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The Epitopes Targeted by the Rheumatoid Arthritis-Associated Antifilaggrin Autoantibodies are Posttranslationally Generated on Various Sites of (Pro)Filaggrin by Deimination of Arginine Residues

Elisabeth Girbal-Neuhauser,* Jean-Jacques Durieux,* Michel Arnaud,† Pascal Dalbon,† Mireille Sebbag,* Christian Vincent,* Michel Simon,* Tatsuo Senshu,‡ Christine Masson-Bessière,* Colette Jolivet-Reynaud,† Michel Jolivet,† and Guy Serre3*

Antifilaggrin autoantibodies (AFA) are a population of IgG autoantibodies associated to rheumatoid arthritis (RA), which includes the so-called “antikeratin” Abs and antiperinuclear factor. AFA are the most specific serological markers of RA. We previously showed that they recognize human epidermal filaggrin and other profilaggrin-related proteins of various epithelial tissues. Here, we report further characterization of the protein Ags and epitopes targeted by AFA. All the Ags that exhibit numerous neutral/acidic isolectric variants were immunochromatically demonstrated to be deiminated proteins. In vitro deimination of a recombinant human filaggrin by a peptidylarginine deiminase generated AFA epitopes on the protein. Moreover, two of three filaggrin-derived synthetic peptides with a citrulline in the central position were specifically and widely recognized by AFA affinity-purified from a series of RA sera. These results indicate that citrulline residues are constitutive of the AFA epitopes, but only in the context of specific amino acid sequences of filaggrin. In competition experiments, the two peptides abolished the AFA reactivity of RA sera, showing that they present major AFA epitopes. These data should help in the identification of a putative deiminated AFA-inducing specific amino acid sequences of filaggrin. They could also open the way toward specific immunosuppressive and/or preventive therapy of RA.

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§ Department of R & D Immunossays, bioMérieux, Marcy l’Étoile, France;
* Abbreviations used in this paper: AFA, antifilaggrin autoantibodies; RA, rheumatoid arthritis; APF, antiperinuclear factor; AKA, antikeratin Abs; pI, isoelectric point; GST, glutathione-S-transferase; MBP, myelin basic protein; MS, multiple sclerosis; IEF, isoelectrofocusing; NEpHGE, nonequilibrium pH gel electrophoresis; PAD, peptidylarginine deiminase; AHF, anti-human filaggrin.

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show that citrulline is constitutive of AFA epitopes, but only
nized by AFA purified from a panel of RA sera only when their
lational modification that generates the epitopes recognized by
proteins. Moreover, we demonstrate that in vitro deimination of a
(pro)filaggrins and show that they correspond to deiminated pro-
phorylation of serine residues (30, 31) and/or deimination of basic
charge of (pro)filaggrins could be involved: incomplete dephos-
hypothesis. According to the current model of profilaggrin pro-
filaggrin unit consensus sequence (24) were recognized by AFA-
synthetic peptides, 14–19 aa in length, encompassing an entire
basic filaggrin. This strongly suggested that the epitopes targeted
deficit anti-serum to modified citrulline was produced by injection of deiminated and chemically modified calf thymus histones. An IgG fraction was ob-
tained by ammonium precipitation and gel filtration chromatography of the anti-serum. Then, IgG specific to modified citrulline were purified by af-
finity-chromatography using a modified citrulline-Cellulofine column pre-
pared as previously described (33).

Protein extraction
Normal human breast epidermis was cleaved from dermis by heat treat-
ment and sequentially extracted as previously described (29). Briefly, hu-
man epidermis was homogenized on ice in a low-salt 40 mM Tris-HCl
buffer, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet
P-40, and 0.1 mM PMSF. The lysate was centrifuged at 15,000 g for 15
min at 4°C and the supernatant (“low-salt extract”) was kept at 4°C. In
cluded, the immunoprecipitated pellets were treated with 20°C until used.

