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Characterization of recombinant type II collagen: arthritogenicity and tolerogenicity in DBA/1 mice

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SUMMARY

Recombinant human type II collagen (rhCII) was produced using both the HT1080 mammalian cell expression system ($rhCII^{ht}$) and a baculovirus expression system ($rhCII^{bac}$). The biosynthesis of CII requires extensive post-translational modifications, such as the hydroxylation of prolyl and lysyl residues and glycosylation of hydroxylysyl residues. Amino acid analyses indicated that the $rhCII^{bac}$ was adequately hydroxylated at prolyl residues but underhydroxylated at lysyl residues and underglycosylated compared with tissue-derived hCII, while $rhCII^{ht}$ was hyperhydroxylated and hyperglycosylated at lysyl residues. When the murine collagen-induced arthritis (CIA) model was used to investigate the immunological properties of the two forms of recombinant CII, each induced a high incidence of arthritis following immunization of susceptible mice when emulsified with complete Freund's adjuvant (CFA). However, the severity of the arthritis, as assessed by the number of affected limbs, was significantly higher in mice immunized with $rhCII^{ht}$ than in mice immunized with $rhCII^{bac}$. These data indicate that the degree of hydroxylysine glycosylation may play a role in the induction of the arthritogenic response to CII. Each of the recombinant collagens was comparable to tissue-derived hCII in their ability to induce tolerance and suppress arthritis when given as intravenous or oral tolerogens. Taken together, our data suggest that recombinant CII can be prepared in adequate amounts for therapeutic uses and that the material is immunologically comparable to tissue-derived hCII when used to induce tolerance.

INTRODUCTION

Strong theoretical reasons suggest that patients with autoimmune arthritis should benefit from treatment with cartilaginous proteins, such as type II collagen (CII). The destruction of cartilage that occurs in rheumatoid arthritis (RA) involves an autoimmune response to cartilage-specific proteins.[1] Therefore, inducing the state of tolerance to these autoantigens should down-regulate the inflammatory T-cell cytokine and antibody response, resulting in decreased joint inflammation and cartilage destruction. In fact, the induction of tolerance to CII by either parenteral, intranasal or oral administration of antigen caused a profound suppression of arthritis in the animal model of collagen-induced arthritis (CIA).[2–7] Recently, Barnett and co-workers published the results of a double-blind control study in which oral administration of low doses of CII to RA patients resulted in improvement in their disease activity, presumably by induction of tolerance to CII.[8,9] In addition, Barnett *et al.* reported that oral administration of CII was beneficial to patients with juvenile rheumatoid arthritis (JRA) in an open-labelled trial.[10] Although conflicting results were obtained in a study of early RA patients by Sieper *et al.*,[11] none of these studies reported any appreciable toxicity following oral administration of CII. These data suggest that further studies are warranted to clarify the possible efficacy of CII in the therapy of patients with RA and JRA.

The availability of a recombinant CII would be extremely useful for preparing adequate amounts of CII suitable for therapeutic use. However, the extensive post-translational modifications required for the biosynthesis of CII, such as the

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Abbreviations: CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CII, type II collagen; hCII, human type II collagen; IFN-γ, interferon-γ; JRA, juvenile rheumatoid arthritis; Ova, ovalbumin; RA, rheumatoid arthritis; $rhCII^{bac}$, recombinant human type II collagen derived from insect cells; $rhCII^{ht}$, recombinant human type II collagen derived from HT1080 cells.

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hydroxylation of prolyl and lysyl residues and glycosylation of hydroxylysyl residues, raise questions as to whether recombinant CII proteins would have immunological properties comparable to native tissue-derived CII. Insect cells are relatively deficient in the enzymes, naturally occurring in mammalian cells, which catalyse both hydroxylation and glycosylation of the collagen molecule. In the present paper, we describe recombinant CII produced using both the HT1080 mammalian cell expression system (rhCII^{ht}) and a baculovirus expression system (rhCII^{bac}). We utilize the murine CIA model to investigate the immunological properties of the two forms of recombinant CII, both in their ability to induce arthritis and to induce tolerance and suppress arthritis.

MATERIALS AND METHODS

Development of recombinant type II collagens: rhCII^{ht} and rhCII^{bac}

A cosmid clone containing a COL1 A1/COL2 A1 hybrid gene construct was assembled in a modified cosmid vector as previously described.[12,13] The hybrid gene contained a 5′-fragment from the human COL1 A1 gene that included the promoter, the first exon and most of the first intron. The COL1 A1 fragment was linked to a 29·5-kb sequence of the human COL2 A1 gene that extended from the 3′-end of the second intron of the gene to about 3·5 kb beyond the major polyadenylation signal of the gene. The hybrid gene construct was cleaved from the vector and transfected into human kidney tumour cells (HT1080; American Type Culture Collection CCL121; ATCC, Rockville, MD) using the calcium phosphate precipitation method as previously described.[12,13] Cells were selected by growing them in the presence of G418. The cells were grown to confluence, lysed and assayed for the presence of recombinant procollagen II by Western blot analysis with rabbit antihuman procollagen II antiserum. In a second set of experiments, the cDNA cassette encoding the amino-terminal propeptide of human type III collagen was cloned in-frame with a cDNA construct encoding the α1(II) chain and carboxyl-terminal propeptide of human type II collagen. The entire construct was then cloned into the baculovirus shuttle vector pVL1392 (pVLC/3 A1NP/C2 A1) and recombined with linearized baculovirus DNA. Viruses were plaque purified, amplified and high-titres were produced. Expression of rhCII involved infection of *Trichoplusia ni* (High Five®) cells or *Spodoptera frugiperda* (Sf-9) cells with two virus types; one encoding human type II collagen and the other encoding the enzyme prolyl-4-hydroxylase.[14,15]

