Autoantibodies to type II collagen: occurrence in rheumatoid arthritis, other arthritides, autoimmune connective tissue diseases, and chronic inflammatory syndromes

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SUMMARY Serum IgG antibodies to native and denatured human type II collagen (Col II) were measured using an enzyme linked immunosorbent assay (ELISA). One hundred and thirty one patients with various forms of arthritis such as rheumatoid arthritis (RA), ankylosing spondylitis (AS), psoriatic arthritis (PSA), Reiter’s Syndrome (RS), osteoarthritis (OA), and gout, 60 with autoimmune connective tissue disease, and 37 with the chronic inflammatory conditions—graft versus host disease and leprosy—were studied. With the exception of RS, PSA, OA, and gout, significant levels of Col II antibodies were detected in each disease group. Blocking studies with types I and II collagen on selected serum samples confirmed the specificity to native Col II, though some cross reactivity was apparent with denatured collagen. The patients with RA who were Col II antibody positive tended to fall into stage III of disease progression. There was, however, no correlation with rheumatoid factor, erythrocyte sedimentation rate, or disease duration and this, together with the finding that Col II antibodies are present in a wide array of diseases, makes their role in the pathogenesis of RA questionable. They may arise as a secondary disease perpetuating mechanism in some patients, or in turn may be an epiphenomenon secondary to generalised disturbed immunoregulation or B cell hyperreactivity, or both, that characterises these clinical conditions.

Key words: autoimmunity, collagen antibodies, systemic lupus erythematosus, scleroderma, lepromatous leprosy, graft versus host disease.

Rheumatoid arthritis (RA) is an inflammatory disease of unknown aetiology, though it is widely believed that disturbed immunological mechanisms play a part in its pathogenesis.1 Clear cut causative autoimmune mechanisms remain to be identified, however. Type II collagen, a major constituent of joint cartilage, has received much recent attention as a possible autoantigen, to which individuals of a particular genetic background may make an autoimmune reaction responsible for initiation and pathogenesis of the articular disease.

Evidence in support of this hypothesis comes from both animal and human studies. Thus injection of native Col II into rats and mice results in the development of a RA like polyarthritis and production of circulating antibodies to collagen in these animals.2-5 In addition, functional T cells are required for the development of arthritis in these models.6-8 and it is possible to show genetic restriction at the T cell level of antigen recognition, both in the intact animals and at a clonal level in vitro.9-14 Immunohistological studies suggest that T cells may play a part in the effector phase of the Col II induced arthritis,9 though other studies clearly indicate a role for humoral mechanisms, albeit T cell dependent.

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Thus in rats there is a strain dependent correlation between Col II antibodies and development of arthritis.\textsuperscript{15} 16 The arthritis can be transferred by injection of immunoglobulin concentrate or purified Col II antibodies from immunised arthritic rats to healthy recipients.\textsuperscript{4} 17 Collagen induced arthritis in rats and mice has been shown to be mediated by complement-fixing Col II antibodies.\textsuperscript{4} 18 These findings suggest that such antibodies may play a primary part in the initiation and pathogenesis of some forms of arthritis in experimental animals.

Antibodies as well as T cell reactivity to Col II have been described in patients with RA.\textsuperscript{19-26} These findings imply that autoimmunity to Col II may have a role in disease initiation and pathogenesis of RA, as can be shown in animal models.

A number of groups have identified native or denatured, or both, Col II antibodies in patients with RA and have speculated on their potential pathogenetic role. The role of autoimmunity to Col II remains to be clarified in human disease, however, as several factors have emerged which cause its primary role in disease pathogenesis to be questioned. For example, it has become apparent that these antibodies are not confined to RA but can be detected in other rheumatic diseases,\textsuperscript{22-24} and in the case of antibodies to denatured Col II, in a substantial proportion of normal individuals.\textsuperscript{26}

The present study was designed to assess the incidence of antibodies to both native and denatured human Col II in RA, other forms of arthritis, other autoimmune connective tissue diseases, and certain chronic inflammatory conditions. Such a study, including a wider range of diseases than those usually chosen, should help clarify any role of autoimmunity to Col II in the pathogenesis of RA.

