

Ph.D. Theses

**Investigation of possible immunological
mechanisms in the pathogenesis of a murine
model of rheumatoid arthritis**

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SUMMARY

Rheumatoid arthritis (RA) is a systemic autoimmune disease and its targeting of the joints indicates the presence of a candidate autoantigen(s) in synovial joints. Patients with RA show cellular immune responses in their peripheral blood to cartilage matrix components such as proteoglycan aggrecan. The most evident source of these autoantigens is the immunoprivileged articular cartilage, which is avascular, and is therefore not subject to immunologic surveillance. One of the most relevant animal models of rheumatoid arthritis appears to be proteoglycan (aggrecan)-induced arthritis. Although the underlying mechanisms are still unclear, T cells, particularly CD4⁺ lymphocytes, seem to play a crucial role in the initiation of the disease. Earlier studies explored numerous T cell epitopes along the core protein of human aggrecan, and one of the epitopes (called peptide p135H) present in the G3 domain at the C-terminus of the human aggrecan appeared to be involved in arthritis induction.

In the first part of the study, we made an effort to study the immunologic function and determine the fine epitope structure of this synthetic peptide p135H ((2373)TTYKRRLQKRSSRHP), which contains a highly homologous sequence motif of the “shared epitope” (QKRAA), a sequence that is overrepresented in numerous RA-associated HLA-DR4 alleles and common human pathogens. To further investigate whether the T cell epitope represented by p135H might be directly involved in the pathogenesis of the disease, we transferred p135H-stimulated lymphocytes from p135H-primed BALB/c mice into SCID mice. Despite differences in the amino acid sequences between the human and mouse peptide and the cryptic character of the epitope, we were able to show that the transfer of p135H-reactive lymphocytes into “presensitized” SCID mice leads to a rapid development of arthritis. Since the G3 domain of cartilage proteoglycan aggrecan with the p135 sequence is “lost” during the normal metabolic turnover of cartilage proteoglycan or in pathologic conditions, an antigen-oriented T cell migration into joints of presensitized (susceptible) individuals may contribute to the organ-specificity of RA.

In the second part of the study, we compared the effects of dimethyldioctadecylammonium bromide (DDA), a potent nonirritant adjuvant with those of Freund’s complete adjuvant (CFA) in arthritis-susceptible murine strains. As a result, the overall immune responses (antibody production and antigen-specific T cell responses) were highly comparable when human proteoglycans (PG) in CFA or in DDA were used, however the use of DDA accelerated the development of a more severe arthritis, and suboptimum doses of PG or type II collagen antigens were able to induce inflammation. We showed that DDA exerts a strong stimulatory effect via the activation of nonspecific (innate) immunity and forces the immune regulation toward Th1 dominance. Since a number of potential autoantigens (*e.g.* type II collagen, proteoglycan aggrecan) can be identified in various subsets of RA patients, it may be an attractive hypothesis that seemingly innocuous compounds may exert an adjuvant effect in humans and may create the pathophysiologic basis of autoimmunity in susceptible individuals via the activation/stimulation of innate immunity.

INTRODUCTION

1. Structure and function of the aggrecan molecule

Proteoglycans (PG) are protein polysaccharide molecules, form 10–20% wet weight and provide a compressive strength to the articular cartilage. They are produced inside the chondrocytes and secreted in the matrix, and their function is to maintain the fluid and electrolyte balance in the articular cartilage. Aggrecan is a modular proteoglycan with multiple functional domains. Its core protein consists of three globular regions, termed G1, G2 and G3. The G1 and G2 regions are separated by a short interglobular domain, and the G2 and G3 regions are separated by a long glycosaminoglycan-attachment region, which consists of adjacent domains rich in keratan sulphate and chondroitin sulphate. Aggrecan molecules do not exist in isolation within the extracellular matrix, but as PG aggregates. Each aggregate is composed of a central filament of hyaluronic acid (HA) with up to 200 aggrecan molecules radiating from it.

Loss of aggrecan is a major feature of cartilage degradation associated with arthritis. There is also an age-related loss of the G3 domain of aggrecan, and 92% of the G3 domain is lost as part of the normal turnover of the PGs, whereas the rest of the molecule, which is bound to HA, is retained in the cartilage.

2. Immunogenicity of cartilage components

Cartilage is one of the few immunologically privileged tissues in the body in that it is essentially avascular and therefore not subjected to close “internal” immunological surveillance. When it gets degraded, however, uniquely antigenic molecules become exposed, released and subsequently recognized by the immune system. Thus, articular components may trigger and maintain immune responses to these antigens.

The immune attack on the joints could also be initiated by a cross-reactive immune reaction in response to unrelated antigens by the mechanism of “molecular mimicry”. The net result of such autoimmune reactions could be further destruction of cartilage and release of more autoantigens. This could lead to a chronic, self-perpetuating inflammation in genetically predisposed individuals who are prone to develop these autoimmune reactions.

Patients with RA show cellular immune responses in their peripheral blood to PG, and immunoreactive fragments of PG have been demonstrated in the synovial fluids of patients with RA. These facts suggest that PG aggrecan may play an important role in the development of autoimmunity against peripheral joints.

3. Rheumatoid arthritis and its experimental animal models

Although RA is a systemic autoimmune disease, its targeting of the joints indicates the presence of a candidate autoantigen(s) in synovial joints. The most relevant animal models of rheumatoid arthritis appear to be those induced by cartilage matrix components such as proteoglycan aggrecan or type II collagen. The pathologic basis in both model systems appears to be cross-reactive immune reactions, that is, the T cells and antibodies raised against the immunizing heterogeneous (human, bovine) cartilage antigens recognize and subsequently attack the mouse’s own tissues.

3.1. Proteoglycan-induced arthritis (PGIA)

Systemic immunization of genetically susceptible strains of mice (*e.g.* BALB/c) with human cartilage proteoglycan, that has been depleted of glycosaminoglycan side chains, leads to the development of progressive polyarthritis. PGIA shows many similarities to RA in humans, as indicated by findings of clinical assessments, radiographic analyses,

scintigraphic bone scans, laboratory tests and histopathologic studies of diarthrodial joints.

PGIA was first described in the BALB/c strain, but certain C3H colonies (e.g., C3H/HeJCr) were also found to be susceptible to PGIA. During the immunization protocol female mice are injected intraperitoneally (i.p.) with 100 µg of cartilage PG in Freund's complete adjuvant (CFA), followed by injection of the same doses of PG in Freund's incomplete adjuvant (IFA), on days 21 and 42. The initial clinical manifestations of joint inflammation appear after the third to fourth i.p. injection of antigen, depending on the source of PG antigen, adjuvant, and BALB/c or C3H colony used. Joint inflammation starts as polyarticular synovitis in small peripheral joints. During the early phase, lymphocytes and polymorphonuclear leukocytes invade the synovium. This is followed by gross, "tumorlike" proliferation of synovial lining cells and fibroblasts. Once an animal develops arthritis, repeated "spontaneous" episodes of inflammation result in complete destruction of the articular cartilage and erosion of the subchondral bone, which leads to severe deformities of the peripheral joints.

PGIA in BALB/c mice is a T cell-dependent and (auto)antibody/B cell-driven disease. CD4⁺ T cells have been implicated in the development of PGIA by observations that anti-CD4 monoclonal antibody (mAb) treatment prevents arthritis, and that the transfer of disease requires T cells from arthritic animals. BALB/c mice are genetically predisposed to a T helper (Th)2-type immune response, however, immunization of these mice with PG induces a higher ratio of interferon (IFN)-γ to interleukin (IL)-4, indicating that PGIA is a Th1-type response, and immunization with PG in CFA is a sufficient Th1 stimulus to overcome the genetic inclination toward development of a Th2-type response.

3.2. Collagen-induced arthritis (CIA)

Collagen-induced arthritis was first elicited in rats following a single intradermal injection of type II collagen (CII) emulsified in Freund's adjuvant. Further studies demonstrated that a similar pathology could also be induced in primates and in susceptible strains of mice. CIA can be induced using native autologous or heterologous CII. Immunization with CII/CFA results in a rapid and severe polyarthritis of the peripheral articular joints that first appears around 3–4 weeks after antigen challenge and becomes progressively worse for approximately 2–4 weeks before slowly waning. There are considerable data to implicate CII-reactive CD4⁺ T cells as the primary mediators of disease induction, and complement-fixing anti-CII autoantibody production by B cells as the major immune mechanism leading to the localized chronic inflammatory response. CIA is classified as a Th1-mediated disease based on the abundant IFN-γ production. Autoreactivity to cartilage CII in human RA patients has been clearly demonstrated.

