CONCISE REPORT

High anti-collagen type-II antibody levels and induction of proinflammatory cytokines by anti-collagen antibody-containing immune complexes in vitro characterise a distinct rheumatoid arthritis phenotype associated with acute inflammation at the time of disease onset

Mohammed Mullazehi, Linda Mathsson, Jon Lampa, Johan Rönnelid

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Objective: To investigate whether the cytokine-inducing properties of surface-bound collagen type II (CII)-containing immune complexes (IC), which were reported earlier, have any clinical impact.

Methods: Anti-CII serology was analysed in 274 patients with early rheumatoid arthritis (RA). Patients with increased levels of anti-CII were followed serially for 1–5 years with regard to anti-CII IC-induced levels of tumour necrosis factor (TNF) α , interleukin (IL)1 β and IL8. Levels of antibodies and IC-induced cytokines were compared with clinical indices over 5 years of follow-up.

Results: 5/100 healthy controls and 24/274 (8.8%) patients with RA exhibited increased levels (>29 arbitrary units (AU)/ml) of anti-native CII antibodies, a non-significant difference. 9/274 (3.3%) patients with RA and no controls comprised a discrete group with high anti-CII levels >450 AU/ml. These high anti-CII level sera were associated with induction of pro-inflammatory cytokines by anti-CII-containing IC formed in vitro. 8/9 patients with high baseline anti-CII levels exhibited a parallel decline in antibody levels, IC-induced cytokines, C reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Anti-CII-positive patients had significantly increased levels of CRP and ESR at baseline, but not later during the follow-up.

Conclusions: Anti-native CII-positive patients with RA have a distinct clinical phenotype characterised by an early acute phase response that might be driven by anti-CII-containing IC in joint cartilage.

Collagen type II (CII) is the predominant hyaline cartilage collagen. Anti-native CII antibodies occur in between 3% and 27% of patients with RA.^{1–6} Clinical studies have demonstrated that anti-CII-positive patients with RA have increased disease activity, more severe symptoms,⁶ higher serum levels of C reactive protein (CRP), tumour necrosis factor (TNF) α and interleukin (IL)6, and also higher erythrocyte sedimentation rates (ESR).⁷

We have recently described how solid-phase immune complexes (IC) with human native CII and anti-CII antibodies from arthritis sera induced TNF α , IL1 β and IL8 from monocytes via Fc γ RIIa, with a close correlation between anti-CII and cytokine levels.⁸ To determine the clinical significance of these findings, we have now investigated a clinically well-characterised early cohort of patients with RA with regard to anti-CII serology and CII-IC-induced cytokine responses.

PATIENTS AND METHODS

Patients and controls

Two hundred and seventy four patients with RA from a prospective early RA cohort described in Rönnelid *et al.*,⁹ and diagnosed according to American College of Rheumatology were included. Baseline sera were no longer available for 5 of the initial 279 patients. Forty-six of the cases had been included in Mullazehi *et al.*⁸ Serum sampling was performed at baseline, after 3 months and after 1 year in all patients, and also after 2, 3 and 5 years for 74 patients. Clinical data were available from all patients at inclusion, 97.1% at 3 months, 87.5% at 1 year, 86.4% at 2 years, 83.2% at 3 years and 54.1% at 5 years. Table 1 presents the baseline data. One hundred blood donors constituted the control group. All participants gave informed consent to participate in the study that had been ethically approved.

Antibody and cytokine measurements, peripheral blood mononuclear cell preparation

Maxisorp plates were incubated at 4°C overnight with 100 μ l of human native CII (ELISA grade, Chondrex, Redmond, Washington, DC, USA) diluted to 2.5 μ g/ml in ice-cold phosphate-buffered saline (PBS) immediately before coating. Plates were blocked with 150 μ l of 1% bovine serum albumin (BSA) in PBS and subsequently incubated with 100 μ l of sera diluted 1:100. The calibration curve was constructed from an RA serum defined as containing 10⁴ AU anti-CII/ml. The detection antibody against the IgG γ chain (Jackson, Cambridgeshire, UK) was diluted 1:10 000.