**Patients and methods**

**Subjects**

The patient group comprised 76 individuals with classic or definite RA: 55 individuals with other forms of arthritis, including 15 with ankylosing spondylitis (AS), five with Reiter's syndrome (RS), four with psoriatic arthritis (PSA), three with gout, and 28 with osteoarthritis (OA); 60 individuals with other autoimmune connective tissue diseases, including 33 with systemic lupus erythematosus (SLE), 13 with scleroderma (SCL), eight with polymyositis (PM), and six with mixed connective tissue disease (MCTD). After the observation of antibodies in the latter group, 20 individuals with lepromatous leprosy (LL) and 17 with graft versus host disease (GVHD), both chronic inflammatory conditions, were also included in the study. The normal control group consisted of 58 unrelated individuals randomly chosen from healthy blood donors and laboratory personnel.

**Clinical assessment of rheumatoid arthritis**

The patients with RA were all from one clinic and were assessed at the time of venepuncture by one of the two rheumatologists (NWMcG, JRY) according to a standardised protocol. The patients all had classical or definite RA according to American Rheumatism Association criteria.\textsuperscript{27} The progression of the disease was classified as early (stage I), moderate (stage II), severe (stage III), or terminal (stage IV).\textsuperscript{28} Note was made of their erythrocyte sedimentation rate (ESR) and the treatment being received. This information was recorded without knowledge of the Col II antibody results.

The patients were 23 men and 53 women with an age range of 19 to 89 years. All had definite or classical RA\textsuperscript{27} with a disease duration ranging from several months to 50 years. No patient was receiving more than 10 mg prednisone and 59 patients were receiving disease modifying agents (penicillamine, chloroquine, or gold). There was a tendency for patients with antibodies to native Col II to be clustered in stage III of disease progression. The clustering was a little less striking with antibodies to denatured Col II, but still apparent (data not shown).

**Sera**

Blood was obtained by venepuncture, allowed to clot at room temperature, the serum separated by centrifugation at 1000 g and stored in aliquots at \(-70\)°C.

**Preparation of type II collagen**

Human rib cartilage was obtained from infants undergoing postmortem examination for the sudden infant death syndrome. Native Col II was prepared by pepsin solubilisation and selective salt precipitation.\textsuperscript{29} The purity of the collagen was assessed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. The preparation gave a single band at the $\alpha_1$ chain position in the polyacrylamide gel; uronic acid determination\textsuperscript{30} was negative, suggesting that the preparation was free from proteoglycan contamination. The purified collagen was lyophilised and stored at 4°C. Stock collagen solution was prepared by dissolving purified collagen in 0-1 M acetic acid at a concentration of 0-5 mg/ml at 4°C and stored at \(-70\)°C until use. Denatured Col II was prepared by heating an aliquot of stock collagen solution to 56°C for one hour.
ANTICOLLAGEN ANTIBODY ASSAY

An ELISA modified from Engvall and Perlmann was used to measure serum antibodies to collagen. The assay was performed in 96-well flat bottomed, microtitre trays (Linbro 76–301–05, Titertek, Flow Laboratory, VA, USA). Stock native Col II was diluted to 10 μg/ml in 0.05 M carbonate/bicarbonate buffer pH 9.6 (coating buffer). Aliquots of 75 μl were incubated in the wells overnight at 4°C. Denatured Col II was diluted to 5 μg/ml in coating buffer and coating was carried out at 50°C overnight. At the end of the incubation wells were washed with phosphate buffered saline (PBS) pH 7.3 containing 0.05% Tween 20 (PBS-Tween). Non-specific binding sites were blocked by incubating 200 μl of 2% bovine serum albumin in PBS in the wells for one hour at 37°C. After two washes in PBS-Tween, 50 μl of serum diluted 1 in 100 in 0.5% bovine serum albumin in PBS-Tween (ELISA diluent buffer) was then added; this was followed by an incubation of two hours at room temperature. After three washes in PBS-Tween, 50 μl of goat antihuman IgG antibody conjugated with alkaline phosphatase (Tago, Burlingame, CA, USA), diluted 1 in 1000 in ELISA diluent buffer, was added. After overnight incubation at room temperature, wells were washed three times with PBS-Tween and twice with distilled water. Enzyme activity was determined by incubating 100 μl of 1 mg/ml of p-nitrophenyl phosphate (Sigma, St Louis, MO, USA) in carbonate/bicarbonate buffer in the wells at 37°C. Absorbance at 405 nm was measured with an automated Titertek/multiscan spectrophotometer (Flow Laboratory, Helsinki, Finland). In a preliminary series of experiments serum samples were diluted from 1:12.5 to 1:800 and the optical density (OD) measured and plotted against dilution. The dilution chosen above of 1:100 lay within the linear region of the dose-response curve in all serum samples tested. Results were expressed as a ratio of the absorbance of the sample to that of the positive control. The positive control in the two different assays was selected from serum samples showing reactivity to antigen in a preliminary series of experiments with varying doses of antigen and dilutions of serum. The selected serum samples were then used in each assay as an interassay control. The absorbance obtained with these sera was always greater than 10 times the background. The intraassay coefficient of variation for native Col II was 7.5%, for denatured Col II 6%, and the interassay coefficients of variation were 22% and 16% respectively.

