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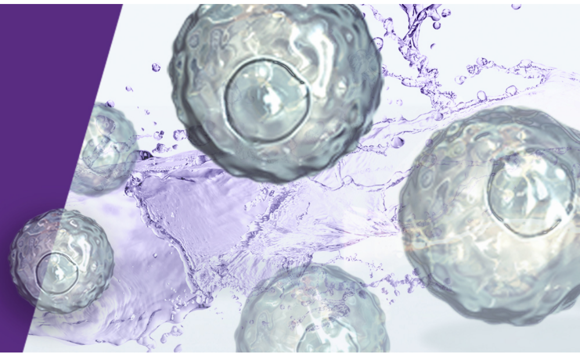

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The Epitopes Targeted by the Rheumatoid Arthritis-Associated Antifilaggrin Autoantibodies are Posttranslationally Generated on Various Sites of (Pro)Filaggrin by Deimination of Arginine Residues[1,2]

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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 isoelectric variants were immunochemically 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 or cross-reactive articular autoantigen and provide new insights into the pathogenesis of RA. They could also open the way toward specific immunosuppressive and/or preventive therapy of RA. *The Journal of Immunology,* **1999, 162: 585–594.**

Rheumatoid arthritis (RA)[4] is the most frequent of human systemic autoimmune diseases. It is characterized by the formation, in synovial membranes, of an inflammatory and invasive tissue, the rheumatoid pannus, that leads to the destruction of synovial joints. Both cellular and humoral autoimmune mechanisms have been implicated in its still poorly understood pathogenesis. The presence of circulating IgM rheumatoid factor in RA patients is widely considered as a diagnosis criterion (1). Nevertheless, a large variety of autoantibodies have been described in the sera of these patients, such as antiperinuclear factor (APF) (2), "antikeratin" (AKA) (3), anticollagen (4), antinuclear (5), anti-Sa (6), anticalpastatin (7, 8) Abs, and also autoantibodies directed against chondrocyte (9) or synovial membrane (10) proteins. Among these Abs, including rheumatoid factor, AKA have been largely demonstrated to be the most specific serological marker of RA because their diagnosis specificity was reported to be from 95 to 100% (11–14). They are increasingly recognized as being a major diagnosis tool.

AKA were originally described by Young et al. (3) as IgG labeling, by indirect immunofluorescence, the cornified layer (stratum corneum) of rat esophagus epithelium. They were shown to be genuine autoantibodies that also label the stratum corneum of human epidermis (15–18). AKA are possibly involved in the pathophysiology of RA because 1) they are highly specific for the disease, 2) they are associated to the more active and severe forms (11, 19), 3) they may appear at very early stages (20) and even before the clinical symptoms (21), and 4) their ratio to global IgG is increased in synovial membranes with regard to the serum or the synovial fluid, and they are synthesized by plasmocytes of the rheumatoid pannus (C.M.-B. et al., manuscript in preparation).

Identification of the Ag(s) and more particularly of the targeted epitope(s) could allow elucidation of the mechanism of AKA production and provide more insights into the pathophysiology of RA. In this prospect, we first identified the rat esophagus epithelium Ags recognized by AKA as three late-differentiation proteins of 210 kDa, 120–90 kDa, and 130–60 kDa exhibiting a large charge

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[1] This work was presented at the VIIth and VIIIth annual meetings of the Association pour la Recherche sur la Polyarthrite held in Paris on October 1996 and 1997. It subsequently appeared in abstract form (see Refs. 55 and 56).

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[4] 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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heterogeneity with isoelectric point (pI) ranging mainly from 4.5 to 7.2 and clearly different from cytokeratins (13, 22). In human epidermis, AKA recognize a 37- to 40-kDa protein that also exhibits numerous pI variants (pI from 5.8 to 7.4) (23). We demonstrated that this autoantigen is a neutral/acidic isoform of filaggrin, a well-known cytokeratin filament aggregating protein of epidermis (23). During the final steps of epidermal differentiation, filaggrin is synthesized as a high molecular mass precursor (>200 kDa), profilaggrin, stored in cytoplasmic dense bodies termed keratohyalin granules. Profilaggrin is an acidic phosphorylated protein, consisting of 10 to 12 tandemly repeated filaggrin units separated by linker peptides and exhibiting large sequence heterogeneity, because 10–39% of their 324 amino-acids (aa) can vary from one unit to another (24). During cornification, profilaggrin is dephosphorylated and hence becomes accessible to specific proteases that release functional cationic 37-kDa filaggrin units (for a review, see Ref. 25). These basic filaggrin units are secondarily processed to give acidic variants by extensive conversion of their arginine residues into citrulline residues. This conversion is probably mediated by a peptidylarginine deiminase (PAD) (26, 27). Interestingly, AKA did not recognize epidermal profilaggrin by immunoblotting (18).

