Autoimmunity to Collagen in Adjuvant Arthritis of Rats

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Abstract Arthritis can be induced in rats by intradermal injection of oil containing bacterial derivatives (adjuvant-induced arthritis) or cartilage collagen (type II collagen-induced arthritis). It was of interest, therefore, to determine whether collagen functions as an autoantigen in rats with adjuvant arthritis. Blood mononuclear cells from the majority of rats with adjuvant arthritis exhibited enhanced thymidine incorporation to homologous types I and II collagens, as well as to purified protein derivative of tuberculin. In contrast, cells from rats remaining nonarthritic after injection of adjuvant did not respond to collagen, although they did react to tuberculin. Similar results were obtained with a radiometric ear assay used to quantify intradermal delayed-type hypersensitivity in vivo. Using passive hemagglutination, autoantibodies to these collagens and their denatured α-chains were frequently detected in the sera of rats late in the course of adjuvant arthritis. Rats with inflammation of a hindlimb induced by turpentine did not acquire sensitivity to collagen. These data indicate that autoimmunity to collagen is a common feature of adjuvant- and collagen-induced arthritis, both of which are considered to be mediated by immunologic mechanisms.

Introduction Adjuvant disease of rats is an experimental polyarthrits induced by intradermal injection of an oily vehicle containing bacterial components, i.e., complete Freund’s adjuvant (1–16). Adjuvant arthritis can be passively transferred by lymphoid cells (17–19), implicating immunologic mechanisms in its pathogenesis. Recently, an arthritis morphologically similar to classical adjuvant disease has been induced in rats by injection of oil containing an apparently nonimmunogenic alkylidiamine, N,N-dioctadecyl-N’,N-bis-(2-hydroxyethyl)propanediamine (CP-20961) (20). Like complete Freund’s adjuvant, a dispersion or solution of this synthetic compound in oil possesses potent adjuvant properties for humoral and cell-mediated processes in rats (20). This recognition has furnished additional support for earlier suggestions (8, 21–25) that the immunogen(s) responsible for the induction of adjuvant arthritis is endogenous, perhaps a constituent of normal host tissue (20).

Intradermal injection of oil containing type II collagen derived from cartilage (26–31) or the vitreous of the eye (32) can also produce a polyarthritis in rats. Since the clinical (26, 31, 32) and histologic (26, 28, 31) features of type II collagen-induced arthritis are similar to those of adjuvant arthritis, it was of interest to determine whether collagen functions as an autoantigen in adjuvant arthritis. In this paper, we report that rats with adjuvant arthritis can exhibit cellular and humoral sensitivities to homologous type I and type II collagens. These data are consistent with the possibility that autoimmune responses to collagen participate in the pathogenesis of adjuvant-induced as well as collagen-induced arthritis in rats.

Methods Rats. We used outbred female Wistar rats weighing 175–225 g or weanling rats of the same strain (Charles River Laboratories, Wilmington, Mass.).

Collagens. Native type I and type II collagens were purified by neutral salt extraction from the tail tendons and xiphoid cartilages, respectively, of weanling rats rendered lathyritic by the administration of β-aminopropionitrile fumarate (Sigma Chemical Co., St. Louis, Mo.) (26). Differential salt precipitation removed type I collagen from our cartilage collagen preparation (33). Results from a uronic acid assay, carboxymethyl-cellulose chromatography and amino acid analysis confirmed the purity of each preparation (26). Because endotoxin may be mitogenic for rat lymphocytes (34), we analyzed our collagens for endotoxin with a Limulus amebocyte lysate test kit (Microbiological Associates, Walkersville, Md.). No endotoxin was detected. We also prepared the denatured constituent α1 (I) and α2 chains of rat type I collagen and the α1 (II) chains comprising rat type II collagen by carboxymethyl-cellulose chromatography (26).
Reference antisera. We obtained reference antisera to native homologous type II collagen by bleeding rats that had been injected intradermally 21 d earlier with 0.5 mg of type II collagen dissolved in 0.5 ml of 0.1 M acetic acid at 4°C for 24 h and emulsified in 0.5 ml cold incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) (26). Using an otherwise identical protocol, antibodies to rat type I collagen and α-chains could be produced only by use of complete Freund's adjuvant (27). For the injection of complete Freund's adjuvant containing Mycobacteria butyricum (Difco Laboratories, code 6038), because intradermal injection of 0.5 ml of this type of adjuvant does not induce arthritis in this strain of rats (26).