Materials and Methods
Human sera and Abs
Human sera were obtained from healthy blood donors (control sera) and
from patients with definite RA according to the criteria of the American Rheumatism Association (1). Their AKA titer and reactivity to neutral/
acidic human epidermal filaggrin were evaluated by indirect immunoflu-
orescence and immunoblotting, respectively (12, 14). All the control sera
and some RA sera were AFA-negative. Other RA sera were selected for
their high titer of AFA. AFA were purified from 45 high-titered RA sera by
finity chromatography on the neutral/acidic human epidermal filaggrin as
previously described (29). Briefly, 1 ml of each serum diluted to 1:2 in PBS
were loaded onto a 5-ml N-hydroxysuccinimide Hi Trap column (Pharma-
cia, Uppsala, Sweden) coupled with 3 ml of neutral/acidic filaggrin ex-
tacted and purified as previously described (23, 29). After a 3-h incubation
at room temperature, the column was washed with 5 volumes of 1 M NaCl,
10 mM phosphate buffer, pH 7.4, then with 5 volumes of PBS. Bound Abs
were eluted with 0.2 M glycine-HCl, pH 2.5, and immediately neutralized by
the addition of 2 M Tris. A fraction of each sample of purified AFA
(samples 1–45) was stored at −80°C until used. In addition, equal volume
fractions of the 45 samples were pooled, then further purified and concen-
trated onto a protein G affinity column (Hi Trap G, Pharmacia) because
they were faintly contaminated by human serum albumin. The pool of AFA
(AFAp) was stored at −80°C until used.

The mAbs AHF (anti-human filaggrin) 1, 2, 4, and 6 belong to a series of murine mAbs, produced and characterized in our laboratory, which are
directed to (pro)filaggrins. They recognize four different epitopes borne by
the various forms of the protein (32). As previously described (33), a rabbit
anti-serum to modified citrulline was produced by injection of deiminated and chemically modified calf thymus histones. An IgG fraction was ob-
tained by ammonium precipitation and gel filtration chromatography of the anti-serum. Then, IgG specific to modified citrulline were purified by af-
finity-chromatography using a modified citrulline-Cellulofine column pre-
pared as previously described (33).

Production of a human recombinant filaggrin
The coding sequence of a single repeat unit of human filaggrin was
amplified from human genomic DNA extracted from Raji cells using a set of
two synthetic oligonucleotides: forward, 5’-TTCCTATACCCAGCCTAG
CACTCATG-3’; reverse, 5’-AGACCCCTGAAGCAGGACGTTGAG

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Table I. Amino acid sequence of the recombinant human filaggrin*  

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>6</th>
<th>7</th>
<th>8</th>
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<td>MSPIL</td>
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<td>PTRLLLEYE</td>
<td>EKYEEHLYER</td>
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<td>PYIYDDGDKL</td>
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<td>ADKHMMLGC</td>
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<td>PRKAEISL</td>
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<td>LPMLENKRMD</td>
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<td>RLCYHTKLY</td>
<td>DHTHPGMEL</td>
<td>YOALDLYVN</td>
<td>DPCMLDAFPK</td>
<td>LVCFPKRLEA</td>
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</tbody>
</table>

* The sequence of the whole fusion protein encoded by the plM163 clone, deduced from nucleotide sequence (GenBank accession number AF043380), tandemly includes GST (italic), a cleavage site for Factor Xa protease (°), and the filaggrin unit (2-323). In the filaggrin unit, by comparison to the previously published consensus sequence (24), the NH2-terminal and COOH-terminal aa are lacking. The positions of the COOH-terminal aa of the eight polypeptides generated by site-specific proteolytic cleavage of the filagrin unit, approximately localized according to the molecular mass of the peptides estimated by SDS-PAGE (Fig. 3), are underlined (bold-type numbers 2–9). Standard one-letter code is used for aa and, in the filagrin sequence, arginines are bold-typed.
deprotection of the last coupled aa-fluorenylmethoxycarbonyl group as previously described (36). The integrity and identity of the peptides were checked by aa analysis and mass spectrometry. The peptides were at least 90% pure, as evaluated by reverse-phase HPLC.

