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Identification and characterization of a major tolerogenic T-cell epitope of type II collagen that suppresses arthritis in B10.RIII mice

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SUMMARY

Tolerization of B10.RIII mice (H-2^r) with intravenously injected type II collagen (CII) renders the animals resistant to induction of collagen-induced arthritis (CIA). In order to clarify H-2^r-restricted T-cell responses that modulate CIA, we have analysed the T-cell proliferative response of B10.RIII mice against cyanogen bromide (CB) peptides of CII, and detected the strongest response to α1(II)-CB10 (CII 552–897). A panel of chemically synthesized overlapping peptide homologues was used to deduce the minimum structure of this determinant which was found to be CII 610–618. A 15-residue synthetic peptide flanking this region, CII 607–621, was found to effectively suppress arthritis when administered as a tolerogen. Collectively, these data identify the structural component within α1(II)-CB10 which is capable of inducing tolerance in B10.RIII mice. A similar approach to the treatment of autoimmune arthritis, involving the institution of self-tolerance, has potential applicability to human rheumatoid arthritis.

INTRODUCTION

Collagen-induced arthritis (CIA) is a model of autoimmune-mediated arthritis induced by immunizing susceptible strains of mice with type II collagen (CII).[1,2] Disease can be prevented in an antigen-specific manner by prior intravenous administration of CII.[3–5] Mice treated in this manner have been shown to mount decreased T-cell responses and significantly lower antibody titres to CII than mice pretreated with ovalbumin.[3–6] Our previous work has shown that CD4$^+$ cells are active in mediating tolerance to CII.[7] Therefore, the induction of self-tolerance has potential applicability to human autoimmune arthritis. In order to design specific therapies, however, T-cell antigenic determinants important to the regulation of arthritis must be identified, and those T cells reactive with these determinants and ultimately responsible for the manifestations of disease regulation must be characterized.

We have selected the B10.RIII mouse, which bears H-2^r, to identify structural determinant(s) of the CII molecule critical for the induction of tolerance and the subsequent suppression of arthritis. Although mice bearing H-2^q or H-2^r allotypes are highly susceptible to CIA,[8] the B10.RIII mouse is of particular interest, inasmuch as our recent data show that the T-cell determinants that induce tolerance are different from those that induce arthritis.[9] Specifically, we have found that bovine α1(II)-CB10 (CII 552–897) is effective as a tolerogen to B10.RIII mice, but does not induce arthritis. Conversely, bovine α1(II)-CB8 (CII 403–551) induces arthritis in B10.RIII mice, but is a poor tolerogen.[9] These observations imply that epitopes on CII are disparate in their abilities to induce tolerance or arthritis in CIA.[4] Therefore, characterization of the T-cell determinants on CII responsible for the induction of tolerance should prove important in developing novel therapeutic approaches for the treatment of human autoimmune diseases.

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Abbreviations: APC, antigen-presenting cells; CB, cyanogen bromide; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CII, type II collagen; MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Animals

B10.RIII mice obtained from Jackson Laboratories (Bar Harbor, ME) were maintained in groups of six in polycarbonate cages and fed standard rodent chow (Ralston Purina Co., St Louis, MO) and water *ad libitum*. The environment was specifically 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.[5]

Preparation of type II collagen and CNBr peptides

Native CII was solubilized from bovine articular and nasal cartilage by limited pepsin digestion and purified as described

earlier.[10] α1(II) chains were obtained by carboxymethyl-cellulose chromatography of thermally denatured CII. Purified α1(II) chains were subjected to cleavage with CNBr. After samples of α1(II) were dissolved in 70% formic acid, the solution was flushed with nitrogen and CNBr added. This reaction mixture was incubated at 40° for 4 hr, diluted 10-fold with cold distilled water and lyophilized. The peptides were redissolved in 50 ml of water and relyophilized. The resulting peptides were separated and purified by a combination of ion-exchange and molecular sieve chromatography as previously described in detail.[11]

