Research article

Induction of IL-10-producing CD4+CD25+ T cells in animal model of collagen-induced arthritis by oral administration of type II collagen

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Abstract

Induction of oral tolerance has long been considered a promising approach to the treatment of chronic autoimmune diseases, including rheumatoid arthritis (RA). Oral administration of type II collagen (CII) has been proven to improve signs and symptoms in RA patients without troublesome toxicity. To investigate the mechanism of immune suppression mediated by orally administered antigen, we examined changes in serum IgG subtypes and T-cell proliferative responses to CII, and generation of IL-10-producing CD4+CD25+ T-cell subsets in an animal model of collagen-induced arthritis (CIA). We found that joint inflammation in CIA mice peaked at 5 weeks after primary immunization with CII, which was significantly less in mice tolerized by repeated oral feeding of CII before CIA induction. Mice that had been fed with CII also exhibited increased serum IgG1 and decreased serum IgG2a as compared with nontolerized CIA animals. The T-cell proliferative response to CII was suppressed in lymph nodes of tolerized mice also. Production of IL-10 and of transforming growth factor-β from mononuclear lymphocytes was increased in the tolerated animals, and CD4+ T cells isolated from tolerized mice did not respond with induction of IFN-γ when stimulated in vitro with CII. We also observed greater induction of IL-10-producing CD4+CD25+ subsets among CII-stimulated splenic T cells from tolerated mice. These data suggest that when these IL-10-producing CD4+CD25+ T cells encounter CII antigen in affected joints they become activated to exert an anti-inflammatory effect.

Keywords: collagen-induced arthritis, IL-10, oral tolerance, type II collagen

Introduction

Oral tolerance is a state of absent or minimal immune responsiveness to protein antigens that were repeatedly administered by oral feeding [1]. Induction of peripheral tolerance by oral administration of antigen has been applied to the treatment of autoimmune diseases such as rheumatoid arthritis (RA) [2,3], multiple sclerosis, systemic sclerosis, type I diabetes and iritis [4], but the mechanisms by which orally administered antigen can induce peripheral tolerance have not yet been elucidated. Studies conducted in animal models have suggested that the possible mechanism may involve secretion of anti-inflammatory cytokines including IL-4, IL-10 and transforming growth factor (TGF)-β by mucosal T lymphocytes that have differentiated into T-helper (Th)2 or Th3 cells after encountering the antigen [5–7]. However, individual studies often report conflicting findings, depending on the route, dose and timing of antigen administration [8].

Although much of the RA pathogenesis remains to be elucidated, it has been reported that joint proteins, probably type II collagen (CII), play a key role in the instigation of T-cell mediated immune responses.
Administration of CII to DBA/1 mice induces polyarthritis with pathological symptoms similar to those observed in human RA [9,10]. In order to investigate the cellular mechanisms that underlie oral tolerance, we studied an animal model of collagen-induced arthritis (CIA), in which mice undergo repeated oral administration of CII, and monitored changes in immune cell function and factors associated with inflammation. We found that serum levels of IgG subtypes, as well as the production of IL-10, TGF-β and IFN-γ, were affected in tolerized mice. We also noticed greater proportions of IL-10-producing CD4+CD25+ T cells in the Peyer's patch, mesenteric lymph nodes and the spleen of tolerized mice. Production of these cells, exhibiting the characteristics of the Treg subset, was associated with inflammation observed in affected joints.

Materials and methods

Induction of oral tolerance in DBA/1 mice

DBA/1 mice used in this study were fed either with 100 µg bovine CII (a kind gift from Prof. Andrew Kang, University of Tennessee) dissolved in 0.05 N acetic acid at 2 µg/ml (50 µl solution plus 150 µl acetic acid) or with an equal volume of phosphate-buffered saline (PBS) using an oral Zonde needle (Natsume, Japan) every 2 days for 2 weeks. All experimental procedures were examined and approved by the Animal Research Ethics Committee at The Catholic University of Korea.

