T Cell Proliferative Response to Type II Collagen in the Inflammatory Process and Joint Damage in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To investigate the role of T cell responses to type II collagen (CII) in disease progression in patients with rheumatoid arthritis (RA).

Methods. T cell proliferative responses to bovine CII by peripheral blood mononuclear cells (PBMC) from patients with early RA (duration < 5 yrs) were assayed by mixed lymphocyte culture. Clinical and laboratory variables including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were examined at the time of sampling. Radiographic damage on hand radiographs was evaluated by the method of Steinbrocker and Sharp.

Results. In a cross sectional study, patients (n = 22) with positive T cell responses (stimulation index ≥ 2) had higher levels of CRP and ESR than those (n = 21) not showing T cell responses. The number of damaged joints (by Steinbrocker's method) and damaged joint scores (by Sharp's method) were significantly higher in patients with positive T cell responses than in those without. The joint space narrowing scores correlated well with T cell responsiveness to CII. Patients (n = 15) with both positive T cell responses and RA-susceptible allotypes HLA-DR1 or DR4 had higher damaged joint scores than the remainder of the patients (n = 24).

Conclusion. T cell proliferative responses to CII are associated with inflammatory activity and radiographic severity in RA. RA-susceptible allotypes positively relate to the radiographic progression associated with T cell responses to CII. Our data suggest that CII-reactive T cells may play a role in the pathogenic process of joint damage, especially in genetically susceptible patients. (J Rheumatol 2005;32:225–30)

Key Indexing Terms:
RHEUMATOID ARTHRITIS TYPE II COLLAGEN T CELLS
INFLAMMATORY ACTIVITY RADIOGRAPHIC PROGRESSION

Autoimmunity to type II collagen (CII) has been suggested to play a role in the pathogenesis of rheumatoid arthritis (RA). Immunization of susceptible mice with CII leads to the development of an autoimmune polyarthritis[1]. Lymphocytes responding to CII have been identified in the peripheral blood and joints of patients with RA[2,3]. Antibodies to CII have also been found in the sera, synovial fluid, and cartilage in patients with RA[4,5].

It is generally accepted that T cell mediated autoimmunity plays a pivotal role in the pathogenesis of RA. In collagen induced arthritis, an experimental model of RA, CII-reactive CD4+ T cell lines have been reported to transfer disease to naive mice[6,7]. We reported that T cell responses to CII or the immunodominant synthetic peptide CII (255–274) were enhanced in human RA, especially in early-stage disease[3]. T cell responses to CII were more frequent and vigorous in synovial fluid than in peripheral blood, suggesting that CII-reactive T cells play an important role in the inflammatory process of RA.

Rheumatoid synovitis is characterized by synovial hyperplasia and infiltration by inflammatory cells such as lymphocytes including autoreactive T cells and monocytes into the synovium, which eventually lead to cartilage and bone destruction. It has been reported that autoreactive T cells are strongly associated with the clinical and pathological progression of several T cell mediated autoimmune diseases[8-10]. If we assume that CII is a pathogenetic autoantigen, CII-reactive T cells also could be a close marker of disease progression. However, it has not yet been determined whether CII-reactive T cells play a part in the progression of joint destruction or whether they predict clinical exacerbation of RA. To investigate the role of CII-reactive T cells in disease progression of RA, we studied T cell responses to CII in

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peripheral blood mononuclear cells (PBMC) from RA patients and compared them to patients' clinical, laboratory, and radiographic findings.

MATERIALS AND METHODS

Patients. Forty-three RA patients whose disease duration was less than 5 years were studied (33 women, 10 men). They all fulfilled the revised criteria of the American Rheumatism Association for RA[11]. Patients' mean age was 45.1 years (range 18–65). The mean disease duration was 30.1 months (range 5–58 mo). All medications were stopped 48 h before the T cell assays.

