Carbamylation-Dependent Activation of T Cells: A Novel Mechanism in the Pathogenesis of Autoimmune Arthritis

Piotr Mydel, Zeneng Wang, Mikael Brisslert, Annelie Hellvard, Leif E. Dahlberg, Stanley L. Hazen and Maria Bokarewa

*J Immunol* published online 19 May 2010
http://www.jimmunol.org/content/early/2010/05/19/jimmunol.1000075

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/05/19/jimmunol.1000075_5.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Carbamylation-Dependent Activation of T Cells: A Novel Mechanism in the Pathogenesis of Autoimmune Arthritis

Piotr Mydel,* Zeneng Wang,† Mikael Brisslert,* Annelie Hellvard,* Leif E. Dahlberg,‡ Stanley L. Hazen,§ and Maria Bokarewa*

The posttranslational modification of proteins has the potential to generate neoepitopes that may subsequently trigger immune responses. The carbamylation of lysine residues to form homocitrulline may be a key mechanism triggering inflammatory responses. We evaluated the role of carbamylation in triggering immune responses and report a new role for this process in the induction of arthritis. Immunization of mice with homocitrulline-containing peptides induced chemotaxis, T cell activation, and Ab production. The mice also developed erosive arthritis following intra-articular injection of peptides derived from homocitrulline and citrulline. Adoptive transfer of T and B cells from homocitrulline-immunized mice into normal recipients induced the induction of arthritis. Immunization of mice with homocitrulline-containing peptides induced chemotaxis, T cell activation, and Ab production. The mice also developed erosive arthritis following intra-articular injection of peptides derived from homocitrulline and citrulline. Thus, the T cell response to homocitrulline-derived peptides, as well as the subsequent production of anti-citrulline and citrulline, is critical for the induction of autoimmune reactions against citrulline-derived peptides and provides a novel mechanism for the pathogenesis of arthritis. The Journal of Immunology, 2010, 184: 000–000.

Following synthesis, proteins undergo the process of post-translational modification (PTM), thus extending their range of function through the modification of amino acids with various functional groups (1, 2). These modifications have a critical influence on protein structure and biological function, especially in the context of aging, physiological stress, and inflammation. The role of PTM in the generation of neoepitopes on self-proteins that are subsequently responsible for the pathogenesis of autoimmune diseases, such as multiple sclerosis, diabetes mellitus, systemic lupus erythematosus, and rheumatoid arthritis (RA), has only recently been recognized (3–6).

Citrullination, the posttranslational conversion of arginine (Arg) residues to citrulline (Cit) residues by peptidylarginine deiminase enzymes, has been extensively studied in relation to autoimmune arthritis (Fig. 1A) (7). The potentially harmful effects of these Cit-modified proteins have attracted a lot of attention recently because of the close association between the production of Abs to Cit-peptides and the development of RA (8, 9). Despite the immense value of these anti-Cit Abs as an early and specific predictive marker for RA (anti-citrullinated Abs are detectable before the onset on clinical disease) (10, 11), the specific role played by citrullination in the development of arthritis remains elusive. Increased levels of Cit-modified residues have been found in myelin basic protein, which is now used as an Ag for the induction of experimental autoimmune encephalomyelitis (12); however, similar attempts using anti-Cit Abs to trigger experimental arthritis have yielded only modest results. Therefore, the existence of another triggering factor has been postulated (13, 14). Factors that have been identified as predisposing to the production of anti-Cit Abs include HLA-DR alleles and smoking (15–17).

Carbamylation (homocitrullination) is a PTM that has been studied for many years in the context of uremia (18–20). It involves the nonenzymatic reaction of urea-derived cyanate with free NH₂-groups on lysine (Lys) residues to yield homocitrulline (Hcit) (Fig. 1B). This process can be mediated in vivo by myeloperoxidase (MPO), the enzyme responsible for the inflammation-driven carbamylation of proteins via the MPO/H₂O₂(SCN−) system. Thus, MPO has recently attracted attention as a potential target factor for atherogenesis and inflammation (21). Hcit residues affect the charge distribution within a peptide in a way that may result in impaired or even loss of function. Loss of enzymatic function upon carbamylation has been reported for matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-2, and insulin (22–24). Plasma SCN− levels are known to be significantly higher in smokers, leading to increased carbamylation of proteins (21, 25); furthermore, carbamylation has emerged as a potential pathogenic factor in renal insufficiency (26), cardiovascular disease (21, 27), and cataracts (28). Extracellular matrix proteins, such as collagen and fibrinogen, are thought to accumulate structural damage elicited by PTMs because of their long half-life and low turnover rates. Notably, it has been shown that collagen is easily carbamylated in vivo and that such modification may be directly linked to granulocyte activation and protease release (29, 30).