Mimotope peptide synthesis

A panel of 116 individual 15-mer peptides spanning the sequence of bovine CB10, CII 552–897, was synthesized, each overlapping by three residue shifts using the Mimotope cleavable pin technology (Cambridge Research Biochemicals, Wilmington, DE), essentially as described by Geysen.[12] Active ester Fmoc amino acids were coupled via a diketopiperazine linkage to solid polyethylene supports. The Fmoc protecting group was removed by treatment with 20% piperidine in dimethyl formamide (v/v), and after several washes in methanol, the subsequent Fmoc amino acids were added in a stepwise fashion. On completion of synthesis, the side groups were deprotected with trifluoroacetic acid and anisole (95 : 5, v/v) and NH_2-terminal groups were acetylated with acetic anhydride in dimethyl formamide and triethylamine (2 : 5 : 1, v/v/v). The pins were then washed thoroughly to remove residual trifluoroacetic acid and scavengers and the peptides were subsequently cleaved from the pins into standard 96-well microtitre plates by overnight incubation in 150 μl of 50 mM HEPES buffer.

Chemical synthesis of oligopeptides of CB10

Oligopeptides containing sequences corresponding to known sequences of α1(II)-CB 10, (CII 552–897),[13,14] were chemically synthesized by a solid-phase procedure described previously[15] using an Applied Biosystem (model 430) Peptide Synthesizer (Applied Biosystems, Foster City, CA).

Immunization

For routine immunization, CII was dissolved in 0·01 N acetic acid and CNBr peptides were dissolved in phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA) as described previously.[5] 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 of *Mycobacterium tuberculosis* and 100 μg of antigen.

Tolerization

B10.RIII mice were tolerized neonatally within 48 hr of birth by giving each mouse 100 μg of antigen, (CB peptides or α1(II) chains), emulsified with incomplete Freund's adjuvant intraperitoneally. Mice were immunized with bovine CII at 8-weeks of age and observed for the incidence of arthritis. Incidence and severity shown represent data taken 5 weeks after immunization when the controls reached their peak. In some experiments, adult mice were tolerized intravenously with 0·33 mg of antigen, (synthetic peptides), daily for three days for a total dose of 1 mg. These mice were immunized with CII one week after initiation of the tolerization.

Measurement of the incidence of arthritis

The presence of arthritis was determined by examining and scoring each of the forepaws and hindpaws on a scale of 0–4 as described previously.[16] There were two separate examiners, one of whom was unaware of the identity of the treatment groups. Each mouse was scored 3 times a week by visual examination beginning 3-weeks post-immunization and continuing for 8 weeks. The incidence of arthritis (number of animals with one or more arthritic limbs) was analysed at each time point.

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 at 4 and 8 weeks after immunization to test for antibodies reactive with native CII or CB peptides of CII using a modification of an enzyme-linked immunoassay (ELISA) previously described.[5] A standard serum was added to each plate in serial dilutions. From these values, a standard curve was derived by computer analysis using a four-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, 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 /well. Normal syngeneic spleen cells were used as the source of antigen-presenting cells (APC). Before their use, spleen cells were exposed to 3000 rads of gamma irradiation and were added to the T cells at 5×10^5/well. Cell cultures were maintained in 300 μl of Click's medium and supplemented with 0·5% mouse serum at 37° and 5% CO_2 for 4 days. Peptides 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 fiber filters and counted on a Matrix 97 direct ionization β-counter (Packard Instrument Co., Meridan, CT). Results of all experiments are expressed as disintegrations per minute (d.p.m.).

RESULTS

T-cell responses to the bovine CB peptides

One critical component of the tri-molecular complex involved in the induction of tolerance is the processed peptide fragment(s) which are presented to CII-specific T cells and ultimately cause T-cell downregulation. Therefore, to identify immunodominant sequences of CII which play an immunoregulatory role, bovine CII was cleaved with CNBr and the major CB peptides selected for analysis of T-cell responses. Pooled splenocytes and lymph node cells from B10.RIII mice immunized with bovine CII were cultured with the CNBr peptides of CII and the proliferative responses measured by a [^{3}H]thymidine incorporation assay. As shown in Fig. 1, the greatest T-cell responses were generated against CB10.

