Incidence of antibodies to native and denatured cartilage collagens (types II, IX, and XI) and to type I collagen in rheumatoid arthritis

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SUMMARY The frequencies of antibodies to the cartilage type IX and XI collagens and to type I collagen were determined in 188 patients with rheumatoid arthritis, of whom 76 were positive for antibodies to native type II collagen. A higher proportion of patients with antibodies to native type II collagen had antibodies to these other collagens, but about one third of patients without antibodies to native type II collagen had antibodies to one or more denatured collagens. The patterns of antibodies present in individual sera suggested that there was a selective response to the collagens in an individual patient. The incidence of patients having antibodies to these native and denatured collagens in a random group of patients with rheumatoid arthritis was calculated.

Antibodies to native type II collagen occur in the sera and synovial fluids of some patients with rheumatoid arthritis (RA).¹⁻⁶ Type II collagen is restricted to a few tissues such as cartilage, vitreous humour, and intervertebral discs as compared with the more universal distribution of type I and III collagens. Thus immunity to type II collagen is of particular relevance in the chronic inflammatory arthritides where destruction of articular cartilage occurs.

Immunity to native type II collagen has been linked to the induction of an inflammatory arthritis in rats and mice.⁷⁻¹² Some features resemble RA.¹³ Also we have shown that type XI (1 α 2 α 3 α) collagen, another cartilage collagen isolated from human articular and bovine nasal cartilage,^{14 15} can be immunogenic and arthritogenic in rats.¹⁶ Several short chain, disulphide bonded collagens have been isolated from cartilaginous tissues,¹⁷ but all are now known to be derived from the same larger molecule, type IX collagen.¹⁸

We have therefore investigated whether antibodies to type IX and XI collagens occur in patients with RA and whether they are linked to the presence of antibodies to native type II collagen.

Similarly, we have investigated whether antibodies to type I, a non-cartilage collagen, found in tendons, synovial tissue, bone, and skin, also occur. From these data we have calculated the incidence of patients with RA having antibodies to these native and denatured collagens.

Patients and methods

PATIENTS

The 188 patients were outpatients or inpatients of the Royal Devonshire Hospital, Buxton; of Noble Hospital, Douglas, Isle of Man; of the Rheumatology Department, Withington Hospital, Manchester; or of the Rheumatology Department, Manchester Royal Infirmary. All patients had definite or classical rheumatoid arthritis and were chosen for the presence or absence of anti-native type II collagen antibodies irrespective of their age, sex, or of their disease duration or severity.

Control sera were obtained from 90 normal healthy subjects attending the Regional Blood Transfusion Service.

BLOOD SAMPLES

Blood was collected, allowed to clot at room temperature, and centrifuged at 1500 g for seven minutes. The serum was removed and frozen in aliquots at -20°C until used.

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COLLAGENS

Acid soluble native type I collagen was extracted from fetal calf skin and purified by the method of Jackson and Cleary.¹⁹ Native bovine type II collagen was extracted from nasal septa by pepsin digestion after prior treatment with 2 M magnesium chloride in 0.05 M TRIS-HCl, pH 7.4, to remove the proteoglycans, and purified further.¹⁶ Native IX and XI collagens were extracted from fetal bovine articular cartilage by similar methods and extensively purified by differential salt precipitation and by dialysis against phosphate buffer.^{15 20}

The collagens were stored at -20°C until used. Acid soluble type I collagen was dissolved at 2 mg/ml in 0.1 M acetic acid and dialysed into 0.45 M sodium chloride/0.02 M TRIS buffer, pH 7.5, before use. The other collagens were dissolved directly in the sodium chloride/TRIS buffer at the same concentration. They were diluted in the same buffer to 10 µg/ml for coating the plates in the anticollagen antibody assay (see below). To denature the collagens the solutions of collagens were heated in a water bath at 46°C for 20 minutes immediately before use.