Isolation and purification of rhCII^{ht} and rhCII^{bac}

For large-scale production of rhCII^{ht}, transfected HT1080 cells were grown in a CelliGen-Packed Bed Bioreactor (New Brunswick Scientific Co., Inc., Edison, NJ) The conditioned medium was collected by centrifugation, and solid $(NH_4)_2SO_4$ was added with stirring at 4°, allowing procollagen to precipitate overnight. The pellet was harvested by centrifugation and subjected to limited pepsin digestion to remove propeptides. The rhCII^{ht} was purified by repeated precipitation with NaCl, as described previously.[16]

For baculovirus CII expression, infected insect cells were grown in shaker flasks containing TNM-FH medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (BioClear, Canton, OH) containing ascorbate phosphate (80 μg/ml). After 24–96 hr, cells were harvested and homogenized in cold 0·05 M Tris, pH 7·4, containing 0·1% Triton-X-100 and 0·2 M NaCl. The supernatant was chromatographed on a column of DEAE-Cellulose (DE-52; Whatman, Hillsboro, OR), equilibrated with 0·05 M Tris pH 7·4/0·2 M NaCl. The procollagen sample was then digested with pepsin to remove propeptides. The rhCII^{bac} was purified by salt precipitation and by chromatography on a Sephacryl HR-500 gel filtration column (Pharmacia, Piscataway, NJ).[15]

Preparation of tissue-derived type II collagen

Native CII was solubilized from human cartilage (from individuals under 18 years of age), fetal calf cartilage or murine sternal cartilage, by limited pepsin digestion and purified as described previously.[17] The collagen was dissolved in cold 0·01 M acetic acid at 4 mg/ml and stored frozen at −70° until used.

Melting profile of recombinant human collagen

The thermal unfolding profile of hCII^{bac} was determined by circular dichroism. Purified human type I collagen was used as a control as it was more readily available and because it has been amply documented, from previous studies, that the thermal denaturation temperature of type I and type II collagens is identical.[16] Collagen solutions were prepared at a concentration of 50 μg/ml in 50 mM acetic acid. Solutions were heated in a thermostatted 1-cm quartz cell (Gilford, Berlin, Germany) at a rate of 0·5°/min in a JASCO J-500 A circular dichroism spectropolarimeter and the ellipticity at 221 nm was recorded as a function of temperature. To convert the ellipticities to percentage triple helix content, the ellipticity at 20° was assumed to reflect 100% triple helix content, and the ellipticity at 45° was set to be 0% triple helix.

Determination of lysine hydroxylation and glycosylation

Collagen samples were dissolved in 2 M KOH, deoxygenated by purging with nitrogen gas and heated in sealed polypropylene tubes to 110° for 24 hr. Hydrolysates were diluted with nine volumes of water, and 0·4 volumes of 5 M acetic acid was added to the dilution. The pH was adjusted to 1·5–2·0 with 6 M HCl, and basic components were purified by cation exchange chromatography on Dowex 50 W-X8.[18] The purified basic amino acids were then separated on a Beckman 6300 automated amino acid analyser with a modified elution programme and detected by ninhydrin reaction. The ninhydrin colour factor of hydroxylysine was also used for the hydroxylysine glycosides. Standards for glucosyl–galactosyl–hydroxylysine and galactosyl–hydroxylysine were prepared from an alkaline hydrolysate of marine sponge, as described previously.[18]

Amino acid analysis

For amino acid analysis, collagen samples were hydrolyzed in 6 N HCl for 22 hr at 110° under an atmosphere of nitrogen. Hydrolysates were dried *in vacuo* over potassium hydroxide, and dissolved in sodium citrate sample buffer (Na-S; Beckman, Fullerton, CA). Separation and quantification was performed with the Beckman 6300 amino acid analyser using a modified gradient that allowed determination of hydroxyproline and hydroxylysine together with all other amino acids.[16,19] No corrections for hydrolytic losses or incomplete peptide bond cleavages were applied.

Animals

DBA/1 mice obtained from Jackson Laboratories (Bar Harbor, ME) were maintained in groups of five or six in polycarbonate cages and fed standard rodent chow (Ralston Purina Co., St. Louis, MO) and water *ad libitum*. The environment was specific pathogen-free for the mice obtained from Jackson Laboratories, and sentinel mice were tested routinely for mouse hepatitis and Sendai viruses. Mice were immunized at 8–12 weeks of age as described previously.[20]

Immunization

For routine immunization, CII was dissolved in 0·01 M acetic acid at 4 mg/ml and emulsified with an equal volume of complete Freund's adjuvant (CFA) as described previously.[20] The resulting emulsion was injected intradermally into the base of the tail. Each mouse received a total volume of 0·05 ml, containing 100 μg *Mycobacterium tuberculosis* and 100 μg of antigen.

Tolerization

DBA/1 mice were tolerized by either intravenous or oral administration of CII. For intravenous tolerization, CII was solubilized in 0·01 M acetic acid and dialysed against several changes of phosphate-buffered saline (PBS) in the cold (at 4°). Mice were then injected intravenously with 33 μg or 330 μg hCII or rhCII, daily for 3 days, for a total dose of 0·1 or 1 mg. Ovalbumin (Ova) was injected intravenously as a control. These mice were immunized with CII 1 week after the initiation of the tolerization and observed for the incidence of arthritis. This protocol is a modification of one previously used to suppress arthritis.[4]

In oral tolerization experiments, mice were administered different amounts of CII, ranging from 1 μg to 1000 μg per dose of CII, by oral gavage using ball-tipped gavage needles (Popper & Sons, Inc., New Hyde Park, NY). Ova was administered as a control. Each mouse was given four doses per week (on Monday, Tuesday, Thursday and Friday) for 2 weeks, for a total of eight doses. These mice were immunized with hCII 3 days after the last oral dose and observed for the development of arthritis. This protocol is a modification of one used by Khare and co-workers.[7]

Measurement of the incidence and severity of arthritis

The incidence and severity of arthritis were determined by examining and scoring each of the forepaws and hindpaws on a scale of 0–4, as described in detail previously.[20] There were two separate examiners, one of whom was unaware of the identity of the treatment groups. Each mouse was scored three times a week by visual examination, starting 3 weeks postimmunization and continuing for 8 weeks. The incidence of arthritis (number of animals with one or more arthritic limbs) was analysed at each time-point. The incidence and severity shown represent data taken 8 weeks after immunization.

