Primary research

Interferon-γ production in response to in vitro stimulation with collagen type II in rheumatoid arthritis is associated with HLA-DRB1*0401 and HLA-DQ8

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Statement of findings

Interferon-γ was measured in supernatants after in vitro stimulation of peripheral blood mononuclear cells with collagen type II (CII), purified protein derivative or influenza virus. Interferon-γ production in response to CII was similar in rheumatoid arthritis (RA) patients and healthy control individuals. The interferon-γ response to purified protein derivative and influenza virus was lower in RA patients, reflecting a general T-cell hyporesponsiveness in RA. After recalculating the response to CII taking this hyporesponsiveness into account the CII response was higher in RA patients, and was associated with human leucocyte antigen (HLA)-DRB1*0401 and HLA-DQA1*0301-DQB1*0302 (HLA-DQ8). Rheumatoid arthritis patients with elevated serum levels of immunoglobulin (Ig)G anti-CII antibodies had lower CII-induced interferon-γ production than patients with low anti-CII levels. The relative increase in CII-reactivity in RA patients as compared with healthy control individuals, and the association of a higher response with RA-associated HLA haplotypes, suggest the existence of a potentially pathogenic cellular reactivity against CII in RA.

Keywords: collagen type II, human leucocyte antigen-DR, interferon-γ, rheumatoid arthritis, T cell

Abstract

Introduction: Despite much work over past decades, whether antigen-specific immune reactions occur in rheumatoid arthritis (RA) and to what extent such reactions are directed towards joint-specific autoantigens is still questionable. One strong indicator for antigenic involvement in RA is the fact that certain major histocompatibility complex (MHC) class II genotypes [human leucocyte antigen (HLA)-DR4 and HLA-DR1] predispose for the development of the disease [1]. In the present report, collagen type II (CII) was studied as a putative autoantigen on the basis of both clinical and experimental data that show an increased frequency of antibodies to CII in RA patients [2–4] and that show that CII can induce experimental arthritis [5].

It is evident from the literature that RA peripheral blood mononuclear cells (PBMCs) respond poorly to antigenic stimulation [6–8], and in particular evidence for a partial tolerization to CII has been presented [9]. The strategy of the present work has accordingly been to reinvestigate T-cell reactivity to CII in RA patients, to relate it to the response to commonly used recall antigens and to analyze interferon-γ responses as an alternative to proliferative responses.

Aims: To study cellular immune reactivity to CII in patients with RA and in healthy control individuals and to correlate this reactivity to HLA class II genotypes and to the presence of antibodies to CII in serum.

CII = collagen type II; HLA = human leucocyte antigen; HLA-DQ8 = HLA-DQA1*0301-DQB1*0302; Ig = immunoglobulin; MHC = major histocompatibility complex; PBMC = peripheral blood mononuclear cell; PBS = phosphate buffered saline; PPD = purified protein derivative; RA = rheumatoid arthritis.
Rheumatoid arthritis (RA) peripheral blood mononuclear cells (PBMCs) respond similarly to collagen type II (CII) as healthy control individual PBMCs, but they are hyporesponsive to other antigens. PBMCs from RA patients \( n = 45 \) and healthy control individuals \( n = 25 \) were stimulated with antigen for 7 days, and supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) for concentration of interferon-\( \gamma \). The background interferon-\( \gamma \)-production in wells without added antigen was subtracted in each case. (a) Levels of interferon-\( \gamma \) after stimulation with native or denatured CII, and (b) after stimulation with purified protein derivative (PPD) or killed influenza virus are shown. The box plots show the median as a line and the 25th and 75th centiles limiting the box, with the 10th and 90th centiles indicated with bars.

Methods: Forty-five patients who met the 1987 American College of Rheumatology classification criteria for RA \[10\] and 25 healthy control individuals of similar age and sex were included. Twenty-six of these patients who had low levels of anti-CII in serum were randomly chosen, whereas 19 patients with high anti-CII levels were identified by enzyme-linked immunosorbent assay (ELISA)–screening of 400 RA sera. Heparinized blood was density gradient separated and PBMCs were cultured at \( 1 \times 10^6/\text{ml} \) in RPMI-10% fetal calf serum with or without antigenic stimulation: native or denatured CII (100 \( \mu \)g/ml), killed influenza virus (Vaxigrip, Pasteur Mérieux, Lyon, France; diluted 1:1000) or purified protein derivative (PPD; 10 \( \mu \)g/ml). CII was heat-denatured in 56°C for 30 min. Cell supernatants were collected after 7 days and interferon-\( \gamma \) contents were analyzed using ELISA. HLA-DR and HLA-DQ genotyping was performed utilizing a polymerase chain reaction-based technique with sequence-specific oligonucleotide probe hybridization. Nonparametric statistical analyses were utilized throughout the study.