Induction of arthritis. Adjuvant arthritis was produced by injecting rats intradermally with 0.1 ml CFA H37Ra. This adjuvant was made to contain desiccated, heat-killed Mycobacteria tuberculosis strain H37Ra (Difco Laboratories, code 3114), which had been finely ground with a tissue pulverizer and added to incomplete Freund's adjuvant at a concentration of 10 mg/ml oil (26). Collagen arthritis was induced by the procedure used to obtain reference antisera to type II collagen. The injection sites were on the back in both protocols.

Production of a nonspecific hindlimb inflammation. As a control for the adjuvant- and collagen-induced arthritis, we incited periarticular inflammation in age-matched rats by injecting the volar pad of the right hindpaw with 0.3 ml turpentine oil (Fisher Scientific Co., Pittsburgh, Pa.) (35, 36) at weekly intervals. We injected only one paw, because adjuvant- or collagen-induced arthritis frequently involved a single hindlimb and because the considerable inflammation created by this regimen. After 3 wk, these rats were studied to determine whether they had become sensitized to collagen.

Evaluation of arthritis. The severity of adjuvant-, collagen-, and turpentine-induced arthritis was quantified by an arthritic index (26, 28, 37). In brief, each paw was graded from 0 to 4 based on the degree of periarticular erythema and swelling (26). A summated index was then derived for each rat. Light microscopic and radiographic evaluations (26) of adjuvant arthritis and the turpentine-induced hindlimb inflammation were also performed in selected rats. Thymidine-incorporation assay. Cellular sensitivities to collagen, as well as to the mycobacteria present in CFA H37Ra, were quantified in vitro by antigen-induced tritiated thymidine ([3H]TdR) incorporation responses by blood mononuclear cells. Blood was obtained by cardiac puncture, and mononuclear cells were isolated on gradients of Ficoll-Hypaque. 2 × 10^6 cells were then cultured in quadruplicate for 4 d in the presence or absence of antigens by a previously described microtechnique (27). Based on prior dose-response studies (27), assays from almost all rats included cells exposed to concentrations of 100 and 250 μg/ml of each collagen and 50 μg/ml of purified protein derivative of tuberculin (PPD, kindly supplied by the National Institute of Allergy and Infectious Diseases, Bethesda, Md., through the Joint United States-Japan Tuberculosis Program). Concentrations employed for cultures with α-chains were identical to those used for native collagens. As controls, assays for each rat also included cells cultured with the mitogens phytohemagglutinin-M (Difco Laboratories) and concanavalin A (Miles Laboratories, Inc., Elkhart, Ind.) (27). Incorporation of [3H]TdR by cells was expressed as mean counts per minute ± SE of the mean for the quadruplicate cultures. A stimulation index (SI) was then derived where SI = (mean counts per minute of [3H]TdR incorporated by cultures stimulated with collagen, PPD, or mitogen)/(mean counts per minute of [3H]TdR incorporated in unstimulated cultures). SI in this paper represent the maximal response induced by either of the two doses of collagen (38) or the single dose of PPD or mitogen. To derive values that represent statistically significant proliferations, cells from 23 immunologically naive rats were cultured with types I and II collagens, as well as PPD. The SI from these assays were used to determine the random distribution. SI that exceed the mean + 3 SD of these control values are considered significant proliferations in this paper. By these criteria, SI ≥ 2 were clearly significant responses to collagen or PPD.