Cell isolations and antigens. Heparinized peripheral blood was collected under sterile conditions and diluted 1:1 with RPMI-1640. Mononuclear cells in peripheral blood were isolated by density gradient centrifugation on Ficoll-Hypaque (SG 1077). Cell viability was > 95% by trypan blue exclusion. Lyophilized bovine CII, a generous gift from Dr. A.H. Kang (University of Tennessee, Memphis, TN, USA), was dissolved in 0.1 N acetic acid at 1 mg/ml, dialyzed against 50 mM Tris, 0.2 M NaCl, and then sterilized by filtering through a 0.2 µm micropore filter.

Assay for T cell proliferative response to CII. Cells were resuspended in complete medium consisting of RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Mononuclear cells were then separated immunomagnetically into T cells and non-T cells using anti-CD3 micro-beads (Miltenyi Biotec, Auburn, CA, USA). Non-T cells were γ-irradiated with 3000 rad and used as antigen-presenting cells. Each culture was performed in triplicate at a density of 1×10^5/well for both T cells and non-T cells in 96 round-bottom microtiter wells (Nunc, Roskilde, Denmark). Forty micrograms/well of native CII were then added to the wells. Forty micrograms/well of ovalbumin and 1 µg/well of phytohemagglutinin (PHA, Gibco BRL, Grand Island, NY, USA) were used as the negative and positive controls, respectively. Ten micrograms/well of synthetic peptide CII (255–274; ^{255}TGEBGIAGFKGEQGPKGEBG^{274}; Emory University Microchemical Facility, Atlanta, GA, USA) were also used as the immunodominant peptide of CII[12]. The plates were incubated at 37°C in 5% CO_2 for 5 days. Before the last 12 h of culture, 0.5 µCi of ^{3}H-thymidine (NEN, Boston, MA, USA) was added to each well. Cells were harvested onto nitrocellulose, and the incorporated radioactivity was measured in a ß-scintillation counter. The data are presented as stimulation indices (SI), calculated as the ratio of cpm in the presence of antigen/cpm without antigen. T cell proliferative responses were considered positive if the stimulation index (SI) was ≥ 2 and if the increase of cpm (Δ cpm) was > 1000.

Clinical and laboratory assessment. Clinical and laboratory assessments were done at the time of sampling. Clinical variables consisted of the pattern of RA, functional status according to the American College of Rheumatology revised criteria[13], the presence of rheumatoid nodules, and medications used previously. The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), and hemoglobin were measured as laboratory variables. The RF titer was determined by nephelometry and values > 20 IU/ml were considered positive. ESR was measured by the recommendation of the International Committee for Standardization in Hematology by the Westergren method.

Radiographic examination. The following peripheral joints were assessed radiographically at the time of sampling: the wrists, carpal bones, and metacarpophalangeal, proximal interphalangeal and distal interphalangeal joints. Articular damage was scored according to the methods of Steinbrocker[14] and Sharp[15]. By the Steinbrocker method, the joints were scored on a scale of 1–4, where 1 = soft tissue swelling, 2 = erosions only, 3 = erosions and joint space narrowing (JSN), and 4 = total joint destruction or ankylosis. According to the modified Sharp method, 17 areas of each hand and wrist were scored for erosions and 18 areas were scored for JSN. Hand radiographs were read by a radiologist and 2 rheumatologists who were blind to the patients' information and the results for T cell reactivity to CII. The intraobserver coefficient of variation (CV) for radiographic findings was below 6%.

HLA typing. Each HLA-DR allele was genotyped by reverse-dot hybridization. The primers and probes for genotyping of generic DR types were designed according to the 11th International Histocompatibility Workshop[16].

Statistical analysis. Since the various data sets were not normally distributed, results were expressed as medians (range). Comparisons of numerical data between groups were by the Mann-Whitney rank-sum test or Kruskal-Wallis test, and categorical data by a chi-square test, when appropriate. Correlation between 2 variables was performed using Spearman's rank correlation coefficient. P values less than 0.05 were considered statistically significant.