To better define the molecular forms of (pro)filaggrins (this term including the precursor and its various posttranslationally modified products) recognized by AKA, we studied their expression in human epidermal keratinocytes cultured in differentiating conditions (28). They consisted of the 37- to 40-kDa neutral/acidic isoform of filaggrin and additional neutral/acidic proteins of higher molecular mass (40–200 kDa). We also characterized the autoantigen targeted by the IgG APF. This autoantigen is located in perinuclear granules of superficial human buccal mucosa cells. It was identified as a low-salt soluble 200- to 400-kDa protein, closely related to, but nevertheless different from, epidermal profilaggrin (29). We showed that this protein is also an autoantigen targeted by AKA. Thus, APF and AKA, which were previously considered as two different RA-associated Abs, were demonstrated to be largely the same autoantibodies that we proposed to name antifilaggrin autoantibodies (AFA) (29).

Therefore, all the AKA and APF Ags identified in the various epithelial tissues were related to (pro)filaggrins and exhibited a neutral/acidic pI but differed both from epidermal profilaggrin and basic filaggrin. This strongly suggested that the epitopes targeted by AFA are generated by a posttranslational modification of profilaggrin and/or filaggrin. The fact that none of 33 overlapping synthetic peptides, 14–19 aa in length, encompassing an entire filaggrin unit consensus sequence (24) were recognized by AFA-positive RA sera (our unpublished observations) reinforced this hypothesis. According to the current model of profilaggrin processing (25), two metabolic modifications able to influence the charge of (pro)filaggrins could be involved: incomplete dephosphorylation of serine residues (30, 31) and/or deimination of basic arginine residues to give neutral citrullines (26, 27).

In the present study, we report further physicochemical and biochemical characterization of the AFA-targeted epithelial (pro)filaggrins and show that they correspond to deiminated proteins. Moreover, we demonstrate that in vitro deimination of a recombinant human filaggrin by PAD is an absolute prerequisite to its recognition by AFA, showing that deimination is the posttranslational modification that generates the epitopes recognized by these autoantibodies. Finally, we show that, among three 14-aa filaggrin-derived synthetic peptides, two are specifically recognized by AFA purified from a panel of RA sera only when their central arginine residue is substituted with a citrulline. Thus, we show that citrulline is constitutive of AFA epitopes, but only within specific filaggrin sequences, and we identify two peptides that bear major AFA epitopes.

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 immunofluorescence 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 affinity 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 (Pharmacia, Uppsala, Sweden) coupled with 3 mg of neutral/acidic filaggrin extracted 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 concentrated 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 obtained by ammonium precipitation and gel filtration chromatography of the anti-serum. Then, IgG specific to modified citrulline were purified by affinity-chromatography using a modified citrulline-Cellulofine column prepared as previously described (33).

Protein extraction

Normal human breast epidermis was cleaved from dermis by heat treatment and sequentially extracted as previously described (29). Briefly, human 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. The pellet underwent the same treatment twice. Then, it was homogenized in ice-cold 50 mM Tris-HCl, pH 7.6, 6 M urea, and 0.1 mM PMSF. The suspension was centrifuged at 15,000 × *g* for 15 min at 4°C and the supernatant (“urea extract”) collected. Proteins of the “low-salt extract” were precipitated by ethanol and redissolved in distilled water. As previously reported (23), this method gives rise to an extract enriched in the neutral/acidic isoform of epidermal filaggrin.

Normal human keratinocytes were cultured in differentiating conditions as previously described (28). The stratified and cornified epithelial sheet was detached by scraping, homogenized on ice in the low-salt buffer mentioned above, and the extracted proteins recovered in the supernatant after centrifugation.

Rat esophagus epithelium was obtained from 6-wk-old male Wistar rats (Iffa-Credo, Lyon, France). After cleavage from the underlying connective tissue by heat treatment, antigenic proteins were extracted by homogenization in an ice-cold low-salt 40 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 10 mM EDTA, 0.1 mM PMSF, and 0.1% sodium azide and were recovered in the supernatant after centrifugation, as previously described (22).

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′-TTCCTATACCAGGTGAG CACTCATG-3′; reverse, 5′-AGACCCTGAACGTCCAGACCGTCCC-3′. The PCR products were blunt-ended, cloned into the *Sma*I site of pUC 19, and transformed into *Escherichia coli* DH5α cells (Life Technologies, Cergy-Pontoise, France). Clones were screened for the presence of inserts, and one clone was selected, sequenced, and subcloned in frame in the *Eco*RI-*Hin*dIII sites of the pGEX vector (Pharmacia), which allows expression of glutathione-*S*-transferase (GST) fusion proteins. The resulting clone, pBM163, allowed the production of a GST-filaggrin fusion protein