Induction of CIA and evaluation of arthritis

Bovine CII was dissolved in 0.05 N acetic acid at 2 mg/ml and emulsified with an equal volume of complete Freund’s adjuvant (CFA). As primary immunization, 0.1 ml of the emulsion containing 100 µg CII, was injected into the tails of DBA/1 mice (both tolerized mice and nontolerized control mice; n = 6 per group). Two weeks later, a booster injection consisting of 200 µg CII similarly dissolved and emulsified 1:1 with incomplete Freund’s adjuvant was injected into a hind leg. Starting from 2.5 weeks (18 days) after primary immunization, three independent observers examined the degree of arthritis three times a week for up to 11 weeks. The severity of arthritis was represented as mean arthritic index on a 0–4 scale according to the following criteria [11]: 0 = no oedema or swelling; 1 = slight oedema and erythema limited to the foot and/or ankle; 2 = slight oedema and erythema from the ankle to the tarsal bone; 3 = moderate oedema and erythema from the ankle to the tarsal bone; and 4 = oedema and erythema from the ankle to the entire leg. The sum of values from three legs, excluding the hind leg into which CII/incomplete Freund's adjuvant was injected, was determined at the time of the second injection. The final average of the indices recorded by three independent observers. All experimental procedures were examined and approved by the Animal Research Ethics Committee at The Catholic University of Korea.

Analysis of IgG antibody subtypes

Blood samples obtained from each mouse at 3, 5 and 7 weeks after primary immunization were used to investigate IgG antibody subtype concentrations using the mouse IgG1/IgG2a ELISA quantitation kit (Bethyl Lab Co., Montgomery, TX, USA). Levels of IgG1 and IgG2a were measured in sera diluted 50,000- to 400,000-fold.

Determination of collagen-specific T-cell proliferative response

The draining lymph nodes and the spleen were removed from each mouse and washed twice with PBS. Tissues were minced and the cells were filtered through a cell strainer and centrifuged at 1500 rpm at 4°C for 10 min. The cell pellet was resuspended in RPMI-1640 medium to a concentration of 1 × 10^5 cells/ml. Cells were then plated in 96-well microtitre plates at a concentration of 2 × 10^5 cells/well concentration and cultured with 40 µg/well CII in 0.3 ml Click’s medium supplemented with 0.5% mouse serum for 3 days. The same amount of ovalbumin was added instead of CII to control wells. Eighteen hours before the termination of culture, 0.5 µCi [3H]thymidine (NEN, Boston, MA, USA) was added to each well. Cells were harvested onto glass fibre filters and counted on a Matrix-96 direct ionization β counter (Packard Instrument Co., Downers Grove, IL, USA). The degree of T-cell proliferation is presented as the stimulation index, which is calculated by dividing the counts/min in the presence of CII by the counts/min in the presence of ovalbumin.

Analyses of cytokine production by ELISA

Mononuclear cells were isolated from the Peyer’s patch and spleen, and cultured at a density of 0.5 × 10^6 cells/ml in flat-bottomed, 48-well tissue culture plates (Corning, Corning, NY, USA). After 2 days, culture supernatants were harvested and stored at −70°C. To determine the amount of IL-10 and TGF-β in each supernatant, 96-well ELISA plates were coated with rat antimurine IL-10 and TGF-β monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) for 24 hours at 4°C. After incubating with blocking agents, the plates were incubated with previously collected supernatants for 1 hour at room temperature. The plates were then washed and incubated for 1 hour at room temperature with biotin-conjugated rat antimurine IL-10 and TGF-β monoclonal antibodies, followed by an alkaline phosphatase-conjugated goat antibinin monoclonal antibody (R&D Systems). The fluorescent substrate for alkaline phosphatase (R&D Systems) was used for colour development, and fluorescence was measured using a microtitre plate reader (Dynex, Chantilly, VA, USA) at excitation and
emission wavelengths of 450 nm. The amounts of cytokines present in test samples were determined from standard curves established with serial dilutions of recombinant murine IL-10 and TGF-β.

Fluorocytometric analysis of T cells
Single-cell suspensions were prepared from each lymphoid organ and cultured in 24-well plates at a concentration of 1 × 10⁶/well with or without 40 µg/well CII for 3 days. Golgi Stop (Pharmingen, San Diego, CA, USA) was added 4 hours before the termination of culture. Cells were subsequently washed and resuspended in fluorescence-activated cell sorting (FACS) staining buffer (PBS plus 0.1% bovine serum albumin plus 0.09% sodium azide), and probed with anti-CD4-perCP and/or anti-CD25-FITC (Pharmingen) for 30 min at 4°C. Next, cells were fixed with cytoperm/cytofix (Pharmingen) for 20 min and probed for intracellular cytokines using phycoerythrin-labelled anti-IL-10 antibody, anti-IFN-γ antibody, or isotype control antibody (Pharmingen) for 30 min at room temperature. Finally, cells were washed with PBS and analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis
Experimental findings are presented as mean ± standard deviation. Statistical significance was determined by Student’s t-test using the SPSS program (version 10.0; SPSS Inc, Chicago, IL, USA). P < 0.05 was considered statistically significant.