RESULTS

T cell proliferative response and demographic features of patients. T cell responses to CII were positive (SI ≥ 2) in 22 (51.2%) of 43 patients with early RA (disease duration < 5 yrs). Comparing demographic features between the patients with positive T cell responses (n = 22) and those with negative T cell responses (n = 21), there were no significant differences in sex, age at diagnosis, and disease duration (Table 1). Patients with positive T cell responses to CII had a higher SI to CII (255–274) than those with negative responses [median (range) SI with CII (255–274): 2.12 (1.06–5.38) vs 1.40 (0.58-2.40); p < 0.001; Table 1]. T cell responses to CII (n = 42) strongly correlated with responses to the immunodominant peptide of CII (255–274) (r = 0.574, p < 0.001). No differences were found in SI in cells exposed only to medium, ovalbumin, or PHA between patients with positive responses to CII and those without (Table 1).

Correlation of T cell responses to CII with inflammatory markers. To determine the clinical significance of CII-reactive T cells in RA, we compared several clinical and laboratory indicators and the medications used previously between patients with and without positive T cell responses. As shown in Table 2, there were no differences in the pattern of RA (intermittent vs continuous), functional status, presence of rheumatoid nodules, anemia, RF titers, prednisone dosage, the number and kinds of disease modifying antirheumatic drugs (including methotrexate, antimalarials, sulfasalazine, bucillamine, and gold) taken previously, and HLA-DR4 or DR1 positivity between the 2 groups. However, patients with positive T cell responses had higher median levels of CRP and ESR than those without [median CRP 22.3 (range 3.4–137.0) vs 9.9 (range 3.1–134.0), p = 0.026; median ESR 55 (range 17–110) vs 41 (range 5–104), p = 0.043; Table 2].

Correlation of T cell responses to CII with radiographic severity. Using Steinbrocker's classification, the median number of joints with JSN (score 3) and overall damaged joints (score 2–4) was significantly higher for the patients with positive T cell responses to CII (n = 22) than those without (n = 21) [11 vs 2 for JSN (p = 0.022), 16 vs 5 for overall damaged joints (p = 0.019); Table 3]. The median

Table 1. Demographic features and T cell proliferative responses in patients. Data are presented as median (range), except sex.

Group Tested, Demographics, and T Cell Response	Positive T Cell Response, n = 22	Negative T Cell Response, n = 21	p†
Female/male, n	16/6	17/4	NS
Age at diagnosis, yrs	46 (18–65)	48 (22–64)	NS
Disease duration, mo	31 (5–58)	33 (11–54)	NS
T cell responses			
Medium only, cpm	6345 (2120–15500)	5980 (3400–14200)	NS
CII (255–274)**, SI	2.1 (1.06–5.4)	1.4 (0.6–2.4)	< 0.001
Ovalbumin response, SI	1.1 (0.8–1.8)	1.1 (0.9–1.7)	NS
PHA response, SI	8.8 (2.5–25.2)	11.4 (2.4–26.2)	NS

** Synthetic peptide of a T cell reactive sequence. † Significance of differences in patients with positive T cell response to CII versus those with negative responses. NS: not significant. SI: stimulation index, cpm in wells containing antigen/cpm in wells without antigen. SI ≥ 2 and increase in cpm (Δ cpm) > 1000 is defined as positive T cell response to bovine type II collagen (CII).

Table 2. Comparison of clinical and laboratory features and medications used previously between patients with positive T cell responses to CII and those with negative T cell responses.

Group Tested, Clinical and Laboratory Characteristics	Positive T Cell Response, n = 22	Negative T Cell Response, n = 21	p
Pattern of RA, n (%)			
Intermittent	5 (22.7)	6 (28.6)	NS
Continuous	17 (77.3)	15 (71.4)	NS
Functional status, n (%)			
Class I	5 (22.7)	6 (28.6)	NS
Class II	16 (72.7)	14 (66.6)	NS
Class III	1 (4.5)	1 (4.8)	NS
Rheumatoid nodules, n (%)	7 (31.8)	4 (19.0)	NS
Laboratory features			
Anemia, n (%)	7 (31.8)	7 (42.9)	NS
Rheumatoid factor, IU/ml*	80.3 (10.3–749.0)	52.0 (9.9–789.0)	NS
ESR, mm/h*	55 (17–112)	41 (5–104)	0.043
CRP, mg/l*	22.3 (3.4–137.0)	9.9 (3.1–134.0)	0.026
Prednisone dose, mg/day*	5 (0–10)	5 (0–10)	NS
Percentage treated with			
Methotrexate	68.2	76.2	NS
Antimalarial	59.1	57.1	NS
Sulfasalazine	36.4	23.8	NS
Bucillamine	27.3	23.8	NS
Gold	9.1	4.8	NS
DR4 positive, % (n)	38.5 (8/21)	55.6 (10/18)	NS
DR4 or DR1 positive, % (n)	71.4 (15/21)	72.2 (13/18)	NS

