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References

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Critical Roles of Glycosaminoglycan Side Chains of Cartilage Proteoglycan (Aggrecan) in Antigen Recognition and Presentation

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Systemic immunization of BALB/c mice with proteoglycan (aggrecan) from fetal human cartilage induces progressive polyarthritis, an experimental disease similar to human rheumatoid arthritis. The development of the disease in this genetically susceptible murine strain is based on cross-reactive immune responses between the immunizing fetal human and mouse self-proteoglycans. One of the cross-reactive and arthritogenic T cell epitopes (GR/QVRVNSA/IY) is localized in the G1 domain of human/murine proteoglycan. Susceptible BALB/c mice, however, develop arthritis only if both the chondroitin sulfate (CS) and keratan sulfate (KS) side chains of the arthritogenic human proteoglycans are removed. The function of these two glycosaminoglycan side chains is opposite. The presence of a KS side chain in adult proteoglycan inhibits the recognition of arthritogenic T cell epitopes, prevents the development of T cell response, and protects animals from autoimmune arthritis. In contrast, the depletion of the CS side chain generates clusters of CS stubs and provokes a strong B cell response. These carbohydrate-specific B cells are the most important proteoglycan APC. Taken together, proteoglycan-induced progressive polyarthritis is dictated by three major components: genetic background of the BALB/c strain, highly specific T cell response to epitope(s) masked by a KS chain in aging tissue, and the presence of proteoglycan (CS stub)-specific B cells required for sufficient Ag presentation.

Immuno

immunization of BALB/c mice with chondroitin sulfate (CS)-depleted high density proteoglycan (PG) (aggrecan) from fetal human cartilage induces progressive polyarthritis (1, 2). This animal model shows many similarities to human rheumatoid arthritis, as indicated by clinical assessments such as radiographic analysis and scintigraphic bone scans, and by histopathologic studies of diarthrodial joints (1, 3). During the course of arthritis, more and more joints are involved, and repeated inflammatory episodes result in a complete deterioration of articular cartilage and lead to severe deformities of the peripheral joints (1, 2, 4, 5). The development of arthritis is based on cross-reactive immune responses between the immunizing fetal human and mouse self-PGs (2, 6). While the mouse PG-specific Ab level precedes the inflammation and shows a correlation with the incidence of arthritis, these Abs do not transfer the disease (4, 7). An effective transfer requires the presence of both T and B cells from arthritic animals, and a rapid accumulation of T cells in the synovium appears to be the most critical component of the development of arthritis (6–8).

Our previous studies have compared the chemical composition and expression of various PG epitopes in cartilage, and have screened the arthritogenic potential of PGs isolated from fetal, newborn, and adult cartilages of 10 species (2, 9). In essence, PGs (Fig. 1) from fetal or newborn human and canine cartilages were the only ones able to induce a cross-reactive T cell response to mouse PG and subsequent arthritis in BALB/c mice, but only if the CS side chains were removed from the immunizing fetal-type PGs (2, 3, 9). As the depletion of the CS side chains was essential to reveal the arthritogenic effect of either human or canine fetal PGs (9), it was believed that a critical T cell epitope is located in, or close to, the CS attachment region of the core protein (Fig. 1) and expressed only in a splice variant present in fetal cartilage. Studies based on this compelling hypothesis resulted in the discovery of splice variants of human and canine PGs (10–12); however, the presence or absence of alternatively spliced exons of the core protein did not associate with arthritis induction in BALB/c mice (13).

Glycosylation differences between fetal and adult PGs in the same species (14–16) may explain, at least in part, the arthritogenic potential of fetal vs nonarthritogenic adult molecules. Recently, we have found that the in vitro response of a PG (aggrecan)-specific, and arthritogenic, T cell hybridoma (17) was significantly higher if the PG from adult cartilage was depleted of keratan sulfate (KS) side chains (18) and, especially, if the glycosaminoglycan (GAG)-depleted core protein is presented by either a B lymphoma (A20) or B cells from arthritic animals (19). Taken together, these experiments indicated that the presence of...
that remained of different glycosidases, and the primary structure of KS in adult cartilage PG. Mammalian hyaluronidase degrades CS side chains of PG, and the CS stubs resulting from unsaturated bonds between C4 and C5 of the terminal glucuronic acid residues. Clusters of these CS stubs are the most dominant B cell epitopes of PG in mice. Standard chemical abbreviations used: Ser = serine; Thr = threonine; Xyl = xylose; Gal = galactose; GlcUA = glucuronic acid; GalNAc = N-acetylgalactosamine; GlcNAC = N-acetylgalcosamine; and SA = stialic acid; CRP = complement regulatory protein.

