Citrulline is an Essential Constituent of Antigenic Determinants Recognized by Rheumatoid Arthritis–specific Autoantibodies

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Abstract

Only a few autoantibodies that are more or less specific for RA have been described so far. The rheumatoid factor most often tested for is not very specific for RA, while the more specific antiperinuclear factor for several reasons is not routinely used as a serological parameter. Here we show that autoantibodies reactive with synthetic peptides containing the unusual amino acid citrulline, a posttranslationally modified arginine residue, are specifically present in the sera of RA patients. Using several citrulline-containing peptide variants in ELISA, antibodies could be detected in 76% of RA sera with a specificity of 96%. Sera showed a remarkable variety in the reactivity pattern towards different citrulline-containing peptides. Affinity-purified antibodies were shown to be positive in the immunofluorescence-based antiperinuclear factor test, and in the so-called antikeratin antibody test, and were reactive towards filaggrin extracted from human epidermis. The specific nature of these antibodies and the presence of these antibodies early in disease, even before other disease manifestations occur, are indicative for a possible role of citrulline-containing epitopes in the pathogenesis of RA. (*J. Clin. Invest.* 1998. 101:273–281.) Key words: antiperinuclear factor • antikeratin antibodies • rheumatoid factor • autoantigen • autoimmunity • filaggrin

Introduction

RA is a systemic autoimmune disease of unknown etiology. The disease is characterized by chronic inflammation of the joints eventually resulting in tissue degradation and joint deformation that can lead to severe disability. RA is the most common of the inflammatory joint diseases, affecting 1–2% of the world population.

The diagnosis of RA depends primarily on clinical manifestations of the disease, with only limited serological support. The only serological test routinely used is to determine the presence of rheumatoid factors (RF)[1] in the serum. RF are antibodies directed to the constant region of immunoglobulins of the IgG subclass, and their presence is determined by agglutination assays, nephelometry, or ELISA-based tests. RF can be detected in up to 70–80% of RA patients, but is also detected in relatively high percentages in other autoimmune and infectious diseases, and in up to 15% of healthy individuals (1).

Antibodies of a more specific nature have also been found in sera of RA patients. Such antibodies were first described by Nienhuis en Mandema (2), who discovered that antibodies in RA sera specifically label the perinuclear factor, a component of a number of so-called keratohyaline granules surrounding the nucleus in indirect immunofluorescence (IIF) using buccal mucosa (BM) cells. These antiperinuclear factor (APF) antibodies are reported to be present in 49–91% of RA patients with a specificity between 73 and 99% (3). Not all BM cells donated are suited as an antigenic substrate. A high percentage of APF-positive BM cells can only be found in ~ 5% of the donors. This dependence on availability of suitable donors and the inconvenience of the IIF test format are the main reasons why the APF test is not routinely used.

A third class of antibodies specifically present in RA sera are the so-called antikeratine antibodies (AKA). Their presence is determined by IIF on cryosections of rat esophagus. AKA label the cells of the stratum corneum of rat esophagus epithelium (4). The sensitivity of the AKA test reported varies between 36 and 59%, with a specificity between 88 and 99% (3). Again, the presence of AKA is not routinely tested for because of the inconvenient antigenic substrate and the use of IIF that is regarded as a problem in a routine laboratory setting.

The nature of the antigen recognized by both the AKA and APF antibodies has until recently been unclear. It was shown that the APF and a monoclonal antibody specific for human (pro)filaggrin produce an identical staining pattern in IIF (5). A 40-kD protein extracted from human epidermis, specifically labeled by RA sera in Western blots, was identified as a neutral/acidic isoform of filaggrin (6). Moreover, it was demonstrated that the IgG fraction, affinity-purified from patient sera by using the 40-kD protein, was reactive in both the APF and AKA test, indicating that the AKA and APF antibodies are at least partly the same (7). The immunological relationship between APF and AKA had already been proposed based on the strong correlation of the presence of APF and AKA in patient sera (3). The high specificity of the APF/AKA antibodies and their presence early in disease, often before apparent disease manifestations are noted (8, 9), are indicative for a possible role of these antibodies in the pathogenesis of RA.

Filaggrin (filament-aggregating protein) is produced during the late stages of terminal differentiation of epithelial cells in

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1. *Abbreviations used in this paper:* AKA, antikeratin antibodies; APF, antiperinuclear factor; BM, buccal mucosa; IIF, immunofluorescence; RF, rheumatoid factor.

mammals. It is synthesized as a heavily phosphorylated precursor protein (profilaggrin) that consists of 10–12 filaggrin repeats (10). Profilaggrin is deposited in granules, and filaggrin is released by proteolytic cleavage during differentiation of the cells. During this stage the protein is dephosphorylated, and ~20% of the arginine residues are converted into citrulline by the enzyme peptidylarginine deiminase. The large heterogeneity in amino acid sequence of filaggrin (30–40% of the amino acid residues of the filaggrin repeat units are variable) and the partial citrullination of arginine residues result in an extensive charge heterogeneity (11).

Although the antigen recognized by the APF antibodies in IIF of BM cells has been identified as filaggrin, it remains unclear from where these antibodies present in RA sera originate. Based on the observation that the AKA/APF antibodies are reactive with the acidic/neutral isoform of filaggrin (6), the possibility that citrulline residues might be present in the epitopes was investigated. To study this possibility, several peptides were synthesized in which arginine residues encountered in the cDNA-derived profilaggrin sequence were substituted with citrulline. The prevalence of antibodies against these peptides in RA sera and their relation with the APF/AKA antibodies are investigated in this study. Furthermore, possible mechanisms for the specific occurrence of these antibodies in RA sera are discussed.