Peptide ELISA

**Binding of AFA.** Synthetic peptides were diluted to 5 μg/ml in PBS, pH 7.4, and 96-well MaxiSorp microtiteration plates (Nunc, Roskilde, Denmark) were coated by an overnight incubation at 4°C with the solutions. After blocking for 30 min at 37°C in PBS containing 0.05% Tween 20 (PBST) and 2.5% teleostean gelatin (Sigma), AFA individually purified from 12 RA sera (samples 1–12) and the pool of AFA purified from 45 RA sera (AFAp) were diluted to 10 μg of protein/ml in PBST containing 0.5% teleostean gelatin and incubated for 1 h at 37°C then overnight at 4°C. After washing in PBSTG, complexed IgG were visualized with peroxidase activity was revealed by 2 mg/ml orthophenylene diamine dihydrochloride, H2O2 3% in 35 mM trisodium citrate, and 40 mM Na2HPO4 at a pH adjusted to 4. The plates were coated with NeutrAvidin (Pierce, Rockford, IL) at 5 μg/ml in PBS and incubated overnight at 4°C then 1 h at 37°C. After washing in PBST, the biotinylated peptides diluted to 0.1 μg/ml in PBS were incubated for 1 h at 37°C. Blocking was performed for 30 min at 37°C in 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, and 0.1% Tween 20 (TBST), and 1/10 (v/v) casein-based blocking buffer (Genosys, Cambridgehire, U.K.). After washing in TBST, a serial dilution of the competing peptide was added or not to RA sera diluted 1/400 in PBST containing 0.125% teleostean gelatin and incubated for 1 h at 37°C then overnight at 4°C. The other steps were as described above.

**Results**

The human epidermal Ags defined by AFA are deiminated forms of (pro)filaggrins

The various (pro)filaggrins extracted from human epidermis, using a sequential procedure, were immunodetected after two-dimensional NEpHGE/SDS electrophoresis with AHF1, a mAb specific for profilaggrin and the various isoforms of filaggrin, with AFA-positive RA sera, and with purified IgG specific for modified citrulline (Fig. 1).

As shown in previous work (28), in the “low-salt extract” (Fig. 1A), both AHF1 and the RA sera detected the comma-shaped 37- to 40-kDa neutral/acidic isoform of filaggrin. Among the other molecular forms of (pro)filaggrin extracted in a second step with a high urea concentration (Fig. 1B), AHF1 detected three groups of molecules: 1) the acidic profilaggrin, >200 kDa, 2) the 37-kDa basic isoform of filaggrin, and 3) 40- to 200-kDa intermediates between profilaggrin and filaggrin. Among these three forms, the RA sera neither labeled the acidic profilaggrin nor the most basic variants of the 37-kD basic filaggrin but only recognized the 40–200-kDa intermediates and the most acidic variants of the 37-kD basic filaggrin (Fig. 1B). In the “low-salt extract,” the Ab to citrulline labeled the 37- to 40-kDa neutral/acidic isoform of filaggrin targeted by the RA sera (Fig. 1A). Similarly, in the “urea extract,”

Table II. Filaggrin-derived synthetic peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequencea</th>
<th>Positionb (homology with consensus’ filaggrin)c</th>
<th>Positionc (homology with recombinant filaggrin)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12DE12Dcit</td>
<td>ESSRDGRGcitHRPSHD</td>
<td>108(57), 113(36), 184(86)</td>
<td>22(36), 108(57), 113(36), 184(71), 237(50)</td>
</tr>
<tr>
<td>T12E/T12Ecit</td>
<td>TGGSTTGGGcitGSHHE</td>
<td>18(93), 53(86)</td>
<td>18(79), 53(79), 311(36)</td>
</tr>
<tr>
<td>E12HE12Hcit</td>
<td>EQSADSSScitHSSGSH</td>
<td>31(64), 108(79), 125(57), 204(64), 246(86)</td>
<td>31(57), 108(79), 125(50), 204(57)</td>
</tr>
</tbody>
</table>

a Standard one-letter code is used for aa residues except for citrulline, which is abbreviated as cit. The central pentapeptides chosen as potential epitopes are bold typed.