Radiometric ear assay. Intradermal delayed-type hypersensitivity to collagen or PPD was quantified in vivo by a radiometric ear assay (39–41). Our assay (30) was modified from one previously described by Kostiala (41). In brief, type I or type II collagen was dissolved at a concentration of 1.0 mg/ml in 0.1 M acetic acid at 4°C for 24 h and then dialyzed overnight against cold 0.05 M calcium acetate. PPD was dissolved at an identical concentration in 0.9% sodium chloride. To radiolabel dividing cells that might accumulate at the site of a delayed reaction (39–41), a rat was injected subcutaneously with [3H]TdR (specific activity 6.7 Ci/mmol, New England Nuclear, Boston, Mass.) at a dose of 0.34 microcurie/μg body wt. 24 h later, 20 μl of the collagen or PPD solution was injected intradermally into the right ear. To control for any inflammation attributable to the buffer, an identical volume of calcium acetate or sodium chloride solution alone was injected into the left ear. After 24 h (39–41), we sacrificed the rat and obtained punch biopsies 6 mm in diameter from both ears. This assay does not detect a humoral response at this time (41). After digestion of the tissue (41), the radioactivity present in each biopsy was counted, and the radiometric ear index (REI) was expressed as the ratio of the counts per minute in the antigen-challenged ear vs. the control ear (41). As in our [3H]TdR incorporation assays, REI derived from 10 naive rats tested with type I collagen, type II collagen, and PPD were determined from random distributions. REI exceeding the mean + 3 SD of the control values for each antigen are considered significant responses. By these criteria, REI ≥ 1.6 indicate cellular sensitization to any of these antigens.

Hemagglutination assay. Blood was collected from rats by cardiac puncture or distal tail amputation (27), and sera were stored at −70°C. Antibodies to native collagens and α-chains were quantified by passive microhemagglutination using human (blood group O, Rh+) erythrocytes fixed by glutaraldehyde and coated with collagen (27). Titers are expressed as serial-log dilutions. Antibody specificities were assessed in reference antisera by passive hemagglutination techniques (42).

Statistical analysis. Comparisons of mean group data were performed by use of Student's t test.

RESULTS

Clinical features of adjuvant arthritis. 148 of 328 rats (45%) injected with CFA H37Ra experienced an explosive onset of arthritis 11–17 d after injection (mean ± SEM 13.2 ± 0.3). The remaining rats showed no macroscopic evidence of arthritis during observation periods of up to 6 wk after injection. Histologic and radiographic evaluation of the hindlimbs of 20 rats clinically adjudged to be nonarthritic 24 d after in-
jection of CFA H37Ra showed no evidence of adjuvant disease. The arthritic index of the first 46 adjuvant arthritic rats was calculated 1 wk after the onset of arthritis. The mean index (6.5±0.5) was similar to those obtained in our previous study of adjuvant- and collagen-induced arthritis (26). As in our earlier assessment (26), we could discern no clinical differences between these two arthropides. In agreement with another study (43), clinical evidence of active joint inflammation persisted 3–4 wk. Ankles from nine rats evaluated by light microscopy 28–42 d (mean 35.0 ±2.3) after the onset of adjuvant arthritis revealed fibrous and bony ankylosis of the joint, but only a few mononuclear cells remained in the periarticular tissues.

**Cellular sensitivity to native collagens in adjuvant arthritis.** The majority of rats with adjuvant-induced arthritis exhibited cellular sensitivity to homologous type I and type II collagens. In [³H]TdR incorporation assays (Fig. 1), cells from 24 and 28 of the 32 arthritic rats responded with SI ≥ 2 to types I and II collagens, respectively. The mean SI to both collagens was significantly higher in cells from arthritic rats compared with cells from rats remaining nonarthritic after injection of CFA H37Ra (in arthritic vs. nonarthritic rats, mean SI±SEM to type I collagen was 3.0±0.3 vs. 1.2±0.2, and mean SI to type II collagen was 3.8±0.4 vs. 1.0±0.1, P < 0.0001 for both responses). Cells from 16 of 30 (53%) arthritic and 13 of 27 (48%) nonarthritic adjuvant-injected rats responded with SI ≥ 2 to PPD. However, the mean SI to PPD did not differ significantly between the two groups (mean SI, 5.2±1.0 arthritic vs. 3.0±0.7 nonarthritic, P not significant).

