Abstract: Autoantibodies reacting against citrullinated peptides derived from C-telopeptides of type I and type II collagens are found in patients with rheumatoid arthritis. They detect sequences -YYXA from a1 or -FYXA from a2 chain of type I collagen or -YMXA from a1 chain of type II collagen, where X is citrulline. The antibodies are different from anti-filaggrin antibodies. The peptides of the invention can be used in diagnosis of rheumatoid arthritis. Oral administration of citrullinated peptides can induce tolerance and lead to the treatment of rheumatoid arthritis.
Method for detecting autoantibodies formed in rheumatoid arthritis

The invention relates to a method for detecting autoantibodies found in patients with rheumatoid arthritis (RA).

Rheumatoid arthritis is a common autoimmune disease affecting about 1% of the world population. It is difficult to recognize in the early stages, and the disease subsequently proceeds to irreversibly damage in the joints by destroying the cartilage and leading to the erosion of the periarticular bones.

Citrulline is an amino acid that is post-translationally formed from arginine in peptides or proteins by the enzyme peptidylarginine deiminases (PADs). This structure has attracted considerable attention in rheumatology, as several autoantibodies in RA patients are directed to proteins that contain citrulline. The first such autoantibody was the antiperinuclear factor (APF) described by Neinhuis and Mandema as early as 1964 (Ann Rheum Dis 1964; 23: 302-305). This antigen is present in the keratohyaline granules surrounding the nucleus of human buccal mucosa cells. In 1979, Young et al (BMJ 1979; 2: 97-99) discovered an antikeratin antibody (AKA) by indirect immunofluorescence using sections of rat oesophagus. Schellekens et al (J Clin Invest 1998; 101: 273-281) and Girbal-Neuhauser et al (J Immunol 1999; 162: 585-594) later showed that both APF and AKA specifically bind (pro)filaggrin, which contains citrulline residues. These autoantibodies are more specific for RA than certain traditional autoantibodies, e.g. the rheumatoid factor, which constitutes one of the classification criteria proposed by the American College of Rheumatology (Arnett et al., Arthritis Rheum 1988; 31: 315-324). On the other hand, it has been speculated that, because of their specificity, the antibodies to citrullinated proteins could be involved in the aetiology and/or pathogenesis of RA (van Boekel et al. Arthritis Res 2002; 4: 87-93). However, the fact that the production of anti-citrullinated filaggrin antibodies precedes disease onset contradicts the simple hypothesis that citrullination and/or anti-citrullinated filaggrin antibodies are pathogenic on their own (Yamada et al. Front Biosci 2005; 10: 54-64). E.g. deep destruction of cartilage and erosion of bone in periarticular tissue cannot be induced by these autoantibodies.

The bindings of autoantibodies to citrullinated proteins can be detected by several methods. The immunostaining methods used at first were not suitable for routine use in the
clinical laboratory, but the introduction of enzyme-linked immunosorbent assays (ELISA), later with cyclic, citrullinated peptides as antigens (anti-CCPs), has greatly simplified the determination of such antibodies with good reproducibility (Schellekens et al. et al: Arthritis Rheum 2000; 43: 155-163). The presence of anti-CCP antibodies has been reported to predict the development of RA in apparently healthy individuals (Rantapaa-Dahlqvist et al. Arthritis Rheum 2003; 48: 2741-2749 and Nielen et al. Arthritis Rheum 2004; 50: 380-386) and the progression of undifferentiated arthritis to RA (van Gaalen et al: Arthritis Rheum 2004; 50: 709-715). Possible clues concerning the pathogenetic role of these antibodies include an association of citrullination with apoptosis, the appearance of anti-CCP antibodies before the occurrence of clinical symptoms and their specificity for RA and the suggested genetic risk factor that leads to increased citrullination associated with RA (see van Gaalen FA et al. Arthritis Rheum 2004; 50: 709-715).

