

AUTOIMMUNITY TO TYPE II COLLAGEN: AN EXPERIMENTAL MODEL OF ARTHRITIS*

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Immunologic hypersensitivity to collagen, the major structural component of connective tissue, could explain both the systemic nature and chronicity of the inflammation occurring in rheumatoid arthritis. Recent demonstrations of antibodies to collagen in sera from patients with rheumatoid arthritis support this premise (1-8). Also consistent with this hypothesis is the distribution of collagen in structurally distinct types in various tissues. For example, types I and III collagens are found in the skin and parenchyma of several organs, whereas type II exists in cartilage (9). Thus, an immune response to the cartilage type of collagen (type II) could explain the predilection of rheumatoid arthritis to involve diarthrodial joints.

Native collagens consist of three polypeptide chains linked in triple helices. Terminal peptides (telopeptides) do not have a helical structure and are more variable in amino acid content (10). Type I collagen combines two $\alpha 1$ -type I chains with one $\alpha 2$ -chain and is depicted as $[\alpha 1(I)]_2\alpha 2$. Types II and III collagens are comprised of three $\alpha 1$ -type II chains and three $\alpha 1$ -type III chains, respectively. Thus, type II is depicted as $[\alpha 1(II)]_3$ and type III as $[\alpha 1(III)]_3$.

Injection of heterologous type I and II collagen in complete Freund's adjuvant has been reported to elicit type-specific antibody responses in rats (11) and mice (12). This paper reports that approximately 40% of rats injected intradermally with native type II collagen, derived from human, chick, or rat cartilage, in either complete or incomplete Freund's adjuvant, develop an inflammatory arthritis. In contrast, type I and III collagen are not arthritogenic. This new animal model suggests that immune responses to type II collagen could play a role in inciting or perpetuating joint inflammation in other arthritides.

Materials and Methods

Rats. Outbred female Wistar, Sprague-Dawley and inbred Wistar-Lewis rats were obtained from Microbiological Associates (Bethesda, Md.), Harlan (Indianapolis, Ind.) or Charles River Breeding Laboratory (Wilmington, Mass.). These rats were housed in metal cages and given water

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and standard rat chow ad lib. They weighed 150-225 g and were 10-24 wk of age at the time of immunization.

Tissues. The xiphoid cartilage of 3 wk old White Leghorn chicks, rendered lathyrictic by administration of β -aminopropionitrile fumarate (BAPN,¹ Sigma Chemical Co., St. Louis, Mo.) (13), was used as the source of chick type II collagen. Similarly, weanling outbred female Wistar rats were given BAPN (0.4%) in water for 3 wk, and their xiphoid and hip cartilage was used after careful dissection to avoid contamination by bone. Human collagens were prepared from neonatal skin and cartilage obtained at autopsy. Skin and cartilage were pulverized in liquid nitrogen with a freezer mill (Spex Industries, Inc., Metuchen, N.J.). All subsequent procedures were performed at 4°C.

Skin Collagens. Type I collagen was extracted from skin powder by overnight suspension with gentle shaking in 5 vol of 0.05 M Tris/1 M NaCl, pH 7.4. After centrifugation (20 min at 20,000 g), collagen in the supernate was precipitated by gradual addition of NaCl to a final concentration of 3 M. The precipitate was redissolved in 0.05 M Tris/1 M NaCl and again precipitated in an identical manner. After redissolving in the neutral salt buffer, the collagen was precipitated by the addition of 0.1 vol of 1 M acetic acid. The precipitate was collected and dissolved with 0.5 M acetic acid, reprecipitated with NaCl to a final concentration of 1 M, redissolved with 0.5 M acetic acid, dialyzed exhaustively against 0.1 M acetic acid, and lyophilized.

Additional type I collagen was obtained from the skin pellet after neutral salt extraction by suspending the pellet in 5 vol of 0.5 M acetic acid for 24 h. After an identical subsequent extraction, collagen in both supernates was precipitated by the addition of NaCl to a final concentration of 1 M, redissolved in 0.5 M acetic acid, precipitated again with NaCl, dissolved in acetic acid, dialyzed, and lyophilized. Pepsin-solubilized type I collagen and type III collagen were prepared from the skin residues by methods previously described (14).