Anti-CII-positive samples were re-investigated with parallel control wells only blocked with PBS-bovine serum albumin. Anti-CII-reactive samples having higher optical density (OD) values in CII-coated wells were regarded as positive and the original anti-CII value was used, whereas samples with higher OD levels in control wells were regarded as negative. Among 100 normally distributed control sera, the upper 95th centile yielded a cut-off level of 29 AU/ml.

Methods for purification and culture of peripheral blood mononuclear cell (PBMC), cytokine stimulation by solid-phase IC and supernatant levels of IL1 β , IL8 and TNF α have been described recently.⁸ For practical reasons, cell culture

Abbreviations: AU, arbitrary unit; BSA, bovine serum albumin; CII, collagen type II; CRP, C reactive protein; DAS, disease activity score; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; IC, immune complex; IL, interleukin; OD, optical density; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; RF, rheumatoid factor; TNF, tumour necrosis factor

Table 1 Baseline characteristics of the 274 included patients with rheumatoid arthritis

	All patients with RA (n = 274)	Anti-CII-negative patients with RA (n = 250)	Anti-CII positive (>29 AU/ml) patients with RA (n = 24)	p Value (between anti-CII negative and all anti-CII positive patients)	Anti-CII highly positive (>470 AU/ml) patients with RA (n = 9)	p Value (between anti-CII negative and highly anti-CII positive patients)
Mean age at inclusion (years)	56	55.8	59.4	NS	63.3	NS
Females (%)	70 (193/274)	70 (176/250)	71 (17/24)	NS	67 (6/9)	NS
Mean disease duration at inclusion (months)	5.8	5.8	4.2	0.007	4.4	NS
RF positive (%; number positive/total number)	63 (172/272)	64 (160/249)	52 (12/23)	NS	56 (5/9)	NS
Anti-CP positive (%; number positive/total number)	57 (157/274)	59 (148/250)	37.5 (9/24)	0.040	33 (3/9)	NS
CRP (mg/l)	24	21	53	<0.001	46	0.001
ESR (mm/h)	28	27	36	0.046	41	0.005
Physician's assessment of disease activity (0–4)	2.1	2.1	2.3	NS	2.4	NS
Number of swollen joints	9	9	12	NS (0.071)	10	NS
Number of tender joints	8	8	8	NS	8	NS
DAS28	5	5	5.2	NS	5.5	NS
Global VAS	43	44	39	NS	46	NS
Pain VAS	45	45	38	NS	37	NS
HAQ	0.98	0.98	1.02	NS	1.33	NS
Patients starting DMARD treatment at baseline (%)	84 (230/274)	84 (210/250)	83 (20/24)	NS	89 (8/9)	NS

AU, arbitrary unit; CII, collagen type II; CP, citrullinated protein/peptide; CRP, C reactive protein; DAS, disease activity score; DMARD, disease-modifying antirheumatic disease; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; RA, rheumatoid arthritis; RF, rheumatoid factor; VAS, Visual Analogue Scale.

Values are given as mean values and as percentages (ratios).

Differences between anti-CII-negative and anti-CII-positive patients were analysed using unpaired *t* tests, whereas differences between proportions were analysed using the χ^2 test or Fisher's exact test. Data for anti-citrullinated protein/peptide measured as cyclic citrullinated peptide antibodies have been published earlier.² Data for rheumatoid factor at the time of diagnosis are missing for two patients.

experiments were performed on different days with different PBMC responder cells.

Statistics

Differences between anti-CII-positive and anti-CII-negative patients were analysed using an unpaired *t* test, whereas differences between proportions were analysed using the χ^2 test or Fisher's exact test. For evaluation of changes with time in the group of nine patients with high anti-CII levels, the Wilcoxon signed rank test was performed. *p* Values <0.05 were regarded as significant.

