COLLAGEN-INDUCED ARTHRITIS IN MICE

Localization of an Arthritogenic Determinant to a Fragment of the Type II Collagen Molecule

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Collagen-induced arthritis (CIA)[1] is a chronic inflammatory arthropathy induced by immunizing rodents with type II collagen, the major matrix protein of hyaline cartilage (1, 2). Histopathologically, CIA in rats and mice is characterized by a proliferative synovitis that erodes the adjacent cartilage, ultimately producing articular injury and ankylosis (3, 4). In some rats, the auricles of the ear are also affected by an inflammatory chondritis (5, 6). Both articular and auricular lesions have been closely associated with an intense immune response to type II collagen that is controlled by genes within or closely linked to the major histocompatibility loci (7–9). Because CIA shares clinical, histological, immunological, and genetic features with human rheumatoid arthritis and relapsing polychondritis, it may serve as an important model of these human diseases.

The immunologic events resulting in arthritis have not been fully elucidated. Although the role of cell-mediated immunity in the pathogenesis of CIA is debated (10, 11), most investigators agree that antibodies to type II collagen are critically involved. We have found that CIA can be transferred from arthritic rats and mice to unimmunized recipients with concentrates of sera and affinity-purified antibody specific for type II collagen (12). Antibody can be identified on the cartilage surface in arthritic joints, and apparently localizes to the joint by binding to autologous type II collagen (13, 14). While these findings demonstrate the importance of antibody in initiating CIA, they do not explain why some inbred strains of rats and mice develop high titers of anticollagen antibodies but are resistant to arthritis (7, 15, 16).

The response to type II collagen may involve several different epitopes on the molecule, and susceptibility to arthritis may be dependent upon reaction with a limited number of these epitopes. To identify regions of the molecule containing arthritogenic epitopes, we cleaved chick type II collagen (CII) with cyanogen

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[1] *Abbreviations used in this paper:* CII, chick type II collagen; CB, cyanogen bromide; CIA, collagen-induced arthritis; CB-peptides, peptides derived from type II collagen by CB cleavage; ELISA, enzyme-linked immunoassay; IFA, incomplete Freund's adjuvant; MII, mouse type II collagen; PBS-Tween, phosphate buffered saline containing 0.05% Tween 20.

bromide (CB), isolated the resulting fragments, and renatured them. We tested sera from arthritis-susceptible DBA/1 mice immunized with CII for reaction with each fragment.

Materials and Methods

Animals. Male DBA/1J mice were obtained from the Jackson Laboratories (Bar Harbor, ME), maintained in groups of six in polycarbonate cages, and fed standard rodent chow (Ralston Purina Co., St. Louis, MO) and water ad libitum.

Preparation of Type II Collagen and Isolation and Renaturation of CB-produced Peptide Fragments (CB-peptides). Native type II collagen was solubilized from the sternae of adult chickens by limited pepsin digestion, as described earlier (17). CB-peptides were prepared and isolated as described by Miller (18), and renatured to their original triple-helical conformation by stepwise cooling. The renaturation procedure, and studies describing the physicochemical and immunological properties of the CB-peptides are reported in detail elsewhere.[2]

Native mouse type II collagen (MII) was prepared from the auricular cartilage of mice. Ears were excised, the overlying skin was removed by blunt dissection, and the cartilage was pulverized using a liquid nitrogen freezer mill. The cartilage powder was extracted twice for 48 h in 0.5 M acetic acid at 4°C to remove contaminating type I collagen. The cartilage residue was washed in distilled water, stirred in 4 M guanidine-HCl for 24 h to remove contaminating proteoglycans, washed twice in 0.5 M HCl, and digested with pepsin (19). Pepsin-solubilized MII collagen was then purified in the same manner as CII. The purity of CII and MII was determined by electrophoresis using 6% polyacrylamide gels.

Immunization of Mice. All mice were 8–10 wk old when immunized. Native type II collagen was dissolved in 0.1 N acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Heat-killed *Mycobacterium tuberculosis* (Fisheries and Food Central Veterinary Lab., Weybridge Surrey, United Kingdom) was ground with a mortar and pestle and added to incomplete Freund's adjuvant (IFA). Equal volumes of adjuvant and collagen solution were emulsified for 2 min at high speed with a homogenizer. Each mouse received an intradermal injection of 0.1 ml of the resulting emulsion in the tail. Other groups of mice were immunized in the same manner with 100 μg of denatured CII, or CB-peptides 8, 9, 11. A booster injection of 100 μg of antigen emulsified in IFA was given by intradermal injection in the dorsal skin on day 28. A control group received an equal volume of 0.1 N acetic acid emulsified with IFA and containing 250 μg of *Mycobacterium tuberculosis* as a primary injection, and acetic acid in IFA alone as a booster on day 28.