Cartilage Collagens. Pepsin-solubilized type II collagen was prepared by washing the cartilage powder with 20 vol of 0.05 M Tris/2 M MgCl₂, pH 7.4, at 4°C. The extract was stored at -20°C for subsequent use as a crude proteoglycan complex. The residue was washed twice with distilled water, then suspended in 0.5 M acetic acid, and the pH of the suspension was adjusted to 2.5 by the addition of formic acid. Pepsin (two times crystallized, Worthington Biochemical Corp., Freehold, N.J.) was then added (1/50 g wet weight), and digestion was allowed to proceed for 72 h at 4°C with gentle shaking. The undigested residue was separated by centrifugation (20,000 g for 20 min) and extracted two more times with pepsin under identical conditions. The supernates from the three extractions were dialyzed against 0.05 M Tris/0.2 M NaCl, pH 7.6, diluted 10-fold with the buffer and passed through a column of fresh DEAE cellulose (DE 52, Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England) that had been equilibrated with the same buffer. Collagen was eluted with the buffer and precipitated by the addition of NaCl to make a final concentration of 3 M. The precipitate was dissolved in 0.5 M acetic acid, dialyzed against 0.01 M Na₂HPO₄, and the collagen precipitate was harvested by centrifugation. After resolubilization in acetic acid and precipitation with 1 M NaCl, it was redissolved in acetic acid, dialyzed against 0.1 M acetic acid, and lyophilized.

Soluble type II tropocollagen was obtained from the cartilage of lathyrictic chicks or rats. After pulverization, the powder was extracted with a 0.4 ionic strength phosphate buffer, pH 7.6, for 24 h. After an additional extraction, the two supernates were dialyzed against 0.05 M Tris/0.2 M NaCl, pH 7.6, passed through DEAE, and purified in a manner described above for pepsin-extracted collagen.

α -Chains. The constituent α -chains of human or chick type II collagen were prepared by ion exchange chromatography on columns of carboxymethyl-cellulose (CM-cellulose, CM-52, Whatman, Inc., Clifton, N.J.) as previously described (14).

Collagen Analysis. The purity of each collagen preparation was assessed by CM-cellulose chromatography and amino acid analysis by using an automatic analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif., model 121) as previously described (14).

Uronic acid assay (15) indicated that proteoglycan contamination of the collagen samples (1 mg/ml 0.1 M acetic acid) was less than the 5 μ g/ml minimum detectable by the assay.

Adjuvants. The complete Freund's adjuvant (CFA) used for all collagen immunizations

¹ Abbreviations used in this paper: BAPN, β -aminopropionitrile fumarate; CFA, complete Freund's adjuvant; CM-cellulose, carboxymethylcellulose; ICFA, incomplete Freund's adjuvant.

(Difco, code 0638, Difco Laboratories, Detroit, Mich.) contained *Mycobacterium butyricum* at a concentration of 0.5 mg/ml. This preparation failed to induce adjuvant arthritis (16) in 30 Wistar rats when 0.5 ml was emulsified with 0.5 ml 0.1 M acetic acid and injected intradermally on the back.

As a control for the collagen-induced arthritis, adjuvant arthritis was induced by intradermal injection of 0.1 ml complete Freund's adjuvant H37Ra (CFA H37). This preparation contained desiccated, heat-killed *M. tuberculosis* H37 Ra (Difco Laboratories, code 3114) at a concentration of 10 mg/ml in incomplete Freund's adjuvant (ICFA, Difco Laboratories, code 0639).