FIGURE 1. Schematic presentation of the high density cartilage PG (aggrecan) structure. This cartilage PG (Mw ~2.2 x 10^6 Da) consists of a central protein core (Mw ~2.3 x 10^6 Da) to which glycosaminoglycan side chains of CS (Mw ~2.3 x 10^6 Da) and KS (Mw ~1.2 x 10^6 Da) are attached together with O-linked and N-linked oligosaccharides. The various domains/subdomains of the central protein core are: G1 domain with A, B and B' loops; IGD (interglobular domain between G1 and G2 domains); G2 domain with B and B' loops; KSrr (a KS-rich region between the G2 domain and CS attachment region); CS (CS1 + CS2) attachment regions and G3 domain containing EGF (epidermal growth factor-like), LB (lectin-binding), and CRP (complement regulatory protein-like) subdomains. Approximately 100 to 120 CS side chains are attached to a long but restricted region of the core protein (CS attachment region). Although most of the KS chains are localized in a special region (KSrr), KS side chains are also present along the entire core protein. Corresponding recombinant proteins (~hPG) used in these experiments are indicated by horizontal lines underneath the PG (aggrecan) structure. A more detailed structure of the N-terminal end of the PG molecule is shown at the left top corner with arrows indicating the positions of the 5/4E8 T cell epitope and the stromelysin cleavage site in the IGD. Two panels at the bottom right corner show the linkage region of the CS stubs remained O-linked to serine after the degradation of different glycosidases, and the primary structure of KS in adult cartilage PG. Mammalian hyaluronidase degrades CS side chains of PG, and the CS stub that remained O-linked to the core protein is essentially a very short CS chain. Bacterial amylases (e.g., chondroitinase ABC) generate shorter stubs with unsaturated bonds between C^5 and C^6 of the terminal glucuronic acid residues. Clusters of these CS stubs are the most dominant B cell epitopes of PG in mice. Standard chemical abbreviations used: Ser = serine; Thr = threonine; Xyl = xylose; Gal = galactose; GlcUA = glucuronic acid; GalNAc = N-acetylgalactosamine; GlcNAC = N-acetylgalcosamine; and SA = stialic acid; CRP = complement regulatory protein.

both GAG side chains (CS and KS) of PG (Fig. 1) somehow interfered with the immune response to T cell epitopes of the core protein, and inhibited the development of PG-induced arthritis. Here we present evidence that the depletion of the CS side chains of cartilage PG dramatically increases the B cell response to carbohydrate epitopes (clusters of CS stubs), and that these professional PG-presenting B cells play a crucial role in PG-induced arthritis. In contrast, the most critical arthritogenic T cell epitope(s) located in the G1 domain become masked with a KS chain in cartilage during aging. As the KS is resistant to in vivo degradation in the mammalian system, this GAG side chain has a protective effect and prevents susceptible animals from the development of an (auto)immune T cell response to PG and arthritis.

Materials and Methods
Preparation of PG monomer (aggrecan)
PGs of human fetal (HFC-PG) (27–30-wk gestation), newborn, maturing, and adult articular cartilages (HAC-PG) as well as from bovine fetal (FBC-PG) (21–24-wk gestation) and adult (18-mo-old steers) articular cartilages were prepared as previously described (20). Human tissue was obtained from autopsies within 2 to 8 h after death through either the Cooperative Human Tissue Network (Columbus, OH), Comprehensive Cancer Center (Birmingham, AL), or Department of Pathology, University of Medicine (Debrecen, Hungary). Human fetal and newborn cartilage samples were received from spontaneously aborted premature fetuses or from immature newborns with congenital heart malformation. Bovine articular cartilages were obtained from a local slaughterhouse, and mouse cartilage from the knee joints of 5- to 7-day-old BALB/c mice. Cartilage pieces were frozen and 20-μm cryostat sections extracted with 4 M guanidinium chloride in 50 mM sodium acetate, pH 5.8, containing protease inhibitors at 4°C (20). High buoyant density PG monomers (aggrecan) (Fig. 1) were prepared by dissociative cesium chloride gradient centrifugation (20). PGs for immunization were digested with protease-free chondroitinase ABC (Seikagaku America, Rockville, MD), one or all of the three endo-β-galactosidases (endo-β-galactosidase, keratanase, or keratanase II; all from Seikagaku), testicular hyaluronidase (Sigma Chemical, St. Louis, MO), N-glycosidase F (PNGase F; New England BioLabs, Beverly, MA), or O-glycosidase (Boehringer Mannheim, Indianapolis, IN) overnight at 37°C and then further purified on a Sephacryl S-200 (Pharmacia Biotec, Uppsala, Sweden).
column as described (1, 20). The absence of the KS side chains was confirmed by three anti-KS mAbs (EFG1, 5D4, and EFG4) (15, 20, 21). Core protein of PG was hydrolyzed by papain, an enzyme that cleaves the core protein in small peptides and releases single GAG chains coupled to a few amino acids (15, 20). Papain was inactivated by iodoacetamide and the digest used after dialysis or GAG chains purified by column chromatography as described (20). Protein was measured by Pierce’s biocinchonic acid assay (Pierce, Rockford, IL), uronic acid by Bitter and Muir’s (22) method, and the KS content by ELISA or radioimmunoassay (RIA) (15, 20, 21).