ANTICOLLAGEN ANTIBODIES

Antibodies to specific collagens were measured in the sera by a solid phase, double antibody, enzyme labelled immunoassay. Linbro 96 well ELISA (enzyme linked immunosorbent assay) plates were coated overnight at 4°C with 200 µl/well of a 10 µg/ml solution of collagen in 0.45 M sodium chloride/0.02 M TRIS buffer, pH 7.5. The procedure was the same for both native and denatured collagens. Control plates were coated with 200 µl of 0.1% (w/v) bovine serum albumin in phosphate buffered saline containing 0.05% (w/v) Tween 20, pH 7.5 (BSA/PBS/Tween). After washing six times with phosphate buffered saline, pH 7.5, containing 0.05% (w/v) Tween 20 (PBS/Tween) the remaining binding sites in the wells were blocked with 300 µl of BSA/PBS/Tween for four hours at room temperature. After washing the plates six times with PBS/Tween the sera diluted 100 times in BSA/PBS/Tween were added to the wells (200 µl) in duplicate. The plates were left overnight at 4°C and then washed six times with PBS/Tween. The second antibody (200 µl/well), which was alkaline phosphatase labelled, was added at a dilution of 1:2000 in BSA/PBS/Tween. After four hours at room temperature the plates were washed six times in PBS/Tween and six times in distilled water.

The substrate, *p*-nitrophenyl phosphate (Sigma 104), was added (200 µl/well) at a concentration of 1 mg/ml in 10% diethanolamine buffer, pH 9.8. The reaction was stopped by the addition of 50 µl/well of

3 M sodium hydroxide and the developed colour read at 405 nm on a Titertek MC densitometer.

Each plate had wells containing BSA/PBS/Tween instead of serum as a blank for non-specific binding of the second antibody and background colour of the reagents. The mean absorbance value for the blank was subtracted from the mean absorbance value for each sample on the same plate. The level of binding to each collagen was then determined by subtraction of the resultant value of each sample on a BSA/PBS/Tween coated plate from the mean of the same sample on the collagen coated plate. The results were expressed as arbitrary units between 0 and 1900 (maximum measurable absorbance being 2000 before subtraction of blank).

Screening assay

Samples of one date for each of the 188 patients with RA and of the 90 blood donors were screened in one assay for antibodies to native type I, II, IX, and XI collagens. Each plate in the assay had 15 control sera and 32 patients' sera on it. Antihuman IgGAM (DAKO Ltd) was used as the second antibody. The assay was repeated using denatured collagens.

Class of anticollagen antibodies

Samples of one date for each of 30 sera from patients with RA that were positive for antibodies to native type II collagen were tested for IgG, IgA, or IgM antibodies to native and denatured type II and XI collagens separately in one assay. The second antibodies were antihuman IgG, anti-IgA, or anti-IgM (DAKO Ltd).

STATISTICS

Antibodies to different antigens were tested for correlation by the Spearman rank correlation coefficient test.

Results

ANTIBODIES TO NATIVE COLLAGENS

The mean absorbance value of the 90 donors was 22

Table 1 *Incidence of sera containing antibodies which bind to native type I, IX, or XI collagens in the three groups tested*

Collagen	Donors (n=90)	RA anti-native II negative (n=112)	RA anti-native II positive (n=76)
Native I	1* (1.1)†	1 (0.9)	9 (11.8)
Native IX	1 (1.1)	2 (1.8)	3 (3.9)
Native XI	0 (0)	2 (1.8)	9 (11.8)

*Number of samples above the mean plus three standard deviations of the blood donor group (normal controls) for that particular antigen.

