Cartilage antigen dependent lymphotoxin release

Immunopathological significance in articular destructive disorders

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The proteoglycans comprising the extracellular matrix of articular cartilage have been shown to be antigenic, determinants residing in the protein moieties comprising their subunit and link fractions (Loewi and Muir, 1965; Sandson, Rosenberg, and White, 1966; Baxter and Muir, 1972; Keiser, Shulman, and Sandson, 1972). Cartilage allograft transplantation studies (Heyner, 1969) and experimentation using syngeneic chondrocytes and cartilage fragments (Langer, Gross, and Greaves, 1972) suggest chondrocyte antigenicity as well, occurring not only in the form of expression of histocompatibility antigens but also perhaps as surface constituents unique to this cell type (Westhues, 1971; Langer and others, 1972). Yet, because the cartilage is avascular it may be regarded as an immunologically privileged tissue, its chondrocytes, surrounded by dense proteoglycan and collagen matrix, being sequestered from the immune system; whereas its proteoglycan antigens are so sequestered because of masking of determinants afforded by their aggregate structure. However, upon enzymatic degradation of matrix, potential antigenic constituents could be released (Herman, Wiltse, and Dennis, 1973) and indigenous proteoglycan and chondrocyte surface antigens exposed, affording host sensitization. A resulting immune response could facilitate further cartilage degradation and joint inflammation.

Evidence is currently lacking to support a role for antibody to such cartilage antigens in the pathogenesis of articular destructive disease. However, our previous investigations (Herman and Dennis, 1973; Herman and others, 1973) using lymphocyte transformation and macrophage aggregation as *in vitro* correlates of delayed hypersensitivity have shown a cell mediated immune response to proteoglycan and chondrocyte

synthesized antigens. This was observed in association with inflammatory cartilage degradation in diseases such as relapsing polychondritis, rheumatoid arthritis, chronic tophaceous gout, and infectious and psoriatic arthritis.

In the present study, a third *in vitro* correlate of cell mediated immunity, antigen dependent release of a lymphotoxin, has been used to study immune response to constituents of cartilage in articular destructive diseases. By means of this test parameter, we not only corroborate our earlier observations but implicate such an immune response in degenerative joint disease as well.

Materials and methods

CARTILAGE ANTIGENS

The cartilage antigens used consisted of (1) crude proteoglycan complex (PPC) isolated from human costal cartilage by water extraction with high speed homogenization (Malawista and Schubert, 1958); (2) proteinpolysaccharide light fraction (PPL) prepared by differential centrifugation of PPC (Gerber, Franklin, and Schubert, 1960); (3) products of synthesis of human articular chondrocytes isolated by collagenase digestion (Manning and Bonner, 1967) and maintained in monolayer cultures as previously described (Herman and others, 1973). This chondrocyte synthesized antigen (S-antigen) was derived by ultrafiltration (membrane retentivity > 10,000 MW) of growth media of newly established cultures. On immunodiffusion a single line reactive with anti-PPL specific antisera could be identified, indicating the capacity of these chondrocytes to synthesize *in vitro* at least one of the recognized proteoglycan antigens.

LYMPHOTOXIN ASSAY

A modification of the Granger and Kolb (1968) technique was used. Peripheral buffy coat isolated by dextran

Accepted for publication March 3, 1974.

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Supported in part by National Institute of Arthritis and Metabolic Diseases Training Grant AM 05044, an Arthritis Foundation Clinical Research Center Grant, and U.S. Public Health Service Grant AM 16894.