Methods

Sera and IgG purification. Sera were collected from the outpatients clinic of the Department of Rheumatology of the University Hospital Nijmegen. During a 3-wk period, a total of 288 sera were collected from patients, of which 134 were diagnosed as RA according to the revised criteria formulated by the American Rheumatology Association (ARA; 12). The remaining 154 sera, hereafter referred to as control sera, were taken from patients with various rheumatic complaints (23 ankylosing spondylitis, 15 systemic sclerosis, 11 psoriatic arthritis, 8 SLE, 7 undifferentiated connective tissue disease, 7 polyarticular arthrosis, 5 juvenile chronic arthritis, 5 polymyalgia rheumatica, 4 mixed connective tissue disease, 4 polymyositis, and 65 miscellaneous rheumatic complaints). Sera were stored at −80°C until use, and were tested for autoantibody activity without prior knowledge of the diagnosis. In addition, 200 sera (50 SLE, 50 systemic sclerosis, 50 dermatomyositis/polymyositis, and 50 primary Sjögren's syndrome) were used to further assess specificity. IgG was purified chromatographically from patient sera using protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) by standard procedures (13).

Peptide synthesis. Peptides were selected for synthesis from amino acid sequences deduced from known cDNA sequences of human profilaggrin (10, 11). All complete human filaggrin sequences found in the SwissProt and EMBL databases were used for a computer analysis of probable antigenic sites. Regions with a high antigenicity index according to Jameson and Wolf (14) and a high probability of containing turns according to secondary structure predictions (15) were identified. Peptides were synthesized by the solid-phase strategy using fmoc chemistry as described previously (16). The peptides were at least 90% pure as deduced from their elution pattern on reversed-phase HPLC and the relative absorption at 214 nm. The identity of the peptides was confirmed by mass spectrometry (matrix-assisted laser desorption/ionization mass spectrometry). Key peptides in this study (cfc1 and cf0; Table I) were resynthesized on a larger scale and purified using preparative reversed-phase HPLC. Purity of these peptides was at least 98%. All peptides were synthesized as peptide amides. Peptides used in this study are listed in Table I.

Immunoblot on epidermal filaggrin. Filaggrin was extracted from human epidermis as described previously (6) and after SDS-PAGE was electroblotted to nitrocellulose (0.5 μg/strip). Blot strips were blocked with 0.5% skimmed milk powder in TBST (50 mM Tris-HCl, pH 7.4; 150 mM NaCl 0.05% Tween-20) and incubated with sera diluted 1:50 in the same buffer for 2 h. After washing, the strips were incubated with anti–human IgG conjugated to alkaline phosphatase (D0336, Dako SA, Glostrup, Denmark) diluted 1:1000 for 1 h. For detection, the strips were developed using NBT/BCIP (Research Organics, Cleveland, OH; 13).

Indirect immunofluorescence. Detection of APF antibodies by IIF on buccal mucosa cells was performed as described (5). The serum dilution used was 1:10. Buccal mucosa cells from a single donor were used throughout this study. Detection of AKA antibodies was performed on cryosections of rat esophagus prepared as described previously (17).

Peptide ELISA. Peptides were coupled covalently to 96-well assay plates (Costar Corp., Cambridge, MA) at 1 μg/well. Coupling was performed at 4°C for 16 h at pH 9.0. For blank controls, wells were coupled with 2 μg BSA/well. All wells were blocked with 2% BSA for 1 h. Sera were diluted 200-fold in RIA (1% BSA, 350 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% [vol/vol] Triton X-100, 0.5% [wt/vol] Na-deoxycholate, 0.1% SDS) supplemented with 10% normal rabbit serum, and incubated for 1.5 h at room temperature. After washing (6 times with PBS/0.05% [vol/vol] Tween-20), 100 μl of anti–human IgG conjugated to peroxidase (P0214, Dako SA) diluted 1:1,000 in RIA was

Table I. Sequence of Synthetic Peptides

Peptide name	Sequence*	Corresponding filaggrin region and substitutions
cf0	SHQESTRGRSRGRSGRSGS	306-324
cfA	SHQESTAGRSRGRSGRSGS	306-324 Arg_{312}→Ala
cfE	SHQESTEGRSRGRSGRSGS	306-324 Arg_{312}→Glu
cfQ	SHQESTQGRSRGRSGRSGS	306-324 Arg_{312}→Gln
cfZ	SHQESTZGRSRGRSGRSGS	306-324 Arg_{312}→Orn
cfc1	SHQESTXGRSRGRSGRSGS	306-324 Arg_{312}→Cit
cfc2	SHQESTRGXSRGRSGRSGS	306-324 Arg_{314}→Cit
cfc3	SHQESTRGRSXGRSGRSGS	306-324 Arg_{316}→Cit
cfc4	SHQESTRGRSRGXSGRSGS	306-324 Arg_{318}→Cit
cfc5	SHQESTRGRSRGRSGXSGS	306-324 Arg_{321}→Cit
cfc6	SHQESTXGXSRGRSGRSGS	306-324 $Arg_{312+314}$→Cit
cfc7	SHQESTXGRSXGRSGRSGS	306-324 $Arg_{312+316}$→Cit
cfc8	SHQESTXGRSRGXSGRSGS	306-324 $Arg_{312+318}$→Cit
cfc9	SHQESTXGRSRGRSGXSGS	306-324 $Arg_{312+321}$→Cit
cfc1-319	SHQESTXGRSRGRS	306-319 Arg_{312}→Cit
cfc1-318	SHQESTXGRSRGR	306-318 Arg_{312}→Cit
cfc1-317	SHQESTXGRSRG	306-317 Arg_{312}→Cit
cfc1-316	SHQESTXGRSR	306-316 Arg_{312}→Cit
cfc1-315	SHQESTXGRS	306-315 Arg_{312}→Cit
cfc1-314	SHQESTXGR	306-314 Arg_{312}→Cit
f12-31	ESSHGWTGPSTRGRQGSRHE	12-31
cf18-31	TGPSTRGRQGSXHE	18-31 Arg_{29}→Cit
cf18-34-1	TGPSTRGRQGSXHEQAQ	18-34 Arg_{29}→Cit
cf18-34-2	TGPSTRGXQGSRHEQAQ	18-34 Arg_{25}→Cit
cf18-34-3	TGPSTXGRQGSRHEQAQ	18-34 Arg_{23}→Cit
cf48-65-1	TIHAHPGSXRGGRHGYHH	48-65 Arg_{56}→Cit
cf48-65-2	TIHAHPGSRXGGRHGYHH	48-65 Arg_{57}→Cit
cf48-65-3	TIHAHPGSRRGGXHGYHH	48-65 Arg_{60}→Cit
cf48-65-4	TIHAHPGSXXGGRHGYHH	48-65 Arg_{56+57}→Cit
cf260-276-1	GQASSAXDRGHRGSRGS	260-276 Arg_{266}→Cit
cf260-276-2	GQASSARDXGHRGSRGS	260-276 Arg_{268}→Cit