**Preparation of GI domain and G1-free PG fragments**

PG samples were pretreated with endo-β-galactosidase (100 mg of PG/0.1 U of enzyme) in 50 mM sodium acetate buffer (pH 5.8) in the presence of protease inhibitors (20) for 16 h at 37°C, dialyzed against stromelysin buffer (20 mM sodium acetate, 10 mM NaCl, 0.1% Brij, 0.02% NaN3), and then digested with 10 mg of PG protein/275 U of recombinant human stromelysin (EC 3.4.24.17) (a gift from Dr. M. Lark, Merck Research Laboratories, Rahway, NJ) (5, 23). The GI domain (Fig. 1) was separated from the rest of the PG fragments by DEAE-cellulose chromatography using stepwise elution with NaCl. GI domain and G1-free core protein fragments were identified by Western blotting using polyclonal Abs to core protein-generated neoepitopes of either the C-terminal (ΔVDIPEN) or N-terminal (Δ2FFGFGVVGE) cleavage sites of the core protein (Fig. 1) and a panel of mAbs against various protein and carbohydrate epitopes of human PG (15, 20, 24) (all from Chemicon International, Tamecula, CA). Protein cross-contamination between GI domain and G1-free fragment preparations after DEAE-cellulose chromatography was less than 1%. G1-free fragments of PGs were further digested with protease-free fragment preparations after DEAE-cellulose chromatography was less than 1%. G1-free fragments of human PGs (sp. act. ranging from 6 to 12 μC/μg protein), and the Ag-Ab complex pelleted either with protein A-bearing Staphylococcus aureus (Zysorbin; Zymed Laboratories, San Francisco, CA) for IgG subclasses, or pig anti-mouse IgM, followed by Zysorbin (15). The radioactivity of pellets was measured using a gamma counter (Beckman Instruments, San Francisco, CA) for IgG subclasses or, pig anti-mouse IgM, followed by Zysorbin (15). The radioactivity of pellets was measured using a gamma counter (Beckman Instruments, San Francisco, CA) for IgG subclasses or, pig anti-mouse IgM, followed by Zysorbin (15). The radioactivity of pellets was measured using a gamma counter (Beckman Instruments, San Francisco, CA) for IgG subclasses or, pig anti-mouse IgM, followed by Zysorbin (15).

**Recombinant PG core proteins**

Originally, a 2.7-kb human PG cDNA fragment (pSA003; nucleotide 2641-3539) (11) coding for almost the entire GI domain region was cloned into pGEX-3X expression vector (Pharmacia, Piscataway, NJ). The products of subclones of p2684-hPG represent the CS attachment regions, either the CS1 or CS2 domains (11) of the human PG core protein (Fig. 1). The PG-specific T cell hybridoma 5/4E8 was obtained following fusion of in vitro-stimulated lymphocytes from an arthritic mouse with B10.A(2R) thymoma cells as described (17). This hybridoma expresses CD4, TCRβ (Vβ4), and belongs to the Th1 subset (17).

**Isolation of APCs and T cells from mice**

**Splenic and lymph node B cells.** Splenic and lymph node B cells were isolated using miniMACS magnetic separation columns (27) (Miltenyi Biotech, Sunnyvale, CA) or PG-coated surfaces. Lymphocytes separated on a Lympholyte-M gradient (Accurate Chemical and Scientific, Westbury, NY) were incubated with a biotinylated mAb to CD45R (clone RA3-3A1/6.1), Lyt 2.2 (CD8; clone 2.43), L3T4 (CD4; clone 145-2C11), and P388D1 macrophage cell lines) and rat B cell hybridomas producing Abs to mouse Mac-1 (CD11b, clone M1/70.15.11.5HL), CD45R (BE-8D7), and the Ia region of H-2 (I-Ab, I-Ak reactive; clone M5/114,15,2), were obtained from American Type Culture Collection (Rockville, MD). The PG-specific T cell hybridoma 4E8 was obtained following fusion of in vitro-stimulated lymphocytes from an arthritic mouse with BW517 thymoma cells as described (17). This hybridoma expresses CD4, TCRβ (Vβ4), and belongs to the Th1 subset (17).