Sensitization Procedures. Collagen was dissolved in 0.1 M acetic acid at a concentration of 1 mg/ml. Equal volumes of collagen solution and CFA or ICFA were mixed, and a stable emulsion was made with an emulsifier (VirTis 45, VirTis Co., Inc., Gardiner, N.Y.). 1 ml of the cold emulsion was then immediately injected intradermally in four to six sites on the backs of the rats (11). Small ulcers frequently formed at the injection site, but these healed without sequelae in 7-10 days. Control injections consisted of the acetic acid emulsified in CFA or ICFA or human or chick type II collagen dissolved in acetic acid but injected intradermally without adjuvant. As an additional control, 1.0 ml of MgCl₂-extractable cartilage proteoglycans containing approximately 100 µg uronate per ml was mixed with 0.5 ml of CFA or ICFA, emulsified, and injected in a manner identical to the collagens. Unless otherwise specified, booster doses consisting of 0.5 mg collagen dissolved in 0.5 ml 0.1 M acetic acid were given intraperitoneally (i.p.) without adjuvant 21 days after primary immunization. 1 ml of the MgCl₂ extract was given i.p. after an identical interval to the proteoglycan control animals. Adjuvant arthritis was induced by intradermal injection of 0.1 ml CFA H37 at the base of the tail.

Arthritis Evaluation. Animals were observed daily for the onset of arthritis, and an arthritic index was derived by grading the severity of involvement of each paw from 0 to 4. Scoring was based on the degree of periarticular erythema and edema as well as deformity of the joints, as previously described (16). Swelling of hindpaws was also quantitated by measuring the thickness of the ankle from medial to lateral malleolus with a constant tension caliper (B.C. Ames Co., Waltham, Mass.). Results could be reproducibly expressed to the nearest 0.1 mm.

Histopathology. Animals were sacrificed, and involved paws were amputated on the day of onset of arthritis or at later periods ranging up to 6 mo after onset. After immersion in 10% neutral formalin, the joints were decalcified, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Joint Roentgenography. Sequential joint roentgenograms were made on dental X-ray film (Kodak DF45, Eastman Kodak Co., Rochester, N.Y.)

Bacteriology. Blood was obtained aseptically by cardiac puncture from eight rats within 24 h of the onset of arthritis and inoculated onto sheep blood agar plates. Inflamed synovia obtained from five rats at the onset of arthritis were similarly inoculated onto identical nutrient media and observed for 72 h. Finally, paired sera and synovia from an additional four rats were collected on the day of onset of arthritis and cultured for *Mycoplasma arthritidis* by Microbiological Associates (Bethesda, Md.).

Results

Incidence and Specificity of Collagen-Induced Arthritis. A chronic inflammatory arthritis developed in 144 of 348 rats (41%) injected intradermally on the back with type II collagen derived from human, chick, or rat cartilage and either CFA or ICFA. These data are summarized in Table I. The arthritis could be induced with approximately equal frequency in outbred female Wistar, Sprague-Dawley or inbred Wistar-Lewis strains whether CFA or ICFA was employed. In contrast, type I collagen derived from human, chick, or rat skin or type III collagen derived from human skin were not arthritogenic in a total of 181 rats. In the other control groups, type II collagen without adjuvant, MgCl₂-extractable cartilage proteoglycans, denatured α1(II) chains, and the type and dose of CFA or ICFA injected without collagen also did not cause arthritis in a total of 140 rats. Table II summarizes these data in 321 control rats.

TABLE I
Preparations which Induced Arthritis

Preparation injected	Rat strain	Adjuvant		Total
		CFA*	ICFA*	
				%
Human type II‡	Wistar	43/78	14/47	46
Chick type II	Wistar	4/10	15/33	44
Chick type II	Wistar-Lewis		24/60	40
Rat type II‡	Wistar	6/20	17/50	33
Chick type II	Sprague-Dawley		5/10	50
CFA H37	Wistar			40§

* Number of rats developing arthritis/number of rats injected with the preparation in the specified adjuvant. There was no significant difference in the incidence of arthritis induced by heterologous or homologous type II collagens by Chi square analysis.

‡ Pepsin-modified.

§ Incidence (16/40) of adjuvant-induced arthritis, produced by intradermal injection of 0.1 ml adjuvant oil containing *M. tuberculosis* H37Ra (10 mg/ml).

TABLE II
Preparations which Did Not Cause Arthritis

Preparation injected	No. of rats injected		Total
	Adjuvant		
	CFA	ICFA	
Human type I*	45	10	55
Human type I	10	10	20
Chick type I	20	25	45
Rat type I	10	10	20
Human type III*	31	10	41
Human $\alpha 1$ (II) chains*	20	10	30
Chick $\alpha 1$ (II) chains	10	10	20
Human cartilage proteoglycans	10	10	20
Human type II* without adjuvant			20
Chick type II without adjuvant			10
Acetic acid‡	30	10	40

* Pepsin-modified.