Results
Inhibition of arthritis development in tolerized CIA mice
The severity of arthritis remained low both in the tolerized and nontolerized CIA mice until 4 weeks after primary immunization with CII/CFA (Fig. 1). The average arthritis index in the nontolerized mice began to increase after week 4, reaching a peak between weeks 5 and 6 after primary immunization, and then started to decrease by week 8, and then persisted at an almost constant level until the 11th week. The arthritis index in the tolerized group also peaked between 5 and 6 weeks after primary immunization, but the level was significantly lower than that in the nontolerized CIA mice throughout the period of examination. Mice tolerized by oral CII feeding also exhibited an earlier decrease in their mean arthritis index, which began at week 7 and continued gradually thereafter.

Changes in the serum level of IgG subtypes in tolerized mice
Among three subtypes of IgG, IgG₁ is reported to be associated with anti-inflammatory actions, whereas IgG₂a is known to be a mediator of inflammation. Likewise, the level of IgG₂a has been shown to be significantly increased in DBA/1 mice with CIA [12,13]. We investiga-

Proliferation response to CII by T cells isolated from the draining lymph nodes and spleens of tolerized mice
We then investigated the proliferation of T cells isolated from the draining lymph node and spleen on in vitro antigenic simulation with CII. At 3 and 5 weeks after primary immunization with CII/CFA, the T-cell proliferative response to CII was about 25% lower in cells isolated from the tolerized group when examined at both 3 weeks and

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A significant difference was noticed between draining lymph node T cells from the two groups (Fig. 3a). Splenic T cells from orally tolerized mice also exhibited a lower proliferative response to CII than did nontolerized mice at all three time points, whereas the IgG2a level was lower in the tolerized group. The data represent average values from 15 mice in each group.

Changes in the production of IL-10, TGF-β and IFN-γ by lymphocytes of orally tolerized mice

To examine the effect of oral CII administration on the activity of immune cells, we measured the production of IL-10 and TGF-β by mononuclear lymphocytes isolated from the Peyer’s patch and spleen of each mouse. As shown in Fig. 4, production of both IL-10 and TGF-β was increased in lymphocytes isolated from the Peyer’s patch and the spleen of tolerized mice as compared with lymphocytes from nontolerized CIA mice or from normal DBA/1 mice. Although the level of TGF-β production in cells from nontolerized CIA mice was similar to that in cells from normal DBA/1 mice, the production of IL-10 was slightly greater in cells from nontolerized CIA than in cells from normal DBA/1 mice.

We then compared the level of IFN-γ production from antigen stimulated CD4+ T cells isolated from the Peyer’s patch, mesenteric lymph nodes and spleens of tolerized and nontolerized mice (Table 1) by FACS analyses using
monoclonal antibodies against CD4 and intracellular IFN-γ. When examined ex vivo, no apparent differences in IFN-γ production were observed between the two groups. When these cells were stimulated in vitro with 40 µg/ml CII for 3 days, the level of IFN-γ synthesis increased up to 10-fold in nontolerized CIA mice but remained virtually unchanged in tolerized animals.