4. Dimethyldioctadecylammonium bromide (DDA) as a potential adjuvant in PGIA and CIA

A number of animal models can simulate human RA, but all require antigen challenge with adjuvant. Mycobacterial heat-shock proteins (HSP) present in CFA are known to be very potent nonspecific immunostimulators, and these proteins significantly contribute to, and potentiate the systemic T cell response. However, these bacterial HSPs of CFA cause numerous undesired side effects, including CFA-induced irritation and granuloma formation with subsequent adhesions in the peritoneal cavity, and immune reactions to mycobacterial components.

Dimethyldioctadecylammonium bromide (DDA) is a powerful, nonirritant adjuvant and, via T cell stimulation, significantly enhances antigen-specific B cell activation and immunoglobulin production. A special benefit of the use of DDA in numerous rodent models of autoimmunity is that this adjuvant forces the immunoregulation toward Th1 type response. However, DDA as an adjuvant has never previously been tested in PGIA or CIA.

5. The rheumatoid arthritis *HLA-DRB1* “shared epitope”

Previous studies have shown that RA is closely associated with *HLA-DRB1* alleles that code a five amino acid sequence motif in residues 70–74 of the DR β chain – commonly referred to as the “shared epitope” (SE). The better-known SE-coding alleles include members of the *HLA-DRB1*04* allele group, *HLA-DRB1*0101* or **0102*, *HLA-DRB1*1402* and *HLA-DRB1*1001*. The disease in SE-positive patients begins earlier and is more erosive than in SE-negative individuals. Based on the known role of MHC molecules in antigen presentation, the prevailing hypotheses postulate that either selective binding and presentation of arthritogenic self-peptides, molecular mimicry with foreign antigens, or T cell repertoire selection could be involved. It has been recently demonstrated that SE also acts as a potent immune-stimulatory ligand that activates a nitric oxide-mediated pathway in other cells and can polarize T cell differentiation toward Th17 cells.

6. T cell epitope mapping study of aggrecan

In an epitope mapping study using a total of 143 synthetic peptides containing predicted T cell epitopes, we identified 27 peptide sequences that induced T cell responses in PG-immunized BALB/c mice. Four of these T cell epitopes were dominant (designated “arthritogenic”), whereas the others were either subdominant or cryptic. Synthetic peptides (15 amino acids long) containing the predicted T cell epitopes, alone as a single peptide or in combination with other peptides (4 \times 1–100 μ g of peptide per mouse) were used for hyperimmunization of BALB/c mice, coimmunization with cartilage PG, or for eliciting arthritis (inflammation) in mice that had been preimmunized with cartilage PG (“prearthritic” state). While positive control groups immunized with cartilage PG consistently developed arthritis (95–100% incidence), none of the peptides used in any combinations for immunization induced disease. Some peptides even induced immunologic tolerance.

Since (hyper)immunization with synthetic peptides, including those designated as arthritogenic, did not induce arthritis in repeated experiments, we administered a single dose of cartilage PG (100 μ g of protein) to peptide-immunized mice 4 weeks after the fourth (last) peptide injection. This single injection of cartilage PG does not induce arthritis, and only moderate antibody and T cell responses can be detected 2–3 weeks after the injection. There was no arthritis in peptide-immunized and PG-challenged BALB/c mice, except in a group that had been hyperimmunized with peptide p135H. Of 10 p135H-hyperimmunized mice, 6 developed arthritis within 9–12 days and 4 developed arthritis within 3 weeks after a single injection of human PG.

The p135H peptide sequence (²³⁷³TTYKRRLQKRSSRHP) is located in the C-terminal, carbohydrate side chain-free, G3 domain of PG, and this peptide was originally identified as cryptic rather than arthritogenic. Interestingly, p135H shows high sequence homology with proteins of common human pathogens (*e.g.* several bacterial heat-shock proteins) and the “shared epitope”, the most common sequence motif (QKRAA) in *HLA-DR4* alleles, which predispose humans to the development of RA.

AIMS OF THE STUDY

I. Investigation of the role of peptide p135-specific lymphocytes and T cell hybridomas in the induction of arthritis

The aim of the study was to further investigate whether the T cell epitope represented by peptide p135H might be directly involved in the pathogenesis of the disease, and reveal the precise immunological mechanisms in the background. To reach our goals, we performed the following experimental studies:

1. (Hyper)immunization experiments using p135 and peptide priming of lymphocytes.
2. Generation of p135-reactive T cell hybridomas.
3. Determination of the peptide specificity and antigen specific cytokine profile of lymph node cells and T cell hybridomas.
4. Characterization of cell surface markers of p135-specific lymphocytes and p135-reactive T cell hybridomas.
5. Determination of the MHC restriction of T cell hybridomas.
6. Identification of the core epitope and amino acid residues critical for antigenicity of peptide p135.
7. Determination of the MHC-binding residues of p135H.
8. Identification of T cell receptors of p135-reactive T cell hybridomas.
9. Transfer of arthritis into SCID mice using p135H-primed lymphocytes and p135H-specific T cell hybridomas.

II. Investigation of the adjuvant effect of dimethyldioctadecylammonium bromide (DDA) in proteoglycan-induced arthritis and collagen-induced arthritis

PGIA and CIA were induced using standard immunization protocols with cartilage PGs or human type II collagen (CII) emulsified with CFA, and compared with PGIA and CIA generated using immunization protocols in which the same antigens were administered in combination with the adjuvant DDA. Immune responses to immunizing and self PGs and CII, the incidence, severity and onset of arthritis were monitored throughout the experiments. In this study we performed the following experiments:

1. Isolation of antigenic components from cartilage of different species.
2. Immunization of different inbred mouse strains according to the standard PG/CFA protocol or using DDA as adjuvant.
3. Assessment of arthritis onset, incidence and severity.
4. Measurement of antigen-specific T cell responses, antibodies and cytokines.
5. Characterization of cell surface markers.
6. Cell isolation and transfer of arthritis into SCID mice.

MATERIALS AND METHODS

1. Isolation of antigenic components from cartilage

Total cartilage extracts were obtained by 4M guanidinium chloride extraction from newborn and adult human and bovine articular cartilage, bovine nasal cartilage, ewe and swine articular cartilage, chicken sternal cartilage, cartilaginous skeletal tissue from newborn mice and rat chondrosarcoma. These cartilage extracts were dialyzed against water and lyophilized (crude cartilage extract), or further purified by repeated cesium chloride gradient ultracentrifugation to obtain high density cartilage proteoglycans (PG). Purified cartilage PGs were deglycosylated for immunization or were left untreated. Type II collagen was purified by sequential and repeated precipitation with NaCl in 0.5M acetic acid at 4°C.

2. Synthetic peptides

Fifteen amino acid long peptides representing the human aggrecan core protein sequence (p135H) or corresponding sequences of aggrecan from other species, such as mouse, bovine, canine, and porcine, were synthesized by Research Genetics (Carlsbad, CA). Peptides of p135H having 1-mer offset shifts in sequence or single amino acid substitutions (Ala or Gly) were synthesized by Chiron Mimotopes (Clayton, Victoria, Australia).

3. Animals

Mice of different strains, ages and both sexes were purchased from the National Cancer Institute (NCI; Frederick, MD), Charles River Laboratories (Kingston, NY, Raleigh, NY, and Portage, MI colonies), Harlan (Madison, WI), Taconic Farm (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the same room of the Comparative Research Center at Rush University (Chicago, IL). Female SCID mice of BALB/c background (NCI/NCrC.B-17-scid/scid) were obtained from the NCI and maintained under germ-free conditions; these mice were used for transfer experiments.

4. Immunization protocols

4.1. Peptide p135 (hyper)immunization experiments and peptide priming of lymphocytes

Female BALB/c mice were injected intraperitoneally (IP) with 3 different doses of peptide p135 (1.0 µg, 10 µg, and 100 µg/injection) in CFA followed by similar injections containing the peptide in IFA on days 21, 42, and 63. Since no joint inflammation was observed in these p135 peptide-immunized mice after the fourth injection, peptide-hyperimmunized animals also received a single dose of proteoglycan (100 µg protein in IFA) IP on day 84.

Female BALB/c mice were injected once into the footpad with 100 µg of human p135 (p135H) or mouse p135 (p135M) peptide in CFA. Mice were sacrificed 9 days later, and popliteal lymph nodes were harvested. The peptide-primed lymph node cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% normal syngeneic mouse serum and 5% fetal calf serum (FCS) in the presence of peptide p135H or p135M (50 µg/ml). Viable lymphocytes were harvested 3 days later by gradient centrifugation over Lympholyte M (Cedarlane, Hornby, Ontario, Canada) and further cultured for an additional day in the presence of IL-2. Conditioned medium from

EL-4.IL-2 cells stimulated with 10 ng/ml of phorbol 12-myristate 13-acetate was used as a source of IL-2. These peptide p135H-specific and peptide p135M-specific *in vitro*-restimulated lymphocytes were used for cell transfer into SCID mice or for fusion to generate T cell hybridomas.