Measurement of the Severity of Arthritis. The severity of arthritis was determined for each mouse by separately scoring the fore- and hindpaws on a scale of 0–3, as described elsewhere (7). A maximum arthritic index was derived by combining the score for all four paws recorded for each mouse, regardless of when the measurement was made. It was used to compare the severity of arthritis achieved in mice immunized with each antigen.

Measurement of Serum Antibody Titers. Serum antibody titers to type II collagen and CB-peptides were determined by enzyme-linked immunoassay (ELISA), as described previously (19). Native collagen or CB-peptides were dissolved in 0.4 ionic strength phosphate buffer, pH 7.6, and diluted to a final concentration of 5 μg/ml. ELISA plates were coated with collagen by adding 100-μl aliquots to each well and incubating overnight. When native collagen or renatured peptides were studied, the temperature of the assay was maintained at 4°C to prevent denaturation. On the other hand, for the determination of antibody to denatured peptides, all manipulations were done at room temperature to prevent renaturation.

Sera for analysis were diluted with 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.4, containing

[2] Terato, K., M. A. Cremer, K. A. Hasty, A. H. Kang, D. L. Hasty, and A. S. Townes. Physiological and immunologic studies of the renatured α1 (II) chains and isolated CB peptides of type II collagen. (Manuscript submitted for publication).

0.5% Tween 20 (PBS-Tween). Diluted antisera were added to each well of collagen-coated microtiter plates (Nunc, Roskilde, Denmark) in 100-μl aliquots. After incubation overnight, the wells were washed, and 100 μl of peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) were added. Incubation was continued for 4 h, and the plates were again washed. Plates were developed using 40 mg of orthophenylenediamine dissolved in 100 ml of citrate-phosphate buffer, pH 5.5, to which 40 μl of 30% H_2O_2 was added immediately before use. After 1 h, the absorbance at 490 nm was measured using an automated reader (model 580; Dynatech, Alexandria, VA)

For absorption experiments, chick CII was dissolved in 0.4 ionic strength phosphate buffer, pH 7.6. After heating at 56°C for 15 min, the collagen was coupled to CB-activated Sepharose, as previously described (12). Absorption was performed by adding 1 μl of undiluted antiserum to 1.0 ml of a 10% suspension of collagen-Sepharose in PBS-Tween. After incubation at 4° overnight, unbound antibody was eluted and the column was washed with an additional milliliter of PBS-Tween. The total recovered volume was measured and considered to be the dilution of absorbed antibody. The absorbed antibody was then used diectly in the ELISA assay.

Results

To ascertain whether antibodies reactive with individual CB-peptides could be detected, microtiter plates were coated with either renatured or denatured CB-peptides, and used in an ELISA assay with sera obtained at 8 wk after immunization. To ensure that conformation-dependent epitopes were detected, sera were absorbed with denatured CII covalently bound to Sepharose before analysis using renatured peptides. As shown in Fig. 1, reaction with renatured peptides was in the order CB-11 > 10 > 8 > 7 > 9. No reactivity was detected to peptides CB-6 and CB-12. A similar pattern of recognition was seen when the unabsorbed sera were reacted with denatured peptides (CB-11 > 10 > 8 > 9 > 7 > 12), except that reactivity with CB-9 was greater than CB-7, and reaction with CB-

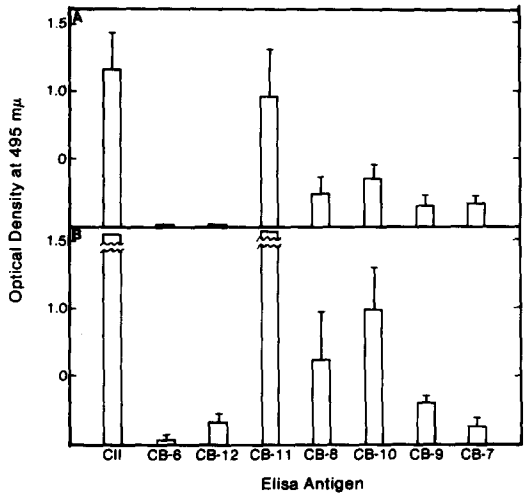

FIGURE 1. Relative levels of antibody to each CB-peptide of type II collagen. Sera were obtained from mice 8 wk after immunization with native type II collagen, and tested by ELISA. Plates were individually coated with native collagen and each renatured peptide (*a*), or denatured collagen and each unrenatured peptide (*b*). Aliquots of a 1/1,000 dilution of serum were tested in triplicate. The vertical bar and crosshatch represent SD of the values for eight mice.