In this study, we show that carbamylation provides the missing link between Cit-peptides and the development of erosive arthritis. Using experimental models and patient material, we show that the...
presence of carbamylated Lys residues triggers primary immune responses, including the proliferation and chemotaxis of CD4+ T cells and the production of IL-10 and the proinflammatory cytokines IFN-γ and IL-17. Our results provide a novel mechanism for the pathogenesis of arthritis involving activation of T cells in combination with a powerful Ab response, which leads to the local recognition of Cit-peptides in the joints and the subsequent development of erosive arthritis.

Materials and Methods

Patients with RA and control individuals

Plasma and synovial fluid were collected from 72 consecutive RA patients (age range, 34–82 y; 17 males and 55 females; disease duration, 1–36 y) admitted to Rheumatology Clinics at Sahlgrenska University Hospital for acute joint effusion. Clinical and demographic characteristics of the patients are presented in Supplemental Table I. Plasma of 41 healthy individuals, matched to RA patients by age and gender, and synovial fluid of patients (age range, 28–82 y, 17 males and 23 females) with knee trauma (n = 24) and osteoarthritis (n = 16) were used as control. Collected samples were centrifuged at 800 × g for 15 min, aliquoted, and stored frozen at −70°C until use. The study was approved by the Ethical Committee of the University of Göteborg. Informed consent was obtained from all patients. Erosive arthritis was defined by the presence of bone erosions on recent posterior-anterior radiographs of hands and feet. The presence of Ab to cyclic citrullinated peptides (aCCPs) was measured by ELISA (Immunoscan AB, Uppsala, Sweden).

Animals, immunization procedure, and experimental arthritis

For in vivo experiments, NMRI, BALB/c, and C57Bl/6 mice were purchased from B & K International (Sollentuna, Sweden). An Asn breeding pair was adopted to the local recognition of Cit-peptides in the joints and the subsequent development of erosive arthritis.

Adoptive transfer of T cells

CD3+ cells were isolated from spleens of BALB/c mice on day 21 following immunization with Hcit peptide (sequence B) using the Dynal Mouse T cell Negative Isolation Kit (Invitrogen, Stockholm, Sweden). Flow cytometry proved 98% of cells to be CD3+. A total of 2 × 10^6 of CD3+ cells were injected i.v. into naive BALB/c mice. Controls received the same amount of CD3+ cells isolated from unmanipulated BALB/c mice. Following di−

Cell stimulation and proliferation assay

Spleens were excised on immunization day 21, and a single−cell suspension was prepared as described (33). The cultures (1 × 10^6 cells/ml) were maintained in 96−well plates (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2 and 95% humidity and stimulated with peptides and anti−CD3 Abs (BD Pharmingen, San Diego, CA). The supernatants were collected after 24 and 48 h.

Mass spectrometry of proteins recovered from blood and synovial fluid

Stable isotope−dilution liquid chromatography mass spectrometry analysis was performed on Arg and Lys and their derivatives, Cit and Hcit, in proteins recovered from blood and synovial fluid, as described (21). In short, [13C6]Arg, [13C6, 15N2]Lys, and [13C6, 15N]Hcit were added before hydrolysis and used as internal standards to quantify Arg, Lys, Cit, and Hcit. Proteins were hydrolyzed with 6 N HCl at 110°C under vacuum. After hydrolysis and cleanup with a mini solid−phase DSC−SCX extraction column (Discovery DSC−SCX SPE tubes; 1 ml; Sigma−Aldrich), analytes were resolved on a Phenyl column (4.6 × 250 mm, 5 μm Exekrom Phenyl, Regis Technologies, Morton Grove, IL) using a gradient generated between aqueous ammonium formate versus methanol/0.1% formic acid/5 mM ammonium formate/40 mM acetic acid mobile phases. Ammonia was analyzed on an API 365 triple−quadrupole mass spectrometer with Ions Ep 10+ upgrade (Concord, Ontario, Canada) interfaced to a C18 column Aria LX Series HPLC multiplexing system using electrospray ionization on positive−ion mode with multiple reaction monitoring of parent and characteristic daughter ions specific for each analyte monitored. The results for ammonia acids are reported in absolute concentrations.

In vitro migration assay

The migratory capacity of splenocytes was tested using the Transwell system with a pore size of 3 μm (AH Diagnostics, Stockholm Sweden), as described previously (34). In short, splenocytes (5 × 10^5/well) were placed in the upper chamber and migrated for 12 h toward CXCL13 (100 ng/ml) or 0.1% BSA−PBS dilution buffer as negative control added to the lower chamber. To exclude chemokinesis, 100 ng/ml CXCL13 was included in a set of wells in the lower and upper chambers. Migrated cells were collected from the lower chamber and subjected to phenotype analyses by flow cytometry, as described below.