*X, citrulline; Z, ornithine; standard one-letter code is used for the other amino acid residues.

added to the wells. After incubation (1 h at room temperature), plates were washed (6 times PBS/Tween-20), and bound antibodies were detected with 3,3′-5,5′- tetramethyl-benzidine (Organon-Teknika, Boxtel, the Netherlands) as a substrate. The reaction (10 min) was stopped by adding 100 μl 2 M sulfuric acid/well. Plates were read at a wavelength of 450 nm. All sera were tested in duplicate, and the results were averaged. Sera resulting in an OD_{450} of 0.2 after subtraction of the blank for the particular serum (well lacking peptide), were considered positive. A control serum was included on all plates to monitor plate-to-plate variation. Variation never exceeded 5%, and values were therefore not corrected.

Competition assay. Peptides were coupled to the plates as described above. Patient sera were used in a dilution resulting in an OD_{450} of 1–2 as determined by an endpoint titration of the serum. A serial dilution of the competing peptide was added to the serum incubations in the ELISA format described. Noncompeting peptides were used as a negative control and the same peptide as that coupled to the plate was used as a positive control. Competition was plotted as percentage inhibition against peptide concentration. The OD_{450} signal of the blank was defined as 100% inhibition, and the signal without peptide added to serum incubation was defined as 0% inhibition. After serum/peptide incubation for 2 h, plates were treated as described above.

Affinity chromatography. For purification of peptide-binding antibodies from RA sera, an affinity matrix was prepared. Peptide (2 mg) was coupled to 0.3 g of CH-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.1 M Na_2HCO_3/NaH_2CO_3 (pH 8.0) containing 0.5 M NaCl. The efficiency of coupling was determined by using a sample of the peptide solution (before coupling) and the supernatant after coupling in the competition assay. Coupling efficiencies were estimated to be at least 90%. Excess reactive groups were blocked by 0.1 M Tris-HCl/0.5 M NaCl. A control column was prepared by blocking the reactive groups with Tris/NaCl using the same amount of resin. Chromatography was carried out at room temperature at a flow rate of 0.2 ml/min. Protein-A–purified IgG, 20 mg in PBST (PBS/0.05% Tween-20) obtained from RA sera, was applied to the column for 2 h. The fraction not bound by the column was saved for analysis. Hereafter, the column was washed extensively with PBST followed by the same buffer supplemented with 0.35 M NaCl. During elution of bound material with 0.1 M glycine-HCl, pH 2.5, fractions of 0.2 ml were collected and immediately neutralized with 10 μl 2 M Tris (unbuffered) and subsequently dialyzed against PBS. Alternatively, a batch-wise procedure was used in which peptide resin was added to purified IgG and incubated for 2 h. After centrifugation the supernatant was saved for analysis, and the resin was washed several times with PBST. The peptide-bound antibodies were eluted from the resin with glycine/HCl, neutralized with 2 M Tris, and dialyzed against PBS. These antibodies were used in blotting procedures and IIF on BM cells or cryosections of rat esophagus.

Results

Peptide synthesis. Regions within the deduced amino acid sequences of human profilaggrins with a high antigenicity index and a large number of residues with high turn probability were identified. The most promising were found in the extreme COOH-terminal regions of the filaggrin subunits (amino acid residues 306–324). The filaggrin sequence that contained the largest number of arginine residues within this region, and therefore the most likely to contain citrulline in the in vivo situation, was selected for peptide synthesis (cf0; Table I). Several peptide variants in which the arginine residues were substituted with citrulline were synthesized (Table I). Peptides derived from other regions with high antigenicity index and a large number of residues with high turn probability (amino acid residues 18–31, 48–65, and 260–276) in which arginine residues were substituted with citrulline were also synthesized (Table I). Again, the sequences were based on the filaggrin subunit that contained the largest number of arginine residues within the particular region.