There are no anti-CCP antigens in joints, which means that any autoantibodies reacting with these antigens most probably only reflect an immunological cross-reaction (Yamada et al. Front Biosci 2005; 10: 54-64). Possible candidates for the original immunogen include citrullinated α and β chains of fibrin in synovial tissue (Masson-Bessiere et al: J Immunol 2001; 166: 4177-4184) and the Sa antigen, identified as citrullinated vimentin (Vossehaar at al. Arthritis Res Ther 2004; 6:R142-R150).

It has long been supposed that autoimmunity against collagens might be involved in the pathogenesis of RA. However, the evidence is only circumstantial. In certain animal species, e.g. rats, mice, rabbit and monkeys, immunisation with type II collagen results in the development of polyarthritis that resembles human RA. Type II collagen is post-translationally modified, and some of these modifications (e.g. glycosylations of lysine at position 264) may be recognised by T cells (Holmdahl et al. Ageing Res Rev 2002; 1: 135-147). Anti-collagen antibodies are not formed only against cartilage collagen, but also against bone (type I collagen) and soft tissue collagens (e.g. type III and V collagens) (Stuart et al. Arthritis Rheum 1983; 26: 832-840). Antibodies against denatured collagens are more frequent and present in higher concentrations in RA sera than antibodies against native collagens (Nijenhuis et al. Clin Chem Acta 2004; 350: 17-34 and Nomura K: Arch Biochem Biophys 1992; 293: 362-369). These anti-collagen antibodies are by no means specific for RA, and their formation could be secondary to the destruction of connective tissues rather than a cause of the disease.
Several years ago many papers were published dealing with the treatment of rheumatoid arthritis with orally administered type II collagen (Trentham et al. Science 1993;262:1727-1730). However, the experiments did not show any benefit (Cazzola et al. Clin Exp Rheumatol 2000; 18:571-577) or only small disease improvement of RA (Choy et al. Arthritis Rheum 2001;44:1993-1997), and interest in this hypothesis has since been lost. Burckhardt et al. (Eur J. Immunol 2005. 35: 1643 - 1652) tested the hypothesis, whether collagen II (CII) might be a substrate for structural modification by PAD enzymes, which could result in a breakage of self tolerance to this cartilage—specific auto-antigen. The results gave evidence that PAD2 (PAD isotype 2) catalyzes conversion of arginine to citrulline in a CII region of immunodominance for autoantibody formation and showed the presence of circulating autoantibodies that specifically bind to the citrullinated triple helical collagen peptide CII amino acid residues 359-369 in early RA patients. According to Burckhardt et al. their results indicate that autoimmune recognition of citrullinated collagen is a frequent event in early RA. The authors further noted that the IgG antibody to citrullinated CII correlates with the occurrence of anti-CCP autoantibodies, which are used as early markers of RA.

Suzuki et al. (Bioch Biophys Res Commun 2005;33:418-426) found out that human type I collagen could be one of the autoantigens, using a RA synoviocyte cDNA library by immunoscreening. They tested the hypothesis, whether type I and type II collagen could be used as substrates for citrullination. In contrary to the findings of the previous paper (Burckhardt et al. Eur J Immunol 2005; 35:1643-1652), there were no specific antibodies against citrullinated type II collagen in RA, but antibodies were found against citrullinated type I collagen.

The anti-CCP autoantibodies, although very specific for rheumatoid arthritis, are probably harmless, since they cannot be responsible for the destruction of cartilage and periarticular bone. The first assay published for anti-CCP (mark 1) detects citrullinated filaggrin, which has a sequence of -STXG-, where X is citrulline (Schellekens et al: Arthritis Rheum 2000;43:155-163). The second assay published for anti-CCP (mark 2) is based on several peptide sequences that have been selected from randomly generated synthetic peptides on the basis of discriminating between RA and control sera (Zendman A et al. Rheumatology
The sequences have not been published, but they are not related to either filaggrin or any other proteins found in human bodies.

Lundberg et al. (Arthritis Res Ther 2005;7:R 458-R467) examined the responses of rat T and B cells to citrullinated rat serum albumin (Cit-RSA) in comparison with those of unmodified rat serum albumin (RSA). It was found out that Cit-RSA leads to breakdown of immunological tolerance, since antibodies were produced against both Cit-RSA and RSA antigens. However, RSA alone did not induce any antibodies. Citrullinated collagen II (Cit-CII) induced arthritis with higher incidence and earlier onset than did the native counterpart. The amount of citrullinated proteins and the enzyme PAD4 correlated, according to Lundberg et al, with severity of inflammation and were not detectable in healthy joints.

Examples of known collagen amino acid sequences can be found in databases, for example in databases EMBL-EBI, AC Q8N473, OI-OCT-2002, UniProtKB/TrEMBL, Alpha 1 type I collagen, preprotein, EMBL-EBI, AC Q7Z5S6, OI-OCT-2003, UniProtKB/TrEMBL, Alpha 2 type I collagen, EMBL-EBI, AC Q96IT5, OI-DEC-2001, UniProtKB/TrEMBL, COL2A1 protein (Collagen type II alpha 1).

An assay for the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) has been shown to reflect increased type I collagen degradation in some pathological conditions as rheumatoid arthritis. The antigenic determinant recognized by the ICTP assay was shown by Sassi M.-L. et al. (Bone, April 2000; 26(4):367 -373 to reside within the hydrophobic phenylalanine-rich regions of the carboxyterminal telopeptides of the two α1 chains of human type I collagen (AGFDFSFL in human type I collagen). Nordic Bioscience diagnostics CrossLaps ® ELISA http://www.nbdiagnostics.com assays can be used for assessment of bone resorption. It detects collagen type I fragments generated during osteoclastic bone resorption and employs monoclonal antibodies recognizing C-telopeptide fragments of collagen type I α1 chains containing the epitope Glu-Lys-Ala-His-Asp- β-Gly-Gly-Arg in an isomerised form.

There are several patent publications, which disclose peptides suggested for use in diagnosing rheumatoid arthritis. International Patent Publication WO 95/081 15 describes a
method for determining collagen fragments in a body fluid. The method uses synthetic peptides derived from collagen and containing potential sites for cross-linking. Also in US 6,355,442 a method is disclosed for determining collagen fragments. International Patent Publication WO 03/050542 describes a method for detecting autoantibodies from patients suffering from rheumatoid arthritis. The method comprises the use of a peptide unit comprising XG motif and XnonG, wherein X is citrulline residue or an analogue thereof, G is the amino acid glycine and nonG is an amino acid other than glycine. International Patent Publication WO 2004/07898 disclose citrullinated peptides having increased affinity to a MHC class II molecules with the shared epitope. Such peptides are suggested to be used in diagnosing rheumatoid arthritis. International Patent Publication WO 2004/087747 disclose linear citrullinated synthetic peptides and multiple antigen peptides comprising them. The peptides are also suggested to be used in diagnosing rheumatoid arthritis.

It is an aim of the present invention to provide an improved method for diagnosing rheumatoid arthritis. It is also an aim of the present invention to provide a new method for the treatment of rheumatoid arthritis and for monitoring such treatment.

SUMMARY OF THE INVENTION

One object of the present invention is a method for detecting autoantibodies associated with rheumatoid arthritis. The method can be used in the diagnosing of the disease.

The method is based on the finding that in patients having rheumatoid arthritis autoantibodies were formed against parts of collagen peptides. In particular, such parts of collagen peptides are telopeptides, more specifically the carboxyterminal telopeptides. It was found that the C-telopeptides comprised peptide sequences, which were selected from the group of —YYXA, —FYXA, and —YMXA, where X stands for arginine converted to citrulline. —YYXA was found in α1 chain of type I collagen, —FYXA was found in α2 chain of type I collagen and —YMXA was found in α1 chain of type II collagen. These autoantibodies were different from those detected with the sequence based on filaggrin. The anti-CCP method suggested in the prior art (Schellekens et al: Arthritis Rheum
One further object of the invention is peptides and pharmaceutical compositions for treating RA. It was found in the present invention that parts of citrullinated collagens can induce immunological tolerance against potentially harmful citrullination of collagens. RA patients could thus be treated by administering them parts of citrullinated collagens. In particular, such parts of collagen were from telopeptides, more specifically the carboxyterminal telopeptides. It was found that the C-telopeptides comprised peptide sequences, which were selected from the group of —YYXA, —FYXA, and —YMXA, where X stands for arginine converted to citrulline.

The prior art publications do not disclose studies of the role of collagen telopeptides in autoantibody formation in patients having RA. The reason is that such autoantibodies have mainly been tested by using collagen preparations rendered soluble by pepsin digestion. This protease removes the carboxy telopeptides of collagens and the authors of the prior art publications thus could not make any observances of telopeptides.

Furthermore, the present invention can be used to monitor patients with RA treated with peptides of the present invention.

The assays developed in this study can be used both to detect the autoantibodies and diagnose RA in patients and to monitor patients with RA treated with peptides of the present invention.

Anti-CCP method functions well in diagnosis of rheumatoid arthritis. However, the autoantibodies detected by the method cannot be responsible for cartilage and bone degradation. Therefore, it is important to recognize those patients who need the most aggressive treatment with drugs.

Many drugs used for treating patients having or suspected to have rheumatoid arthritis cause several side effects. Therefore, the drugs cannot be given to all patients. If a patient is shown to have antibodies against citrullinated collagens, this is a reason to treat the patient in a most aggressive manner. The most recent drugs are also very expensive, which
prevents their broad use. By using the method of the invention those patients who need the most effective treatment can be recognized and the treatment can be directed to the right groups of patients.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the sequences of and locations of the chosen peptides in the primary structures of human type I and II collagens.

Figure 2. A serum sample was tested on three different ELISAs: binding of citrullinated carboxytelopeptides of the α1 chain of type I (A) and II collagen (B) and reaction in the anti-CCP 2 assay (C).

DETAILED DESCRIPTION OF THE INVENTION

"Rheumatoid arthritis" (RA) is a prevalent autoimmune disease characterized by synovial inflammation and pannus formation, which can lead to cartilage and bone degradation.

Three classes of human collagen have been described. The first group contains several collagen types, for example types I, II, III and V. From these, type I collagen accounts for more than 90% of the organic matrix bone. Type II collagen is an abundant molecule in articular cartilage. The collagen types I and II are synthesized as procollagens, containing N-terminal and C-terminal propeptide sequences, which are attached to the core collagen molecules. When the propeptides are removed, the remaining core of the collagen molecules consists of a triple-helical domain having short terminal telopeptide sequences, which are non-helical.

By the term "autoantibodies" is meant antibodies, which are formed against arginine containing peptides or their citrullinated forms in a patient having rheumatoid arthritis. These autoantibodies are formed in association to cartilage and bone degradation.

In this invention it has been found that in particular the carboxyterminal telopeptides comprise sequences which can be used in detection of autoantibodies. Such sequences
have been found specifically from the α\textsubscript{l} chain of type I collagen, the α\textsubscript{2} chain of type I collagen and the α\textsubscript{l} chain of type II collagen. Furthermore, it has been found that peptide sequences comprising sequences from these carboxyterminal parts can be used in detection of autoantibodies, if the last arginine residue in the peptide sequence is converted to citrulline.

The length of the carboxyterminal telopeptide of the α\textsubscript{l} chain of type I collagen is 26 amino acids. The length of the carboxyterminal telopeptide of the α\textsubscript{2} chain of type I collagen is 17 amino acids and the length of the carboxyterminal telopeptide of the α\textsubscript{l} chain of type II collagen is 27 amino acids.

The present invention encompasses in particular peptides which comprise the amino acid sequence of the C-terminal parts of the carboxyterminal telopeptides. Of special interest is that part of the telopeptide(s) which can induce the formation of an autoantibody complex when contacted with a biological fluid sample under suitable conditions for the formation of immunological complexes.

The peptide preferably ends with a sequence selected from the group comprising -YYXA, -FYXA and -YMXA, wherein X is citrulline.

According to the most preferred embodiment of the invention, the peptide comprises or has a sequence selected from the group comprising EKAHDGGRYYXA (SEQ ID NO:1), YDFGYDGDFYXA (SEQ ID NO:2), and EKGPDPLQYMXA (SEQ ID NO:3), where X is citrulline.