Flow cytometry

Migrated cells were stained with allophycocyanin−conjugated monoclonal rat anti−mouse CD3 Abs, PE−conjugated monoclonal rat anti−mouse CD19 Abs, PerCP−conjugated monoclonal rat anti−mouse CD4 Abs, and Pacific Blue−conjugated monoclonal rat anti−mouse CD8 Abs (all Abs were from BD Biosciences, Erembodegem, Belgium) for 30 min on ice in FACS buffer (PBS, 1% FCS, and 0.02% azide [pH 7.2]). Cells were washed, collected in the cytometer (FACSCanto II, BD Biosciences), and analyzed with FACS−Diva software using FlowJo software (Tree Star, Ashland, OR). Gating of
FIGURE 1. Schematic illustration of PTMs of Arg and Lys. A. Deamination (citrullination) is an enzymatic modification characterized by cleavage of NH$_3$ groups from Arg residues mediated by peptidylarginine deiminases. B. Carbamylation (homocitrullination) is characterized by the addition of a CONH$_2$ group to Lys residues.

the cells was based on the isolate control values, as well as fluorochrome minus one settings when needed.

**Immunohistochemistry and histopathology**

Lower paws of sacrificed mice were removed, fixed, decalcified in Parengy’s buffer, embedded in paraffin, and cut into 4-μm thin sections. Tissue sections were stained with H&E. For immunohistochemical evaluation, paws were decalcified using 0.5 M EDTA (pH 7.1). Tissue sections were deparaffinized and stained using rat anti-mouse CD3 Abs (10 μg/ml, MCA1477, Serotec, Oxford, U.K.), followed by biotinylated goat anti-rat Abs (2.5 μg/ml, Vector Laboratories, Burlingame, CA). Color reaction was completed with the Vectastain Elite ABC kit (Vector Laboratories) and 3-amino-9-ethyl-carbazole containing H$_2$O$_2$. Sections were counterstained with hematoxylin. Rat serum constituted negative control. The sections were evaluated with respect to synovitis and erosion of bone/cartilage by a blinded examiner. Synovitis was defined as a membrane thickness of more than two cell layers (35) and scored as follows: 1, mild; 2, moderate; and 3, severe synovitis and joint damage (36). One half point was added to the score if erosions were present. Final arthritis index in the group was calculated as a sum of all scores divided by the number of joints.

**Detection of peptide-specific Abs**

The 96-well plates (Maxisorp, Nunc) were coated with Cit-peptides (sequence E, 0.5 μg/ml) or Hcit-peptides (sequence B, 0.1 μg/ml) in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Following blocking with 2% skim milk/PBS and washing with 0.05% Tween/PBS, the samples (diluted 1/50 and 1/100 in blocking solution) were applied and incubated for 6 h at room temperature. Rabbit anti-human IgG/HRP (100 ng/ml, Dako A/S, Glostrup, Denmark) was used as detection Ab. Incubated thin sections. Tissue sections were stained with H&E. For immunohistochemical evaluation, the samples (diluted 1/50 and 1/100 in blocking solution) were applied and incubated overnight at 4°C. Rabbit anti-mouse IgG/HRP 10 ng/ml in blocking solution were used as detection Abs. Secretion of Ig by splenocytes from mice that underwent adoptive transfer of T and B cells was detected using ELISPOT, as described (37).

**Measurements of cytokine levels**

IL-2, -6, -10, and -17A and INF-γ levels in supernatants of cells cultured in the presence or absence of Cit-peptide or Hcit-peptide were assessed by Quantikine ELISAs (R&D Systems, Minneapolis, MN).

**Statistical analysis**

Continuous parameters were expressed as mean ± SEM. Comparisons between the matched blood and synovial fluid samples were analyzed by the Kruskal–Wallis test, followed by Dunn post hoc comparisons. The sections were evaluated with respect to synovitis and erosion of bone/cartilage by a blinded examiner. Synovitis was defined as a membrane thickness of more than two cell layers (35) and scored as follows: 1, mild; 2, moderate; and 3, severe synovitis and joint damage (36). One half point was added to the score if erosions were present. Final arthritis index in the group was calculated as a sum of all scores divided by the number of joints.

**Results**

**Immunization with carbamylated peptides predisposes to the development of erosive arthritis**

NMRI mice were immunized on days 0 and 14 with synthetic peptide sequences derived from filaggrin, an epidermal skin protein (Table I). On day 21, the knee joints were injected with carbamylated/Hcit-peptide (sequence B) or deiminated/Cit-peptide (sequence E). Examination of knee joints injected with peptides identical to the one of immunization showed that Hcit-immunized mice had clear signs of arthritis (arthritis index, 1.05 ± 0.19) and cartilage damage. In contrast, the Cit-immunized mice injected intra-articularly with Cit-peptide developed arthritis only occasionally (arthritis index, 0.56 ± 0.11) (Fig. 2A, 2B). Interestingly, the Hcit-immunized mice injected with Cit-peptide developed the most pronounced arthritis (arthritis index, 1.98) (Fig. 2A, 2C), with a prevalence of 92%. The severity of arthritis in the latter case was 2.21-fold greater than that seen in nonimmunized or Cit-immunized mice given subsequent intra-articular injections of Cit-peptide (Fig. 2C). We found these results to be consistent over five independent experiments using NMRI, BALB/c, and A/Sn mice strains (n = 126). The severity of the arthritis seen in the Hcit-immunized mice varied among the strains, being greatest in BALB/c (H2-A$^d$ haplotype) and outbred NMRI mice (arthritis index, 2.23 ± 0.49 and 2.06 ± 0.21, respectively). A/Sn mice developed less severe arthritis (arthritis index, 1.09 ± 0.27), whereas the arthritis index for the C57BL/6 (H2-A$^b$ haplotype) mice was significantly lower (0.45 ± 0.28), about the same as for nonimmunized controls. Taken together, these findings suggest that the development of arthritis in this particular model is dependent upon the MHC-H2 haplotype.

Intra-articular injection of Hcit into nonimmunized or Cit-immunized mice (Fig. 1) led to the development of synovitis (arthritis index, 1.33 ± 0.2 and 0.77 ± 0.17, respectively) (Fig. 2B, 2C3). This was less severe than the arthritis that we saw previously, with the development of erosions in only a few cases compared with the mice immunized with Hcit and intra-articularly injected with Cit-peptide (p < 0.0001) (Fig. 2C7). No significant difference was observed in the frequency or severity of arthritis between the Cit-immunized and nonimmunized mice that were intra-articularly injected with Cit-peptide (Fig. 2C2, 2C4, respectively), nor did we see any evidence of cartilage destruction.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence Name</th>
<th>Arthritis Index</th>
<th>Erosions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SHQESTK-----------------GKSKGKSKGS</td>
<td>Lys linear</td>
<td>25</td>
</tr>
<tr>
<td>B</td>
<td>HOCQESTKHCit-GKSKGKC</td>
<td>Carbamylated, cyclic (Hcit)</td>
<td>1.98 ± 0.27*</td>
</tr>
<tr>
<td>C</td>
<td>SHQESTKHCit-------GKSKGKSKGS</td>
<td>Carbamylated, linear</td>
<td>0.97 ± 0.23**</td>
</tr>
<tr>
<td>D</td>
<td>HOCQESTK--GKSKGKGS</td>
<td>Lys cyclic</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>HOCQESTK-Cit--GSRGRCG</td>
<td>Deiminated, cyclic (Cit)</td>
<td>0.28 ± 0.15</td>
</tr>
</tbody>
</table>

Mice were immunized with peptides A–E (75 μg/mouse on days 0 and 14); all mice received Cit-peptide intra-articularly (1 μg/knee). *p < 0.005; **p < 0.05; compared with nonimmunized mice injected with Cit-peptide intra-articularly (arthritis index 0.33 ± 0.13; n = 18).
To confirm that the injected peptide must contain carbamylated residues to induce arthritis, we immunized mice with peptides containing unmodified Lys residues (Table I, sequences A and D), Cit-containing peptide (sequence E), or with peptides containing cyclic or linear Hcit-peptides (sequences B and C). On day 21, all animals received intra-articular injections of Cit-peptide (sequence E) (1 μg/knee). Histological evaluation of the injected joints showed significant levels of synovitis and cartilage erosion only in mice immunized with the cyclic or linear Hcit-peptides (sequences B and C, respectively) (Table I), although the histological changes were less severe in the mice immunized with linear Hcit compared with those immunized with cyclic Hcit. The arthritis index of mice immunized with the Lys-containing peptides (sequences A and D) or with the Cit-containing peptide (sequence E) was significantly lower than for mice immunized with either cyclic or linear Hcit (Table I).

Hcit-peptides induce Hcit-specific chemotaxis and T cell activation in immunized mice

Immunohistological staining of the Cit-injected joints from Hcit-immunized mice showed that substantial numbers of CD3+ cells had infiltrated the synovia (Fig. 2C5, 2C6), but none were found in the synovia of Cit-immunized mice. This interesting observation prompted us to look at the effects of Hcit-immunization on the activity and function of T cells.

We examined the ability of splenocytes harvested from Hcit- and Cit-immunized mice to migrate in response to a chemotactic agent (CXCL13) in vitro. Flow cytometric analysis of the transmigrated cells showed a preponderance of CD4+ cells compared with CD8+ or CD19+ cells (p < 0.001). This increased migration of CD4+ cells occurred in cultures derived from Hcit-immunized mice, but not in those from Cit-immunized mice or nonimmunized controls (Fig. 3A). Interestingly, the addition of Hcit- or Cit-peptides (500 ng/ml) to the lower chamber resulted in no difference in splenocyte migration between immunized and nonimmunized mice.

We next examined whether splenocytes isolated from Hcit- and Cit-immunized mice were able to proliferate and produce cytokines in response to CD3 stimulation. Lymphocytes isolated from Hcit-immunized mice had a significantly greater proliferative capacity compared with those from Cit-immunized mice or nonimmunized controls (p < 0.0001) (Fig. 3B). The culture supernatants from CD3-stimulated cells isolated from Hcit-immunized mice also contained substantially greater concentrations of IL-10 and -17 and IFN-γ compared with those of Cit-immunized mice or nonimmunized controls (Fig. 3C). However, IL-6 levels were similar in all culture supernatants.

The adoptive transfer of T and B lymphocytes from Hcit-immunized mice induces specific Ab response and arthritis in unmanipulated recipients

To evaluate the role of T cells in Cit-induced arthritis, CD3+ T cells were isolated by negative selection from the spleens of BALB/c Hcit-immunized mice and injected i.v. into unmanipulated BALB/c recipients (5 × 10^6 cells/mouse, n = 5). Control BALB/c mice received the same number of CD3+ T cells isolated from unmanipulated BALB/c mice (n = 5). Two weeks later, all mice were challenged with an intra-articular injection of Cit-peptide (1 μg/knee). Histological examination of the injected joints of mice that received T cells from Hcit-immunized mice showed evidence of severe arthritis, with cartilage destruction (erosion) and bone loss (Fig. 4A1). Mice that received T cells from unmanipulated mice showed no morphological signs of arthritis (Fig. 4A2). Also, we found that splenocytes isolated from the Hcit-immunized T cell recipients had significantly greater proliferation rates and produced more IFN-γ in response to CD3 stimulation than did control mice that received naive T cells (Fig. 4B). Transfer of CD19+ B cells from Hcit-immunized animals triggered arthritis in a manner similar to T cell transfer, with an arthritis index of 1.41 ± 0.23. Interestingly, arthritis triggered by adoptive transfer

FIGURE 2. Immunization with carbamylated peptide predisposes to the development of erosive arthritis. Mice were immunized with Hcit-peptide (Hcit) and Cit-peptide (Cit) (75 μg/mouse) on days 0 and 14. Intra-articular injections of Cit-peptide (A) and Hcit-peptide (B) (1 μg/knee) were given on day 21. Histological evaluation of injected knees was undertaken on day 28; data are presented as arthritis index. Values from three independent experiments using NMRI mice were pooled (n = 60). The arthritis index for Hcit-immunized mice was 2.21-fold higher compared with Cit-immunized and nonimmunized mice (p < 0.001). Values represent the mean ± SEM. Horizontal lines indicate the medians. C, Representative histological changes seen in the injected joints of mice immunized with Hcit (1) and Cit (2) followed by intra-articular injection of Cit-peptide, and nonimmunized controls (3, 4). Arthritis induction was performed in five independent experiments, giving a 92% incidence of arthritis in three wild-type strains (NMRI, BALB/c, and Asn) (n = 126). Immunohistological staining of Hcit-immunized and Cit-immunized mice was done using rat anti-mouse CD3 Ab. Large numbers of CD3+ cells (brown staining) were observed in the synovia of Hcit-immunized mice (5) compared with Cit-immunized mice (6). Original magnification ×500. Tissue sections were stained with H&E (C1–C4). Immunostaining for CD3 was developed with 3-amino-9-ethylcarbazole. Hematoxylin was used as counterstain (C5, C6).

To confirm that the injected peptide must contain carbamylated residues to induce arthritis, we immunized mice with peptides containing unmodified Lys residues (Table I, sequences A and D), Cit-containing peptide (sequence E), or with peptides containing cyclic or linear Hcit-peptides (sequences B and C). On day 21, all
of B cells was only occasionally destructive for joint cartilage and bone. Adoptive transfer of T and B cells also triggered peptide-specific Ab production in the recipients. In both cases, we observed significantly more specific Abs to Hcit-peptide in our ELISPOT assay compared with control mice that received unmanipulated lymphocytes (Fig. 4C).

Immunization with carbamylated peptide induces efficient Ab production

When we analyzed blood samples taken from mice on day 0 and on days 7, 21, and 28 postimmunization, we found that the anti–Hcit-Ab titers rapidly increased after the second round of immunization and that the Ab titer remained high throughout the course
of the experiment. In contrast, mice immunized with the Cit-peptide produced low levels of Abs. Specificity studies showed that the IgG Abs produced by the Hcit-immunized (cyclic) mice bound exclusively to the Hcit-peptides and not to Cit-peptide (E). We decided to analyze the specificity of these anti-Hcit Abs further, using plates coated with peptides A–E. The anti-Hcit Abs cross-reacted to some extent with peptides C and D (Table II). Despite the fact that mice immunized with peptides A and D produced high levels of Abs that cross-reacted with the Hcit-peptide, they showed no morphological signs of arthritis following intra-articular injections of the Cit-peptide (Table I). Immunization with Cit-peptide resulted in low Ab titers that had almost no cross-reactivity with any of the peptides studied (Table II).