4.2. Adjuvant studies: comparison of DDA with CFA

Female and male mice (12–16 weeks of age) of different inbred strains as well as their F₁ hybrids were used to compare selected immunization protocols. In experiments in which the effects of different cartilage PGs and/or crude cartilage extracts, with or without treatment of different glycosidases, were compared, we immunized female retired breeder BALB/c mice that had been purchased from the NCI.

Mice were injected intraperitoneally (IP) with 100 µg of deglycosylated cartilage PGs or crude extracts or with human cartilage CII. A fine cationic liposome form of DDA (micelle) was obtained by heating a 10 mg/ml DDA suspension (Sigma-Aldrich, St. Louis, MO) in PBS (pH 7.4) to 56–63°C for 15–20 minutes and then cooling on ice. This DDA micelle form was mixed with an equal volume of antigen (1 mg of CII/ml or 1 mg of PG core protein/ml of PBS), and the antigen/DDA micelle emulsion (200 µl total) was injected IP or, where indicated, subcutaneously (SC) or intradermally (ID).

Four major groups of animals were treated according to one of the following “standard” immunization protocols. In protocol 1 (PG/CFA), an IP injection of 100 µg of PG protein in 100 µl of PBS (pH 7.4) emulsified with 100 µl of CFA (Difco, Detroit, MI) was given on day 0. On days 21 and 42, the same dose of PG in IFA (Difco) was injected IP. In protocol 2, the same antigen dose, injection time points, and IP approach were used, but the 100 µg of PG in 100 µl of PBS was mixed with 1 mg of DDA micelle in 100 µl of PBS. In protocol 3, 100 µg of CII was dissolved in 0.1M acetic acid, and the volume was adjusted to 1 mg of CII/ml of PBS to prepare an emulsion with an equal volume of CFA. The emulsion containing 100 µg of CII was then injected either IP or ID into the proximal tail on days 0 and 21. In protocol 4, the same dose of CII (100 µg) in 100 µl of DDA micelle (1 mg) was also used for either IP or ID immunization.

5. Assessment of arthritis

The paws of peptide p135 specific lymphocyte or T cell hybridoma transferred mice were examined daily. The paws of all mice in DDA/CFA comparison related experiments were examined twice weekly until day 21, and then daily thereafter. Abnormalities due to arthritic changes of the joints were recorded. The appearance of the first 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; maximum possible score 16 per animal) was used for the assessment of disease severity. The limbs and spine of arthritic and nonarthritic mice were dissected, fixed, decalcified, sectioned, and the tissue sections were stained with hematoxylin and eosin for histopathologic examination.

6. Cell lines and culture media

Cell lines BW5147.G.1.4 (TCR α/β +), A20 (H-2^d), CTLL-2 and EL-4.IL-2 were obtained from American Type Culture Collection (Manassas, VA). The culture medium for all cell types was Dulbecco’s modified Eagle’s medium (DMEM) and either heat-inactivated (56°C for 30 minutes) 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO) or 1% pooled normal mouse serum (unless indicated otherwise). All cultures were performed at 37°C in a humidified atmosphere of 5% CO₂ in air.

7. Generation of p135-reactive T cell hybridomas

Peptide p135H- or peptide p135M-specific lymphocytes were generated as described above. These lymphocytes were fused with BW5147.G.1.4 thymoma cells as described elsewhere. Fused cells were seeded into 96-well plates, and hybridomas were selected in medium containing hypoxanthine/aminopterin/thymidine (Sigma-Aldrich) and 15% FCS. Hybrids were screened for reactivity to peptides p135H and p135M using irradiated (140 Gy) (JL Sheperd & Associates, San Fernando, CA) A20 lymphoma cells as APCs. T cell hybridomas reactive to the corresponding peptide were frozen, and cells that retained their antigen (p135) specificity after the second testing were cloned by limiting dilution.

8. Measurement of antigen-specific cell proliferation, cytokine secretion and antibody production

Spleen and lymph node cells were collected at the end of the experiments. Sera were collected from immunized mice during the immunization period (once or twice each week from the retroorbital venous plexus) and at the end of the experiments.

To assess cell proliferation, p135H- and p135M-primed lymph node cells (3×10^5 cells/well) or T cell hybridomas (5×10^4 cells/well) were cultured with or without peptides p135H and p135M or analog peptides (25–50 $\mu\text{g/ml}$) in quadruplicate wells of 96-well plates for 36 hours. Irradiated A20 B lymphoma cells (10^5 cells/well) were used as APCs for T cell hybridomas in the presence or absence of 25–50 $\mu\text{g/ml}$ of p135H, p135M or analogous peptides.

In the adjuvant comparison study, antigen-specific T cell responses were measured in quadruplicate samples of spleen cells or PG/adjuvant-primed lymph node cells (3×10^5 cell/well) cultured in the presence of 25 μg of PG protein/ml or 25–50 μg of CII. In both study antigen-specific IL-2 production was measured in 2-day-old supernatant by CTLL-2 bioassay. The result was expressed as stimulation index, which was calculated by dividing the counts per minute (cpm) of peptide- or PG-stimulated cells by the cpm of unstimulated cells.

Production of antigen-specific IFN γ , IL-4, IL-5, IL-10, IL-12, and TNF α (BD Biosciences, San Diego, CA, or R&D Systems, Minneapolis, MN) was measured in cell culture supernatants (3×10^6 cell/ml) on day 4 using capture enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Cytokines (IFN γ , IL-1 β , IL-6, IL-10, IL-12, and TNF α) in the sera of immunized animals were measured by ELISA at the end of the experiments.

Antigen-specific antibodies were measured by ELISA. Maxisorp immunoplates (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). The total serum IgG fraction was determined by ELISA using mouse κ -chain-specific peroxidase-labeled rat mAb for detection (BioSource, Camarillo, CA). The color reaction was quantified with an ELISA reader (Coulter, Hialeah, FL) at 405 nm.

9. Flow cytometric analysis of cell surface markers

The expression of cell surface markers was assayed by flow cytometry. Briefly, either 10^6 unseparated spleen cells obtained at different time points of immunization, lymph node cells from antigen-primed mice, or cells harvested from peritoneal lavage fluid were incubated with fluorescein isothiocyanate- or phycoerythrin-labeled, or biotinylated mAbs to CD3, CD4, CD8, CD25, CD28, CD44 and CD69 (T cell and T cell activation markers), mAbs to T cell receptor (TCR) α/β , TCR γ/δ and to various TCR V_β chains, mAb to CD45/B220 (B cell marker), mAb to CD68 or F4/80 (monocyte/macrophage marker), mAb to Gr-1 (myeloid cell lineage marker), and mAb to CD11c (activated dendritic cell marker). Isotype control antibodies labelled with the corresponding fluorescent dyes were used as negative controls. Samples were analyzed on a FACScan flow cytometer using CellQuest software (both from Becton Dickinson, San Jose, CA).

10. MHC restriction of p135-reactive T cell hybridomas

Hybridoma cells were cultured with A20 APCs in the presence of peptide p135H or p135M, and either anti-I-A^d (BD PharMingen) or anti-I-E^d (Cedarlane Laboratories) mAb at concentrations of 5–20 $\mu\text{g/ml}$. The effect of mAb on IL-2 production was determined with the CTLL-2 bioassay. Isotype-matched mAbs were used as controls.

11. Determination of the core epitope and amino acid residues critical for antigenicity of peptide p135

To further characterize the critical peptide sequence required for MHC and TCR binding, we used peptides with a 1-mer offset covering the sequence p2367–2396 to define the minimum length of the peptide recognized by a p135H-specific T cell hybridoma H74/4. Subsequently, single-amino acid (Ala or Gly)-substituted peptides of p135H were used for the identification of the essential residues involved in the antigenicity of this epitope. In both cases, H74/4 hybridoma cells were cultured with or without the above-mentioned panels of peptides (20 $\mu\text{g/ml}$ or 40 $\mu\text{g/ml}$) in the presence of irradiated A20 APCs, and IL-2 secretion in the supernatants was measured by the CTLL-2 assay.

12. Determination of the MHC-binding residues of p135H

All peptides, including those that did not stimulate T cell hybridoma H74/4 cells, were also tested for their MHC-binding capability. Briefly, A20 lymphoma cells (2×10^5) were incubated with 2–4 μg of biotinylated p135H or synthetic single-residue-substituted p135H peptides for 2 hours at 37°C in 100 μl of PBS. After 2 washes with PBS containing 1% FCS, cells were incubated with 1.5 μg of streptavidin-R-phycoerythrin at room temperature for 30 minutes. The cells were washed again and analyzed by FACSCalibur (Becton Dickinson). Results were expressed as geometric mean values calculated by CellQuest Software (Becton Dickinson). Serial dilutions of nonbiotinylated peptides of identical sequence were used for competitive inhibition or nonbiotinylated anti-avidin IgG were used as specificity controls.