Delayed-type hypersensitivity to collagen was also frequently found in vivo in rats with adjuvant arthritis using the radiometric ear assay (Fig. 2) (in arthritic vs. nonarthritic rats previously injected with CFA H37Ra,

**Figure 1** Comparison of collagen- and PPD-induced [³H]TdR incorporation by cells from 32 arthritic vs. 28 nonarthritic rats injected ~21 d earlier with CFA H37Ra, as well as cells from 23 age-matched, uninjected rats. Insufficient cell yields precluded testing four nonarthritic rats with type I collagen and two arthritic and one nonarthritic rat with PPD. Mean background counts per minute (727±82 arthritic vs. 983±122 nonarthritic vs. 1,631±269 normal) and mitogen responses (for arthritic vs. nonarthritic vs. normal, mean SI to phytohemagglutinin 58±12 vs. 45±8 vs. 21±4, mean SI to concanavalin A 165±36 vs. 97±22 vs. 42±10) did not differ significantly for these groups. In all figures, horizontal lines represent group means±SEM.
mean REI to type I collagen was 1.6±0.1 vs. 1.1±0.1, P < 0.002, mean REI to type II collagen was 2.4±0.2 vs. 1.0±0.1, P < 0.0001, and mean REI to PPD was 4.6±0.6 vs. 4.0±0.3, P not significant). By this assay, the magnitude of the cellular response to type II collagen was significantly higher than that observed to type I collagen in rats with either adjuvant- (P < 0.006) or collagen-induced (P < 0.0002) arthritis. The absence of reactivity to PPD in the ear assays of rats with collagen-induced arthritis (Fig. 2) provides evidence against the possibility that a nonspecific enhanced cell migration to the skin accounted for our positive REI to collagen in arthritic rats, and indicates that collagen and PPD do not share cross-reacting cellular antigenic determinants.

**Cellular sensitivity to denatured collagen in adjuvant arthritis.** Cells from 14 arthritic rats, studied during the first 3 wk of adjuvant disease, exhibited less [³H]TdR incorporation to the α-chains of type I collagen than to the native molecules (mean SI, 3.4 ±0.5 to type I collagen vs. 0.9±0.2 to α1 (I) chains, P < 0.001, and 2.0±0.4 to α2 chains, P < 0.03). However, the [³H]TdR incorporation responses to type II collagen vs. α1 (II) chains were not significantly different (mean SI, 2.9±0.5 to type II collagen vs. 2.1±0.4 to α1 (II) chains, P not significant).

**Serial studies of cellular autoimmunity to collagen in adjuvant arthritis.** The relationship of cellular sensitivities to collagen and PPD with the course of adjuvant arthritis is depicted in Fig. 3. Cells obtained from 15 rats before the expected time of onset of adjuvant arthritis exhibited little [³H]TdR incorporation to collagen or PPD. Histologic examination of the ankles of five of these rats showed no synovitis. In contrast, during the first 2 wk of arthritis, the responses to collagen and PPD previously noted in Fig. 1 occurred. Reactivities were minimal in cells from the nine rats whose articular inflammation had subsided...
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arthritic rats collected after
the 1st wk of disease (Fig. 4).
No hemagglutinating antibodies
to
collagen were found in sera from
46
nonarthritic rats studied at varying
times after injection
of
CFA H37Ra or in sera from 23
unjected rats.

