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Mice Lacking Mature T and B Lymphocytes Develop Arthritic Lesions after Immunization with Type II Collagen

David Plows,* George Kontogeorgos,† and George Kollias²

Collagen-induced arthritis in DBA/1 mice is a widely used experimental model of rheumatoid arthritis. The induction phase of the disease is thought to be dependent upon MHC-restricted T and B cell-mediated immune responses to type II collagen, but an influence of additional non-MHC-restricted mechanisms has also been proposed. In this study, we report that type II collagen immunization of DBA/1 mice lacking mature T and B lymphocytes resulted in the development of arthritic lesions, which were characterized by synovial hyperplasia with occasional inflammation as well as cartilage and bone destruction. The specificity of disease induction to type II collagen was confirmed, because arthritis could not be induced when control preparations of OVA or adjuvant alone were administered. A delay in clinical disease onset and a reduction in severity between lymphocyte-positive and -negative DBA/1 mice confirmed that lymphocytes play an important role in disease; however, similar pathologic features and normal incidence suggest that lymphocyte-independent mechanisms of disease induction also operate in the standard collagen-induced arthritis model. We conclude that adaptive immune responses are not the only arthritogenic mechanism and hypothesize that the nonantigenic properties of type II collagen can also lead to arthritis.


Collagen-induced arthritis (CIA) is a well-established model for human rheumatoid arthritis that is induced after the immunization of susceptible rat or mouse strains with homologous or heterologous type II collagen in adjuvant. Disease initiation is described as dependent upon both humoral and cellular immunity to the immunizing Ag (2), but the influence of additional non-MHC-restricted genes has been reported that suggests a nonlymphocyte component in disease (3–7). An autoimmune hypothesis is supported by the observations that CIA can be attenuated by treatment with mAbs to CD4 and TCR, (8–10) and that recipient mice develop arthritis after adoptive transfer of collagen-specific T cell lines (11) or anticollagen Abs (12–15). Evidence has been presented that the induction of CIA is associated with the dominant expression of a Th1 cytokine pattern, suggesting that the specific cellular type involved in disease is CD4⁺ T cells (16); however, disease induction in targeted “gene knockout” mice has failed to provide a clear indication as to T cell involvement. CD4-deficient mice developed CIA with unaltered incidence and severity, whereas CD8-deficient mice showed a decreased incidence but unaltered severity (17).

Despite the lack of definitive experimental evidence for an essential role of the T cell compartment in the initiation of CIA, the notion that disease onset is dependent upon the MHC class II-restricted antigenic properties of type II collagen remains the prevalent concept. However, due to the conflicting evidence both for and against B or T lymphocyte involvement in the induction of CIA, we tested the possibility that a lymphocyte-independent mechanism may be involved. We challenged DBA/1 mice lacking functional mature T and B lymphocytes with rat type II collagen in the presence of adjuvant. These mice were generated by backcrossing recombination activation gene (RAG-1)-deficient mice into the DBA/1 background. This generated disease-susceptible (H-2b) mice lacking both mature B and T lymphocytes as a result of the inactivation of the RAG-1 enzyme that catalyzes the V(D)J-recombination reaction of Ig and TCR genes (18). Our investigations showed that arthritis can be initiated in the absence of mature, functional B and T lymphocytes. Analysis of the disease profile revealed a delay in clinical disease onset and a reduction in severity, confirming a positive contribution by T and B lymphocytes to disease progression. However, similar pathologic features and normal incidence suggest that lymphocyte-independent mechanisms of disease induction also operate in the standard CIA model. We conclude that adaptive immune responses are not the only mechanism of CIA initiation and hypothesize that the nonantigenic properties of type II collagen can also lead to arthritis.