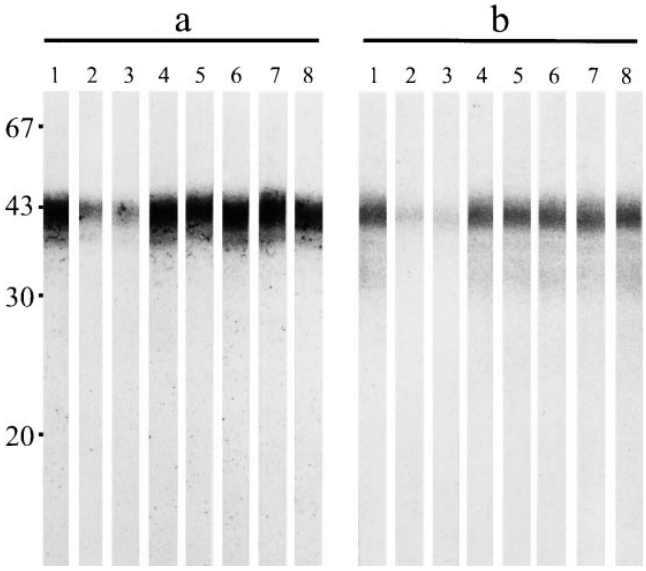

Figure 1. Binding of RA antibodies to human filaggrin is inhibited by citrullinated peptides. Peptide (10 μg/ml) was added during serum incubation on blotstrips containing filaggrin extracted from human epidermis. A strong reactive serum (*a*) and a moderately reactive serum (*b*) diluted 50-fold were incubated in the presence of no peptide, lane *1*; cfc1, lane *2*; a mixture of the peptides cfc1, cfc2, and cfc3, lane *3*; cf0, lane *4*; cfA, lane *5*; cfE, lane *6*; cfQ, lane *7*; and cfZ, lane *8*. Note that filaggrin, due to its charge heterogeneity, migrates as a broad band in SDS-PAGE. The relative migration of molecular size markers in kD is indicated at the left.

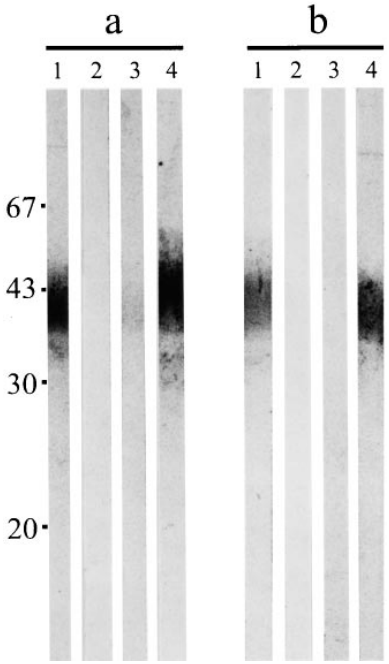

Figure 2. Antibodies purified using a citrullinated peptide coupled to an affinity matrix are reactive with filaggrin. Antibodies were fractionated from an IgG preparation derived from two different RA sera (*a* and *b*) by chromatography. Antibodies eluted from a column prepared by coupling of peptide cfc1 (lane *1*), and a control column (lane *2*) with no peptide coupled were incubated on blotstrips containing filaggrin extracted from human epidermis. As a control, blotstrips were incubated with the fractions not retained by the peptide column (lane *3*) and the control column (lane *4*). The relative migration of molecular size markers in kD is indicated at the left.

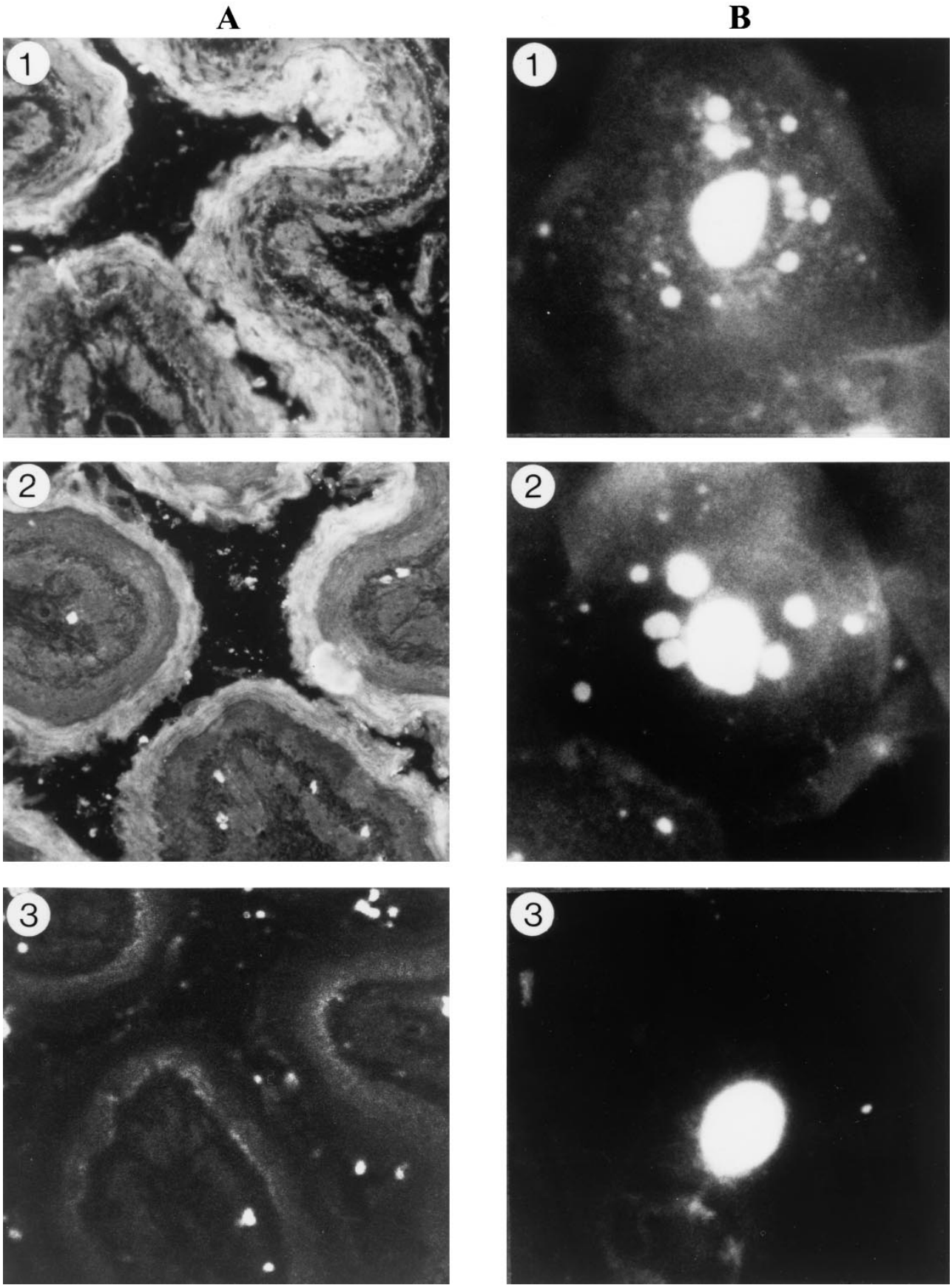

Figure 3. Antibodies purified from a citrullinated peptide coupled to an affinity matrix are reactive towards the stratum corneum of rat esophagus and keratohyalin granules in BM cells. Cryosections of rat esophagus (*A*) and unfixed buccal mucosa cells (*B*) were incubated with (*1*) an AKA and APF-positive serum diluted 1:10, (*2*) IgG antibodies eluted from a column containing peptide cfc1, and (*3*) IgG antibodies eluted from a control column with no peptide coupled. The nuclei of the buccal mucosa cells were counterstained with ethidium bromide.

The citrulline-substituted peptide cfc1 is reactive with the APF/AKA antibodies. Addition of peptide cfc1 (Table I) during incubation of RA sera on immunoblots containing human filaggrin partially inhibited the binding of antibodies to filaggrin (Fig. 1, strips 2). The peptide containing an unmodified arginine at the same position (cf0) did not show any inhibition (Fig. 1, strips 4). Control peptides in which citrulline was substituted by glutamine (cfE), glutamic acid (cfQ), alanine (cfA), or ornithine (cfZ; a hydrolysis product of citrulline) did not inhibit binding (Fig. 1, strips 5–8), suggesting that not the absence of arginine, but the presence of citrulline specifically rendered the peptide autoantigenic. The absence of inhibition with peptide cfZ shows that citrulline itself, and not a hydrolysis product, is responsible for recognition by autoantibodies. A mixture of equimolar amounts of the citrullinated peptides cfc1, cfc2, and cfc3 was an even more potent inhibitor of serum reactivity towards filaggrin (Fig. 1, strip 3). Most RA sera reactive towards filaggrin on blot (about 50% of the sera; data not shown) were inhibited by the addition of cfc1, but the degree varied with the serum used independently of the signal strength of a particular serum on blot.

Antibodies eluted from an affinity column containing cfc1 peptide coupled to the matrix were reactive with filaggrin on blot. No reactive antibodies could be eluted from a control column to which no peptide had been coupled (Fig. 2). These affinity-purified antibodies also gave the characteristic staining pattern on rat esophagus cryocoupes (Fig. 3 *A*) in the AKA-IIF test, and of the keratohyalin granules in BM cells in the APF-IIF assay (Fig. 3 *B*).

To investigate whether the recognition of cfc1 by the autoantibodies could be used in a direct binding assay, RA sera (n = 134) and control sera (n = 154) were tested on peptide cfc1 in ELISA. With a cutoff value of 0.2 (well above the signal of negative sera + 3 × SD), 49 of the RA sera (36%) were reactive with cfc1, while only one of the control sera (< 1%) was positive. The control peptides cf0, cfA, cfE, cfQ, and cfZ were not reactive with RA sera in direct ELISA (data not shown),

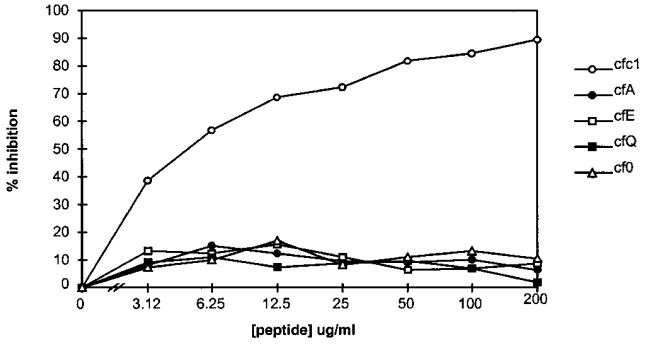

Figure 4. Substitution variants of the citrulline-containing peptide cfc1 do not compete with anti-cfc1 antibody binding in ELISA. Substitution variants cfA (Ala), cfE (Glu), cfQ (Gln), cf0 (Arg, the non-citrullinated peptide) and cfc1 (positive control) were added at different concentrations during serum incubation on ELISA with the citrulline-containing peptide cfc1 coupled to the plates. Relative inhibition was calculated and plotted as a function of peptide concentration on a linear scale between 100% inhibition (OD_{450} of the background signal with no serum added) and 0% inhibition (OD_{450} with no peptide added).

Table II. Reactivity of RA Sera on Shortened Variants of cfc1

Peptide*	No. of positive sera
cfc1	12
cfc1-319	12
cfc1-318	9
cfc1-317	7
cfc1-316	7
cfc1-315	4
cfc1-314	1

*Peptide sequences can be found in Table I.

and were not able to inhibit antibody binding in an ELISA competition assay as is illustrated in Fig. 4.