13. Identification of T cell receptors of p135-reactive T cell hybridomas

Total cellular RNAs from T cell hybridomas were isolated using the TRIzol reagent method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The first-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using an oligo(dT) primer and a SuperScript reverse transcription kit (Invitrogen). The cDNA were used as templates to amplify the transcripts of the V_α and V_β regions of the

TCR. To amplify unknown V_{β} genes expressed by T cell hybridomas, a degenerate upstream primer (5'-ATGTACTGGTATCAGCAG-3') was paired with a C_{β} downstream primer (5'-GCCAAGCACACGAGGGTAGCC-3'). For amplification of V_{α} genes, a consensus upstream primer (5'-TGGTACNDVCAGCATCCYGGVGAAGGCC-3') was used with a C_{α} downstream primer (5'-AGCTTTCATGTCCAGCACAG-3') [123]. After 35 cycles at 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes, polymerase chain reaction products were extracted from 1.5% agarose gels using Qiaex II gel extraction kit (Qiagen, Valencia, CA) and sequenced on an ABI 310 Genetic Analyzer (Perkin Elmer).

14. Cell isolation and transfer of arthritis

14.1. Transfer of arthritis using p135H-primed lymphocytes and p135H-specific T cell hybridomas

For adoptive transfer experiments, SCID mice were “presensitized” by injection with a suboptimum amount of spleen cells (insufficient for arthritis induction) from acutely arthritic BALB/c mice and PG antigen. Unseparated spleen cells (4×10^6) were injected IP into syngeneic recipient SCID mice, together with 100 μ g of PG on day 0, as described previously. These presensitized SCID mice that received only 1 injection of spleen cells plus proteoglycan on day 0 were considered a baseline control group and were used as a reference for all other experimental groups.

The first experimental group received 5×10^6 p135H-specific *in vitro*-stimulated lymphocytes from p135H-primed BALB/c mice along with 80 μ g of p135H as a second injection on day 7. The second group received 5×10^6 *in vitro*-stimulated lymphocytes from mice primed with irrelevant peptide p130H (¹⁵³⁰STLVEVVTASTASE) along with 80 μ g of synthetic p130H. p130H contains a cryptic T cell epitope in the glycosaminoglycan-attachment region of the core protein of human aggrecan, and served as a negative control. The third experimental group (subgroups for each p135H-specific T cell hybridoma) received 1×10^6 hybridoma cells intravenously and 80 μ g of p135H IP on day 7. The positive control group received a second IP injection of 1×10^7 spleen cells from acutely arthritic BALB/c mice on day 7. In each experiment, at least 5 SCID mice per group were used simultaneously, and transfer experiments were repeated 3–5 times.

14.2. Transfer of arthritis using spleen cells from PG/CFA or PG/ DDA immunized mice

Single-cell suspensions were prepared from the spleens of arthritic BALB/c mice. Donor arthritic mice were immunized with cartilage PG in CFA or in DDA as described above for protocols 1 and 2. Mononuclear cells were isolated on Lympholyte M (Zymed) and used for adoptive transfer of arthritis as described previously. In all transfer experiments, 1.5×10^7 spleen cells were injected IP on day 0 with 100 μ g of cartilage PG (measured as protein), and 1×10^7 spleen cells from arthritic donors were injected on day 7 without cartilage PG into SCID mice.

15. Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 10.0.5 and 7.5, SPSS, Chicago, IL). Since the results of all *in vitro* tests showed a normal distribution, Student’s 2-sample *t*-test was used to compare the results from 2 groups in peptide p135 related experiments. Significance was set at $P \leq 0.05$. In DDA/CFA

immunization experiments the Mann-Whitney and Wilcoxon tests were used for intergroup comparisons. Both the 5% significance level and the 1% significance level were used.

RESULTS

I. Investigation of the role of peptide p135-specific lymphocytes and T cell hybridomas in the induction of arthritis

I.1. Induction of arthritis in p135H-immunized BALB/c mice by a single dose of cartilage proteoglycan

All BALB/c mice that had been immunized with p135H or analogous aggrecan peptides of various species and then given a single injection of human PG developed arthritis. The incidence and severity varied, being the lowest in p135M (mouse) peptide-injected animals. Since the p135H sequence contains a conserved motif QKRSS, which is similar to the “shared epitope” (QKRAA) present in *HLA-DR* of patients with RA, as well as to DnaJ from *Escherichia coli*, we also used synthetic peptide analogs of p135 with altered sequences. The conservative amino acid replacement (SS to AA) did not change the arthritogenicity of peptide p135H, while the “shared epitope” present in either the DnaJ or *HLA-DR4* peptide, could presensitize BALB/c mice for arthritis. These results confirmed that the p135H sequence, or peptides containing the “shared epitope”, can prime BALB/c mice, although none of the peptide-hyperimmunized mice developed arthritis without additional challenge with proteoglycan.

I.2. *In vitro* T cell responses in p135H- and p135M-primed BALB/c mice

Since all of the p135 synthetic peptides were able to sensitize BALB/c mice, the next question was whether T cells primed by human p135H synthetic peptide could cross-react with those primed by mouse (self) p135M peptide. The mouse-specific p135M peptide could prime BALB/c mice, and lymphocytes from either p135H- or p135M-primed mice cross-reacted with analog peptides of different species. However, none of the p135 peptide-primed lymphocytes could be stimulated with cartilage PG, indicating the cryptic character of the T cell epitope incorporated into the p135 sequence. This was the case not only for p135M and p135H peptide-primed lymphocytes, but also for all other p135 peptides, DnaJ from *E. coli* and p135H-AA-substituted analog peptides.

I.3. Cell surface markers and cytokine secretion of p135H- and p135M-primed and *in vitro* restimulated lymphocytes

Peptide p135-primed lymph node cells were mostly CD4⁺ cells (63% of the CD3⁺ cells) and were TCR α/β ⁺ (70%). Sixteen percent of the CD3⁺ cells were CD25⁺, and 30% of the T cells were V β 8⁺. The ratio of CD3⁺ T cells (73%) to CD45/B220⁺ B lymphocytes (25%) in peptide-primed lymph nodes was very similar in both p135H- and p135M-primed animals. Peptide-primed CD4⁺ cells were predominantly of the Th1 type, producing large amounts of IFN- γ , twice as much in p135H-stimulated as in p135M-stimulated cultures, and only trace amounts of IL-4. TNF- α was essentially undetectable, and production of peptide-specific IL-5 and IL-10 was detected only in lymph node cultures of p135H-primed animals. Taken together, these data indicate that both self and non-self p135 peptide sequences can elicit T cell reactions in BALB/c mice, and peptide priming induces a predominant Th1 type of response.

I.4. Induction of arthritis in SCID mice by p135H peptide-primed lymphocytes

We used *in vivo* cell transfer to determine which aggrecan epitope-specific lymphocyte population is capable of inducing arthritis. To bring the recipient SCID mice to a prearthritic state and reduce the time needed for the development of arthritis, mice were “presensitized” by injecting a suboptimum number of spleen cells (4×10^6) from acutely arthritic BALB/c donors, along with a single dose (100 μ g) of human proteoglycan. Presensitization of recipient SCID mice was particularly useful when T cell hybridomas with a metastatic behavior were used for the transfer experiments. SCID mice that received only a single injection of 4×10^6 unseparated spleen cells from arthritic BALB/c donors did not develop arthritis within 2 weeks after the transfer, and even later, fewer than 50% of the recipients developed arthritis with very low severity scores. These presensitized SCID mice were considered baseline control animals.

Experimental groups (except the baseline control group) were reinjected with 5×10^6 peptide-primed lymph node cells restimulated *in vitro* for 3 days with the corresponding synthetic peptide. Peptide p135H-primed lymphocytes induced arthritis in all presensitized SCID mice with a relatively high severity score (mean \pm SD 5.0 ± 2.2), which was similar to those receiving 1×10^7 cells from acutely arthritic BALB/c mice (positive control). In contrast, only 50% of recipient SCID mice developed arthritis in a mild (score of 1.8 ± 0.8), occasionally transient form both in the baseline control group and in the group injected with irrelevant peptide (p130)-primed and *in vitro*-stimulated lymphocytes (62% incidence with an arthritis score of 2.4 ± 0.5). In summary, these transfer experiments clearly indicated that peptide p135H is able to activate lymphocytes, making these cells capable of inducing arthritis in an adoptive-transfer system. It is unclear why the peptide-immunized BALB/c mice did not develop arthritis, but the cryptic character of the p135H epitope might be a likely reason.