Blocking Studies

Cross reactivity between different types of collagen has been reported. Indeed, with the degree of chain sharing between collagen molecules and the similarity of the individual chains one would expect this. To study the cross reactivity between types I and II collagen and between native and denatured forms blocking studies were carried out on selected positive serum samples. Human Col I and Col II were dissolved in 0.1 M acetic acid at 0.5 mg/ml. To 250 μl of serum, diluted 1 in 50 in ELISA diluent buffer, an equal volume of inhibitor, appropriately diluted in the same buffer, was added. The mixture was incubated at room temperature for two hours and residual antibody activities to native and denatured Col II were determined by ELISA. Results were expressed as percentage inhibition, which was calculated as follows:

\[ \frac{OD_{\text{nt} \ of \ serum - (OD_{\text{nt} \ of \ serum + inhibitor})}}{OD_{\text{nt} \ of \ serum}} \times 100 \]

Inhibitor was used at 10 and 30 μg/ml final concentration.

DNAse Treatment of Serum

Double stranded deoxyribonucleic acid (DNA) has been shown to bind to native Col II. To ascertain that the antibody activity detected in the SLE sera was not due to binding of DNA-anti-DNA immune complexes to the collagen used in the ELISA, positive SLE sera were treated with DNase to release any bound anti-DNA antibody and then tested for residual antibody activity.

DNase I (Sigma, St Louis, MO, USA) was dissolved in PBS at 200 μg/ml and 50 μl of serum was incubated with an equal volume of PBS containing 10 μg DNase and 0.3 μmol MgCl₂ for one hour at 37°C, followed by overnight at 4°C and four hours at 37°C. Five microlitres of 1 M tetrabasic sodium edetate in distilled water was added to stop the enzyme action. PBS (400 μl) was then added to create a 1 in 10 dilution of the original serum samples. Control serum was incubated with PBS containing no DNase. After addition of 1 M tetrabasic sodium edetate 400 μl of PBS containing an equal amount of DNase and MgCl₂ was added. Treated and control sera were further diluted 1 in 10 in ELISA diluent buffer to give a final dilution of 1 in 100 and assayed for antibody activity. To ensure that DNase treatment did not interfere with the ELISA, serum samples from RA and normal controls were incorporated in the study.

RHEUMATOID FACTOR (RF) DETERMINATION

Serum samples from patients with RA were titrated for rheumatoid factor (RF) with the Ortho RA test kit (Ortho Diagnostic System, Beerse, Belgium). The method recommended by the manufacturer was followed.
STATISTICAL METHODS
Spearman rank correlation coefficient, Kruskal-Wallis test, and Mann-Whitney rank sum test were performed using the Biosoft statistics package on a Kanga microcomputer. Where multiple comparisons were made the Bonferroni adjustment was incorporated.

Results
COL II ANTIBODIES IN RA AND OTHER ARTHRITIDES
Patients with RA, AS, RS, PSA, OA, and gout are included in this category (Fig. 1). The upper limit of normal was set at three standard deviations (SD) above the mean antibody level of normal controls to Col II. Previous studies have set different levels and this is equivalent to the most stringent. Any serum samples showing a value higher than this were regarded as positive.

