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Incauragarat et al.(54) **METHOD FOR DETECTING RHEUMATOID
ARTHRITIS-SPECIFIC AUTOANTIBODIES**(52) **U.S. Cl. 435/7.9**(76) **Inventors: Brigitte Incauragarat, Lentilly (FR);
Michel Jolivet, Saint Bonnet de Mure
(FR); Odile Letourneur, Sainte Foy les
Lyon (FR); Maria Leonor Nogueira,
Toulouse (FR); Mireille Sebbag,
Toulouse (FR); Guy Serre, Toulouse
(FR); Christian Vincent, Lauzerville
(FR)****Correspondence Address:**
Oliff & Berridge
PO Box 19928
Alexandria, VA 22320 (US)(21) **Appl. No.: 10/479,571**(22) **PCT Filed: Jun. 13, 2002**(86) **PCT No.: PCT/FR02/02032**(30) **Foreign Application Priority Data****Jun. 13, 2001 (FR)..... 01/08068****Publication Classification**(51) **Int. Cl.⁷ G01N 33/53; G01N 33/542**(57) **ABSTRACT**

The invention concerns a method for detecting rheumatoid arthritis-specific autoantibodies, in a biological sample suspected of containing said autoantibodies and other autoantibodies not specific of rheumatoid arthritis, comprising the following steps: providing filaggrin (FNC) or a citrulline-containing filaggrin derivative (FNC) or a filaggrin peptide (PFNC) comprising at least an arginine residue and said citrulline-containing filaggrin (FC) or said citrulline-containing filaggrin derivative (FC) or said citrulline-containing peptide (PFC); contacting said biological sample with said filaggrin (FNC) or said filaggrin derivative (FNC) or said peptide (PFNC) and said filaggrin (FC) or said filaggrin derivative (FC) or said peptide (PFC) in conditions suitable for the formation of immune complexes with the autoantibodies; detecting and quantifying the immune complexes formed between the autoantibodies or the other antibodies present in the sample and said filaggrin (FNC) or said filaggrin derivative (FNC) or said peptide (PFNC) and said citrulline-containing filaggrin (FC) or said citrulline-containing filaggrin derivative (FC) said peptide (PFC), said quantification being expressed by a value respectively X_{NC} and X_C , and in reducing the value of X_{NC} to that of X_C .

METHOD FOR DETECTING RHEUMATOID ARTHRITIS-SPECIFIC AUTOANTIBODIES

[0001] Rheumatoid arthritis (RA) is the most common of the chronic inflammatory rheumatisms. It is a systemic autoimmune disease characterized by an inflammation of the joints, which affects more than 500 000 individuals in France and more than 2 million in the United States. The serum of affected patients contains autoantibodies, some of which are specific and may constitute a marker for the disease allowing diagnosis thereof even at an early stage.

[0002] Autoantibodies which are specifically present in patients suffering from RA and which react with a rat esophageal epithelial antigen were described for the first time by B. J. J. Young et al., in *Br. Med. J.* 2:97-99 (1979). At the time, these autoantibodies were named "anti-keratin antibodies" (AKAs), but it has subsequently been shown that these antibodies are in fact antifilaggrin autoantibodies (AFAs). The antigens for these antibodies are the neutral/acidic forms of filaggrins (Simon et al., *J. Clin. Invest.*, 92, 1387, (1993)).

[0003] Filaggrin is generated from profilaggrin, which is a phosphorylated polypeptide of epidermal keratohyalin granules. Profilaggrin has a high molecular weight (approximately 400 000 in humans), and it is soluble in the presence of high concentrations of salts or of urea. It has a high content of basic amino acids (arginine and histidine), and also of glycine, serine and glutamic acid. It has a low content of nonpolar amino acids and contains neither methionine, nor cysteine, nor tryptophan. It is highly phosphorylated on serine residues, which confers on it an isoelectric point too close to neutrality.

[0004] Profilaggrin is cleaved into filaggrin units in the course of a complex process of maturation involving dephosphorylation followed by cleavage by proteases in the interdomain segments. This cleavage first generates fragments of intermediate size, and then the functional filaggrin molecules.

[0005] Filaggrins derived from the dephosphorylation and cleavage of profilaggrin are basic proteins, the amino acid content of which is similar to that of the profilaggrins. They contribute to keratin filament organization and undergo gradual maturation during which the arginine residues, which are basic, are converted to citrulline residues, which are neutral, under the action of peptidylarginine deiminase (Harding C. R. and Scott I. R., *J. Mol. Biol.* 170, p. 651-673 (1983)). This leads to a decrease in their affinity for the keratins, from which they detach; they are then completely degraded by various proteases.

[0006] The properties of filaggrins and of pro-filaggrins have been particularly well studied in rats, in mice and in humans. The size of profilaggrin varies, according to species, from 300 to 400 kD, and that of the filaggrins from 27 to 64 kD.

[0007] The gene encoding profilaggrin is made up of repeated subunits, each one of which encodes a filaggrin molecule, separated by portions encoding the interdomain peptide segments. All the repeat units encoding each of the human filaggrins have the same length (972 base pairs in humans); however, in humans, considerable sequence variations (10-15%) are observed from one subunit to the other. While most are conservative, some of these variations

induce amino acid changes and, in some cases, changes in the electric charge of the protein. Thus, human filaggrins form, independently of the post-transcriptional modifications, a heterogeneous population of molecules of similar size but of different sequences and charges (pHi equal to 8.3 ± 1.1) (Gan et al., *Biochem.* 29, p. 9432-9440 (1990)).

[0008] The polymorphism observed in humans between the sequences of the filaggrin units within the same profilaggrin gene is not apparent in rats and mice. Filaggrins also exhibit a great inter- and intraspecific variability in terms of their sequence. This variability does not however affect their functional properties, nor their overall amino acid composition or their biochemical properties. Similarly, the tissue localizations of profilaggrin and of filaggrins are identical in the various mammals studied.

[0009] The autoantibodies (AFAs) are associated with the clinical and biological parameters which define the most active and the most severe forms of the disease, and are thought to precede the appearance of the clinical signs. The detection thereof therefore proves to be an essential step in the diagnosis of RA.

