Serum Antibodies Against Intact Human Collagen IX Are Elevated at Onset of Rheumatoid Arthritis But Are Not Related to Development of Erosions

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ABSTRACT. ***Objective***. To measure the presence of autoantibodies binding to intact human recombinant collagen IX and assess their usefulness as a diagnostic marker and an indicator of disease activity in rheumatoid arthritis (RA).

Methods. Recombinant human full-length collagen IX (rCIX) was produced in a baculovirus expression system and purified for use in ELISA developed to detect antibodies to native and denatured collagen IX. Fifty-three patients with recent-onset rheumatoid factor-seropositive RA were analyzed for the presence of rCIX antibodies of the IgG type at the time of initial diagnosis and after 3, 6, 12, and 24 months of followup. The RA sera were accompanied by 30 controls. Associations were determined between patients' antibody titers, development of erosions in the hands and feet, and various clinical and laboratory markers.

Results. Serum antibody levels among patients with RA at time of diagnosis were 1.78 times higher against native rCIX ($p < 0.001$) and 1.71 times higher against denatured rCIX ($p < 0.001$) than in the controls, and they remained high during the followup. No correlation was seen between antibody levels and clinical and laboratory findings.

Conclusion. Our data show that patients with recent-onset RA have significantly elevated levels of autoantibodies to human rCIX. These autoantibodies were observed already at the early stages of the disease, which may reflect their diagnostic potential in RA. (First Release Mar 15 2008; J Rheumatol 2008;35:745–51)

Key Indexing Terms:
COLLAGEN IX RHEUMATOID ARTHRITIS AUTOANTIBODY
DIAGNOSIS ENZYME LINKED IMMUNOSORBENT ASSAY

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation that typically destroys joint structures and may lead to severe disability. It currently affects about 0.8% of the world's adult population[1]. Early diagnosis and treatment of RA may be difficult because of the variable and often unpredictable course of the disease. New information and new markers regarding the pathogenetic process are therefore needed to improve the diagnostics and predictability of RA[2].

Anticollagen antibodies are implicated in the pathogenesis of RA. For instance, susceptible strains of rodents and nonhuman primates immunized either with collagen II or XI have been found to develop autoreactive antibodies resulting in a collagen-induced arthritis that bears immunological, pathological, and phenotypic similarities to RA[3-6]. The presence of collagen II (CII) antibodies in the sera of humans with RA was noted as early as 1976[7], and high frequencies of these antibodies have also been detected in rheumatoid synovial fluid[8,9] and cartilage specimens[10,11]. Subsequently, antibodies binding to CII have been detected in RA sera on multiple occasions[12-15] and quite recently those binding to the citrullinated form of CII also[16,17]. Further, IgG-producing B cells specific to CII are present in the rheumatoid synovium[18] and synovial fluid[19,20], suggesting the presence of a local antigen-driven immune process. This concept is supported by the fact that the synovitis in RA is known to remit within a particular joint after the removal of cartilage at

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arthroplasty[21], while the rest of the affected joints remain inflamed. In addition, the pathogenic potential of these antibodies has been demonstrated in experiments in which purified human or murine anti-CII antibodies were shown to be able to transfer arthritis passively to susceptible recipients[22-24]. It has recently been shown that CII-C1 antibodies, recognizing the C1 conformational epitope of CII, impair cartilage formation in cultured chondrocytes, strongly inhibit the self-assembly of CII *in vitro*, and cause disorganization of CII fibrils and loss of proteoglycan and CII in the extracellular matrix[25-28], providing evidence that these antibodies can contribute directly to cartilage destruction. Further, immune complexes containing anti-CII antibodies from patients with arthritis have been shown to induce the production of inflammatory cytokines such as tumor necrosis factor-α, interleukin 1ß (IL-1ß), and IL-8 *in vitro*[29]. The role of immune complexes containing anti-CII has also been shown in a clinical setting, as the patients with very high anti-CII levels seem to represent a distinct phenotype characterized by significantly elevated cytokine profiles as well as elevated C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) at baseline[30]. Interestingly, the C1 epitope targeted by these antibodies is also recognized by human IgG antibodies in RA sera[31]. Antibodies to CII are also known to correlate with inflammatory activity and disease progression in RA[32,33].