b The various positions of the 14-aa peptides on the consensus sequence of filaggrin and on the filaggrin unit encoded by the pBM163 clone are indicated by the number of d.

c The consensus sequence of filaggrin was determined from all the various published sequences of genomic DNA and cDNA clones (24, 35) and from that of the pBM163 clone (Table I).

d At each position, the percentage of homology with the synthesized peptide is shown in parentheses.

FIGURE 1. The epidermal (pro)filaggrins defined by AFA are deiminated proteins. After sequential extraction, proteins of a “low-salt extract” (A) and of a “urea extract” (B) obtained from human epidermis were separated by NEpHGE in the first dimension and by SDS-PAGE in the second dimension. After transfer onto nitrocellulose membranes, immunoblotting analysis was performed either directly with AHF1, a mAb specific for the various molecular forms of (pro)filaggrin, and a representative RA serum with a high titer of AFA or indirectly after previous incubation of the membrane in a citrulline modification medium, with an Ab to modified citrulline (AMC). P1 and molecular mass of immunodetected proteins were approximately estimated using a set of appropriate markers.

A

B

AHF1

RA

AMC

SDS

NEpHGE

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Deimination generates the AFA-targeted epitopes on recombinant human filaggrin

The recombinant filaggrin produced in *E. coli* as a fusion protein with GST consisted of a series of nine polypeptides from 28 kDa to 66 kDa (Fig. 3C). The molecular mass of polypeptide 1 (66 kDa) and 9 (28 kDa) corresponded to the expected molecular mass of the whole fusion protein and of the GST protein alone, respectively. The mAbs AHF2, 4, and 6, directed to three different epitopes of (pro)filaggrin, exhibited a variable reactivity toward the recombinant polypeptides. AHF2 recognized polypeptides 1–8 and not polypeptide 9 (AHF2 panel). AHF4 and AHF6 recognized polypeptides 1–7 and 1–3, respectively (not shown). The reactivity of AHF2, 4, and 6 confirmed that polypeptides 1–8 all bear one to three filaggrin epitopes and thus contain a filaggrin portion. Because affinity purification of the fusion protein required the integrity of the GST part, it is highly probable that the polypeptides are degradation products of the fusion protein generated in *E. coli* by site-specific proteolytic cleavages of the filaggrin part and that polypeptide 9 almost entirely corresponds to GST (Table I).

Recombinant GST-filaggrin was deiminated using rabbit skeletal muscle PAD and the kinetics of deimination was assessed by immunoblotting with the Ab to citrulline. After a 5-min deimination, citrulline residues were fairly detectable, but their labeling regularly increased over the 1-h period analyzed (AMC panel, lanes 4–7). As expected (34, 37), the deiminated polypeptides exhibited a modified SDS-PAGE mobility and therefore appeared as increasingly diffuse bands with apparent molecular mass increasing up to 97 kDa for the longest incubation times. Polypeptides 1–5 progressively became highly reactive, polypeptides 6 and 7 remained weakly reactive, and polypeptides 8 and 9 were unreactive. When a PAD inhibitor was added (AMC panel, lane 8), no immunoreactivity was observed. Whatever the length of incubation, AHF2 detected the quantitatively predominant undeiminated forms of polypeptides 1–8. This showed both that no polypeptide degradation occurred during the enzymatic treatment and that deimination concerned only a subtraction of each polypeptide (AHF2...
Recognition of deiminated recombinant filaggrin by human sera is related to their AFA activity. Human recombinant GST-filaggrin was incubated for 1 h at 50°C with 7.5 U/mg protein of PAD, separated by SDS-PAGE, and analyzed by immunoblotting with AHF2 (lane 1), a pool of AFA affinity-purified from 45 RA sera (lane 2, AFAp), RA sera with high titers of AFA (lanes 3–12), AFA-negative RA sera (lanes 13–15), or control sera (lanes 16–18).