**Splenocytes, Th1, and Th2 cells.** Spent cell-free culture medium from arthritic mice was combined with the spent cell-free culture medium of nonarthritic mice and the mixture was incubated with a biotinylated mAb to CD4, TCRβ (Vβ4), and CD8 (clone 53-5.7), and P388D1 macrophage cell lines) and rat B cell hybridomas producing Abs to mouse Mac-1 (CD11b, clone M1/70.15.11.5HL), CD45R (BE-8D7), and the Ia region of H-2 (I-Ab, I-Ak reactive; clone M5/114,15,2), were obtained from American Type Culture Collection (Rockville, MD). The PG-specific T cell hybridoma 4E8 was obtained following fusion of in vitro-stimulated lymphocytes from an arthritic mouse with BW517 thymoma cells as described (17). This hybridoma expresses CD4, TCRβ (Vβ4), and belongs to the Th1 subset (17).

**Immunization of BALB/c mice with cartilage PGs (HFC-PG, HAC-PG, or FBC-PG), core protein fragments, or recombinant proteins**

Female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA; either Portage P07 or Kingston K51 colonies) weighing 17 to 20 g were injected i.p. with 100 μg of cartilage PGs (either intact (native) or degraded, measured as protein), 15 μg of G1 domain, 85 μg of G1-free core protein, or 50 μg of purified recombinant core protein (Defco Laboratories, Detroit, MI) by a standard immunization method described elsewhere (1, 2, 8). The first i.p. injection was given in Freund’s complete adjuvant followed by three identical Ag injections in Freund’s incomplete adjuvant on days 7, 28, and 49.

**Assessment of arthritis**

The limbs of all mice were examined daily to record abnormalities due to arthritic changes of the joints and documented as described previously (1, 6, 8). The appearance of joint swelling was recorded as the time of onset of arthritis. An “acute” clinical score, which is a cumulative arthritis score of the four limbs of each animal, was determined by the same person over the entire experimental period. Each paw was given a grade ranging from 0 to 4. Maximum redness and swelling of all four paws thus resulted in an arthritis score of 16 (8, 17). For standard histopathologic assessments of arthritis, the paws were fixed, decalcified, embedded in paraffin, sectioned, and stained as described (1, 4).

**Measurement and characterization of anti-PG Abs in sera of immunized mice**

Serum Abs (Fig. 2) were measured by solution RIA as described (1, 2, 20). Serial dilutions of sera were incubated with 125I-labeled PGs (mouse or human), GI domain, recombinant proteins, or chondroitinase ABC-digested G1-free fragments of human PGs (sp. act. ranging from 6 to 12 μC/μg protein), and the Ag-Ab complex pelleted either with protein A-bearing Staphylococcus aureus (Zysorbin; Zymed Laboratories, San Francisco, CA) for IgG subclasses, or pig anti-mouse IgM, followed by Zysorbin (15). The radioactivity of pellets was measured using a gamma counter (Beckman Instruments, San Francisco, CA) for IgG subclasses or, pig anti-mouse IgM, followed by Zysorbin (15). The radioactivity of pellets was measured using a gamma counter (Beckman Instruments, San Francisco, CA) for IgG subclasses or, pig anti-mouse IgM, followed by Zysorbin (15).
Either T or B cell response, an observation comparable with that shown in animals immunized with untreated human PGs (data not shown).

Abs by ELISA (Genzyme, Cambridge, MA).

Purification of the cell surface antigens was performed by adherence to tissue culture-treated petri dishes (Becton Dickinson, Rutherford, NJ).

Flow cytometry

Adherent peritoneal macrophages, spleen or lymph node cells, P388D1 macrophage and A20 lymphoma cell lines (both H-2b haplotypes), as well as B and T cells from normal, immune, or arthritic BALB/c mice were tested for cell surface molecules by flow cytometry as previously described (6, 8). Purified B cells were examined for CD45R, CD3, Mac-1, Ia (MHC class II), ICAM-1, B7-1, and B7-2 expression; T cells for CD3, CD4, CD8, TCRαβ, TCRγδ, CD25, or CD44; and adherent mononuclear cells for Mac-1, Ia, and CD40. Surface determinant-specific rat mAbs were purchased from either PharMingen (San Diego, CA) or Life Technologies, or were purified on a protein G column from ascites fluids of homozygous nude mice (Charles River) injected with hybridomas, as described (8).