RA is characterized by an inflammatory process that is accompanied by degradation of articular cartilage. As the alterations in cartilage matrix turnover take place early in the disease process, long before any significant damage can be detected radiographically, it is crucial to find sensitive, noninvasively detectable biochemical markers of cartilage matrix homeostasis. We hypothesized that collagen IX (CIX) would be an excellent marker candidate because of its location on the surfaces of CII heterotypic fibrils and its susceptibility to enzymatic digestion. This idea was supported by the findings of Kojima and colleagues, who demonstrated using a rabbit model of inflammatory arthritis that loss of the NC4 domain of CIX is an early event that precedes any detectable damage to CII[34]. In line with these findings, synovial fluids from patients with arthritic diseases have also been shown to contain fragments of CIX[35]. Moreover, it has been demonstrated that CIX is able to effectively stimulate the production of prostaglandin E_2 and IL-1 by monocytes[36], and that transgenic mice lacking CIX develop a more severe form of autoimmune arthritis[37]. Finally, it has been shown that rCIX is able to induce tolerance and suppress arthritis in a murine model of collagen-induced arthritis[38]. These observations also support the idea that CIX may play a role in RA.

Our aim was to investigate the presence of autoantibodies binding to intact human rCIX in RA, and to assess if they could be used as a diagnostic tool or as an indicator of disease activity.

MATERIALS AND METHODS

Patients and controls. The series consisted of 53 patients, 36 women and 17 men, with a mean age of 50 years, all of whom had received a baseline diagnosis of early RA at the Rheumatism Foundation Hospital, Heinola, Finland, in 1988-1991 and fulfilled the classification criteria of the American College of Rheumatology (ACR) for RA[39]. The duration of their symptoms at the time of the initial diagnosis was less than 12 months (median duration 6 mo) and the patients had received no previous antirheumatic medication. Only rheumatoid factor (RF)-positive patients were included. Baseline clinical and demographic data are shown in Table 1. The patients then had followup visits at 3, 6, 12, and 24 months, and were treated with disease modifying antirheumatic drugs (DMARD) in accord with the "saw-tooth strategy," reflecting the possibilities available at that time (the initial drug being either aurothiomalate or sulfasalazine) and with glucocorticoids when necessary. The study from which our samples derive was initially launched by Prof. M. Nissilä (retired) in the Rheumatism Foundation Hospital to compare the effects of aurothiomalate and sulfasalazine in the treatment of RA. The results of that study have not been published. The original serum samples were discarded in 2003. However, a small quantity diluted 1:10 with 1% bovine serum albumin in phosphate buffered saline was available.

The control samples consisted of consecutively blindly chosen samples of 20 women and 10 men [mean age 63 yrs, standard deviation (SD) 10] whose sera were sent to be tested for *Helicobacter pylori* infection at the Department of Medical Microbiology, University of Oulu. There are no data available on their possible symptoms or diseases. Our study was approved by the ethical committee of the Päijät-Häme Hospital District in which the Rheumatism Foundation Hospital is located.

Laboratory tests and clinical examination. Laboratory tests for ESR, CRP, RF, and thrombocyte count were performed. RF was determined both by latex fixation and by Waaler-Rose tests and was regarded as positive when ≥ 32. Clinical measures including duration of morning stiffness, number of swollen and tender joints, and patient's global assessment on a visual analog scale (VAS) were assessed by a trained rheumatologist. Physical function as measured by a Health Assessment Questionnaire (HAQ) was also recorded. Radiographs of the hands and the feet were obtained at the entry into the study and at 24 months, scored by the method of Larsen in sequence by 1 rheumatologist who was unaware of the treatment and clin-

Table 1. Baseline demographic, clinical, and radiographic characteristics of 53 seropositive patients with RA.

Variables	Values
Demographic	
Sex (female/male)	36/17
Age, yrs, mean (SD)	50 (10)
Duration of disease, mo, median (IQR)	6 (4, 0)
Measures of disease activity	
Erythrocyte sedimentation rate, mm/h, median (IQR)	30 (17, 64)
C-reactive protein, mg/l, median (IQR)	14 (2, 38)
Thrombocyte count, 10^9/l, median (IQR)	336 (281, 409)
Duration of morning stiffness, min, median (IQR)	90 (60, 120)
No. of swollen joints, mean (SD)	14 (9)
No. of tender joints, mean (SD)	16 (10)
Patient's global assessment (VAS; 0–100 mm), mean (SD)	43 (20)
Physical function (HAQ; 0–3), median (SD)	0.25 (0.00, 0.81)
Radiographic	
No. of erosive joints in hands and feet, median (IQR)	0 (0, 1)

SD: standard deviation; IQR: interquartile range; VAS: visual analog scale; HAQ: Health Assessment Questionnaire.

ical features of the patients. Joints with a Larsen score ≥ 3 were regarded as erosive and were taken into account in the further analyses.

