

An HLA-DR1 Transgene Confers Susceptibility to Collagen-induced Arthritis Elicited with Human Type II Collagen

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Summary

Rheumatoid arthritis (RA) is an autoimmune disease that is strongly associated with the expression of several HLA-DR haplotypes, including DR1 (DRB1*0101). Although the antigen that initiates RA remains elusive, it has been shown that many patients have autoimmunity directed to type II collagen (CII). To test the hypothesis that HLA-DR1 is capable of mediating an immune response to CII, we have generated transgenic mice expressing chimeric (human/mouse) HLA-DR1. When the DR1 transgenic mice were immunized with human CII (hCII), they developed a severe autoimmune arthritis, evidenced by severe swelling and erythema of the limbs and marked inflammation and erosion of articular joints. The development of the autoimmune arthritis was accompanied by strong DR1-restricted T and B cell responses to hCII. The T cell response was focused on a dominant determinant contained within CII(259–273) from which an eight amino acid core was defined. The B cell response was characterized by high titers of antibody specific for hCII, and a high degree of cross-reactivity with murine type II collagen. These data demonstrate that HLA-DR1 is capable of presenting peptides derived from hCII, and suggest that this DR1 transgenic model will be useful in the development of DR1-specific therapies for RA.

It is well established that strong correlations exist between the expression of specific MHC alleles and increased susceptibility to certain immune-mediated diseases. Some of these diseases, such as ankylosing spondylitis, have been shown to be associated with the expression of a specific class I allele (HLA-B27), whereas many other diseases are strongly linked with the expression of certain class II alleles. One autoimmune disease that has been studied extensively for its association with class II alleles is rheumatoid arthritis (RA)¹. Many years ago it was noted that a strong correlation existed between the expression of HLA-DR1 or DR4 and an increased risk of developing RA (1–3). We now know that in most cases, this increased susceptibility is associated with the DRB1 locus, and more specifically with the

presence of the DRB1*0101, DRB1*0401, DRB1*0404, or DRB1*0405 genetic allotypes (4). Sequence analysis of these alleles revealed that each encodes a similar amino acid sequence within residues 67–74, termed the shared epitope (5, 6). The role of this shared epitope has been the focus of several hypotheses (5–8); however, little is known about how it affects the function of the DR molecules and confers susceptibility to RA.

Although the etiology of RA remains unsolved, several antigens have been proposed to be involved in the stimulation of pathogenic T cells in RA. Viral proteins such as CMV and EBV have been implicated (9, 10), as well as autologous proteins normally expressed in diarthroidal joints. One autologous protein, type II collagen (CII), has received considerable attention as a potential antigen in RA because of both its ability to induce experimental autoimmune arthritis in several animal models (11–13), and the demonstration of CII-specific immunity in the diseased synovium and cartilage of RA patients (14–17). Although it

¹Abbreviations used in this paper: CII, type II collagen; CIA, collagen-induced arthritis; DMF, dimethyl formamide; hCII, human CII; mCII, murine CII; RA, rheumatoid arthritis; Tg, transgenic.

has not been proven that autoimmunity to CII initiates RA, it seems clear that at least some of the autoimmunity in RA is directed towards CII. The difficulty, however, has been in determining the relationship between immunity to CII and the role of the class II alleles that confer susceptibility to RA.

One approach to analyzing the role of the HLA-DR molecules in RA is to establish them as transgenes in animals. Using transgene models, antigens proposed to be recognized by DR-restricted T cells can be tested for their ability to induce an immune response as well as elicit an experimental autoimmune disease. In addition, HLA transgenic (Tg) mice facilitate the identification of peptides presented by DR molecules from an antigen in question, especially from human proteins. Toward these aims, we have established transgene expression of a chimeric (human/mouse) HLA-DR1 molecule (DRB1*0101, DRA1*0101) in the arthritis-resistant B10.M (H-2^d) mouse in an attempt to determine if the DR1 molecule is capable of presenting peptides derived from human CII (hCII), and if this presentation would lead to the development of an experimental autoimmune arthritis. Here we report that this chimeric DR1 transgene is fully functional as a class II restriction element in the mouse and strongly confers susceptibility to an autoimmune, collagen-induced arthritis (CIA) after immunization with hCII. In contrast, non-Tg control mice were totally resistant to arthritis induction and mounted only a negligible immune response to hCII. The autoimmune arthritis that develops in the DR1 Tg mice is accompanied by strong T and B cell responses to hCII. Virtually all of the DR1-restricted T cell responses to hCII are directed to two antigenic determinants, with the core of the dominant determinant located within CII(263–270). Antibodies produced by DR1 Tg mice after hCII immunization bound strongly to both the immunogen, hCII, and the autoantigen murine CII (mCII). These data indicate that the DR1 molecule is capable of binding and presenting peptides derived from hCII, supporting the hypothesis that autoimmunity to CII plays a role in the pathogenesis of RA.

Materials and Methods

Animals. (C57BL/6 × SJL/J)F2 and B10.M/Sn (H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Generation of Tg Mice Expressing DR1. Chimeric (human/mouse) DRB1*0101 constructs were made using the chimeric DRB1*0401 β chain gene construct that has been previously described (18). To generate the chimeric DRB1*0101 gene construct, the plasmid containing the chimeric DRB1*0401 β chain gene was partially digested with BamHI and completely digested with EcoRI to remove a gene fragment encompassing exon 2. A PCR product was obtained by amplification of DRB1*0101 β chain cDNA with primers flanking exon 2 of the DRB1*0101 β chain (5'-AAC-CGG-ATC-GTT-CTT-GTC-CCC-CCA-GCA-CGT-TTC-TTG-TGG-CAG-CTT-AAG-T-3' and 5'-CGG-CCC-GCC-CCG-GCG-CCG-TGC-TCA-C-3'). Intron/exon splice junctions along with BamHI and EcoRI cloning sites were ligated onto this gene fragment by a second PCR using overlapping primers (5'-GAC-TTG-AAT-TCC-GGA-GGC-GCT-TCT-GTA-

ACC-GGA-TCG-TTC-TTG-TCC-CCC-C-3' and 5'-GAC-TTG-GAT-CCG-CCG-CTC-ACA-GGG-CTC-GGC-CCG-CCC-CGG-CGC-CGT-GC-T-C-3'). The resulting 372-bp PCR product was digested with BamHI and EcoRI and cloned into the partially digested chimeric β chain gene construction described above. To generate Tg mice, fertilized (C57BL/6 × SJL/J)F2 oocytes were microinjected with an XhoI/NotI DNA fragment containing the chimeric DRB1*0101 β chain gene along with a DNA fragment containing the chimeric DRA1*0101 gene (18). Eight Tg founders were obtained. The founder with the highest cell surface DR1 expression levels was backcrossed for three generations with B10.M mice and then intercrossed to fix the H-2^d locus and DR1 transgenes to homozygosity.

Tg control mice were derived from DR negative litter mates during the backcross breeding and express only I-A^d from the class II locus. For these experiments all mice were bred and maintained at the Veterans Affairs Medical Center of Memphis (Memphis, TN) in a specific pathogen-free environment, and sentinel mice were tested routinely for the presence of mouse hepatitis and Sendai viruses.

Collagen Preparation. Native hCII was solubilized from sternal cartilage harvested from donors <20 yr of age by limited proteolysis with pepsin and purified by repeated differential salt precipitation as described by Miller (19).

Immunizations. 6–8-wk-old mice were immunized with hCII for the induction of arthritis. hCII was dissolved in cold 0.01 M acetic acid by stirring overnight at 4°C, and emulsified at a 1:1 (vol/vol) ratio with complete Freund's adjuvant (GIBCO BRL, Gaithersburg, MD), as previously described (20). Mice were immunized subcutaneously at the base of the tail with 100 μg of hCII. For some experiments, mice were boosted 2–3 wk later with 100 μg of hCII in incomplete Freund's adjuvant. Each paw was evaluated and scored for the degree of inflammation on a scale of 0–4 (21).

Peptide Synthesis. Peptides were synthesized by Fmoc chemistry using either an automated peptide synthesizer (model 430; Applied Biosystems, Foster City, CA), or manually using the Mimotope cleavable pin technology (Chiron Mimotopes, San Diego, CA), essentially as described (22, 23). For the Mimotope synthesis, the active ester Fmoc protecting groups were removed by treatment with 20% piperidine in dimethyl formamide (DMF; vol/vol), and after several washes in methanol, the subsequent Fmoc amino acids were added in a step-wise fashion to generate 15-mer peptides. Hydroxyproline (BACHEM Bioscience Inc., Philadelphia, PA) was obtained as a standard Fmoc, necessitating the use of 1-hydroxybenzotriazole (Calbiochem, La Jolla, CA) when coupling this amino acid to the peptide chains. Upon completion of the synthesis, the side groups were deprotected with TFA and anisole (95:5, vol/vol), and terminal amino groups were acetylated with acetic anhydride in DMF and triethylamine (2:5:1; vol/vol/vol). The pins were then washed thoroughly to remove residual TFA and scavengers, and the peptides were subsequently cleaved from the pins by overnight incubation in 750 μl of 50 mM Hepes buffer. Selected Mimotope peptides were analyzed and quantitated by reverse phase HPLC, and all were >80% pure and contained 2–4 μg/μl of peptide.

Immunofluorescence. Cell surface expression of class II molecules DR1 and I-A^d was evaluated by immunofluorescence and flow cytometry. Lymphocytes were obtained from either spleens, lymph nodes, or purified from peripheral blood using Lympholyte M (Accurate Chemical, Westbury, NY). 2 × 10⁵ mononuclear cells were incubated with a 100 μl of anti-DR antibody (L243; Becton Dickinson, San Jose, CA) or anti-I-A antibody

(10-3.6) conjugated with FITC. The antibody 10-3.6 was purified from a culture supernatant using protein A affinity chromatography (Sigma Chemical Co., St. Louis, MO). For two-color immunofluorescence of I-A^f and DR1, anti-DR (L243; Becton Dickinson) conjugated to PE was used. After a 30 min incubation on ice, cells were washed several times with PBS containing 0.1% sodium azide. Fluorescence was analyzed by a flow cytometer (Coulter Profile II; Coulter Electronics, Miami, FL), and all data are based on a minimum of 5,000 gated cells analyzed.

Proliferation Assays. Draining lymph nodes were removed from animals at 10 d after immunization, dissociated, and washed in RPMI 1640. Lymphocytes were cultured in 96-well plates at 4×10^5 /well in either 300 μ l of Click's medium (24) supplemented with 0.5% mouse serum, or in HL-1 medium (BioWhittaker, Inc., Walkersville, MD) at 37°C, 5% humidified CO₂ for 4 d. 18 h before the termination of the cultures, 1 μ Ci of [³H]thymidine (Dupont NEN, Boston, MA) was added to each well. Cells were harvested onto glass fiber filters, and counted on a Matrix 96 direct ionization β -counter (Packard Instrs., Meriden, CT). Proliferation assays using Mimotope synthetic peptides were performed at one well per peptide and 10 μ l of peptide per well. Results were confirmed by replicate experiments, and all data are expressed as decays per minute.

T Cell Hybridomas and Antigen Presentation Assays. T cell hybridomas were established by polyethylene glycol (Boehringer Mannheim, Indianapolis, IN) induced fusion of lymph node cells with TCR- α^-/β^- BW5147 thymoma cells (25, 26). Lymph node cells were obtained from B10.M-DR1 Tg mice immunized 10 d previously with hCII emulsified with complete Freund's adjuvant and cultured with human α 1(II) for 5 d, followed by IL-2 for 3 d before fusion. Resulting hybridomas were screened in antigen presentation assays for their ability to recognize human α 1(II) chains presented by DR1 and I-A^f. Antigen presentation experiments were performed in 96-well microtiter plates in a total volume of 0.3 ml containing 10^5 APCs or 4×10^5 syngeneic spleen cells and 10^5 T hybridoma cells. The following APCs were used: L57.23, L cells (L66) transfected with wild-type DRA1*0101 and DRB1*0101 (27); DRAB 10, MUD45 cells transfected with chimeric DR1 constructs as described for DR4 (18); and 43.2.1, a B cell hybridoma that expresses I-A^f and I-A^d. Cell cultures were maintained at 37°C in 5% humidified CO₂ for 20–24 h, after which twofold serial dilutions were made for determination of IL-2 titers. 4,000 HT-2 cells were added to each supernatant dilution, and after 16–20 h, HT-2 cell viability was evaluated by visual inspection and cleavage of MTT (28, 29). IL-2 titers were quanti-

fied by the reciprocal of the highest twofold serial dilution maintaining 90% viability of the HT-2 cells. Results are presented as units of IL-2 per milliliter of undiluted supernatant as described by Kappler et al. (30).

ELISA. Antibody titers specific for hCII and mCII were determined using a solid phase ELISA as previously described (31). In brief, microtiter plates were coated with either 500 ng of hCII or mCII at 4°C overnight. After extensive washing with 0.15 M saline/0.05% Tween 20, dilutions of sera ranging from 1:4,000 to 1:24,000 in 2% normal goat sera were added to each well and incubated overnight at 4°C. After washing with saline and Tween 20, a goat anti-mouse Ig (1:5,000) was added for 2 h. The plates were then washed and developed by the addition of *o*-phenyldiamine (Sigma Chemical Co.). After stopping the reaction with 2.5 N H₂SO₄, the degree of color development was measured at 490 nm with background absorbance of 650 nm subtracted. Data are expressed as mean relative units of activity based on a standard anti-hCII serum. Sera were collected at 6 wk after hCII immunization, and quantity of specific antibody was measured for each animal.

Histology. Arthritic and nonarthritic limbs were removed at 6 wk after hCII immunization and fixed in 10% buffered (PBS) formaldehyde, decalcified in 5% formic acid, and embedded in paraffin. Serial sections (5 μ m) of the joints were stained with either hematoxylin and eosin, or toluidine blue.

Results

Production and Characterization of HLA-DR1 Tg Mice. DR1 Tg founders were backcrossed to the CIA nonsusceptible strain B10.M (H-2^f), and homozygous DR1, I-A^f mice were established and inbred. The DR1 transgene is expressed in a tissue-specific manner identical to that of the chimeric DR4 transgene previously described (18). As shown in Fig. 1, the DR1 transgene is expressed only by lymphocytes that express I-A^f, and all lymphocytes expressing I-A^f express the chimeric DR1. Based on immunofluorescence intensity, the DR1 molecule is expressed at an \sim 10-fold higher level than the previously described chimeric DR4 transgene, using the same anti-DR fluorescein-conjugated antibody (L243, data not shown).

Susceptibility of DR1 Tg Mice to CIA. HLA-DR1 Tg mice were immunized with hCII to determine if expression of

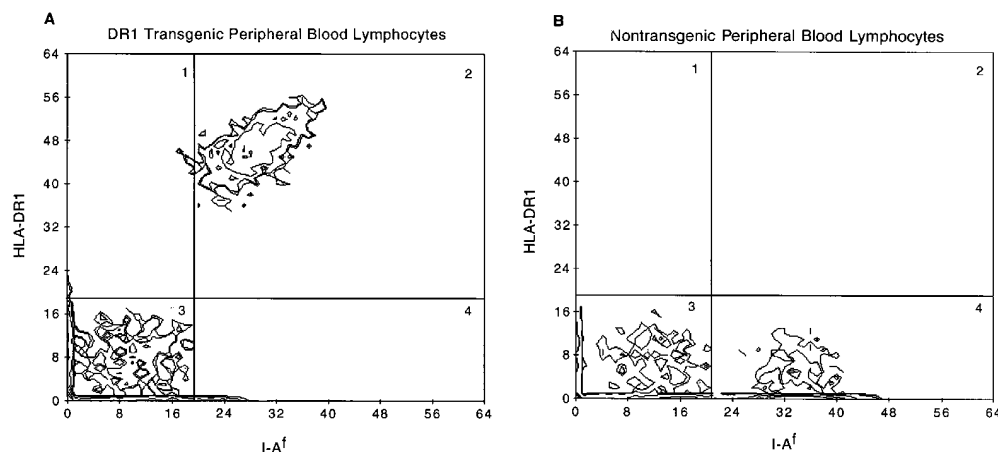


Figure 1. Expression of chimeric HLA-DR1 by lymphocytes from Tg mice. Two-color flow cytometry was performed to analyze the expression of DR1 and I-A^f using mAbs L243 (anti-DR, PE labeled) and 10-3.6 (anti-I-A^f, FITC labeled). In the DR1 Tg mice, virtually all of the cells that express I-A^f also express DR1. (A) Lymphocytes from Tg DR1 mice express both I-A^f and DR1. (B) Lymphocytes from non-Tg B10.M mice expressing only I-A^f. Contour plots are based on 5,000 cells analyzed.

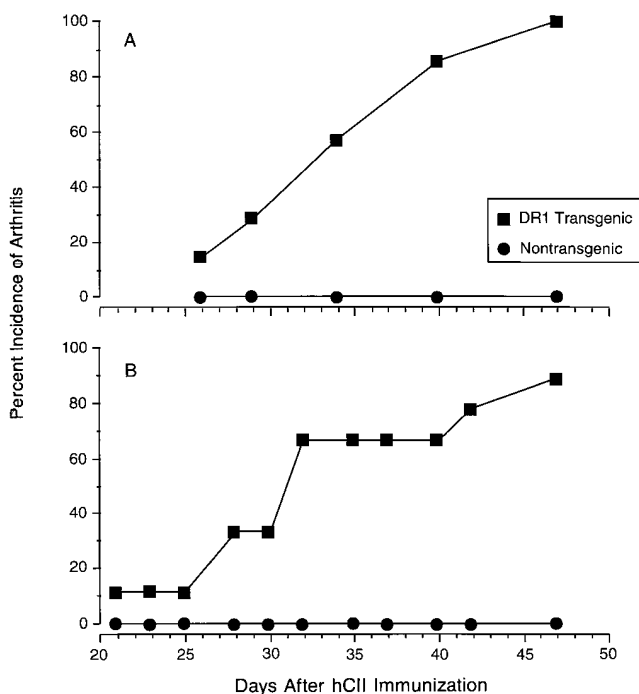


Figure 2. HLA-DR1 expression confers susceptibility to CIA in DR1 Tg mice. In two separate experiments, mice were immunized subcutaneously at the base of the tail with 100 μ g of human CII in CFA. (A) Mice ($n = 7$ per group) were boosted with 100 μ g of human CII in incomplete Freund's adjuvant at 21 d. (B) Mice ($n = 9$ per group) were not boosted. Beginning at 3 wk after immunization, mice were monitored for inflammation of fore and hind paws. ■, DR1 Tg mice; ●, non-Tg mice.

DR1 confers susceptibility to CIA in the murine model. As shown in Fig. 2, DR1 Tg mice are highly susceptible to CIA induced by immunization with hCII. In two separate experiments (Fig. 2, A and B), the incidence of arthritis was 100 and 88% ($n = 7$ and 9 per experimental group, respectively). These mice developed severe arthritis, evidenced by marked swelling and erythema of the hind and fore paws. Inflammation in these paws frequently included the wrist or ankle and extended distally through the limb and into the digits (Fig. 3), resulting in the majority of arthritic scores being 3 or 4 (most severe). Both the severity and the time of development of arthritis were very similar to that previously described for CIA in the DBA/1 mouse (12, 21). In contrast, non-Tg control mice, I-A^f positive, DR1 negative, were totally resistant to hCII-elicited CIA (Fig. 2).

Histological examination of arthritic DR1 joints revealed a pathology that was also very similar to that previously described for CIA in DBA/1 mice (12, 21). As shown in Fig. 4 A, massive infiltrations of inflammatory cells were frequently evident in the articular joints as well as erosions of articular cartilage surfaces. Additionally, proliferation of the synovial lining as well as necrosis of the superficial layer of the synovium were also evident in some of the arthritic joints (Fig. 4 B).

The susceptibility of the DR1 Tg mice to CIA was accompanied by both a strong T cell proliferative response to hCII (Table 1) and a strong antibody response to both the immunogen, hCII, and the autoantigen, mCII (Fig. 5). In both instances, the immune response of the DR1 Tg mice

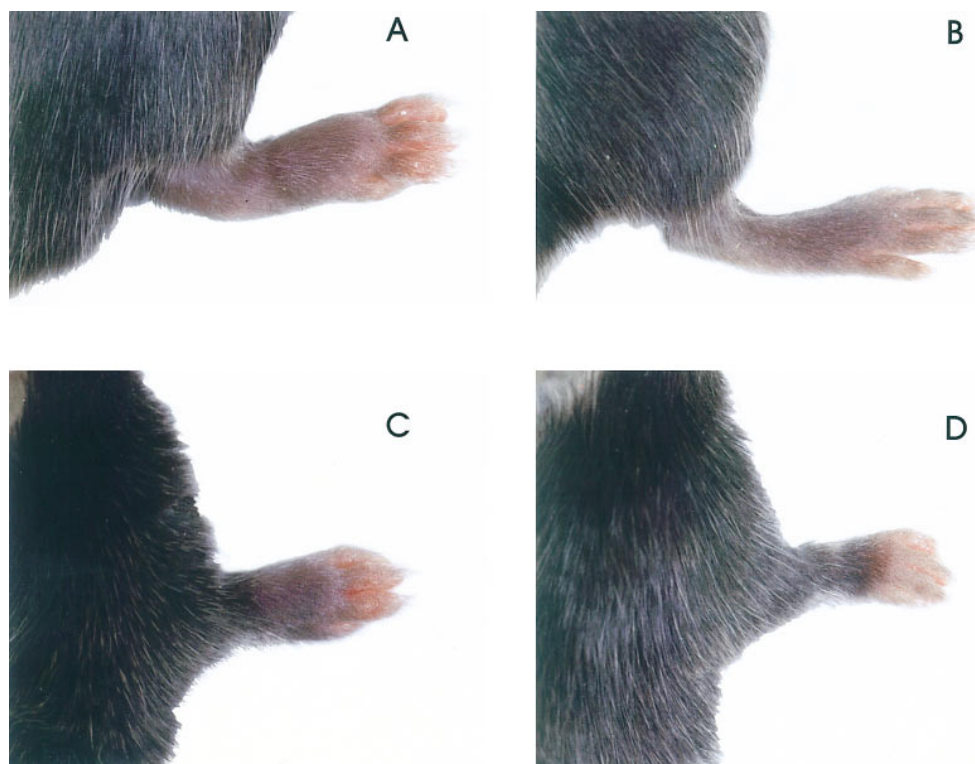


Figure 3. Development of arthritis in DR1 Tg mice immunized with hCII. (A) Marked swelling of the tarsal and metatarsal joints, extending from the ankles through the digits of a DR1 Tg mouse immunized with hCII. (B) Normal hind paw of immunized non-Tg mouse. (C) Arthritic fore paw from DR1 Tg mouse. (D) Normal fore paw from an immunized non-Tg mouse.

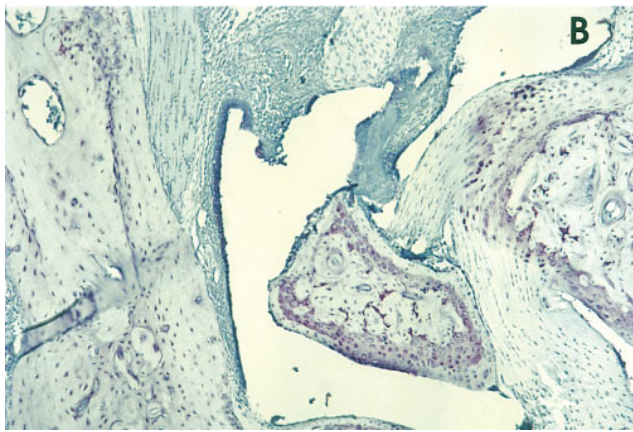
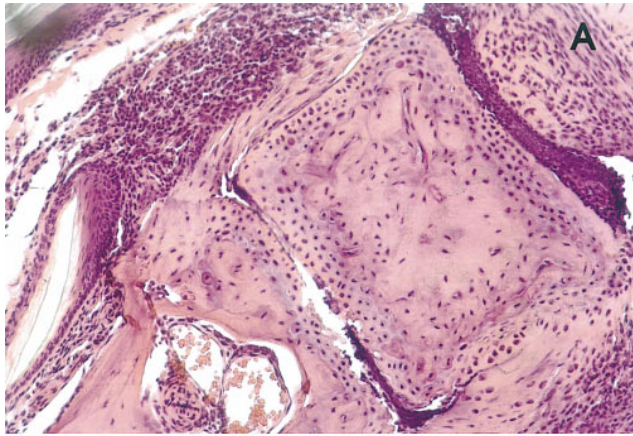


Figure 4. Histopathology of arthritic joints from DR1 Tg mice immunized with hCII. (A) Hematoxylin and eosin stain of sections from an arthritic hind limb joint. Massive infiltration of inflammatory cells (upper left) and erosion of articular surfaces (lower articular joint) are clearly evident. (B) Toluidine blue stain of sections from an arthritic hind limb joint. Staining reveals proliferation of the synovial lining on the left side of the joint space as well as necrosis of the synovial lining (upper right portion of the joint). Samples were obtained 6 wk after hCII immunization.

to hCII was considerably stronger than that of the non-Tg control mice. 10 d after immunization, the T cell proliferative response of the DR1 Tg mice was >5 times that of the control mice (Table 1). At the peak of arthritis incidence (6 wk after hCII immunization) DR1 Tg mice produced high levels of hCII-specific antibody, whereas the non-Tg control mice produced only a small amount of hCII-specific antibody (Fig. 5). Immunization of DR1 Tg mice with hCII also induced high titers of antibody that recognized the autoantigen, mCII, whereas little or no mCII-specific antibody was detectable in the serum of non-Tg control mice (Fig. 5).

Identification of DR1-restricted T Cell Determinants in hCII. Based on the susceptibility of DR1 Tg mice to CIA and their T cell proliferative response to hCII, we proceeded to identify the hCII antigenic determinants bound and presented to T cells by the DR1 molecule. A

Table 1. T Cell Proliferative Response of DR1 Tg⁺ and Tg⁻ Mice to hCII

Strain	[³ H]Thymidine incorporation (Δ DPM)*	
	Human α1(II) chain	
	50 μg	25 μg
B10.M DR1 Tg ^{+/+}	81,463 ± 3,866	58,030 ± 319
B10.M Tg ^{-/-}	14,906 ± 1,993	12,592 ± 2,576

*T cell proliferation assays were performed 10 d after immunization with hCII as described in Materials and Methods. Data are expressed as Δ decays per minute (DPM), (experimental - control) ± SEM. Control stimulations: B10.M DR1 Tg^{+/+}, 1,796 DPM; B10.M Tg^{-/-}, 3,850 DPM.

panel of Mimotope peptides, 15 mer overlapping by 12 amino acids, representing the entire length of the hCII α1(II) chain, was synthesized and tested for their ability to stimulate hCII-primed T cells from the DR1 Tg mice. Using this approach, two definitive DR1-restricted T cell determinants were identified (Fig. 6 A). The dominant DR1 determinant was identified by three consecutive peptides, spanning amino acid residues 256–276 of hCII, that strongly stimulated hCII-primed T cells. Identical data were also obtained by the use of a second DR1 Tg mouse derived from the SWR strain (data not shown; 32). When these peptides were aligned (Table 2), a determinant core of hCII(262–270) was deduced. This determinant core was verified and refined by the use of a second set of Mimotope peptides, advancing 15 mer with a 14-amino acid overlap (Fig. 7). Only peptides containing hCII residues 263 (F) through 270 (K) were capable of stimulating T cell proliferation, thus defining an 8-amino acid determinant core, hCII(263–270). A second subdominant, but clearly stimulatory determinant

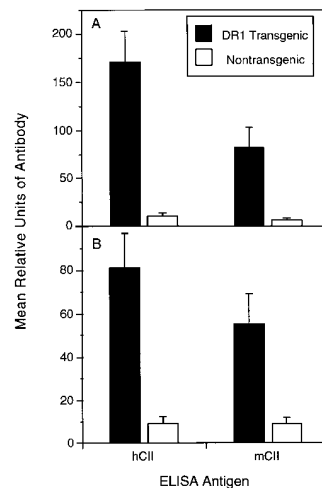


Figure 5. Detection of antibody specific for hCII and mCII after immunization with hCII. Serum antibody specific for hCII and mCII was measured using solid phase ELISA as described in Materials and Methods. DR1 Tg mice (solid bars) produced high levels of antibody specific for both hCII and mCII in comparison to non-Tg control mice (open bars). A and B represent independent experiments and correspond respectively to A and B in Fig. 2. Sera were collected at 6 wk after immunization, and quantity of antibody was determined for individual mice. Data are expressed as mean relative units based on a standard anti-hCII sera. Error bars indicate the standard error of the mean.

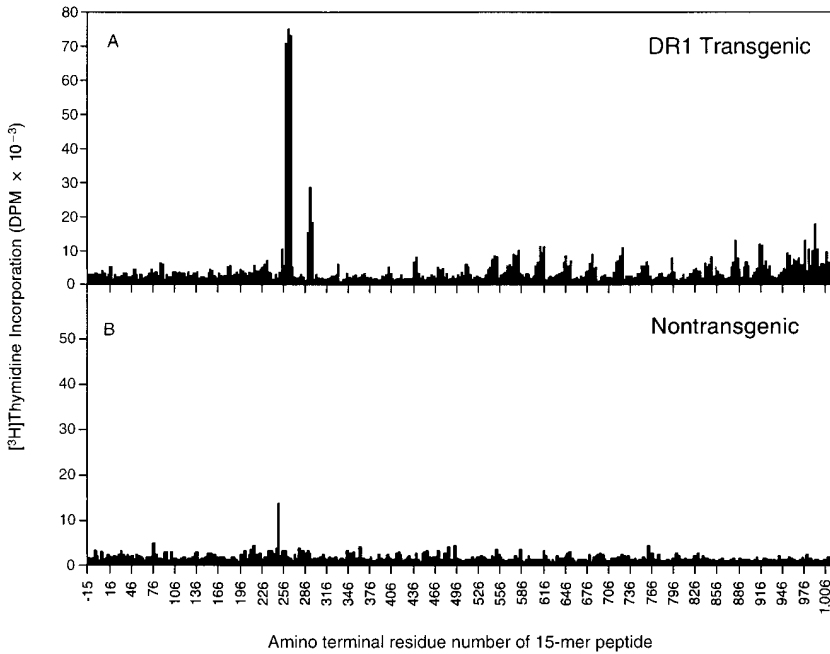


Figure 6. Identification of the hCII immunodominant T cell determinants recognized by T cells from DR1 Tg and non-Tg mice. Mice were immunized with hCII and tested for their ability to respond to a panel of Mimotope peptides, 15 mer overlapping by 12 amino acids, spanning the entire length of the human $\alpha 1(\text{II})$ chain. The abscissa indicates the NH_2 -terminal residue number of the synthetic peptide. 10 μl of synthetic peptide (10–40 μg) was used in each proliferation assay. The data are expressed as DPM; (A) DR1 Tg mice, (B) non-Tg mice. Mean ^3H thymidine incorporation in the absence of antigen was 3,696 DPM in A and 2,095 DPM in B. Data are representative of three independent experiments.

was also identified for DR1 (Fig. 6 A). This determinant was present in three consecutive peptides that encompass hCII(286–306), again allowing deduction of a 9–amino acid core, hCII(292–300) (Table 2). None of the other minor peaks were consistently reproducible in repeat experiments with the possible exception of two very minor determinants lying within peptides 826 through 856, and 976 through 1,006 (Fig. 6 A).

Analysis of hCII T cell determinants bound and presented by the I-A^f molecule, expressed by the non-Tg control mice, identified a single peptide that stimulated T cells weakly, but significantly above background (Fig. 6 B). This peptide lies close to the NH_2 terminus of the dominant determinant identified for DR1-restricted T cells (Fig. 6 A), and lies within hCII(250–264) (Table 2). The fact that this I-A^f determinant is represented in only a single 15-mer peptide is consistent with its weak antigenic capacity for stimulating T cells. This I-A^f-restricted determinant is also detected as a single peptide in the Mimotope proliferative response of DR1 Tg mice (Fig. 6 A), located two Mimotope peptides to the NH_2 terminus of the dominant determinant. Since the DR1 Tg mice were derived from backcrosses to B10.M mice, they also express I-A^f. Based on the T cell responses observed with both human $\alpha 1(\text{II})$ chains (Table 1) and the Mimotope peptides, the stimulatory capacity of the I-A^f determinant for the murine T cells is considerably less than that of the DR1 dominant determinant.

Although the dominant determinant was only present in the proliferative response of the DR1 Tg T cells, it is possible that a mixed isotype molecule between chains of the I-A^f and the DR1 molecule might have mediated the T cell response to hCII. To address this issue, T cell hybridomas derived from DR1 Tg mice immunized with hCII were tested for their ability to respond to hCII(249–281) presented by APCs expressing transfected DR1 molecules. A

representative sample of all the hybridomas tested are shown in Table 3. None of the T cell hybridomas in our panel were stimulated when the antigen was presented by I-A^f. However, each was strongly stimulated by the two DR1 transfected cell lines used, indicating that the T cell response to the dominant determinant is clearly DR1 restricted. Additionally, as shown in Table 4, the antigen specificity of these hybridomas is identical to that defined in the T cell proliferative assay (Fig. 6 and Table 2). Interestingly, optimal stimulation of the T cell hybridomas in vitro did not require that the peptide be presented by the chimeric form of DR1 (Table 3). Each DR1-restricted T cell

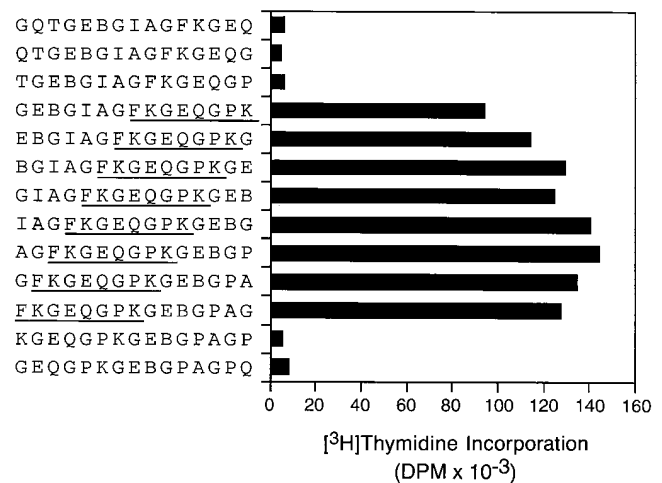


Figure 7. Identification of the core of the immunodominant determinant. hCII-primed T cells from DR1 Tg mice were tested for their ability to recognize a panel of Mimotope peptides, 15 mer, overlapping by 14 amino acids. Underlined residues indicate the deduced core of the T cell determinant. Data are expressed as DPM. T cell proliferation in the absence of antigen was 1,247 DPM.

Table 2. Human CII Determinants Recognized by T Cells from B10.M and B10.M-DR1 Mice

T cell determinant	Peptide*	Peptide sequence [‡]	T cell stimulation [§]
B10.M determinant	hCII (247–261)	GPLGPKGQTGEBGIA	–
	hCII (250–264)	<u>GPKGQTGEBGIAGFK</u>	+
	hCII (253–267)	GQTGEBGIAGFKGEQ	–
B10.M-DR1	hCII (256–270)	<u>GEBGIAGFKGEQQPK</u>	+
Dominant determinant	hCII (259–273)	<u>GIAGFKGEQQPKGEB</u>	+
	hCII (262–276)	<u>GFKGEQQPKGEBGPA</u>	+
	hCII (265–279)	GEQQPKGEBGPAGPQ	–
B10.M-DR1	hCII (283–297)	GPAGEEGKRGARGE	–
	hCII (286–300)	GEEGKRGARGE <u>BGGV</u>	+
	Subdominant determinant	hCII (289–303)	GKRGARGE <u>BGGV</u> GPI
Subdominant determinant	hCII (292–306)	<u>GARGE</u> BGGVGP <u>IGPB</u>	+
	hCII (295–309)	CEBGGVGP <u>IGPB</u> GER	–

*Mimotope synthetic peptides. Sequence numbers are based on assigning the first amino acid of the human α 1(II) chain as residue number 1.

[‡]Synthetic peptides are aligned according to sequence overlap used in the Mimotope analysis. Alignment allows for deduction of determinant core (underlined text) when more than one peptide is stimulatory for T cells. Amino acids are represented by single letter code with *B* denoting hydroxyproline.

[§]Stimulation data summarized from T cell proliferation data in Fig. 6.

hybridoma examined responded equally well to the CII peptide presented by the chimeric DR1 molecule as with the wild-type DR1 molecule.

Discussion

The relationship between the expression of specific HLA-DR alleles and the predisposition to developing RA has been the subject of numerous hypotheses aimed at determining the functional role of these class II molecules in RA. One hypothesis proposes that the DR molecules that confer susceptibility to RA do so by means of the shared epitope located in the α -helical region of the β chain (6, 33, 34) and that this shared epitope selects pathogenic T cells that promote the autoimmune response (8). Initial stimulation of these pathogenic T cells would still be dependent on the presence of an antigenic peptide of unknown origin, bound and presented by the DR molecule. Recently, Zanelli et al. proposed an alternate hypothesis for the role of the shared epitope in conferring susceptibility to RA (7). Based on linkages between specific DRB and DQB genes, they have proposed that an extended DQ/DR haplotype confers susceptibility to RA, and that the polymorphisms of the DRB1 chain control this susceptibility (35). The basis for this protection/susceptibility is proposed to be through the presentation of a peptide derived from the shared epitope region of the DRB1 molecule. If derived from a nonsusceptible DR allele, this peptide binds tightly to an HLA-DQ8 molecule and the DQ8-peptide complex alters the T cell repertoire, resulting in protection from RA (35). In contrast, when this peptide is derived from DR alleles associated with increased susceptibility to RA, it binds poorly to the DQ molecule, thus allowing the potentially autore-

active T cells to survive. In contrast to the shared epitope hypothesis described above, this hypothesis predicts that the DQ molecule, and not the DR molecule, mediates the autoimmune response in RA, and indeed Tg HLA-DQ8 mice are susceptible to CIA elicited by bovine CII (36).

Although it appears that the HLA-DQ8 molecule can participate in the autoimmune response in RA, the data we have presented here indicates that the DR1 molecule may also participate as an antigen presenter to pathogenic T cells. Our studies demonstrate that the DR1 molecule is fully capable of binding and presenting peptides derived from the processing of hCII, and that in a DR1 Tg mouse model, the DR1-restricted anti-hCII response leads to the development of a severe autoimmune arthritis. Although these data do not address the issue of what initiates the autoimmune response that leads to the development of RA, hCII is appealing as one of the antigens involved in the autoimmune response in RA for several reasons. First, autoimmune arthritis models of RA have been described for several species including mouse (12), rat (13), and monkey (11, 37), all as a result of immunization with CII. Second, CII is a major component of articular cartilage, the site of inflammation in RA, and anti-CII antibodies have been eluted from a high percentage of RA cartilages (17). Lastly, several studies have demonstrated the existence of T (15) or B cell (16, 38–40) immunity to CII in RA patients. Consequently, these data suggest that at least some of the autoimmune response in RA is DR-restricted and that these anti-CII responses may be mediated directly by the DR1 molecule presenting an hCII peptide to T cells.

The DR transgenes used in these studies encode chimeric molecules in which the second domains of the DR1 chains were replaced with corresponding domains from the

Table 3. The Response of DR1-Tg⁺ T Cells to hCII Is Restricted by HLA-DR1

T cell hybridoma	APC*				
	Chimeric DR1		DR1		I-A ^f
	+ Antigen [‡]	- Antigen	+ Antigen	- Antigen	+ Antigen
DR1hCII-2.0	>2,560	- [§]	>2,560	-	-
DR1hCII-3.0	>2,560	-	>2,560	-	-
DR1hCII-14.0	>2,560	-	>2,560	-	-
DR1hCII-16.0	2,560	-	>2,560	-	-
DR1hCII-19.0	2,560	-	>2,560	-	-
DR1hCII-22.0	>2,560	-	>2,560	-	-

*APCs: DRAB10, transfected cells that express the chimeric DR1; L57, transfected L cells expressing wild-type DR1; and, the B cell hybridoma 43.2.1 that expresses I-A^f.

[‡]Antigen presentation assay performed as described in Materials and Methods. hCII(249–281) was used as antigen at 150 µg/ml final concentration.

[§]-, less than 20 U/ml of IL-2 produced.

murine I-E chains, thereby enhancing the interaction of murine CD4 with the DR1 molecule. This was done to enable murine CD4 interaction with the DR1 molecule since, at least for some antigens, there appears to be a species barrier to the interaction of murine CD4 with human class II (41, 42). Surprisingly, all of the T cell hybridomas produced in these studies using the chimeric DR1 mice are not dependent on the presence of the chimeric second domain for stimulation. The hybridomas are stimulated equally well with CII peptides presented by the chimeric DR1 or by the wild-type DR1, expressed either as a transfectant or on EBV-transformed B cells. Regardless of whether this indicates that murine CD4 interacts weakly with DR1 or that the immune response to CII in the DR1 Tg mice is CD4 independent, it appears that all of these hybridomas

express high affinity TCR. This conclusion is supported by the fact that they respond very well to as little as 100 nM concentrations of antigen (data not shown). Whether this is due to the levels of transgene expression or the characteristics of hCII as an antigen is not clear, but similar sensitivities to antigen stimulation have been reported for a chimeric DR4 transgene (18).

It is interesting to note that most of the dominant T cell determinants that have been described for CII, regardless of the MHC restriction, are clustered within a small region of the CII molecule. The dominant determinant, CII(260–267), for I-A^g, one of the natural murine susceptibility alleles, overlaps with the DR1 determinant, CII(263–270), although they clearly use different class II binding motifs (43). The I-A^f dominant peptide identified in these studies,

Table 4. DR1-restricted T Cell Hybridomas Are Specific for the hCII(262–270) Core Determinant

T cell hybridoma	IL-2*					
	Human CII Mimotope Peptides [‡]					
	CII(250–264)	CII(253–267)	CII(256–270)	CII(259–273)	CII(262–276)	CII(265–279)
DR1hCII-2	- [§]	-	2,560	2,560	1,280	-
DR1hCII-3	-	-	2,560	2,560	2,560	-
DR1hCII-5	-	-	640	1,280	1,280	-
DR1hCII-14	-	-	1,280	2,560	1,280	-
DR1hCII-19	-	-	2,560	>2,560	640	-

*Production of IL-2 was measured as an indicator of T cell hybridoma stimulation in an antigen presentation assay using DR1-expressing APCs, (cell line L57). After 24 h of culture, supernatants were collected and tested for the presence of IL-2-dependent cell line HT-2, as described in Materials and Methods. Data are expressed as units of IL-2 per milliliter.

[‡]Peptides were derived from the same Mimotope peptide synthesis used in Fig. 6.

[§]-, <20 U/ml of IL-2 produced.

CII(250–264) also overlaps with the DR1 and I-A^q determinants, but again is clearly different. Similarly we have also identified a subdominant I-A^q T cell determinant in the same region as the DR1 subdominant determinant (Rosloniec, E., and D. Brand, unpublished observations). Why this should be is not clear, and may be more representative of the primary structure of the collagen molecule itself. Since these determinants are all derived from the α -helical portion of CII, they all have a repetitive primary amino acid sequence of Gly-X-Y, where X and Y are frequently hydrophobic residues. Since Gly has no side chain with which to interact with a binding pocket within the I-A^q molecule (or TCR), the number of potential antigenic peptides contained in a Gly-rich protein would likely be lower than the number of antigenic peptides in a noncollagenous protein.

Indeed, given its size (>1,000 amino acids), very few antigenic peptides have been identified in CII, regardless of the class II molecule in question. It would appear that either this area of the collagen molecule contains amino acids frequently used by class II binding motifs, or the diversity of amino acids within this region of CII is much greater than the rest of this highly repetitive molecule. Finally, the core of the T cell determinant identified for DR1, CII(263–270), appears to be identical to that described for DR4 (DRB1*0401) and bovine CII (44). If this similarity in peptide binding motifs occurs among all of the DR susceptibility alleles, it may be possible to construct an analogue peptide that effectively interferes with many, if not all, of these susceptibility alleles, thus simplifying an immunotherapeutic approach to RA.

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References

1. Stastny, P., E.J. Ball, P.J. Dry, and G. Nunez. 1983. The human immune response region (HLA-D) and disease susceptibility. *Immunol. Rev.* 70:113–154.
2. Stastny, P., E. Ball, M. Kahn, N. Olsen, T. Pincus, and X. Gao. 1988. HLA-DR4 and other genetic markers in rheumatoid arthritis. *Br. J. Rheumatol.* 27:132–138.
3. Nepom, G.T., P. Byers, C. Seyfried, L.A. Healey, K.R. Wilks, D. Stage, and B.S. Nepom. 1989. HLA genes associated with rheumatoid arthritis. Identification of susceptibility alleles using specific oligonucleotide probes. *Arthritis Rheum.* 32:15–21.
4. Wordsworth, B.P., J.S. Lanchbury, L.I. Sakkas, K.I. Welsh, G.S. Panayi, and J.I. Bell. 1989. HLA-DR4 subtype frequencies in rheumatoid arthritis indicate that DRB1 is the major susceptibility locus within the HLA class II region. *Proc. Natl. Acad. Sci. USA.* 86:10049–10053.
5. Gregersen, P., J. Silver, and R. Winchester. 1987. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum.* 30:1205–1213.
6. Winchester, R., E. Dwyer, and S. Rose. 1992. The genetic basis of rheumatoid arthritis. The shared epitope hypothesis. *Rheum. Dis. Clin. N. Am.* 18:761–783.
7. Zanelli, E., M.A. Gonzalez-Gay, and C.S. David. 1995. Could HLA-DRB1 be the protective locus in rheumatoid arthritis? *Immunol. Today.* 16:274–278.
8. Penzotti, J.E., D. Doherty, T.P. Lybrand, and G.T. Nepom. 1996. A structural model for TCR recognition of the HLA class II shared epitope sequence implicated in susceptibility to rheumatoid arthritis. *J. Autoimmun.* 9:287–293.
9. Roudier, J., G. Rhodes, J. Petersen, J.H. Vaughan, and D.A. Carson. 1988. The Epstein-Barr virus glycoprotein gp110, a molecular link between HLA DR4, HLA DR1, and rheumatoid arthritis. *Scand. J. Immunol.* 27:367–371.
10. Fujinami, R.S., J.A. Nelson, L. Walker, and M.B. Oldstone. 1988. Sequence homology and immunologic cross-reactivity of human cytomegalovirus with HLA-DR beta chain: a means for graft rejection and immunosuppression. *J. Virol.* 62:100–105.
11. Yoo, T.J., S.Y. Kim, J.M. Stuart, R.A. Floyd, G.A. Olson, M.A. Cremer, and A.H. Kang. 1988. Induction of arthritis in monkeys by immunization with type II collagen. *J. Exp. Med.* 168:777–782.
12. Courtenay, J.S., M.J. Dallman, A.D. Dayan, A. Martin, and B. Mosedale. 1980. Immunisation against heterologous type II collagen induces arthritis in mice. *Nature (Lond.)* 283:666–668.
13. Trentham, D.E., A.S. Townes, and A.H. Kang. 1977. Autoimmunity to type II collagen: an experimental model of arthritis. *J. Exp. Med.* 146:857–868.
14. Banerjee, S., H.S. Luthra, S.B. Moore, and W.M. O'Fallon. 1988. Serum IgG anti-native type II collagen antibodies in rheumatoid arthritis: association with HLA DR4 and lack of clinical correlation. *Clin. Exp. Rheumatol.* 6:373–380.
15. Londei, M., C.M. Savill, A. Verhoef, F. Brennan, Z.A. Leech, V. Duance, R.N. Maini, and M. Feldman. 1989. Persistence of collagen type II-specific T-cell clones in the synovial membrane of a patient with rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA.* 86:636–640.
16. Stuart, J.M., E.H. Huffstutter, A.S. Townes, and A.H. Kang. 1983. Incidence and specificity of antibodies to type I, II, III, IV, and V collagen in rheumatoid arthritis and other rheumatic diseases as measured by ¹²⁵I-radioimmunoassay. *Arthritis Rheum.* 26:832–840.

17. Watson, W.C., R.E. Tooms, P.G. Carnesale, and J.P. Dutkowsky. 1994. A case of germinal center formation by CD45RO T and CD20 B lymphocytes in rheumatoid arthritic subchondral bone: proposal for a two-compartment model of immune-mediated disease with implications for immunotherapeutic strategies. *Clin. Immunol. Immunopathol.* 73: 27–37.
18. Woods, A., H.Y. Chen, M.E. Trumbauer, A. Sirotna, R. Cummings, and D.M. Zaller. 1994. Human major histocompatibility complex class II-restricted T cell responses in transgenic mice. *J. Exp. Med.* 180:173–181.
19. Miller, E.J. 1971. Isolation and characterization of the cyanogen bromide peptides from the $\alpha 1$ (II) chain of chick cartilage collagen. *Biochemistry.* 10:3030–3035.
20. Stuart, J.M., M.A. Cremer, A.S. Townes, and A.H. Kang. 1982. Type II collagen induced arthritis in rats. Passive transfer with serum and evidence that IgG anticollagen antibodies can cause arthritis. *J. Exp. Med.* 155:1–16.
21. Wooley, P.H., H.S. Luthra, J.M. Stuart, and C.S. David. 1981. Type II collagen induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. *J. Exp. Med.* 154:688–700.
22. Maeji, N.J., A.M. Bray, and H.M. Geysen. 1990. Multi-rod peptide synthesis strategy for T cell determinant analysis. *J. Immunol. Methods.* 134:23–33.
23. Brand, D.D., L.K. Myers, K.B. Whittington, J.M. Stuart, A.H. Kang, and E.F. Rosloniec. 1994. Characterization of the T cell determinants in the induction of autoimmune arthritis by bovine $\alpha 1$ (II)-CB11 in H-2^a mice. *J. Immunol.* 152: 3088–3097.
24. Click, R.E., L. Benck, and B.J. Alter. 1972. Immune response in vitro. I. Culture conditions for antibody synthesis. *Cell. Immunol.* 3:264–270.
25. Marrack, P. 1982. Production of antigen-specific H-2 restricted T cell hybridomas. In *Isolation, Characterization, and Utilization of T Lymphocyte Clones*. C.G. Fathman and F. Fitch, editors. Academic Press, New York. 119–126.
26. White, J., M. Blackman, J. Bill, J. Kappler, P. Marrack, D.P. Gold, and W. Born. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J. Immunol.* 143:1822–1825.
27. Klohe, E.P., R. Watts, M. Bahl, C. Alber, W.Y. Yu, R. Anderson, J. Silver, P.K. Gregersen, and R.W. Karr. 1988. Analysis of the molecular specificities of anti-class II monoclonal antibodies by using L cell transfectants expressing HLA class II molecules. *J. Immunol.* 141:2158–2164.
28. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55–63.
29. Denizot, F., and L. Rita. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods.* 89:271–277.
30. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198–1214.
31. Myers, L.K., J.M. Stuart, and A.H. Kang. 1989. A CD4 cell is capable of transferring suppression of collagen-induced arthritis. *J. Immunol.* 143:3976–3980.
32. Altmann, D.M., D.C. Douek, A.J. Frater, C.M. Hetherington, H. Inoko, and J.I. Elliott. 1995. The T cell response of HLA-DR transgenic mice to human myelin basic protein and other antigens in the presence and absence of human CD4. *J. Exp. Med.* 181:867–875.
33. Winchester, R., and E. Dwyer. 1991. MHC and autoimmune diseases: susceptibility to rheumatoid arthritis associated with a hydrophobic strip of alpha helix encoded by several MHC alleles. *Immunol. Ser.* 55:203–219.
34. Albani, S., D.A. Carson, and J. Roudier. 1992. Genetic and environmental factors in the immune pathogenesis of rheumatoid arthritis. *Rheum. Dis. Clin. N. Am.* 18:729–740.
35. Zanelli, E., C.J. Krco, J.M. Baisch, S. Cheng, and C.S. David. 1996. Immune response of HLA-DQ8 transgenic mice to peptides from the third hypervariable region of HLA-DRB1 correlates with predisposition to rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA.* 93:1814–1819.
36. Nabozny, G.H., J.M. Baisch, S. Cheng, D. Cosgrove, M.M. Griffiths, H.S. Luthra, and C.S. David. 1996. HLA-DQ8 transgenic mice are highly susceptible to collagen-induced arthritis: a novel model for human polyarthritis. *J. Exp. Med.* 183:27–37.
37. Terato, K., H. Arai, Y. Shimozuru, T. Fukuda, H. Tanaka, H. Watanabe, Y. Nagai, K. Fujimoto, F. Okubo, F. Cho et al. 1989. Sex-linked differences in susceptibility of cynomolgus monkeys to type II collagen-induced arthritis: evidence that epitope-specific immune suppression is involved in the regulation of type II collagen autoantibody formation. *Arthritis Rheum.* 6:748–758.
38. Jasin, H.E. 1985. Autoantibody specificities of immune complexes sequestered in articular cartilage of patients with rheumatoid arthritis and osteoarthritis. *Arthritis Rheum.* 28:241–248.
39. Watson, W., M. Cremer, P. Wooley, and A. Townes. 1986. Assessment of the potential pathogenicity of type II collagen autoantibodies in patients with rheumatoid arthritis. *Arthritis Rheum.* 29:1316–1321.
40. Terato, K., Y. Shimozuru, K. Katayama, Y. Takemitsu, I. Yamashita, M. Miyatsu, K. Fujii, M. Sagara, S. Kobayashi, M. Goto et al. 1990. Specificity of antibodies to type II collagen in rheumatoid arthritis. *Arthritis Rheum.* 33:1493–1500.
41. Vignali, D.A., J. Moreno, D. Schiller, and G.J. Hammerling. 1992. Species-specific binding of CD4 to the beta 2 domain of major histocompatibility complex class II molecules. *J. Exp. Med.* 175:925–932.
42. Lamarre, D., A. Ashkenazi, S. Fleury, D.H. Smith, R.P. Sekaly, and D.J. Capon. 1989. The MHC-binding and gp120-binding functions of CD4 are separable. *Science (Wash. DC).* 245:743–746.
43. Rosloniec, E.F., K.B. Whittington, D.D. Brand, L.K. Myers, and J.M. Stuart. 1996. Identification of MHC class II and TCR binding residues in the type II collagen immunodominant determinant mediating collagen induced arthritis. *Cell. Immunol.* 172:21–28.
44. Fugger, L., J.B. Rothbard, and G. Sonderstrup-McDevitt. 1996. Specificity of an HLA-DRB1*0401-restricted T cell response to type II collagen. *Eur. J. Immunol.* 26:928–933.