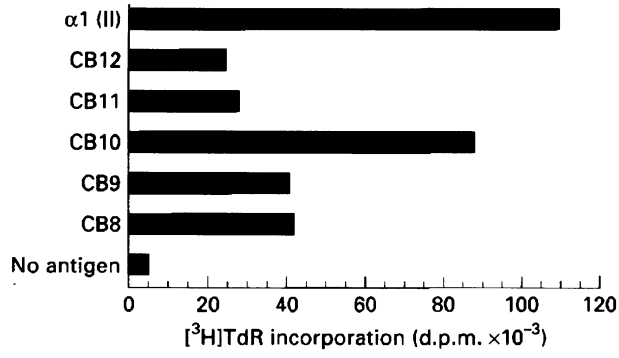

Figure 1. Identification of T-cell responses to bovine CNBr peptides. T cells from B10.RIII mice immunized 10 days earlier with bovine CII in CFA were tested for their ability to respond to CNBr peptides of bovine α1(II) chain. Details are described in the Materials and Methods section. Results are expressed as DPM.

Responses to other peptides were significantly lower over a range of antigen concentrations on repeated studies. These data are in agreement with results reported by Nabozny and co-workers using porcine CB peptides,[17] as well as with data obtained using a lymphokine production assay.[12] Collectively, these results suggest that CB10 contains the major T-cell epitope recognized by CII-immune T cells from B10.RIII mice.

Tolerization and suppression of arthritis in B10.RIII mice with CNBr peptides of bovine CII

Having characterized the T-cell responses to the CB peptides of CII, neonatal mice were injected with the peptides within 48 hr of birth to identify tolerogenic determinants capable of suppressing arthritis. Mice were subsequently immunized when they reached immunologic maturity and were observed for arthritis. As expected, denatured α1(II) chains were effective tolerogens (14% incidence versus 83% incidence in untolerized controls, Table 1). However, of the CB peptides, only CB10 was similarly effective (17% incidence, Table 1). Antibody levels to bovine CII were significantly lower in mice given either α1(II) chains or CB10 ($P \leqslant 0{\cdot}005$, Table 1), unlike mice given CB10, CB11, or CB12. CB8 did cause a significant but more modest decrease in antibody levels, ($P \leqslant 0{\cdot}05$), however, the incidence of arthritis was not statisitically different from controls (44% versus 83% incidence arthritis, P = NS).

Table 1. Tolerization Of B10.RIII mice with CNBr peptides

Tolerogen	Incidence of arthritis	Antibodies to bovine CII
None	15/18 (83%)	69·5 ± 37·3
α1(II)chains	**2/14 (14%)***	**11·0 ± 9·9 ($P \leqslant 0{\cdot}005$)**
CB8	4/9 (44%)	**23·0 ± 1·8 ($P \leqslant 0{\cdot}05$)**
CB9	4/6 (67%)	40·6 ± 13·2
CB10	**2/12 (17%)***	**15·3 ± 17 ($P \leqslant 0{\cdot}005$)**
CB11	9/12 (75%)	63·5 ± 11
CB12	5/6 (83%)	51·6 ± 18

Groups of from six to 18 B10.RIII mice were tolerized neonatally within 48 hr of birth by giving each mouse 100 μg of antigen emulsified with incomplete Freund's adjuvant intraperitoneally. Mice were immunized with bovine CII at 8 weeks of age and observed for the incidence of arthritis. Incidence and severity shown represent data taken 5 weeks after immunization when the controls reached their peak. As shown above, both denatured bovine CII and CB10 effectively suppressed the incidence and severity of arthritis while other CNBr peptides were ineffective. Boldface indicates positive correlation by Fisher's exact test.

* $P \leqslant 0{\cdot}05$ by Fischer's Exact Test.