Production and purification of collagen IX. Recombinant human collagen IX was produced as described[40]. Briefly, *Trichoplusia ni* insect cells (High Five, Invitrogen) grown in suspension at 27°C were seeded at 1.0–1.5 × 10^6 cells/ml in Sf900 II SFM medium (Invitrogen) and supplemented with 5% fetal bovine serum. The cells were coinfected with 3 viruses coding for the α1(IX), α2(IX), and α3(IX) chains and a double-promoter virus 4PH αß coding for the α- and ß-subunits of human prolyl 4-hydroxylase with multiplicities of infection of 2:2:2:2, respectively. Ascorbate (80 µg/ml) was added to the culture medium daily. After 72 h of infection, the cells were harvested from the culture medium by centrifugation. Proteins were precipitated from the culture medium by adding solid ammonium sulfate to 25% saturation and placing the mixture on ice for 1 h with mixing. The precipitate was collected by centrifugation at 15,000 × g for 1 h at 4°C, and the pellet was dissolved in 0.1 M Tris, 10 mM EDTA, 0.4 M NaCl, 2 M urea buffer, pH 7, with proteinase inhibitors (Roche Applied Science) overnight. The dissolved recombinant protein was applied to a Superdex 200 gel filtration column (Amersham Biosciences) in the same buffer and subsequently purified by Resource S or HiPrep CM (Amersham Biosciences) cation exchange chromatography in a 50 mM PIPES, 20 mM NaCl, 2 M urea buffer, pH 6.5, eluting with an increasing salt concentration (0.02–1 M NaCl). The purified rCIX was analyzed by SDS-PAGE and subjected to amino acid analysis in an Applied Biosystems 421 analyzer. The concentrated rCIX (Amicon Ultra, 30,000 molecular weight cutoff, Millipore) was stored in 50 mM acetic acid at –20°C.

ELISA for anticollagen IX antibodies. Native or denatured rCIX (1 µg/ml) in PBS were incubated in Costar 3591 microplates (Corning) overnight at 4°C in order to detect antibodies to rCIX. Denatured rCIX was prepared by heating at 55°C for 30 min. After coating, the plates were washed 3 times for 1 min with PBS containing 0.05% Tween (PBST) and blocked for 45 min with Stabilguard commercial blocking agent (Surmodics). The plates were then incubated with patient sera at a 1:200 dilution for 1 h at 4°C with mild shaking. The bound immunoglobulins were detected by using HRP-labelled anti-human IgG secondary antibody (Jackson Immuno Research Laboratories) and Blue Star commercial HRP substrate, TMB (Diarect AG, Freiburg, Germany). The reaction was stopped after 5 min by adding 50 µl of 0.3 M sulfuric acid and the optical densities were measured with a Dynex plate reader at 450 nm. All samples were analyzed in duplicate. If a difference > 15% was detected, the samples were reassayed. IgG binding to bovine serum albumin (nonspecific or background binding) was determined for each sample in parallel wells and the results were expressed as arbitrary optical density units. The specificity of the antibody binding to rCIX was determined in a competition ELISA. Aliquots of 2 different sera were incubated in the absence or presence of indicated amounts of rCIX or ovalbumin (10 or 30 µg) overnight at 4°C, and the IgG binding to rCIX was measured in an ELISA as described above. The interassay coefficients of variation for native and denatured collagen IX were 18.2% and 15.6%, respectively.

Statistical analysis. The results were expressed as means or medians, with SD or ranges and 95% confidence intervals (CI) obtained by accelerated bias-corrected bootstrapping (5000 replications). Statistical comparisons between the groups were made using a bootstrap-type t-test. Correlation coefficients were calculated by the Spearman method, using Sidak-adjusted probabilities.