[0010] According to document WO-A-98/08946 in the applicant's name, a method has been developed for detecting AFAs, in a biological sample, based on the discovery that citrullination of filaggrin is necessary for detection of AFAs present in sera from patients suffering from RA, this citrullination generating epitopes recognized by the AFAs. This method comprises the following steps:

[0011] the biological sample analyzed is brought into contact with a citrulline-containing recombinant filaggrin or a fragment thereof, under conditions suitable for the formation of immune complexes with the AFAs, and

[0012] the immune complexes possibly formed with the AFAs are detected.

[0013] The results demonstrate that the method is highly sensitive, most of the sera from patients suffering from RA recognizing the citrulline-containing recombinant filaggrin.

[0014] It was however observed, in the course of the steps for diagnosing RA in the patients whose sera were found to be positive in this method, that not all were RA cases. This method in fact leads to the detection of false positives, as will be illustrated in the following examples, and cannot therefore be used reliably, due to an insufficient specificity.

[0015] The present inventors have now developed a new ELISA assay for diagnosing RA in patients, which has great specificity while at the same time conserving the high sensitivity of the abovementioned method.

[0016] According to this method, rheumatoid arthritis (RA)—specific autoantibodies (AFAs) are detected in a biological sample liable to contain said autoantibodies (AFAs) and other autoantibodies not specific for RA, as follows:

[0017] firstly, a filaggrin (FNC) or a derivative of filaggrin (FNC) or a filaggrin peptide (PFNC) comprising at least one arginine residue and, secondly, said citrulline-containing filaggrin (FC) or a derivative of said citrulline-containing filaggrin (FC) or said citrulline-containing peptide (PFC) are provided,

[0018] said biological sample is brought into contact with, firstly, said filaggrin (FNC) or said derivative of said filaggrin (FNC) or said peptide (PFNC) and, secondly, said filaggrin (FC) or said derivative of said filaggrin (FC) or said peptide (PFC), under conditions suitable for the formation of immune complexes with the autoantibodies (AFAs),

[0019] the immune complexes formed between the auto-antibodies (AFAs) or the other antibodies present in the sample and, firstly, said filaggrin (FNC) or said derivative of said filaggrin (FNC) or said peptide (PFNC) and, secondly, said citrulline-containing filaggrin (FC) or said derivative of said citrulline-containing filaggrin (FC) or said peptide (PFC) are detected and quantified, this quantification being expressed by a value respectively X_{NC} and X_C ,

[0020] the value of X_C is reduced by the value of X_{NC} .

[0021] To implement the method of the invention, the filaggrin (FNC) or the derivative of a filaggrin (FNC) or the peptide (PFNC) is immobilized on the solid support and the filaggrin (FC) or a derivative of the filaggrin (FC) or the peptide (PFC) is immobilized on another solid support. They may be, inter alia, two wells on the microtitration plate or two tips of the Vidas device (registered trademark) marketed by the applicant, but also strips or any other suitable support.

[0022] Before describing the invention in greater detail and presenting the preferred variants thereof, some terms used in the description and the claims are defined below.

[0023] Citrullination is the reaction of conversion of an arginine residue to a citrulline residue by conversion of the $—C(NH)NH_2$ group of the arginine to $—CONH_2$. The reaction is advantageously carried out in vitro with the peptidylarginine deiminase (PAD) enzyme. This enzyme may be a rabbit muscle PAD; it is however within the scope of those skilled in the art to select another suitable PAD. It is of course possible to envision any other means of citrullination, such as chemical reagents or microorganisms.

[0024] According to the invention, when reference is made to a citrulline-containing filaggrin, this means that a proportion of approximately at least 20% of the arginine residues have been converted to citrulline residues, this conversion taking place randomly. Preferably, this degree of citrullination is at least 30%, and advantageously at least 50%. It has been observed that the immunoreactivity of a citrulline-containing filaggrin for which the degree of citrullination is approximately 80% is similar to that of a filaggrin for which the degree of citrullination is approximately 50%. Under these conditions, it is therefore unnecessary to seek to achieve very high degrees of citrullination.

[0025] If the citrullination is not carried out randomly, but is region-specific, and in particular if the conversion reaction can be limited to an arginine residue belonging to an epitope of the filaggrin after citrullination, the degree of citrullination can be lower than the abovementioned values, without the immunoreactivity of the filaggrin thus citrullinated being effected by this.

[0026] The expression “derivative of a filaggrin (FNC)” or “corresponding citrulline-containing derivative” means: a chimeric filaggrin which consists of a combination of frag-

ment(s) of sequences of human and rat filaggrin and/or a combination of fragment(s) of sequences of human and/or rat filaggrin and of consensus peptide(s).

[0027] To implement the method of the invention, the filaggrin is preferably chosen from human filaggrin and a filaggrin of animal origin. It advantageously consists of a recombinant rat filaggrin having the sequence SEQ ID No. 1.

[0028] In place of the filaggrin, it is possible to choose a filaggrin peptide (PFNC), the sequence of which comprises at least one arginine residue, and the corresponding citrulline-containing peptide (PFC) for which said arginine residue at least of PFNC has been converted to a citrulline residue. According to a variant of the method of the invention, the peptide contains an epitope of filaggrin after citrullination. Thus, a peptide (PFNC) may in particular be chosen from the following peptides:

[0029] +the S47S peptide, the sequence of which is represented in SEQ ID No. 2, corresponding to amino acids 71 to 119 of the sequence of a human filaggrin unit and containing 6 arginine residues,

[0030] +the E12H peptide, the sequence of which is represented in SEQ ID No. 3, as determined with reference to the nucleotide sequences of the human profilaggrin gene described by Gan S. Q. et al. (Biochemistry, 29: 9432-9440, (1990)) and comprising 1 arginine residue,

[0031] +the E12D peptide, the sequence of which is represented in SEQ ID No. 4, as determined with reference to the nucleotide sequences of the human profilaggrin gene described by Gan S. Q. et al. (Biochemistry, 29: 9432-9440, (1990)) and comprising 3 arginine residues,

[0032] +the G22Q peptide, the sequence of which is represented in SEQ ID No. 5, corresponding to a consensus sequence of a rat filaggrin unit comprising 4 arginine residues,

[0033] +the G26E peptide, the sequence of which is represented in SEQ ID No. 6, corresponding to a consensus sequence of a rat filaggrin unit comprising 10 arginine residues.