Statistical analysis

The incidence of arthritis in various groups of mice was compared using χ^2-analysis with Yates' correction. Antibody levels were compared using the Student's *t*-test.

Measurement of serum antibody titres

Mice were bled 4 weeks after immunization and sera were analysed for antibodies reactive with native hCII and murine (m)CII using a modification of an enzyme-linked immunosorbent assay (ELISA), previously described.[4] A standard serum was added to each plate in serial dilutions. From these values, a standard curve was derived by computer analysis using a 4-parameter logistic curve. Results are reported as units of activity, derived by comparison of test sera with the curve derived from the standard serum, which was arbitrarily defined as having 50 U of activity.

Measurement of T-cell responses by proliferation

Draining lymph nodes were removed from animals at 10 days after immunization with either rhCII^{bac} or tissue-derived CII, minced, and cells washed in RPMI-1640. T cells were purified by nylon wool chromatography and placed in 96-well plates at 5×10^5 cells/well. Normal syngeneic spleen cells were used as a source of antigen-presenting cells (APC). Before their use, spleen cells were exposed to 3000 rads of gamma irradiation and then added to the T cells at 5×10^5 cells/well. Cell cultures were maintained in 300 μl of HL-1 medium and supplemented with 50 μM 2-mercaptoethanol (2-ME) and 0·1% bovine serum albumin (BSA, fraction V, IgG free, low endotoxin; Sigma Chemical Co.) at 37° and 5% CO_2 for 4 days. Antigens were tested at two concentrations: 40 μg/ml and 20 μg/ml. Eighteen hours before the termination of the cultures, 1 μCi of [^{3}H]-thymidine was added to each well. Cells were harvested onto glass fibre filters and counted on a Matrix 97 direct ionization β-counter (Packard Instrument Co., Meridan, CT). Results of all experiments are expressed as a stimulation index, which is the disintegrations per minute (DPM) response to each antigen, divided by the DPM response to media alone.

RESULTS

Production of recombinant hCII (rhCII)

A construct of the human gene for procollagen II (COL2 A1) was developed and previously used to produce recombinant CII from the human kidney tumour cell line (HT1080).[12,13] When the baculovirus construct (pVL3C/A1NP/C2 A1) was transfected into Sf-9 insect cells and assayed for expression of protein by Western blot, large quantities of rhCII protein were produced and recognized by hCII specific antibody (Fig. 1). The conformational stability of the rhCII^{bac} was evaluated using circular dichroism to determine the thermal transition temperatures. Both the recombinant human CII from the baculovirus and the tissue-derived type I collagen had melting temperatures of 41° (Fig. 2), which is consistent with that

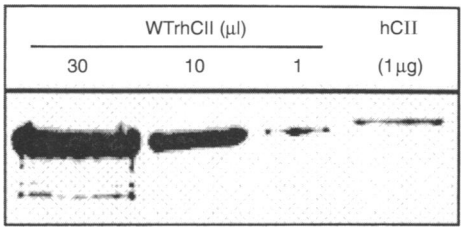

Figure 1. Western blot analysis of rhCII protein produced in insect cells. rhCII was resolved on SDS–PAGE, blotted, and probed with hCII affinity-purified antibody derived from DBA/1 mice immunized with tissue-derived hCII.

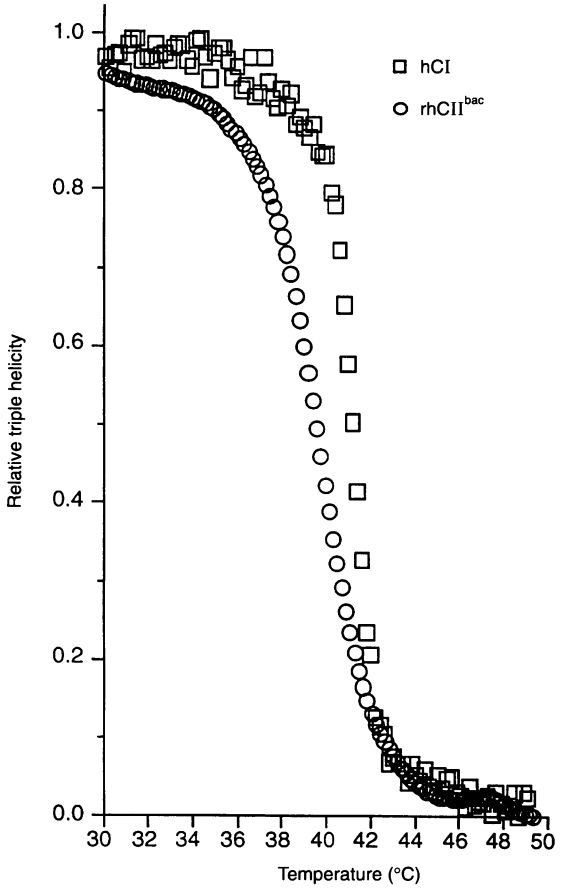

Figure 2. Melting curve analysis of the rhCII^{bac} and tissue-derived type I collagen. Each collagen sample was analysed by circular dichroism using a JASCO J-500 spectropolarimeter as described in the Materials and methods. Type I collagen, which has a melting curve identical to type II collagen, was used as a control.