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

		MSPIL	*GYWKIKGLVQ*	*PTRLLLEYLE*	*EKYEEHLYER*
	DEGDKWRNKK	*FELGLEFPNL*	*PYYIDGDVKL*	*TQSMAIIRYI*	*ADKHNMLGGC*
	PKERAEISML	*EGAVLDIRYG*	*VSRIAYSKDF*	*ETLKVDFLSK*	*LPEMLKMFED*
	RLCHKTYLNG	*DHVTHPDFML*	*YDALDVVLYM*	*DPMCLDAFPK*	*LVCFKKRIEA*
				****	
	IPQIDKYLKS	*SKYIAWPLQG*	*WQATFGGGDH*	*PPKSDLIEGR*	*DPRSEFEL*GT
					9
2	LYQVSTHEQS	ESTHGQTAPS	TGG**R**QGS**R**HE	QA**R**NSS**R**HSA	SQDGQDNI**R**G
		8			**7**
52	HPGSS**R**GG**R**Q	GSYHEQSVD**R**	SGHSGYHHSH	TTPQG**R**SDAS	HGQSGP**R**SAS
		6	**5**	**4**	
102	**R**QT**R**NEEQSG	DGS**R**HSGS**R**H	HEAST**R**AGSS	**R**HSQVGQGES	AGSKTS**RR**QG
					3
152	SSVSQD**R**DSE	GHSEDSE**RR**S	ESAS**R**NHYGS	S**R**EQS**R**HGS**R**	NP**R**SHQED**R**A
		2			
202	SHGHSAESS**R**	QSGT**R**HAETS	SGGQAASSQE	QA**R**SSPGE**R**H	GS**R**HQQSADS
252	STDSGTG**RR**Q	DSSVVGDSGN	**R**GSSGSQASD	SEGHSEESDT	QSVSAHGQAG
302	PHQQSHQEFT	**R**GQSGG**R**SG**R**	SG		

[a] The sequence of the whole fusion protein encoded by the pBM163 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 NH_2-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 filaggrin unit, approximately localized according to the molecular mass of the peptides estimated by SDS-PAGE (Fig. 3, *lanes C*), are underlined (bold-type numbers 2–9). Standard one-letter code is used for aa and, in the filaggrin sequence, arginines are bold-typed.

(Table I) by stimulation of a mid-log phase culture of transformed DH5α cells with 0.2 mM isopropyl thio-β-galactoside for 3 h at 37°C. After induction, the cells were pelleted by centrifugation, resuspended in 1/10 of the initial volume in lysis buffer (1 M Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, and 1mM PMSF), and submitted twice to a 30-s ultrasonic treatment at 4°C. Insoluble material was eliminated by centrifugation and the soluble GST-filaggrin was purified on a glutathione-Sepharose column according to the manufacturer's instructions (Pharmacia). The eluted fraction was analyzed by SDS-PAGE. Surprisingly, before and after the purification procedure the fusion protein consisted of numerous polypeptides. After purification, nine polypeptides from 28 to 66 kDa were identified (see Fig. 3). This pattern was repeatedly observed even when protease inhibitors were added to the extraction and purification buffers.

Protein deimination

Purified PAD from rabbit skeletal muscle was purchased from Takara Biomedicals (Shiga, Japan). BSA (Pierce, Rockford, IL) and the recombinant GST-filaggrin were incubated at 0.75 mg/ml with PAD (7.5 U/mg protein) in 0.1 M Tris-HCl, pH 7.4, 10 mM $CaCl_2$, and 5 mM DTT with or without 10 mM of the PAD inhibitor *N*-ethylmaleimide (Sigma, St. Louis, MO), for 5 min, 15 min, 30 min, or 1 h at 50°C. Deimination was stopped by addition of 2% SDS and incubation for 3 min at 100°C.

Electrophoreses

One-dimensional electrophoresis. The recombinant GST-filaggrin and the partially purified neutral/acidic epidermal filaggrin (23) were separated by SDS-PAGE using PhastSystem (Pharmacia) on precast 12.5% and 8–25% polyacrylamide gels, respectively. Low range molecular mass protein markers from Bio-Rad Laboratories (Richmond, CA) and high molecular mass markers from Pharmacia were used as references.

Two-dimensional electrophoreses: first dimension. Proteins of the low-salt buffer extracts from human epidermis and cultured keratinocytes and from rat esophagus epithelium were precipitated by ethanol and redissolved in distilled water with 0.01% bromphenol blue. They were separated by nonequilibrium pH gel electrophoresis (NEpHGE)—epidermis—or by isoelectrofocusing (IEF)—cultured keratinocytes and rat esophagus epithelium—using precast PhastGels with ampholytes generating a 3–9 pH gradient. Proteins of the "urea extract"—epidermis—were precipitated by acetone and redissolved in 50 mM Tris-HCl, pH 7.6, 8 M urea, and 0.01% bromphenol blue then separated by NEpHGE using IEF PhastGels that were previously washed, dried, and rehydrated in 6 M urea, 0.5% Nonidet P-40, and ampholytes generating a 3–9 pH gradient (28).