Systemic injection of anti-Hcit Abs or intra-articular injection of the Hcit-Ab/Cit-peptide mixture does not induce susceptibility to arthritis

We used two approaches to elucidate the role of Hcit-Abs in the development of arthritis. First, purified IgG from Hcit-immunized mice was injected i.p. into unmanipulated NMRI mice (0.2 mg/mouse, n = 5), followed by an intra-articular injection of Cit-peptide (1 µg/knee) 1 d later. Control mice (n = 6) were treated in the same way but received IgG from nonimmunized mice. When we examined the injected joints histologically, we saw no signs of arthritis in the test or control mice (data not shown). In the second approach, anti–Hcit-Ab/Cit-peptide complexes were formed ex vivo and then injected directly into the knee joints of naive mice (total protein content 1 µg/knee, n = 8). Control mice (n = 8) received intra-articular injections of Cit-peptide complexed with the monoclonal anti–Cit-Ab, ACC3 (32). Histological evaluation of the injected joints showed mild synovitis in two of eight joints injected with the Hcit-Ab/Cit-peptide complex (arthritis index, 0.25 ± 0.12), whereas no signs of arthritis were seen in the joints injected with ACC3/Cit-peptide complex. Because of the low affinity of Hcit-Ab to Cit-peptide, only a restricted complex formation is expected. However, the experiments showed that the simultaneous presence of Hcit-Ab and Cit-peptide, as well as fully formed complexes of Cit-peptide with its mAb (ACC3), in the joint cavity was not sufficient to induce arthritis.

Carbamylated proteins have arthritogenic properties

To try to establish the role of carbamylation in triggering autoimmune arthritis and to exclude the possibility of specific reactions against the synthetic peptides themselves, we immunized mice with exogenous (BSA) and endogenous (mouse albumin [mAlb]) proteins carbamylated using potassium cyanate (KNOC). Forty-seven NMRI mice were immunized on days 0 and 14 with carbamylated BSA (Hcit-BSA) or mAlb (Hcit-mAlb), followed by intra-articular injection of Cit-peptide on day 28. Histological examination of the injected joints showed that mice immunized with Hcit-mAlb displayed signs of severe arthritis, with a prevalence of 91% (arthritis index, 1.45 ± 0.18) compared with control animals immunized with unmodified albumin (arthritis index, 0.26 ± 0.17; p = 0.0024). Immunization with Hcit-BSA showed a similarly high prevalence of erosive arthritis (87%; arthritis index, 1.56 ± 0.23) compared with controls (arthritis index, 0.5 ± 0.24; p = 0.04). We also detected high levels of Abs specific for the Hcit-protein following immunization with Hcit-mAlb or Hcit-BSA. These results clearly show that carbamylated proteins can trigger processes that lead to the development of arthritis in vivo. Taken together, our findings strongly suggest that this mechanism is not peptide specific and that the immune responses that result in joint destruction are activated by carbamylated residues within proteins.

Quantitative analysis of carbamylated and deiminated residues in the blood and synovial fluid from RA patients

We then looked at the levels of carbamylated Lys (Hcit) and deiminated Arg (Cit) residues in the proteins recovered from the blood and synovial fluid of RA patients (n = 72), patients with knee trauma (n = 24), and patients with osteoarthritis (n = 16), using mass spectrometry, and compared them with the levels of unmodified Lys and Arg residues. The RA patients were further stratified according to radiological findings (erosive RA versus nonerosive RA) and the presence of anti-Cit Abs (aCCPs). We found that the circulating levels of Cit- and Hcit-peptides were greater in patients with erosive RA than in those with nonerosive RA or controls (p = 0.001; Fig. 5A, 5B). In contrast, the levels of unmodified Arg and Lys residues in the blood and synovial fluid were not significantly different (data not shown). Furthermore, the levels of Hcit were greatest in patients with erosive RA plus positive anti–Cit-Ab titers (p = 0.023; Fig. 5B). The levels of Cit were high in all patients with erosive RA compared with the nonerosive group (p = 0.0008), and they were not influenced by whether the patient was anti–Cit-Ab⁺ or anti–Cit-Ab⁻ (Fig. 5A). The levels of Hcit-Abs were significantly greater in patients with erosive RA than in those with nonerosive RA or in controls (Fig. 5C).

Discussion

In this study, we showed that the carbamylation of Lys residues may be the missing link in the chain of pathogenesis of autoimmune arthritis. Indeed, immunization of mice with carbamylated peptides (Hcit-immunization) induced T and B cell activation, followed by high levels of proliferation and cytokine and autoantibody production. This clearly suggests that Hcit-modification changes immunologically inert peptides into autoantigens. More importantly, Hcit-immunized mice became susceptible to arthritis induced by the intra-articular injection of Cit-peptides.

We found that splenocytes isolated from Hcit-immunized mice showed a 2-fold increase in proliferation in response to CD3 stimulation compared with those from nonimmunized or Cit-immunized mice. In contrast, mice immunized with the Cit-peptide produced low levels of Abs. Specificity studies showed that the IgG Abs produced by the Hcit-immunized (cyclic) mice bound exclusively to the Hcit-peptides and not to Cit-peptide (E). We decided to analyze the specificity of these anti-Hcit Abs further, using plates coated with peptides A–E. The anti-Hcit Abs cross-reacted to some extent with peptides C and D (Table II). Despite the fact that mice immunized with peptides A and D produced high levels of Abs that cross-reacted with the Hcit-peptide, they showed no morphological signs of arthritis following intra-articular injections of the Cit-peptide (Table I). Immunization with Cit-peptide resulted in low Ab titers that had almost no cross-reactivity with any of the peptides studied (Table II).

### Table II. Ab production following immunization with filagrin-derived peptides and their cross-reactivity in solid phase

<table>
<thead>
<tr>
<th>Plate Coating</th>
<th>Lys Lineal (A)</th>
<th>Hcit, Cyclic (B)</th>
<th>Hcit, Lineal (C)</th>
<th>Lys Cyclic (D)</th>
<th>Cit, Cyclic (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><strong>0.514</strong></td>
<td>0.075</td>
<td>0.112</td>
<td>0.617</td>
<td>0.086</td>
</tr>
<tr>
<td>B</td>
<td>0.187</td>
<td><strong>0.522</strong></td>
<td>0.386</td>
<td>0.048</td>
<td>0.039</td>
</tr>
<tr>
<td>C</td>
<td>0.094</td>
<td>0.149</td>
<td><strong>0.471</strong></td>
<td>0.057</td>
<td>0.054</td>
</tr>
<tr>
<td>D</td>
<td>0.755</td>
<td>0.145</td>
<td>0.231</td>
<td><strong>0.574</strong></td>
<td>0.074</td>
</tr>
<tr>
<td>E</td>
<td>0.096</td>
<td>0.041</td>
<td>n.a.</td>
<td>0.037</td>
<td><strong>0.228</strong></td>
</tr>
</tbody>
</table>

Bold font indicates level of specific Ab.

n.a., not available.
mice. Additionally, IL-10 and -17 and IFN-γ levels were significantly higher in the culture supernatants of splenocytes isolated from Hcit-immunized mice. Phenotypic analysis of spleen cells from Hcit-immunized mice migrating along a CXCL13 gradient showed a high proportion of CD4+ cells (Th lymphocytes) compared with those from Cit-immunized and nonimmunized mice, whereas the numbers of transmigrated CD8+ (cytotoxic T lymphocytes) and CD19+ cells (B cells) were similar. IL-10 and IFN-γ are cytokines produced by CD4+ T cells and are known to play a pivotal role in Ag presentation (38–40).

We went on to investigate the pathogenic significance of Hcit-immunization in a murine model of experimental arthritis. The results of these experiments showed that the injection of Hcit-peptide into nonimmunized mice gave rise to moderate arthritis; this was not the case for Cit-peptide. Surprisingly, intra-articular injection of Cit-modified peptides into Hcit-immunized mice resulted in the greatest arthritis index and severe erosive arthritis. Immunization with Hcit-peptides, followed by intra-articular injection of Cit-peptides, triggered a severe erosive arthritis in >90% of mice. In contrast, Cit-immunization followed by the intra-articular injection of Cit-peptide had no arthritogenic effects. This predisposition to Cit-induced arthritis was independent of the structure (linear or cyclic) of the Hcit peptide and supports the role of carbamylation in the induction of this disease.

Our model of arthritis shows a clear MHC class II dependency, being most severe in BALB/c mice (H-2^d haplotype), whereas C57BL/6 mice (H-2^a haplotype) are resistant. This strong association of Hcit-triggered arthritis with MHC class II emphasizes the clinical relevance of our model to human RA, because RA susceptibility in humans is known to be strongly associated with HLA-DRB1 (14).

We have also provided evidence for the pivotal role of Hcit-sensitized T cells in the etiology of Cit-induced arthritis. Immunostaining of the Cit-injected joints of Hcit-immunized mice showed high numbers of CD3+ cells. In comparison, we found no T cell infiltration of the synovia following Cit-immunization. To further confirm the role of T cells in the development of disease, we performed the adoptive transfer of T cells from Hcit-immunized mice into unmanipulated BALB/c mice. Our results consistently showed that the arthritis index of the Cit-injected knees was significantly higher in recipients of Hcit-immunized mice compared with recipients of unstimulated CD3+ cells. In line with the histological findings, recipients of the Hcit-immunized CD3+ cells exhibited greater T cell proliferation rates and IFN-γ production, mimicking the characteristics found in mice directly immunized with Hcit-containing peptides.