[0034] As mentioned above, while a filaggrin (FNC) is provided for implementing the method of the invention, the citrulline-containing filaggrin (FC) used has the peptide sequence of filaggrin (FNC), in which at least 20%, preferably at least 30%, and better still at least 50%, of the arginine residues of said FNC are converted to citrullines.

[0035] The autoantibodies (AFAs) are circulating antibodies, and a biological sample to be analyzed according to the method of the invention may be chosen from blood, plasma and serum.

[0036] The detection and quantification of the immune complexes formed are carried out by any techniques well known to those skilled in the art, and in particular using an

enzyme for the detection and its calorimetric or fluorescent substrate for the quantification (examples: alkaline phosphatase/4-methylumbelliferyl phosphate, peroxidase/orthophenylenediamine).

[0037] As will be illustrated in the examples, it is thus possible to bring the immune complexes formed into contact with a conjugate consisting of a labeled antibody directed against human immunoglobulins, under conditions suitable for the formation of labeled immune complexes, and then the labeled immune complexes are detected and quantified. The conjugate is advantageously an anti-human immunoglobulin antibody, whole or fragmented (examples: Fab', Fab₂' fragments), labeled with peroxidase or with alkaline phosphatase, and the formation of labeled immune complexes is then detected and quantified by colorimetry or fluorimetry, the quantification being expressed as optical density or fluorescence values X_{NC} and X_C .

[0038] A positive assay revealing the presence of RA-specific autoantibodies (AFAs) in the sample analyzed is characterized by the value X_C being greater than the value X_{NC} .

[0039] The invention is illustrated below and its characteristics and advantages are brought to light.

EXAMPLE 1

Production of a Recombinant Rat Filaggrin

[0040] A) Cloning of the Rat Profilaggrin Gene

[0041] The studies by Haydock and Dale, 1986 (J. Biol. Chem., 261, 12520-12525) and by Rothnagel et al., 1987 (J. Biol. Chem., 262, 15643-15648) have shown that the rat profilaggrin gene is made up of 20 ± 2 repeat units of approximately 1 200 base pairs (bp) and has no intron in its coding region, each profilaggrin unit being made up of a filaggrin domain and a binding sequence.

[0042] Furthermore, the coding sequence for two incomplete profilaggrin units has been published by Haydock and Dale, 1990 (DNA Cell Biol., 9, 251-261).

[0043] Based on these data, oligonucleotides intended for the PCR amplification of the coding sequence for a complete rat profilaggrin unit were designed. The rat genomic DNA matrix was prepared from whole blood of a Wistar rat. Three different PCR products were obtained with three different pairs of oligonucleotides. These PCR products were cloned into a vector pCAPs (PCR cloning kit, Boehringer) and sequenced. They exhibit a high degree of homology (of more than 99%) with one another and also a 95% and 98% homology with the two incomplete profilaggrin units published by Haydock and Dale, 1990.

[0044] These data confirm the nature of the DNA obtained and suggest that the available sequences are representative of the existing rat filaggrin domains, a certain inter- and intra-individual variability being expected.

[0045] One of the sequences obtained was cloned into an expression vector pMR (Cheynet et al., 1993, Prot. Exp. Purif., 4, 367-372) as a fusion with a coding sequence for 6 histidines in the 5' position. The construct identified as pOL041 was used to transform *E. coli* bacteria. It encodes a 399 amino acid protein with a theoretical molecular weight of 42 210, having the sequence SEQ ID No. 1.

[0046] B) Expression and Purification of the Recombinant Rat Filaggrin

[0047] Culturing:

[0048] To place the transformed *E. coli* strain (BL21 or DH5 α) in culture, an isolation from the library conserved at -80°C . is carried out on agar medium with 0.1 mg/ml ampicillin.

[0049] From an isolated colony, a preculture is prepared in liquid medium (yeast extract, 2% glucose and 0.1 mg/ml ampicillin) in Erlenmeyer flasks placed at $+37^\circ\text{C}$. and with orbital shaking (280 rpm).

[0050] This preculture then makes it possible to seed 5 l Erlenmeyer culture flasks, each containing 1 l of the same medium optionally substituted with PMSF at a final concentration of 0.5 mM. These Erlenmeyer flasks are cultured at $+37^\circ\text{C}$. with shaking at 280 rpm, until an OD at 600 nm of 0.8 is obtained. Once this OD value has been reached, the culturing is continued for a period of 3 hours.

[0051] The culture is then stopped and centrifuged for 25 minutes at 6-000 g. The bacterial pellets are then collected and conserved at -25°C .

[0052] Purification:

[0053] The bacterial pellet is lysed by the action of lysozyme in the presence of protease inhibitor in a buffer consisting of 0.1 M NaH_2PO_4 /0.6 M NaCl/0.2% Tween/1 mM Na azide/6 mM imidazole, pH 7.3, and by sonication. This lysate is then clarified for 30 minutes at 23 700 g and the supernatant is conserved.

[0054] The recombinant filaggrin is purified by metal (nickel) chelate affinity chromatography at $+4^\circ\text{C}$.

[0055] Briefly, after re-equilibration of the gel in lysis buffer, the lysis supernatant is injected onto the column, followed by washing (and monitoring of the UV 280 nm detection signal) with the buffer consisting of 0.1 M NaH_2PO_4 /0.6 M NaCl/1 mM Na azide/6 mM imidazole, pH 7.3, until the UV 280 nm detection signal has returned to base line.

[0056] The recombinant filaggrin (rFlg), adsorbed onto the gel, is eluted with the buffer consisting of 0.1 M NaH_2PO_4 /0.6 M NaCl/1 mM Na azide/0.5 M imidazole, pH 7.15.