12 was also detected. These results indicated that most of the conformation-dependent and -independent determinants were located on CB-11, 10, and 8.

The kinetics of the antibody response in mice immunized with native CII was also studied (Fig. 2). Immunized mice produced a rapid and strong IgG response

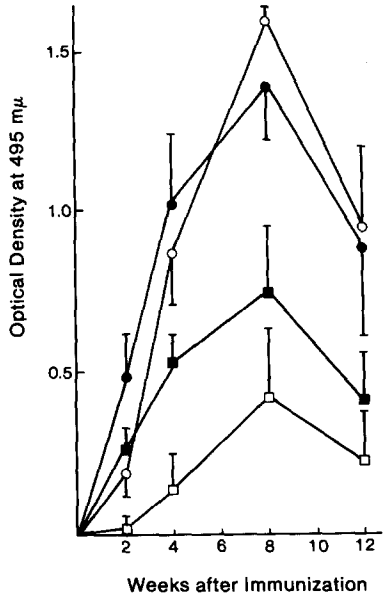

FIGURE 2. Kinetics of the antibody response to CII and MII after immunization with native CII. ELISA was performed using plates coated with native CII (●), denatured CII (○), native MII (■), and denatured MII (□). Sera were diluted 1/4,000 for assay against CII, and 1/1,000 for assay against MI. Mean and SD for eight mice are shown.

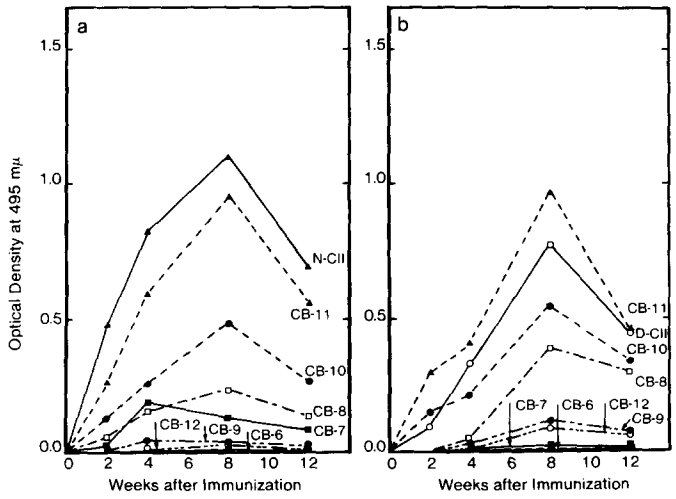

FIGURE 3. Kinetics of the antibody response to each CB-peptide after immunization with native CII. ELISA was performed using plates coated with native collagen and renatured peptides at 4°C (*a*), or denatured collagen and unrenatured peptides at 25°C (*b*). Mean values for eight sera diluted 1/1,000 are shown.

easily detected 2 wk after immunization. The incidence of arthritis was 100%, with onset at 4–5 wk, as we have found in previous experiments. Antibody reactive with MII rose in parallel with that to CII, and by 4 wk there was a significant amount present in the sera, although peak levels were not achieved until 8 wk after immunization.

Shown in Fig. 3 are the kinetics of the response to each of the CB-peptides. These data confirm the important contribution of peptides 11, 10, and 8 to the overall response. Antibody levels to CB-11 were higher than levels to other peptides at all time points. However, at 4 wk, when arthritis first became evident, significant levels were also detected to CB-7, 8, and 10.

Because reaction with autologous collagen appears to be important to the development of arthritis, we analzyed sera from individual arthritic mice for reactivity with each peptide and with MII. A significant positive correlation was observed between level of reactivity with CB-11 and MII (Fig. 4). No correlation was found between reaction with other CB peptides and MII. These data suggest that epitopes responsible for crossreactivity are primarily located on CB-11 and therefore CB-11 may contain determinants important for the development of arthritis.

To confirm the importance of CB-11 in the induction of arthritis, we immunized mice with purified CB-11. We modified our usual immunization regimen by increasing the amount of mycobacterium given and by administering a subcutaneous booster of CB-11 in incomplete Freund's adjuvant at day 28. Arthritis developed in five of eight mice 6–8 wk after the primary immunization. A maximum arthritic index of 6.6 ± 2.4 was observed, which is comparable to an index of 6.2 ± 1.2 seen in another group of mice immunized with native CII under our usual regimen. Immunization using the modified regimen but employing CB-8 and CB-10 failed to induce arthritis. Sera obtained 8 wk after primary immunization contained antibodies reactive with the peptide used for