Hcit-immunization led to relatively strong Ab responses, whereas immunization with the Cit-peptide did not. Evaluation of Hcit-Ab specificity showed binding to the linear Hcit-peptide, as well as the linear and cyclic forms of the Lys-containing peptides. However, despite high Ab titers, immunization with the unmodified Lys-containing peptides did not result in arthritis. With the help of Ag-specific T cells, B cells can produce isotype-switched Abs with high specificity to Ags (41–43). The adoptive-transfer experiments showed that the immunization of mice generated T cells with the ability to stimulate B cells for production of Hcit-specific Abs. Low cross-reactivity between the Abs induced by immunization with Hcit or Cit and the monoclonal anti-Cit Ab (ACC3) was detected. Taken together, these results show the importance of carbamylation in the initiation of autoimmune responses.

To rule out the possibility that immune complexes may be responsible for the development of arthritis in our model, we injected Hcit-Ab/Cit-modified peptide immune complexes into the joints of naïve mice. Our results suggested that immune complex formation between Hcit-Ab/Cit-peptide is not the major mechanism responsible for arthritis in Cit-injected joints. Similarly, the intraperitoneal

**FIGURE 5.** Quantitative evaluation of carbamylated (Hcit) and citrullinated (Cit) peptides in proteins recovered from blood and synovial fluid of patients with RA. A, Quantitative evaluation of Hcit and Cit proteins recovered from blood and synovial fluid was done by mass spectrophotometry and expressed as absolute concentrations (μmol). Hcit Ab levels are presented as OD at 450 nm with 1:100 serum dilution. Levels of Hcit-Abs in serum and synovial fluid were measured using ELISA. Patient material was stratified according to radiological findings into erosive, nonerosive, and control groups. The erosive group has higher levels of Hcit-Ab compared with controls (p = 0.019) and the nonerosive group (p = 0.024). B, Levels of Hcit-peptides measured by mass spectrometry in proteins recovered from blood are increased in erosive and aCCP RA patients compared with erosive and aCCP patients and the control group (p < 0.05). C, Cit-peptide levels in blood of RA patients with erosive changes are higher compared with the control group but not within the nonerosive group. D, Hcit-Abs in synovial fluid of erosive RA patients are higher that in patients with nonerosive RA (p < 0.05). E, Absolute levels of Hcit-peptides are identical among the groups. F, Cit-peptide levels in synovial fluid are higher than in control group, regardless of aCCP status of RA patients. Blood and synovial fluid samples were collected from RA patients (n = 72) and controls with knee trauma (n = 41) and osteoarthritis (n = 40). Values are presented as mean ± SEM. Horizontal lines indicate the median.
transfer of Hcit-specific Ig prior to intra-articular injection of the Cit-peptide was not sufficient to trigger arthritis.

We have also provided evidence supporting the importance of Cit-peptides, rather than anti-Cit Abs, in the pathogenesis of arthritis. Cit-peptides are highly arthritogenic in animals that have been exposed to peptide/proteins containing carboxamylated Lys residues (Hcit-peptide). In agreement with previous reports (44, 45), we showed that injection of Cit-peptides into the joints of naive mice failed to induce arthritis and that immunization with Cit-peptide elicited only a modest Ab response. To exclude any potential bias related to the synthetic origin of the peptides used in the study, we conducted experiments using carboxamylated endogenous mAlb (Hcit-mAlb) and exogenous BSA (Hcit-BSA) as immunizing agents. Immunization with Hcit-mAlb and Hcit-BSA resulted in levels of T cell activation similar to those seen with the Hcit-peptide and predisposed the animals to Cit-induced arthritis. Thus, taken together, our experimental data support the role of carboxamylated Lys residues within peptides as the trigger for a chain reaction that results in an increased sensitivity to Cit-peptide at the site of its accumulation. To test our hypothesis, this sequence of events was challenged in a human model. Quantitative evaluation of carboxamylated and citrullinated residues in blood and synovial fluid-recovered proteins, as well as levels of specific IgG against these PTMs, was done in patients with RA. Increased levels of Hcit-peptides and Cit-peptides characterized patients with RA and were associated with an erosive course of arthritis. Although levels of Cit-peptides were found, irrespectively of the Cit-Ab presence, it was our experimental data that support the role of carboxamylated Lys residues within Cit-peptides as the trigger for a chain reaction that results in an increased sensitivity to Cit-peptide at the site of its accumulation.

References