Reactivity of shortened peptide variants. RA sera (n = 12) reactive with cfc1 were assayed in ELISA on shortened variants of peptide cfc1 (Table I). The peptide missing five COOH-terminal residues (cfc1–319) was still reactive with the same number of sera as peptide cfc1, although in most cases the OD_{450} value was lower. Surprisingly, the sera showed a distinct difference in reactivity pattern towards the shortened variants; for some sera truncation of a peptide by a single residue completely abrogated reactivity, while for others the reactivity did not change (Table II). This fact illustrates that even for a relatively small antigenic substrate, there can be large differences in antibody binding characteristics between individual sera.

Reactivity of variant peptides from the COOH-terminal region. Peptide variants were synthesized in which other arginine residues within the sequence of cf0 were substituted by citrulline (cfc2, cfc3, cfc4, and cfc5; Table I) or in which two arginines were simultaneously substituted by citrulline (cfc6, cfc7, cfc8, and cfc9; Table I). These peptides were all assayed in the ELISA test using RA and control sera. Testing of additional connective tissue disease sera confirms the high specificity of the peptides for antibodies present in RA sera (Table III). Combining the data presented in Table III, a sensitivity of

Table III. Reactivity of Sera with Citrullinated COOH-terminal Peptide Variants

Peptide*	RA sera (%) (n = 134)	Control sera (%) (n = 154)	SLE§ (%) (n = 50)	SSC§ (%) (n = 50)	pSS§ (%) (n = 50)	PM/DM§ (%) (n = 50)
cfc1	49 (36)	1 (0.6)	1 (2)	0	0	0
cfc2	27 (20)	4 (2.6)	1 (2)	0	1 (2)	1
cfc3	37 (28)	2 (0.6)	0	0	1 (2)	1
cfc4	32 (24)	2 (1.3)	0	0	0	0
cfc5	64 (48)	1 (0.6)	0	1 (2)	2 (4)	1
cfc6	65 (48)	1 (0.6)	0	0	2 (4)	1
cfc7	60 (45)	1 (0.6)	0	0	1 (2)	1
cfc8	55 (41)	1 (0.6)	0	0	1 (2)	1
cfc9	57 (42)	1 (0.6)	0	0	2 (4)	0
cfc1-cfc9‡	102 (76)	6 (3.9)	2 (4)	1 (2)	3 (6)	1 (2)
APF-IIF	99 (74)	27 (17)	—	—	—	—

*Peptide sequences can be found in Table I. ‡Sera positive on at least one of the peptides. §SSC, systemic sclerosis; pSS, primary Sjögren's syndrome; PM/DM, polymyositis/dermatomyositis.

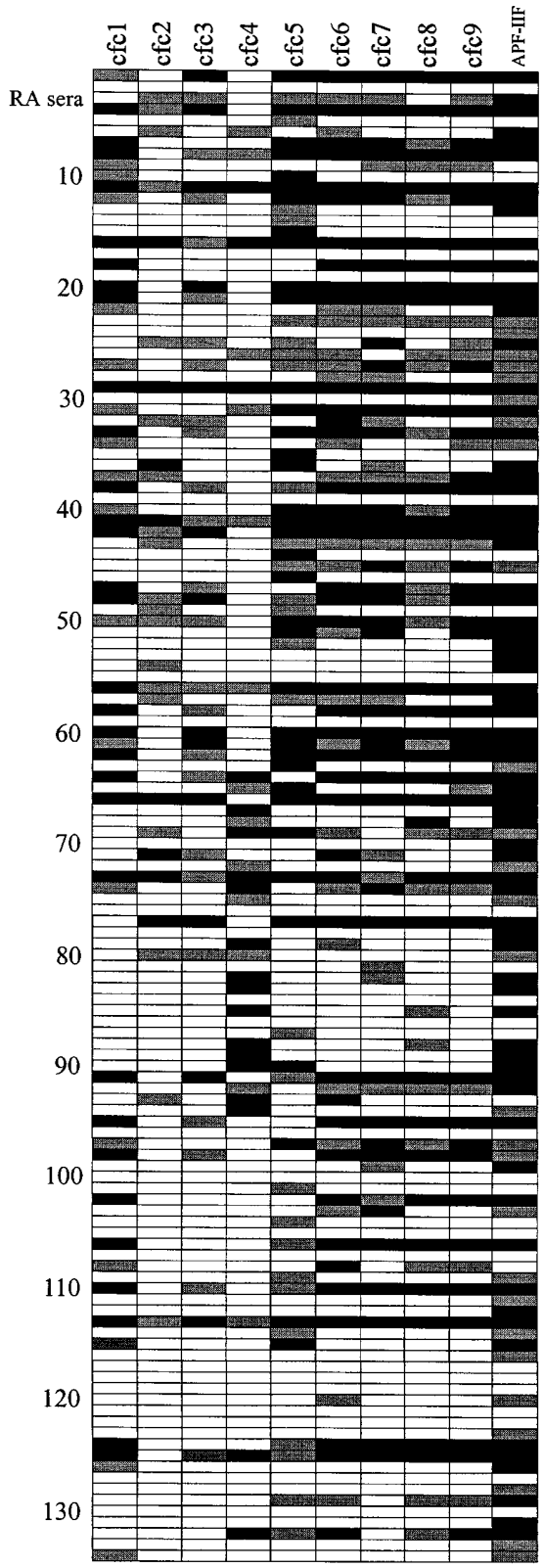

Figure 5. RA sera have different patterns of reactivity with citrullinated peptide variants. The reactivity of 134 RA sera with the citrullinated peptide variants (cfc1–cfc9, see Table I) in ELISA and their ability to stain keratohyalin granules in APF-IIF is depicted. High reactivity ($OD_{450} > 0.5$) is depicted as *black boxes*; lower reactivity ($OD_{450} < 0.5$ but > 0.2) is depicted as *gray boxes*. Reactivity below the cutoff value ($OD < 0.2$) is depicted by *white boxes*. Positive APF-IIF (*right*) is depicted by *black boxes*, weakly positive sera are depicted by *gray boxes,* and a negative result in APF-IIF is depicted by *white boxes.*