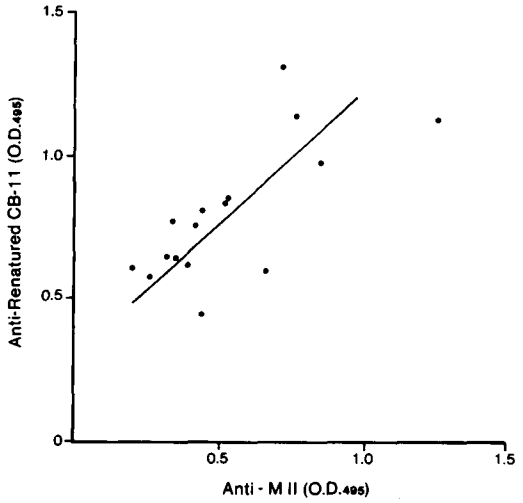

FIGURE 4. Correlation of antibody levels to chick CB-11 and those to MII as measured by ELISA. Sera were obtained 8 wk after immunization. $r = 0.85$, $p < 0.001$.

immunization (Table I). However, the comparative immunogenicity of CB-11 was considerably greater than CB-8 and CB-10. In addition, the antibody generated by immunization with CB-11 also reacted with native CII and MII. In fact, antibody from arthritic mice immunized with CB-11 showed significantly greater reactivity with native MII than either nonarthritic mice immunized with CB-11, or mice immunized with CB-8 or CB-10.

The ability of CB-11 to induce arthritis was somewhat unexpected, since the native conformation of the peptide in solution is unstable at body temperature. Therefore, we decided to test the ability of denatured α chains from type II collagen to induce arthritis under the same conditions used for CB-11. A similar arthritis was induced in 8 out of 14 mice immunized with denatured CII. The onset was observed at the same time as seen after immunization with CB-11, and occurred 8–12 wk after primary immunization. For both CB-11 and denatured CII, the onset of arthritis was delayed as compared to using native CII as the immunogen, the disease was generally milder, and the range of severity was greater. In mice immunized with denatured CII, the maximal arthritic index was 3.9 ± 2.2, compared to 6.2 ± 1.2 in mice receiving native CII.

Kinetic studies of the antibody response to denatured CII shows a prompt response develops to the immunizing antigen (Figure 5). In addition, antibodies crossreactive with native MII were detected. The reactivity with native MII was, however, delayed as compared to that induced by immunization with native CII. It required 8 wk for levels to reach those seen 4 wk after immunization with native CII.

Discussion

Type II collagen is unique among the collagens in its ability to induce an inflammatory arthritis in mice. This arthritis is invariably associated with high levels of antibodies against native type II collagen in the sera of affected animals. In addition, both susceptibility to arthritis and the immune response to type II

TABLE I

Antibody Responses to Heterologous and Autologous Type II Collagens in Mice Immunized with CB-peptides

Immunizing CB-peptide	Arthritis incidence	Antibody level when tested against:*					
		N-CII	D-CII	N-MII	CB-8	CB-10	CB-11
CB-8	0/8	0.06 ± 0.12	0.88 ± 0.50	0.03 ± 0.05	0.84 ± 0.55	0.28 ± 0.22	0.07 ± 0.10
CB-10	0/6	0	0.46 ± 0.54	0	0.03 ± 0.09	0.40 ± 0.56	0.01 ± 0.02
CB-11	5/8	Nonarthritic 0.34 ± 0.15	>1.50	0.30 ± 0.13	0.11 ± 0.13	0.32 ± 0.23	>1.50
		Arthritic 1.22 ± 0.19	>1.50	0.64 ± 0.13	0.11 ± 0.03	0.37 ± 0.13	>1.50

CB-peptides were prepared by digesting chick type II collagen with CB and purifying the resulting peptides.

* Sera were obtained 8 wk after primary immunization, diluted 1:1,000 and tested by ELISA for reactivity with each of the collagens and fragments listed. Results are expressed in optical density units and are the mean ± SD. N-CII, native chick type II collagen; D-CII, denatured chick type II collagen; N-MII, native mouse type II collagen.

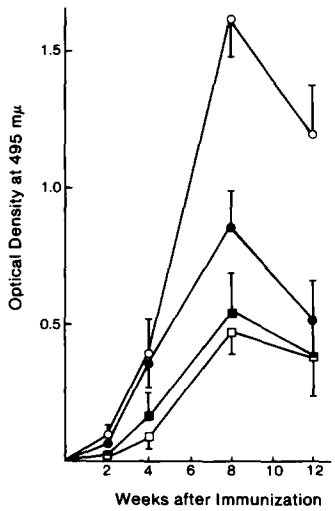

FIGURE 5. Kinetics of the antibody response after immunization with denatured CII as measured by ELISA. Plates were coated with native CII (●), denatured CII (○), native MII (■), or denatured MII (□).

collagen are linked to the H-2 major histocompatibility locus. Not all high-responder mice are susceptible to arthritis, however. This discrepancy may in part be related to differences in the fine specificity of the immune response to type II collagen. We have previously (13) shown that mice from resistant strains will develop arthritis after passive transfer of a serum concentrate from immunized mice of the susceptible DBA/1 strain.