RESULTS

Baseline levels of anti-CII antibodies among 274 patients with RA and 100 controls

In all, 24/274 (8.8 %) of the RA baseline sera were anti-CII antibody positive, a non-significant difference compared with the control group (*p* = 0.14). However, 9/274 (3.3%) of the RA sera had concentrations between 471 and 3521 AU/ml (hereafter denoted "very high anti-CII"). These patients constituted a discrete group of outliers, the next patient having 57 AU/ml. All of the initially anti-native CII-positive RA sera above 29 AU/ml were truly positive—that is, all 24 had higher OD values in CII-coated wells than in wells coated with BSA alone.

All nine patients with initially high anti-CII levels had strikingly diminished anti-CII antibody levels during the 1–5-year follow-up period, with 68–97% reduction compared with baseline (fig 1A). One patient had a late increase in anti-CII antibody levels between 3 and 5 years (patient 3, fig 1A).

Serial investigations of anti-CII and TNF α , IL1 β and IL8 levels induced by anti-CII-containing IC

The borderline anti-CII value for cytokine induction by corresponding solid-phase IC containing CII and anti-CII was between 125 and 500 AU/ml (fig 4B–D in Mullazehi *et al*⁸)—that is, between the controls and most patients with RA on one hand and the nine very high anti-CII RA sera on the other.

When the nine patients with very high baseline anti-CII antibody levels were followed serially with regard to anti-CII IC-induced cytokine responses *in vitro*, eight exhibited marked declines in the production of TNF α (fig 1B). Analysis of IL1 β and IL8 revealed identical patterns (data not included). The patient exhibiting a bimodal appearance of anti-CII also had a parallel increase in the induction of all three cytokines after 5 years (fig 1B, data not included).

The patient with the second highest baseline anti-CII antibody level diverged from the other eight patients with low cytokine levels induced by anti-CII IC on all occasions (patient 2, fig 1A,B). No other RA sera induced significant cytokine production (data not included).

Correlation between clinical parameters and IC-induced cytokine production

All patients but one with very high baseline anti-CII had similar anti-CII levels, solid-phase anti-CII IC-induced cytokine production and clinical response, with falling values for CRP, ESR (fig 1C,D), swollen and tender joint counts, disease activity score using 28 joint counts (DAS)28 and physician's assessment of disease activity (data not included). In patient 3, the biphasic appearance of anti-CII antibody and cytokine induction was accompanied by parallel late increases in CRP, ESR and rheumatoid factor (RF), as well as for DAS28. Patient 2, whose CII-IC did not induce appreciable cytokine levels, had consistently low levels of CRP and ESR (fig 1C,D). All nine patients with very high baseline anti-CII levels exhibited a good (*n* = 4) or moderate (*n* = 5) DAS28 response during the first year. The four patients with the highest anti-CII levels were RF negative and the remaining five were RF positive at baseline.

In all, 20 of 250 initially anti-CII-negative patients turned positive during the follow-up, mostly in the borderline region. Seven patients had sharp increases to >100 U/ml in anti-CII on one or two consecutive occasions. Of these, two showed modest corresponding increases and four decreases or no change in CRP (data not included).

Figure 1 Serial investigations of (A) anti-native human collagen type II (CII) antibodies, (B) induction of tumour necrosis factor (TNF) α by solid-phase anti-CII immune complex (IC) prepared with the same serum anti-CII antibodies according to the Patients and methods section, (C) C reactive protein (CRP) and (D) erythrocyte sedimentation rate (ESR) from the nine patients with rheumatoid arthritis (RA) having the highest anti-CII values at the time of diagnosis (Diagn) in a cohort of 274 patients. The patients are numbered (in B) according to anti-CII levels. Seven patients were followed for 1 year, whereas two patients were followed for 5 years. Owing to practical limitations, all patients could not be investigated using the same peripheral blood mononuclear cell (PBMC) responder cells. Six different PBMC donors were used. Patient sera investigated with the same PBMC donor were patients 2 and 7; 3 and 4; and 5 and 8. ESR was missing for patient 4 at the 1-year visit. CII-IC-induced production of interleukin (IL)1 β and IL8 was comparable to the results for TNF α . To also obtain quantitative CRP values in the normal range, the depicted samples had been reanalysed with high-sensitivity CRP measurement (Immagine, Beckman Coulter, Fullerton, California, USA). Significances (paired comparisons) are given as * $p < 0.05$ or ** $p < 0.01$ compared with baseline. No asterisk implies that no significance was determined.