‡ 0.5 ml 0.1 M acetic acid.

Onset and Course of Arthritis. The clinical features of the collagen-induced arthritis were similar whether heterologous or homologous type II collagen was the immunogen or whether CFA or ICFA was used. The onset of arthritis was explosive occurring 14–60 days postimmunization with a peak onset at 20 days and a median onset at 21 days for a group of 61 rats examined daily. Typically, a hindlimb became severely red and edematous within a 16–24-h period such that the arthritic index was 5.0 ± 0.4 (mean \pm SEM) for a group of 28 rats evaluated on the day of onset of arthritis. 10 had bilateral hindlimb involvement (Fig. 1), whereas in 18 the disease remained unilateral.

The hindlimb swelling could also be easily quantified by measuring the

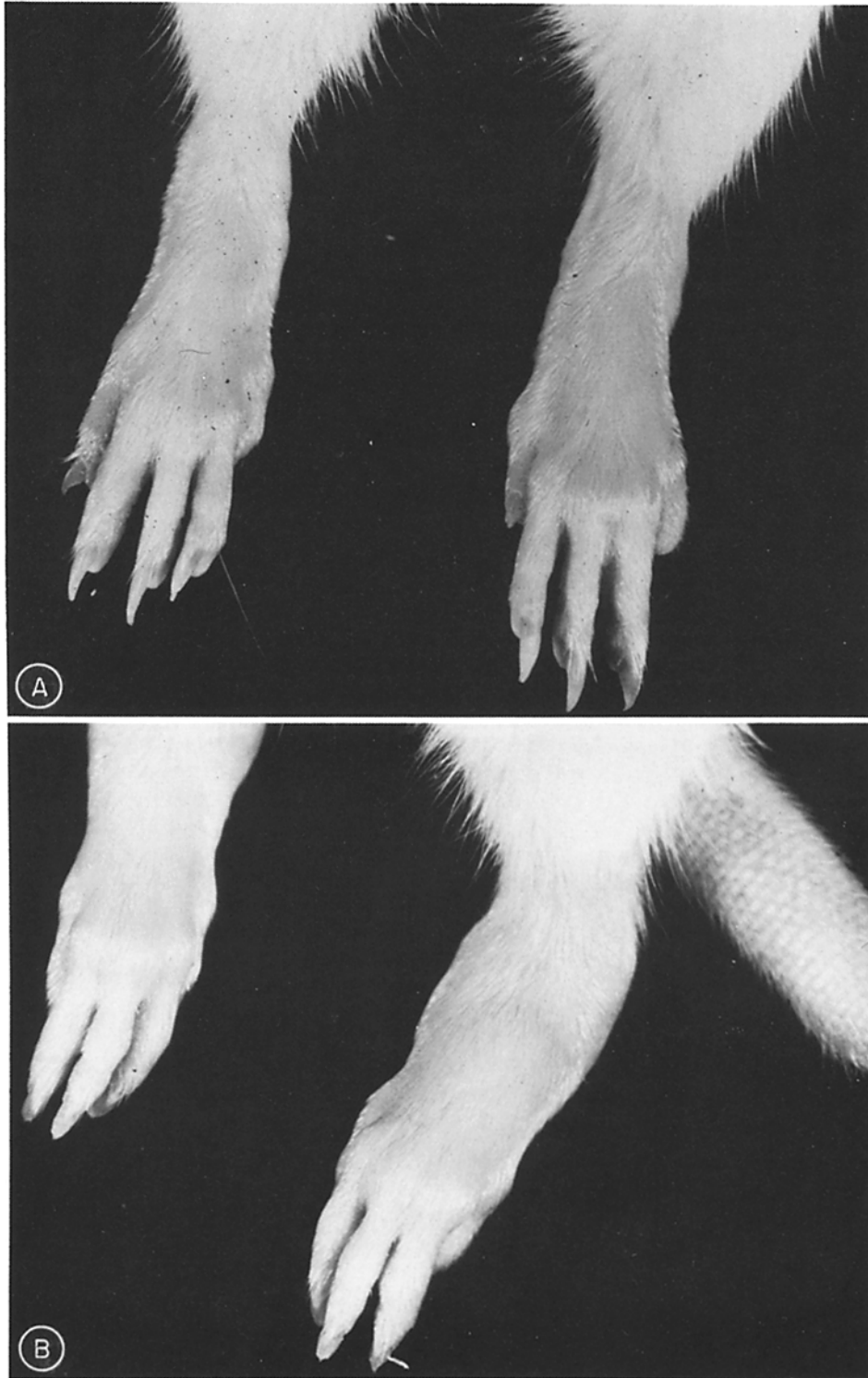


FIG. 1. Comparison of normal rat hindlimbs (A) with those (B) rendered arthritic by intradermal injection of human type II collagen in ICFA. Both arthritic paws were graded as 4 by the arthritic index and had an intermalleolar thickness of greater than 10 mm by constant tension caliper measurement. Ankle thickness of age-matched normal rats was consistently less than 7 mm.

thickness of the ankle between the malleolae with a constant tension caliper. The mean ankle thickness was 10.2 ± 0.3 mm for 10 arthritic joints evaluated on the day of onset of arthritis induced in Wistar-Lewis rats by chick type II collagen in ICFA compared to 6.4 ± 0.1 for 20 ankles from age-matched, uninjected female Wistar-Lewis rats.

No additional limbs became affected after the initial 48 h of disease. Forepaw inflammation occurred in 10% of the entire arthritic population. Involvement was predominately distal with ankle, tarsal, and interphalangeal inflammation. Peak severity occurred within 4 days after the onset with each involved limb usually reaching a maximum score by the arthritic index. Weight bearing on the affected limb was poorly tolerated by the rats, and swelling usually persisted 5–8 wk, gradually culminating in a deformed joint. Nine rats had an identical clinical course but a delayed onset 30–60 days after primary immunization. All had received i.p. booster injections at 21 days.

Spontaneous exacerbations of disease did not occur; however, i.p. booster doses, identical to those given at 21 days, as long as 6 mo after original immunization caused a recrudescence within 3 days in all 11 previously arthritic rats when rechallenged. None of 20 rats which initially remained nonarthritic after injection of type II collagen became arthritic when rechallenged after similar time intervals.

