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*J Immunol* 2006; 177:1421-1425; doi: 10.4049/jimmunol.177.3.1421

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CUTTING EDGE

Cutting Edge: Unique T Cells That Recognize Citrullinated Peptides Are a Feature of Protein Immunization

Jamie Ireland, Jeremy Herzog, and Emil R. Unanue

Abs against citrullinated proteins are present in patients with rheumatoid arthritis. In this study, we describe a unique cohort of T cells that selectively responded to citrullinated variants of two epitopes of hen egg-white lysozyme, a major and a minor one, bound to the MHC molecule, I-Ak. In addition, we show that when given an intact, unmodified lysozyme protein, dendritic cells and peritoneal macrophages presented citrullinated peptides and stimulated modification-specific T cells. Thus, presentation of citrullinated-peptide-MHC complex is a feature of immune responses to protein Ags. The Journal of Immunology, 2006, 177: 1421–1425.

Antibodies to a variety of citrullinated proteins are found in a very high number of patients with rheumatoid arthritis (RA) and can be used as a highly specific clinical index of the disease. Citrullination of arginines is a posttranslational change resulting from the action of peptidylarginine deiminases (PAD), a family of enzymes found in different cells including neutrophils, monocytes, and macrophages (1–6). The immunological consequences of deimination of arginines are of great interest, particularly with respect to their possible pathogenetic significance in RA. The issue of whether citrullinated peptides may be autoantigenic has been raised, not only in RA but also in cases of inflammatory disease of the nervous system where citrullination occurs in myelin basic protein (7, 8). Citrullinated proteins have been found to be antigenic (9, 10).

Posttranslational changes have been reported in MHC-bound peptides (11, 12). We recently called attention to nitration of tyrosine residues (13) and oxidation of tryptophans on peptides from the protein, hen egg-white lysozyme (HEL), bound to the class II histocompatibility molecule I-Ak (14). These changes took place when APCs were activated during immunological reactions including infections. T cells highly specific to the changes were generated in vivo, and some escaped negative selection in vivo.

Citrullinated peptides from the protein, hen egg-white lysozyme (HEL), and oxidized tyrosine peptides were observed in spleens of B10.BR mice, usually 6- to 12-wk-old, of both sexes were obtained from The Jackson Laboratory and maintained in facilities at Washington University. B10.BR-Tg(KLK4mHEL)6Ccg mice expressing membrane HEL under the class II Ero promoter were generated and maintained by our laboratory (15). HEL was obtained from Sigma Chemical and purified to eliminate contaminants and LPS. Peptides were synthesized using Fmoc techniques. LPS (Sigma-Aldrich) was used at a final concentration of 1 µg/ml.

Methods and Materials

Cell culture

CD4 T cell hybridomas were made by fusing cells obtained from popliteal lymph nodes 7 days postimmunization with 10 nmol of HEL in CFA. First, lymphocytes were cultured for 1 wk in the presence of 1 µM peptide. The cells were then stimulated with fresh peptide and irradiated splenocytes as APCs. Three days later, the cells were fused to BW5147α-β-thymoma cells line, as described previously (15).

C3.F6 B lymphoma cells were used as APC for examining the response to the various peptides. Peritoneal macrophages were obtained by i.p. injection with 100 µg of Con A. After 4 days, peritoneal exudate cells (PEC) were harvested. Dendritic cells (DC) were derived from bone marrow cultured for 6 days in GM-CSF containing medium as described previously (16). Hybridoma activation was measured by IL-2 secretion as assayed by CTLL proliferation. ELISPOT assays were conducted according to the BD Biosciences protocol.

For limiting dilution cloning, mice were immunized with 10 nmol of HEL in CFA in the hind footpads. Seven days later, popliteal lymph node nodules were harvested. Lymphocytes were plated in round-bottom plates with 5 × 10^3 irradiated (2000 rad) splenocytes with 50 U/ml rIL-2 and 5 µM 48–62cit61 peptide. Clonal growth-positive wells were maintained in these culture conditions.

Mice and reagents

B10.BR mice, usually 6- to 12-wk-old, of both sexes were obtained from The Jackson Laboratory and maintained in facilities at Washington University. B10.BR-Tg(KLK4mHEL)6Ccg mice expressing membrane HEL under the class II Ero promoter were generated and maintained by our laboratory (15). HEL was obtained from Sigma Chemical and purified to eliminate contaminant proteins and LPS. Peptides were synthesized using Fmoc techniques. LPS (Sigma-Aldrich) was used at a final concentration of 1 µg/ml.

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0022-1767/06/$02.00

Received for publication April 18, 2006. Accepted for publication June 6, 2006.