I.5. Histopathologic features of arthritis adoptively transferred by p135H-specific lymphocytes

The histopathologic features of the arthritic joints of SCID mice were very similar to and technically indistinguishable from those described for either the primary or adoptive-transferred forms of PGIA. Accumulation of mononuclear cells in the synovium was observed at the time of onset of the first clinical symptoms. A few days later, with the progression of inflammation (redness and swelling), lymphocytes became more abundant in the synovium and periarticular connective tissues, and proliferating synovial cells formed a pannus-like structure. The joint space became enlarged due to the accumulation of the synovial exudates rich in lymphocytes and neutrophils. At a more advanced stage of arthritis (\sim 2–3 weeks after onset), there was damage to the articular cartilage, accompanied by subchondral bone erosion. Until day 19, no sign of inflammation was detected in mice that received only a single injection of cells (baseline control) or mice that were injected with irrelevant peptide (p130H)-specific lymphocytes.

I.6. Characteristics of peptide p135-specific T cell hybridomas

The next important step was to identify the critical T cell epitope within the p135 sequence and determine the TCR and MHC binding sites. To achieve this goal, T cell hybridomas with uniform and stable expression of p135-specific TCRs were generated by fusing p135H- or p135M-primed and *in vitro*-stimulated lymphocytes with BW5147 thymoma cells. Stable p135H- and p135M-specific hybridomas were selected after several rounds of cloning. The p135H peptide-specific hybridoma H74/4 responded strongly to stimulation with p135H, and recognized bovine, canine and porcine

sequences with only slight variations in the magnitude of response. Moreover, hybridoma H74/4 recognized a modified peptide (p135H-AA), where alanine was substituted for the 2 serine residues to create a sequence incorporating the “shared epitope”. In contrast to the p135H-specific hybridoma H74/4, the p135M-specific hybridoma M24/4 did not cross-react with p135 peptides containing homologous sequences from other species.

While both p135H- and p135M-specific hybridomas produced relatively high levels of IFN- γ and TNF- α in response to peptide-specific stimulation, the p135M-specific hybridomas also produced significant amounts of IL-5 and IL-10. This difference might be the reason why the p135M-specific hybridomas did not transfer (induce) arthritis in SCID mice.

All stable T cell hybridomas, including H74/4 and M24/4, were tested *in vivo* for arthritis induction, as described for peptide-stimulated lymphocytes. The only difference was that T cell hybridomas were injected intravenously, rather than IP, because of their high metastatic propensity involving the liver. Peptide p135H-reactive T cell hybridomas (H74/4 was used as a prototype for more detailed characterization) induced arthritis consistently in “presensitized” SCID mice, although both the incidence and severity of the disease were lower (70% incidence; severity score of 4.29 ± 3.2) in hybridoma-injected SCID mice than in those injected with peptide p135H-primed and *in vitro*-stimulated lymphocytes, or in those that received PG-stimulated lymphocytes from arthritic donor BALB/c mice.

T cell hybridoma H74/4 was CD4⁺ and demonstrated high levels of the TCR α / β /CD3 complex, with predominant expression of V β 10 and V α 1.2 transcripts. This T cell hybridoma secreted significant amounts of IL-2, TNF- α and IFN- γ , but not IL-4 upon peptide p135H stimulation, indicating that it belonged to the Th1 subset. Recognition of peptide p135H by hybridoma H74/4 was class II MHC-restricted. Peptide p135H was presented in the context of I-E^d molecules, since a mAb against I-E^d completely abrogated the p135H-specific *in vitro* IL-2 secretion of the H74/4 hybridoma.

I.7. Determination of the core sequence of peptide p135H that is critical for recognition by T cell hybridoma H74/4

To determine the fine epitope sequence of arthritogenic T cell hybridoma H74/4 (representing a p135H-specific immortalized lymphocyte), we first examined the minimal core peptide required to stimulate H74/4 hybridoma cells using a set of overlapping peptides with a 1-mer offset (p2367–2396 of human aggrecan). A murine B cell lymphoma cell line (A20) was used as APCs, and IL-2 production was measured with the CTLL-2 bioassay. The results define amino acids 2374–2382 (TYKRRLQKR) as the core region containing the minimal sequence required for the activation of T cell hybridoma H74/4.

I.8. Stimulatory activity of single-amino acid-substituted altered peptides

To study the functional role of each residue in the core sequence of peptide p135H, the stimulatory activities of analog-altered peptides were tested on peptide p135H-specific T cell hybridoma H74/4. Alanine and glycine substitutions of residues at positions P1, P7 and P9 had no major effect on the stimulatory activity compared with the wild-type peptide p135H. However, substitution of a single amino acid at the P2–P6 or P8 positions completely abrogated the T cell response, indicating that these residues are likely to be critical for recognition by T cell hybridoma H74/4.

I.9. Localization of the I-E^d-binding residues within p135H

To define which residues within p135H were involved in the interaction with I-E^d molecules on the A20 antigen-presenting cells, we analyzed the I-E^d binding capacity of single-amino acid (Ala or Gly)-substituted peptide analogs using flow cytometry. Although most of the substitutions were well tolerated at the level of MHC binding, 4 substitutions led to a marked decrease in peptide binding to the I-E^d molecule. The 4 affected substitutions were mostly basic residues at positions P2 (Tyr), P3 (Lys), P4 (Arg) and P5 (Arg). The positively charged residues in p135H could possibly interact with the negatively charged residues at positions 114 (Glu) and 155 (Asp) of the E^d β -chain. With the exception of the P1, P7 and P9 substitutions that had no appreciable effect on T cell stimulation, substitutions at all other residues (P2 [Tyr], P3 [Lys], P6 [Leu] and P8 [Lys]) appeared to interfere with recognition by the TCR.

II. Investigation of the adjuvant effect of dimethyldioctadecylammonium bromide in proteoglycan-induced arthritis and collagen-induced arthritis

II.1. Incidence and severity of PGIA in arthritis-susceptible BALB/c and C3H colonies

A group that received proteoglycan with DDA (PG/DDA) intraperitoneally, developed more severe PGIA significantly earlier than did BALB/c mice immunized with the same dose of PG with CFA and IFA.

PGIA can only be induced in genetically susceptible BALB/c or C3H strains, but since the severity and susceptibility can vary even between different colonies of the same murine strain, we compared the major clinical parameters (arthritis onset, severity and incidence) in commercially available BALB/c and C3H colonies. While the intergroup (substrain) differences (50–100% incidence with arthritis scores of 5.1–12.4) occurred in BALB/c mice, the individual differences among BALB/c colonies disappeared by week 9 in PG/DDA-immunized mice. The incidence was 100%, and the mean \pm SD arthritis score in 9 different BALB/c colonies (n = 462) was 10.6 ± 3.6 at week 9.

In contrast to the BALB/c strain, the extreme differences among C3H colonies, for example, between C3H/HeJ and C3H/HeJCr, 2 colonies which are otherwise derived from the same founder, remained significant in PG/DDA-immunized mice. Only 28% of the C3H/HeJ mice (from The Jackson Laboratory) developed arthritis with very low arthritis scores (1.9 ± 1.2), after up to 4 antigen injections, whereas the incidence was 100% in C3H/HeJCr mice (from the NCI) with an arthritis score of 5.7 ± 2.3 .

Although all BALB/c mice and the highly susceptible C3H colonies uniformly reached 100% incidence of arthritis by weeks 9-10 in response to PG/DDA immunization, there were significant differences in the final arthritis scores in BALB/c mice (arthritis score 11.6 ± 1.6) compared with the highly susceptible C3H colonies (arthritis score 7.2 ± 2.1).

II.2. PGIA susceptibility in inbred murine strains

Susceptibility to PGIA is determined by both MHC and non-MHC genes, and the different colonies of susceptible BALB/c (H-2^d) and C3H (H-2^k) strains exhibit variabilities in both the severity and incidence of PGIA. Therefore, the next evident question was whether other inbred murine strains with the same (H-2^d or H-2^k) or different haplotypes can develop PGIA in response to PG/DDA immunization.

While all murine strains, regardless of their H-2 haplotype or genetic background, responded well to PG/DDA immunization, we could not find any strain other than BALB/c and C3H that was susceptible to PGIA. This was especially interesting because

a number of murine strains carried the same class II alleles (either H-2^d or H-2^k haplotype), and exhibited similar, or occasionally even higher T cell and B cell responses to PG immunization, compared with the susceptible BALB/c or C3H colonies.

II.3. Arthritogenic effect of immunization with DDA and PGs isolated from various species

In previous studies, we tested cartilage PGs from various species for their ability to induce arthritis in BALB/c mice when used with Freund's adjuvants. PGs from fetal human and pig cartilage, and from adult human and canine cartilage were the only ones that could induce arthritis in BALB/c mice, but only if the glycosaminoglycan side chains were removed. Cartilage PGs from other species either did not induce arthritis or induced only a weak and transient inflammation at a very low incidence (<10%) in BALB/c mice immunized with PG in Freund's adjuvants.