The RA sera recognized neither the untreated recombinant protein (RA panel, lane 3) nor the protein incubated with PAD in the presence of its inhibitor (RA panel, lane 8). They only labeled the deiminated protein (RA panel, lanes 4–7). After a 5-min incubation, intense reactivities were observed at 66 kDa and above and at near 41–45 kDa. These zones correspond to polypeptide 1 and polypeptides 2 and 3, respectively. Slight reactivity was observed at 35 and 40 kDa (RA panel, lanes 4–7) i.e., on polypeptides 4 and 5. The reactivity of deiminated polypeptides 1–3 was slightly reinforced after 15 min or 30 min and strongly reinforced after 1 h of incubation. At this time, the reactivity pattern of the RA sera became partly similar to that of the Ab to citrulline, with the same 66- to 97-kDa and 41- to 60-kDa strongly immunoreactive zones corresponding to deiminated polypeptide 1 and to deiminated polypeptides 2 and 3, respectively, but only a slightly reactive zone at 35–40 kDa corresponding to deiminated polypeptides 4 and 5 and no reactivity to deiminated polypeptides 6 and 7.

Interestingly, although deimination of BSA was clearly demonstrated to be efficient (AMC panel, lanes 1 and 2), the faint reactivity of the RA sera to this protein remained unchanged after deimination (RA panel, lanes 1 and 2).

Taken together, these results demonstrate that citrulline residues are necessary to generate the epitopes recognized by AFA. However, the mere presence of citrulline is not sufficient; indeed, the epitopes appeared on some deiminated regions of filaggrin (borne by polypeptides 1 to 3) but not on others (borne by polypeptides 6 and 7) and not on BSA. Therefore, particular sequences in filaggrin, around certain citrulline residues, are necessary to generate the epitopes.

Recognition of deiminated recombinant filaggrin is specific to AFA-positive RA sera

The reactivity to the deiminated GST-filaggrin of 50 sera from RA patients and 20 from healthy donors (control sera) and of a pool of AFA, affinity-purified from the sera of 45 RA patients, was tested by immunoblotting. A subgroup of sera is shown in Fig. 4. The unmodified recombinant protein was not detected by any of the sera (not shown). Conversely, after deimination, the protein became highly reactive both with the pool of AFA and with all the AFA-positive RA sera, but was unreactive with the AFA-negative sera both from RA patients and from healthy donors (compare lanes 3–12 and 13–18). Although all the AFA-positive RA sera detected the entire GST-filaggrin in the 66- to 97-kDa zone (polypeptide 1), their affinity toward the first five polypeptides was variable from one serum to another. Polypeptides in the 41- to 60-kDa zone (polypeptides 2 and 3) were detected by all the sera but with variable intensities (compare lanes 5 and 6) and polypeptides in the 35- to 40-kDa zone (polypeptides 4 and 5) were detected by only 6 of 12 sera (lanes 3, 5, 7, 8, 10, and 12). No sera detected polypeptides 6, 7, and 8 (lanes 3–12).

Therefore, recognition of the deiminated recombinant filaggrin was clearly specific to the AFA-positive RA sera. Their recognition pattern, considered as a whole, was similar to that obtained with the pool of purified AFA (lane 2), demonstrating that their reactivity clearly results from their AFA activity.