Identification of the T-cell epitope within CB10

To identify the T-cell epitope within CB10 that is responsible

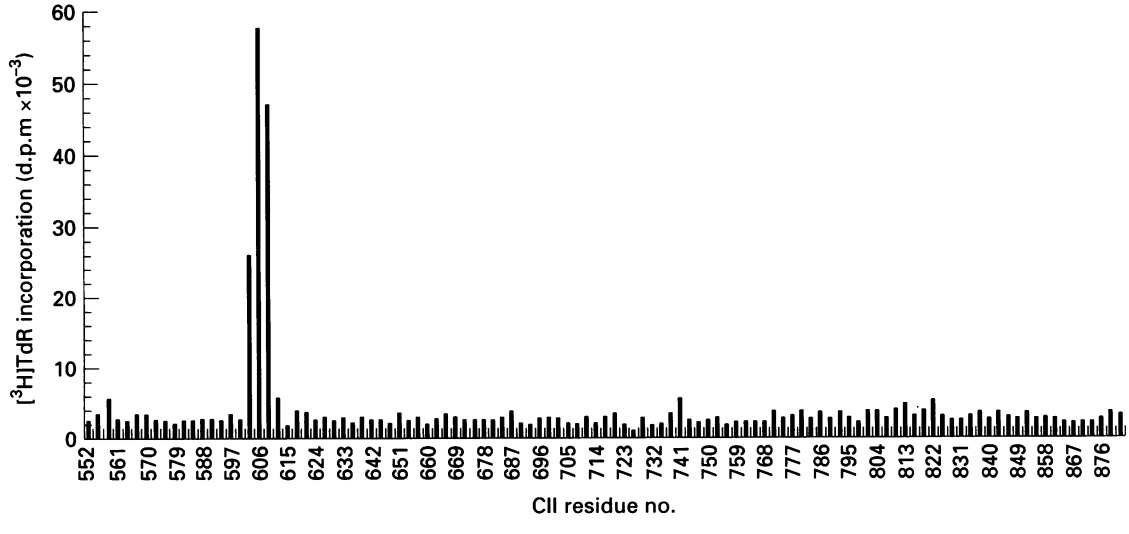

Figure 2. Identification of antigenic determinants in bovine CB10 recognized by B10.RIII mice using Mimotope synthetic peptides. T cells obtained from CII-immunized mice were cultured with a series of overlapping peptides (Mimotope peptides), 15mers, beginning at every third residue, representing the entire sequence of CB10. T-cell proliferation was measured by [^{3}H]thymidine incorporation.

Table 2. Identification of the dominant I-A^r-restricted T-cell determinant in bovine CB10

Peptide antigen*	601	606	611	616	621	626	T-cell proliferation†
CII(601–615)	G E V G P P G P A G P A G T A‡						–
CII(604–618)	G P P G P A G P A G T A G A R						+ +
CII(607–621)	G P A G P A G T A G A R G A P						+ +
CII(610–624)	G P A G T A G A R G A P G E R						+ +
CII(613–627)	G T A G A R G A P G E R G E T						–

* Overlapping Mimotope peptides (15mers) were synthesized as described in the Materials and Methods. This panel of peptides overlapped at every third residue.

‡ Amino acids are represented by single-letter code and residues are numbered from the amino terminus of the α1(II) chain. Prolines in the 'Y' position of the Gly-X-Y repeat are hydroxylated.

† T-cell proliferation data are summarized from Fig. 2. The three stimulatory peptides, CII (604–618), CII (607–621), and CII (610–624) represent the immunodominant determinant identified in Fig. 2.

for the potent suppression of arthritis, a series of 116 mimotope synthetic peptides was generated to overlap the CB10 region of bovine CII. Available amino acid sequence of bovine CB10 was used as the template. This panel of overlapping 15-mer peptides spanned the entire 346 amino acid residues of CB10, and allowed us to map the entire CB10 segment for T-cell determinants. As shown in Fig. 2, a distinct dominant antigenic determinant was identified, when these peptides were tested for their ability to stimulate T cells from B10.RIII mice immunized with bovine CII in CFA. Peptides CII (604–618), CII (607–621) and CII (610–624) strongly stimulated proliferation of bovine α1(II)-primed T cells, indicating that the T-cell determinant was contained within residues CII (610–618) (Table 2). These data suggest that T cells from mice expressing I-A^r respond predominantly to one major determinant of bovine CB10, and consequently this determinant most likely plays a central role in regulation of CIA in H-2^r mice.

