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CUTTING EDGE

Cutting Edge: The Conversion of Arginine to Citrulline Allows for a High-Affinity Peptide Interaction with the Rheumatoid Arthritis-Associated HLA-DRB1*0401 MHC Class II Molecule[1]

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***Rheumatoid arthritis (RA) is genetically associated with MHC class II molecules that contain the shared epitope. These MHC molecules may participate in disease pathogenesis by selectively binding arthritogenic peptides for presentation to autoreactive CD4$^+$ T cells. The nature of the arthritogenic Ag is not known, but recent work has identified posttranslationally modified proteins containing citrulline (deiminated arginine) as specific targets of the IgG Ab response in RA patients. To understand how citrulline might evoke an autoimmune reaction, we have studied T cell responses to citrulline-containing peptides in HLA-DRB1*0401 transgenic (DR4-IE tg) mice. In this study, we demonstrate that the conversion of arginine to citrulline at the peptide side-chain position interacting with the shared epitope significantly increases peptide-MHC affinity and leads to the activation CD4$^+$ T cells in DR4-IE tg mice. These results reveal how* DRB1 *alleles with the shared epitope could initiate an autoimmune response to citrullinated self-Ags in RA patients. The Journal of Immunology,* 2003, 171: 538–541.**

Rheumatoid arthritis (RA)[4] is a prevalent autoimmune disease characterized by synovial inflammation and pannus formation, which can lead to cartilage and bone degradation. Genetic susceptibility to this disease in most populations is associated with MHC class II molecules that contain an amino acid motif known as the shared epitope (1). This shared epitope (encoded by the residues Q/R, K/R, R, A, and A at positions 70–74 of the β-chain) is positively charged and contributes to one of the peptide-anchoring pockets of these MHC class II molecules known as P4. Notably, MHC class II molecules with a negatively charged P4 pocket (such as DRB1*0402) may protect from disease (2), suggesting that the interactions of amino acid side chains from antigenic peptides with this MHC anchoring pocket may be critical in initiating an autoimmune response.

Although many candidate autoantigens have been investigated, an RA-specific Ag targeted by both the CD4$^+$ T cell and B cell immune response has not been identified yet. However, recent studies have identified a subset of IgG autoantibodies that are a sensitive and specific (>90%) diagnostic marker of RA. The target of these autoantibodies is citrulline, a posttranslationally modified arginine found within the context of certain protein/peptide sequences (reviewed in Ref. 3). Citrulline is an essential component of epitopes recognized by anti-perinuclear, anti-keratin, anti-filaggrin, anti-cyclic citrullinated peptide, and anti-Sa Abs (3, 4). Although these Abs target citrulline within a number of different proteins, one of the joint-derived targets appears to be vimentin (5). An additional intriguing finding is that anti-citrulline Ab production is significantly associated with the presence of the MHC shared epitope in RA patients (6, 7).

In this study, we researched the interaction of arginine- or citrulline-containing peptides with MHC class II molecules associated with RA, and the CD4$^+$ T cell response to these peptide Ags in HLA-DRB1*0401 transgenic (DR4-IE tg) mice. We selected candidate T cell epitopes for study, including one from vimentin, using an algorithm to predict MHC-peptide affinity. These peptides were chosen based on the predicted binding to DRB1*0401 in a register that would position either arginine or citrulline at the positively charged P4 anchoring pocket (shared epitope). In this study, we show that the conversion of arginine to citrulline, a process which replaces the charged imino side-chain group with an uncharged carbonyl, dramatically increases the affinity of the vimentin peptide for DRB1*0401. Furthermore, this posttranslational modification is necessary to elicit a CD4$^+$ T cell response to these peptides in

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; Vim, vimentin; Cit, citrulline.

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DR4-IE tg mice. Peptide affinity for a number of *HLA* alleles was assessed and showed that only MHC class II molecules with the shared epitope had an increased affinity for the citrulline-containing peptide. These studies, which identify a novel peptide-MHC interaction, may help to explain the molecular basis of disease-associated *HLA* alleles in RA.

Materials and Methods

Animals

HLA-DR4-IE transgenic, murine MHC class II-deficient mice were used in these experiments (8). These mice were bred and maintained as previously described (9).