†Percentage positive sera.

units (standard deviation 15.2 units) when using the anti-IgGAM antibody with native bovine type II (NBII) collagen. When three standard deviations above this mean value were taken (i.e., 67.6 units), 88 of these 90 normal sera (97.8%) were below this value. With the patients' sera 76 were above this value (anti-NBII positive patients) and 112 were below (anti-NBII negative patients). This, of course, does not reflect the incidence in a random RA group, in which about 10% of the patients would be positive (personal observations). The mean absorbance value of the 112 anti-NBII negative patients was 22.7 (SD 13.6) units. The 76 anti-NBII positive patients had values ranging from 71 to 1095 units (median value 216.5).

The incidence of antibodies to native type I collagen (a non-cartilage collagen) and to native type IX and XI collagens (minor cartilage collagens) is shown in Table 1 for the three groups. The levels of antibody to these other collagens are shown in Table 2. Sera from patients with RA who had antibodies to native type II collagen had the highest incidence of antibodies to the other native collagens.

Table 2 Levels of the antibodies to the native collagens (types I, IX, and XI) in the positive sera shown in Table 1

Serum	Native antigens			
	I	IX	XI	II
Upper limit of normal	83.8	109.8	129.6	67.6
Normal sera				
A	—*	147	—	—
B	122	—	—	—
C	—	—	—	71
D	—	—	—	90
RA patients anti-II negative				
A	99	122	150	—
B	—	220	201	—
RA patients anti-II positive				
A	98	—	—	184
B	98	—	—	409
C	100	—	—	157
D	123	137	131	358
E	127	—	—	303
F	130	—	—	152
G	136	—	—	155
H	460	—	—	72
I	888	—	—	162
J	—	—	132	595
K	—	—	135	189
L	—	—	149	505
M	—	—	155	314
N	—	143	197	224
O	—	—	235	1095
P	—	—	256	465
Q	—	335	577	650

*The dash (—) indicates below the mean of normals plus three standard deviations (i.e., below the upper limit of normal).

Table 3 Incidence of sera containing antibodies which bind to denatured type I, II, IX, or XI collagens

Collagen	Donors (n=90)	RA anti- native II negative (n=112)	RA anti- native II positive (n=76)
None	85 (94.5)*	77 (68.8)	3 (3.9)
Denatured I	1 (1.1)	15 (13.4)	27 (35.5)
Denatured II	1 (1.1)	20 (17.9)	71 (93.4)
Denatured IX	1 (1.1)	4 (3.6)	9 (11.8)
Denatured XI	2 (2.2)	16 (14.3)	30 (39.5)

*Values are number of sera (%).

The 17 patients with RA who were positive for antibodies to native type II collagen and also positive for other native collagens fell into two distinct groups. One group showed low to high levels of antibodies to native type I collagen (sera A–I) and the other group low to high levels of antibodies to type XI collagen (sera J–Q). The levels of these other antibodies was unrelated to the level of anti-type II collagen antibody. Also the other 59 anti-NBII positive patients had levels of binding to native type II collagen from 71 to 842 units but no antibodies to the other native collagens.

ANTIBODIES TO DENATURED COLLAGENS

The number of sera having antibodies to the denatured collagens is shown in Table 3, and the pattern of binding is shown in Table 4. Only five of the 90 sera from the blood donors showed any binding to the denatured collagens, and this was at low levels only. In the two groups of patients with RA there was a higher incidence of sera containing antibodies binding to the denatured collagens. About 1/3 of the sera from the RA patient group who had no antibodies to native type II collagen had antibodies to one or more of the denatured collagens. There were similar frequencies of antibodies to denatured type I, II, and XI collagens with a much lower frequency of antibodies to denatured type IX collagen. From Tables 3 and 4 it can be seen that many sera contained antibodies to more than one type of denatured collagen.