Autoantibodies to collagens in adjuvant arthritis.
Although there were no hemagglutinating antibodies
to
collagen in sera from 20
arthritic rats obtained
during the first 3 d of disease, passive hemagglutination
assays detected antibodies to homologous
types I and II collagens and their α-chains in the sera of
~40% of the rats with adjuvant arthritis collected after
the 1st wk of disease (Fig. 4). No hemagglutinating antibodies
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rats with a chemically induced hindlimb inflammation
were examined to determine whether they acquired sensitivity to collagen. Weekly injections of
turpentine into a hindpaw of 20 age-matched rats caused persistent swelling and erythema graded 4 by
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arthritic index. Radiographic evaluation after
3–4 wk revealed severe joint destruction and an ex-


te type II collagen). Similarly, sera from 10
arthritic rats that had been injected with type II collagen
21 d
earlier agglutinated type II collagen-coated erythrocytes, but not those coated with type I collagen (mean
−log₂ antibody titer, 6.0±0.2 to type II collagen vs. 0
for type I collagen). In addition, antiserum to these native
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collagens failed to react with the α-chains of either
collagen, indicating that the antibodies were directed

toward helical antigenic determinants. The specificities
of antibodies to denatured rat collagen could not be
studied satisfactorily because our immunization
regimen produced only minimal antibody titers to α-
chains.

Absence of sensitivity to collagen in turpentine-induced hindlimb inflammation. The reactivities to
collagen in adjuvant arthritis could be explained by
immunocompetent cells becoming exposed to collagen
as a consequence of periarticular inflammation. Therefore,
rats with a chemically induced hindlimb inflammation
were examined to determine whether they acquired sensitivity to collagen. Weekly injections of
turpentine into a hindpaw of 20 age-matched rats caused persistent swelling and erythema graded 4 by
the
arthritic index. Radiographic evaluation after
3–4 wk revealed severe joint destruction and an ex-
tensive periosteal reaction of the bones in the hindpaw. Histologically, there was an intense infiltration of the periarticular tissues by, predominantly, polymorphonuclear leukocytes. Flagrant damage to cartilage and bone was evident. At this time, cells from these rats did not exhibit enhanced $^{3}H$Tdr incorporation to collagens, $\alpha$-chains, or PPD (mean background counts per minute $1,849 \pm 315$; mean SI to phytohemagglutinin, $11.6 \pm 2.7$; to concanavalin A, $17.6 \pm 9.4$; to type I collagen, $1.1 \pm 0.1$; to type II collagen, $1.3 \pm 0.2$; to $\alpha_1$ (I) chains, $0.8 \pm 0.1$; to $\alpha_2$ chains, $0.5 \pm 0.1$; to $\alpha_1$ (II) chains, $0.9 \pm 0.1$; and to PPD, $1.0 \pm 0.1$). Their sera contained no hemagglutinating antibodies to native or denatured collagens.

Nonspecific inflammatory processes, such as those induced by carrageenan, may impair humoral (44, 45) or cellular (46–48) functions. Immunosuppression, possibly mediated by prostaglandins (49), could have prevented the development of collagen immunity in the turpentine model. We therefore injected 20 rats with type II collagen in incomplete Freund’s adjuvant and simultaneously began the turpentine injections. After 4 wk, antibodies to type II collagen were present in the sera of 16 rats (mean titer, $6.4 \pm 0.6$); cells from 9 rats responded to type II collagen (mean SI, $2.2 \pm 0.4$); and arthritis was evident in the un.injected hindlimb of 6. 5 of 20 additional rats subjected to the turpentine protocol and injected with CFA H37Ra developed polyarthritis. These experiments show that rats with turpentine-induced hindlimb inflammation are capable of becoming sensitized to collagen and developing collagen or adjuvant arthritis. However, they provide only indirect evidence to suggest that, if it were appropriate, rats with turpentine-induced arthritis would be capable of acquiring sensitivity to collagen endogenously.

**DISCUSSION**

Adjuvant disease of rats is a systemic polyarthritis involving articular and dermal tissues (6, 11). While it has been presumed that a cellular response (17–19, 50–54) to one or more exogenous antigens derived from bacterial cell walls (9, 16, 37, 55–57) induces adjuvant arthritis, some investigators have considered that this disease comprises, at least partially, a response to one or more endogenous antigens (20, 21). Pearson (8) first suggested that adjuvant disease might represent an autoimmune reaction to collagen altered at the site of injection of CFA. Ryżewska (22) provided evidence for this hypothesis by reporting that intradermal injection of lymph node cells from rats with adjuvant arthritis into immunologically naive syngeneic recipients incited a species-specific delayed skin test reaction.
He concluded that immunocompetent cells from the arthritic rats were reacting against antigens present in dermal connective tissue. The observation that lathyritic rats appeared to be less susceptible to adjuvant arthritis (23) prompted Steffen and Wick (24, 25) to investigate more directly whether collagen might function as an autoantigen in this model. Delayed-type skin reactions to denatured (24, 25) and native (25) rat skin (type I) collagen were elicited in some rats with adjuvant arthritis, although they failed to detect antibodies to skin collagen by passive hemagglutination (25).