Peptides

Peptides used in these studies were synthesized and purified by the manufacturer (Genemed Synthesis, San Francisco, CA). Peptides were selected based on their predicted affinity for DRB1*0401 according to the method of Hammer et al. (10). Underlined amino acids indicate the residues interacting with the nine MHC class II binding pockets (P1–P9), while those that appear in bold interact at the P4 shared epitope position. The sequences of the peptides used from the proteoglycan aggrecan are as follows: P4D, human aggrecan peptide 280–292, AGWLA**D**RSVRYPI; P4R, altered human aggrecan peptide 280–292, AGWLA**R**RSVRYPI; and P4citrulline (Cit), altered human aggrecan peptide 280–292, AGWLA**Cit**RSVRYPI. Because citrulline is not accounted for in the predictive algorithm of Hammer et al., the value of glutamine was substituted for arginine when identifying a candidate T cell epitope from vimentin (glutamine has the same terminal side-chain group as citrulline). The sequences of the vimentin peptides used are as follows: vimentin $(Vim)_{65–77}$, human vimentin peptide 65–77, SAVRA**R**SSVPGVR; and Vim_{R70Cit}, altered human vimentin peptide 65–77, SAVRA**Cit**SSVPGVR.

Immunizations

DR4 tg mice were immunized intradermally at the interior side of both hind legs with 100 μl of peptide (1 μg/μl) emulsified in CFA (Difco Laboratories, Detroit, MI) in a 1:1 volume ratio. After 10 days, mice were sacrificed, and their draining lymph nodes were removed for in vitro proliferation and cytokine assays.

T cell cultures

Cell suspensions were prepared from the draining lymph nodes and cultured in 96-well plates at a concentration of 4×10^5 cells/well in the presence or absence of peptide Ag for 4 days. Anti-DR Ab (BD PharMingen, Mississauga, ON) was added to some cultures (1 μg/ml) to confirm DR-restricted T cell responses, as described previously (11). Culture supernatants were removed after 78 h to test IFN-γ production by ELISA (BD PharMingen), as described previously (9). Cytokine production was measured in duplicate and represents the average Ag-specific cytokine production (cytokine production in control samples plus 2 SD were subtracted from the peptide-specific cytokine production) $\pm$ SD. Eighteen hours before culture termination, 1 μCi of [^{3}H]thymidine (ICN Biomedicals, Montreal, Quebec) was added to each well to assess T cell proliferation. Proliferation experiments were conducted in triplicate, and results are presented as average proliferation in cpm $\pm$ SD or stimulation index (cpm of experimental sample/cpm of control sample) $\pm$ SEM.

Peptide-binding assay

Peptide-binding affinity to purified HLA-DRB1*0101, *0401, *0404, *0301, *0701, *0802, *1101, and *1302 molecules was determined relative to radiolabeled peptide probes as described previously (12). The nanomolar concentration of unlabelled vimentin peptide necessary for 50% inhibition of the labeled peptide to the purified HLA-DRB1 molecules (IC_{50}) was used as an approximation of the affinity of interaction (K_D). Results are expressed as the inverse of the IC_{50} values measured in nanomolar concentration.

Results and Discussion

The third hypervariable region of MHC class II molecules associated with RA contains the amino acid sequence Q/R, K/R, R, A, A, spanning positions 70–74 of the β-chain. This shared epitope region forms the peptide-anchoring pocket known as P4, is positively charged due to the K or R at position 71, and can make direct contact with side-chain residues from the antigenic peptide (13, 14). Previous studies on peptide-MHC affinity have shown that K or R at position 71 influences the properties of the amino acid that can interact at this P4 pocket (15). In general, MHC with the shared epitope have a high affinity for negatively charged or uncharged polar amino acids, whereas positively charged amino acids (e.g., arginine) inhibit peptide binding (10, 15). Because the process of deimination (performed by the enzyme peptidylarginine deiminase in a number of tissues and cell types) converts positively charged arginine to polar but uncharged citrulline, we reasoned that this posttranslational modification may increase affinity to the shared epitope P4 pocket. Because amino acid interactions at MHC anchoring pockets are not only dependent on the charge of the residue but also the size, we wanted to confirm that the P4 pocket formed by the shared epitope was large enough to accommodate the side chain of citrulline. This was verified by molecular modeling using the crystal structure of DRB1*0401 and DRB1*0101 (data not shown). Therefore, based on the charge properties of the P4 shared epitope, and the size of this pocket, peptide-bound citrulline should interact favorably at the P4 anchoring pocket of *0401 and *0101.