Materials and Methods

Animals

RAG-1-deficient mice (kindly provided by Dr. Spanopoulou, Rockefeller University, New York, NY) were backcrossed into the disease-susceptible DBA/1 background (H-2b) for at least five generations; a change in MHC haplotype was confirmed by FACSscan analysis (Abs were obtained from PharMingen, San Diego, CA). Genotype was determined by Southern blot hybridization as described previously (18) and was confirmed by FACSscan analysis for CD4 and CD8 lymphocytes (Abs were obtained from PharMingen). Mixed groups of virgin male littermates, which had been separated from females at 3 wk of age, were housed at low numbers of mice per cage. Experimental mice comprised males of between 8 and 10 wk of age at the time of immunization. Mice were fed water and food ad libitum in accordance with European Union guidelines on animal welfare.

Induction of CIA

Rat type II collagen from a rat chondrosarcoma (19) (kindly donated by Dr. R. Holmdahl, Lund, Sweden) was prepared by pepsin digestion and subsequent purification as described previously (20). Native type II collagen was dissolved in 0.1 M acetic acid at a concentration of 1 mg/ml. Mice were immunized intradermally at the base of the tail with a single injection

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of 100 μg of collagen emulsified in an equal volume of CFA containing 100 μg of H37Ra Mycobacterium tuberculosis (Sigma, St. Louis, MO). Where stated, chicken egg OVA (Sigma, USA) was dissolved in 0.1 M acetic acid at a concentration of 1 mg/ml and used as the immunogen instead of type II collagen.

Clinical analysis of disease

Arthritis was scored three times per week in each of four paws based on the following arthritis severity index: grade 0, normal appearance and flexion; grade 1, mild swelling and/or erythema; grade 2, moderate swelling and erythema; grade 3, severe swelling and erythema. Scores were compiled for each mouse at each timepoint by adding the score for each of the four paws, thereby giving a maximum potential score of +12 and a minimum score of 0. To derive the arthritic index for each experiment, maximal arthritic scores were divided and multiplied by the number of arthritic mice in the group.

Histopathologic examination

Joint tissues from freshly dissected mice were immersion-fixed in 10% formalin in 0.15 M PBS (pH 7.4) overnight, decalcified in 30% formic acid/0.28 M sodium citrate for 48 h, and subsequently dehydrated in graded alcohol and embedded in paraffin (BDH, Dorset, U.K.). Serial sections throughout the joint were cut at 4–6 μm on a microtome and stained with hematoxylin and eosin. Sections were evaluated in a blinded manner. The sections were studied for histologic signs of arthritis and scored as follows: 1, synovial cell proliferation, synovial hypertrophy with villus formation and/or fibrin deposition; 2, inflammation, synovitis and/or generalized inflammation; 3, cartilage disruption, chondrocyte degeneration and/or ruffling of cartilage surface and/or dystrophic cartilage; and 4, joint destruction, cartilage erosion with abundant inflammation and pannus formation with bone erosion.

Evaluation of serum Ab levels

Serum Ab levels against type II collagen were measured by a standard ELISA assay. Briefly, a 96-well Immuno-Maxisorp plate (Nunc, Roskilde, Denmark) was coated with rat type II collagen (10 μg/ml) overnight at 4°C and blocked with 1% BSA in PBS. Plates were washed, and sample sera (at a dilution of 1/200) were incubated for 3 h at 37°C. Subsequently, plates were washed and incubated with biotin-conjugated polyclonal rabbit anti-mouse IgG (heavy and light chain) or IgM Abs (Sigma, St. Quentin Falavier, France) followed by incubation with horseradish peroxidase-streptavidin (Sigma). The ELISA was developed with o-phenylenediamine dihydrochloride (Sigma) containing 0.03% H2O2, and the reaction was terminated with 50 μl of 2 M H2SO4. OD was read at 490 nm using an MRX microplate reader (Dynatech Labs, Chantilly, VA). Data generated were used to confirm the presence of Ab response to the Ag.

Statistical analysis

Data were analyzed using the unpaired Student t test. p values of <0.05 were considered statistically significant. Data are presented as the mean ± SEM where appropriate.