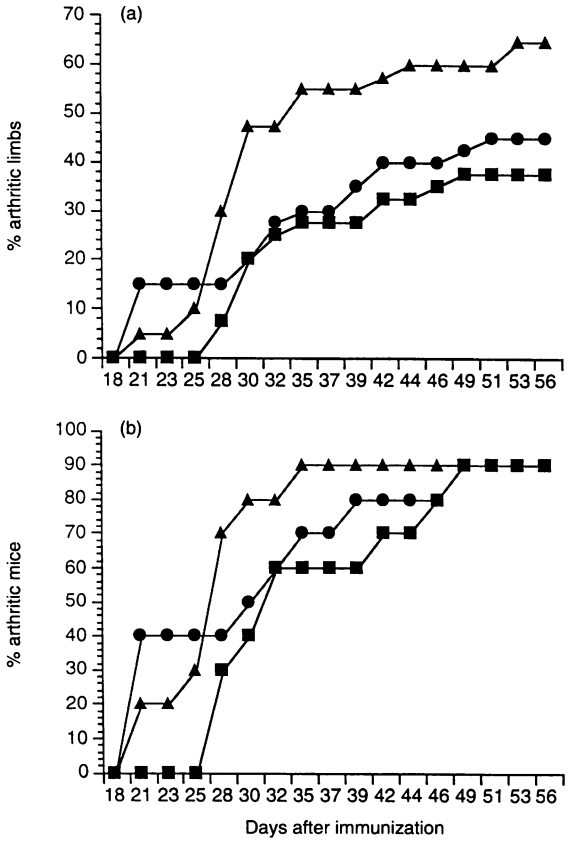

Figure 3. Arthritis following immunization with various collagens. Groups of 10 DBA/1 mice were immunized with tissue-derived hCII (●), rhCII^{bac} (■) or rhCII^{ht} (▲). Mice were evaluated individually for arthritis from day 18 until day 56 after immunization. Data shown in (a) represents the total number of arthritic limbs in each group of mice at each time-point. Data shown in (b) represents the percentage incidence of arthritis in each group of mice at each time-point. The group immunized with rhCII^{bac} differed significantly from the group immunized with rhCII^{ht} in percentage of arthritic limbs (65% compared to 38% at 8 weeks after immunization, $P \leq 0.05$).

found by Nokelainen *et al.*[15] and that reported for the HT1080-derived recombinant collagen.[12,13]

While HT1080 is a mammalian cell line and contains adequate prolyl and lysyl hydroxlase activities as well as

Table 1. Selected amino acid composition and carbohydrate content of the collagens*

	rhCII^{bac}	CII†	rhCII^{ht}
Hydroxylysine (Hyl)	5	9·2	12
Galactosylhydroxylysine (GH)	1	4·2	2·6
Glucosylgalactosylhydroxylysine (GGH)	4	1·9	9·5
Total (Hyl+GH+GGH)	10	15·3	24·1
Hydroxyproline	89	99	91‡
Proline	124	120	102‡

*The content of hydroxyproline, proline, and total hydroxylsine was determined on collagen samples, after hydrolysis in 6 N HCl, using a Beckman 6300 amino acid analyser and a modified gradient, as previously described.[16,19] Hydroxylysine glycoside contents were analysed after hydrolysis of collagen samples in 2 N KOH. Values are expressed as number of residues per thousand rsidues.

†Data adopted from.[27]

‡Data adopted from.[28]

CII, cartilage-derived CII; rhCII^{bac}, recombinant CII produced in the baculovirus expression system; rhCII^{ht}, recombinant CII produced in the HT 1080 mammalian-cell expression system.

hydroxylysine glycosyl transferase, Sf-9 cells are insect cells and poor in these enzyme activities.[12,13] Amino acid analysis indicated that the hydroxyproline content of the rhCII^{bac} produced in the presence of recombinant human prolyl-4 hydroxylase was comparable within experimental error to that of the tissue-derived CII (Table 1). These data are consistent with the observed melting temperature. In contrast, the content of hydroxylysine and hydroxylysineglycosides of rhCII^{bac} was lower than that of tissue-derived hCII (Table 1), while the HT1080-derived recombinant collagen was hyperhydroxylated and hyperglycosylated (Table 1).

Evaluation of arthritogenicity

The effectiveness of the recombinant collagens in inducing arthritis was determined by immunizing susceptible DBA/1 mice with rhCII^{bac}, rhCII^{ht} or CII, and observing for the incidence and severity of arthritis. As shown in Table 2, both forms of rhCII elicited a high incidence of arthritis (90%). On the other hand, the severity of the arthritis varied significantly among the three groups, with mice receiving rhCII^{ht} having 65% of the limbs affected compared to 38% of the limbs affected in the group receiving rhCII^{bac} ($P \leq 0{\cdot}05$) (Table 2 and Fig. 3). Antibody titres to human CII and murine CII tended to correlate with the severity of the arthritis although the differences did not achieve statistical significance (Table 2). Similarly, T-cell proliferation responses to human CII correlated with severity: T cells from mice immunized with rhCII^{bac} cultured *in vitro* with hCII gave a stimulation index of 3 compared with a stimulation index of 6 for T cells from mice immunized with tissue-derived material (data not shown). Unfortunately, T cells from rhCII^{ht}-immunized mice were not available for proliferative assays owing to insufficient availability of rhCII^{ht}. Taken together, these data reveal that both types of recombinant collagen are immunologically competent, capable of inducing severe autoimmune arthritis and significant antibody titres to both hCII and the autologous murine CII, but the severity of the arthritis produced varied significantly,

Table 2. Arthritogenicity of rhCII in DBA/1 mice

Immunogen†	% Arthritic mice‡	% Arthritic limbs‡	Antibodies to hCII§	Antibodies to mCII§
rhCII^{ht}	9/10 (90%)	26/40 (65%)	77±26	35±15
rhCII^{bac}	9/10 (90%)	15/40 (38%)*	46±22	20±19
hCII	9/10 (90%)	18/40 (45%)	56±25	32±12

*The group immunized with rhCII^{bac} differed significantly from the group immunized with rhCII^{ht} relative to percentage of arthritic limbs (65% versus 38%, $P \leq 0{\cdot}05$).