[0057] The fraction thus obtained is concentrated on a 10 kDa membrane.

[0058] The final purification step is carried out by gel filtration chromatography on a Superdex 200 support, in 20 mM Tris/0.3 M NaCl buffer, pH 11. The concentrate is injected onto the column, the fractions being selected by monitoring the UV signal at 280 nm and 214 nm. This step makes it possible to isolate and recover the immunologically active recombinant filaggrin of interest, in the second elution peak ($MW \approx 42\ 000$).

EXAMPLE 2

Citrullination of the Recombinant Filaggrin

[0059] After assaying the total proteins by the Bradford method (Biorad reagents), the citrullination is performed, by the action of peptidylarginine deiminase, for 4 hours at $+37^\circ\text{C}$. with magnetic stirring, at a rate of 4 enzyme units/mg of

total proteins, in the presence of 0.1 M EDTA/1 M DTT/0.5 M CaCl₂. The amount of total proteins is then re-assayed on the deiminated fraction.

[0060] The citrulline-containing filaggrin is purified by Ni-NTA chromatography and then gel filtration chromatography.

[0061] The degree of citrullination detected is 53%.

[0062] Examples 3 and 4 below illustrate the diagnosis of RA in a method of detection of the prior art and in a method of detection according to the invention, so as to allow the comparison thereof.

[0063] The method of detection of the prior art consists of an ELISA assay, of the type such as that described in WO-98/08946 in the applicant's name, using citrulline-containing filaggrin and based on the detection of immune complexes formed between the AFA antibodies possibly present in the sample and the citrulline-containing filaggrin.

[0064] The method of detection according to the invention is an ELISA assay using citrulline-containing filaggrin and non-citrulline-containing filaggrin and which is based on the parallel detection of the immune complexes formed, firstly, with the citrulline-containing filaggrin and, secondly, the non-citrulline-containing filaggrin, and on the difference in values between the two.

EXAMPLE 3

[0065] A) Samples

[0066] The assays were carried out on the following samples:

[0067] 63 control sera affected by the following pathological conditions: algodystrophy, gout, malignant hemopathy, Paget's disease, neuralgia, mechanical pathology, peri-arthritis nodosa, polymyositis, lupus-related rheumatism, psoriatic rheumatism, scleroderma, ankylosing spondylitis, spondylarthropathy, Gougerot's syndrome, Sharp's syndrome, vasculitis.

[0068] 65 sera from patients suffering from clinically diagnosed rheumatoid arthritis.

[0069] B) Material

[0070] Nunc Maxisorp 468667 plate

[0071] Incubator at 35-39° C.

[0072] Axia microwasher U4402

[0073] Biotek (commercial trademark) plate reader

[0074] C) Protocol

[0075] Deposition in wells: 100 μ l per well of 2.5 μ g/ml of filaggrin for 2 hours at 37° C. in PBS buffer, pH 7.2;

[0076] 3 washed with 300 μ l per well of PBS/0.05% Tween buffer, pH 7.2;

[0077] blocking for 1 hour at 37° C. with 250 μ l per well of PBS/1% BSA buffer, pH 7.2;

[0078] 3 washed with 300 μ l per well of PBS/0.05% Tween buffer, pH 7.2;

[0079] Incubation for 1 hour at 37° C. with 100 μ l per well of serum diluted to 1/100 in buffer consisting of

10 mM Tris, 350 mM NaCl, 1% TritonX100, 1% BSA, 10% rabbit serum, pH 7.6;

[0080] 3 washed with 300 μ l per well of PBS/0.05% Tween buffer, pH 7.2;

[0081] incubation of an anti-human IgG conjugate diluted in buffer consisting of 10 mM Tris, 350 mM NaCl, 1% TritonX100, 1% BSA, 10% rabbit serum, pH 7.6; 100 μ l per well;

[0082] 3 washed with 300 μ l per well of PBS/0.05% Tween buffer, pH 7.2.

[0083] Enzymatic Revelation:

[0084] 100 μ l of ortho-phenylenediamine (OPD) per well

[0085] incubation for 10 min at 18-25° C.

[0086] blocking with 100 μ l H₂SO₄

[0087] reading at 492 nm.

[0088] The samples are assayed in parallel on a plate coated with non-citrulline-containing filaggrin and on another plate coated with citrulline-containing filaggrin.

[0089] The samples are assayed unduplicated.

[0090] The results are expressed as optical density. The signals indicated at an optical density of 3 are equal to or greater than 3.

[0091] D) Results

[0092] The results are given in tables 1 and 2 below.

[0093] With the results obtained on citrulline-containing filaggrin according to WO-A-98/08946 (table 1), the performance levels are as follows:

100% specificity	threshold at 1.337	34% sensitivity	(22/65)
98% specificity	threshold at 0.937	38% sensitivity	(22/65)
95% specificity	threshold at 0.335	61.5% sensitivity	(40/65)

[0094] With the results obtained on citrulline-containing filaggrin/non-citrulline-containing filaggrin according to the invention (table 2), the performance levels are as follows:

100% specificity	threshold at 0.731	38% sensitivity	(25/65)
98% specificity	threshold at 0.009	69% sensitivity	(45/65)
95% specificity	threshold at -0.004	77% sensitivity	(50/65)

[0095] In both cases, at 95% specificity, 3 sera are false positives.

[0096] All the samples detected on the citrulline-containing filaggrin phase are detected on the differential system with 100% and 98% specificity. A single serum is addition-

ally detected with the differential system. At 95% specificity, 7 samples are additionally detected with the differential system.

[0097] The differential system is more effective than the citrulline-containing filaggrin phase on this population, with 69% sensitivity for 98% specificity.

[0098] For this population, the performance levels of the assays normally used are as follows:

[0099] GIFOR (immunofluorescence on section of rat esophagus)

98% specificity	threshold at 2	49% sensitivity	(32/65)
Gblot			
98% specificity	threshold at 2.25	58% sensitivity	(38/65)

[0100] The performance levels obtained by ELISA on rat filaggrin are better than the techniques currently used, GIFOR and GBlot.

TABLE 1

Results as OD at 492 nm of the ELISA assays on citrulline-containing filaggrin (FC)			
Negative controls	FC	RA sera	FC
58	0.050	78	0.051
57	0.056	76	0.059
10	0.058	72	0.077
33	0.060	74	0.077
51	0.062	81	0.079
53	0.062	79	0.088
44	0.064	73	0.091
54	0.066	71	0.098
47	0.069	80	0.102
49	0.069	75	0.109
38	0.070	65	0.113
39	0.070	86	0.121
40	0.071	66	0.124
50	0.072	83	0.132
29	0.073	69	0.141
45	0.073	77	0.147
25	0.076	84	0.151
55	0.076	85	0.156
28	0.079	90	0.157
42	0.083	67	0.158
46	0.083	68	0.158
60	0.085	70	0.202
43	0.087	89	0.207
31	0.088	88	0.276
34	0.088	92	0.299
37	0.090	93	0.387
41	0.090	87	0.409
16	0.092	64	0.434
35	0.092	82	0.472
48	0.092	95	0.496
26	0.093	98	0.499
52	0.093	96	0.524
17	0.097	94	0.535
61	0.098	100	0.611
27	0.111	99	0.659
19	0.113	91	0.667
20	0.113	103	0.726
4	0.116	101	0.729
22	0.116	102	0.801
36	0.123	97	0.853
18	0.124	104	0.939
24	0.124	105	1.203

TABLE 1-continued

Results as OD at 492 nm of the ELISA assays on citrulline-containing filaggrin (FC)			
Negative controls	FC	RA sera	FC
14	0.128	106	1.240
12	0.129	107	1.526
23	0.130	108	1.694
7	0.133	109	1.900
5	0.134	110	2.076
9	0.135	111	2.387
30	0.142	114	2.593
59	0.146	112	2.610
6	0.158	113	3.000
21	0.159	115	3.000
11	0.166	116	3.000
32	0.167	117	3.000
15	0.169	118	3.000
62	0.193	119	3.000
56	0.222	120	3.000
3	0.253	121	3.000
13	0.269	122	3.000
1	0.335	123	3.000
8	0.369	124	3.000
63	0.937	125	3.000
2	1.337	126	3.000
		127	3.000
		128	3.000

[0101]

TABLE 2

Results as OD at 492 nm of the ELISA assays on citrulline-containing filaggrin (FC) and non- citrulline-containing filaggrin (FNC)							
Negative controls	FNC	FC	FC - FNC	RA sera	FNC	FC	FC - FNC
1	0.780	0.335	-0.445	64	0.777	0.434	-0.343
2	1.747	1.337	-0.410	65	0.202	0.113	-0.089
3	0.433	0.253	-0.180	66	0.204	0.124	-0.080
4	0.268	0.116	-0.152	67	0.222	0.158	-0.064
5	0.271	0.134	-0.137	68	0.221	0.158	-0.063
6	0.294	0.158	-0.136	69	0.195	0.141	-0.054
7	0.262	0.133	-0.129	70	0.255	0.202	-0.053
8	0.492	0.369	-0.123	71	0.131	0.098	-0.033
9	0.231	0.135	-0.096	72	0.109	0.077	-0.032
10	0.149	0.058	-0.091	73	0.115	0.091	-0.024
11	0.256	0.166	-0.090	74	0.099	0.077	-0.022
12	0.218	0.129	-0.089	75	0.131	0.109	-0.022
13	0.351	0.269	-0.082	76	0.072	0.059	-0.013
14	0.209	0.128	-0.081	77	0.157	0.147	-0.010
15	0.248	0.169	-0.079	78	0.055	0.051	-0.004
16	0.158	0.092	-0.066	79	0.090	0.088	-0.002
17	0.162	0.097	-0.065	80	0.104	0.102	-0.002
18	0.188	0.124	-0.064	81	0.079	0.079	0.000
19	0.174	0.113	-0.061	82	0.466	0.472	0.006
20	0.173	0.113	-0.060	83	0.124	0.132	0.008
21	0.219	0.159	-0.060	84	0.137	0.151	0.014
22	0.171	0.116	-0.055	85	0.137	0.156	0.019
23	0.179	0.130	-0.049	86	0.101	0.121	0.020
24	0.172	0.124	-0.048	87	0.372	0.409	0.037
25	0.123	0.076	-0.047	88	0.236	0.276	0.040
26	0.139	0.093	-0.046	89	0.137	0.207	0.070
27	0.153	0.111	-0.042	90	0.057	0.157	0.100
28	0.120	0.079	-0.041	91	0.514	0.667	0.153
29	0.113	0.073	-0.040	92	0.058	0.299	0.241
30	0.181	0.142	-0.039	93	0.134	0.387	0.253
31	0.126	0.088	-0.038	94	0.240	0.535	0.295
32	0.204	0.167	-0.037	95	0.156	0.496	0.340
33	0.096	0.060	-0.036	96	0.122	0.524	0.402