Using DDA as adjuvant, we retested a few, relatively easily accessible cartilage PGs of various species. Quite unexpectedly, PGs from fetal and adult bovine cartilage, and from adult pig and sheep cartilage proved as arthritogenic as PGs isolated from newborn or adult human cartilage, when they were injected with DDA. Moreover, crude cartilage extract from osteoarthritic cartilage proved to be excellent arthritogenic material if both the chondroitin sulfate and keratan sulfate side chains of PG were removed; it was as effective as the highly purified human cartilage PG.

Thus, when using lipophilic adjuvant DDA for immunization, PGs with relatively poor arthritogenicity in Freund's adjuvants could induce arthritis with maximum incidence and high severity in mice of the BALB/c strain.

II.4. Histopathologic features of the peripheral joints and spine of PGIA-susceptible mice

In the mice immunized with PG in DDA, the clinical scores for the paws corresponded well to the histopathologic abnormalities in the small peripheral joints, as described for the "classic" PG/CFA-induced form of PGIA. Joint inflammation started with mononuclear (mostly lymphocyte) and polymorphonuclear infiltration, which was soon accompanied by massive cartilage degradation, followed by bone erosion.

A remarkable difference was that simultaneously with the synovial joint inflammation, massive spondylitis was detected in all arthritic BALB/c mice that had been immunized with PG/DDA. This was especially unusual, because spondylitis typically only appeared 2–4 months after peripheral (synovial) joint inflammation in BALB/c mice that had been immunized with PG/Freund's adjuvants.

II.5. Mechanisms of action of DDA as adjuvant

The overall results of T cell- and B cell-mediated immune responses to human PGs in PG/CFA- or PG/DDA-immunized BALB/c mice were highly comparable, and serum levels of proinflammatory cytokines were even more pronounced in PG/CFA-immunized animals than in those injected with PG/DDA.

The serum antibody levels to either PG or CII were similar or even lower when injected with DDA than with CFA. In contrast, we found extensive differences in antigen-specific IgG1:IgG2a, and IFN- γ :IL-4 ratios when the 2 adjuvants were compared throughout the immunizations. Again, while the overall effects of DDA and CFA on the T cell response were highly comparable, antigen (PG)-specific cytokine production by either PG-primed spleen cells or peripheral lymph node cells was significantly different, and was clearly shifted toward Th1 type immune response.

To gain insight into the local mechanisms of DDA action and to understand why DDA supported PGIA more powerfully, BALB/c mice were injected IP with PBS, PBS/IFA, PBS/CFA or PBS/DDA, with or without cartilage PG antigen, and cells obtained from a peritoneal lavage and from the spleen were harvested at 6 hours, 12 hours, every 24 hours thereafter until day 9, on days 14 and 21 after the first injection, and 9 days after the second or third injection. As expected, the cell number was significantly higher, and reached a peak after 24-48 hours, in the peritoneal lavage fluid from all adjuvant-injected groups compared with the PBS- or PG/PBS-injected groups, and these levels never returned to normal. The cell influx contained predominantly neutrophilic granulocytes (70-85%), and the ratio of the neutrophils was consistently highest in the CFA-injected groups at every time point evaluated. The cell number in peritoneal lavage fluid from DDA-injected groups was approximately two-thirds to one-half the cell number in fluid from CFA-injected animals by days 9-14. The cells consisted almost exclusively of F4/80⁺ macrophages (mean \pm SD 79 \pm 11%) and CD3⁺ T cells (7.0 \pm 2.6%), and more than 60% of the CD4⁺ cells were activated (CD4⁺/CD69⁺). Moreover, the ratio and the total number of CD11c⁺ cells (dendritic cells) in the peritoneal lavage fluid were highest in the PG/DDA-injected group.

II.6. Application of DDA in the induction of collagen-induced arthritis

The onset of CIA was highly comparable in various groups of DBA/1 mice, but 100% incidence was reached only in CII/DDA-immunized mice after 2 antigen injections, with a significantly higher arthritis score in CII/DDA-immunized mice than in CII/CFA-immunized mice. The route of immunization (ID versus IP), however, was a critical component of arthritis induction, and the incidence reached only 60% even after the third IP injection of CII given either in CFA or DDA. It is important to note here that although both murine strains (BALB/c and DBA/1) showed immune response to immunization with either human PG or CII, DBA/1 mice did not develop arthritis in response to PG/DDA immunization, and BALB/c mice did not show signs of arthritis after CII/DDA or CII/CFA immunization.

NOVEL FINDINGS

I. Investigation of the role of peptide p135-specific lymphocytes and T cell hybridomas in the induction of arthritis

1. Following a T cell epitope mapping study of human cartilage proteoglycan (aggrecan), peptide p135H (²³⁷³TTYKRRLQKRSSRHP), located in the G3 domain of human aggrecan and containing a highly homologous sequence motif of the “shared epitope” (QKRAA), could induce arthritis in BALB/c mice after a single injection of human proteoglycan. It is important to note, that a single injection of cartilage proteoglycan does not induce arthritis.

2. T cells primed by human p135H synthetic peptide could cross-react with those primed by mouse (self) p135M peptide. However, none of the p135-primed lymphocytes could be stimulated with cartilage proteoglycan, indicating the cryptic character of the T cell epitope incorporated into the p135 sequence.

3. Peptide p135-primed lymph node cells were mostly CD4⁺ cells and were TCR α / β ⁺, producing large amounts of IFN- γ , indicating that both self and non-self p135 peptide sequences could elicit T cell reactions in BALB/c mice, and peptide priming induced a predominant Th1 type of response.

4. Using an adoptive-transfer system, p135H-primed lymphocytes induced arthritis in “presensitized” SCID mice, with a relatively high incidence and severity score. The histopathologic features of the arthritic joints of SCID mice were indistinguishable from those described for either the primary or adoptive-transferred forms of PGIA. It is unclear why the p135H-immunized BALB/c mice did not develop arthritis before, but the cryptic character of the p135H epitope might be a likely reason.

5. Applying p135H-specific T cell hybridoma H74/4, we determined the fine epitope structure of p135H. We defined amino acids 2374–2382 (TYKRRLQKR) as the core region of the peptide. Recognition of p135H by hybridoma H74/4 was class II MHC-restricted, since p135H was presented in the context of I-E^d molecules. Centrally located basic amino acids at positions P2 (Tyr), P3 (Lys), P4 (Arg) and P5 (Arg) proved to be responsible for binding of p135 to I-E^d, while residues P2 (Tyr), P3 (Lys), P6 (Leu) and P8 (Lys) appeared to play a role in the recognition by the TCR. Peptide p135-reactive T cell hybridoma H74/4 belonged to the Th1 subset, and induced arthritis consistently in “presensitized” SCID mice, although both the incidence and severity of the disease were lower than in those injected with p135H-primed and *in vitro*-restimulated lymphocytes.

II. Comparison of the adjuvant effects of dimethyldioctadecylammonium bromide (DDA) and Freund’s complete adjuvant (CFA) in proteoglycan-induced arthritis and collagen-induced arthritis

1. The overall immune responses (antibody production and antigen-specific T cell responses) were highly comparable when human PGs in CFA or in DDA were used. The notable differences were as follows:

2. In PG/CFA-injected mice significantly higher levels of serum proinflammatory cytokine and PG-specific IgG1, but not IgG2a were measured.

3. In PG/DDA-immunized mice significantly higher ratios of antigen-specific IFN- γ to IL-4 and significantly lower ratios of IgG1 to IgG2a were observed than in PG/CFA-immunized mice, indicating a more pronounced shift of the Th1/Th2 balance toward a Th1 type of response in PG/DDA-injected mice.

4. A highest ratio of activated CD3⁺, CD44^{High} cells in PG/DDA-primed lymph nodes; an increased ratio of CD4⁺ to CD8⁺ cells in the spleens of arthritic mice but not in PG/DDA-primed lymph nodes, and a significantly increased number of CD4⁺/CD69⁺ cells in peritoneal lavage fluid from PG/DDA-primed BALB/c mice, were detected.

5. Moreover, at least a 2-4-fold increase in macrophage influx into the peritoneal cavity, accompanied by more CD11c⁺ dendritic cells but significantly fewer polymorphonuclear cells, was characteristically observed in DDA-injected mice.

6. The use of DDA accelerated the development of a more severe arthritis, and suboptimum doses of cartilage PG or CII antigens, or PGs having only a suboptimal arthritogenic effect when injected with CFA, were able to induce inflammation, via a more potent activation of the innate immunity.