†DBA/1 mice (10 per group) were immunized with hCII, mammalian cell (HT1080)-derived rhCII^{ht} or insect cell (baculovirus)-derived rhCII^{bac}. Each mouse received 100 μg collagen, emulsified in CFA, subcutaneously at the base of the tail and a second dose of 100 μg of protein emulsified in incomplete Freund's adjuvant, 3 weeks after the first dose, for a total 200 μg.

‡The incidence and severity of arthritis was reported at 8 weeks postimmunization.

§Antibodies represent the mean units per group using sera collected 4 weeks after immunization. ELISAs were performed using human CII (hCII) and murine CII (mCII) and results are reported as units of activity, derived by comparison of test sera with the standard serum, which was arbitrarily defined as having 50 U of activity. Sera were analysed individually and results shown represent the mean±SD for each group of animals with statistical analysis performed using the Student's *t*-test.

Table 3. Prevention of collagen-induced arthritis by induction of intravenous tolerance with mammalian and insect cell-derived rhCII

Tolerogen†	% Arthritic mice‡	Antibodies to hCII§	Antibodies to mCII§
Ova (1000 μg)	18/20 (90%)	55·6±20	20·0±9
rhCII^{ht} (1000 μg)	0/10 (0%)	4·6±5*	2·5±1*
rhCII^{ht} (100 μg)	0/10 (0%)	5·0±3*	3·2±2*
rhCII^{bac}(1000 μg)	0/10 (0%)	2·3±2*	3·2±2*
rhCII^{bac} (100 μg)	0/20 (0%)	4·6±2*	5·0±4*
hCII (1000 μg)	0/10 (0%)	2·7±1*	2·0±3*

*$P \leq 0{\cdot}0005$.

†DBA/1 mice (10–20 per group) were injected intravenously with ovalbumin (Ova, negative control), tissue-derived hCII (positive control), mammalian cell-derived rhCII^{ht}, or insect cell-derived rhCII^{bac}. Either 33 or 333 μg of protein was administered daily for 3 days so that each mouse received a total of either 100 or 1000 μg protein. Mice were immunized with hCII emulsified in complete Freund's adjuvant 4 days after the last intravenous dose.

‡The incidence of arthritis was reported 6 weeks after immunization.

§Antibodies represent the mean units per group using sera collected 4 weeks after immunization. ELISAs were performed and results are reported as units of activity, derived by comparison of test sera with the standard serum, which was arbitrarily defined as having 50 U of activity. Sera were analysed individually and results shown represent the mean±SD for each group of animals with statistical analysis performed using the Student's *t*-test, comparing each group with the Ova control group.

correlating with the degree of glycosylation of the collagen utilized for immunization.

Evaluation of tolerogenicity following intravenous administration

The ability of each type of collagen to suppress arthritis was evaluated by intravenous injection into DBA/1 mice prior to immunization with hCII. Mice were administered either 100 or 1000 μg of each protein (Table 3), immunized with hCII and observed for the development of arthritis. Both recombinant collagens induced a profound suppression, preventing the induction of arthritis with even the lowest dose. Antibody titres to hCII were significantly lower compared with Ova-tolerized, control sera and were comparable with titres obtained when hCII was administered as the tolerogen. These data suggest that either of the recombinant CII given as a tolerogen is as effective as tissue-derived CII in suppressing experimental arthritis.

Evaluation of recombinant native CII for its effectiveness as an oral tolerogen

Each recombinant collagen was tested for its effectiveness as an oral tolerogen. However, in order to conserve the available supply of recombinant collagens, preliminary dose–response studies were first carried out using tissue-derived bovine CII to determine the optimal dose for oral tolerance. Bovine CII has 98% homology with the human CII and was more readily available in greater quantities than the recombinant materials. CII was administered in varying doses to DBA/1 mice prior to immunization with CII. Figure 4(a) shows that native CII given in doses of 5–500 μg per dose significantly reduced the incidence of arthritis compared to controls, with the most effective suppression occurring at 5–10 μg per dose. Similarly, antibody titres to CII from mice tolerized with different doses of CII were significantly suppressed in mice given doses of either 5 μg or 10 μg ($P \leq 0{\cdot}05$) (Fig. 4b). Based on these data we elected to use the 10 μg and 100 μg doses for testing the two recombinant CII proteins.

Recombinant collagens were then administered as oral tolerogens. As shown in Table 4, rhCII^{bac} and rhCII^{ht} administered at 10 μg/dose were as effective in tolerance and suppression of arthritis as tissue-derived hCII when incidence was evaluated at 5 weeks postimmunization. The differences in incidence remained significant throughout the evaluation period. When mice were evaluated at 8 weeks after immunization, mice given rhCII^{ht} had an incidence of 4/10 (40%, $P = 0{\cdot}001$), mice given rhCII^{bac} had an incidence of 11/24 (45%, $P = 0{\cdot}007$) and mice given tissue-derived CII had an incidence of 4/10 (40%, $P = 0{\cdot}001$) compared to mice fed Ova with an incidence of 21/24 (87%). Similarly, an analysis of the severity of arthritis revealed that groups of mice fed each of the collagen preparations varied significantly in the percentage of arthritic limbs from mice fed Ova by 5 weeks after immunization (Fig. 5); these differences continued until the end of the evaluation period (8 weeks postimmunization).