To test the hypothesis that the conversion of arginine to citrulline would increase peptide affinity for DRB1*0401, we first chose to study a peptide sequence that we have previously shown to activate $CD4^+$ T cells from DR4-IE tg mice. This peptide (from the cartilage proteoglycan aggrecan) normally contains a negatively charged aspartic acid (D) that interacts with the P4 shared epitope (P4D). We synthesized two additional peptides based on this sequence: one had aspartic acid substituted by arginine (P4R), and the other had citrulline substituted at this position (P4Cit). DR4-IE tg mice were then immunized with these peptides, and T cell responses were assessed 10 days later. The peptide P4D induced a strong proliferative response that was accompanied by IFN-γ production (Fig. 1, *A* and *B*) as shown previously (9). However, the peptide containing the arginine substitution (P4R) did not induce T cell proliferation or cytokine production in these mice. In contrast to the lack of response for P4R, P4Cit could induce T cell proliferation and IFN-γ production. To confirm that P4Cit was activating T cells in a DR-restricted manner, anti-DR Ab was used to inhibit TCR interaction with the peptide-MHC complex (Fig. 1*B*). This treatment inhibited the proliferative response to P4Cit and P4D.

We next wanted to identify potential T cell epitopes from an actual target of anti-citrulline Abs in RA patients. We chose to study vimentin, because autoantibodies to this citrullinated protein are frequently found in patients expressing the shared epitope (6). A candidate T cell epitope from human vimentin was identified using a predictive model for peptide-MHC affinity (10). This peptide was selected based on the properties of having favorable interactions with the MHC anchoring pockets P1, P6, and P9, and having an arginine at the P4 shared epitope. Two peptides were synthesized, the unmodified peptide $Vim_{65–77}$ and Vim_{R70Cit}, in which arginine was substituted by citrulline. T cell responses to these peptides were then characterized using DR4-IE tg mice. As predicted, the unmodified peptide $Vim_{65–77}$ did not induce T cell activation; however, Vim_{R70Cit} stimulated a strong proliferative response that was accompanied by IFN-γ production (Fig. 2, *A* and *C*). T cell responses to Vim_{R70Cit} could also be inhibited using anti-DR Abs, confirming the MHC class II-restricted immune response (Fig. 2*B*). We also found that T cells primed by Vim_{R70Cit} could