sedimentation and washed with Eagle's minimum essential media (MEM) was added in a concentration of 750,000 mononuclear cells/ml, 4 ml/tube, to sterile polystyrene culture tubes. Glutamine, gentamicin sulphate, and 15% decomplemented fetal calf serum were added. Cartilage antigens pretreated with hyaluronidase—500 µg PPC, 50 µg PPL, 1 mg chondrocyte supernate (not enzymatically pretreated)—in doses yielding maximal blastogenic response as based on earlier experiments (Herman and others, 1973) were added. Triplicate cultures were used. Control tubes consisted of buffy coat without cartilage antigen and as a positive toxic control, buffy coat plus phytohaemagglutinin-M. Cultures were maintained under anaerobic conditions at 37°C for 5 days after which cells were pelleted, supernatants were removed, pooled, and lyophilized. Mouse L-929 fibroblasts were used as target cells for detection of released cytotoxin. Lyophilized supernatants were reconstituted with sterile distilled water to effect an eightfold concentration and 100 µl of this material added to 2 ml MEM at a concentration of 100,000 L-cells/ml. Cultures were continued for 48 hrs after which cells were gently pelleted, media replaced with 2 ml MEM without leucine, 1 µCi ³H-leucine (specific activity, 38 Ci/mmol) added, and a 6-hour pulse performed at 37°C in a 5% CO₂ atmosphere. At time of harvest, supernates were removed, carrier protein was added, and trichloroacetic acid (TCA) precipitable counts/minute (cpm) were determined. In 25% of experiments pellets as well were processed for TCA precipitable counts, results being comparable. Results were expressed as a toxicity ratio derived as follows:

$$\frac{\text{CPM L-Cells + Buffy Coat and Cartilage Antigen Supernates}}{\text{CPM L-Cells + Buffy Coat Supernate Alone}}$$

Values of less than 1 would be indicative of a cytotoxic effect elicited by cartilage antigen.

PATIENTS

Studies were performed on 29 patients meeting the criteria of the A.R.A. (Ropes, Bennet, Cobb, Jacox, and Jessar, 1959) for definite or classical rheumatoid arthritis (RA), on 22 patients with degenerative joint disease (DJD), and on eight patients with crystal documented gouty arthritis. Thirteen normal individuals (six male, seven female; ages 26–64 years, mean 44.2 years) served as controls.

Results

L-CELL CYTOTOXICITY INDUCED BY BUFFY COAT SUPERNATANT ALONE

As illustrated in Fig. 1, variability was obtained in L-cell protein synthesis after exposure to aliquots of buffy coat supernate from both normal and patient categories. However, the majority of specimens from each group, with the exception of gout, revealed a toxic response. As a consequence, these values were used as a base in determining toxicity ratios in

FIG. 1 *L-cell toxicity response in 3 patient groups and normals after exposure to buffy coat supernatant. DJD, degenerative joint disease*

experiments using cartilage antigens rather than using figures derived from L-cells cultured alone.

EFFECT OF CARTILAGE ANTIGEN ADDITION ALONE ON L-CELL PROTEIN SYNTHESIS

Two types of experiments were conducted to determine this response. In the first, as illustrated in Fig. 2, varying quantities of the 3 cartilage antigens were added directly to L-cells and incubation and pulsing were undertaken as described above. Results of L-cell protein synthesis were comparable in supernate and pellet determinations. A dose response relationship was apparent for each antigen. PPC exhibited a persistent cytotoxic response, whereas additions of PPL and S antigens gave either a stimulatory or inhibitory effect as determined by the quantity of antigen added. Extrapolating this data to the quantity of antigen employed in the patient assay system, PPC alone could be expected to induce a low grade toxic response as contrasted with mild stimulation for the other 2 antigens.

In a second series of experiments, the full culture schema was followed using usual test quantities of antigens with the exceptions that buffy coat was omitted and in one group the initial phase of 5-day incubation of cartilage antigen was conducted without addition of fetal calf serum. PPC used in the same quantity as in our test assay system exhibited a mild toxic response (toxicity ratio, 0.95) whereas a minimal stimulation was observed with PPL (toxicity ratio, 1.08) and negligible toxicity with the chondrocyte supernatant antigen (toxicity ratio, 0.98). Addition of fetal calf serum to the antigens had no significant effect. Addition of PHA alone to L-cells similarly gave no appreciable response.