Sera were collected from these mice 4 weeks after immunization and analysed individually for antibody titres to both human and murine CII by ELISA (Table 4). Both heterologous and autologous antibody titres correlated closely with the reduction in the incidence of arthritis. The antibody titres from the mice fed the various collagen preparations did not differ statistically.

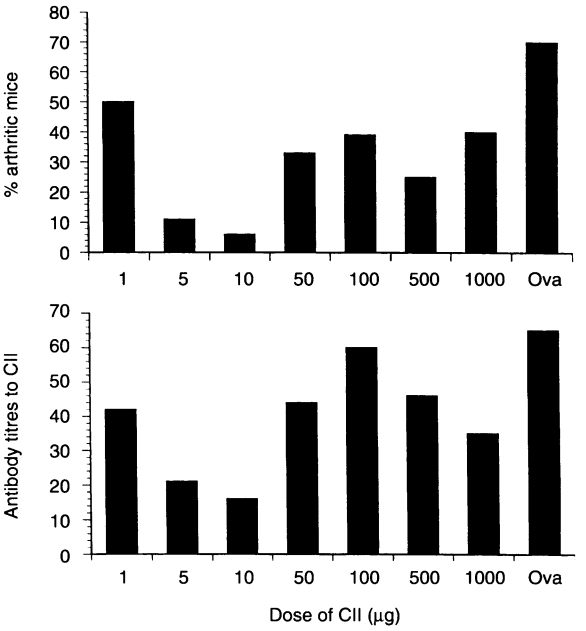

Figure 4. Incidence of arthritis and anti-CII antibody response following oral tolerance with various doses of CII. Groups of 10–24 DBA/1 mice were given native bovine CII, 1 to 1000 μg per dose, by gavage, administered as four doses per week for 2 weeks. Control mice received 1000 μg Ova. Mice were injected subcutaneously with CII emulsified in CFA 3 days after the last gavage. The incidence of arthritis occurring at 8 weeks postimmunization is shown in (a). Statistical analysis of arthritis incidence calculated using the χ^2-test with Yates' correction revealed that groups of mice given 5 μg/dose (3/24, $P = 0{\cdot}0001$), 10 μg/dose (1/20, $P < 0{\cdot}0001$), 50 μg/dose (8/24, $P = 0{\cdot}02$), 100 μg/dose (9/24, $P = 0{\cdot}03$) or 500 μg/dose (6/24, $P = 0{\cdot}0003$) were significantly different from controls given Ova (14/20). Sera were obtained at 4 weeks postimmunization and evaluated for anti-CII antibodies by ELISA (b). Results are reported as units of activity, derived by comparison of test sera with the standard serum, which was arbitrarily defined as having 50 U of activity. Sera were analysed individually and results shown represent the mean for each group of animals with statistical analysis performed using the Student's *t*-test, comparing each group to the Ova control group. Groups of mice given either 5 μg/dose or 10 μg/dose had significantly lower antibody titres than controls given Ova ($P \leq 0{\cdot}05$).

DISCUSSION

Recombinant CII offers several practical advantages over tissue-derived CII. The technology is applicable to preparation of large quantities of protein. This is of particular importance given the difficulty of obtaining human tissues for research purposes. Another advantage is the lack of contamination with pathogenic viruses. Our data suggest that recombinant CII can be prepared in adequate amounts for therapeutic use and that the material is immunologically comparable to tissue-derived hCII. The proteins induce a high incidence of arthritis following immunization of susceptible mice when emulsified with CFA. Moreover, rhCII is reasonably effective in the induction of tolerance and suppression of arthritis in CIA.

Table 4. Oral tolerance with rhCII^{ht} and rhCII^{bac}

Antigen fed	% Arthritic mice	Antibodies to hCII	Antibodies to mCII
Ova (100 μg)	18/24 (75%)	55±15	32±8
rhCII^{ht} (100 μg)	8/12 (67%)	27±17	19±14
rhCII^{ht} (10 μg)	2/10 (20%)*	22±15*	15±9*
rhCII^{bac} (100 μg)	12/24 (50%)	36±16	24±15
rhCII^{bac} (10 μg)	5/24 (21%)*	23±8*	21±4*
hCII (100 μg)	6/12 (50%)	25±9*	18±7*
hCII (10 μg)	2/10 (20%)*	21±11*	13±5*

Groups of 10–24 DBA/1 mice were administered, orally, ovalbumin (Ova), human type II collagen (hCII), mammalian cell-derived recombinant type II collagen (rhCII^{ht}), or insect cell-derived recombinant type II collagen (rhCII^{bac}). The collagens were dissolved in 0·01M acetic acid and administered four times per week for 2 weeks for a total of eight doses. Either 10 μg or 100 μg was administered daily so that mice received a total of either 80 μg or 800 μg protein. Mice were immunized with CII 3 days after the last dose. The incidence of arthritis was evaluated 5 weeks after immunization and statistical analysis carried out using Fisher's exact test. Antibodies represent the mean level/group (expressed in units) against native human type II collagen (hCII) or native murine type II collagen (mCII) using sera collected 4 weeks after immunization. Statistical analysis was performed using the Student's *t*-test comparing each group with the Ova control group.

*$P \leq 0{\cdot}05$.