Proliferation assays

Quadruplicate samples of various APCs (2 × 10^5 cells/200 μl/well) were cultured with purified T cells from normal, immune nonarthritic, or immune arthritic mice in 96-well microtiter plates (Flow Laboratories, McLean, VA) in DMEM. Cells were cultured alone (nonstimulated cultures) or with Con A (5 μg/ml) or various PG Ags (5–50 μg protein/ml). A 16-h incorporation of [H]thymidine (1 μCi/well; Amersham, Arlington, Heights, IL) was measured on day 5, as described elsewhere (2, 4, 6). Lymphocyte proliferation was expressed as the stimulation index, which is the ratio of incorporated cpm in cells stimulated with Ag or mitogen to incorporated cpm in nonstimulated cultures (2). For T cell hybridoma 5/4E8, either irradiated A20 B lymphoma cells (as standard APCs) or B cells isolated on a PG-coated surface (2 × 10^5 cells/well) were used, and the supernatants assayed for cytokine activities using CTL cells or the IL-2 content measured by ELISA (Genzyme, Cambridge, MA).

Statistical analysis

Results were analyzed in a two-factor repeated measures multivariate ANOVA. The Pillai’s trace criterion was used to detect multivariate significance. Subsequently, paired Student’s t tests were performed with Bonferroni correction. All statistical analyses were performed using personal computer-based statistical software (SPSS/PC+ version 4.0.1; SPSS, Chicago, IL).

Results

Arthritogenic effect of HFC-PG

Immunizations with intact (nondeglycosylated) HFC-PG (Table I, first lane) or HFC-PG treated with endo-β-galactosidase, O-glycanase, or N-glycanase provoked only a weak Ab response and essentially no detectable T cell responses and did not induce arthritis in susceptible BALB/c mice. In contrast, any form of the CS depletion of HFC-PG induced a strong Ab response to CS stubs (Table I), and these Abs cross-reacted with mouse PG. Approximately 60% of Abs reacted with nonsulfated, 22% with C4S, and less than 5% with C6S stubs, but only if the stubs were immobilized to the core protein. Moreover, immunization of animals with CS-depleted HFC-PG also induced a T cell response and resulted in the development of progressive polyarthritis (Table I and Fig. 2A). An anti-mouse (self-) PG-specific T cell response, however, became evident only after the onset of arthritis (Fig. 2) and was detected primarily in joint-draining lymph nodes (data not shown).

Papain digestion of the core protein dramatically reduced (to less than 5%) the Ab binding to the target PG molecule, completely abolished the T cell response and, when this papain-treated and CS-depleted HFC-PG were used for immunization, it has never induced immune responses or arthritis.

Arthritogenic effect of PGs from adolescent or adult human cartilages

Intact (nondeglycosylated) HAC-PG induced a high Ab response (Table I). Neither intact HAC-PG nor those depleted of either CS (Table I) or KS side chains induced a detectable T cell response to mouse PG, and all remained nonarthritogenic (Fig. 3, second, third, and fourth columns). An age-related T cell response and arthritogenic effect of cartilage PGs, however, appeared to correlate inversely with the KS content: the more KS of the PG, the less arthritis appeared to develop in susceptible BALB/c mice. In contrast, any form of the CS depletion of HAC-PG induced a strong Ab response to CS stubs (Table I and Fig. 2A). An anti-mouse (self-) PG-specific T cell response, however, became evident only after the onset of arthritis (Fig. 2) and was detected primarily in joint-draining lymph nodes (data not shown). Papain digestion of the core protein dramatically reduced (to less than 5%) the Ab binding to the target PG molecule, completely abolished the T cell response and, when this papain-treated and CS-depleted HAC-PG were used for immunization, it has never induced immune responses or arthritis.

Table I. Effect of deglycosylation of HFC-PG and HAC-PG on anti-PG immune responses and arthritis

<table>
<thead>
<tr>
<th>Experimental Groups Immunized with Human Cartilage PG</th>
<th>Incidence of Arthritis</th>
<th>Abs (cpm ± SD) to&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T Cell Response (SI ± SD) to&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Native (untreated) HFC-PG</td>
<td>1/16</td>
<td>210 ± 86</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Treatments of HFC-PG&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testicular hyaluronidase</td>
<td>15/16</td>
<td>3200 ± 490</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Chondroitinase ABC&lt;sup&gt;c&lt;/sup&gt; (ABC)</td>
<td>24/24</td>
<td>4460 ± 490*</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>Native (untreated) HAC-PG</td>
<td>0/24</td>
<td>3600 ± 180&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Treatments of HAC-PG&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testicular hyaluronidase</td>
<td>0/8</td>
<td>6340 ± 680</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Chondroitinase ABC&lt;sup&gt;c&lt;/sup&gt; (ABC)</td>
<td>0/8</td>
<td>7220 ± 1220&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Incidence of arthritis: arthritic animals/treated animals. Only 1 of 16 native HFC-PG-immunized BALB/c mice developed transient and mild arthritis (score was 2).