**Induction of IL-10-producing CD4+CD25+ population from splenic T cells of orally tolerized mice by in vitro CII stimulation**

To identify any possible involvement of particular T-cell subsets that are known to exert immune suppressive functions [6,14], we compared the proportion of IL-10-producing CD4+CD25+ T lymphocytes between tolerized and nontolerized mice. For this, single-cell preparations from the Peyer’s patch, mesenteric lymph node and spleen extracted at 5 weeks after primary immunization were cultured in vitro with or without CII stimulation for 3 days, and analyzed using triple-colour FACS. The proportion of IL-10-producing CD4+CD25+ cells was higher in tolerized mice than in nontolerized CIA mice and normal DBA/1 mice in all three organs examined (Table 2). In normal DBA/1 mice the proportion of IL-10-producing CD4+CD25+ cells remained unchanged from baseline in all three organs examined, regardless of whether in vitro CII stimulation was applied. The proportion of IL-10-producing CD4+CD25+ cells in tolerized mice exhibited a modest increase in the Peyer’s patch and mesenteric lymph nodes when stimulated in vitro with CII; however, the degree of enrichment was not significantly different from that in nontolerized CIA mice. On the other hand, in vitro CII stimulation induced a much steeper elevation in IL-10-producing CD4+CD25+ population in the spleens of tolerized mice (from 2.95 ± 1.62% to 15.5 ± 4.50%) than in the nontolerized group (from 0.15 ± 0.07% to 3.85 ± 0.63%). Differences between the enrichment in T-cell subsets in the spleen are shown in a representative dot plot (Fig. 5).

**Discussion**

Treatment of autoimmune diseases by induction of oral tolerance is attractive because of the few side effects and easy clinical implementation of this approach. Although protein antigens administered orally are degraded quickly in the digestive system and their effects do not last long, we previously overcame these drawbacks by encapsulating CII – the major cartilage autoantigen in RA – in a polymer complex [15]. In the present study we attempted to improve our basic understanding of the immune cell functions that participate in the process of induction of oral tolerance. Because most of the previous studies that addressed the mechanism underlying oral tolerance were conducted in transgenic animal models that overexpress T-cell clones that recognize experimental antigens such as ovalbumin, it is difficult to relate their findings to the
clinical situation [16]. In the present study we examined the changes in immune function that are involved in the induction of peripheral tolerance in an orally tolerized mouse model of arthritis.

Our findings indicate that the serum levels of pro-inflammatory and anti-inflammatory IgG subtypes are differentially modulated in mice that have undergone repeated oral administration of CII. We also found that, although the production of IFN-γ by CD4+ T cells from tolerized mice on CII stimulation is reduced, the production of IL-10 and TGF-β is increased not only in cells from the site of initial contact with the oral antigen (i.e. the Peyer's patch) but also in cells from lymph nodes and the spleen.

That in vitro restimulation with CII generated significant enrichment in IL-10-producing CD4+CD25+ T cells among lymphocytes from tolerized spleen is particularly interesting. We hypothesize that by the time these IL-10-producing cells have reached the spleen through peripheral blood, they have also been transferred to the affected joints where CII is accumulated from cartilage degradation and have begun to alleviate inflammation.

Many recent studies have reported that IL-10, a major anti-inflammatory cytokine secreted by Th2 lymphocytes, contributes to the process of peripheral tolerance [1,6]. Our data suggest that the establishment of CII-mediated tolerance to arthritic inflammation involved, among other mechanisms of immunosuppression, participation of CD4+CD25+ T cells and their production of IL-10 in peripheral lymphoid organs. However, roles for Th3 cells (known for their production of TGF-β [17,18]) and for Tr1

### Table 1

| Site                      | % IFN-γ-positive cells Without CII stimulation | With CII stimulation |
|---------------------------|-----------------------------------------------|----------------------|
| Peyer’s patch             |                                               |                      |
| CIA                       | 3.4 ± 0.22                                    | 26.6 ± 0.6           |
| Tolerized                 | 4.6 ± 0.1                                     | 8.9 ± 0.2            |
| Mesentric lymph node      |                                               |                      |
| CIA                       | 3.3 ± 0.4                                     | 34 ± 0.2             |
| Tolerized                 | 2.3 ± 0.5                                     | 5.6 ± 0.8            |
| Spleen                    |                                               |                      |
| CIA                       | 5.4 ± 0.01                                    | 42.6 ± 0.1           |
| Tolerized                 | 4.5 ± 0.2                                     | 5.3 ± 0.02           |

Values are expressed as mean ± standard deviation, calculated from three independent measurements. CIA, collagen-induced arthritis; CII, type II collagen.