Seventy three of the 76 sera from the anti-NBII positive patients had antibodies to denatured collagens. Seventy one of these had antibodies to denatured type II collagen either alone (36 sera) or in combination with antibodies to other denatured collagens (35 sera). There was a correlation between the level of antibodies to native and denatured type II collagens in the 71 sera ($\rho=0.69$, $p<0.02$). Thirty sera had antibodies to denatured type XI collagen, and all but one of these had antibodies to

Table 4 Pattern of binding to denatured collagens shown by sera

Collagen	Donors (n=90)	RA anti-native II negative (n=112)	RA anti-native II positive (n=76)
Denatured II only	1 (1.1)*	7 (6.3)	36 (47.4)
Denatured I only	1 (1.1)	3 (2.7)	1 (1.3)
Denatured IX only	1 (1.1)	3 (2.7)	0
Denatured XI only	2 (2.2)	9 (8.0)	0
Denatured II and I	0	6 (5.4)	5 (6.6)
Denatured II and IX	0	1 (0.9)	1 (1.3)
Denatured II and XI	0	1 (0.9)	7 (9.2)
Denatured I and XI	0	0	1 (1.3)
Denatured II, I and XI	0	5 (4.5)	14 (18.4)
Denatured II, IX and XI	0	0	2 (2.6)
All four denatured antigens	0	0	6 (7.9)

*Values are number of sera (%).

denatured type II collagen. The levels of antibodies to denatured type II collagen in the patients' sera were correlated with the levels of antibodies to denatured type XI ($\rho=0.67$, $p<<0.02$) but not to denatured type I collagen ($\rho=0.25$, $p=0.04$). No patient in this group had antibodies only to denatured type XI, whereas in the anti-NBII negative group nine sera had antibodies only to denatured type XI collagen.

Table 5 shows the levels of antibodies to the different denatured collagens in the sera. The highest levels of antibodies to denatured type I and II collagen were seen in the anti-NBII positive patients. There was little difference between the groups in the levels of antibodies to denatured IX or to denatured XI collagens, but the highest level to denatured type IX collagen was seen in the anti-NBII positive group.

ESTIMATED INCIDENCE OF SERA HAVING ANTICOLLAGEN ANTIBODIES

In a group of 100 sera taken from patients with RA at random 10 would be expected to have antibodies to native type II collagen and 90 would be negative (personal observations). Using the above results, we can calculate the expected incidence of sera with antibodies to each of the individual native or denatured collagens (Table 6).

Many patients' sera, of course, will fall into more

than one group. Thus we can calculate that of 100 patients with RA, 62 would have *no* antibodies to these native or denatured collagens, 26 patients would have antibodies to one or more denatured collagen only (I, II, IX, XI), two would have low levels to native type IX, XI and/or type I collagens with antibodies to these denatured collagens too, five would have antibodies to native and denatured type II collagen only, and five would have antibodies to native type II collagen with antibodies to one or more denatured collagens.

CLASS OF ANTIBODIES TO TYPE II AND XI COLLAGENS

Thirty sera from anti-NBII positive patients were tested for IgG, IgA, and IgM anticollagen antibodies separately. All 30 sera had IgG antibodies to native type II collagen, while 25 had IgA and 12 IgM antibodies to this antigen. Similarly, 29 sera had IgG antibodies to denatured type II collagen, while 11 had IgA and 10 IgM antibodies to this antigen. Thus IgG antibodies were most common, but a large number of sera had IgA antibodies as well. The only correlation in antibody levels was shown between IgG antibodies to native and denatured type II collagen.

Nine sera had antibodies to native type XI collagen and 25 to denatured type XI collagen. Of the nine sera positive for antibodies to native type

Table 5 Comparison of antibody levels to denatured collagens in the RA patient groups

Collagen	Upper limit of normal	Highest level in positive normal	RA anti-native II negative		RA anti-native II positive	
			Range	Median	Range	Median
Denatured I	255	341	259- 790	384	275-1532	477
Denatured II	199.2	229	200- 990	341	203-1397	630
Denatured IX	33.6	41	34- 41	37	39- 186	44
Denatured XI	398.3	458	400-1300	509	410-1018	572

Table 6 Calculated incidence of sera containing antibodies to native and denatured type I, II, IX, and XI collagens in a rheumatoid arthritis patient population

Collagen	Incidence (%)	Collagen	Incidence (%)
Native II	10.0	Denatured II	25.5
Native I	2.0	Denatured I	15.6
Native IX	2.0	Denatured IX	4.4
Native XI	2.8	Denatured XI	16.8

XI collagen, one had IgM antibodies only, five had IgG antibodies only, and three had both IgG and IgA antibodies. Of the 25 sera with antibodies to denatured type XI collagen, 20 had IgG antibodies only, four had IgG and IgA antibodies, and one had IgG and IgM antibodies. Thus again IgG antibodies were predominant.