Two-dimensional electrophoreses: second dimension. SDS-PAGE—epidermis and cultured keratinocytes—or nondenaturing PAGE—rat esophagus epithelium—were performed with precast 8–25% polyacrylamide gels.

The IEF calibration kit standards from Pharmacia, the two-dimensional calibration kit standards from Bio-Rad, and the high molecular mass markers from Pharmacia were used as references.

Immunoblotting

After electrophoresis, proteins were electrotransferred onto reinforced nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). They were probed with human RA sera, control sera, or a pool of 5 RA sera all diluted to 1/2000, with the pool of AFA purified from 45 RA sera (AFAp) diluted to 4 μg/ml or with the AHF mAbs diluted to 0.1 μg/ml. Human and murine IgG were detected with peroxidase-conjugated goat Abs to human IgG (Southern Biotechnology, Birmingham, AL) or with peroxidase-conjugated rabbit $F(ab')_2$ to mouse IgG (Biosys, Compiègne, France), respectively. Peroxidase activity was visualized using ECL Western blotting reagents (Amersham International, Aylesbury, U.K.) following the procedure suggested by the manufacturer. On some nitrocellulose membranes, after electrotransfer, the citrulline residues were modified as previously described (34). Briefly, membranes were incubated overnight at 37°C in a modification medium containing 0.0125% $FeCl_3$, 0.25% diacetyl monoxime (Prolabo, Paris, France), 0.125% antipyrine (Fluka, Buchs, Switzerland), 12.5% H_2SO_4, and 8.5% H_3PO_4. The membranes were then probed with the rabbit IgG to modified citrulline diluted to 0.125 μg/ml and then with peroxidase-conjugated goat $F(ab')_2$ to rabbit IgG (Biosys) and ECL reagents.

For competition assays, nitrocellulose strips blotted either with deiminated recombinant GST-filaggrin (0.2 μg/lane) or with partially purified neutral/acidic epidermal filaggrin (0.2 μg/lane) were incubated overnight at 4°C with RA sera diluted to 1/4000 and containing or not the competing peptides at 40 μg/ml. Human IgG were then detected as described above.

Synthetic peptides

For peptide selection, we first identified all the pentapeptides centered by an arginine residue, which are present in the aa sequences of human filaggrin units deduced from all the various published sequences of genomic DNA and cDNA clones (24, 35) and from that of the pBM163 clone described above (Table I). The frequency of each pentapeptide was evaluated, then reevaluated after grouping all the arginine-centered 80% homologous pentapeptides. Among the most frequent pentapeptides, three that were encoded by the pBM163 clone were chosen. Three 14-aa peptides consisting of the chosen pentapeptide, preceded and followed by the most frequent aa encountered at each of the five positions upstream and four positions downstream, were synthesized (Table II). Each peptide was synthesized with an arginine as the central residue (E12D, T12E, and E12H) and, in a substituted form, with a citrulline as the central residue (E12Dcit, T12Ecit, and E12Hcit). Synthesis was performed using fluorenylmethoxycarbonyl and t-butyl protecting groups and trifluoroacetic acid deprotection. Biotinylation of peptides was selectively performed at the N terminus after

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Table II. *Filaggrin-derived synthetic peptides*

Peptide	Sequence[a]	Position[b] (homology with consensus[c] filaggrin[d])	Position[c] (homology with recombinant filaggrin[d])
E12D/E12Dcit	ESSRD**GS<u>R/cit</u>HP**RSHD	108(57), 113(36), 184(86)	22(36), 108(57), 113(36), 184(71), 237(50)
T12E/T12Ecit	TGSST**GG<u>R/cit</u>QG**SHHE	18(93), 53(86)	18(79), 53(79), 311(36)
E12H/E12Hcit	EQSAD**SS<u>R/cit</u>HS**GSGH	31(64), 108(79), 125(57), 204(64), 246(86)	31(57), 108(79), 125(50), 204(57)

[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 the first aa.
[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.