RESULTS

Characterization of rCIX. The structural properties of the purified rCIX were analyzed by SDS-PAGE gel, which showed 1 band in nonreducing conditions and 3 equivalent bands in reducing conditions indicating the structural integrity of collagen. The amino acid content was also analyzed and found to correspond to that of native tissue-extracted collagen IX (data not shown).

rCIX autoantibodies at the baseline. The demographic and disease-related characteristics of the 53 patients are shown in Table 1. Figure 1 shows the baseline (before treatment) serum IgG antibody levels to native and denatured rCIX in patients with RA and control subjects. The mean (SD) baseline IgG antibody binding to native rCIX was significantly higher in the sera of RA patients (0.361 ± 0.124) than in the controls (0.203 ± 0.117) (95% CI 0.102–0.212: $p < 0.001$; Figure 1A). Also, the IgG binding to denatured rCIX was significantly higher in the patients with RA (0.926 ± 0.230) than in the controls (0.541 ± 0.241) (95% CI 0.278–0.491: $p < 0.001$; Figure 1B). There were no measurable IgM or IgA autoantibodies binding to native or denatured rCIX in RA or control samples, when measured in a subset of 15 samples (data not shown). The IgG antibodies binding to the native and denatured collagen were associated with each other ($\rho = 0.642$, $p < 0.001$).

Analysis of specificity. To investigate the specificity of the IgG binding to rCIX a competition ELISA was performed (Figure 2). First, a serum dilution curve of RA patient IgG binding to rCIX was carried out (Figure 2A). Then the ability of RA patient IgG to bind to rCIX in the presence or absence of soluble rCIX as a competitor was measured. Figure 2B demonstrates that incubation with rCIX resulted in over 90% reduction in the IgG binding to rCIX when 10 or 30 µg of rCIX was added as a competitor, while incubation with nonspecific ovalbumin showed no effect, indicating high specificity of IgG binding to rCIX.

rCIX autoantibodies during the followup. To assess if the IgG antibody levels to rCIX among the patients with RA were related to the disease activity the patients were followed for 24 months. The median count of joints with erosion was 0 [interquartile range (IQR) 0, 1] at baseline and the increase during the 2-year followup was 3 (95% CI 1–5). The IgG antibody levels to rCIX among all the patients remained highly elevated throughout the 24-months followup and remained notably higher against denatured compared to native antigen (Figure 3). The autoantibody levels to native ($p = –0.07$) and denatured ($p = –0.03$) rCIX (area under the curve) did not correlate with radiological progression during the 2-year followup as judged by the number of erosive joints in the hands and feet. Also, no relationships between the antibody levels to rCIX and the disease activity could be demonstrated during the followup when the disease activity was measured by ESR, CRP, thrombocyte count, duration of morning stiffness, number of swollen or tender joints, VAS, or HAQ (data not shown).

DISCUSSION

We found significantly elevated levels of autoantibodies towards rCIX in patients with RA. All the patients included in our study had presented with recent-onset RF-seropositive disease. This suggests that analysis of rCIX autoantibodies, possibly accompanied by other markers such as RF

