CD4 and the HLA allele or the incongruity between the mouse TCR and HLA. The differences between the 2 transgenic/congenic lines might be due to either the different levels of expression of the HLA transgene or the different epitope repertoire presented by the 2 HLA alleles.

Further studies should focus on the in vivo consequences of the interaction of mouse versus human CD4 with HLA alleles, the effect of the coexpression of RA-predisposing DR4 and DQ8 alleles on an arthritis-susceptible genetic background, and the localization of peptide-binding sites within the hypervariable regions of the V\(_\alpha\)/V\(_\beta\) chains of the TCR. Thus, combined transgenic lines simultaneously expressing human CD4/HLA-DR4/DQ8 alleles, as well as appropriate TCR chains, on the BALB/c background may be useful tools for better understanding the disease mechanisms by revealing an autoimmune/arthritogenic epitope(s) of human cartilage PG and the non-MHC locigenes involved in pathologic mechanisms of RA.

**Summary**

In our work human cartilage PG was used as an arthritis-inducing antigen in humanized mice expressing different HLA alleles, instead of their own class II molecules, on an arthritis-susceptible or an arthritis-resistant genetic background.

We found that the human PG was presented effectively to mouse T cells in the context of HLA class II transgenes.
The experimental system provided us with an opportunity to identify epitopes of human cartilage PG that are presented by RA-predisposing HLA molecules. Beside the predicted peptide epitopes, some unpredicted sequences in the core protein of the PG molecule were recognized as being strong T cell epitopes in HLA-DR4.Ab\(^0\)- and HLA-DQ8.Ab\(^0\)-transgenic mice.

Using DDA, a potent nonirritant adjuvant, which eliminates the undesired side effects of the Freund’s adjuvant, we could induce PGIA in HLA-DR4.Ab\(^0\) and HLA-DQ8.Ab\(^0\) transgenic BALB/c mice, but not in transgenic mice with mixed genetic background. We could establish, that the histologic features of arthritic joints seemed slightly milder in HLA transgenic/congenic mice than in wild-type BALB/c mice, there were no qualitative differences in appearance of inflamed joints. While the epitopes of cartilage PG presented by HLA-DR4 or DQ8 can induce arthritis only in the presence of an appropriate genetic background, we could confirm the critical role of non-MHC-associated genes in the disease mechanisms.

**Thesis based on the following publications:**

**In English:**

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In Hungarian:

4. Szántó S, Glant TT, Szekanecz Z. Arthritises állatmodellek. Magyar Immunológia (accepted for publication)

5. Szántó S. Rheumatoid arthritis. Granum (accepted for publication)

Other publications:

In English:


In Hungarian:


Published abstracts supported the thesis:


19. Szanto S, Bardos T, Gonda A., David CS, Mikecz K, Glant TT. Peptide epitopes and arthritogenicity of human cartilage proteoglycan (PG) in human MHC class II transgenic mice (HLA-DR4 and HLA-DQ8. (EULAR 2004, oral presentation, Abott Abstract Award Winner)

Other published abstracts:


