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Collagen-induced arthritis in B10.RIII mice (H-2^r): identification of an arthritogenic T-cell determinant

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SUMMARY

Susceptibility to collagen-induced arthritis (CIA), a murine model of autoimmune arthritis, is strongly linked to only two major histocompatibility complex (MHC) haplotypes, H-2^q and H-2^r. In order to identify the determinants of type II collagen (CII) required to induce arthritis in H-2^r-bearing mice, B10.RIII mice were immunized with bovine, chick or human CII. Only bovine CII induced significant arthritis and autoantibodies. When the major CNBr peptides of bovine collagen were isolated and used for immunization, only mice immunized with CB8, representing CII 403–551, developed arthritis. To identify immunogenic epitope(s) within CB8, a panel of synthetic peptides representing overlapping sequences of the bovine peptide was generated. When each peptide was cultured with T cells from B10.RIII mice immunized with CII, one peptide, representing CII 430–466, contained a major T-cell epitope. By using an *in vitro* lymphokine production assay, the T-cell epitope was further narrowed to CII 442–456. These findings suggest that a T-cell determinant important for the initiation of arthritis in B10.RIII (H-2^r) mice is located within a 15 amino acid sequence, residues 442–456 of bovine CII.

Immunization of susceptible mice with type II collagen (CII) leads to the development of an autoimmune polyarthritis, collagen-induced arthritis (CIA).[1,2] Disease susceptibility is strongly linked to only two major histocompatibility complex (MHC) haplotypes, H-2^q and H-2^r.[3] However, many other strains respond to immunization with CII without developing arthritis, and the basis of disease susceptibility is unknown.[4] It has been shown previously that arthritis can be transferred passively with serum from arthritic DBA/1 mice, and that transfer to resistant strains is possible.[5] This has led to the supposition that there is a qualitative difference in the immune response of susceptible strains. Susceptible strains may respond to epitopes that are uniquely exposed *in vivo*, or may develop a stronger autoimmune component of the overall response to heterologous collagen, or other factors such as the T-cell receptor (TCR) repertoire may be important.

We have shown previously that the cyanogen bromide (CB) peptide, CB11, can induce disease in H-2^q mice, and have identified a dominant T-cell epitope and several subdominant T-cell epitopes within this peptide.[6–8] However, it is not known which determinants of CII are necessary to induce disease in mice bearing the H-2^r haplotype. B10.RIII mice (H-2^r) have a different arthritis-susceptibility pattern following immunization with collagens from various species than do H-2^q mice.[3] B10.Q (H-2^q) mice develop a high incidence of arthritis following immunization with bovine, chick and human CII, but not porcine CII. On the other hand, B10.RIII (H-2^r) mice develop arthritis following immunization with bovine or porcine CII, but not with chick or human collagen.[3] The present studies were undertaken to determine whether the arthritogenic epitopes recognized by B10.RIII mice are in the same or different region of CII as those recognized by B10.Q, and to determine if there is a correlation between the development of autoreactive antibodies and arthritis.

In order to determine which regions of CII are recognized by T cells from CII-immune mice, bovine CII was cleaved with CNBr and the major CB peptides were tested for analysis of T-cell responses. B10.RIII mice were immunized with bovine CII by injecting 100 μg CII emulsified in complete Freund's adjuvant. Pooled splenocytes and lymph node cells from immunized animals were cultured with the various CNBr peptides. When supernatants were evaluated for the T-cell lymphokine interferon-γ (IFN-γ), the greatest T-cell responses were generated against CB10 (33 U IFN-γ, Fig. 1). Less, but still significant, responses were generated to other peptides, especially CB8. The fact that CB11 generated the weakest T-cell response was strikingly different from the prominent T-cell responses to CB11 previously reported in DBA/1 and B10.Q (H-2^q) mice. These data suggest that amino acid sequences of

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Abbreviations: B, hydroxyproline; CB, cyanogen bromide; CII, type II collagen; CIA, collagen-induced arthritis; IFN-γ, interferon-γ.

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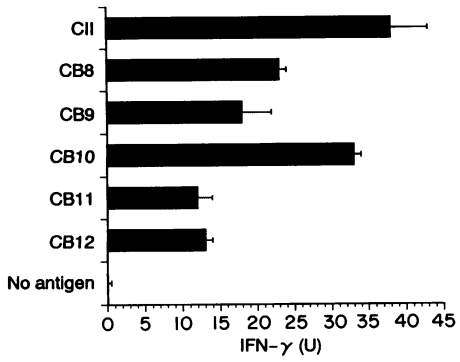

Figure 1. T-cell responses to bovine CB peptides. Native CII was solubilized from bovine nasal cartilage by limited pepsin digestion and purified as described elsewhere.[20] α1(II) chains were obtained by carboxymethyl-cellulose chromatography of thermally denatured CII and subjected to cleavage with CNBr. The resulting peptides were separated and purified by a combination of ion-exchange and molecular sieve chromatography, as previously described in detail.[21] For the T-cell stimulation assays, spleens and lymph nodes from mice immunized with CII 14–21 days previously were minced individually into single-cell suspensions and washed. 5×10^6 cells/ml were cultured with 100 μg/ml of antigen (CNBr peptides or collagen) in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). Supernatants were collected from 72 to 120 hr later and 200 μl aliquots were analysed for IFN-γ production. Quantitative measurement of murine IFN-γ (Gibco BRL, Grand Island, NY) was done using a solid-phase ELISA.[22] The absorbance was measured at 405 nm with a spectrophotometer and a standard curve was obtained by plotting the absorbance versus the corresponding concentration of the standards. Units of IFN-γ were calculated based on the National Institute of Health (NIH) standard number Gg02-901-533. Each sample was tested in duplicate wells. Data shown represent the mean ± SD of three separate experiments and results are expressed in units (U/ml) of IFN-γ. CB8 contained CII 403–551; CB9, CII 908–980; CB10, CII 552–897; CB11, CII 124–402; and CB12, CII 40–123.

bovine CII which are critical for initiation and regulation of arthritis in B10.RIII mice may be different from those required in DBA/1 mice.

In order to localize arthritogenic determinants, groups of B10.RIII mice were immunized with the individual CNBr peptides from bovine CII. Mice administered denatured α1(II) chains from bovine collagen developed overt persistent arthritis (40% incidence; Table 1). Of the groups of mice given the individual CNBr peptides, only the mice given CB8 (CII 403–551) developed arthritis (35% incidence). Surprisingly, CB10 was unable to induce disease (0/17 animals; Table 1). The onset of disease in mice administered CB8 was similar to that of mice immunized with denatured α1(II) chains of bovine CII (38 days compared to 40 days post-immunization; data not shown). Mice continued to develop arthritis until 7 weeks after immunization, and the arthritis persisted until the end of the observation period. The severity index was comparable in the two groups, ranging from digital swelling to synovitis involving the entire paw and ankle. These data suggest that bovine CB8 alone contains sufficient T- and B-cell determinants to induce arthritis in B10.RIII (H-2^r) mice. Levels of antibodies to bovine CII and autoantibodies to murine CII were higher in sera from mice immunized with CB8 compared to those from mice immunized with CB9, 10, 11 or 12 (Table 1).

In order to identify T-cell epitopes within CB8, a series of overlapping peptides, 32–37 amino acid residues in length, corresponding to bovine CB8, were chemically synthesized by a solid-phase procedure described elsewhere[9] using an Applied Biosystem (Foster City, CA) peptide synthesizer (model 430). T cells from B10.RIII mice immunized with bovine CII were cultured with each of these peptides. As shown in Fig. 2, when T cells were cultured with the peptides and assayed for T-cell lymphokine IFN-γ production (Fig. 2a), CII 430–466 generated the greatest response. Identical results were obtained by assaying proliferative responses using [^{3}H]thymidine incorporation (Fig. 2b). Taken together, these data demonstrate that a T-cell epitope important for the initiation of arthritis in B10.RIII mice is located within residues 430–466. The sequence of CII 430–466 is shown in Table 2. Surprisingly, it had only one sequence difference compared with homologous human and chick peptides at residue 450, and one sequence difference compared with its murine homologue at residue 447. Residue 450 had an alanine in the bovine CII but a valine in the human and chick CII, suggesting the possibility that residue 450 might be a critical residue for an important arthritogenic T-cell epitope. When T cells from B10.RIII mice immunized with bovine CII were cultured with synthetic peptides representing the sequences of either bovine, murine, chick or human CII 430–466, and supernatants were analysed for levels of the T-cell lymphokine IFN-γ, the bovine and murine CII 430–466 peptide generated significant levels of IFN-γ (22 and 15 U, respectively) compared to 1 U IFN-γ generated by the chick and human peptides. A 15-mer peptide encompassing CII 442–456 was as effective as the longer CII 430–466 in generation of IFN-γ (Table 2), supporting the possibility that residue 450 is critical

Table 1. Immunization of B10.RIII mice with bovine CB peptides*

Antigen	Incidence of arthritis	Antibodies to bovine CII	Antibodies to murine CII
α1(II) chains	2/5 (40%)	11·0 ± 6	5·0 ± 2
CB8	6/17 (35%)	14·3 ± 5	3·4 ± 2
CB9	0/5 (0%)	3·5 ± 1	1·0 ± 0·4
CB10	0/17 (0%)	4·0 ± 4	1·4 ± 1
CB11	0/22 (0%)	0·3 ± 0	0·6 ± 0·4
CB12	0/7 (0%)	2·5 ± 2	0·8 ± 0·8

* Groups of three to 22 B10.RIII mice were immunized with each of five bovine CNBr peptides or α1(II) chains, and observed for the incidence of arthritis. Results shown represent the incidence of arthritis observed at 7 weeks after immunization. Mice were bled at 6 weeks after immunization and sera analysed individually by ELISA as described previously[19] to determine antibody levels against both bovine CII and murine CII. Results shown represent the mean ± SD for each group of animals and are expressed in units of activity.

For immunization, peptides were dissolved in phosphate-buffered saline and emulsified with an equal volume of complete Freund's adjuvant, as described previously.[19] The resulting emulsion was injected intradermally into the base of the tail when mice were 8 weeks of age. Each mouse received 0·05 ml containing 100 μg of antigen and 100 μg of *Mycobacterium tuberculosis*. The presence of arthritis was determined by examining and scoring each of the forepaws and hindpaws as described previously,[4] and the incidence of arthritis (number of animals with one or more arthritic limbs) reported.

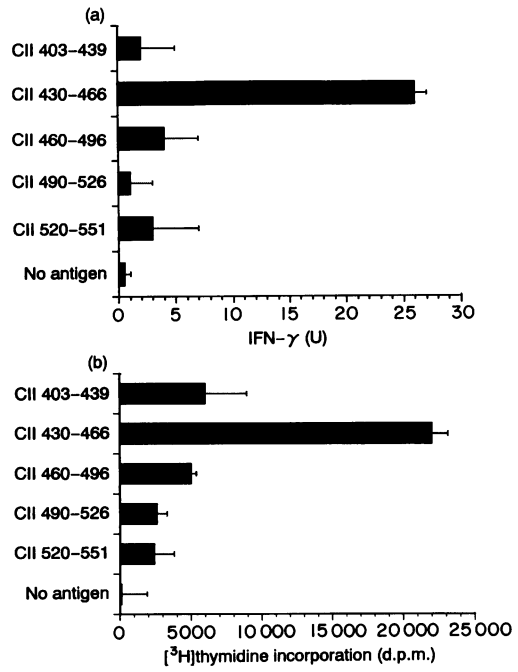

Figure 2. T-cell responses to synthetic peptides representing CB8. Lymph node cells from B10.RIII mice previously immunized with bovine CII were cultured with each of five synthetic peptides representing overlapping sequences of CB8.[23] The T-cell lymphokine, IFN-γ, assay was performed in the manner described in the legend to Fig. 1. For the lymphocyte proliferation assay, draining lymph nodes were removed from animals at 10 days after immunization with 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/well. Normal syngeneic spleen cells were used as the source of antigen-presenting cells. Before their use, spleen cells were exposed to 3000 rads of γ-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 humidified CO_2 for 4 days. 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 shown are the means of two separate experiments, expressed as disintegrations per minute (D.P.M.) ± SD.

for arthritis and that an important T-cell epitope is located within 442–456.

These data demonstrate that B10.RIII mice respond differently to CII than B10.Q, as might be expected from the considerable differences in their H-2 molecules. For B10.Q mice, CB11 contains an immunodominant epitope and can induce arthritis. For B10.RIII mice, the predominant T-cell epitope that stimulates the greatest IFN-γ production and proliferative response is contained within CB10. However, arthritis in B10.RIII can only be induced by CB8 under the conditions used in this study. Both strains developed a substantial cross-reactive response to homologous CII and there was a correlation between the level of autoantibodies produced and the development of arthritis.

Two components appear to be necessary for foreign proteins to initiate an autoantibody response. One is the presence of a T-cell epitope capable of initiating the appropriate T-cell lymphokine responses to produce autoantibodies, and the other is the presence of B cells cross-reactive with homologous epitopes. T-cell help, initiated by immunization with a foreign protein, can cause B cells to produce antibodies that cross-react with native protein.[10,11] Since both CB8 and CB11 elicit disease in mice bearing the appropriate MHC, both peptides must contain sufficient B-cell determinants to induce autoreactive antibodies and arthritis. Therefore, the requirements for T-cell determinants to bind I-A and initiate T-cell responses must represent the critical difference between the two haplotypes. Similarly, in the animal model experimental allergic encephalomyelitis (EAE), each disease-susceptible H-2 haplotype required immunization with a different myelin proteolipid protein (PLP) in order to induce encephalitis.[12] PLP 139–151 was encephalitogenic in H-2^s mice, while PLP 103–116 was encephalitogenic in H-2^q mice, PLP 215–232 in H-2^k mice, and PLP 43–64 in H-2^u mice.