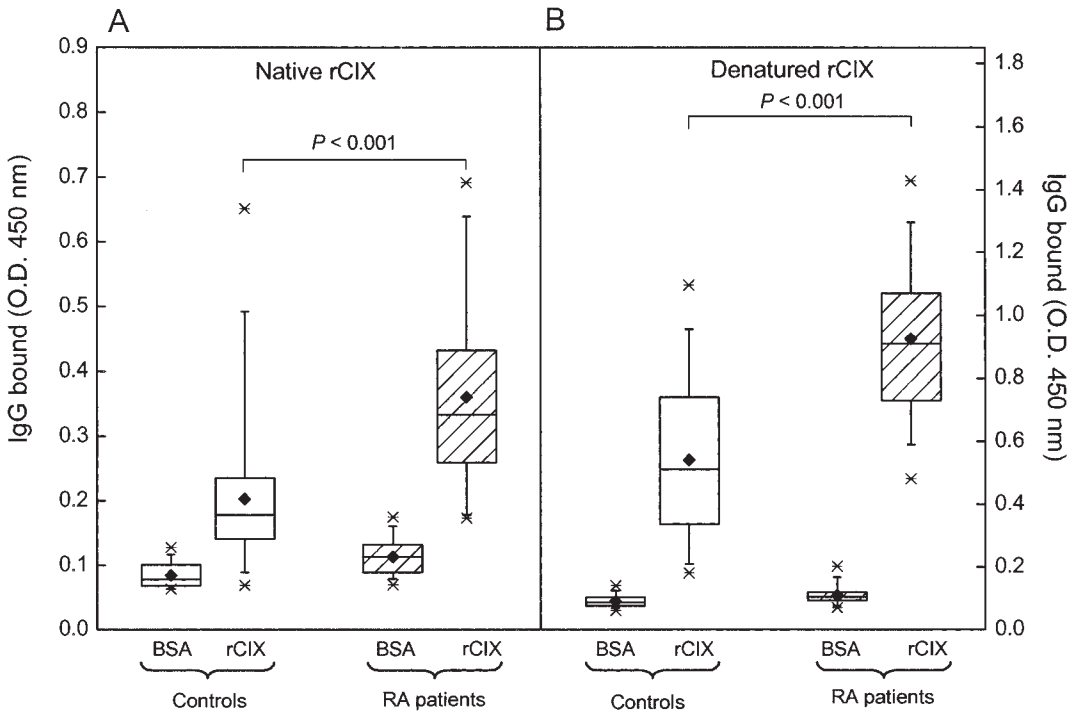

Figure 1. Serum levels of IgG autoantibodies to native (A) and denatured (B) rCIX among 53 seropositive patients with RA and 30 controls. Box plot identifies the middle (50%) of the data, the median (line across the box), and the mean (the solid square). Whiskers represent 5% to 95% of samples; stars show minimum and maximum. The difference between patients and controls was 1.78-fold for native rCIX (95% CI 0.102–0.212: $p < 0.001$) and 1.71-fold for denatured rCIX (95% CI 0.278–0.491: $p < 0.001$).

or cyclic citrullinated peptide, may aid in the diagnosis in cases with early RA.

There are several lines of evidence to indicate that cartilage-specific collagens may play a role in autoimmune-mediated arthritis[3-6,8-11,16-24,29-35,37]. One of the first critical steps in the disease process is the breakdown of the collagenous network that is essential for the maintenance and integrity of the tissue. This process is either accompanied by or preceded by the formation of collagen-related autoantibodies. It is not known, however, whether these antibodies initiate the inflammatory process in the joint or whether they are formed during cartilage breakdown, being unrelated to the initiation of arthritis. Experiments with certain antibodies binding to collagen II[25-28] suggest that they may interfere with the structural integrity of cartilage and precede or possibly initiate the inflammatory expression of disease, thus representing an important component of pathogenesis. Despite some controversy concerning the role of collagen antibodies in the pathogenesis of RA, these observations favor the view that collagen-related autoimmunity occurs during the early initiating events in the development of RA. Nevertheless, independent of our understanding of the disease mechanism, autoantibodies binding to collagen or degradation products of collagen may reflect the biochemical status of the joint and thus serve as diagnostic or prognostic tools.

To our knowledge there are no previous studies published on autoantibodies binding to intact full-length collagen IX in humans, and only 1 report showing increased antibodies against pepsin-extracted bovine CIX in patients with RA[41]. We utilized recombinant human-like collagen as an antigen instead of pepsin-digested tissue-derived material. With this approach we aimed to have an antigen that resembles that encountered *in vivo,* being intact and containing the nonhelical regions that may function as natural epitopes. This is particularly important in the light of results by Wotton and colleagues, who found collagen IX fragments containing significant amounts of noncollagenous material in the synovial fluids of arthritic patients[35]. This observation suggests that these fragments may serve as antigenic targets for autoimmunity. The fact that CIX antibodies were significantly elevated among the patients with RA in our study contrasts to 2 previous reports[42,43] suggesting that the noncollagenous regions in collagen IX show a potential as RA epitopes. The recombinant material is also devoid of impurities that can cause cross-reactivity, such as other cartilage collagens that might be present in tissue-extracted collagen preparations.