Of the 76 patients with RA studied, antibody activity to native Col II was detected in 11, of whom five were negative for antibody to denatured Col II. Denatured Col II antibody was also detected in 12 patients, of whom six showed no antibody activity to native Col II.

There was no correlation between antibody activity and the presence or titre of RF. Of the four patients who showed high antibody activity to native Col II, two were negative for RF and the other two had RF titres of 1/40 and 1/160. In addition, some sera with high RF titre did not have native Col II antibody activity. Similar findings were noted in patients with denatured Col II antibody. There was also no correlation between ESR or disease duration with antibody activity, and patients who were antibody positive did not differ from others with respect to drug treatment (data not shown). These findings are consistent with those reported by others.21 23 except for the incidence of the antibodies, which was slightly lower in the present study.21 23-25 This discrepancy can be explained by the fact that the criterion used to determine a positive sample in the present study was more stringent (3SD) than that used by most others (2SD). Selection of the lower cut off point produced a higher incidence of positives, but this resulted in some positive sera in the normal control group. The upper limit was therefore retained at the 3SD level.

Interestingly, low antibody activities to both native and denatured Col II were detected in patients with AS, and the mean antibody levels were significantly higher (p<0.01) in this group than in the normal control group. These values were only just above the cut off values, and the biological significance of this reactivity remains to be determined.

Antibody activity was not detectable in patients with RS, PSA, and gout. Only a small number of serum samples were studied, however, and it may be premature to conclude that such antibodies are always absent from this group of patients. No antibodies were found in any of the 28 patients with OA.

Fig. 1 Serum IgG antibody activities to (A) native and (B) denatured human type II collagen in patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS), Reiter’s syndrome (RS), psoriatic arthritis (PSA), osteoarthritis (OA), and gout. The mean antibody activity (SEM) and the number of sera studied in each group are shown. The dotted line represents the upper limit, which is 3SD above the mean antibody activity of the normal control group. Positive samples are indicated by black circles.
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**COL II ANTIBODIES IN AUTOIMMUNE CONNECTIVE TISSUE DISEASES**

A total of 60 patients with SLE, SCL, PM, and MCTD were included in this category. Antibodies to native and denatured Col II were detectable in each disease group (Fig. 2). Antibody activity to native Col II was shown by seven patients with SLE, two with SCL, one with PM, and two with MCTD. Antibody activity was, in general, low except for two patients with SLE who had high antibody activity. Mean antibody levels with the exception of SLE were lower in these disease groups than in RA.

The two patients with high antibody to native Col II included one patient who had marked synovitis with a non-erosive arthritis affecting many joints, but, in contrast, the other patient had multisystem disease with mild arthritis. There were other patients within the series with substantial arthritis without antibody to native Col II.

More patients showed antibody activity to denatured Col II, and the activity, in general, was higher. Antibody activity to denatured Col II was found in 14 patients with SLE, four with SCL, two with PM, and three with MCTD. Mean antibody levels were significantly higher in patients with SLE (p<0.005) and SCL (p<0.005) than in the RA group.

The antibodies detected in patients with SLE did not appear to be related to anti-DNA antibody as patients with high anti-DNA antibody did not necessarily have antibodies to Col II, and patients with high antibody activity to Col II did not always have high anti-DNA antibody. In addition, DNase treatment of the serum samples also showed no change in antibody activities to Col II in both native and denatured forms (data not shown).

**COL II ANTIBODIES IN OTHER CHRONIC INFLAMMATORY DISEASES**

As Col II antibodies could be found in such a wide array of diseases it was of interest to investigate other chronic inflammatory conditions, such as GVHD and LL. Arthropathy is not a prominent feature of GVHD and although it can occur in LL particularly after treatment, it was not reported in these patients. Gross immune disturbance is a feature of both.

We studied 17 patients with GVHD and 20 with LL; interestingly, native Col II antibodies were found in 11 of the patients with leprosy and the mean antibody level was significantly higher (p<0.0025) in this group than in the RA group. Low antibody activity was observed in a single GVHD patient. Fig. 3 summarises these results.