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 microtitration 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 (PBSTG) and incubated for 1 h at 37°C then overnight at 4°C. After washing in PBSTG, complexed IgG were visualized with peroxidase-conjugated goat Abs to human IgG (Southern Biotechnology) diluted to 1/2000 and incubated for 1 h at 37°C. Peroxidase activity was revealed by 2 mg/ml orthophenylene diamine dihydrochloride, H_2O_2 3×10^{-2}% in 35 mM trisodium citrate, and 40 mM Na_2HPO_4 at a pH adjusted to 5 with orthophosphoric acid. The reaction was stopped by 1.7 M H_2SO_4 and OD were read at 492 nm. All the samples were tested at least two times in duplicate and the results averaged. The specific reactivity to the citrulline-substituted peptides was taken as the difference between the OD obtained with the substituted peptide and that obtained with the related, unsubstituted peptide. Only differences above 0.1 were considered positive.
Competition assays. 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, Cambridgeshire, U.K.). After washing in TBST, a serial dilution of the competing peptide was added or not to RA sera diluted to 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. 1*A*), 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. 1*B*), 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. 1*B*). 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. 1*A*). Similarly, in the "urea extract,"

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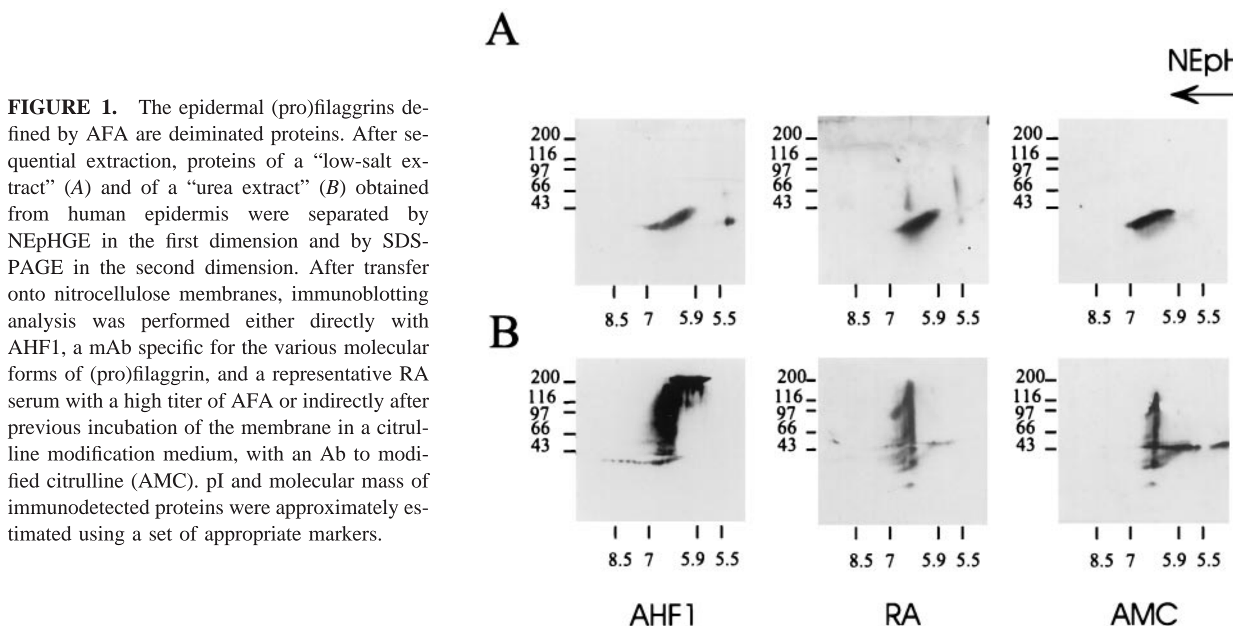

FIGURE 1. The epidermal (pro)filaggrins defined by AFA are deiminated proteins. After sequential extraction, proteins of a "low-salt extract" (*A*) and of a "urea extract" (*B*) obtained from human epidermis were separated by NEpHGE in the first dimension and by SDS-PAGE in the second dimension. After 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). pI and molecular mass of immunodetected proteins were approximately estimated using a set of appropriate markers.