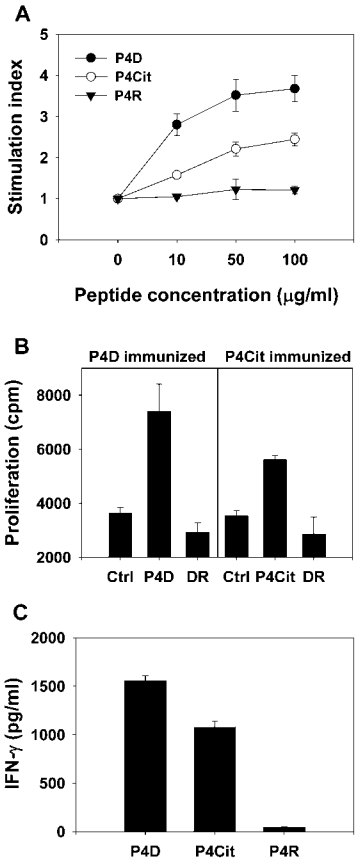

FIGURE 1. Analysis of the T cell immune response in DR4-IE tg mice to peptides containing arginine (P4R), citrulline (P4Cit), or aspartic acid (P4D) at the position that interacts with the P4 pocket formed by the shared epitope. *A*, DR4-IE tg mice were immunized with the indicated peptides, and 10 days later, draining lymph node cells were challenged in vitro with the same peptide at various concentrations. Data represent the average proliferative response ± SEM of eight mice for each peptide tested. *B*, HLA-DR restriction of the recall T cell response was determined by incubating draining lymph node cells without Ag (Ctrl), with 10 μg/ml the immunizing Ag (P4D, *left*; P4Cit, *right*), or in the presence of immunizing Ag (10 μg/ml) and anti-DR Ab (DR). Results represent the average proliferative response ± SD of four mice for each immunizing Ag. *C*, IFN-γ production in response to in vitro challenge with 10 μg/ml the immunizing peptide (P4D, P4Cit, or P4R). Cytokine production was determined by ELISA and represents the average Ag-specific IFN-γ production ± SD of four mice for each peptide tested.

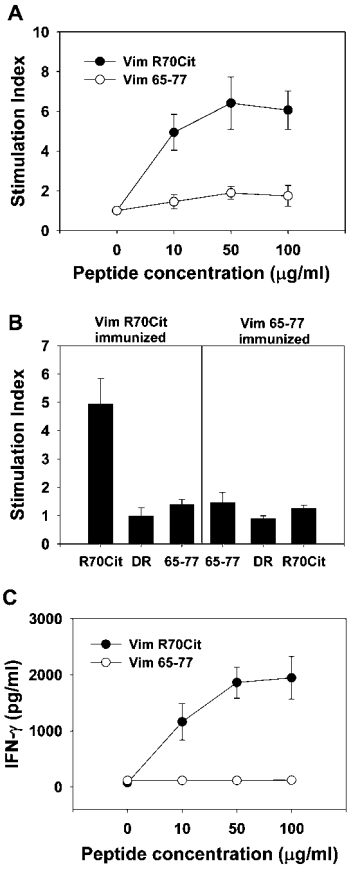

FIGURE 2. Analysis of the T cell immune response in DR4-IE tg mice to the unmodified vimentin peptide ($Vim_{65–77}$) or the citrulline-containing vimentin peptide (Vim_{R70Cit}). *A*, DR4-IE tg mice were immunized with the indicated peptides, and 10 days later, draining lymph node cells were challenged in vitro with the same peptide at various concentrations. Data represent the average proliferative response ± SEM of eight mice for each peptide tested. *B*, Specificity and HLA-DR restriction of the T cell recall response from Vim_{R70Cit}-immunized mice (*left*) and $Vim_{65–77}$-immunized mice (*right*). Draining lymph node cells were challenged with the immunizing peptide, the immunizing peptide plus anti-DR Ab (DR), the unmodified vimentin peptide (65–77; *left*), or the citrulline-containing vimentin peptide (R70Cit; *right*). The concentration used for in vitro challenge was 10 μg/ml, and the results represent the average stimulation index ± SEM of four mice for each immunizing Ag. *C*, IFN-γ production in response to in vitro challenge with varying concentrations of the immunizing peptide (Vim_{R70Cit} or $Vim_{65–77}$). Cytokine production was determined by ELISA and represents the average Ag specific IFN-γ production ± SD of four mice for each peptide tested.

not be activated by the unmodified peptide, further supporting the notion that $Vim_{65–77}$ does not interact productively with the DR4 binding groove (Fig. 2*B*).

Finally, we wanted to formally confirm that the conversion of arginine to citrulline could increase peptide affinity for MHC class II molecules that contained the shared epitope. Peptide competition assays were conducted to determine the relative affinity of $Vim_{65–77}$ and Vim_{R70Cit} for purified MHC that were either shared epitope positive (DRB1*0101, *0401, and *0404) or shared epitope negative (DRB1*0301, *0701, *0802, *1101, and *1302) (Fig. 3). Whereas $Vim_{65–77}$ had a low to intermediate affinity for all MHC tested, Vim_{R70Cit} bound *0101, *0401, and *0404 with a strikingly high affinity. Compared with the unmodified peptide, the citrulline-containing peptide bound with 100-, 90-, and 20-fold higher affinity to *0101, *0401, and *0404, respectively. Most importantly, the conversion of arginine to citrulline did not increase peptide affinity for any shared epitope-negative MHC tested.