Even more striking was the proportion of individuals with antibodies to denatured Col II. The mean antibody levels were significantly higher (p<0.0025) in both groups of patients than in the RA group.

**BLOCKING STUDIES**

Blocking studies using native and denatured forms...
of Col I and Col II were carried out on selected positive serum samples. Ten RA and 10 LL serum samples were tested. It was observed that antibody activity to native Col II could be effectively inhibited by native Col II, but not by denatured Col II or either form of Col I. This confirmed the specificity of the native Col II antibody and, further, showed that the antibody was mainly directed to the conformational determinants dependent upon the triple helix of the native Col II molecule and that these determinants are unique to native Col II, not being shared by Col I.

In contrast, antibody to denatured Col II could be inhibited by both denatured Col II and denatured Col I, though the latter inhibited less effectively than the former; inhibition by native Col II was occasionally observed. Fig. 4 shows examples of such experiments performed on RA and LL serum samples.

Discussion

The data presented here raised a number of issues with regard to antibodies to Col II and their role in human disease. It is necessary to discuss in turn their role in RA and other arthritides, their occurrence and role in other diseases, and to consider the implications of the latter on the former. This study has confirmed the presence of antibodies to native and denatured Col II in RA.22-24 Other investigators have demonstrated antibodies to Col II in autoimmune connective tissue diseases, including SCL and SLE.24 and the results described here support this. Antibodies were not detected to either native or denatured Col II in levels above normal in OA. We did not find antibodies to either native or denatured Col II in normal individuals, though it is worth emphasising that the cut off used in our series was fairly stringent; 3SD above the mean normal value. A less stringent cut off could have been chosen at 2SD above the mean normal value, in which case we would have seen a few positives among the normal controls, but none would have had OD values similar to those of most of the positives seen with RA or the other clinical conditions studied. In this regard our results differ substantially from those...
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Inhibition studies were carried out on selected positive serum samples, and results of such studies performed on a representative RA serum (panel I) and a representative LL serum (panel II) are shown here. Aliquots of the sera were preincubated with native Col II (●), denatured Col II (○), native Col I (■), or denatured Col I (□) and assayed for residual antibody activities to (A) native Col II and (B) denatured Col II by an ELISA. Antibody activity to native Col II was strongly inhibited by native Col II, but not by other collagen types; whereas antibody activity to denatured Col II was inhibited by both denatured Col II and denatured Col I; denatured Col II inhibited the activity to a greater extent than did denatured Col I.

reported by Rowley et al.26 These workers used bovine collagen and a radioimmunoassay and found antibodies to both native and denatured Col II in patients and normal controls. The reason for this discrepancy is not apparent, though a recent study by Ellingsworth et al has shown no correlation between the presence of antibodies to bovine collagen and to human collagen in individuals injected with reconstituted bovine collagen for cosmetic therapy.36 In contrast, other workers have shown a good correlation between antibodies to bovine and human collagen in RA.37 The pattern of our results in RA resembles that found by a number of groups, all of whom used human Col II in their assays.22,24,25 and the reports of their occurrence in normals remain to be satisfactorily explained.

Blocking studies indicate the specificity of the antibodies, with those to native Col II being blocked effectively by native Col II and little, if any, blocking activity occurring with denatured Col II and native or denatured Col I. This suggests that the native Col II antibodies are directed against conformational determinants dependent upon the whole triple helix rather than those seen on individual α₁.
chains. In contrast, antibodies to denatured Col II were occasionally blocked by native Col II, suggesting shared determinants between the triple helix and individual α1 chains. Some blocking was also seen with denatured Col I. Again this is not surprising as denatured Col I contains two α1 chains very similar in structure to those of Col II.\(^{32,36}\)

Neither native nor denatured Col II antibodies show any correlation with rheumatoid factor titres, ESR, or disease duration. There is, however, a suggested relation with the stage of disease progression. Most of the positive antibody results, both to native and denatured Col II, are found in the patients with stage III of disease progression, suggesting that the antibodies parallel extensive, but still active, disease. The antibody activity may be lost as ankylosis occurs in stage IV. In a previous study antibody activity was reported to predominate early in the disease and decline as the disease progressed to chronicity.\(^{39}\) In the study reported here we have not had the opportunity to examine patients in a serial fashion and a direct comparison cannot be made with these findings.