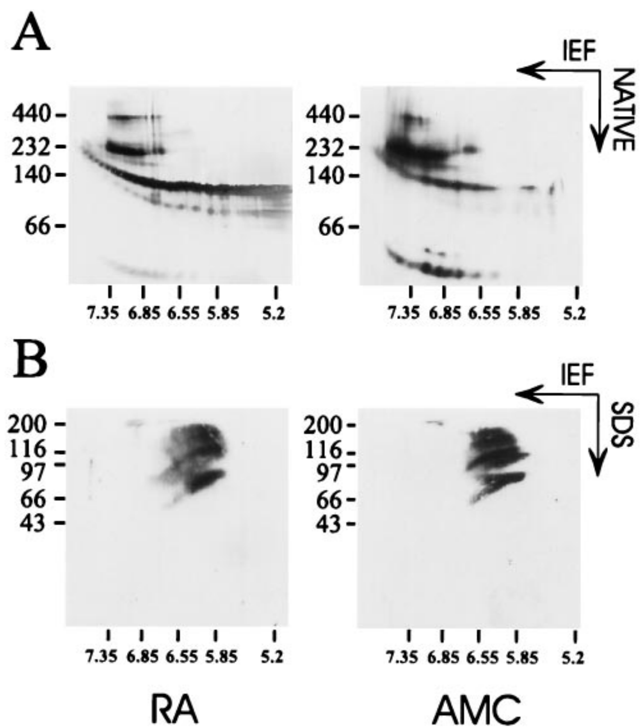

FIGURE 2. The Ags defined by AFA in rat esophagus epithelium and human cultured epidermis are also deiminated proteins. Proteins extracted in low-salt buffers from rat esophagus epithelium (*A*) and cultured human epidermis (*B*) were separated by IEF in the first dimension and by nondenaturing (NATIVE)-PAGE or SDS-PAGE in the second dimension, respectively. After transfer onto nitrocellulose membranes, immunoblotting analysis was performed either directly with a representative RA serum showing 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). pI and molecular mass of immunodetected proteins were approximately evaluated using a set of appropriate markers.

the Ab to citrulline precisely recognized the whole subset of neutral/acidic (pro)filaggrins in the same way as RA sera (Fig. 1*B*).

Thus, the low-salt soluble and urea-soluble neutral/acidic (pro)filaggrins from human epidermis that bear the epitopes targeted by AFA are all deiminated proteins.

The various other epithelial Ags defined by AFA are also deiminated proteins

The low-salt soluble AFA Ags from rat esophagus epithelium and human cultured keratinocytes were separated by two-dimensional electrophoresis and immunodetected with AFA-positive RA sera and with the Ab to citrulline (Fig. 2). The three AFA-defined rat esophagus epithelial Ags of 210 kDa, 120–90 kDa and 130–60 kDa appearing after separation in native conditions as forms with a molecular mass of 440 kDa, 232 kDa, and between 140 and 67 kDa, respectively, were clearly shown to be deiminated proteins (Fig. 2*A*). Similar results were obtained with the AFA-defined (pro)filaggrins extracted from cultured keratinocytes because all these autoantigens were also labeled by the Ab to citrulline (Fig. 2*B*).

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. 3*C*). 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)filaggrins, 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 subfraction of each polypeptide (*AHF2*

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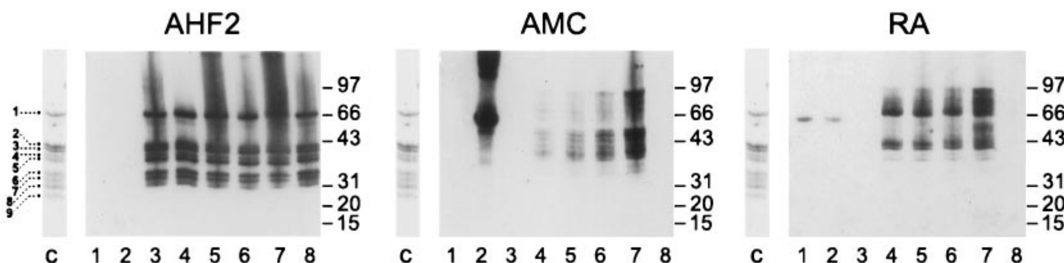

FIGURE 3. Deimination of human recombinant filaggrin induces its recognition by AFA. Human recombinant GST-filaggrin was incubated at 50°C with PAD (7.5 U/mg protein) for different periods of time: 5 min (*lane 4*), 15 min (*lane 5*), 30 min (*lane 6*), 1 h (*lane 7*), or without PAD for 1 h (*lane 3*). *Lane 8* corresponds to recombinant GST-filaggrin incubated with PAD for 1 h at 50°C in the presence of 10 mM *N*-ethylmaleimide, a PAD inhibitor. As a control, BSA was incubated at 50°C for 1 h without (*lane 1*) or with PAD (*lane 2*) at 7.5 U/mg protein. Proteins were then separated by SDS-PAGE. Coomassie blue staining of the polyacrylamide gel (C) allows visualization of the various untreated affinity-purified recombinant GST-filaggrin polypeptides (*dots 1 to 9*). After electrotransfer onto nitrocellulose membrane, GST-filaggrin and BSA, deiminated or not, were probed either with the Ab to modified citrulline (AMC) after previous incubation of the membrane in the citrulline modification medium or directly with AHF2, a mAb specific for (pro)filaggrins, and a pool of five RA sera with a high titer of AFA.

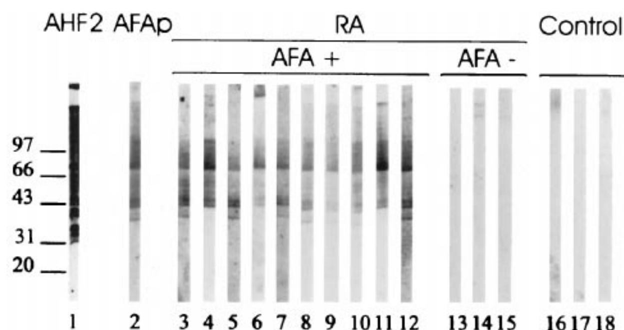

FIGURE 4. Recognition of deiminated human recombinant filaggrin by human sera is related to their AFA activity. Human recombinant GST-filaggrin was incubated for 1 h at 50°C with 7.5 U/mg protein of PAD, separated by SDS-PAGE, and analyzed by immunoblotting with AHF2 (*lane 1*), a pool of AFA affinity-purified 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*).