No difference in treatment between anti-CII-positive and anti-CII-negative patients with RA

There was no difference between percentage of anti-CII-positive and anti-CII-negative patients starting with disease-modifying anti-rheumatic disease (DMARD) treatment at the time of diagnosis (table 1), or with regard to choice of DMARDs (data not included; for DMARD distribution, see Rönnelid *et al*⁹).

Anti-CII antibody-positive patients are characterised by high baseline inflammatory activity

Anti-CII positive patients had higher CRP and ESR than anti-CII-negative patients at baseline, but not later during follow-up (table 1, fig 2). Besides a few weakly significant differences (all

with anti-CII positive patients having the lowest responses), anti-CII-positive and anti-CII-negative patients did not differ in other parameters (fig 2). Anti-CII-positive patients had shorter disease duration before diagnosis (table 1).

DISCUSSION

A minority of patients with RA have highly increased levels of anti-CII clearly distinguished from the otherwise normal distribution, thereby comprising a separate outlier subgroup. These patients have a distinct phenotype with significantly increased CRP and ESR values at baseline, paralleled by CII-IC-induced cytokine production in vitro. In parallel with declines in antibody levels with time, such patients exhibit reductions in

Figure 2 Line graphs depicting changes in mean clinical indices detailed in table 1 for anti-collagen type II (CII)-positive (anti-CII+, n = 24) and anti-CII-negative (anti-CII-, n = 250) patients treated separately. Statistical comparisons were performed between anti-CII+ and anti-CII- patients at the indicated time points, where * denotes $p < 0.05$ and *** denotes $p < 0.001$. No asterisk implies that no significant difference was determined.

CII-IC-induced production of pro-inflammatory cytokines, in parallel with reductions in laboratory and clinical disease activity. Collectively, these data suggest that high baseline anti-CII levels are associated with acute inflammation generated through IC-stimulated cytokine production. Anti-CII-positive patients have significantly decreased disease duration before diagnosis, probably due to this acute inflammation.

The fraction of high anti-CII antibody-positive patients with RA corresponds to the most conservative prevalence (3%) of anti-CII published.¹ Both haemagglutination¹ and our functional cell stimulation assays use techniques requiring cellular attachment via anti-CII, indicating that few patients have sera with such properties.

Anti-CII antibodies are produced in RA joints,^{10, 11} but not in the periphery.¹¹ The lower incidence of RF among anti-CII-positive RA in this and other⁶ studies might be caused by retention of RF in the joint space by binding to anti-CII-containing IC. The serum with the next highest baseline anti-CII level did not induce pro-inflammatory cytokines. This patient had low levels of CRP and ESR throughout the follow-up period. This might be due to differences in epitope specificities, hypotheses that we will study further. Patients with RA differ considerably with regard to anti-CII epitope specificities.^{5, 12–15}

Patients with and without anti-citrulline antibodies investigated in the same RA cohort did not differ with regard to clinical indices at baseline, but thereafter showed increasing differences relating to swollen joint count.⁹ It is intriguing that anti-CII and anti-citrulline associate with different clinical signs and symptoms at diverse time points after diagnosis, and also associate negatively with one another (table 1), thereby representing distinct clinical phenotypes.

In conclusion, our data support the hypothesis that patients with early RA with anti-native CII have a unique phenotype where the early inflammation might be driven by cartilage-bound IC.