Results

CIA in DBA/1 mice lacking functional mature lymphocytes

To assess whether the initiation of CIA is dependent upon mature T and B cells, RAG-1-deficient mice in a DBA/1 background were challenged with rat type II collagen emulsified in adjuvant. Clinically, we found that, whereas lymphocyte-positive RAG+/+ DBA/1 littermate controls presented severe swelling with peak incidence around week 5 postchallenge, RAG−/− DBA/1 mice developed mild swelling, with peak incidence around week 7 (Fig. 1; Table I). In the RAG-1-deficient mice, both the delay in disease onset (p = 0.0001) and the reduction in disease severity (p = 0.0007) were deemed to be statistically significant. However, the overall degree of incidence between RAG+/+ and RAG−/− DBA/1 mice was not significantly different (p = 0.8614; Table I). No arthritis was observed in the control, non-immunized DBA/1 group. In all mice, the functional deletion of the RAG-1 gene product was confirmed by the absence of anticollagen Abs in the sera of mice after challenge with type II collagen, in comparison with positive control RAG-1 heterozygous littermates, as determined by ELISA analysis (Table II). Additional characterization by FACScan analysis (anti-CD4 and anti-CD8 Abs by PharMingen) was conducted on selected RAG-1-deficient and RAG-1 heterozygous DBA/1 mice to confirm the presence or absence of CD4+ and CD8+ single-positive T lymphocytes (Table II).

Histopathologic examination was in agreement with the clinical scoring and showed typical signs of ongoing arthritis (Fig. 2). Arthritic lesions were observed in 11 of 18 RAG−/− DBA/1 mice, of which 4 were characterized as suffering from mild focal arthritis. In this group of mice, synovial hyperplasia initiating at the cartilage/synovium junction was apparent, in some cases extending down the shaft of the bone cortex (Fig. 2A). The synovium showed signs of activation characterized by fibrin deposition along the hypertrophic synovium with occasional villus formation. There was no evidence of inflammation in these mice, with the soft tissue remaining free of cellular infiltration (Fig. 2B). The articular cartilage showed areas of proteoglycan loss as determined by toluidine blue and safranin O staining (data not shown). In addition, histologic examination of the articular cartilage showed a variable

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<th>Table 1. Incidence of CIA in mice^a</th>
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^a Summary of clinical scores after immunization with the designated immunogen for each experimental group of mice. Data shown are the combined results from three separate experiments.

^b Percent incidence of disease is not significantly different; p = 0.86.

^c The reduced arthritic index is statistically significant; p = 0.0077.

^d Results from a single blinded experiment designed to assess whether the observed arthritis is Ag-specific.

^e Arthritic index is shown as mean ± SEM.
The degree of cartilage disruption characterized by chondrocyte degeneration and necrosis. Ruffling and fragmentation of the articular cartilage was also obvious. In areas of greatest synovial expansion, erosion of the subchondral bone was observed.

The remaining seven arthritic mice were characterized as suffering from advanced arthritis, with characteristic arthritic features such as pannus formation, cartilage, and bone erosion. Cartilage destruction was more advanced, occurring proximal to the hyperplastic synovium (Fig. 2C). Subchondral bone erosion was also more pronounced. One interesting feature found in four of seven mice was the development of dystrophic cartilage that was characterized by the deposition of extracellular matrix proteins within the hyperplastic synovium (Fig. 2D) and confirmed by toluidine blue and safranin O staining (data not shown). In two of seven mice with advanced arthritis, an inflammatory infiltrate was observed. Histologic analysis of this inflammatory lesion revealed major differences in the inflammatory component compared with normal CIA. In lymphocyte-positive control mice with CIA, heavy inflammation in both the subsynovium and the soft tissue was observed (Fig. 2E). This inflammation consisted of lymphocytes interspersed with polymorphonuclear cells. In RAG-1-deficient DBA/1 mice with mild focal arthritis, there was no inflammatory component in the hyperplastic synovium (Fig. 2B); only synovial cells were detectable. In RAG-1-deficient DBA/1 mice with advanced arthritis that showed inflammation, the inflammatory component contained virtually no lymphocytes as determined by histologic examination; although polymorphonuclear cells were present, they were greatly reduced in number (Fig. 2F).