Table III. *ELISA reactivity to the citrulline-substituted synthetic peptides of AFA purified from sera of RA patients*[a]

Purified AFA	E12Dcit	T12Ecit	E12Hcit
AFAp	460	0	1566
1	201	0	1219
2	111	0	154
3	864	0	0
4	0	0	131
5	0	0	0
6	376	0	1786
7	150	0	717
8	903	0	1073
9	0	0	0
10	174	0	1069
11	0	0	102
12	190	0	1725

[a] The values shown are the differences between OD ($\times 10^3$) on citrulline-substituted and unsubstituted peptides. Only differences above 100 were considered positive. AFAp, pool of AFA purified from 45 different sera; 1–12, AFA purified from 12 different sera.

panel). 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 E12Hcit was higher than that toward E12Dcit. 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.

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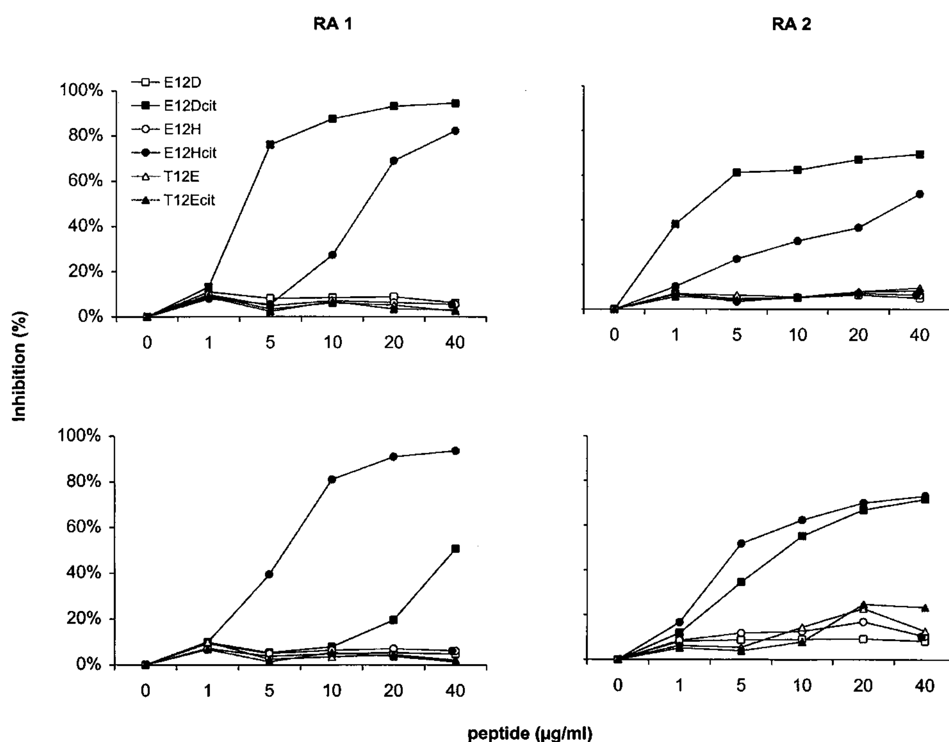

FIGURE 5. In some RA sera, AFA are cross-reactive with the two citrulline-substituted peptides E12Dcit and E12Hcit. The peptides with an arginine (E12D, E12H, T12E) or a citrulline (E12Dcit, E12Hcit, T12Ecit) as the central residue were added at various concentrations (0, 1, 5, 10, 20, and 40 μg/ml) during incubation of two RA sera (RA1 and RA2) assayed by ELISA on either the peptide E12Dcit (*upper panels*) or the peptide E12Hcit (*lower panels*). Competition was plotted as the percentage of OD inhibition, the OD obtained without peptide added being considered as 100% as the reference.

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 showed that all the molecules targeted by AFA are deiminated. Moreover, superimposable results were obtained with the other AFA-targeted epithelial Ags, namely the rat esophagus epithelium Ags and the (pro)filaggrin molecular forms expressed by cultured keratinocytes. This close colocalization of citrulline and AFA epitopes on the same molecular forms of the proteins strongly suggested the involvement of deimination in the formation of the epitopes. Biochemical evidence was reported for physiological deimination of human epidermal filaggrin (26, 27) and the Ab to modified citrulline used in this work previously indicated that deiminated proteins are localized in the stratum corneum of human epidermis (27). This deimination is very probably catalyzed by PAD, because the enzyme was reported to be highly expressed in human epidermis (38).