<sup>b</sup> Abs to immunizing PGs (HFC-PG or HAC-PG, untreated or deglycosylated) and mouse cartilage PG (treated as the corresponding human PG) were measured by RIA at the serum dilutions indicated. Most of these Abs recognized carbohydrate (clusters of CS stubs) epitopes. Significantly higher Ab levels to chondroitinase ABC-treated samples (p < 0.01) relative to the testicular hyaluronidase-treated PGs were Abs to the unsaturated group of the terminal uronic acid of the CS stub (Fig. 1).

<sup>c</sup> SI, stimulation index; nd, not detected.

<sup>d</sup> ST ± SD was calculated in quadruplicate spleen cell cultures (on wk 24 of immunization) stimulated with 50 μg of Ag protein of chondroitinase ABC-digested PGs.

<sup>e</sup> A single glycosidase treatment of HFC-PG with keratanase, keratanase II, endo-β-galactosidase, N-glycanase, or O-glycanase (four animals in each group did not modify either T or B cell response, an observation comparable with that shown in animals immunized with untreated human PGs (data not shown).

<sup>f</sup> Incidences were the same, and immune responses were comparable (data not shown), if chondroitinase ABC-digested HFC-PG was further treated with keratanase, keratanase II, endo-β-galactosidase, N-glycanase or O-glycanase (four animals in each group); thus, none of the KS, W-linked, or O-linked oligosaccharides was involved in the immune responses to HFC-PG or mouse PG.

<sup>g</sup> Abs to native HAC-PG recognized KS epitopes.

<sup>h</sup> Treatments of HAC-PG with CS-degrading enzymes (Fig. 1) were the same as used for deglycosylation of HFC-PG.
Recombinant core proteins

A set of human recombinant core proteins of various PG domains (Fig. 1) (all expressed in \(E. coli\)) have been tested in BALB/c mice for arthritis induction. These nonglycosylated recombinant proteins were believed to represent the most variable and immunogenic regions of the core protein. Although these recombinant proteins have induced an Ab response, none of these Abs reacted with mouse PG, and essentially no reaction was detected against intact or chondroitinase ABC-treated HFC-PG (data not shown). Moreover, no T cell responses to cartilage PGs were detectable in BALB/c mice immunized with recombinant proteins, and none of the recombinant proteins induced arthritis.

Epitope mapping of an arthritogenic T cell hybridoma with synthetic peptides

An initial epitope mapping of our PG-specific and arthritogenic T cell hybridoma 5/4E8 using different fragments of human cartilage PGs indicated that the epitope sequence of this arthritogenic T cell hybridoma is located somewhere in the G1 domain. As the recombinant G1 domain expressed in \(E. coli\) was completely insoluble in physiologic condition, for the epitope mapping of 5/4E8 hybridoma we have used synthetic peptides. The epitope mapping of the 5/4E8 hybridoma confirmed that at least one of the “arthritogenic” T cell epitopes (92 GR/QVRVNSA/IY) (Fig. 4) is located in the G1 domain of the PG molecule (Fig. 1).

Arthritogenic T cell epitope(s) in the G1 domain of human cartilage PG

For in vivo studies, we have purified the G1 domain from stromelysin-cleaved cartilage PGs (Fig. 1). BALB/c mice immunized with chondroitinase ABC-digested HFC-PG (Fig. 2A), G1-free fragments of HFC-PG (Fig. 2B), and the endo-\(\beta\)-galactosidase-treated G1 domain (Fig. 2C) exhibited immune responses to various T and B cell epitopes of HFC-PG, and a number of animals
have 98% identity, the negative reactions with 12 additional B control (G1 domain of HFC-PG at 5 μg/ml of concentration). The amino acid sequences of synthetic peptides 24-28 are shown with a shaded area of the 5/4E8 epitope. The 5/4E8 epitope (GRGRVNSAY) is located on the top of the A loop of aggrecan (Fig. 1).

developed arthritis. Although a B cell response was induced by the G1 domain as well (Fig. 2C), and some T cell epitopes were also present in the G1-free fragments of fetal human PG (Fig. 2B), the onset and the incidence of arthritis appeared to correlate with the T cell response to an epitope(s) present in the G1 domain (Fig. 2, A and C, third columns). This was further confirmed when a single injection of 25 μg of G1 domain of HFC-PG (injected on wk 18) induced arthritis in 7 of the 10 nonarthritic animals immunized with chondroitinase ABC-digested G1-free fragments of HFC-PG, shown in Figure 2B. In contrast, the purified G1 domain from FBC-PG did not induce a cross-reactive immune response to mouse PG, or arthritis in BALB/c mice (data not shown).