Results: PBMCs from both RA patients and healthy control individuals responded with interferon-\( \gamma \) production to the same degree to stimulation with native and denatured CII (Fig. 1a), giving median stimulation indexes with native CII of 4.6 for RA patients and 5.4 for healthy control individuals, and with denatured CII of 2.9 for RA patients and 2.6 for healthy control individuals. RA patients with elevated levels of anti-CII had a weaker interferon-\( \gamma \) response to both native and denatured CII than did healthy control individuals \( (P = 0.02 \) and 0.04, respectively). Stimulation with the standard recall antigens PPD and killed influenza virus yielded a median stimulation index with PPD of 10.0 for RA patients and 51.3 for healthy control individuals and with influenza of 12.3 for RA patients and 25.7 for healthy, control individuals. The RA patients displayed markedly lower responsiveness to both PPD and killed influenza virus than did healthy control individuals (Fig. 1b). Interferon-\( \gamma \) responses to all antigens were abrogated when coincubating with antibodies blocking MHC class II.

The low response to PPD and killed influenza virus in RA patients relative to that of healthy control individuals reflects a general downregulation of antigen-induced responsiveness of T cells from RA patients \[6–8\]. That no difference between the RA group and the control group was recorded in CII-induced interferon-\( \gamma \) production therefore indicates that there may be an underlying increased responsiveness to CII in RA patients, which is obscured by the general downregulation of T-cell responsiveness in these patients. In order to address this possibility, we calculated the fraction between individual values for the CII-induced interferon-\( \gamma \) production and the PPD-induced and killed influenza virus-induced interferon-\( \gamma \) production, and compared these fractions. A highly significant difference between the RA and healthy control groups was apparent after stimulation with both native CII and denatured CII when expressing the response as a fraction of that with PPD (Fig. 2a). Similar data were obtained using killed influenza virus-stimulated interferon-\( \gamma \) values as the denominator (Fig. 2b).

When comparing the compensated interferon-\( \gamma \) response to denatured CII stimulation between RA patients with different HLA genotypes, highly significant differences were evident, with HLA-DRB1*0401 patients having greater CII responsiveness than patients who lacked this genotype (Fig. 3a). HLA-DQ8 positive patients also displayed a high responsiveness to CII as compared with HLA-DQ8 negative RA patients (Fig. 3b). These associations between the relative T-cell reactivity to denatured CII and HLA class II genotypes were not seen in healthy control individuals. Similar results were achieved using influenza as denominator \( (P = 0.02 \) for HLA-DRB1*0401 and \( P = 0.01 \) for HLA-DQ8).
Discussion: No reports have previously systematically taken the general T-cell hyporesponsiveness in RA into account when investigating specific T-cell responses in this disease. In order to address this issue we used the T-cell responses to PPD and killed influenza virus as reference antigens. This was made on the assumption that exposure to these antigens is similar in age-matched and sex-matched groups of RA patients and healthy control individuals. The concept of a general hyporesponsiveness in RA T cells has been documented in several previous reports, in which both nominal antigens [6–8] and mitogens [11–13] have been used. The fact that a similar functional downregulation in RA PBMCs was obtained with both PPD and killed influenza virus as reference antigens strengthens the validity of our approach.

Conclusion: CII, a major autoantigen candidate in RA, can elicit an interferon-γ response in vitro that is associated with HLA-DRB1*0401 and HLA-DQ8 in the RA patient group, which is of obvious interest because both these MHC class II alleles have been associated with high responsiveness to CII in transgenic mice that express these human MHC class II molecules [14,15]. There was no association between high anti-CII levels and shared epitope (HLA-DRB1*0401 or HLA-DRB1*0404).

Rheumatoid arthritis (RA) patients with disease-associated human leucocyte antigen (HLA) genotypes had a higher relative reactivity to CII than RA patients with other HLA genotypes. Purified protein derivative (PPD) compensated interferon-γ production in response to denatured type II collagen are shown: (a) HLA-DRB1*0401-positive (n = 13) or DRB1*0401-negative (n = 32) RA patients and healthy control individuals (n = 9 and 15, respectively); and (b) HLA-DQA1*0301-DQB1*0301 (HLA-DQ8)-positive (n = 15) and HLA-DQ8 negative (n = 30) RA patients and healthy control (HC) individuals (n = 5 and 15, respectively). The box plots show the median as a line and the 25th and 75th centiles limiting the box, with the 10th and 90th centiles indicated with bars.