Previous reports have suggested that a distinct feature of a putative pathogenic peptide involved in RA may be the presence of a negatively charged side chain at P4 (interacting with the shared epitope) (15). This is based on the fact that *0401 and *0404 have a substantially higher affinity for aspartic and glutamic acid at the P4 pocket than the RA-nonassociated *0402 molecule. However, after analysis of multiple DRB1 pocket profiles, it can be found that some RA-nonassociated alleles have a higher affinity for negatively charged amino acids at their P4 pockets than even *0101, *0401, and *0404, such as *0301 (16). It has also become clear that some MHC may actually be protective against disease (e.g., *0402), rather than simply nonassociated, suggesting that a passive role for these alleles in peptide binding may not occur (2). Instead, protective alleles may bind a putative pathogenic peptide with a high

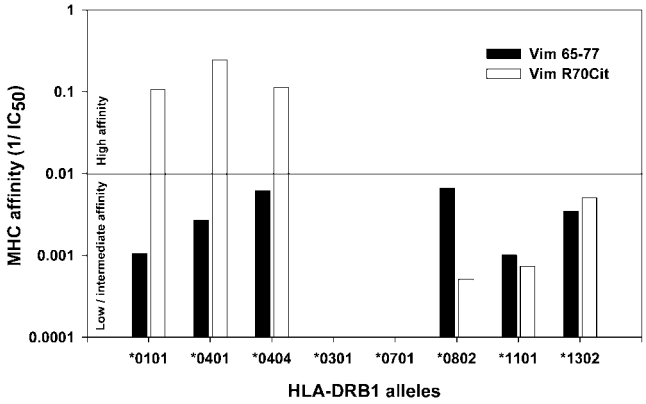

FIGURE 3. Relative affinities of $Vim_{65–77}$ and Vim_{R70Cit} for purified MHC class II molecules. Binding affinities to shared epitope-positive (**0101*, **0401*, **0404*) and -negative (**0301*, **0701*, **0802*, **1101*, **1302*) alleles were determined as described in *Materials and Methods*. Peptides with IC_{50} values of <100 nM are considered to be high-affinity binders.

enough affinity to induce negative selection, or to establish peripheral tolerance.

Based on our results, we propose that the differential binding properties of *DRB1* alleles to either arginine or citrulline at P4 could help explain how MHC class II molecules are disease associated, nonassociated, or protective. First, the conversion of peptide-bound arginine to citrulline can cause a 100-fold increase in affinity for MHC with the shared epitope. This could result in a higher density of peptide-MHC complexes on APCs, which may exceed the biochemical margin of safety necessary for T cell activation (17–19). A process similar to this occurs in celiac disease, in which deamidation of a gliadin peptide (modified at a position interacting with a MHC anchoring pocket) leads to a 50-fold increase in peptide affinity and the activation of DQ2-restricted T cells (20). Second, nonassociated MHC class II molecules (e.g., *0301) may contain P4 pockets that lack the proper size or charge to productively accommodate the large polar side chains of arginine or citrulline, and would therefore be unable to bind and present peptides regardless of the state of modification (21). Finally, disease-protective MHC may interact productively with both arginine and citrulline at P4, resulting in peptide-MHC ligands that could induce negative selection, lead to the production of $CD4^+CD25^+$ regulatory T cells (22), or simply remain within the biochemical margin of safety (17).

Although these studies have focused on a restricted set of peptides, we have identified additional sequences within vimentin and the α- and β-chains of fibrinogen, another target of anti-citrulline Abs in RA patients (23), that are predicted to bind *0401 in a register that would position arginine or citrulline at P4. This suggests that a number of unique pathogenic peptides could give rise to activated T cells with a heterogeneous array of specificities, a characteristic typical of T cells found within the rheumatoid joint (24).

In conclusion, we have identified a novel peptide-MHC interaction that is dependent on both a posttranslational modification and the presence of the RA shared epitope. This peptide-MHC interaction, and subsequent Th activation, may be responsible for driving autoantibody production. Future work will determine whether these modified peptides are targets of the T cell response in RA patients.

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