Post-translational modifications of proteins may play a significant role in defining antigenic determinants. The high incidence of arthritis induced with insect cell-derived rhCII^{bac} was of particular interest in light of its relatively lower lysine hydroxylation and glycosylation as compared with cartilage-derived hCII (Table 1). Jensen and co-workers reported that T cells from CBA/J mice recognize a haemoglobin-derived decapeptide haemaglobin (Hb) 67–76) only when it contains the tumour-associated carbohydrate at position 72, while the peptide remained non-immunogenic if unglycosylated.[21] Other investigators have reported that DBA/1 mice immunized with periodate-treated CII showed a significant decrease in the incidence and severity of arthritis,[22] possibly because of the periodate destruction of the sugar residues. In our current study, immunization of DBA/1 mice with a recombinant collagen that was under-glycosylated, induced a less severe arthritis than that induced in mice immunized with hyperglycosylated collagen. Similarly, total antibody titres to human and autologous murine CII correlated with the number of affected limbs.

Another important immunological aspect of a given protein is its ability to induce tolerance. We report that recombinant hCII produced either by mammalian or insect cells is as effective as tissue-derived hCII in suppressing arthritis and down-regulating antibody titres to CII. Either type of rhCII is effective whether administered orally or parenterally. Although the oral route of administration offers practical advantages, oral tolerance offers several significant challenges as well. One is the proper selection of a dose. Our data indicates that the low dose of 10 μg/dose is more effective than the higher doses. Although these data are consistent with dose–response curves generated by others,[23–25] it is not clear why this occurs. It has been suggested that high doses of oral antigen lead to deletion or anergy of T cells whereas low doses induce regulatory T cells that secrete T helper 2 (Th2) cytokines (interleukin (IL)-4/IL-10) and transforming growth factor-β (TGF-β).[24] Perhaps the induction of a cytokine response of the Th2 type results in more effective suppression of arthritis than that induced by anergy alone.

Recently, attempts to use oral tolerance to treat autoimmune diseases in humans have yielded conflicting results. In a preliminary blinded study, bovine myelin basic protein (MBP) was given orally to 30 multiple sclerosis patients who subsequently experienced a decrease in the number of relapses of disease over a period of 1 year.[26] Oral MBP was not associated with measurable toxicity although long-term studies have not been completed.[26] Results from studies using CII to treat RA patients have also been conflicting. When chick CII was given orally to patients with RA, patients treated with the lowest dose (20 μg/dose) of CII had a 'significant' ($P < 0{\cdot}035$) improvement in response rate compared with placebo-treated, control patients.[8] In this CII treatment study, there was no evidence of antibody formation to chick CII.[8,9] On the other hand, Sieper and co-workers administered bovine CII orally to early RA patients for 12 weeks using doses of either 1 mg/day or 10 mg/day.[11] Unlike the previous study, there was no significant difference between patients treated with collagen and placebo. However, a higher prevalence of responders in the type II collagen-treated groups occurred and more patients in the 10-mg group had a very good response compared with placebo controls. A third study involving open-label treatment of JRA patients with oral chick CII[10] reported that eight of 10 patients improved with treatment.

Theoretically, the induction of an immune response targeted to a specific joint tissue, such as type II collagen, should effectively down-regulate joint swelling while avoiding the toxicities associated with a broad-based immune suppression. More work will be necessary to clarify the best antigens, routes of administration and dosing strategies effective for the treatment of human arthritis. Our data indicate that recombinant CII can be successfully generated and is effective in suppressing experimental collagen-induced arthritis when administered as a tolerogen, either intravenously or orally. Our data also suggest that the degree of glycosylation of the collagen

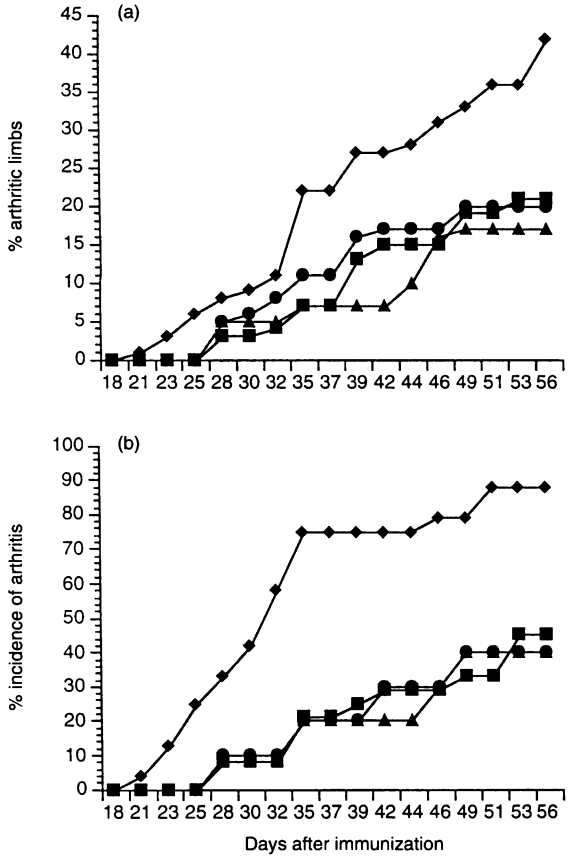

Figure 5. Arthritis following oral tolerance with various collagens. Groups of 10–24 DBA/1 mice were given 10 μg per dose of tissue-derived hCII (●), rhCII^{bac} (■) or rhCII^{ht} (▲), by gavage, administered as four doses per week for 2 weeks. Control mice received 1000 μg Ova (◆). Mice were injected subcutaneously with CII, emulsified in CFA, 3 days after the last gavage. Numbers shown in (a) represent the percentage of arthritic limbs observed at each time-point postimmunization. Numbers shown in (b) represent the percentage incidence of arthritis at each time-point postimmunization.

preparation can affect the potency of both T and B-cell responses to the immunogen. Because recombinant CII offers several practical advantages over tissue-derived CII, recombinant CII should play an important role in deciphering the mechanisms of oral tolerance and in development of immune-specific therapies for treatment of human autoimmune arthritis.