Of the mice that were scored as clinically nonarthritic, three of seven showed signs of synovial activation (characterized by mild hyperplasia with either villus formation or fibrin deposition). Consequently, there remains the possibility that disease would have developed in these mice over a longer period. Studies into the chronicity of CIA in the absence of T and B lymphocytes are now underway.

**Disease induction in DBA/1 mice lacking functional mature lymphocytes is collagen-specific**

Single-blinded induction experiments were conducted to investigate the specificities of disease. Grouped RAG-1−/− DBA/1 males were injected with either rat type II collagen, chicken egg OVA, or diluent (0.1 M acetic acid) emulsified in adjuvant. These results (Table I) showed an induction of CIA only in those mice challenged with type II collagen. Histologic analysis confirmed the clinical scores and showed characteristic features of arthritis only in those mice challenged with heterologous type II collagen. Mice challenged with either OVA or diluent showed normal histologic features in the joint (data not shown).

**Discussion**

In this study, we show that mice lacking mature, functional T and B cells can initiate CIA-like disease at normal incidence when backcrossed into a disease-susceptible genetic background. It has been documented previously that the initial event after CIA induction in DBA/1 mice is fibrin deposition and synovial hyperplasia followed by the induction of synovitis (21). Simultaneously, a disruption of cartilage characterized by proteoglycan depletion and erosion at the cartilage/bone marginal junction occurs. In more advanced disease, pannus formation is observed that is accompanied by cartilage and subchondral bone destruction. Histologic analysis of disease in lymphocyte-deficient DBA/1 mice revealed extensive synovial hyperplasia, fibrin deposition, and cartilage/bone disruption but a low incidence of inflammation. Thus, lymphocyte-deficient DBA/1 mice show pathologic lesions found in the standard CIA model upon induction in DBA/1 mice. Using a nonarthriogenic Ag such as OVA or adjuvant alone, we demonstrated that the disease remained collagen-specific. Spontaneous arthritis, previously reported to occur in DBA/1 male mice (22), was excluded as a possible mechanism, because the disease was demonstrably collagen-specific and the nonimmunized control group failed to show detectable arthritis upon clinical and histologic examination.

Taken together, our results imply that the arthritis-triggering properties of collagen can act independently of the adaptive arm of host immunity. However, the reduced severity argues that the pathologic changes observed in the joint are accentuated by the action of lymphocytes. Therefore, it is possible that there are additional mechanisms of disease induction at work in CIA. This conclusion would explain the partial protection from CIA induction and severity observed in a diverse range of mice deficient in gene products that are known to play immunomodulatory roles. For example, in mice lacking major components of the immune response such as CD4/CD8 (17), ICAM-1 (23), IL-12 (24), IFN regulatory factor-1 (25), 5-lipoxygenase-activating protein (26), or the p55 TNF receptor (27), protection from CIA was never complete. Interestingly, the only study so far that reported total protection from CIA concerned IL-6 knockout mice (28), but the exact...
mechanism for this protection remains unclear. In contrast to our report, recent experiments have shown that B cell-deficient mice do not develop clinical signs of CIA (29). This contradictory finding may be due to the difference in genetic background between the DBA/1 and the B10 mouse strains used by Svensson et al. or to a difference in the immunizing protocol. An alternative explanation may be that B cells are important in eliciting an inflammatory response, the absence of which may mask the clinical signs of disease. As shown in our study, a histopathologic analysis of CIA should be helpful in defining disease states, especially in immunodeficient strains in which clinical/inflammatory lesions may be dampened.