FIG. 2 *L*-cell toxicity response after direct addition of cartilage antigens. PPC = crude proteoglycan; PPL = proteinpolysaccharide light fraction; S = chondrocyte supernatant antigen

L-CELL TOXICITY RESPONSE IN PATIENT GROUPINGS

Fig. 3 shows *L*-cell toxicity ratios to PPC antigens in the 4 clinical groupings. As illustrated, only 1 of 13 normal subjects displayed a minimal toxic ratio. The toxic effect observed in control experiments after the addition of PPC alone to *L*-cells was negated in the presence of normal human buffy coat. Values indicative of a toxic response were obtained in twelve of the 29 RA patients, in eleven of 22 with DJD, and in four of eight gouty patients.

Fig. 4 illustrates toxicity ratios to PPL antigen. Though toxicity was not observed in normal controls, such a response occurred in eleven of 29 RA patients, in twelve of 22 with DJD, and in four of eight with gout.

L-cell response to the S-antigen is shown in Fig. 5. A single normal individual displayed a minimal toxic response, whereas such an effect was observed in seventeen of 29 RA patients, in nine of 22 with DJD, and in four of eight with gout.

The mean toxicity ratio response to the 3 cartilage antigens in the rheumatoid and degenerative disease groups is shown in Tables I and II, respectively. Patients have been arbitrarily divided into 3 groups based on *L*-cell response.

Table III correlates toxicity results obtained in rheumatoid patients with clinical and laboratory parameters. Twelve of the 29 patients had a toxicity ratio to 1 or more cartilage antigens of less than 0.90. All had clinical evidence of an active synovitis,

positive rheumatoid factor reactivity, and *x*-ray evidence of severe, destructive articular changes. Of the nine patients with ratios between 0.9 and 1.0, there was a comparable age distribution, disease duration, and degree of clinical activity. Antinuclear

Table I Mean *L*-cell cytotoxic response to cartilage antigens in 29 patients with rheumatoid arthritis

	<i>L</i> -cell cytotoxicity ratio		
	<0.90 (12 patients)*	0.90-0.99 (9 patients)	>1.0 (8 patients)
PPC	6 (0.72)	5 (0.95)	(1.34)
PPL	7 (0.63)	1 (0.90)	(1.24)
S	8 (0.72)	6 (0.95)	(1.43)

* Seven patients responded to 2 or more antigens with ratio <0.90.

Table II Mean *L*-cell cytotoxic response to cartilage antigens in 22 patients with DJD

	<i>L</i> -cell cytotoxicity ratio		
	<0.90 (9 patients)*	0.90-0.99 (8 patients)	>1.0 (5 patients)
PPC	5 (0.79)	5 (0.96)	(1.26)
PPL	5 (0.79)	6 (0.95)	(1.16)
S	1 (0.54)	4 (0.94)	(1.32)

* Two patients responded to 2 antigens with ratio <0.90.

FIG. 3 L-cell toxicity response in 3 patient groups and normals after exposure to buffy coat-PPC supernatant*

FIG. 5 L-cell toxicity response in 3 patient groups and normals after exposure to buffy coat-chondrocyte culture supernatant*

FIG. 4 L-cell toxicity response in 3 patient groups and normals after exposure to buffy coat-PPL supernatant*

antibody (diffuse staining pattern indicative of deoxyribonucleoprotein response) was present more frequently, rheumatoid factor less so. It should be noted that only three of the nine patients had x-ray evidence of destructive articular changes. Similarly, though the eight patients who failed to show L-cell toxicity all manifested clinical evidence of active synovitis, only three had x-ray evidence of cartilage involvement. Treatment was comparable in each of

Table III Clinical and laboratory findings correlated with L-cell cytotoxicity response in 29 patients with RA

	L-cell cytotoxicity ratio		
	<0.90	0.90-0.99	>1.0
No. of patients	12	9	8
Sex	4F, 8M	4F, 5M	6F, 2M
Age (yrs)	33-62	19-65	48-62
(mean)	(52.6)	(49.1)	(56.1)
Disease duration (yrs)	2-30	1-31	0.6-20
(mean)	(11.3)	(12)	(11.1)
ESR (Westergren)	14-108	6-83	14-96
(mean)	(58.5)	(41.9)	(53.9)
Latex	100%+	66.6%+	87.5%+
SCAT	83%+	66.6%+	75%+
(mean)	(1:224)	(1:224)	(1:224)
ANA	3/10+	4/7+	0/6+
X-ray signs of severe cartilage damage	12	3	3

*Figs 3-5: the responses of the normal subjects are statistically significantly different ($P < 0.001$) from response in each of the 3 patient groups; differences within those three groups were not statistically significant. Data were analysed using the Wilcoxon-Mann-Whitney rank test (Remington and Schork, 1970).