We identified an association between the interferon-γ response to CII and HLA-DRB1*0401 and HLA-DQ8 in the RA patient group, which is of obvious interest because both these MHC class II alleles have been associated with high responsiveness to CII in transgenic mice that express these human MHC class II molecules [14,15]. There was no association between high anti-CII levels and shared epitope (HLA-DRB1*0401 or HLA-DRB1*0404).

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Introduction

Despite much work over past decades, whether antigen-specific immune reactions occur in rheumatoid arthritis (RA) and to what extent such reactions are directed towards joint-specific autoantigens is still questionable. The fact that certain major histocompatibility complex (MHC) class II genotypes [human leucocyte antigen (HLA)-DR4 and HLA-DR1] predispose to the development of RA [1] points to the possibility of antigenic involvement. The presence of an increased frequency of autoantibodies to cartilage-specific molecules such as collagen type II (CII) in RA [2–4] and the fact that CII and other joint-derived proteins can cause arthritis in experimental animals after immunization [5] indicate a possible pathogenic role of autoimmune reactions towards these molecules. It has been difficult, however, to convincingly demonstrate cellular reactivity against joint-derived autoantigens such as CII in RA. This has contributed to the widespread notion that specific T-cell reactivities may not be all that important in RA, and that autoimmunity to joint antigens such as CII may only be of importance in very limited subgroups of RA patients.

A few lines of partly new evidence have now led us to reinvestigate the issue of cellular reactivity to CII in RA. First, there is increasing evidence that immunoreactivity to CII can indeed be associated with the RA-associated HLA allele DRB1*0401 and the closely linked allele DQA1*0301-DQB1*0302 (HLA-DQ8). The best evidence comes from studies in DRB1*0401 [14,16] or HLA-DQ8 [15] transgenic mice, which have a high susceptibility to CII-induced arthritis. There are also studies of antibody production to CII in joints that indicate that this is a relatively common feature among RA patients [17,18] and is associated with the presence of HLA-DR4 [18,19]. Second, at least two possible mechanisms by which an existing T-cell responsiveness to CII may have remained undetected using conventional methods (such as determination of proliferation in response to CII in vitro) have been suggested. One mechanism may reside in the low T-cell reactivity to recall antigens in RA patients as compared with healthy control individuals [6–8]. Another mechanism may reside in the partial tolerization to CII that can occur in vivo, as reported in transgenic mice in which the DNA sequence of an immunodominant peptide from rat CII was expressed in the mouse CII gene [9]. In that study, T-cell responses to the rat CII peptide was measured after immunization of rat CII. Proliferative responses were not detected but peptide-induced interferon-γ production was evident in vitro, indicating that the mouse was tolerized to the antigen but that this tolerization only affected the proliferative response.

The strategy behind the present work has accordingly been to reinvestigate T-cell reactivity to CII in RA patients, taking the potential partial tolerization of CII-specific T cells and the general hyporesponsiveness of RA T cells into consideration. We investigated interferon-γ responses after CII stimulation of peripheral blood mononuclear cells (PBMCs) in vitro and compensated for the general T-cell hyporesponsiveness by expressing the CII response as a fraction of the response to standard recall antigens. We also investigated possible associations of the recorded cellular reactivity to CII with HLA genotypes. Finally, we compared CII-induced interferon-γ responses in RA patients with and without antibodies to CII.

Materials and methods

Patients

Initially 400 RA patients, including both those with early arthritis and those with more advanced stages of disease, were screened for the occurrence of high levels of anti-CII antibodies. From this screening 23 patients were identified as having more than 15 U/ml anti-CII antibodies [for definition of these units, see separate method description of CII enzyme-linked immunosorbent assay (ELISA)]. These patients were asked to return and participate in the present study. Of these 19 also had elevated anti-CII levels (> 9 U/ml) at inclusion into the study and comprised the group of RA patients with high anti-CII levels (median age 54 years, range 31–86 years). In addition, 26 RA patients (median age 55 years, range 23–78 years) with low anti-CII levels were randomly chosen. All patients met the 1987 American College of Rheumatology classification criteria for RA [10]. Twenty-five healthy staff members of similar age and sex from the Department of Rheumatology, Karolinska Hospital, served as healthy control individuals. Informed consent was obtained from all patients and control individuals included in the study. Patient characteristics are given in Table 1.