TABLE 2-continued

Results as OD at 492 nm of the ELISA assays on citrulline-containing filaggrin (FC) and non- citrulline-containing filaggrin (FNC)							
Negative controls	FNC	FC	FC - FNC	RA sera	FNC	FC	FC - FNC
34	0.122	0.088	-0.034	97	0.424	0.853	0.429
35	0.125	0.092	-0.033	98	0.063	0.499	0.436
36	0.156	0.123	-0.033	99	0.208	0.659	0.451
37	0.122	0.090	-0.032	100	0.117	0.611	0.494
38	0.101	0.070	-0.031	101	0.207	0.729	0.522
39	0.101	0.070	-0.031	102	0.165	0.801	0.636
40	0.098	0.071	-0.027	103	0.072	0.726	0.654
41	0.117	0.090	-0.027	104	0.142	0.939	0.797
42	0.110	0.083	-0.027	105	0.231	1.203	0.972
43	0.112	0.087	-0.025	106	0.087	1.240	1.153
44	0.089	0.064	-0.025	107	0.118	1.526	1.408
45	0.097	0.073	-0.024	108	0.268	1.694	1.426
46	0.107	0.083	-0.024	109	0.136	1.900	1.764
47	0.092	0.069	-0.023	110	0.095	2.076	1.981
48	0.113	0.092	-0.021	111	0.127	2.387	2.260
49	0.089	0.069	-0.020	112	0.256	2.610	2.354
50	0.091	0.072	-0.019	113	0.555	3.000	2.445
51	0.081	0.062	-0.019	114	0.128	2.593	2.465
52	0.112	0.093	-0.019	115	0.518	3.000	2.482
53	0.081	0.062	-0.019	116	0.338	3.000	2.662
54	0.083	0.066	-0.017	117	0.315	3.000	2.685
55	0.092	0.076	-0.016	118	0.295	3.000	2.705
56	0.233	0.222	-0.011	119	0.224	3.000	2.776
57	0.065	0.056	-0.009	120	0.184	3.000	2.816
58	0.058	0.050	-0.008	121	0.184	3.000	2.816
59	0.153	0.146	-0.007	122	0.181	3.000	2.819
60	0.089	0.085	-0.004	123	0.171	3.000	2.829
61	0.095	0.098	0.003	124	0.160	3.000	2.840
62	0.184	0.193	0.009	125	0.145	3.000	2.855
63	0.206	0.937	0.731	126	0.081	3.000	2.919
				127	0.062	3.000	2.938
				128	0.056	3.000	2.944

EXAMPLE 4

[0102] This example makes it possible to compare a diagnostic test according to the invention, the ArFA-ELISA assay, and several diagnostic tests of the prior art which are commercially available or which use known methods, namely the AKA by immunofluorescence, AhFA-IB, AhFA-ELISA and CCP-ELISA assays.

[0103] A) Samples

[0104] The assays were carried out on 711 sera, 240 of which came from patients suffering from rheumatoid arthritis (RA) and 471 of which came from non-RA patients. Among the non-RA patients, 157 were suffering from an inflammatory disease chosen from progressive lupus erythematosus (21), systemic scleroderma (9), psoriatic rheumatism (43), ankylosing spondylitis (40) and other inflamma-

tory diseases (44), and 314 were suffering from a non-inflammatory disease chosen from arthrosis (104), compressive neuropathy (25), Paget's disease (68), reflex sympathetic dystrophy (29), serious bone diseases (49) and other non-inflammatory diseases (39).

[0105] The filaggrin is a recombinant rat filaggrin; the non-citrulline-containing filaggrin is obtained in accordance with example 1, and the citrulline-containing filaggrin is obtained by citrullination (40% degree of citrullination of arginine residues) of the non-citrulline-containing filaggrin in accordance with example 2.

[0106] B) Protocol

[0107] The detection of the antibodies according to the AKA by immunofluorescence and AhFA-IB assays was carried out according to the methods described in C. Vincent et al., Ann. Rheum. Dis. (1989) 48, 712-722, and C. Vincent et al., J. Rheumatol. (1998) 25, 838-846.

[0108] The AhFA-ELISA assay is used according to the method described by L. Nogueira et al., Ann. Rheum. Dis (2001) 60, 882-887.

[0109] The CCP-ELISA assay is carried out with the Immunoscan™ RA material (Euro-diagnostica, Arnhem, The Netherlands) used according to the manufacturer's recommendations.

[0110] The ArFA-ELISA assay according to the invention was used in accordance with protocol C) described in example 3 with the exception of the fact that, in the present example, the samples were assayed in quadruplicate. The average variation between the assays for the same samples was less than 6%.

[0111] The results obtained for each of the assays are given in table 3 below.

TABLE 3

	95% specificity	98.5% specificity	99% specificity
'AKA'	0.52 (0.45-0.58)	0.45 (0.38-0.51)	0.40 (0.34-0.46)
AhFA-IB	0.59 (0.53-0.65)	0.48 (0.42-0.55)	0.37 (0.31-0.43)
AhFA- ELISA	0.53 (0.46-0.59)	0.38 (0.32-0.44)	0.37 (0.31-0.43)
CCP-ELISA	nd	0.58 (0.52-0.65)	0.50 (0.44-0.57)
ArFA- ELISA	0.76 (0.70-0.81)	0.67 (0.60-0.73)	0.65 (0.59-0.71)

[0112] It emerges from this table that the assay according to the invention is the most reliable of the assays examined in the detection of RA.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 6

<210> SEQ ID NO 1

<211> LENGTH: 399

<212> TYPE: PRT

-continued

<213> ORGANISM: recombinant rat flaggrin

<400> SEQUENCE: 1

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 1           5           10           15

Met Gly Ile Gln Gln Gln Arg Gln Arg Arg Gln His Glu Gln Glu Arg
          20           25           30

Glu His Glu His Gln Gln Pro Glu Ser Ser His Arg Gln Gln Gly Ser
          35           40           45

Ser Gly Arg Thr His Arg Ala Ala Arg His Glu Gln Glu Ser Asp Ser
          50           55           60

Thr Arg Gln Arg Gly Ser His Gln Ala His Ser Ser Ala Arg Thr Gln
          65           70           75           80

Glu Glu Ile Ala Arg Gly Arg Ser Gly Ala Ser Ala Ser Glu Gly Pro
          85           90           95

Gly Pro Gln Arg Glu Ala Ala Arg Asp Ser Ser Glu His Ala Gln Ser
          100          105          110

Arg Arg Ser Glu Thr Ile Ser Arg Gly Arg Ser Gly His Ser Thr Gly
          115          120          125

Arg Ala His Glu Asp Arg His Glu Gln Ala Thr Asp Arg Ser Ala Arg
          130          135          140

Ser Gly Ser Arg Gly Gly Gln Ala Gly Ser His Ser Glu Ser Glu Ala
          145          150          155          160

Ser Gly Gly Gln Ala Gly Arg Arg Gly Thr Ala Ala Thr Arg His Thr
          165          170          175

Ser Arg Pro Glu Gln Ser Pro Asp Thr Ala Gly Arg Thr Gly Ser Ser
          180          185          190