### Table 2

| Site                      | % CD4+CD25+ IL-10-positive cells Without CII stimulation | With CII stimulation |
|---------------------------|--------------------------------------------------------|----------------------|
| Peyer’s patch             |                                                         |                      |
| Normal                    | 0.17 ± 0.12                                            | 0.14 ± 0.06          |
| CIA                       | 1.6 ± 0.7                                              | 3.6 ± 0.2            |
| Tolerized                 | 2.4 ± 1.6                                              | 6.8 ± 1.2            |
| Mesentric lymph node      |                                                         |                      |
| Normal                    | 0.35 ± 0.35                                            | 0.5 ± 0.28           |
| CIA                       | 0.85 ± 0.35                                            | 1.8 ± 0.5            |
| Tolerized                 | 2.95 ± 1.06                                            | 7.9 ± 1.48           |
| Spleen                    |                                                         |                      |
| Normal                    | 0.13 ± 0.04                                            | 0.25 ± 1.3           |
| CIA                       | 0.15 ± 0.07                                            | 3.85 ± 0.63          |
| Tolerized                 | 2.95 ± 1.62                                            | 15.5 ± 4.5           |

Values are expressed as mean ± standard deviation, calculated from three independent measurements. CIA, collagen-induced arthritis; CII, type II collagen.

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**Table 1**

| Site                      | % IFN-γ-positive cells |
|---------------------------|------------------------|
| Without CII stimulation   | With CII stimulation   |
| Peyer’s patch             |                        |
| CIA                       | 3.4 ± 0.22             |
| Tolerized                 | 4.6 ± 0.1              |
| Mesentric lymph node      |                        |
| CIA                       | 3.3 ± 0.4              |
| Tolerized                 | 2.3 ± 0.5              |
| Spleen                    |                        |
| CIA                       | 5.4 ± 0.01             |
| Tolerized                 | 4.5 ± 0.2              |

**Table 2**

| Site                      | % CD4+CD25+ IL-10-positive cells |
|---------------------------|----------------------------------|
| Without CII stimulation   | With CII stimulation             |
| Peyer’s patch             |                                  |
| Normal                    | 0.17 ± 0.12                      |
| CIA                       | 1.6 ± 0.7                        |
| Tolerized                 | 2.4 ± 1.6                        |
| Mesentric lymph node      |                                  |
| Normal                    | 0.35 ± 0.35                      |
| CIA                       | 0.85 ± 0.35                      |
| Tolerized                 | 2.95 ± 1.06                      |
| Spleen                    |                                  |
| Normal                    | 0.13 ± 0.04                      |
| CIA                       | 0.15 ± 0.07                      |
| Tolerized                 | 2.95 ± 1.62                      |

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**Figure 5**

Induction of IL-10-producing CD4+CD25+ T cells in the spleen of tolerized mice. The proportion of IL-10-producing CD4+CD25+ T cells was analyzed by triple-colour fluorescence-activated cell sorting (FACS) analyses. Single-cell suspension was prepared from the spleens of normal DBA/1, and tolerized and nontolerized collagen-induced arthritis (CIA) mice at 5 weeks after primary immunization. Cells were first gated for CD4+ proportions, and subsequently analyzed for surface expression of CD25+ and for the presence of intracellular IL-10. A representative dot plot chosen from three independent measurements (summarized in Table 2) is shown.
cells (which are characterized by low proliferation and high production of IL-10 [19–21]) are equally implicated in the process of induction of peripheral tolerance. We observed elevated production of TGF-β from tolerized lymphocytes as well, and it would therefore be very interesting to investigate the role of Th3 cells. In order to further define the characteristics of cells that exert immunosuppressive functions, we are in the process of examining the expression of CD62L and CD45RB, which are accepted as additional phenotypic markers for regulatory T cells [22,23], and the effect of transferring T cells into nontolerized CIA mice.

Conclusion
We observed a greater proportion of IL-10-producing CD4+CD25+ T cells in mice that had undergone repeated oral administration of CII. Together with increased production of IL-10 and TGF-β in peripheral lymphoid organs, induction of this suppressor population is believed to block the induction of IFN-γ from CII-stimulated CD4+ cells. Because little is known about the cellular mechanisms that underlie orally induced tolerance in nontransgenic systems, our findings provide important basic information on the generation of peripheral immune tolerance. Once merged with findings on possible intercellular cooperation between CII-specific T cells and other cell types such as dendritic cells, this data will provide a basis for more effective application of oral tolerance induction in RA patients.

Competing interests
None declared.

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