Cell separation and stimulation

Peripheral blood was collected into heparinized tubes and diluted 1:2 with phosphate buffered saline (PBS). Mononuclear cells were isolated by density gradient centrifugation and diluted to $1 \times 10^6$/ml in RPMI-1640 (Flow Laboratories, Irvine, Scotland, UK) supplemented with glutamine, HEPES buffer (Life Technologies, Paisley, Scotland, UK), penicillin, streptomycin and 10% of a defined batch of fetal calf serum (Flow Laboratories). Chick CII (Sigma, St Louis, MO, USA) or human CII [a kind gift from Alvar Grönberg (formerly Pharmacia), Upp sala, Sweden] diluted in 0.1 mol/l acetic acid, or just 0.1 mol/l acetic acid as a buffer control, was added to 1 ml of the cell suspension, giving a final concentration of 100 µg/ml of CII and 2 mmol/l acetic acid. When stimulating with denatured CII, a stock solution of CII was incubated at 56°C for 30 min and then added to the cell suspension.
As standard recall antigens, purified protein derivative (PPD; Statens Smittskyddsinstitut, Solna, Sweden; final concentration 10 µg/ml) and killed influenza virus (Vaxigrip, Pasteur Mérieux, Lyon, France; diluted 1 : 1000) was added to 1 ml of the cell suspension.

For blocking of HLA class II antigens, three different sets of antibodies were used in parallel: a mixture of 1 µg/ml of each of three monoclonal antibodies: antibody 2.06 [anti-DR, mouse immunoglobulin (Ig)G1], IVA12 (anti-DR, DP, mouse IgG1) and 9.3F10 (anti-DR, DQ, mouse IgG2a; all from ATCC, Rockville, Maryland, USA). This mixture of anti-class II antibodies has earlier been described to specifically block antigen-induced cytokine responses [20].

Cells were incubated in round bottomed 96-well plates (NUNC A/S, Roskilde, Denmark) for 7 days using a protocol that has been optimized for antigen induced interferon-γ detection in supernatants ([24], our unpublished data). Supernatants were collected and frozen at −20°C for later analyses of cytokine content.

**Interferon-γ measurements**

ELISA plates (Maxisorp; NUNC A/S) were coated with 50 µl of 2 µg/ml of each of three monoclonal antibodies: antibody 2.06 [anti-DR, mouse immunoglobulin (Ig)G1], IVA12 (anti-DR, DP, mouse IgG1) and 9.3F10 (anti-DR, DQ, mouse IgG2a; all from ATCC, Rockville, Maryland, USA). This mixture of anti-class II antibodies has earlier been described to specifically block antigen-induced cytokine responses [20]. As control, an isotype-weighted mixture of the antikeyhole limpet haemocyanin monoclonal antibodies HS (mouse IgG1 [21]) and 7B4 (mouse IgG2a [21]) were used. In a second set of experiments, 10 µg/ml of L243 (mouse IgG1; ATCC [22]) were used with HS as control antibody. In some confirmatory experiments 10 µg/ml of a F(ab)′₂ rabbit polyclonal antibody against human class II antigens were used with rabbit F(ab)′₂ antibody against human IgG as control [23].

Data are given as median values and ranges for the various groups. Daily prednisolone dosages are presented as mean values in mg. Disease modifying antirheumatic drugs (DMARDs) include sulfasalazin (0/18 high anti-CII patients treated, 6/24 low anti-CII patients treated), cyclosporine (1/18 high anti-CII, 1/24 low anti-CII), Reumacon (a podophyllin derivative, 1/18 high anti-CII, 1/24 low anti-CII), chloroquine (0/18 high anti-CII, 2/24 low anti-CII), aurathiomalase (0/18 high anti-CII, 3/24 low anti-CII), auranofin (1/18 high anti-CII, 0/24 low anti-CII) and azathioprin (1/18 high anti-CII, 1/24 low anti-CII). Figures for sex distribution, erosiveness and medications are given both as fraction of number positive/number investigated, and as percentage. Data on erosive disease are based on X-ray evaluations. Patient charts were not available for three patients, one with high anti-CII levels and two with low anti-CII levels. F, female; CRP, C-reactive protein; NSAID, nonsteroidal anti-inflammatory drug; MTX, methotrexate; ND, not done; CII, collagen type II.