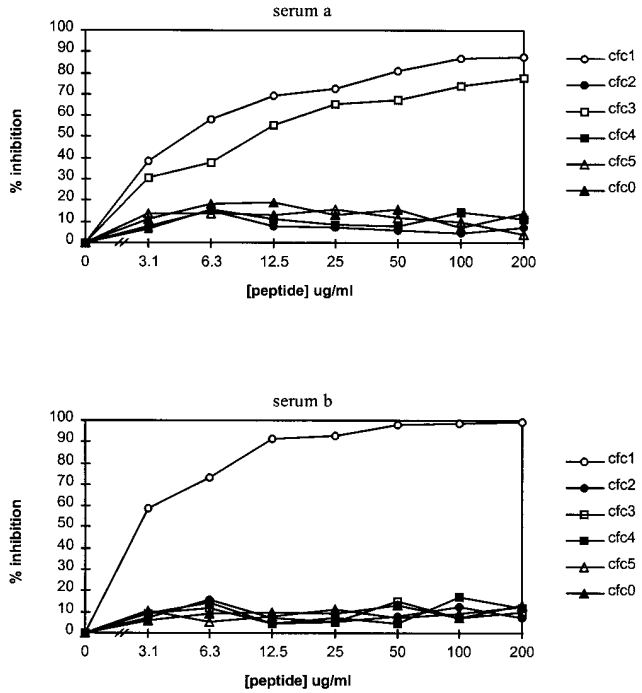

Figure 6. Competition profiles of RA sera. The citrulline substituted peptides (cfc2–cfc5), cf0 (Arg, the noncitrullinated peptide) and cfc1 (positive control) were added at different concentrations during serum (a and b) incubation on ELISA with the citrulline-containing peptide cfc1 coupled to the plates. Relative inhibition was calculated and plotted as a function of peptide concentration on a linear scale between 100% inhibition (OD_{450} of the background signal with no serum added) and 0% inhibition (OD_{450} with no peptide added).

76% (102/134) with 3.7% (13/354) of false positives (specificity 96%) is obtained.

Striking differences in the reactivity pattern against the monosubstituted peptides were encountered between individual sera (Fig. 5). These results suggest that each serum contains a specific subset of antibodies recognizing related citrulline-containing epitopes. Moreover, the antibodies differ in their fine specificity towards the citrulline-containing epitopes, which is becoming particularly evident in competition assays using the various peptides (Fig. 6). The results show, for example, that for only a certain subset of sera the peptide cfc3 is able to compete for binding to cfc1.

All double-citrullinated peptide variants (cfc6–cfc9) show an enhanced sensitivity in ELISA as compared with the monosubstituted variant cfc1 (Table III). The degree of enhancement is more or less the same for all four peptides, pointing to a specific conformational effect. Possibly by the introduction of a second citrulline, and thereby the removal of a positively charged arginine, the peptide can more easily adopt to a conformation that is favorable for antibody binding.

The strong relation between the reactivity towards the citrullinated peptides and the APF-IIF test suggests that these peptides represent the major epitopes recognized by the APF/

Table IV. Relation Between Anticitrullinated Peptide Antibodies (ELISA), APF, and RF in RA

Serum characterization			
ELISA	APF	RF	No. of positive sera (%)
+	+	+	79 (59)
+	+	−	8 (6.0)
+	−	+	8 (6.0)
+	−	−	7 (5.2)
−	+	+	12 (9.0)
−	+	−	0 (0)
−	−	+	12 (9.0)
−	−	−	8 (6.0)

*Sera reactive with at least one of the peptides cfc1-cfc9.

AKA antibodies (Table IV). The distribution of ELISA positivity over RF-positive (n = 111) and RF-negative (n = 23) sera is given in Table IV. The sensitivity of the ELISA (78% for RF-positive sera) seems to be somewhat lower (65%) for RF-negative sera. The number of RF-negative sera is, however, rather small in the group of sera tested.

Reactivity of peptides based on other filaggrin regions. Peptides based on sequences of other regions of the filaggrin repeats were selected. Criteria for selection were a high incidence of turn probability, a high antigenicity index, and an arginine pattern that differed to some extent from that of the cfc peptides (Table I; peptides cf18–31; cf18–34-1 to 3; cf48–65-1 to 4; cf260–276 1 and 2). To test if these peptides had an additional value for test performance, the RA sera (n = 32) that were negative on cfc1–cfc9 were tested in ELISA for reactivity with these peptides (Table V). Except for peptide cf48–65-4, on which an additional 8 sera were positive, these peptides had little or no additional value, since sera positive on cf18–31 (n = 3), cf18–34-1 (n = 1), and cf18–34-3 (n = 2) were also positive on cf48–65-4. Interestingly, all sera positive on these peptides were also positive in the APF-IIF test, which suggests that peptide cf48–65-4 may expose APF/AKA epitopes that are not presented by peptides cfc1-cfc9. Taken together, the additional peptide cf48–65-4 may lift the test sensitivity above the 80% level.