Our data show that serum levels of IgG binding to rCIX were significantly higher in the RA patient sera as compared to the controls. This difference was almost 2-fold in magnitude and was not affected by age. The levels of rCIX anti-

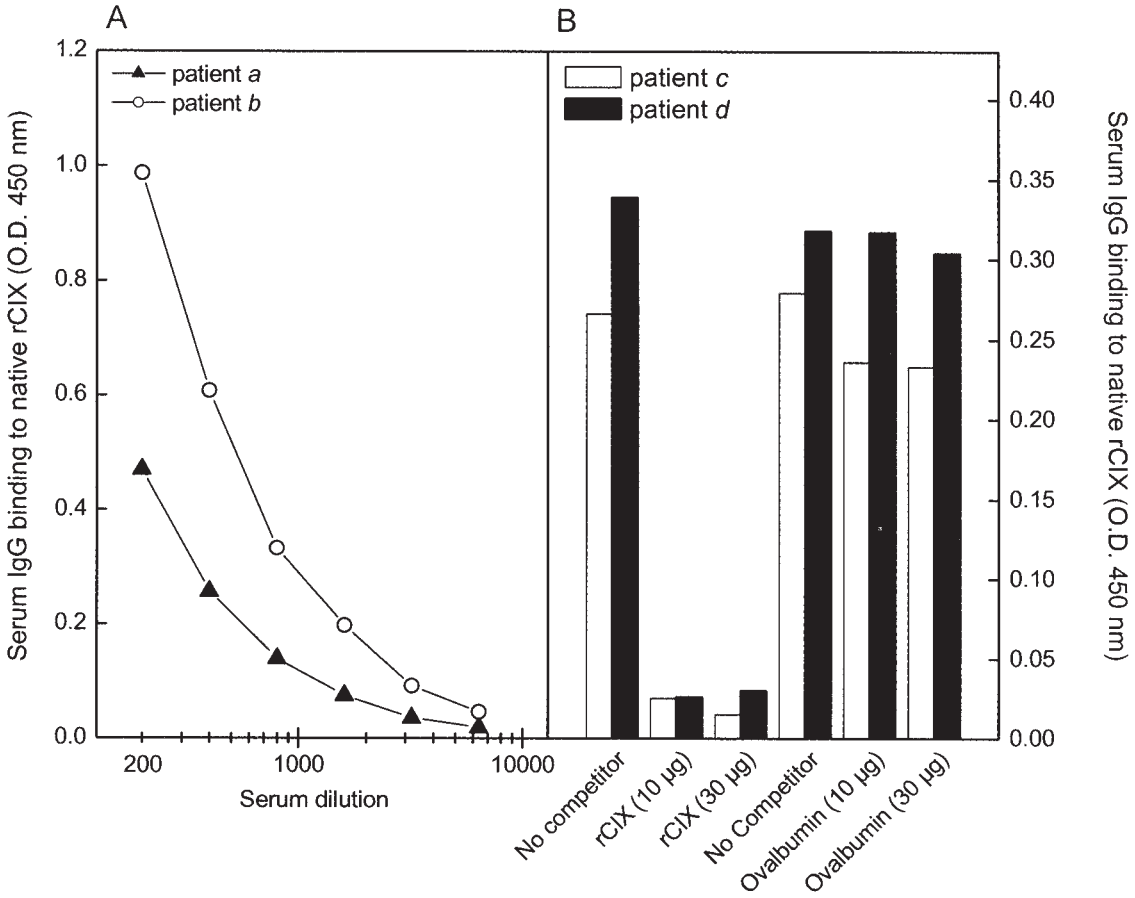

Figure 2. A. Serum dilution curves of IgG binding to native rCIX of 2 patients with RA. B. Specificity of IgG binding to rCIX, measured in a competition assay with 1:200-diluted patient sera in the presence or absence of indicated amounts of competitors. rCIX was able to specifically abolish almost all the binding, while ovalbumin showed no effect, reflecting the high specificity of IgG binding to rCIX.

bodies remained elevated, showing a very slight decrease towards the end of the followup, but did not approach those of the controls. This kind of attenuation of collagen antibodies over time is frequently observed in RA[14,44]. In the control population very low or low levels of rCIX antibodies were commonly detected, but few sera displayed high reactivity. The latter finding might be due to unknown joint pathology, since no exclusion criteria were applied to the controls at study entry.