Discussion

From the incidences and patterns of antibodies to native collagens we conclude that the patients were not displaying a general reactivity to their collagens but a selective response. Antibodies to the denatured collagens were more widely distributed, occurring mainly in patients who also had antibodies to native type II collagen, but also in a substantial number of patients without these antibodies. Thus distinctive subsets of patients could be identified in terms of the patterns of antibodies to native and denatured collagens.

Antibodies to the different collagens may consist of antibodies to epitopes unique to each individual collagen or there may be antibodies to common epitopes. Antibodies to particular epitopes may be more important than others in the disease process. Therefore we are investigating the nature of the epitopes on the collagen molecule to which the antibodies seen in these patients bind and whether these subsets of patients can be distinguished either by the stage of their disease, the severity of their joint destruction, by their drug treatment, or by laboratory parameters.

The significance of these antibodies in the initiation or development of RA is unknown, though antibodies to native type II collagen seem to be more specifically associated with RA, whereas antibodies to denatured type II collagen occur more widely in the arthritides.⁵ Articular cartilage, which contains type II collagen as its major collagen type with both type IX and XI collagens as minor collagen components, is degraded in patients with RA. Antibodies to these collagens could be formed during breakdown of the cartilage as the collagens

are exposed, and thus antibodies may be unrelated to the initiation of the arthritis but may have a role in its development and be markers of the extent of existing or potential cartilage damage. Alternatively, immunity to collagens or a foreign antigen with similar epitopes could produce antibodies which then could initiate the inflammatory processes in the joint. Recently, the ability of antibodies to type II collagen from a patient with RA to initiate an inflammatory arthritis in mice has been described.²¹

Whatever the role for these antibodies it probably is not a universal one in all patients with RA. Many patients showed no evidence of any antibodies to these collagens whether native or denatured. Also RA patients with antibodies to native type II collagen form a distinct genetic subset,²² though they could not be distinguished on clinical grounds from the other patients with RA. An individual patient with antibodies to native type II collagen or another collagen at one stage in their disease, however, may be negative for these antibodies at another stage (personal observations). Such changes could be due to fluctuations in disease activity or the treatment.

Although these studies give us a very good idea of the incidence of such antibodies in a RA population, they are but a snapshot in time. Changing patterns of antibodies binding to different collagens and to different epitopes in the same collagen molecule in individual patients may be a better reflection of changes in disease activity and progressions of the disease. Such studies are in progress.

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References

- 1 Andriopoulos N A, Mestecky J, Miller E J, Bradley E L. Antibodies to native and denatured collagens in sera of patients with rheumatoid arthritis. *Arthritis Rheum* 1976; **19**: 613-7.
- 2 Clague R B, Shaw M J, Holt P J L. Incidence and correlation between serum IgG and IgM antibodies to native type II collagen in patients with chronic inflammatory arthritis. *Ann Rheum Dis* 1981; **40**: 6-10.
- 3 Trentham D E, Kammer G M, McCune W J, David J R. Autoimmunity to collagen: a shared feature of psoriatic and rheumatoid arthritis. *Arthritis Rheum* 1981; **24**: 1363-9.
- 4 Clague R B, Moore L J. IgG and IgM antibody to native type II collagen in rheumatoid arthritis serum and synovial fluid. *Arthritis Rheum* 1984; **27**: 1370-7.
- 5 Stuart J M, Huffstutter E H, Townes A S, Kang A H. Incidence and specificity of antibodies to types I, II, III, IV and V collagen in rheumatoid arthritis and other rheumatic diseases as measured by ¹²⁵I-radioimmunoassay. *Arthritis Rheum* 1983; **26**: 832-40.
- 6 Mestecky J, Miller E J. Presence of antibodies specific to cartilage-type collagen in rheumatoid synovial tissue. *Clin Exp Immunol* 1975; **22**: 453-6.