Two of the three citrulline-substituted filaggrin-derived synthetic peptides are recognized by affinity-purified AFA

To characterize the citrulline-bearing epitopes defined by AFA, three filaggrin-derived sequences were chosen to synthesize 14-aa peptides (Table II). In the synthesized peptides, each sequence presented a central arginine residue that was either left as such or substituted by a citrulline residue. The reactivity to the peptides of 12 samples of AFA purified from the sera of 12 RA patients (samples 1–12) and of the pool of AFA purified from the sera of 45 RA patients (AFAp) was tested by ELISA (Table III). Neither the samples nor the pool of purified AFA significantly recognized the unsubstituted peptides. By contrast, the pool was highly reactive to the peptides E12Dcit and E12Hcit but not to T12Ecit. Among the 12 AFA samples, 8 recognized E12Dcit, 9 E12Hcit, and none T12Ecit. In most cases, the reactivity toward E12Dcit was higher than that toward E12Hcit. Only one sample of AFA recognized E12Dcit and not E12Hcit, and only two of the 12 did not recognize any of the citrulline-substituted peptides. Globally 10 of 12 AFA samples recognized E12Dcit and/or E12Hcit. These results confirmed that citrulline residues are constitutive of AFA epitopes and that only particular sequences of filaggrin can generate the epitopes. They suggested that E12Dcit and E12Hcit bear major AFA epitopes. The ELISA reactivities of RA sera to E12Dcit and E12Hcit were proved to be specific because they were specifically inhibited by E12Dcit and E12Hcit, respectively, when analyzed with the various peptides (citrulline-substituted or not) as competitors (Fig. 5). Moreover, in competition assays, cross-inhibitions of various degrees from a serum to another were obtained with the two peptides E12Dcit and E12Hcit. This indicated that in RA sera several subsets of AFA with different specificities coexist, some of them being cross-reactive with E12Dcit and E12Hcit.
The two citrulline-substituted filaggrin-derived synthetic peptides E12Dcit and E12Hcit bear major AFA epitopes

Inhibition of the immunoblotting reactivity of RA sera to deiminated recombinant GST-filaggrin and to partially purified neutral/acidic epidermal filaggrin by the various citrulline-substituted peptides was analyzed (Fig. 6). With a first high-titered RA serum (RA1), the reactivities to the two antigens were largely decreased when using E12Dcit or E12Hcit as competitors and entirely abolished when the two peptides were used simultaneously. With a second RA serum (RA2), each of the two peptides allowed complete inhibition of the reactivity to the two Ags. The peptide T12Ecit did not significantly modify the reactivity of the sera. These results clearly demonstrated that E12Dcit and E12Hcit bear major AFA epitopes.

Discussion

In the first part of this work, we reported the physicochemical and biochemical characteristics of the (pro)filaggrin autoantigens recognized by RA sera in human epidermis. RA sera only recognized a neutral/acidic subset of the low-salt soluble and urea-soluble (pro)filaggrins. The use of an Ab specific for citrulline and thus able to recognize the deiminated proteins by the various citrulline-substituted peptides was analyzed (Fig. 6). With a first high-titered RA serum (RA1), the reactivities to the two antigens were largely decreased when using E12Dcit or E12Hcit as competitors and entirely abolished when the two peptides were used simultaneously. With a second RA serum (RA2), each of the two peptides allowed complete inhibition of the reactivity to the two Ags. The peptide T12Ecit did not significantly modify the reactivity of the sera. These results clearly demonstrated that E12Dcit and E12Hcit bear major AFA epitopes.
4 and on the lowest molecular mass polypeptides 5, 6, and 7. Lastly, the sequences are absent from polypeptide 8. This good concordance between the results obtained on the deiminated recombinant polypeptides and on the citrulline-substituted synthetic peptides reinforces the proposition that the AFA epitopes borne by E12Dcit and E12Hcit are immunodominant epitopes and suggests that in the region 1–88 of filaggrin such epitopes are very poorly represented. The immunodominant character of the epitopes was further confirmed because the two peptides abolished the reactivity of RA sera to deiminated recombinant filaggrin. Still more convincing was the similar complete inhibition of their reactivity to epidermal neutral/acidic filaggrin, which clearly demonstrated that E12Dcit and E12Hcit are major epitopes. AFA are probably largely cross-reactive with most of these epitopes and perhaps also with the epitopes borne by E12Dcit and E12Hcit. Future competition experiments will verify this hypothesis. Because 12 filaggrin subunits exhibiting 10–39% heterogeneity in the amino acid sequence are expressed in epidermis, all of them bearing hundreds of potential citrulline-containing epitopes, it is probable that each RA serum contains specific populations of AFA more or less cross-reactive with various subsets of related epitopes, E12Dcit and E12Hcit peptides clearly present such epitopes. Modeling of the various molecular motifs recognized by AFA is a future challenge. However, it is already sure that deiminated recombinant filaggrin and/or citrulline-substituted peptides will permit the development of highly sensitive and specific assays for the detection of AFA (40). Given the presence of the highly specific AFA in early RA (21), at a time when the clinical arguments alone do not permit the diagnosis of the disease (41), and given the recently confirmed efficiency of starting RA-specific therapy early (42), these assays will constitute a major clinical tool.