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Authors' affiliations

Mohammed Mullazehi, Linda Mathsson, Johan Rönnelid, Unit of Clinical Immunology, Department of Oncology, Radiology and Clinical Immunology, Uppsala University, Uppsala, Sweden
Jon Lampa, Unit of Rheumatology, Department of Medicine, Karolinska Institute, Stockholm, Sweden

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Correspondence to: Dr J Rönnelid, Unit of Clinical Immunology, Rudbeck Laboratory C5, SE-75185 Uppsala, Sweden; johan.ronnellid@klinimm.uu.se

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REFERENCES

- Greenbury CL, Skingle J. Anti-cartilage antibody. *J Clin Pathol* 1979;**32**:826–31.
- Gioud M, Meghlaoui A, Costa O, Monier JC. Antibodies to native type I and II collagens detected by an enzyme linked immunosorbent assay (ELISA) in rheumatoid arthritis and systemic lupus erythematosus. *Coll Relat Res* 1982;**2**:557–64.
- Stuart JM, Huffstutter EH, Townes AS, Kang AH. 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.
- Morgan K, Clague RB, Collins I, Ayad S, Phinn SD, Holt PJ. Incidence of antibodies of native and denatured cartilage collagens (types II, IX, and XI) and to type I collagen in rheumatoid arthritis. *Ann Rheum Dis* 1987;**46**:902–7.
- Terato K, Shimozuru Y, Katayama K, Takemitsu Y, Yamashita I, Miyatsu M, et al. Specificity of antibodies to type II collagen in rheumatoid arthritis. *Arthritis Rheum* 1990;**33**:1493–500.

- 6 Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. Antibodies to type II collagen in early rheumatoid arthritis—correlation with disease progression. *Arthritis Rheum* 1996;**39**:1720–7.
- 7 Kim WU, Yoo WH, Park W, Kang YM, Kim SI, Park JH, *et al*. IgG antibodies to type II collagen reflect inflammatory activity in patients with rheumatoid arthritis. *J Rheumatol* 2000;**27**:575–81.
- 8 Mollazehi M, Mathsson L, Lampa J, Rönnelid J. Surface-bound anti-type II collagen containing immune complexes induce production of TNF- α , IL-1 β and IL-8 from peripheral blood monocytes via Fc γ RIIa. A potential patho-physiological mechanism for humoral anti-collagen type II immunity in RA. *Arthritis Rheum* 2006;**54**:1759–71.
- 9 Rönnelid J, Wick MC, Lampa J, Lindblad S, Nordmark B, Klareskog L, *et al*. Longitudinal analysis of anti-citrullinated protein/peptide antibodies (anti-CP) during 5 year follow-up in early rheumatoid arthritis: anti-CP status is a stable phenotype that predicts worse disease activity and greater radiological progression. *Ann Rheum Dis* 2005;**64**:1744–9.
- 10 Tarkowski A, Klareskog L, Carlsten H, Herberts P, Koopman WJ. Secretion of antibodies to types I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. *Arthritis Rheum* 1989;**32**:1087–92.
- 11 Rönnelid J, Lysholm J, Engstrom-Laurent A, Klareskog L, Heyman B. Local anti-type II collagen antibody production in rheumatoid arthritis synovial fluid. Evidence for an HLA-DR4-restricted IgG response. *Arthritis Rheum* 1994;**37**:1023–9.
- 12 Buckee C, Morgan K, Ayad S, Collins I, Clague RB, Holt PJ. Diversity of antibodies to type II collagen in patients with rheumatoid arthritis: detection by binding to alpha-chains and to cyanogen bromide peptides. *Br J Rheumatol* 1990;**29**:254–8.
- 13 Cook AD, Rowley MJ, Stockman A, Muirden KD, Mackay IR. Specificity of antibodies to type II collagen in early rheumatoid arthritis. *J Rheumatol* 1994;**21**:1186–91.
- 14 Burkhardt H, Koller T, Engström A, Nandakumar KS, Turnay J, Kraetsch HG, *et al*. Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse. *Arthritis Rheum* 2002;**46**:2339–48.
- 15 Cook AD, Gray R, Ramshaw J, Mackay IR, Rowley MJ. Antibodies against the CB10 fragment of type II collagen in rheumatoid arthritis. *Arthritis Res Ther* 2004;**6**:R477–83.

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