The expression "peptide sequence comprises a sequence derived from a carboxyterminal telopeptide" means that the peptide sequence comprises a sequence which is the same as that of the carboxyterminal telopeptide or has such amino acid changes that the peptide sequence still functions in the same manner. Preferably, the peptide sequence comprises a sequence which is the same as that of the carboxyterminal telopeptide.

The length of the peptide sequences of the present invention are preferably 10 to 30 amino acids, more preferably 12 to 27 amino acids. Preferably they are at least 12 amino acids, more preferably at least 17 amino acids, most preferably at least 26 amino acids.
The peptide sequences can be isolated from tissue collagens and the arginine can be converted to citruUine \textit{in vitro}. Purification can be achieved by protein purification methods well known for a person skilled in the art. Protein purification methods are, for example, chromatography methods, such as gel-filtration, ion-exchange and immunoaffinity, high-performance liquid chromatography (HPLC), high-performance chromatofocusing and hydrophobic interaction chromatography, or precipitation, in particular immunoprecipitation.

Arginine can be post-translationally modified to citruUine through deimination. This process is catalyzed by the enzyme peptidylarginine deiminase (PAD). The deimination process can be carried out \textit{in vitro}.

The peptide sequences can also be synthesized by methods well known for a person skilled in the art. Such methods are for example chemical synthesis methods, such as solid phase synthesis (Merrifiel, 1964. J. Am. Chem. Assoc. 65:2149; J. Amer. Chem. Soc. 85:2149 (1963) and Int. J. Peptide Protein Res. 35:161-214 (1990)) or the synthesis can be done in homogenous solution (Methods of Organic Chemistry, E. Wansch (Ed. Vol. 15 pts. I and II)Thieme, Stuttgart (1987).

The peptide sequences can also be prepared by recombinant DNA techniques well known for a person skilled in the art. The deimination process can be carried out \textit{in vitro} by using the enzyme peptidylarginine deiminase (PAD).

The body fluid sample is preferably a serum sample or a sample of another biological fluid, such as a synovial fluid sample.

The peptide sequences of the invention may be labelled to with a label in order to facilitate their detection in various assays. Examples of such labels are for example a radioactive label, a fluorescent label, a luminescent label, a lanthanide or an enzyme.

The immunologic reaction between the peptide sequence and the biological fluid sample may be carried out in a solid phase. The peptides may be covalently or non-covalently coupled to a solid carrier, such as a microphere of gold or polystyrene, a slide, a chip or a
wall of a microtitre plate. The peptide sequences can be bound directly or indirectly, for example via streptavidin, to the test tube or plate.

Alternatively, the immunologic reaction between the peptide sequence and the biological fluid sample may be carried out in a liquid phase and the peptide sequence may be bound directly or indirectly, for example via streptavidin, to solid particles, such as magnetic particles.

The present invention encompasses also a pharmaceutical composition. The pharmaceutical composition comprises an effective amount of at least one of the peptide sequences of the invention. The pharmaceutical composition is in biologically compatible form suitable for administration in vivo for patients. Such composition should not have any toxic effects. A pharmaceutical composition comprises an effective amount of peptide sequences and pharmaceutically acceptable additives. By additives is meant pharmaceutically acceptable carriers, such as gelatin, dextrin, pectin, agar, oil, saline, sucrose, lactose, calcium phosphate and water, stabilizers, such as carbohydrates (for example mannitol, starch, sucrose, dextrin, glucose and sorbitol), proteins (for example casein and albumin), and buffers. The pharmaceutical composition may comprise also one or more adjuvants.

"Effective amount" is an amount effective at dosages and for periods of time, necessary to achieve the desired result in a human. The desired result is the immune response in a human and may depend on the age, weight, health, sex or disease state. The pharmaceutical composition may be administrated in the amount of 1 - 50 µg/day. Preferably the amount is 1—25 µg/day. The amount of administration may depend on the route of administration, time of administration and may be varied depending on the immune response of an individual.