- 7 Trentham D E, Townes A S, Kang A H. Autoimmunity to type II collagen: an experimental model of arthritis. *J Exp Med* 1977; **146**: 857-67.
- 8 Morgan K, Clague R B, Shaw M J, Holt P J L. Native type II collagen-induced arthritis in the rat. I. Incidence and humoral response to collagen. *Ann Rheum Dis* 1980; **39**: 285-90.
- 9 Stuart J M, Cremer M A, Kang A H, Townes A S. Collagen-induced arthritis in rats: evaluation of early immunological events. *Arthritis Rheum* 1979; **22**: 1344-51.
- 10 Morgan K, Clague R B, Shaw M J, Firth S A, Twose T M, Holt P J L. Native type II collagen-induced arthritis in the rat: the effect of complement depletion by cobra venom factor. *Arthritis Rheum* 1981; **24**: 1356-62.
- 11 Staines N A, Hardingham T, Smith M, Henderson B. Collagen-induced arthritis in the rat: modification of immune and arthritic responses by free collagen and immune anti-collagen antiserum. *Immunology* 1981; **44**: 737-44.
- 12 Courteney J S, Dallman M J, Dayan A D, Martin A, Mosedale B. Immunisation against heterologous type II collagen induced arthritis in mice. *Nature* 1980; **282**: 666-8.
- 13 Trentham D E. Collagen arthritis as a relevant model for rheumatoid arthritis. Evidence pro and con. *Arthritis Rheum* 1982; **25**: 911-6.
- 14 Burgeson R E, Hollister D W. Collagen heterogeneity in human cartilage: identification of several new collagen chains. *Biochem Biophys Res Commun* 1979; **87**: 1124-31.
- 15 Ayad S, Abedin M Z, Grundy S M, Weiss J B. Isolation and characterisation of an unusual collagen from hyaline cartilage and intervertebral disc. *FEBS Lett* 1981; **123**: 195-9.
- 16 Morgan K, Evans H B, Firth S A, *et al.* 1 α 2 α 3 α collagen is arthritogenic. *Ann Rheum Dis* 1983; **42**: 680-3.
- 17 Mayne R, von der Mark K. Collagens of cartilage. In: Hall B K, ed. *Cartilage*. Vol 1. New York: Academic Press, 1983: 181-214.
- 18 van der Rest M, Mayne R, Ninomiya Y, Seidah N G, Chretien M, Olsen B R. The structure of type IX collagen. *J Biol Chem* 1985; **260**: 220-5.
- 19 Jackson D S, Cleary E G. In: Glick D, ed. *Methods of biochemical analysis*. London: Interscience, 1967: 32-3.
- 20 Ayad S, Abedin M Z, Weiss J B, Grundy S M. Characterisation of another short-chain disulphide-bonded collagen from cartilage, vitreous and intervertebral disc. *FEBS Lett* 1982; **139**: 300-4.
- 21 Wooley P H, Luthra H S, Singh S K, Huse A R, Stuart J M, David C S. Passive transfer of arthritis to mice by injection of human anti-type II collagen antibody. *Mayo Clin Proc* 1984; **59**: 737-43.
- 22 Klimiuk P S, Clague R B, Grennan D M, Dyer P A, Smeaton I, Harris R. Autoimmunity to native type II collagen—a distinct genetic subset of rheumatoid arthritis. *J Rheumatol* 1985; **12**: 865-70.