Mild hair and weight loss but no ocular inflammation, hematuria, or mucosal lesions occurred in the arthritic rats. No rat died during active joint inflammation.

Comparison with Adjuvant Arthritis. The clinical features of the type II collagen-induced arthritis were similar to those of adjuvant-induced disease. A single injection of human type II collagen in CFA or ICFA in another group of 62 Wistar rats caused arthritis in 33 (53%), whereas adjuvant arthritis developed in 16 of 40 Wistar rats (40%) injected with CFA H37. Likewise, the onset of clinical disease was similar (mean day of onset 17.0 ± 0.6 for 28 collagen-induced disease rats vs. 15.6 ± 0.4 for 16 adjuvant arthritis rats) as were their mean arthritic indices studied within 48 h of onset (mean 5.0 ± 0.4 for 28 collagen arthritis rats vs. 3.9 ± 0.4 for 16 adjuvant arthritis rats).

Pathology. Groups of six Wistar-Lewis rats were sacrificed before the onset of arthritis on each of days 11–14 after injection of chick type II collagen in ICFA, and their spleen and inguinal and cervical lymph nodes were examined grossly. Marked progressive hypertrophy of these tissues occurred during this period, which immediately preceded the usual time of onset of arthritis. Histopathologic sections of diarthrodial joints showed that injection of type II collagen with ICFA produced a chronic proliferative synovitis which secondarily destroyed articular cartilage and bone. Synovium obtained within 24 h of the onset of arthritis showed marked edema and infiltration by dense aggregates of mononuclear cells and occasional neutrophils. There was no vessel wall necrosis. Serial sections of joints examined at later stages of inflammation showed proliferation of synovio-cytes and fibroblasts resulting in synovial hypertrophy and fibrosis. This synovial pannus, in turn, eroded the cartilage and subchondral bone (Fig. 2). Periosteal new bone formation was prominent and uniformly culminated in joint ankylosis. Numerous mononuclear cells persisted in the synovium for longer than 6 mo after the onset of the disease. The axial skeleton was not involved,



FIG. 2. Section of ankle joint taken approximately 2 wk after onset of type II collagen-induced arthritis. Synovial proliferation and intense infiltration by mononuclear cells is producing cartilage and bone erosion. Hematoxylin and eosin $\times 200$.

and sections of the trachea showed no evidence of generalized cartilage inflammation. Likewise, histologic examinations of the skin, lung, and kidney were normal.