Arg Gly Gln Gln Ser Ala Gln Arg His Ala Asp Ser Thr Pro Gly Ser
          195          200          205

Thr Arg Thr Gly Ser Arg Gly Arg Gly Glu Ser Pro Ala Gly Gln Gln
          210          215          220

Ser Pro Asp Arg Ala Arg His Ile Glu Ser Arg Arg Gly Arg Thr Arg
          225          230          235          240

Glu Ala Ser Ala Ser Gln Ser Ser Asp Ser Glu Gly His Ser Gly Ala
          245          250          255

His Ala Gly Ile Gly Gln Gly Gln Thr Ser Thr Thr His Arg Arg Ala
          260          265          270

Gly Ser Ser Ser Gly Ser Gln Arg Ala Ser Ala Gly Gly Gln Ala Ala
          275          280          285

Asp Thr Ser Ser Arg Ser Gly Ala Ser Gln Gly Gln Ala Ser Gly Gln
          290          295          300

Gly Arg Ala Gly Ser Arg Ser Arg Glu Ala Gln Gly Ile Ala Arg His
          305          310          315          320

Gly Gln Ser Thr Asp Ser His Arg His Ser Gly Ala Gly Gln Ala Gln
          325          330          335

Ala His Thr Ala Thr Ser Ile Asp Ser Arg Arg Pro Arg Gly Ser Ser
          340          345          350

Glu Ser Gln Ala Ser Asp Ser Glu Gly His Ser Asp Tyr Ser Glu Ala
          355          360          365

His Thr Gln Gly Ala His Gly Gly Ile Gln Thr Ser Gln Arg His Glu
          370          375          380

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-continued

Gln Arg Pro Ser Arg Gly Gln Gln Gly Ser Gly His Pro Gln Val
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<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: human filaggrin peptide

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Arg Ser Asp Ala Ser Arg Gly Ser Ser Gly Ser Arg Ser Thr Ser Arg
20 25 30
Glu Thr Arg Asp Gln Glu Gln Ser Gly Asp Gly Ser Arg His Ser Gly
35 40 45

Ser

<210> SEQ ID NO 3
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: human filaggrin peptide

<400> SEQUENCE: 3

Glu Gln Ser Ala Asp Ser Ser Arg His Ser Gly Ser Gly His
1 5 10

<210> SEQ ID NO 4
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: human filaggrin peptide

<400> SEQUENCE: 4

Glu Ser Ser Arg Asp Gly Ser Arg His Pro Arg Ser His Asp
1 5 10

<210> SEQ ID NO 5
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<212> TYPE: PRT
<213> ORGANISM: rat filaggrin peptide

<400> SEQUENCE: 5

Gly Gln Ala Gly Gly Arg Gln Gly Ser Arg His Glu Gln Gly Ser Ser
1 5 10 15
Arg Gly Arg Ser Gly His Glu Gln
20

<210> SEQ ID NO 6
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: rat filaggrin peptide

<400> SEQUENCE: 6

Gly Ser His Arg Gln Gln Ser Ser Arg Arg Gln Gly Ser Ser Arg Gly
1 5 10 15
Gln Gln Ser Gly Gly Arg Gln Gly Ser Arg His Glu
20 25

1. A method for detecting rheumatoid arthritis (RA)-specific autoantibodies (AFAs) in a biological sample liable to contain said autoantibodies (AFAs) and other antibodies not specific for RA, according to which

firstly, a filaggrin (FNC) or a derivative of a filaggrin (FNC) or a filaggrin peptide (PFNC) comprising at least one arginine residue and, secondly, said citrulline-containing filaggrin (FC) or a derivative of said citrulline-containing filaggrin (FC) or said citrulline-containing peptide (PFC) are provided,

said biological sample is brought into contact with, firstly, said filaggrin (FNC) or said derivative of filaggrin (FNC) or said peptide (PFNC) and, secondly, said filaggrin (FC) or said derivative of said filaggrin (FC) or said peptide (PFC), under conditions suitable for the formation of immune complexes with the autoantibodies (AFAs),

the formation of immune complexes formed between the autoantibodies (AFAs) or other antibodies present in the sample and, firstly, said filaggrin (FNC) or said derivative of filaggrin (FNC) or said peptide (PFNC) and, secondly, said citrulline-containing filaggrin (FC) or said derivative of said filaggrin (FC) or said peptide (PFC) are detected and quantified, this quantification being expressed by a value respectively X_{NC} and X_C ,

the value of X_C is reduced by the value of X_{NC} .

2. The method as claimed in claim 1, characterized in that the filaggrin is chosen from human filaggrin and rat filaggrin.

3. The method as claimed in claim 2, characterized in that the filaggrin is a recombinant rat filaggrin and has the sequence SED ID No.1.

4. The method as claimed in claim 1, characterized in that the peptide comprises a peptide sequence chosen from SEQ ID No.2 to SEQ ID No.6.

5. The method as claimed in claim 1, characterized in that the citrulline-containing filaggrin (FC) or the citrulline-containing peptide (PFC) is obtained by the action of peptidylarginine deiminase.

6. The method as claimed in claims 1 and 5, characterized in that a filaggrin (FNC) is provided and in that at least 20% of the arginine residues of the FNC are converted to citrulline so as to obtain said citrulline-containing filaggrin (FC).

7. The method as claimed in claim 6, characterized in that at least 30%, preferably at least 50%, of the arginine residues of the FNC are converted to citrulline.

8. The method as claimed in claim 1, characterized in that the biological sample is chosen from blood, plasma and serum.

9. The method as claimed in claim 1, characterized in that the immune complexes formed are brought into contact with a conjugate consisting of a labeled antibody directed against human immunoglobulins, under conditions suitable for the formation of labeled immune complexes, and then the labeled immune complexes are detected and quantified.

10. The method as claimed in claim 9, characterized in that the conjugate is an anti-human immunoglobulin antibody labeled with alkaline phosphatase or with peroxidase, and then the formation of labeled immune complexes is detected and quantified by colorimetry or fluorimetry, the quantification being expressed as optical density or fluorescence values X_{NC} and X_C .