Table IV *Clinical and laboratory findings correlated with L-cell cytotoxicity response in 22 patients with degenerative joint disease*

	<i>L-cell cytotoxicity ratio</i>		
	<i><0.90</i>	<i>0.90-0.99</i>	<i>>1.0</i>
No. of patients	9	8	5
Sex	8F, 1M	5F, 3M	3F, 2M
Age (yrs)	51-76	47-74	45-71
(mean)	(63.3)	(59.3)	(61.6)
Disease duration			
(yrs)	4-30	8-33	2-15
(mean)	(10)	(12)	(7.8)
ESR (Westergren)	2↑	3↑	0↑
(mean)	(24.5)	(27.8)	(15.0)
Latex	All Neg	1/8+	All Neg
SCAT	All Neg	1/8+	All Neg
X-ray signs of severe cartilage damage	8	3	2

the 3 groups, basically comprising salicylates and in an occasional patient, low dose steroids or chrysotherapy.

A clinical, laboratory, and toxicity response correlation in the DJD group is shown in Table IV. Nine of 22 patients responded with a toxicity ratio of less than 0.90. Two of these had raised sedimentation rates, none had rheumatoid factor or ANA reactivity. All but one had x-ray evidence of severe degenerative changes. Three of eight patients having ratios between 0.90 and 1.0 had similar severe x-ray changes. In those patients without a toxic response, severe changes were observed in two of the five. The mean toxicity ratio response in those patients having levels of less than 0.90 were noted to be higher to PPC and PPL antigen than in the comparable RA group (Tables I and II), but the difference was not statistically significant. However, only one DJD patient of the 22 studied gave such a response to the S-antigen. This occurred in a 64-year-old black female who eventually underwent bilateral total knee replacements for severe articular destructive disease.

Of the eight gouty patients studied, four with tophaceous disease having destructive x-ray changes gave ratios of less than 0.90 to 2 or more of the antigens. Three of these four responded to PPC (mean toxicity ratio 0.70), two to PPL (mean 0.62), and three to the S-antigen (mean 0.77). The four patients having toxicity ratios greater than 0.90 lacked x-ray evidence of cartilage change. The degree of L-cell toxic response in the gouty category was comparable to the RA patients but greater in degree than that occurring in the DJD group.

Discussion

In the experiments described, peripheral buffy coat derived from patients with articular destructive

disorders, and presumably containing lymphocytes sensitized to antigenic components of human cartilage, released a cytotoxic factor upon exposure to cartilage antigens as determined by impairment of incorporation of radio-labelled amino acid into solubilized protein by mouse L-cell fibroblasts. Although variable toxicity was evident upon exposure of such target cells to supernatants derived from buffy coat alone, by using the latter results as a base line response, cartilage antigenic constituents appeared to be able to mediate an enhanced degree of toxicity. We have not determined the chemical component or components in buffy coat cartilage antigen supernates responsible for the toxic effect observed, but there is strong evidence that it could be lymphotoxin, a recognized mediator of delayed hypersensitivity (Granger and Kolb, 1968; Kolb and Granger, 1968; Russell, Rosenau and Lee, 1972; Granger, Laserna, Kolb, and Chapman, 1973). This lymphokine is released by specifically sensitized lymphocytes in the presence of antigen. Once released, cellular injury may be induced in miscellaneous target cells (Granger, Shacks, Williams, and Kolb, 1969).