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REFERENCES

1. Harris E. (1993) Etiology and pathogenesis of rheumatoid arthritis. In: *Textbook of Rheumatology* (eds W. Kelley, E. Harris, S. Ruddy & C. Sledge), 4th edn, p. 833. W. B. Saunders, Philadelphia.
2. Wooley P.H., Luthra H.S., Krco C.J., Stuart J.M. & David C.S. (1984) Type II collagen-induced arthritis in mice. II. Passive transfer and suppression by intravenous injection of anti-type II collagen antibody or free native type II collagen. *Arthritis Rheum* **27,** 1010.
3. van Vollenhoven R.F., Nagler-Anderson C., Soriano A., Siskind G.W. & Thorbecke G.J. (1988) Tolerance induction by a poorly arthritogenic collagen II can prevent collagen-induced arthritis. *Cell Immunol* **115,** 146.
4. Myers L.K., Stuart J.M., Seyer J.M. & Kang A.H. (1989) Identification of an immunosuppressive epitope of type II collagen that confers protection against collagen-induced arthritis. *J Exp Med* **170,** 1999.
5. Nagler-Anderson C.N., Bober L.A., Robinson M.E., Siskind G.W. & Thorbecke G.J. (1986) Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. *Proc Natl Acad Sci USA* **83,** 7443.
6. Staines N.A. (1991) Oral tolerance and collagen arthritis. *Br J Rheumatol* **30,** 40.
7. Khare S.D., Krco C.J., Griffiths M.M., Luthra H.S. & David C.S. (1995) Oral administration of an immunodominant human collagen peptide modulates collagen-induced arthritis. *J Immunol* **155,** 3653.
8. Barnett M.L., Kremer J.M., St. Clair E.W. *et al.* (1998) Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum* **41,** 290.
9. Trentham D.E., Dynesius-Trentham R.A., Orav E.J. *et al.* (1993) Effects of oral administration of type II collagen on rheumatoid arthritis [see comments]. *Science* **261,** 1727.
10. Barnett M.L., Combitchi D. & Trentham D.E. (1996) A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. *Arthritis Rheum* **39,** 623.
11. Sieper J., Kary S., Sorensen H. *et al.* (1996) Oral type II collagen treatment in early rheumatoid arthritis. A double-blind, placebo-controlled, randomized trial. *Arthritis Rheum* **39,** 41.
12. Ala-Kokko L., Hyland J., Smith C., Kivirikko K.I., Jimenez S.A. & Prockop D.J. (1991) Expression of a human cartilage procollagen gene (COL2A1) in mouse 3T3 cells. *J Biol Chem* **266,** 14175.
13. Sieron A.L., Fertala A., Ala-Kokko L. & Prockop D.J. (1993) Deletion of a large domain in recombinant human procollagen II does not alter the thermal stability of the triple helix. *J Biol Chem* **268,** 21232.
14. Lamberg A., Helaakoski T., Myllyharju J. *et al.* (1996) Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. *J Biol Chem* **271,** 11988.
15. Nokelainen M., Helaakoski T., Myllyharju J. *et al.* (1998) Expression and characterization of recombinant human type II collagens with low and high contents of hydroxylysine and its glycosylated forms. *Matrix Biol* **16,** 329.
16. Miller E.J. & Gay S. (1982) Collagen: an overview. *Methods Enzymol* 82, Part A, 3.
17. Stuart J.M., Cremer M.A., Dixit S.N., Kang A.H. & Townes A.S. (1979) Collagen-induced arthritis in rats. Comparison of vitreous and cartilage-derived collagens. *Arthritis Rheum* **22,** 347.
18. Tenni R., Rimoldi D., Zanaboni G., Cetta G. & Castellani A.A. (1984) Hydroxylysine glycosides: preparation and analysis by reverse phase high performance liquid chromatography. *Ital J Biochem* **33,** 117.
19. Miller E.J. (1985) The structure of fibril-forming collagens. *Ann N Y Acad Sci* **460,** 1.
20. Rosloniec E.F., Kang A.H., Myers L.K. & Cremer M.A. (1997)

Collagen-induced arthritis. In: *Current Protocols in Immunology* (eds R. COICO & E. SHEVACH), p. 15.5.1–15.5.24. Wiley & Sons, New York, NY.

21. JENSEN T., GALLI-STAMPINO L., MOURITSEN S. *et al.* (1996) T cell recognition of Tn-glycosylated peptide antigens. *Eur J Immunol* **26,** 1342.
22. MICHAELSSON E., MALMSTROM V., REIS S., ENGSTROM A., BURKHARDT H. & HOLMDAHL R. (1994) T cell recognition of carbohydrates on type II collagen. *J Exp Med* **180,** 745.
23. WEINER H.L., FRIEDMAN A., MILLER A. *et al.* (1994) Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* **12,** 809.
24. CHEN Y., INOBE J. & WEINER H.L. (1997) Inductive events in oral tolerance in the TCR transgenic adoptive transfer model. *Cell Immunol* **178,** 62.
25. MILLER A., LIDER O., ROBERTS A.B., SPORN M.B. & WEINER H.L. (1992) Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci USA* **89,** 421.
26. WEINER H.L., MACKIN G.A., MATSUI M. *et al.* (1993) Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis [see comments]. *Science* **259,** 1321.
27. YANG C.L., RUI H., MOSLER S., NOTBOHM H., SAWARYN A. & MULLER P.K. (1993) Collagen II from articular cartilage and annulus fibrosus. Structural and functional implication of tissue specific posttranslational modifications of collagen molecules. *Eur J Biochem* **213,** 1297.
28. FERTALA A., SIERON A.L., GANGULY A. *et al.* (1994) Synthesis of recombinant human procollagen II in a stably transfected tumour cell line (HT1080). *Biochem J* **298,** 31.