As a first step in identifying critical epitopes on type II collagen responsible for inducing arthritis in DBA/1 mice, we have cleaved native chick type II collagen with CB, and isolated the resulting fragments. We obtained seven fragments in a pure form, and were able to renature all of them.[2] Sera from arthritic mice preferentially recognized CB-11, although substantial reactivity with other peptides was also detected. Renatured peptides were used because it has previously been found that, whereas native type II collagen will consistently induce arthritis in susceptible animals, denatured type II collagen is not effective. To assure that only antibodies reactive with conformation-dependent determinants were detected, the sera were preabsorbed with denatured α chains.

During the course of these experiments, we found that denatured type II collagen is only relatively ineffective in inducing arthritis. Under slightly modified conditions, it will induce arthritis in at least a portion of the immunized mice. Furthermore, sera from DBA/1 mice immunized with denatured collagen had substantial reactivity with the intact native molecule. A similar phenomenon has been noted (21) in other systems, where immunization with protein fragments results in the production of antibodies reactive with the intact protein. It is also possible that denatured chains regained a partial helical conformation in the water-and-oil emulsion. Regardless, the findings are consistent with the hypothesis that antibody generated against type II collagen localizes to the joint by combining with intact autologous collagen. After immunization with native chick collagen, a rapid increase in antibody crossreactive with mouse collagen is seen.

After immunization with denatured collagen, antibody crossreactive with mouse collagen increases slowly, and the onset of arthritis is delayed. In both cases, arthritis appears only after substantial levels of autoantibodies are present. This data supports the hypothesis that a critical level of antibody to native type II collagen is necessary for the initiation of CIA. As additional evidence, the three mice who received denatured type II collagen, but failed to develop arthritis, had the lowest antibody levels to native mouse type II collagen.

This and previous investigations implicate antibodies to native collagen in the induction of arthritis. To analyze the heterogeneity of such conformation-dependent antibodies, we tested sera for reaction with either renatured CB-peptides that exhibit a native helical conformation, or with denatured CB-peptides. When reactivity was compared using animals immunized with native and denatured collagen, only renatured or denatured CB-11 consistently gave high values, as compared to the other peptides. These data were confirmed by using renatured CB-11 to inhibit binding to native CII or MII of sera from mice immunized with either native or denatured CII. In each case, >50% inhibition was seen (data not shown). The high levels of inhibition, by renatured CB-11, of antibody against MII suggested that many of the crossspecies determinants were present on this peptide.

Our most important finding is that CB-11 is not only the most immunogenic portion of the molecule, but also is capable of inducing arthritis. We have thus localized at least one arthritogenic epitope on type II collagen. The other fragments to which substantial reactivity was detected were also used for immunization, but were incapable of inducing arthritis, suggesting that arthritogenic determinants may be restricted to CB-11. These studies were, however, limited to chick type II collagen and DBA/1 mice. Use of type II collagen from other species, and with mice of different H-2 haplotypes and with different background genes are in progress. The use of renatured fragments of type II collagen to localize antigenic determinants is a powerful technique that should make it possible not only to identify determinants important for the induction of CIA, but also to test sera from patients for reaction with specific fragments to identify potentially arthritogenic regions.

Summary

Purified chick type II collagen was cleaved with cyanogen bromide (CB), and the resulting peptides isolated and renatured. Sera from arthritic DBA/1 mice, immunized with chick type II collagen, were tested for reactivity with each peptide. The sera preferentially recognized peptides 11, 10, and 8, in that order. Some reactivity was also detected to peptides 9, 7, and 12. Because arthritis depends upon binding of antibody to autologous type II collagen in the joint, sera were also tested for reactivity with mouse type II collagen. There was a strong positive correlation between reactivity with peptide 11 and reactivity with mouse collagen, but no correlation was found with any of the other peptides. Peptides 11, 10, and 8 were also used for immunization. Antibodies were detected in response to each of these peptides, but arthritis developed only in mice immunized with peptide 11. We conclude that a major immunogenic and

arthritogenic epitope on type II collagen resides in the region of the molecule represented by CB peptide 11.

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