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; PAD, peptidylarginine deiminase; HEL, hen egg-white lysozyme; PEC, peritoneal exudate cell; DC, dendritic cell; BMDC, bone marrow-derived DC.
RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. The RNA was treated with DNA-free (Ambion), then reverse-transcribed using Superscript First-Strand Synthesis (Invitrogen Life Technologies), and amplified using the following primer sequences: PAD2, TACAGATTCCCGTACACGTTGCGT and AACTGGCCAGAGAATTGAGGACCA with an expected amplicon of 245 bp; and PAD4, CCAA GAAAGCCAAGTGCAAGCTGA and TTCCCGATGAGAATTCTGCCAGT with an expected amplicon of 316 bp.

**RT-PCR**

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. The RNA was treated with DNA-free (Ambion), then reverse-transcribed using Superscript First-Strand Synthesis (Invitrogen Life Technologies), and amplified using the following primer sequences: PAD2, TACAGATTCCCGTACACGTTGCGT and AACTGGCCAGAGAATTGAGGACCA with an expected amplicon of 245 bp; and PAD4, CCAA GAAAGCCAAGTGCAAGCTGA and TTCCCGATGAGAATTCTGCCAGT with an expected amplicon of 316 bp.

**Table I. Peptide binding results**

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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC50 (nM)</th>
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<tr>
<td>48–62</td>
<td>DGST DYGILQINSRW</td>
<td>0.07</td>
</tr>
<tr>
<td>48–62 cit61</td>
<td>DGST DYGILQINScitW</td>
<td>0.06</td>
</tr>
<tr>
<td>114–129</td>
<td>RCKFT DVQAWIRGCRKL</td>
<td>3.8</td>
</tr>
<tr>
<td>114–129 cit125</td>
<td>RCKFT DVQAW1citGCRKL</td>
<td>1.0</td>
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**Results**

**Immunization with HEL gives rise to T cells that are specific to citrullinated epitopes**

Mice were immunized with HEL in CFA, and 1 wk later the draining lymph node cells were isolated and screened for CD4+ T cells to citrullinated HEL epitopes. After a 7-day culture in the presence of citrulline (either HEL 48–62cit61 or HEL 114–129cit125) (Table I), followed by a 3-day stimulation with irradiated splenocytes, the cells were fused to create T cell hybridomas. In a different approach, the lymph node cells of the mice immunized with HEL were placed in an ELISPOT assay against the modified peptides, or were cloned by limiting dilution.

Peptide 48–62 represents the major chemically dominant segment of HEL selected from its processing (18). This family of peptides centers on the nine-residue core from 52 to 60. Arginine, underlined, is the tenth residue on this peptide: DYGILQINSRW. The crystal structure of the 48–62 peptide bound to I-Ak showed that the P10 arginine and the P11 tryptophan were solvent exposed (19). As expected, changing the arginine 61 to citrulline did not change the binding of the 48–62 peptide to purified I-Ak molecule (Table I).

Several hybridomas were found that responded to 48–62 with a citrullinated P10, and not to the wild-type arginine residue. Three representative T cells are shown in Fig. 1A, labeled Pepe, Granny, and Marvin, and each responded with varying degrees of sensitivity to 48–62 but only with a citrulline in place of the arginine at P10. These hybridomas did not secrete IL-2 in response to stimulation with wild-type peptide. All of the citrulline-specific hybridomas showed an absolute dependence on the presence of the tryptophan at P11, that is, responded to 48–62cit61 but not 48–61cit61. This finding is not surprising as the screening process selected for a population of T cells whose receptors recognized C-terminal residues. About half of the T cells that responded to the 48–62 segment of HEL also required the presence of Trp62 (20, 21).

It is known that most of the T cells that interact with the 52–60 core segment interact with three TCR contact residues, Tyr53, Leu56, and Asn59 (19, 21, 22). Such was the case for the
T cells that recognized cit61 (Fig. 1B). None recognized the alanine substitutions for Tyr53 or Asn59. Additionally, the response was severely hindered when leucine at the P5 position, a centrally positioned TCR contact, was replaced by an alanine. Whether the TCR directly interacted with the cit\(^{61}\) and Trp\(^{70}\), or the latter imparted a conformational change of the three TCR contact residues of the core segments is not known.

To further define the specificity of the hybridomas reactive with 48–62cit61, we assessed the effects of several amino acid substitutions at the Arg61 residue (Table II). Replacement of the Arg61 with alanine or glycine resulted in a markedly diminished response. The substitution of glutamine, an amino acid with structural similarities to citrulline, induced a similar or smaller response among the various T cell hybridomas. (Fig. 1A and Table II). In contrast, substitution at P10 with lysine completely abrogated the response in data not shown. T cell hybridomas that recognize unmodified 48–62 were tested for reactivity to the modified peptides: we found no difference in the responses to stimulation with the various substitutions.