Suitable administration routes are subcutaneous injections, intramuscular injections, intravenous injections or intraperitoneal injections, oral and intranasal administration. Most suitable routes are oral administration or injection.
A method for treating rheumatoid arthritis comprises that to a person in need of such treatment is administrated an effective amount of at least one of the peptides of the invention. The administration can be given in the form of a pharmaceutical composition.

A method for inducing immunological tolerance against type I and/or type II collagen, comprises that to a person is administrated an effective amount of at least one of the peptides of the invention.

A kit comprises at least one peptide according to the invention. The test kit can be used in the diagnostic or monitoring method according to the invention.

According to a preferred embodiment of the invention the autoantibodies in a biological fluid sample can be detected by using a solid substrate, such as microtitration plate, coated with the citrullinated peptides of the invention. A sample of a biological fluid is placed in contact and incubated with the peptides absorbed on the substrate. The non-bound material of the biological sample is removed by washing. An indicator antibody capable of binding any antibodies present in the citrullinated peptide/anti-citrullinated peptide antibody complex is added to the solid substrate. The indicator may be a anti-human IgG immunoglobulin. The presence of the citrullinated peptide/anti-citrullinated peptide antibody/indicator antibody complex on the solid substrate is detected. The detection may be based for example on chemiluminesence and the emitted light measured by luminometer.

According to another preferred embodiment of the invention the assay is carried out by ELISA method, where the indicator antibody is conjugated to an enzyme. Suitable enzymes are for example enzymes which can be detected with the use of a chromogenic substrate. Such enzymes are for example alkaline phosphatase, horseradish peroxidase and β-galactosidase. The chromogenic substrate gives rise to a coloured product as a result of the reaction with the enzyme. The coloured product can be detected spectrofotometrically.

The presence of autoantibodies in a biological fluid sample can be detected as an immunological reaction with the peptides of the invention by using for example an ELISA method or a chemiluminesence immunoassay. The abundance of autoantibodies in a biological fluid can be measured as the intensity of the absorbance by spectrofotometer or
the light of a chemiluminescence reaction quantified by luminometer. The treatment of RA with the peptides of the invention can be monitored by comparing the amount of autoantibodies in a patient before and after the treatment.

Since there are also antibodies against normal type I and type II collagens in rheumatoid arthritis, for detecting specific antibodies against citrullinated forms two assays should be performed using both arginine and citrulline containing peptides of the type I or type II collagen carboxytelopeptides. The absorbance of arginine containing peptides is subtracted from the absorbance of citrulline containing peptide or the binding ratio of citrulline and arginine containing peptides is calculated. Alternatively it is possible to use only the assay version with the citrullinated peptide, performing it both with and without addition of the same or similar peptide in soluble form as inhibitor of the immunological binding.

Examples are given below to illustrate the present invention in further detail, but the scope of the present invention is not limited by these examples.

**EXAMPLE 1**

**ELISA assay**

The serum samples from 120 patients with RA were obtained from the Division of Rheumatology of Oulu University Hospital. The controls consisted of 81 sera from healthy people matched for age and sex. Six pairs of biotinylated peptides were synthesised by NeoMPS (Strasbourg, France). Figure 1 shows the sequences and locations of the chosen peptides in the primary structures of human type I and II collagens.

Fig IA-C show the localization of the peptides CCl - CC4 in type II collagen and their sequences; the numbers in brackets refer to arginine residues: CCl (1059), CC2 (1048), CC3 (799) and CC4 (28). Collagenase cleavage site is shown by arrow in Fig. IA -GPPGPQGILAGQQRGE- (SEQ ID NO:9). Fig. 1B shows the sequences CCl EKGPDPLQYMXA (SEQ ID NO:3), CC2 SAFAGLGPXEKGD (SEQ ID NO:6), CC3 LAGQXGIVGLP (SEQ ID NO:7) and CC4 GPGPXGPPGPA (SEQ ID NO:8); X is arginine/citrulline. Fig. 1C shows detailed structure of the carboxy terminal telopeptide of type II collagen (GPP belongs to the helix); C-telopeptide of type II collagen has the sequence GPGIDMSAFAGLGPXEKGPDPLQYMXA. The sequence
GPPGPGIDMSAFAGLGPREKGPDPLQYMXA is shown in the sequence listing as SEQ ID NO: 10. The 12 carboxy terminal amino acids represent the peptide CCl.