Roentgenography. Roentgenographs mirrored the progression from soft tissue swelling, articular bone erosions, and prominent periosteal new bone formation, to bony ankylosis in the carpal, tarsal, metacarpal, metatarsal, and interphalangeal regions (Fig. 3).



FIG. 3. Comparison of roentgenograph of normal rat hindpaw (A) with that (B) from a rat whose arthritis was induced by chick type II collagen in ICFA. Soft-tissue swelling, bone destruction and periosteal new bone formation are prominent 2 wk after the onset of inflammation.

Additional Attempts to Induce Arthritis. Primary and booster injection regimens employing 2.0 mg per rat of chick type I collagen, human type III collagen, or chick $\alpha 1(\text{II})$ chains in CFA failed to induce arthritis in a total of 50 additional rats. Also human type I tropocollagen which had been passed through DEAE in a manner identical to the preparative technique for type II collagen did not cause arthritis in 20 rats. No significant differences in the incidence of arthritis were found with doses of type II collagen ranging from 0.5 to 2.0 mg per rat.

Cultures for Microorganisms. No bacterial or mycoplasmal organisms were detected in blood or synovial cultures taken at the onset of disease.

Discussion

These data demonstrate that heterologous or homologous type II collagen derived from cartilage when injected intradermally on the back with CFA or ICFA induces arthritis in approximately 40% of rats of several strains. The structurally distinct types I and III collagen derived from skin are not arthritogenic. Likewise, the denatured constituent α -chains of type II do not cause disease. Various infections such as *M. arthritidis* (17) or *Salmonella* (18) can cause arthritis in rats. However, an infectious agent or product contaminating our preparations is unlikely to explain our results for several reasons: cultures for microorganisms were negative; the identically prepared collagen failed to induce arthritis when injected in the absence of adjuvant; multiple preparations of type II collagen from human, chick or rat sources induced arthritis; collagens prepared and injected in two geographically separate laboratories caused arthritis at all times of the year; the latent period after injection was longer than that usually observed after inoculation with an infectious agent (17); further, the arthritis had a lengthy course with no spontaneous exacerbations; and finally, the synovial infiltration was mononuclear rather than neutrophilic, even initially.

This type II collagen-induced arthritis is distinct from the previously described adjuvant-induced arthritis of rats (16), since the disease can be produced after injection of type II collagen in oil not containing bacterial preparations. Indeed, the equivalent frequency of arthritis whether CFA or ICFA was employed with the type II collagen is surprising. Possibly, type II collagen per se has an adjuvant capacity, but would still require oil since the protein injected alone was incapable of inducing arthritis. While the precise relationship of the collagen-induced arthritis to adjuvant-induced arthritis is at present uncertain, Steffen and Wick have reported delayed-type skin reactions to rat skin collagen in adjuvant arthritis (19). Moreover, we have found that peripheral blood mononuclear cells from rats with adjuvant arthritis exhibit antigen-induced tritiated thymidine incorporation in vitro to homologous type I and II collagen (20). Hemagglutinating antibodies specific for these native collagens and their α -chains can also be detected in sera from rats with adjuvant arthritis (D. Trentham and J. David, unpublished data). Immunologic hypersensitivity to collagen may thus be a common feature of adjuvant- and collagen-induced arthritis.