Challenge of arthritis in preimmunized BALB/c mice

As described above (Table I and Fig. 3), neither intact nor CS-depleted HAC-PG has induced arthritis. However, when animals immunized with CS-depleted HAC-PG were challenged with the G1 domain of human PGs, almost all animals developed arthritis (Table II, groups 3 and 8). This was the case when CS-depleted HFC-PG (Table II, group 2) or HAC-PG depleted of both CS and KS-side chains (Table II, group 7) was used for challenge. Neither intact (Table II, group 5), nor CS-depleted PGs (group 6) of HAC-PG, nor G1-free fragments (groups 4 and 9) of human PGs were effective for arthritis induction of preimmunized mice.

PG presentation by CS-specific B cells

Although the Ab titers to PGs in HFC-PG-immunized mice showed a strong correlation with the incidence of arthritis (Fig. 2A), these CS stub-specific Abs were also present in HAC-PG-immunized mice (Table I). Thus, we assumed that the key role of PG (CS stub)-specific B cells in arthritis induction is Ag presentation. Indeed, CS stub-anchored B cells from either arthritic (HFC-PG-immunized) or nonarthritic (FBC-PG-immunized) mice proved to be excellent PG Ag presenters for T cells (Fig. 5), whereas the T cell response was highly specific for the PG species (human or bovine) used for immunization.

### Table II. Arthritis development in BALB/c mice preimmunized with CS-depleted HAC-PG and then challenged with PG or PG fragments

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cartilage PG or Fragments of PG Used for Challenge</th>
<th>Dose of Challenge (μg Protein)</th>
<th>Incidence of Arthritis</th>
<th>Score of Arthritic Animals (3 wk) After Challenge (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HFC-PG (native)</td>
<td>100</td>
<td>1/4</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>HFC-PG (ABC)</td>
<td>100</td>
<td>5/8</td>
<td>9.2 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td>G1 domain of HFC-PG</td>
<td>25</td>
<td>3/4</td>
<td>7.8 ± 2.2</td>
</tr>
<tr>
<td>4</td>
<td>G1-free fragments of HFC-PG</td>
<td>25</td>
<td>1/4</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>HAC-PG (native)</td>
<td>100</td>
<td>0/4</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>HAC-PG (ABC)</td>
<td>100</td>
<td>0/4</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>HAC-PG (ABC + endoβ)</td>
<td>100</td>
<td>2/4</td>
<td>4 and 9</td>
</tr>
<tr>
<td>8</td>
<td>G1 domain of HAC-PG</td>
<td>25</td>
<td>4/4</td>
<td>11.2 ± 2.4</td>
</tr>
<tr>
<td>9</td>
<td>G1-free fragments of HAC-PG</td>
<td>2 × 75</td>
<td>0/4</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* BALB/c mice were injected 6 times with chondroitinase ABC-digested (ABC) PG from HAC-PG (100 μg of PG protein/injection). All sera of immunized animals contained high levels of Abs to CS stubs and KS, but none of the 40 mice produced Abs to native mouse PG or showed any sign of joint inflammation prior to challenge. Neither histopathologic abnormalities nor T cell response to mouse PG (stimulation index = 1.1 ± 0.2) or HFC-PG (stimulation index = 1.3 ± 0.4) was detected in 16 additional HAC-PG-immunized mice tested at various time periods prior to the challenge. Immunized animals were challenged with PG (native, ABC, or ABC + endo-β-galactosidase (endoβ)-treated). G1 domain (also treated with endoβ or G1-free fragments of PGs as indicated. Histology confirmed the clinical symptoms of arthritis.

* Only one arthritic animal in group 4 was identified by histology.