In the second part of this study, we demonstrated that AFA epitopes are generated in vitro by deimination of a human recombinant filaggrin. AFA-positive RA sera and purified AFA specifically recognized the deiminated protein while they were unreactive on its native form. These data clearly establish that citrulline residues are necessary for the recognition of (pro)filaggrins by AFA. This was further confirmed by the reactivity of the two citrulline-substituted filaggrin-derived synthetic peptides E12Dcit and E12Hcit that were specifically recognized by a large majority of the samples of affinity-purified AFA, whereas the unsubstituted related peptides E12D and E12H were unreactive. RA sera and purified AFA recognized not only the deiminated whole recombinant GST-filaggrin but also some lower molecular mass polypeptides resulting from its cleavage. Variability in the recognition of the various deiminated polypeptides, from one serum to another, further illustrates the known interindividual heterogeneity in the specificity of AFA. Nevertheless, the three highest molecular mass polypeptides were recognized with a high avidity by all the RA sera, suggesting the presence of one or several immunodominant regions on the polypeptides. The importance of the amino acids neighboring citrullines was confirmed by the fact that the control protein BSA, which contains ~4% arginine residues, did not present any AFA epitopes after deimination and that the citrulline-substituted peptide T12Ecit was unreactive. According to their molecular mass, the differences between polypeptide 1 vs 2, 3, and 4 approximately corresponds to the filaggrin regions 169–323, 144–323, and 88–323, which are lacking in polypeptides 2, 3, and 4, respectively (Table I). Interestingly, the region 88–323 encompasses most of the 14-aa peptides sharing substantial homology with E12D and E12H (Table II). Indeed, sequences homologous to E12D are present five times on polypeptide 1 and three times on polypeptides 2 and 3. Similarly, sequences homologous to E12H are present four times on polypeptide 1 and three times on polypeptides 2 and 3. By contrast, only one sequence homologous to E12H and one homologous to E12D are present on polypeptides

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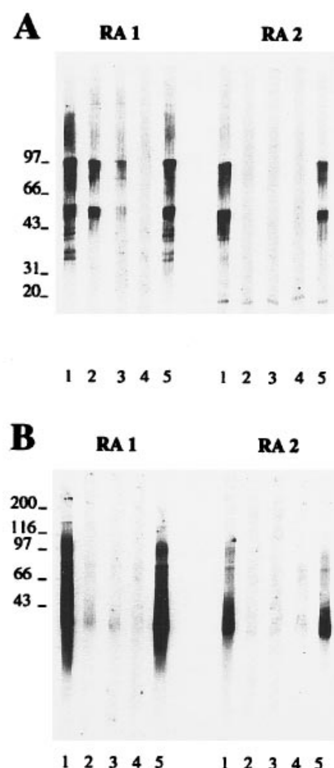

FIGURE 6. Peptides E12Dcit and/or E12Hcit abolish the AFA activity of RA sera. Nitrocellulose strips blotted with deiminated recombinant filaggrin (*A*) or with partially purified neutral/acidic epidermal filaggrin (*B*) were tested with two RA sera (RA1 and RA2) in absence (*lane 1*) or presence of the citrulline-substituted peptides E12Dcit (*lane 2*), E12Hcit (*lane 3*), E12Dcit and E12Hcit (*lane 4*), and T12Ecit (*lane 5*) at a concentration of 40 μg/ml.

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 bear the major AFA epitopes recognized by the sera. Among the 12 samples of purified AFA tested, a majority recognized both E12Dcit and E12Hcit. Because the two peptides show 50% homology and share the central tripeptide, -ser-cit-his-, one could suppose that the two AFA epitopes were targeted by a same mono- or oligo-clonal population of cross-reactive AFA. The existence of such subsets of cross-reactive AFA was confirmed in several sera by competition experiments. Nevertheless, this is not true for all patients because in one of them purified AFA recognized only E12Dcit with a high avidity. Obviously, in the two patients whose purified AFA recognized none of the peptides, AFA are directed against different epitopes.

Recently the reactivity of RA sera to such filaggrin-derived citrulline-substituted peptides was reported (39). The tested peptides were chosen in four distinct regions (aa 12–34, aa 48–65, aa 260–276, and aa 306–324) of published filaggrin subunit sequences (24, 35), and the region bearing immunodominant epitopes was identified as the extreme COOH-terminal part of filaggrin. This is compatible with our results obtained with RA sera on deiminated recombinant filaggrin even if the aa sequences of the reported peptides and our recombinant protein are not exactly superimposable. Comparison of RA sera reactivity toward various substituted peptides in that 306–324 filaggrin region showed the central tripeptides -thr-cit-gly-, -gly-cit-ser-, and -ser-cit-gly-, like the central tripeptide, -ser-cit-his-, we identified here, are constituents of 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 some 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

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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 phosphorylation 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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