Our study demonstrates that humoral and cellular autoimmune responses to homologous type I and type II collagens are present in rats with adjuvant-induced arthritis. Although antibodies to denatured collagens were frequently found in sera from arthritic rats, additional work is required to determine whether cellular responses to α-chains are present in this model. Absorption of adjuvant arthritis sera with mycobacteria did not affect the antibody titers to collagen, and rats injected with type II collagen failed to display delayed hypersensitivity to PPD. Nonetheless we cannot, at present, further exclude the possibility that our findings are attributable to cross-reacting antigenic determinants shared by collagen and the mycobacterial preparation which we used to induce adjuvant arthritis.

It is possible that one collagen type functions as the principal autoantigen in adjuvant arthritis, with the reactivities to other collagen preparations being explained by cross-reacting antigenic determinants. Alternatively, specific humoral and cellular responses to native and denatured collagens could exist in this disease. Immunization with native types I and II collagens induces type-specific humoral (42) and cellular (27) responses in rats, but whether the responses to homologous collagens in adjuvant arthritis are also type specific is unknown.

Finally, our data do not distinguish whether autoimmunity to collagen plays a primary role in the pathogenesis of adjuvant arthritis or whether it represents a secondary response to connective tissue alterations in this inflammatory disease. Hemagglutinating antibodies to collagen appeared in the sera of arthritic rats only after arthritis had been present for several days. This temporal sequence could indicate that the humoral response to collagen in adjuvant arthritis required access of immunocompetent cells to collagen. We would caution that the absence of collagen reactivity in the turpentine model does not discredit this hypothesis, since the magnitude of inflammation induced by turpentine might have degraded collagen before it could function as an autoimmunogen.

Contrasting with the humoral response, cellular sensitivity to collagen was identified during the 1st wk of morphologically evident adjuvant arthritis. This association would be consistent with cellular processes participating in the primary pathogenesis of adjuvant arthritis. However, we have recently shown that delayed hypersensitivity to homologous type II collagen can be induced in rats within 3 d by antigen-presentation techniques in the absence of adjuvant oil (58). This delineation suggests that macrophages may be capable of processing collagen exposed early in the course of adjuvant arthritis and generating the T cell responses which we found during the initial stage of arthritis. Thus, the cellular response to collagen in adjuvant arthritis could also represent a secondary event unrelated to the actual pathogenesis of this disease.

Despite these reservations, our data support the hypothesis that injection of an adjuvant preparation into rats can trigger the expansion of lymphocyte clones which exhibit autoreactivity to collagen. These cells could contribute to the inflammation observed in adjuvant disease. Sensitized T cells, with helper functions, might also recruit B cell responses to collagen and explain the appearance of autoantibodies later in the course of adjuvant arthritis. Our passive transfer experiments (28) indicate that cells sensitized to type II collagen can induce arthritis in immunologically naïve syngeneic rats. The present study provides evidence that cellular sensitivity to collagen may be related to the pathogenesis of adjuvant- as well as collagen-induced arthritis.

ACKNOWLEDGMENTS

We are indebted to Dr. John P. Caulfield for assistance in the histologic evaluation of synovia. We thank Donna Rowland and Roselynn Dynesius for excellent technical assistance.

This work was supported by National Institutes of Health grants AM-21490, AI-07685, AM-05076, AI-07167, AM-05588, RR-05669, and by a grant from the New England Peabody Home Foundation. Dr. Trentham is a Senior Investigator of the Arthritis Foundation.

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