* Data are presented as median (range). See Table 1 for definitions.

number of mildly damaged joints (score 2) or markedly damaged joints (score 4) tended to be higher in patients with positive T cell responses, but it was not statistically significant. Using the modified Sharp criteria, the JSN scores and total damaged joint scores (sum of erosion scores and JSN scores) were also significantly higher for patients with positive T cell responses than for those with negative responses [median (range) JSN scores: 21 (0–59) vs 0 (0–40), p = 0.004; median (range) total damaged joint scores: 25 (0–80) vs 3 (0–54), p = 0.011; Table 3]. The JSN scores correlated well with T cell responsiveness to CII (r = 0.305, p = 0.05). The median erosion scores also tended to be higher in patients with positive T cell responses. However, T cell reactivity with medium only, ovalbumin, or PHA did not correlate with the radiographic severity assessed by either Steinbrocker or Sharp criteria (data not shown).

Effect of HLA-DR4 or DR1 positivity on the radiographic progression associated with positive T cell responses. To investigate the influence of RA-susceptible alleles on the radiographic progression associated with T cell responses,

Table 3. Radiographic severity of the patients with T cell responses to CII versus those without. Data are presented as median (range).

Group Tested, Radiographic Findings	Positive T Cell Response, n = 22	Negative T Cell Response, n = 21	p
No. of damaged joints using Steinbrocker criteria			
Erosions only	5 (0–10)	3 (0–11)	0.159
Joint space narrowing	11 (0–23)	2 (0–23)	0.022
Total joint destruction or ankylosis	0 (0–9)	0 (0–5)	0.120
Any damage**	16 (0–33)	5 (0–37)	0.019
Damaged joint score using modified Sharp method			
Erosion score	4 (0–22)	3 (0–14)	0.152
Joint space narrowing score	21 (0–59)	0 (0–40)	0.004
Total damaged joint score†	25 (0–80)	3 (0–54)	0.011

** Sum of number of joints with erosion, joint space narrowing, and total joint destruction. † Sum of erosion score and joint space narrowing score. See Table 1 for definitions.

we divided the patients (n = 39) into 4 groups according to T cell responsiveness to CII and the presence of HLA-DR4 or DR1: Group 1, patients (n = 15) with both HLA-DR4(+) or DR1(+) and positive T cell response to CII; Group 2, patients (n = 13) with DR4(+) or DR1(+), but negative T cell response to CII; Group 3, patients (n = 6) with DR4(–) and DR1(–), but positive T cell response to CII; and Group 4, patients (n = 5) all negative to the HLA subgroups as well as to response to CII. There was no significant association between HLA-DR4 or DR1 positivity and positive T cell responses. Comparison of radiographic severity using the Sharp method between the 4 groups showed that Group 1 had significantly higher total damaged joint scores than Group 2 ($p < 0.05$), and tended to have higher levels of total damaged joint scores than Group 3 or Group 4 (median levels of total damaged joint scores: Group 1 = 26, Group 2 = 3, Group 3 = 11, Group 4 = 3). In particular, Group 1 had significantly higher levels of erosion, JSN, and total damaged joint scores than the sum of the other 3 groups (Group 2 + Group 3 + Group 4, n = 24) [median (range) erosion scores: 5 (1–21) vs 2.5 (0–14), p = 0.05; median (range) JSN scores: 23 (0–59) vs 2 (0–41), p = 0.03; median (range) total damaged joint scores: 26 (0–80) vs 4.5 (0–54), p = 0.04; Figure 1].