It was unexpected that CB10, the CNBr peptide that induced the greatest T-cell response *in vitro* was unable to induce disease. Proliferative T-cell responses to bovine CB10 (48 000 d.p.m.) were also greater than those elicited against CB8 (22 000 d.p.m.) (Miyahara *et al.*, unpublished data). This immunodominance of CB10 in B10.RIII mice has also been reported by Nabozny *et al.*[13] using porcine CNBr peptides. Despite containing T-cell determinants that were immunodominant when analysed for lymphokine production and

Table 2. Identification of the T-cell epitope within bovine CII 430–466*

	430										442										450							456							466	IFN-γ (U)
Murine	G	L	B	G	K	D	G	E	T	G	A	A	G	P	B	G	P	S	G	P	A	G	E	R	G	E	Q	G	A	B	G	P	S	G	F	Q	G	15
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	22
Chick	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Human	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Bovine													-	-	-	-	-	A	-	-	-	-	-	-	-	-	-											23

* Sequences of CII 430–466 are shown for each of four species of type II collagen. Human and chick CII 430–466 each had two amino acid differences compared with the murine counterpart, while bovine CII 430–466 had only one difference. Synthetic peptides representing each of the above sequences were cultured with pooled splenocytes and lymph node cells from mice previously immunized with CII. Supernatants were assayed for the content of IFN-γ using ELISA.

–, identity of sequence.

proliferation, immunization with CB10 generated lower autoantibody levels than those generated following immunization with CB8. Two possibilities could explain this phenomenon. CB10 may not contain a sufficient number of B-cell epitopes cross-reactive with native CII to generate the quantities of autoantibodies required to induce disease. Alternatively, the T-cell determinant within CB10 may be deficient, either in induction of lymphokines critical for B-cell help or in its ability to induce T cells which cross-react with the murine counterpart. We have demonstrated previously that autoantibody production is important for the initiation of this disease. CIA can be transferred by both polyclonal and monoclonal antibodies[5,14] and the severity of the disease correlated with the level of antibody titres to CII.[2]

Immunization of B10.RIII mice with full-length bovine CII elicits antibodies reactive with CB10 in levels comparable to those generated against CB8 (22 ± 10 U and 15 ± 10 U, respectively; Myers *et al.*, unpublished data). Therefore, we feel that CB10 probably does contain sufficient antibody-binding sites. We favour the possibility that the T-cell epitope is ineffective at the induction of autoreactive responses. Sequence differences with the homologous segment of murine CII may not allow T- and B-cell cross-reactivity with murine collagen. Perhaps the sequence relationship between the determinant within bovine CB10 and murine CB10 is not conducive to the induction of autoimmune responses.[15] Delineation of the T-cell epitope within CB10 should allow clarification of this phenomenon.

We have identified a T-cell determinant within the arthritogenic peptide CB8 from bovine CII. The data suggest that this T-cell determinant is critical for the initiation of arthritis. Within this determinant there is only one sequence difference among bovine, chick and human CII (Table 2). Residue 450 is an alanine in the bovine sequence, whereas the chick and human sequences have a valine in this position. Therefore, residue 450 appears to be critical for the induction of arthritis. Because there may be processing differences between native and denatured CII, we attempted to induce arthritis with chick CB8. Five B10.RIII mice were immunized with chick CB8. Although the group of animals was small, none became arthritic and the mean antibody levels to CII were less than 2 U. Studies using T-cell determinants of several proteins, including tetanus toxin and myelin basic protein, have clearly identified that substitution of one amino acid residue in a critical position can disrupt T-cell function *in vitro*.[16–18] If an amino acid residue is in a critical position for interaction with T-cell receptors, binding to the I-A^r, or even intracellular processing, one substitution could sufficiently disrupt T-cell function to diminish the incidence of arthritis. Bovine CII 430–466 has only one amino acid different from its murine counterpart, whereas chick and human CII 430–466 each have two differences with the murine homologue. It remains unclear how structural differences between foreign and murine T-cell determinants interact to generate cross-reactive T and B cells. Further work identifying residues critical for the T-cell responses to these epitopes should clarify this phenomenon.

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