| Number of swollen joints | Number of tender joints | NSAID therapy |Steroid therapy | Mean daily prednisolone dosage | MTX therapy | DMARD therapy |
|---|---|---|---|---|---|---|
| All patients | 2 (0–17) | 4 (0–17) | 26/42 (62%) | 13/42 (31%) | 2.0 | 13/42 (31%) | 30/42 (71%) |
| Patients with high anti-CII levels | 9 (0–17) | 6 (0–17) | 10/18 (56%) | 4/18 (22%) | 1.1 | 5/18 (28%) | 9/18 (50%) |
| Patients with low anti-CII levels | 2 (0–15) | 3 (0–16) | 16/24 (67%) | 9/24 (38%) | 2.7 | 8/24 (33%) | 21/24 (88%) |
| Healthy controls | – | – | – | – | – | – | – |

Table 1

**Patient characteristics**
(7-B6-1; MabTech) diluted to 1 µg/ml in PBS + 1% bovine serum albumin + 0.05% Tween was added. Incubation was done at 4°C overnight. After washing, the plates were incubated for 1 h at room temperature with 50 µl/well avidine–alkaline phosphatase (Dakopatts, Glostrup, Denmark) diluted 1:1000 in PBS + 0.05% Tween. After washing, 50 µl/well substrate [p-nitrophenyl-phosphate tablets (Sigma) 1 mg/ml in diethanolamine buffer, pH 9.8] was added and the reaction was read at 405 nm in a spectrophotometer. To correct for the general low T-cell reactivity seen in RA PBMCs (Fig. 1a), the CII-induced interferon-γ production was expressed as a fraction of the PPD or killed influenza virus-induced interferon-γ production obtained in parallel PBMC cultures.

Serum anticollagen type II detection by enzyme-linked immunosorbent assay

ELISA plates (Maxisorp; NUNC S/A) were coated with 100 µl/well with native chick CII (Sigma) diluted to 5 µg/ml in ice-cold PBS overnight at 4°C. After blocking with PBS + 1% bovine serum albumin and washing with PBS + 0.05% Tween, 100 µl/well of a high-titre standard serum (added in a dilution series) and the samples, diluted at least 1:5 in PBS + 1% bovine serum albumin, were added in duplicate and incubated for 2 h at room temperature. After washing, 100 µl of a biotinylated goat antihuman IgG antibody (Tago, Burlingame, CA, USA) diluted to 1 µg/ml in PBS + 1% bovine serum albumin was added and incubated overnight at 4°C. The ELISA was developed as above. Elevated levels of IgG anti-CII were defined as mean ± two standard deviations of sera from 39 healthy controls (>9 U/ml). The standard serum was arbitrarily defined as having 1500 U/ml.

Human leucocyte antigen genotyping

HLA-DR and HLA-DQ genotyping was performed utilizing a polymerase chain reaction-based technique with sequence-specific oligonucleotide probes hybridization [25,26]. DRB1*01 subtypes were identified by allele-specific polymerase chain reaction primers [27].

Statistical analysis

Nonparametric methods were used throughout the study. Differences between groups were analyzed using the Mann–Whitney U-test, and analyses for matched pairs were performed using Wilcoxon’s signed-rank test. When analyzing correlations, Spearman rank correlation was used. P< 0.05 was considered statistically significant.

Results

PBMCs from RA patients and healthy control individuals were challenged in vitro with native and denatured CII, and supernatants from these cultures were investigated for interferon-γ content. In both groups, an increased interferon-γ production was recorded after in vitro culture with both native and denatured CII as compared with cultures with no added antigen, giving a median stimulation index with native CII of 4.6 for RA patients and 5.4 for healthy control individuals, and with denatured CII of 2.9 for RA patients and 3.0 for healthy control individuals. No difference in interferon-γ production in response to CII could be demonstrated between RA patients and healthy control individuals (Fig. 1a). As control, PBMCs from 12 RA patients and seven healthy control individuals were stimulated with native collagen type II (CII), influenza virus or purified protein derivative in the presence of antibodies (ab) specific for major histocompatibility complex-class II or isotype-matched control antibodies. Supernatants were collected after 7 days of culture and the concentration of interferon-γ was analysed by enzyme-linked immunosorbent assay. Values are expressed as median % (range) of cytokine present in supernatants of which no antibodies were added.

Parallel cultures were also stimulated with the standard recall antigens PPD and killed influenza virus. These antibodies also induced the production of interferon-γ in PBMCs from both RA patients and healthy control individuals, giving median stimulation indexes with PPD of 10.0 (RA patients) and 51.3 (healthy control individuals), and with killed influenza virus of 23.7 (RA patients) and 25.7 (healthy control individuals). For both PPD and influenza stimulations there was a significant difference between the RA group and the healthy control group in that the RA patients displayed a markedly lower responsiveness to PPD and to influenza (Fig. 1b).