By employing lymphotoxin release as an *in vitro* correlate of delayed hypersensitivity, we have been able to confirm our earlier observations, utilizing systems of blast transformation and macrophage aggregation, that lymphocyte sensitization to constituents of cartilage exists in patients having inflammatory destruction of cartilage (Herman and Dennis, 1973; Herman and others, 1973). In the present report we extend these observations to patients with DJD. The concept that immune mechanisms are potentially operative in the pathogenesis of cartilage degradation in this disease as well as in RA, tophaceous gout, psoriatic arthritis, and protracted septic arthritis is tenable. In severe DJD there may be mononuclear infiltration of synovial membrane. Of interest is the little recognized modification of subchondral intertrabecular marrow (Jaffe, 1972) wherein, in place of the fatty appearance observed in middle-aged or older adults, this marrow site acquires vascularity with collections of plasma cells and lymphocytes. Furthermore, increased exposure of proteoglycan antigenic sites in degenerative cartilage matrix has been shown (Barland, Janis, and Sandson, 1966) and more recently, migration inhibitory factor, a recognized *in vitro* correlate of cell mediated immunity, has been detected in synovial effusions from such patients (Stastny, Rosenthal, Andreis, and Ziff, 1973).

Recognizing the antigenicity of cartilage matrix proteoglycans as well as chondrocyte surface determinants, the question must be raised as to how host sensitization to such antigens may facilitate causation or perpetuation of joint inflammation with concurrent cartilage degradation. The presence of

cartilage antigenic determinants reacting with anti-proteoglycan antisera have been shown in both synovial fluid (Sandson, 1967) and phagolysosomal structures contained within its phagocytic cells secured from a variety of disease states (Herman and others, 1973). There is ample evidence of the capacity of acid and neutral proteases derived from a variety of cellular sources to degrade cartilage proteoglycan, and thus perhaps phagocytosis of proteoglycan constituents themselves or in immune complex form may facilitate such release. It has been shown that antibody directed to cell membrane antigens is capable in the presence of fresh complement of inducing cartilage matrix dissolution in chick limb bud rudiments (Fell, Coombs, and Dingle, 1966; Lachmann, Coombs, Fell, and Dingle, 1969). Presumably the immune response is directed against chondrocyte surface antigenic determinants facilitating a selective release of cathepsins sufficient to induce the reversible response. However, we presently lack sufficient evidence documenting the detection of antibody to cartilage antigens in human articular destructive diseases.

It is possible that the delayed hypersensitivity response to cartilage components that we have identified may accomplish such dissolution of matrix and evoke inflammation. Numerous biological activities have been attributed to soluble lymphokines generated as an expression of cell mediated immunity. Included among the latter are inflammatory, mitogenic, cytopathic, and migration inhibitory factors (Dumonde, Wolstencroft, Panayi, Matthew, Morley, and Howson, 1969). Evidence is accumulating that

such mechanisms may induce alterations in cell membranes and affect metabolic function independently of cell destruction (Williams and Granger, 1969; Koren, Ferber, and Fisher, 1971). Studies are now in progress to document this hypothesis, by evaluating the direct effect of lymphokines generated in response to purified cartilage antigenic constituents, on the metabolic function of chondrocytes and degradation of proteoglycan matrix.

Summary

Preparations of human cartilage crude proteoglycan, its PPL fraction, and products of chondrocyte synthesis have been used as antigenic material in a lymphotoxin assay to determine the presence of cell mediated immunity to such constituents in articular destructive disorders. Positive responses were obtained in patients with rheumatoid arthritis and chronic tophaceous gouty arthritis with active joint inflammation and x-ray evidence of severe articular cartilage degradation. These findings confirmed earlier studies using mitogenic and macrophage aggregation assay systems. Positive responses were also observed in patients with severe degenerative joint disease. The potential immunopathological significance of these observations is discussed.

The authors acknowledge the enthusiastic help of Drs. E. Miller and R. Freiberg, Division of Orthopedic Surgery, in obtaining surgical specimens, and Dr. E. V. Hess for reviewing the manuscript.

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