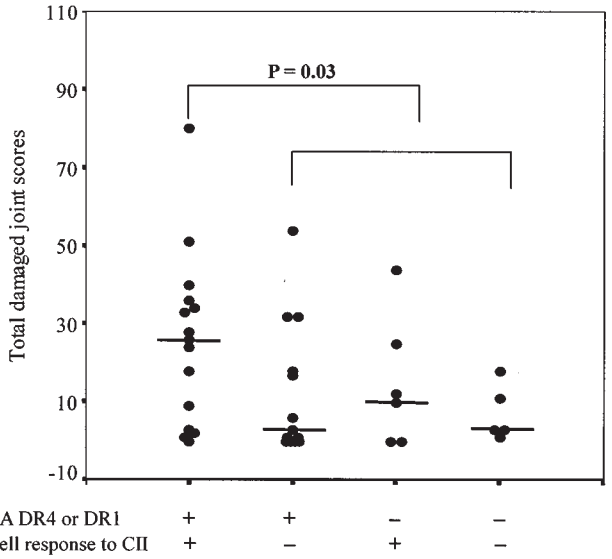

Figure 1. Effect of HLA-DR4 or DR1 positivity on the radiographic progression associated with positive T cell responses. Vertical axis indicates total damaged joint scores using the Sharp method, in patients (n = 15) with HLA-DR4(+) or DR1(+) and positive T cell response to CII; patients (n = 13) with DR4(+) or DR1(+), but negative T cell response to CII; patients (n = 6) with DR4(–) and DR1(–), but positive T cell response to CII; and patients (n = 5) with all negative response. Bars represent medians. P value is the significance of differences in patients with both HLA-DR4(+) or DR1(+) and T cell response to CII(+) compared to other patients.

DISCUSSION

Although the etiologic agent(s) of RA remains unknown, CII is an attractive candidate as an autoantigen because of its abundance in cartilage, and because of its ability to induce destructive polyarthritis in rodents and higher primates[1,12,17]. We reported previously that T cell responses to CII were enhanced in patients with RA compared to patients with osteoarthritis and healthy controls[3]. In addition, T cell responses to bovine CII correlated strongly with responses to human CII, which contains the same CII (255–274) sequence, the major immunodeterminant of T cells in the animal experimental model of RA[12]. Based on the previous reports, we investigated the clinical significance of T cell responsiveness to bovine CII, which was more evident in the early stages of RA but decreased over time.

In this study in RA patients with disease duration of less than 5 years, T cell responses to CII correlated strongly with those to the synthetic peptide CII (255–274), which confirms the previous finding that immunodominant peptide can be recognized by rheumatoid T cells as an autoantigen

like the whole molecule of CII. Importantly, patients with positive T cell responses to CII had higher levels of inflammatory markers including CRP and ESR than patients with negative T cell responses, suggesting that the number of CII-reactive T cells in PBMC depends on the inflammatory status of the RA. It has been reported that CII-stimulated synovial fluid mononuclear cells secrete higher levels of interleukin 6 (IL-6) and IL-1ß in patients with RA than in other forms of inflammatory arthritis[18]. A study in our laboratory also showed that stimulation indexes with CII correlate well with IL-2, interferon-γ, tumor necrosis factor-α, and IL-12 levels in culture supernatants of PBMC from RA patients[19]. Consequently, it seems that CII-reactive T cells may be tightly linked with the production of inflammatory cytokines, possibly by the interaction between T cells and antigen-presenting cells, and thereby provoke exacerbation of RA disease activity and elevated ESR and CRP.

Accumulating evidence indicates the importance of T cell activity to an autoantigen in the progression of several T cell-mediated autoimmune diseases. In patients with insulin-dependent diabetes mellitus, T cell reactivity to ß cell antigens in the peripheral blood reflects disease progression as well as the destruction of the ß cells[8,20,21]. In a study of multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system characterized by infiltration of T cells and macrophages, an increased frequency of activated in contrast to resting myelin basic protein (MBP)-reactive T cells was found in patients with severe disease[9]. Autoreactive T cells responding to MBP are expanded clonally and accumulate in MS lesions[10]. RA resembles the aforementioned diseases in the evidence that T cells, possibly Th1 cells, play an important role in pathogenesis.