Table V. Reactivity of RA Sera Not Reactive on cfc1-cfc9, with Peptides from Other Profilaggrin Regions

Peptide*	Positive sera‡
f12-31§	0
cf18-31	3
cf18-34-1	1
cf18-34-2	0
cf18-34-3	2
cf48-65-1	0
cf48-65-2	0
cf48-65-3	0
cf48-65-4	8
cf260-276-1	0
cf260-276-2	0

*Peptide sequences can be found in Table I. ‡A subset of sera (n = 32) is used that were negative on cfc1-cfc9. §A negative control peptide that does not contain a citrulline (Table I).

Discussion

In this study we show that citrulline is a major constituent of antigenic determinants recognized by antibodies present in RA sera. The observation that a posttranscriptional modification of arginine residues creates autoantigenic B cell epitopes suggests intriguing new possibilities for a mechanism by which autoantibodies (in this case APF/AKA antibodies) may evolve. One possibility is that immunological tolerance has been established for the unmodified protein, but that during some period of the life span of the protein, arginine is modified to citrulline. When presented to the immune system, e.g., after massive cell damage or uncontrolled apoptosis, the modified antigen could evoke an immune response that, via epitope spreading, can lead to a polyclonal autoantibody response against the whole protein or protein-containing complex. It is likely that such a cascade of immune reactions is restricted to genetically susceptible individuals. In this way, the introduction of a modified arginine residue could break tolerance to this kind of neoepitope as postulated recently for another type of posttranslational modification (phosphorylation) of autoantigens that occurs during apoptosis (18).

Genetic susceptibility to RA is associated with the presence of certain subsets of HLA class II DR1 and DR4 (19) that are believed to be involved in presenting T cell epitopes that are associated with joint inflammation. The antibodies recognizing the citrulline-containing epitopes are predominantly of the IgG class, and are of relative high affinity, suggesting an HLA class II–restricted T cell response to the antigen presenting the citrullinated epitopes. Therefore, the identification of an antigen that bears citrulline-containing epitopes and is able to induce an RA-specific HLA class II–restricted T cell proliferation could be highly relevant to the disease processes in RA.

Our study provides further evidence that the antigen recognized in the APF and AKA IIF test is indeed (pro)filaggrin. The origin of the (pro)filaggrin-reactive autoantibodies and their role in the pathology of RA is, however, not clear. Profilaggrin is expressed at a late stage during the differentiation pathway of epidermal cells, and the presence of mature and modified filaggrin is limited to the cornified cell layer of the epidermis. It is therefore hard to imagine how filaggrin could be presented to the immune system. Filaggrin has never been detected in cells other than those encountered in squamous epithelia, and its presence in synovial cells, for example, is unlikely. Therefore, we postulate that the antibodies reactive towards the citrullinated epitopes originate from a response against a yet unidentified, cross-reactive protein (or proteins). Such a protein should contain deiminated arginine residues and some sequence resemblance to the peptides described here.

In general, short synthetic peptides do not have a preferential conformation in solution, but can sometimes adopt moderately stabilized secondary structures (20). Known structures of antibody–peptide complexes show that peptides often adopt a β-turn structure within the complex (21), and such β-turn motifs are frequently encountered within the filaggrin sequence (22). In this scenario the role of filaggrin, due to its special properties, is that of natural library of citrulline-containing

epitopes presented in the right conformational environment. Its sequence heterogeneity, the presence of deiminated arginine residues, and the frequently encountered b-turn motifs in the protein make filaggrin very suited for this role.

Interesting sequence similarities have been encountered in database searches using the sequence on which the cfc peptides are based (Table I). For example, similarities with the characteristic alternating arginine pattern were found in various nucleic acid–binding proteins, some of which were of viral origin (e.g., transcription-regulating proteins of human papilloma and herpes viruses). Papilloma viruses and the Epstein-Barr virus (a gamma herpes virus) can infect epidermal cells, in which the modifying enzyme peptidylarginine deiminase is known to be expressed.

Deimination of arginine residues has been described in detail for two proteins: filaggrin and trichohyalin (22). The latter protein is expressed in cells of the hair follicle and filiform ridges of the tongue, and is reported to have a more globular shape with many α-helical stretches (22). Antibodies reactive to trichohyalin have not as of yet been identified in RA sera.

The enzyme that modifies arginine in a peptide context to citrulline, peptidylarginine deiminase, is expressed in a broad range of tissues and cell types (23, 24), suggesting that deimination of arginine within proteins is not a rare phenomenon. The presence of this ubiquitous modifying enzyme in synovial cells is therefore not unlikely, and its presence in hematopoietic cell types known to infiltrate the synovium during joint inflammation has been shown (24). Little is known about the function of the modification introduced by this enzyme. It was suggested that deimination may play a role in the breakage of inter- and intramolecular interactions, thereby making proteins more susceptible to the activity of proteolytic enzymes (25).

The APF/AKA antibodies that are more specific for RA compared with IgM RF are reported to be present in sera very early in disease (8), even before other disease manifestations can be observed (9). Moreover, the presence of the APF/AKA antibodies are reported to be indicative for a more severe outcome of disease (26). Unfortunately, the inconvenient test format (IIF) and inconvenient antigenic substrates used to determine the presence of the APF/AKA antibodies have hindered their use as a serological marker. In the light of recent findings that an early start of RA therapy with a combination of disease-modifying drugs is very successful in preventing disease progression (27), a more convenient and reliable method of determining the presence of AKA/APF antibodies would be a valuable diagnostic tool. It is shown here that, in principle, the early presence of APF/AKA antibodies, pointing very specifically to the development of RA, can be determined by ELISA using synthetic peptides that contain citrulline. Our future efforts will be directed to the identification of peptides with enhanced binding characteristics to the APF/AKA autoantibodies. The identification of such peptides will not only enhance their diagnostical usefulness, but will also provide more precise information on the nature of the antigenic determinants responsible for the specific occurrence of APF/AKA antibodies in RA sera.

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