The moiety responsible for the arthritogenicity of our cartilage collagen preparations is uncertain, but at present we assume it to be type II collagen. By uronic acid assays and CM-cellulose chromatography, our type II collagen preparations are free from proteoglycan and type I collagen (13) contamination, respectively. A cartilage proteoglycan extract did not cause arthritis. Nonetheless, it is not possible at present to exclude a small amount of proteoglycan linked to collagen being important in the induction of arthritis. The principle responsible for the induction of adjuvant arthritis is of interest in this regard. Initial investigation indicated that arthritogenic activity resided in the wax D fraction which is separated from mycobacteria by ultracentrifugation. Subfractions of wax D containing peptidoglycolipids could also be chromatographically isolated which retained arthritogenic potential (16). Subsequent work suggests that adjuvanticity and arthritogenicity are properties of different glycopeptides contained in these subfractions (21). Finally, it has recently been proposed (22) that the common peptidoglycan backbone of bacterial cell walls explains the ability of extracts from several different bacteria to induce arthritis when emulsified in adjuvant oil. Since these bacterial peptidoglycans may have structural similarities to cartilage proteoglycans, the possible participation of these latter molecules in the induction of our arthritis must be further investigated before it can be firmly concluded that specific determinants on collagen are arthritogenic.

Intact nonhelical telopeptide regions of the type II molecule are not required for arthritogenicity, since pepsin-modified type II collagens were as effective in causing arthritis as the tropocollagen molecules. Although it is difficult to be certain that the helical structure of collagen was maintained during the process of emulsification in oil before injection, the finding that $\alpha 1(\text{II})$ chains did not induce arthritis makes this assumption reasonable. As recently demonstrated for myoglobin, the conformational integrity of immunogenic sites on native proteins can be preserved during emulsification in oil (23). Humoral and cellular immune specificities that further distinguish native type II collagen from its

denatured $\alpha 1(\text{II})$ chains, presented in a subsequent paper,² further support the thesis that sensitization to the helical form of type II collagen was achieved.

Two features of this animal model of arthritis are of particular interest. First, this represents the only experimental model in which disease can be regularly provoked with ICFA injected intradermally with a constituent of homologous tissue, suggesting that an autoimmune process may have been induced with oil not containing bacterial preparations. This is distinct from other models of experimentally-inducible autoimmunity to organ-specific antigens, which usually require injection of CFA with tissue components (24) and demonstrates an unusual property attributable to the type II collagen protein. Second, histopathologic studies show that the primary lesion provoked by type II collagen is a chronic proliferative synovitis. Destruction of articular cartilage and bone appear to be sequelae of synovial inflammation. Mononuclear cells invade and persist in the synovium, suggesting that immune processes may be important in the pathogenesis of disease. These characteristics sufficiently resemble those of rheumatoid arthritis to suggest that this may be an appropriate animal model for the human disease.

In conclusion, type II collagen from either heterologous or homologous sources has been found to be arthritogenic in rats. There is an apparent requirement for type-specific helical determinants in evoking the disease. This conclusion coincides with Hahn et al. (11) concerning the conformationally-dependent antigenic determinants responsible for the humoral specificity of type I and II collagen. By recently achieving passive transfer of this arthritis by spleen and lymph node cells sensitized to type II collagen,³ we have directly implicated immunologic hypersensitivity to collagen in the causation of this autoimmune disease.

Summary

We have found that intradermal injection of native type II collagen extracted from human, chick or rat cartilage induces an inflammatory arthritis in approximately 40% of rats of several strains whether complete Freund's adjuvant or incomplete Freund's adjuvant is used. Type I or III collagen extracted from skin, cartilage proteoglycans and $\alpha 1(\text{II})$ chains were incapable of eliciting arthritis, as was type II collagen injected without adjuvant. The disease is a chronic proliferative synovitis, resembling adjuvant arthritis in rats and rheumatoid arthritis in humans. Native type II collagen modified by limited pepsin digestion still produces arthritis, suggesting that type-specific determinants residing in the helical region of the molecule are responsible for the induction of disease. Since homologous type II collagen emulsified in oil without bacterial preparations regularly causes the disease, this new animal model of arthritis represents a unique example of experimentally-inducible autoimmunity to a tissue component.

² Trentham, D. E., A. S. Townes, A. H. Kang, and J. R. David. Humoral and cellular sensitivity to collagen in type II collagen-induced arthritis in rats. Manuscript submitted for publication.

³ Trentham, D. E., R. A. Dynesius, and J. R. David. Passive transfer of type II collagen-induced arthritis by cells. Manuscript in preparation.

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