ELISPOT analysis of spleen cells from mice immunized with HEL indicated that frequency of T cells reactive with 48–62cit61 is \(~1:36,000\). The ELISPOT, however, did not distinguish those T cells that exclusively reacted with the citrullinated peptides. The frequency of T cells responding to 48–62 was \(~1:25,000\). We therefore conducted cloning of primary T cells from HEL-immunized mice by limiting dilution. Each clone was tested against 48–62 and 48–62cit61. Analysis of 23 primary T cell clones showed that 8, or 35%, reacted only to 48–62cit61, whereas the other 15 responded to both 48–62cit61 and 48–62.

Peptide 114–129 binds weakly to I-A\(^{b}\) molecules, although it induces a relatively strong T cell response (23). The binding core in 114–129 is from residues 119–127: DVQA(W)RGC. Arginine is at P7 in the peptide and is known to be a negative or hindering residue. Peptide 114–129cit125 bound with an IC\(_{50}\) of 1 \(\mu\)M, whereas the unmodified peptide bound at 3.8 \(\mu\)M (Table I). These findings are in agreement with a recent report on the binding of a citrullinated peptide to HLA DRB1*0401 (24).

Immunization with HEL and selection for T cells that recognized a citrulline for the Arg125 resulted in T cells that specifically recognized the modification. Two representative T cells specific for the 114–129 peptide with citrulline at residue 125, C68 and Everardo, are shown in Fig. 2. Unlike the hybridomas that recognize 48–62cit61, these did not react with peptides in which the arginine at 125 was replaced with glutamine. Similarly, hybridomas that recognized the 114–129 epitope did not recognize the citrullinated peptide (Table II). Two ELISPOT assays were done. The number of 114–129-reactive T cells after HEL immunizations were 1:24,539 and 1:36,000. The numbers of 114–129cit125-reactive T cells were much less: 1:70,000 and 1:142,857. Collectively, these data indicated that citrullinated peptides were presented in vivo, from HEL processing, and elicited specific T cell responses.

**Primary APC present modified peptides when given whole HEL**

We tested the abilities of different APC to present the modified peptides when cultured with HEL. To this end, we examined bone marrow-derived DCs (BMDC), adherent PECs, or the C3.F6 B lymphoma line (Fig. 1A), as APC. C3.F6 pulsed with whole HEL did not stimulate the hybridomas that were specific to 48–62 cit61. The hybridomas specific for 48–62cit61 responded to whole HEL when presented by BMDC (Fig. 3A) or PEC (Fig. 3B). The response was increased after addition of LPS to the cultures: with Marvin the response was only detected after LPS stimulation. In addition, DC (CD11b\(^{−}\), CD11c\(^{+}\)) and macrophages (CD11b\(^{+}\), CD11c\(^{−}\)) isolated from B10.BR spleens pulsed with HEL presented 48–62cit61.

In data not shown, less sensitive hybridomas responded weakly or not at all, suggesting that the levels of 48–62cit61 may be limiting. In support of this idea, the 114–129cit125-specific hybridomas, C68 and Everardo, did not respond to HEL presented by BMDC or PEC. Both of these hybridomas are much less sensitive. The levels of the 114–129 epitope of HEL presented on I-A\(^{b}\) are low relative to the levels presented by the 48–62 family. Lastly, in data not shown we found that BMDC and PEC from lysozyme transgenic mice stimulated the 48–62cit61 hybridomas. The B lymphoma line C3.F6 did not

**FIGURE 2.** C68 and Everardo are reactive to citrullinated 114–129. Hybridomas were cocultured with C3.F6 as APC. HEL or peptide was added to the cultures in 1.5 log dilutions, and IL-2 secretion was assayed by proliferation of CTLL cells.
The steps involved in citrullination of Ag require analysis, taking into consideration the properties of the PAD enzymes. The mechanism by which presentation of the modified epitopes occurs is puzzling considering the high level of calcium required and the localization of the enzymes in the cell. A high calcium level could take place during apoptosis, although we have not discerned an obvious apoptotic response in our cultures. The lack of presentation by C3.F6 suggests that PAD4 may be the critical enzyme, but this conclusion needs to be substantiated in experiments where this enzyme is removed from DC or macrophages. Such experiments are in progress.

Having identified specific T cells to citrullinated proteins as part of the response to immunization now begs the question of their immunopathologic role. What is their meaning in the context of the findings in RA? Because citrullination of proteins in the joint is such a striking feature, will T cells to the modified epitopes be enriched there? If such T cells develop in inflamed joints against protein components, will they be pathogenic? The issue of whether these responses are involved in disease pathogenesis or a byproduct of a more general immunological reaction must be clarified before steps to target deimination as a treatment are taken.

Acknowledgments

We thank Shirley Petzold for technical help with the MHC-binding assays.

Disclosures

The authors have no financial conflict of interest.

References