DISCUSSION

Proteoglycan (aggrecan) has been shown to induce progressive polyarthritis in genetically susceptible BALB/c mice. Although the underlying mechanisms are still unclear, T cells, particularly CD4⁺ lymphocytes, seem to play a crucial role in the initiation of the disease. Our earlier studies explored numerous T cell epitopes along the core protein of human aggrecan, most of them being located within the poorly glycosylated globular G1 domain. Interestingly, one of the epitopes present in the G3 domain at the C-terminus of the human proteoglycan also appeared to be involved in arthritis induction. Hyperimmunization of BALB/c mice with synthetic peptide (p135H) representing a segment of the human aggrecan G3 sequence ²³⁷³TTYKRRLQKRSSRHP, followed by the injection of a single dose of proteoglycan, induced progressive polyarthritis. It should be noted, that a single injection of cartilage proteoglycan does not induce arthritis.

To further investigate whether the T cell epitope represented by p135H might be directly involved in the pathogenesis of the disease, we transferred p135H-stimulated lymphocytes from peptide p135H-primed BALB/c mice into SCID mice. The clinical appearance and histopathologic features of adoptively transferred arthritis in SCID mice showed many similarities to PGIA, such as lymphocyte/lymphoblast infiltration in the synovial membrane, pannus formation, synovial hyperplasia, and erosion of articular cartilage and subcortical bone. According to the expression of cell surface markers and the antigen (p135H)-induced cytokine profile, most of the peptide p135H-specific lymphocytes were Th1 cells. These T cells cross-reacted *in vitro* with the human, bovine, canine and porcine, as well as with the corresponding mouse (self), aggrecan sequence. This cross-reactivity between the human and mouse sequences indicated a high degree of epitope spreading that could establish the immunopathologic basis of arthritis in BALB/c mice.

To further characterize T cells with p135H and p135M specificity, we have generated peptide p135H-specific T cell hybridomas and used them to determine the fine epitope specificity of TCR- and MHC-binding sites. Interestingly, while p135H- or p135M-primed lymphocytes cross-reacted well with p135 peptides of the other species, only a few p135H-specific hybridomas were able to transfer arthritis into “presensitized” SCID mice, whereas others, especially those derived from p135M (mouse)-specific T cells, were unable to induce inflammation. This might be explained by the different cytokine profile after peptide stimulation and/or different epitope specificity of the T cell hybridomas. Thus, while the sequence of p135 peptide allows for cross-reactivity between human- and mouse-specific T cell populations, the epitope specificity of individual hybridomas is highly restricted to a single peptide sequence. Essentially, all p135H-specific hybridomas generated in this study recognized the same core sequence and TCR binding sites.

A special feature of this T cell epitope is that the p135H contains a conserved amino acid sequence QKRSS, which shows striking similarity to the “shared epitope” QKRAA. This “shared epitope”, and analogous sequences with conservative replacements (QK/RRA/SA/S) are overrepresented in protein databases, suggesting that this sequence motif may have a fairly ubiquitous function. Previous studies have shown that this 5-amino acid motif is present in the third hypervariable region of certain HLA alleles associated with RA (*e.g.* HLA-DR4/Dw4 and DR9), and that the presence of this “shared epitope” could predict a progressive destructive disease course. HLA-derived peptides encompassing the “shared epitope” sequence can randomly select T cells that bind the self-derived peptide at low avidity. Later in life, these previously quiescent

QKRAA-specific T cells can be activated by binding with high avidity an exogenous peptide containing the “shared epitope”.

Furthermore, it has been recently demonstrated that the “shared epitope” acts as a potent immune-stimulatory ligand that activates a nitric oxide-mediated pathway in other cells (*e.g.* dendritic cells), and can polarize T cell differentiation toward Th17 cells, a T cell subset that has been implicated in the pathogenesis of autoimmune diseases, including RA.

Interestingly, proteins in common human pathogens, such as *Escherichia coli* (DnaJ heat-shock protein), *Lactobacillus lactis* and *Brucella ovis* (DnaJ heat-shock protein), and Epstein-Barr virus (gp110 protein), have been identified that express the “shared epitope” in the context of highly immunogenic proteins, and immune responses to several of these antigens have been evaluated in RA patients. In addition, database searches reveal that peptide p135H also has a high sequence similarity (76%; TTYKRR—RSSR) to the capsid protein VP3 of human JC polyomavirus, which is ubiquitous in the human population and is the etiologic agent of progressive multifocal leukoencephalopathy. The virus can infect children without producing symptoms; it remains latent in the kidneys, tonsils and central nervous system, and may reactivate, causing significant T cell dysfunction. These sequence data suggest that immunologic cross-reactivity might exist between certain RA-associated HLA alleles, the above-mentioned immunogenic proteins of human pathogens, and p135H, a T cell epitope of human cartilage proteoglycan. Indeed, a high cross-reactivity was observed between peptide p135H and p135H-AA, a modified version containing the “shared epitope”.

Intermittent exposure of the immune system to these bacterial or viral antigens at mucosal surfaces might lead to the activation of resting QKRAA-specific T cells, and then the T cell activation is perpetuated by encounters with peptides of self origin, encompassing the “shared epitope” sequence. Why these lymphocytes show a pattern of migration to the peripheral joints remains an open question, and the target of the pathogenic autoimmune process in the joint is still elusive. It seems to be an attractive hypothesis, however, that the sequence of p135H in human cartilage proteoglycan can serve as a homolog of the “shared epitope”, and it may be responsible for the joint-specific homing of QKRAA-reactive T lymphocytes and then be involved in the initiation of the autoimmune process in RA (see Figure).

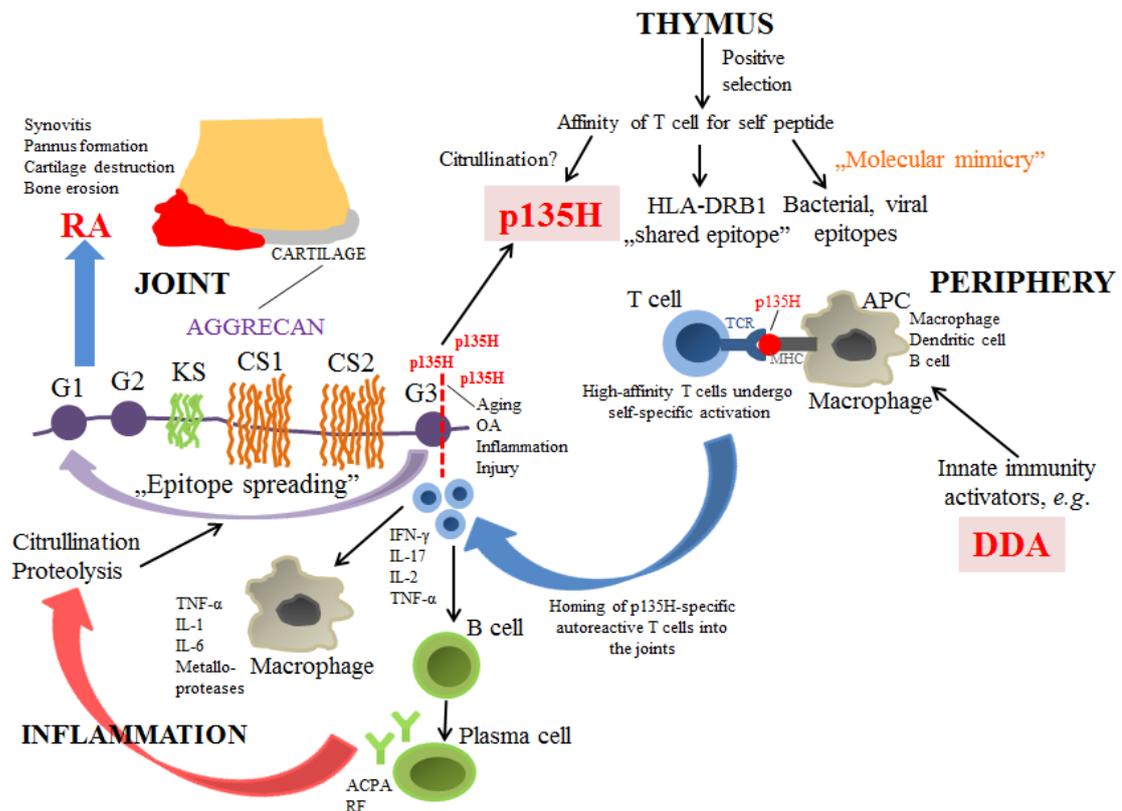
Loss of aggrecan is a major feature of cartilage degradation associated with arthritis. There is an age-related loss of the G3 domain of aggrecan, and 92% of the G3 domain is lost as part of the normal turnover of the proteoglycans, whereas the rest of the molecule, which is bound to hyaluronan, is retained in the cartilage. The C-terminal tail of aggrecan is cleaved first, and 2 tandem boxes (RRXXK and RXXR) of the consensus sequences have been demonstrated to be involved in the earliest cleavage. Interestingly, both cleavage sites described are located within peptide p135H (TTYKRRXXKRXXRHP). Therefore, a significant amount of p135H might be released from articular cartilage due to normal turnover or due to an enhanced proteolytic processing of the G3 domain (*e.g.* in inflammation or in cartilage injury), and then be exposed to the immune system in the joints (see Figure).