A possible connection between CIX antibody status and inflammatory activity in RA is interesting but unexplored. In our study there was no association found between anti-CIX levels and inflammatory or disease activity during an initial 2-year followup (data not shown). Further effective longterm clinical followup was precluded because of substantial dropout of subjects from the cohort. The fact that no correlation was observed between rCIX antibody levels or laboratory or clinical measures and radiological progression is in line with the report by Charrière, *et al*[41] and indicates that antibodies to rCIX may not be a suitable marker to reflect disease activity, the response to treatment, or the outcome in RA, although measurement of rCIX antibodies might be useful in the diagnosis of early RA. This assumption is supported by a number of reports. When antibodies to different types of collagen were studied in the sera of patients with various arthritic diseases, the only antibodies found at high frequencies were those against collagen IX in RA patients[41]. However, low frequencies with collagen IX antibodies have also been demonstrated[42,43]. Further, the frequencies of RA patients with antibodies to CII may be as high as 60%–75% in the early stages of the disease[13,14,44] but fall to 24%–30% later, as shown by a cross-sectional approach in cases of well established RA[32]. Another interesting observation is that CII antibodies precede the appearance of RF in most patients[13], and that a loss of the NC4 domain of CIX, in turn, precedes detectable damage to CII[34].

Early detection of RA is vital, but often problematic, especially because the ACR criteria are best suited for the detection of clinically manifest, well established disease. The optimal time for initiating therapy aimed at achieving a remission is very early in the course of the disease, which

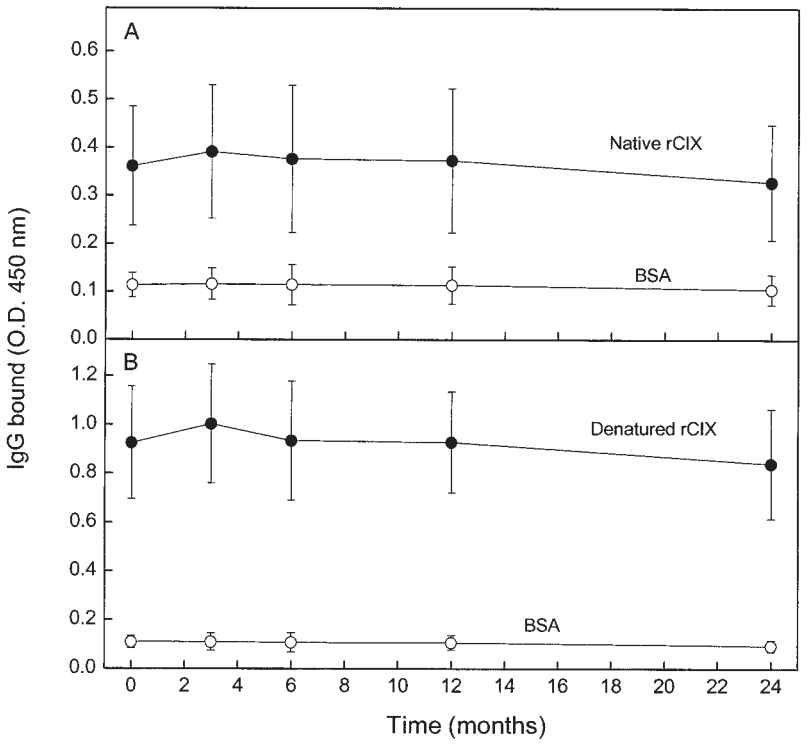

Figure 3. Serum levels of IgG binding to native (A) and denatured (B) rCIX among 53 seropositive patients with RA at the time of diagnosis and during followup. Reactivity toward rCIX remained elevated throughout the course of the disease and was typically higher with denatured than with native rCIX. Data are mean ± SD.

requires early diagnosis[45-47]. If rCIX antibodies were to prove suitable as a predictive marker for early RA, this would be a valuable asset for RA diagnosis, especially in cases where the diagnosis is not clear on the basis of conventional laboratory and clinical findings. It is not currently known, however, whether the rCIX antibodies are associated with RA specifically or with chronic arthritis and joint damage in general.

Our results lead us to conclude that rCIX antibodies emerge early in RF-seropositive RA, being highly elevated already at the time of diagnosis. The presence of these antibodies does not reflect disease activity or outcome, however. These results create a basis for further studies to clarify the potential usefulness of rCIX antibodies as a tool in the diagnosis of early RA or inflammatory arthritis in general, and may contribute to our understanding of the role of collagen autoimmunity in the pathogenesis of RA.

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