Despite these observations any conclusions about the clinical status of patients with RA and either native or denatured Col II antibodies must be tempered by the observation that only a minority of patients, whatever the stage of disease progression, have either class of antibody.

The pathogenetic role of Col II antibodies is also called into question by the observation of their occurrence in various autoimmune connective tissue diseases such as SLE, MCTD, SCL, and PM. Antibodies to Col II and other subtypes of collagen have been described before in SLE and SCL, and our findings extend these observations. No association was observed between the presence of either native or denatured Col II antibodies and the presence of joint disease, with one exception, though the number of cases studied and the proportion of positive patients overall is small and a larger study would be needed to address this question definitively. The occurrence of antibodies to both native and denatured Col II in GVHD and LL was at first sight even more surprising. We chose to examine the antibodies in LL in more detail and to compare them with those seen in RA as it represented disease clearly distinct from the latter. Joint disease was not a prominent manifestation of these patients but skin disease was and the possibility existed that the antibodies were primarily directed against Col I rather than Col II; in fact, antibodies to denatured Col I have been reported in patients with LL.\(^{40}\) Blocking studies, however, showed that this was not the case with native Col II antibodies as a similar pattern was observed for antibodies from patients with RA and LL, and although some cross blocking was seen with denatured Col II antibodies and Col I, this is hardly surprising in view of the similar structure of the α1 chains of these two collagen types. Thus antibodies both to denatured and native Col II occur in patients with LL with no apparent articular involvement.

Nevertheless, some studies, such as the production of arthritis by transfer of human native Col II antibodies to genetically susceptible mice, together with the demonstration of binding of these antibodies to tissue containing Col II in vivo, do support a role for these antibodies in arthritis.\(^{41}\) There may well be significant differences between the epitopes seen by the antibodies from different diseases, a possibility that still remains to be explored. That this may be possible is suggested by the variation in pathogenetic effect of different monoclonal antibodies raised in mice against Col II and transferred passively to genetically susceptible animals.\(^{42,43}\) Furthermore, there is some evidence that epitopes of Col II are exposed in cartilage of individuals with RA in contradistinction to OA.\(^{44}\) Perhaps this sort of lack of exposure of potential antigenic targets occurs in the diseases without arthritis we have described here.

Irrespective of the pathogenetic role of antibodies to Col II, it is of interest to address the question of how tolerance to this self antigen may be broken, and how the findings presented herein support the various possibilities.

It could be argued that the production of these antibodies may occur in an antigen specific fashion. That is, antigen specific T cells see Col II as foreign and provide helper signals to antigen specific B cells, which differentiate and produce antibody. Some workers have suggested that whereas antibodies to native Col II may have an immunogenetically restricted distribution,\(^{26,45,46}\) antibodies to denatured Col II may not be a primary event, but may be secondary to many types of joint disease when fragments of the normally ‘hidden’ Col II are released in denatured form from articular cartilage as it is broken down.\(^{8,26}\)

In this report neither native Col II antibodies nor, especially, denatured Col II antibodies were found in diseases where secondary joint damage is common, for example OA. In contrast, both classes and, particularly, denatured Col II antibodies were detected in LL and in GVHD, neither group of patients showing joint involvement. These findings argue strongly against the concept of the release of hidden determinants in promoting the development of autoimmunity to either native or denatured Col II.

Alternatively, Col II antibodies may represent the
occurrence of B cell immunodisregulation or the presence of B cell polyclonal activators. Such substances may act directly on B cells or indeed polyclonal activation could occur indirectly via T cells. There is a large amount of evidence to support the presence of non-specific activation of the immune system in RA, autoimmune connective tissue diseases, GVHD, and LL.

It is possible that both native and denatured type II collagen antibodies represent some of the many antibodies produced as a consequence of, particularly, B cell hyperreactivity characteristic of these diseases and thus may be epiphenomena rather than pathogenetic. The results we present here are quite consistent with this hypothesis, but definitive proof awaits further studies.

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