### Discussion

Among experimental animal models, which simulate human rheumatoid arthritis, cartilage PG (aggrecan)-induced arthritis is probably the most spectacular murine model due to its high incidence...
(∼100%), irreversible progressivity of the disease, and the histopathology of inflamed joints (1, 3, 8, 29). The unique autoimmune/arthritogenic T cell epitopes were detected only in human, dog, and mouse PGs, whereas PGs or PG fragments from other species showed no (2, 9) or very low incidence (30). As the deletions of the CS side chains in all cases were essential to reveal the arthritogenic effect, it was a compelling hypothesis that a cryptic structure (26, 31) is located in the CS attachment region of this complex molecule (Fig. 1). It was a confusing observation, however, that PG from adult human cartilage, isolated and depleted of CS in exactly the same way as those from fetal tissue, did not induce arthritis (1, 2). Now we report that PG from adult human articular cartilage can be as arthritogenic as that from fetal tissue (Fig. 3), if both the KS and CS side chains of the “matured” (adult-type) molecule are removed. The arthritogenic T cell epitope is masked by a KS side chain in the G1 domain of adult human PG. This GAG completely blocks the development of a cross-reactive T cell response to mouse PG and subsequent joint inflammation in BALB/c mice, but only if the professional, CS stub-specific, Ag-presenting B cells are present. The endo-β-galactosidase-sensitive KS, or KS-like, side chain may inhibit either the internalization of the PG or the intracellular processing arm of Ag presentation. Or, it can physically inhibit the ternary complex formation of MHC, antigenic peptide, and TCR.

KS consists of repeated disaccharide units of N-acetylgalcosamine and galactose (Fig. 1, right bottom panel), and both sugar components can be sulfated at either the C4 or C6 position (32). KS side chains can be O-linked to serine or threonine (Fig. 1) or N-linked to asparagine of the core protein of various PG molecules (33, 34). Thus, KS is an elongated and sulfated form of either an O-linked or N-linked oligosaccharide and, in this term, is one of the most common GAG chains of mammalian PGs. However, the KS is virtually absent in embryonic human (15) and rodent tissues (35). The presence of sulfate groups makes KS resistant to enzymatic degradation in vivo due to the lack of specific endo-β-galactosidases in eukaryotic cells (32, 36). The function of the KS side chains of PGs is unknown. One can speculate that this GAG side chain may provide protection against proteolytic attack on the core protein. The number and size, as well as the degree of sulfation, of the KS side chains vary in different species (37–39) and typically increase during aging in the same species (15, 39).

From a clinical point of view, it is interesting to note that resident chondrocytes in human rheumatoid cartilage switch back to synthesize immature (fetal-type) PG (24, 40), which lack the KS side chains (15, 34). These newly synthesized PGs may immediately undergo proteolytic degradation in diseased cartilage (24); PG fragments could be released into the synovial fluid and then appear in lymphatic fluid and serum (21, 24, 40). Thus, the potential exists that fetal-type core protein fragments, without KS chains, become accessible to the immune system in pathologic conditions (9, 41, 42) and may dictate the organ specificity of autoimmune diseases.

An Ag-specific T cell population seems to play a central role in the mechanisms of both human rheumatoid and murine PG-induced arthritis. As the PG-specific T cell response is rather confined to the joint-draining lymph nodes in PG-induced arthritis (6), it is very likely that an autoantigen-driven mechanism of joint inflammation becomes local and self-sustaining by PG (cartilage) degradation; especially as rodent PGs are free of KS and immediately available for recognition. As the successful transfer of arthritis has required the presence of both B and T cells from arthritic animals (4), in addition to T cells (29), CS stub-specific B cells, but not circulating Abs, might play a crucial role in the initiation and pathology of PG-induced arthritis (4, 7). It has to be mentioned, on the other hand, that degradation of CS side chains induces a significant Ab response to CS stubs immobilized to the core protein (Table I), and most of these Abs cross-react with the intact CS chains as well. A partial degradation of either CS or dermatan sulfate side chains of various PG molecules, for example by hyaluronidase in inflammatory processes or by bacterial amylases during infection, may occur in vivo, and immunogenic carbohydrate structures can be formed (3, 43). Hence, the potential exists that stub-specific, carbohydrate-primed, memory B cells are generated and then involved in the presentation of a PG molecule in a number of human autoimmune diseases (3, 43, 44).

In conclusion, the GAG side chains of cartilage PGs significantly modify, essentially dictate, the immune responses in BALB/c mice, the only susceptible murine strain to PG-induced arthritis identified to date. Although the lack of both CS and KS side chains is required for the induction of autoimmune responses and arthritis in mice, the immunopathologic role of these two GAG chains is opposite. KS, which is absent in fetal human and mouse cartilages, masks a T cell epitope in the G1 domain of human adult PG. Thus, this structure is essentially unavailable for T cell recognition in HAC-PG-immunized animals. In contrast, the depletion of the CS side chains generates a branch of CS stubs immobilized on the core protein, and the clusters of these CS stubs provoke a strong B cell response. Then, these B cells function as the most professional APCs (PG) for T cells if the relevant autoimmune (arthritogenic) T cell epitope is available.

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