In order to investigate whether the observed production of interferon-γ in response to stimulation with CII, PPD and influenza was mediated by MHC class II-dependent T-cell activation, blocking antibodies were added to the cell cultures. As shown in Table 2 these antibodies inhibited the CII as well as the PPD-induced and killed influenza virus-induced interferon-γ production. No such inhibitory effects were observed after incubation with isotype-matched control monoclonal antibodies.

As stated in the introduction to this report, the lower responses to PPD and killed influenza virus in RA patients than in healthy control individuals reflect a general down-regulation of antigen-induced responsiveness of T cells

Table 2

| CII-induced interferon-γ response is major histocompatibility complex (MHC) class II restricted |
|--------------------------------------------------|--|--|
| No ab | Class II ab | Control ab |
| CII   | 100 | 10 (6–22) | 75 (66–125) |
| PPD   | 100 | 13 (8–32) | 109 (63–123) |
| influenza | 100 | 7 (4–15) | 95 (68–125) |

Peripheral blood mononuclear cells from two rheumatoid arthritis patients and three healthy controls were stimulated with native collagen type II (CII), influenza virus or purified protein derivative in the presence of antibodies (ab) specific for major histocompatibility complex-class II or isotype-matched control antibodies. Supernatants were collected after 7 days of culture and the concentration of interferon-γ was analysed by enzyme-linked immunosorbent assay. Values are expressed as median % (range) of cytokine present in supernatants of which no antibodies were added.
from RA patients [6–8]. That no difference between the RA group and the control group was recorded in CII-induced interferon-γ production therefore indicates that there may be an underlying increased specific and MHC class II-dependent responsiveness to CII in the RA patients, which is obscured by the general downregulation of T-cell responsiveness. In order to address this possibility, we calculated the fraction between individual values for the CII-induced interferon-γ production and the PPD-induced and killed influenza virus-induced interferon-γ production, and compared these fractions.

When compensating for the general T-cell hyporesponsiveness in RA-derived T cells in this manner, using the PPD response as the denominator, a highly significant difference between the RA group and the healthy control individuals was apparent after stimulation with both native CII and denatured CII (Fig. 2a). Similar data were obtained using killed influenza virus-stimulated interferon-γ values as the denominator, but no statistical difference between RA patients and healthy control individuals could be determined for native CII (Fig. 2b).

We next investigated whether there was a difference between patients with elevated serum levels of anti-CII antibodies as compared with other RA patients with regard to the T-cell response to CII. Analyzing uncompensated interferon-γ production in response to CII stimulation revealed, contrary to our expectations, that RA patients with high levels of anti-CII had lower interferon-γ responses to denatured CII compared with those of RA patients with low levels of anti-CII (Fig. 4a). A similar tendency, but not statistically significant, could also be demonstrated using native CII. There was no difference in the PPD-induced or killed influenza virus-induced interferon-γ responses between RA patients with high and low anti-CII levels (data not shown).

The results obtained after compensating for the T-cell hyporesponsiveness using PPD as the denominator demonstrate that both the RA patients with low anti-CII antibody levels and those with high levels had significantly greater responses to native CII than did healthy control individuals (Fig. 4b). For the group with low anti-CII levels there was also a highly significant increased response to denatured CII as compared with healthy control individuals, which could not be observed for patients with high anti-CII levels. Similar results were observed using influenza-induced interferon-γ as the denominator (data not shown).

We also correlated the responsiveness to CII to various clinical parameters (C-reactive protein levels, presence of erosions as measured by radiography, numbers of swollen and tender joints, and disease duration). No correlations between C-reactive protein, presence of erosions or number of swollen joints and the compensated interferon-γ response to CII could be demonstrated; the only significant association noted was a weak negative relationship between number of tender joints and the PPD-compensated CII-reactivity ($P = 0.009$, $r_s = -0.46$, data not shown). The RA group with high levels of anti-CII had a significantly higher number of swollen joints than the RA group with low levels of anti-CII ($P = 0.029$, data not shown). A positive correlation was noted between disease duration and responsiveness to native or denatured CII (compensated values; $P = 0.036$, $r_s = 0.33$ for native CII and $P = 0.002$, $r_s = 0.49$ for denatured CII).