As mentioned earlier, numerous clinical arguments suggest that AFA could be involved in the pathophysiology of RA. However, deiminated epidermal (pro)filaggrins cannot be considered as the autoantigen that drives the antifilaggrin response because epidermis is not a target of rheumatoid inflammation and (pro)filaggrins are not considered to be expressed by articular tissues. Moreover, we demonstrated in RA patients that the ratio of AFA to total IgG is higher in extracts of synovial membranes than in the serum or synovial fluid, and that AFA are produced in vitro by plasmocytes of the rheumatoid pannus (C.M.-B. et al., manuscript in preparation). Therefore, it is possible that an AFA-inducing or, at least, cross-reacting Ag is present in the synovial joints of patients with RA. Because PADs were shown to be widely distributed enzymes present not only in keratinocytes (38), but also in many other mammalian cell types (43, 44) like skeletal muscle (45), brain (46), and hemopoietic (47) cells, the presence of a PAD in human synovial membrane and cartilage, and particularly in rheumatoid pannus, can be reasonably suspected. We are now searching for both PAD and AFA-specific deiminated autoantigen(s) in the synovial joints of RA patients. Identification of such deiminated articular Ags might help to elucidate the mechanism of AFA production and would provide new insights into the pathogenesis of RA.

Interestingly, the presence of citrulline residues was described in human myelin basic protein (MBP), a multiple sclerosis (MS) autoantigen (48, 49). An increased proportion of deiminated isoforms of the protein was found both in MS patients (50) and in mice transgenic for the myelin proteolipid gene, recently proposed as a model of MS (51). Because the level of PAD was also significantly increased in the brain of the mice (51), the deimination of MBP probably results from PAD activity (46). Deimination of the protein was proposed as an early event resulting in unstable myelin assemblies and thus producing the initial autoantigenic material (50, 51). Although not disease-specific, anti-MBP autoantibodies are strongly associated with the activity of MS (52). An immunodominant epitope for these autoantibodies was localized to residues 82–100 of MBP (53), but deiminated MBP was never analyzed as an autoantigen. It would be of great interest to test MS
sera on citrulline-substituted MBP peptides. One can speculate that certain similar pathophysiological events, involving PAD, a putative deiminated articular autoantigen and AFA, could occur in RA.

Together, these data about MS and our work raise the question of the biological significance of deimination and its possible involvement in autoimmune responses. It was shown that deimination generates major modifications in the protein structures, contributing to protein unfolding (37, 46). We can also wonder about the physiological consequences of the posttranslational deimination of proteins and whether these contribute to the breakdown of immune tolerance to self-Ags and antigenic structures. More generally, the posttranslational modification of self-proteins could be frequently involved in the generation of autoantigens because protein phoshorylation has also recently been hypothesized to be an important event in the production of autoantibodies found in patients with lupus erythematosus (54).

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