Since peptide p135H possesses a high sequence similarity to the “shared epitope”, it can provide ground for “molecular mimicry”. Peptide p135H of the G3 domain can be recognized by QKRAA-specific autoreactive T cells, which by migrating into the joint and expanding locally, might contribute to the development of arthritis. Therefore, we suggest, that this C-terminal part of the G3 domain plays a role in the initial activation of T cells, then later on the rest of the molecule, including the G1 domain becomes the main target. This process might be interpreted as “epitope spreading”, that is in the

course of autoimmune inflammation neo-epitopes are generated, *e.g.* by citrullination of other parts of the molecule, that subsequently become additional targets for the autoimmune response, or by the enhanced proteolytic activity, exposing other (remnant) epitopes to the immune system (see Figure).

In this study, we made efforts to further examine the role of peptide p135H, a T cell epitope in the C-terminal G3 domain of human proteoglycan, in an experimental animal model. Despite differences in the amino acid sequences between the human and mouse peptide and the cryptic character of the epitope, we were able to show that the transfer of peptide p135H-reactive lymphocytes into “presensitized” SCID mice leads to a rapid development of arthritis. Hence, we have direct evidence that peptide p135H contains an arthritogenic epitope that is recognized by T cells in mice and that it attracts these cells to joints that contain the homologous mouse sequence, thus leading to induction of arthritis.

Before, it has been assumed that all arthritogenic epitopes are located within the G1 domain of aggrecan. Purified G1 domain alone and G1 epitope-specific T cell lines were capable of inducing arthritis when injected into BALB/c mice. More importantly, patients with RA express cellular and humoral immunity to the G1 domain of cartilage proteoglycan aggrecan. To the best of our knowledge, this is the first report of the induction of arthritis by T cells specific for an epitope in the G3 domain of human cartilage proteoglycan.



The hypothetic role of p135H and DDA in the pathogenesis of rheumatoid arthritis.

See discussion for a detailed description. Abbreviations used in the figure: ACPA anti-citrullinated protein/peptide antibody, APC antigen presenting cell, CS chondroitin sulphate, DDA dimethyldioctadecylammonium bromide, G1, 2, 3 globular domains 1,

2, 3 of aggrecan, IFN interferon, IL interleukin, KS keratan sulphate, MHC major histocompatibility complex, OA osteoarthritis, p135H human peptide p135, RA rheumatoid arthritis, RF rheumatoid factor, TCR T cell receptor, TNF tumor necrosis factor.

In the second half of our studies, we have studied the adjuvant effect of dimethyldioctadecylammonium bromide (DDA) on arthritis induction.

DDA belongs to the group of lipophilic quaternary amines that were described more than 35 years ago as a potential adjuvant. DDA as an adjuvant has been used successfully and without side effects in vaccines administered to children and pregnant women. It is also widely used as a detergent in cosmetic compounds and fabric softeners. DDA is a highly potent immunostimulator, especially with negatively charged antigens, provoking strong delayed-type hypersensitivity. It is a powerful, nonirritant adjuvant and, via T cell stimulation, significantly enhances antigen-specific B cell activation and immunoglobulin production. A special benefit of the use of DDA in rodent models of autoimmunity is that this adjuvant forces the immunoregulation toward Th1 direction. DDA can enhance the adjuvant effect of other adjuvants and even potentiate the arthritogenic effect of IFA or CFA in oil adjuvant-susceptible strains of rats. However, DDA as an adjuvant has never previously been tested in any autoimmune model of rheumatoid arthritis.

There are a number of inducible animal models of human autoimmune diseases, all of which require immunization of genetically susceptible strains of rodents and non-human primates with a target organ-specific antigen in adjuvant. Depending on the model and species, 1-4 injections of antigen are required, and at least 1 of these injections should be given with CFA as adjuvant. Therefore, CFA seems to be a critical component in the induction and achievement of a high incidence and severity of autoimmune diseases in rodent models. The use of the same (auto)antigen in IFA, Alhydrogel (aluminum hydroxide gel) or synthetic adjuvants is either insufficient to induce the disease, or the incidence and severity are far below those of the antigen/CFA-induced disease. Thus, the nonspecific activation of innate immunity (*e.g.* macrophages, dendritic cells) and T cells by mycobacterial components such as muramyl dipeptide (peptidoglycan), HSP and trehalose dimycolate (a glycolipid equivalent with lipopolysaccharide of *Escherichia coli*) in mineral oil is a critical component in the provocation of immune reactions to self antigens in autoimmune models.

While a large number of adjuvants can be used for immunization of animals or for vaccination of humans, until now CFA has remained the most potent and powerful adjuvant in all experimental models of autoimmunity. The use of CFA, however, induces several side effects, including immune reactions to mycobacterial components, and as a highly irritating compound, CFA induces sterile inflammation, followed by local granulomatous tissue formation and severe adhesions, especially in the peritoneal cavity.

Here, we have compared the effects of DDA with those of CFA and IFA in arthritis-susceptible murine strains to gain insight as to how the DDA could achieve an even more superior effect than the Freund's adjuvants.

Among the adjuvants tested to date, only CFA and DDA proved to express sufficient power to provoke arthritis in PG- or CII-immunized genetically susceptible strains of mice. In terms of humoral and cellular immune responses to PG or other antigens and production of related cytokines, CFA proved to be an equivalent, or an even more effective adjuvant than DDA, and both CFA and DDA induced significantly higher

immune responses and cytokine production than did PG/IFA. However, the production of antigen-specific Th1 and Th2 cytokines, and the shift of the Th1/Th2 balance toward Th1 type response were significantly more pronounced in animals immunized with PG/DDA, than in those immunized with PG/Freund's adjuvants. Moreover, at least a 2-4-fold increase in macrophage influx into the peritoneal cavity, accompanied by more CD11c⁺ dendritic cells was characteristically observed in DDA-injected mice. Furthermore, suboptimal doses of cartilage PGs or PGs having only a suboptimal arthritogenic effect when injected with CFA, were as effective as the human cartilage PGs in provoking PGIA, and crude extracts obtained from osteoarthritic cartilage, when appropriately deglycosylated, induced arthritis in a manner similar to that of the highly purified human cartilage PG, when DDA was used as adjuvant.

As a result, the use of DDA accelerated the development of a more severe arthritis, and suboptimum doses of PG or CII antigens were able to induce inflammation via a more potent activation of the innate immunity.

In RA there is abundant evidence that the innate immune system is persistently activated, as evidenced by the continual expression of macrophage derived cytokines such as TNF- α , IL-1 and IL-6. Furthermore, the central role of macrophages in RA pathogenesis is supported by the fact that conventional therapies, including methotrexate and cytokine inhibitors, act to decrease the production of cytokines produced primarily by macrophages. A potential or critical role of innate immunity in arthritis induction is also consistent with the findings of studies that used only adjuvants (nonspecific stimulators) in genetically susceptible strains of rodents. Observations from these studies of oil-, pristane- and squalene-induced arthritis together with our observations raise the question of whether the "adjuvants" can also play a role in, and/or contribute to, joint inflammation in genetically susceptible humans. Can immunostimulatory molecules from microbes, environmental compounds (cosmetics, laundry detergents or food additives), or endogenous "self" adjuvants (such as lipid squalene) in fact cause or contribute to joint inflammation? Since a number of potential autoantigens (type II collagen, proteoglycan aggrecan, link protein, gp-39 or glucose-phosphate isomerase) could be identified in various subsets of RA patients, it may be an attractive hypothesis that nonspecific stimulation of innate immunity might be an initial component in the disease mechanism. A defect in immune regulation, the production of cytokines/chemokines, and the possible involvement of joint-derived or joint-independent autoantigens (such as rheumatoid factor) may be only subsequent, although clearly detectable, events in an initial nonspecific activation of innate immunity. While the relevance of this hypothesis requires extensive studies, our observations using an innocuous component as adjuvant, seem to support this possibility (see Figure above).

The two main subjects investigated in our studies, on the one hand peptide p135H, representing a supposed autoantigen in the G3 domain of human cartilage proteoglycan, and on the other hand DDA, a potent activator of innate immunity, might be two players among others, contributing to the initiation, perpetuation and joint-specificity of rheumatoid arthritis.

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