There is a wealth of experimental evidence describing a pivotal role for the adaptive immune response in CIA; however, the data reported here suggest that additional mechanisms may also be present. Several hypotheses can be proposed that rationalize the presence of such additional mechanisms. First, CIA could be a complex disease consisting of two distinct stages, with stage 1 being synovial activation and stage 2 consisting of lymphocytic involvement and leading to readily observable clinical manifestations. Evidence supporting this theory comes from the observations of Caulfield et al., who noted that synovial hyperplasia appears before inflammatory cell influx (21). Second, it is also possible that other mechanisms in disease induction become important in the absence of lymphocytes, and that an arthritic disease distinct from standard DBA/1 CIA is observed in the RAG-1-deficient DBA/1 mouse. However, the similar pathologic features and normal incidence of disease between lymphocyte-deficient and normal DBA/1 arthritic mice do not support this theory. Therefore, to resolve this issue, it is important to define the parameters leading to disease induction in the RAG-1-deficient model. For example, it will be interesting to assess whether disease induction in RAG-deficient mice requires the DBA/1 background and whether it remains specific for type II and not type I or denatured type II collagen. Experiments are now underway to address these questions.

We conclude that the observed pathologic events upon collagen immunization in RAG-deficient mice depend upon the nonantigenic properties of type II collagen. One possible mechanism may use the recently reported capacity of the native triple helical fibrilar collagens (types I, II, III, V, and XI) to act as the specific ligands for the discoidin domain receptor tyrosine kinases DDR1 and DDR2 (30, 31). These receptors are widely expressed in several fetal and adult organs and tissues, but their specific biologic function in mammals remains unclear. Remarkably, activation of the
DDR2 receptor by collagens is found to specifically induce the expression and secretion of matrix metalloproteinases, which are molecules that are implicated in the pathogenesis and perpetuation of destructive inflammatory arthritis (32). In general, receptor tyrosine kinases control a wide variety of cellular responses, including the regulation of cell growth, differentiation, migration, metabolism, and survival. Therefore, it is tempting to speculate that upon the administration of type II collagen, specific collagen receptor-mediated cellular events act as the triggers for an arthritogenic response that could then be exacerbated by the involvement of the adaptive immune response. This hypothesis may explain the unaltered incidence but reduced severity and delayed time of onset of CIA as documented in this study in RAG-1-deficient DBA/1 mice and is further supported by the finding that the activation of macrophages or monocytes can occur in vitro with exogenously added type II collagen or collagen-derived peptides in a dose-dependent manner (33–36).

In the absence of an active T and B lymphocyte population, it is necessary to consider which other cellular types could be involved in disease initiation. It is known that synovial macrophages are activated in the arthritic joints of experimental animal models, and that these cells numbers correlate with disease severity (37). It has also been reported that the transfer of CIA into SCID mice can be blocked by treatment with macrophage-specific anti-CD11b (Mac-1) mAbs (38), and that a clodronate-mediated depletion of phagocytic synovial macrophages ameliorates localized inflammation in CIA (39). In addition, synovial fibroblasts from arthritic patients possess autologous invasive behavior (40). Thus, resident synovial cells are clearly involved in established disease and may also have the potential to act as the pathogenic trigger upon activation. There is much evidence to suggest that the cytokines TNFα and IL-1 play diverse but important roles in arthritis (41). In agreement with its assumed importance, anti-TNFα treatment is one of the most effective therapies developed both for CIA and rheumatoid arthritis alleviation (42, 43), suggesting that pathogenic mechanisms are governed by this cytokine. The activated synovial fibroblast, which is highly responsive to TNFα and shows the properties of a transformed (45) and aggressive cell (40), occupies an a central role in attempts to set up a mechanistic paradigm of disease pathogenesis in rheumatoid arthritis (46). Our evidence of synoviocyte proliferation in the absence of synovius occurring after collagen administration in DBA/1-immunodeficient mice indicates an immediate responsiveness of this specific cell type to collagen. Therefore, it is conceivable that sequestered collagen resulting from diverse insults affecting cartilage integrity may contribute directly to the chronic activation of the synoviocyte in susceptible genetic backgrounds. With further investigation, the non-antigenic properties of collagen may offer new mechanistic clues and therapeutic targets to combat rheumatoid arthritis.

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References


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