Finally, we compared the compensated interferon-γ response to denatured CII stimulation between RA patients with different HLA genotypes. Of the RA patients...
included in this study, 13 were DRB1*0401 positive and 15 were DQ8 positive. Out of these patients, 12 were DRB1*0401/DQ8 double positive. Of the healthy control individuals, nine were DRB1*0401 positive and five were DQ8 positive (four were not genotyped for HLA-DQ). Out of these healthy control individuals, four were DRB1*0401/DQ8 double positive. Highly significant differences were evident, with HLA-DRB1*0401 patients having higher CII responsiveness than patients lacking this genotype (Fig. 3a). HLA-DQ8-positive patients also displayed a high responsiveness to CII as compared with HLA-DQ8-negative RA patients (Fig. 3b). Similar results were achieved using killed influenza virus as the denominator (P = 0.02 for HLA-DRB1*0401 and P = 0.01 for HLA-DQ8). These associations between the relative T-cell reactivity to denatured CII and HLA class II genotypes were not seen in healthy control individuals. No significant relationship between the analyzed HLA-DR or DQ haplotypes and responsiveness to CII remained when the two different groups of RA patients were analyzed separately. No association was detected between serum levels of anti-CII and the shared epitope (DRB1*0401 or DRB1*0404).

**Discussion**

The major findings reported in this paper are as follows. First, an increased interferon-γ production in response to *in vitro* stimulation with CII in RA patients as compared with age-matched and sex-matched healthy control individuals when compensation was made for the general hyporesponsiveness of RA patient PBMCs to nominal antigens. Second, the response to CII was dependent on MHC class II molecules, as demonstrated by the blockade of the response with anti-MHC class II antibodies. Finally, RA patients positive for the genotypes HLA-DRB1*0401 or HLA-DQ8 exhibited a higher interferon-γ response than did RA patients without these alleles.

The choice of interferon-γ production as the parameter for registration of the cellular response to CII was made both from experience in mice immunized with CII and from multiple sclerosis. In mice immunized with autologous CII, an autoimmune arthritis-provoking response may occur that is associated with *in vivo* antibody production to CII and with CII-induced interferon-γ production *in vitro*, but not with any proliferative response to CII [9]. In multiple sclerosis patients, *in vitro* production of T-cell cytokines such as interferon-γ is increased after *in vitro* stimulation with myelin antigens such as myelin basic protein and myelin-oligodendrocyte glycoprotein, despite the difficulties in detecting proliferative responses towards these antigens [28].

Although there is much previous support for the choice of interferon-γ as a readout for T-cell responsiveness to antigens, thus taking the potential partial anergy to CII into account, no reports have previously systematically taken the general T-cell hyporesponsiveness in RA into account when investigating specific T-cell responses in this disease. In the present study we used the T-cell responses to PPD and killed influenza virus as reference antigens for making this compensation. This was based on the assumption that exposure to these antigens is similar in age-matched and sex-matched groups of RA patients and healthy control individuals. The concept of a general hyporesponsiveness of RA T cells has been documented in several previous reports in which both nominal antigens [6–8] and mitogens [11–13] were used. Even though the mechanisms behind this defective T-cell response are not clear, it appears that the hyporesponsiveness is a feature of the disease itself because it has been observed also in patients who are not on disease-modifying antirheumatic drug therapy [6]. The fact that a similar functional down-regulation in RA PBMCs was obtained with both PPD and killed influenza virus as reference antigens strengthens the validity of our approach.

Both native and heat-denatured CII were used in the present study on the basis that native and denatured CII may be processed in different ways by antigen-presenting cells. As shown in Figures 1a and 4a, native CII induced more interferon-γ than did denatured CII, indicating an important role for the structure of CII in cellular stimulation. The difference in the amount of interferon-γ induced by native and denatured CII might depend on differences in uptake or processing of these structurally different CII molecules.

Proliferation in response to *in vitro* CII stimulation of cells from both immunized animals and in human studies have in some instances been found to be a response to contaminating pepsin in the CII preparation [29]. This possibility was excluded in the present study by control experiments that failed to demonstrate any interferon-γ production in response to pepsin (data not shown).

RA patients as well as healthy control individuals responded with interferon-γ production *in vitro* when PBMCs were stimulated with chick or human CII. It has not directly been demonstrated that the interferon-γ-producing cells are T cells, but the fact that blocking of MHC class II blocks the interferon-γ response by RA PBMCs as well as by control PBMCs indicates that this is indeed the case. We also attempted to identify induction of a type 2 cytokine, but failed to detect interleukin-4 in CII stimulated cultures using methods that are capable of detecting interleukin-4 in cultures stimulated with a recall antigen.