Tolerization and suppression of arthritis in B10.RIII mice by CII 607–621

A synthetic peptide of 15 residues (CII 607–621), which contains the immunodominant core T-cell epitope was synthesized to determine its effectiveness in tolerance and suppression of arthritis. Adult mice were each given 1 mg of either CII 607–621 or an irrelevant control peptide, intravenously, divided between three daily doses. One week after the last injection, they were immunized with CII. As shown in Table 3, CII 607–621 significantly reduced the incidence of arthritis and autoantibody levels compared with controls. These data confirm that the 15mer, CII 607–621, which is contained within bovine CB10, is both an immunodominant T-cell determinant recognized by T cells immunized with CII as well as a potent tolerogen, capable of suppressing arthritis.

Table 3. Tolerization of B10.RIII mice with synthetic peptides

Tolerogen	Incidence of arthritis	Antibodies to bovine CII
None	9/10 (90%)	72·8 ± 17·3
CII(245–270)	8/8 (100%)	67·5 ± 17·5
CII(607–621)	2/11 (18%)*	29·6 ± 20·7*

B10.RIII mice were administered 0·33 mg of each antigen intravenously as a tolerogen, daily for 3 days. Seven days later mice were immunized with CII and observed for arthritis. Incidence of arthritis is shown for 6 weeks after immunization. Mice were bled at 6 weeks after immunization and antibodies to CII assayed by ELISA.

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

DISCUSSION

The induction of tolerance and subsequent suppression of autoimmune arthritis holds promise as a potential therapy for human autoimmune disease. Nevertheless, in order to adequately utilize the therapeutic efficacy of this approach we must first be able to identify antigenic determinants which are major players in this basic immunologic phenomenon. For the murine autoimmune model CIA, we have investigated the structural components of collagen peptides which induce tolerance in the B10.RIII mouse. The T-cell proliferative response to the various CB peptides revealed that in B10.RIII mice, CB10 contained the immunodominant T-cell epitope of bovine CII. This determinant was further narrowed using overlapping peptides to identify a core determinant consisting of nine residues, CII 610–618. A 15-mer peptide containing this core sequence was able to induce tolerance and subsequently suppress arthritis as effectively as CB10 itself.

These data are consistent with the observations of other investigators that the immunodominant determinant identified by proliferative assays is often the determinant most effective in inducing tolerance.[18,19] Interestingly, a hierarchy appears to exist in the immunogenicity of various peptides presented by the major histocompatibility complex (MHC). Investigators studying hen egg-white lysozyme observed that determinants recognized as immunodominant by T cells most effectively induced tolerance.[18,20] The determinant hierarchy is possibly due to the relative number of specific peptide-MHC complexes displayed on the APC. Certain peptide/MHC complexes are increased either by competition between peptides for the same class II molecules or by preferential production of certain peptides during processing.[21]

It is unclear at this time whether the suppression of CIA is due to a downregulation of pathogenic T cells that induce arthritis, or whether tolerance requires active participation of tolerogenic T cells. CB10 alone, which contains both a major T-cell epitope as well as antibody-binding sites, cannot induce arthritis when used as an immunogen for immunization in B10.RIII mice.[9] These data argue against a passive down-regulation. In DBA/1 mice, the suppression of arthritis can be transferred to naive mice by the transfer of $CD4^+$ cells.[7] These data support the idea that CIA can be actively suppressed. Either a switch to a more suppressive type of lymphokine profile, [TH2: interleukin (IL)-4, IL-10, TGF-β transforming growth factor-β],[22] or the more recent concept that anergic T cells specifically compete for the APC surfaces and for locally produced IL-2,[23] could explain this phenomenon. A few studies examining T-cell lymphokines produced during neonatal and oral tolerance to CIA have demonstrated decreased secretion of the inflammatory lymphokine interferon-γ (IFN-γ) in response to tolerogenic doses of CII.[24,25] Measurable quantities of the TH2-type lymphokine, IL-4, were not identified. Additional work will be necessary to clarify the mechanism of suppression in CIA.[25]

Tolerance with collagen, using the oral route, has shown promise as a potential therapy for treatment of human rheumatoid arthritis.[26–29] Use of the animal model, CIA, has allowed us to develop methods to identify specific collagen peptides that induce tolerance and suppress arthritis in the B10.RIII mouse. We believe the application of these methods to the study of human disease will ultimately have important therapeutic implications.

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