We demonstrate here that the number of overall damaged joints (Steinbrocker method) and total damaged joint scores (Sharp method) were significantly higher in patients with positive T cell responses to CII. It is difficult to determine whether the presence of a T cell response may play an initiating role in joint destruction or be a secondary effect from the joint damage. Nevertheless, these data provide the clue that T cell responses to CII may be associated with disease severity and progression in RA, as in other Th1-mediated autoimmune diseases[8-10,20,21]. In this context, antigen-driven active suppression of autoreactive T cell responses to CII, such as CII antigen-specific tolerance induction or CII reactive-T cell receptor peptide vaccination, could be useful to prevent the disease progression of RA.

Cartilage breakdown products have been implicated in the progressive cartilage damage of RA[22,23]. In our study, a higher total damaged joint score in patients with positive T cell responses was mainly attributed to a significantly higher number for JSN, indicating cartilage destruction. Moreover, T cell responsiveness to CII correlated well with the severity of JSN. These findings, together with earlier reports, suggest that a higher amount of CII or CII breakdown peptides released from the joints with cartilage destruction may stimulate CII-reactive T cells more potently, followed by oligoclonal expansion of the autoreactive T cells. Because the major source of CII is articular cartilage, it is conceivable that CII-reactive T cells may be expanded specifically in the joints by the repetitive challenge of CII or CII breakdown peptides, with subsequent overflow into the circulatory system. In this case, peripheral CII responsiveness could reflect the pathologic status of cartilage degradation in the multiple involved joints of RA. This possibility is also supported by the findings where ß-chain gene analyses of the CII-reactive T cell receptor showed that some of the clonally expanded T cells responding to CII in the peripheral blood were identical to expanded T cell clones in the synovium[24], and that 40% of the accumulated T cell clonotypes in one joint were found in multiple joints in the same patient[25].

The association between HLA-DRB1 subtypes, particularly DR1 and DR4, and genetic susceptibility to RA is well established[26,27]. Moreover, we and others have reported that the presence of RA-susceptible alleles including HLA-DRB1*0405 is a prognostic marker for severity and radiographic progression of RA[28,29]. Thus, it was important to determine the influence of HLA-DR types on the radiographic progression associated with T cell responses to CII. In this study, a greater radiographic progression was more frequently found in patients with both positive CII responses and DR4/DR1 allotypes than the other groups, suggesting that both factors may be required for the rapid progression of RA.

In our study, the lack of correlation of T cell responses to CII with DR1 and DR4 requires comment. In CII-induced arthritis, an animal model of RA, the DR4 and DR1 expressed as transgenes can confer susceptibility to an otherwise resistant strain of mice by influencing T cell immunity to CII[30]. In human RA, it has been reported that T cell responses to CII are inhibited by DR-specific antibody[31], and that augmented cell-mediated responsiveness to CII is associated with DR4[32]. We did not analyze our patients for the DR4 subtype or RA susceptibility motifs QKRAA and QRRAA. Again, we did not test the T cell responses to whole-molecule human CII or those to 3 portions of the human CII chain, CII (74–93), CII (254–273), and CII (924–943), major immunodeterminants of T cells in an animal experimental model of RA[33], which have been identified in context with DR1 and DR4. Therefore, it is uncertain if the T cell responses detected may or may not be associated with either motif. Further study on a large scale would be required to clarify this issue.

In summary, T cell proliferative responses to CII were associated with inflammatory activity and radiographic progression, especially with JSN indicating cartilage destruction, in patients with early RA. RA-susceptible allotypes positively relate to the radiographic progression associated

with the T cell responses to CII. Our data suggest that peripheral T cell responsiveness to CII in RA could be useful to assess inflammatory activity and to predict disease severity in genetically susceptible patients. CII-reactive T cells could be targets for intervention treatment to avert CII hyperreactivity.

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