We identified an association between the magnitude of the interferon-γ response to CII and HLA-DRB1*0401 and HLA-DQ8 in the RA group, which is of obvious interest because both these MHC class II alleles have been associated with high responsiveness to CII in transgenic mice that express these human MHC class II molecules.
independent association with RA. In in vitro studies of cellular responses to CII in RA patients, some reports have claimed that cellular reactivity to CII (measured as CII-induced proliferation or production of factors capable of stimulation of leucocyte functions) is associated with HLA-DR4 [32–34]. Others, however, have not found this association [35,36]. Anti-CII-producing B cells have been reported to be present in the joint [17,18] and have in some instances been associated with HLA-DR4 [18,19]. It is difficult to separate the influences of HLA-DR4 and HLA-DQ8 in studies of the association between MHC class II alleles and RA or in in vitro associations between cellular reactivity to CII and MHC class II alleles, because of the strong linkage disequilibrium between DRB1*0401 and HLA-DQ8. Finally, it should be noted that the RA patients in the present study were recruited in two different ways; 19 patients were selected on the basis of their high anti-CII antibody levels and 26 patients with low anti-CII levels were randomly recruited from our outpatient clinic. The HLA associations to cellular CII reactivity were only seen when both groups were combined, probably because of the rather low numbers of patients in each group. It appears unlikely to us that the association between HLA haplotype and CII-induced T-cell responsiveness seen in the combined group should be due to a bias introduced by selection of patients with high anti-CII levels for the following reasons: the high anti-CII group had a lower responsiveness to denatured CII than the randomly chosen group of RA patients; and there was no association between anti-CII levels and shared epitope (HLA-DRB1*0401 or HLA-DRB1*0404).

The interferon-γ reactivity to CII seen in most individuals implies that T-cell tolerance to CII is incomplete. The finding that high cellular response to CII associates with HLA-DRB1*0401 and HLA-DQ8 could mean that selection in thymus favours a T-cell repertoire containing T-cell receptors with high specificity for some CII epitope(s). Another explanation for the HLA-associated cellular response to CII is that these class II molecules could be able to present some immunodominant peptides more efficiently than other class II molecules.

One recent study [37] has investigated CII-specific proliferative responses and their relation to the presence of circulating anti-CII antibodies in RA patients. That study could not demonstrate a difference in proliferative response to CII between RA patients and healthy control individuals. In contrast to the present results, however, a lower frequency of responsive RA patients without anti-CII compared with that in RA patients with anti-CII was recorded, which could not be explained by a difference in disease-modifying antirheumatic drug therapy between the two patient groups. This contradiction could be explained by the definition of anergy, as suggested by Mueller et al [38], that proliferative responses are lost whereas effector functions are retained in anergized T cells. Hence, T cells in RA patients without anti-CII might be anergic in the proliferative aspect but are still able to respond by cytokine production.

It has been shown by Cook et al [39] that levels of anti-CII in serum of RA patients decreases over time. This tendency was also apparent in the present study, but it was not designed to address this question (data not shown). We demonstrated a positive correlation between disease duration and interferon-γ response to CII, indicating that the T-cell response to CII (expressed as a fraction of the PPD-induced interferon-γ response) increases with time. As the PPD-induced interferon-γ production decreases with disease duration (data not shown), this implies that general T-cell reactivity decreases gradually during the course of RA. CII-induced interferon-γ production remains stable, however, and it increases when expressing the CII cellular reactivity as a fraction of the PPD cellular reactivity. Consequently, although humoral CII reactivity decreases with time [39], cellular CII reactivity increases. It is thus plausible that serum antibody levels to CII constitute a relatively poor mirror of T-cell responsiveness to CII. Because anti-CII antibodies and T cells reactive with CII may synergize in causing arthritis [40] (at least in rodents), there is an obvious need for further detailed parallel studies of B-cell and T-cell reactivity to CII in RA, taking the fine specificity (not addressed in the present study) into account.

In conclusion, CII, a major autoantigen candidate in RA, can elicit an interferon-γ response in vitro that is associated with HLA-DRB1*0401 and HLA-DQ8 in RA patients. The present study, with a partly new methodological approach to a classical problem in RA, has provided some additional support to the notion that CII may be a target autoantigen of importance for a substantial group of RA patients. Continued efforts to identify mechanisms behind the general hyporesponsiveness to antigens in RA, as well as the mechanisms behind the potential partial anergy to CII, may provide us with better opportunities to study the specificity and pathophysiological relevance of anti-CII reactivity in RA.

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