11. The method as claimed in claim 1, characterized in that, if the value X_C is greater than the value X_{NC} , RA-specific autoantibodies (AFAs) have been detected and quantified in said sample.

12. The method as claimed in claim 5, characterized in that a filaggrin (FNC) is provided and in that at least 20% of the arginine residues of the FNC are converted to citrulline so as to obtain said citrulline-containing filaggrin (FC).

* * * * *

专利名称(译)	检测类风湿性关节炎特异性自身抗体的方法		
公开(公告)号	US20040241767A1	公开(公告)日	2004-12-02
申请号	US10/479571	申请日	2002-06-13
[标]申请(专利权)人(译)	INCAURGARAT BRIGITTE JOLIVET MICHEL LETOURNEUR 奥迪勒 NOGUEIRA MARIA LEONOR 斯贝格MIREILLE SERRE GUY VINCENT CHRISTIAN		
申请(专利权)人(译)	INCAURGARAT BRIGITTE JOLIVET MICHEL LETOURNEUR 奥迪勒 NOGUEIRA MARIA LEONOR 斯贝格MIREILLE SERRE GUY VINCENT CHRISTIAN		
当前申请(专利权)人(译)	BIOMERIEUX		
[标]发明人	INCAURGARAT BRIGITTE JOLIVET MICHEL LETOURNEUR ODILE NOGUEIRA MARIA LEONOR SEBBAG MIREILLE SERRE GUY VINCENT CHRISTIAN		
发明人	INCAURGARAT, BRIGITTE JOLIVET, MICHEL LETOURNEUR, ODILE NOGUEIRA, MARIA LEONOR SEBBAG, MIREILLE SERRE, GUY VINCENT, CHRISTIAN		
IPC分类号	G01N33/564 G01N33/53 G01N33/542		
CPC分类号	G01N33/564		
优先权	2001008068 2001-06-13 FR		
其他公开文献	US8241856		
外部链接	Espacenet USPTO		

摘要(译)

本发明涉及一种检测类风湿性关节炎特异性自身抗体的方法，该方法涉及怀疑含有所述自身抗体和其他非类风湿性关节炎特异性自身抗体的生物样品，包括以下步骤：提供聚丝蛋白（FNC）或含瓜氨酸的聚丝蛋白衍生物（FNC）或包含至少精氨酸残基和所述含瓜氨酸的聚丝蛋白（FC）或所述含瓜氨酸的聚丝蛋白衍生物（FC）或所述含瓜氨酸的肽（PFC）的聚丝蛋白肽（PFNC）；所述生物样品与所述聚丝蛋白（FNC）或所述聚丝蛋白衍生物（FNC）或所述肽（PFNC）和所述聚丝蛋白（FC）或所述聚丝蛋白衍生物（FC）或所述肽（PFC）在适于形成免疫的条件下接触与自身抗体复合物；检测和定量在样品中存在的自身抗体或其他抗体与

所述聚丝蛋白 (FNC) 或所述聚丝蛋白衍生物 (FNC) 或所述肽 (PFNC) 和所述含瓜氨酸的聚丝蛋白 (FC) 或所述瓜氨酸之间形成的免疫复合物 - 含有聚丝蛋白衍生物 (FC) 的所述肽 (PFC) , 所述定量分别用XNC和XC的值表示 , 并且将XNC的值减少到XC的值。

TABLE 2							
Results as OD at 492 nm of the ELISA assays on citrulline-containing flaggrin (FC) and non-citrulline-containing flaggrin (FNC)							
Negative controls	FNC	FC	FC - FNC	RA sera	FNC	FC	FC - FNC
1	0.780	0.335	-0.445	64	0.777	0.434	-0.343
2	1.747	1.337	-0.410	65	0.202	0.113	-0.089
3	0.433	0.253	-0.180	66	0.204	0.124	-0.080
4	0.268	0.116	-0.152	67	0.222	0.158	-0.064
5	0.271	0.134	-0.137	68	0.221	0.158	-0.063
6	0.294	0.158	-0.136	69	0.195	0.141	-0.054
7	0.262	0.133	-0.129	70	0.255	0.202	-0.053
8	0.492	0.369	-0.123	71	0.131	0.098	-0.033
9	0.231	0.135	-0.096	72	0.109	0.077	-0.032
10	0.149	0.058	-0.091	73	0.115	0.091	-0.024
11	0.256	0.166	-0.090	74	0.099	0.077	-0.022
12	0.218	0.129	-0.089	75	0.131	0.109	-0.022
13	0.351	0.269	-0.082	76	0.072	0.059	-0.013
14	0.209	0.128	-0.081	77	0.157	0.147	-0.010
15	0.248	0.169	-0.079	78	0.055	0.051	-0.004
16	0.158	0.092	-0.066	79	0.090	0.088	-0.002
17	0.162	0.097	-0.065	80	0.104	0.102	-0.002
18	0.188	0.124	-0.064	81	0.079	0.079	0.000
19	0.174	0.113	-0.061	82	0.466	0.472	0.006
20	0.173	0.113	-0.060	83	0.124	0.132	0.008
21	0.219	0.159	-0.060	84	0.137	0.151	0.014
22	0.171	0.116	-0.055	85	0.137	0.156	0.019
23	0.179	0.130	-0.049	86	0.101	0.121	0.020
24	0.172	0.124	-0.048	87	0.372	0.409	0.037
25	0.123	0.076	-0.047	88	0.236	0.276	0.040
26	0.139	0.093	-0.046	89	0.137	0.207	0.070
27	0.153	0.111	-0.042	90	0.057	0.157	0.100
28	0.120	0.079	-0.041	91	0.514	0.667	0.153
29	0.113	0.073	-0.040	92	0.058	0.299	0.241
30	0.181	0.142	-0.039	93	0.134	0.387	0.253
31	0.126	0.088	-0.038	94	0.240	0.535	0.295
32	0.204	0.167	-0.037	95	0.156	0.496	0.340
33	0.096	0.060	-0.036	96	0.122	0.524	0.402