Fig 1D shows the sequences of the carboxyterminal telopeptides of the α1 (EKAHDGGRYYXA; SEQ ID NO:1) and α2 (YDFGYDGDFYXA; SEQ ID NO:2) chains of type I collagen. One member of each pair contained the arginine predicted by the respective gene, whereas in the other member of the pair this was replaced by citrulline.

The biotinylated peptides were coupled to streptavidin-coated 96-well assay plates (BioBind Assembly, Thermo Labsystems Oy, Vantaa, Finland) at a concentration of 10 μg/well. The coupling was performed at room temperature, pH 7.5, for two hours. The streptavidin-coated wells had been blocked by the manufacturer to prevent unspecific binding.

The sera to be tested were diluted in assay buffer (10mM Tris-HCl, 350mM NaCl, 1% BSA, 1% [vol/vol] Triton X-100, 0.5% [wt/vol] Na-deoxycholate, 0.1% SDS; pH 7.6) supplemented with 1% rabbit serum and incubated for an hour at room temperature. After washing (3 times with PBS/0.05% [vol/vol] Tween-20), 100 μl of anti-human IgG conjugated to peroxidase (Product # 31412, Pierce, Rockford, IL, USA) diluted 1:7500 in EIA buffer (20mM Tris-HCl, 150mM NaCl, 0.1% BSA, 0.05% Tween-20, pH 7.5) was added. After incubation for an hour at room temperature, the plates were washed (3 times with PBS/Tween-20). The bound antibodies were detected with 3,3’-5,5’-tetramethylbenzidine (Sigma-Aldrich, St.Louis, MN, USA) as a substrate (0.01 mg/100 μl per well in 100 mM sodium acetate trihydrate, 1.5 mM citric acid monohydrate, 0.0015 % H₂O₂). After 30 minutes, the reaction was stopped by adding 100 μl 2M sulphuric acid/well. The plates were read at a wavelength of 450 nm in a Victor² instrument (Wallac, Turku, Finland) and calculated by Multicalc (Wallac). All the sera were tested in duplicate. The coefficients of variation were generally less than 10 %.

When the single peptides were analysed directly, 42 % - 53 % of the patients with RA showed increased binding especially of the peptides derived from type II collagen (table 1).
Table 1. Binding of the peptides to human sera in ELISA.

<table>
<thead>
<tr>
<th></th>
<th>Controls mean ± SD absorbance</th>
<th>Controls over mean + 2 SD number</th>
<th>RA patients over mean + 2 SD number</th>
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<tr>
<td>C-telopeptide of α1(I)</td>
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<tr>
<td>10</td>
<td>Arginine peptide</td>
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<tr>
<td></td>
<td>Citrulline peptide</td>
<td>0.314 ±0.1 17</td>
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<td>0.763 ±0.356</td>
<td>3 /81</td>
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<td>0.184 ±0.071</td>
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ns not significant,  ** p < 0.01 and *** p < 0.001 compared with controls

When each peptide pair was being tested, the means (absorbance value of citrullinated peptide minus that of arginine peptide) and variances of the differences of the control samples were -0.026 (SD 0.032) for CCl, -0.027 (0.029) for CC2, -0.001 (0.026) for CC3 and +0.005 (0.020) for CC4. There was no specific binding to the citrullinated forms of the CC3 and CC4 of the RA sera. However, peptide CC2 and especially peptide CCl in the RA patients showed differences, since two sera in the peptide CC2 group and 12 sera in the peptide CCl group bound more citrullinated peptide than normal peptide. For the α1 chain of type I collagen, the means (absorbance value of citrullinated peptide minus that of arginine peptide) and the variances of the differences of the control samples were around +0.026 (0.074). With the C-telopeptide of the α2 chain from type I collagen, the means and variances of the differences between the control samples were +0.071 (0.252). 20 RA sera bound the citrullinated carboxytelopeptide from the α1 chain of type I collagen (α1(I) telopeptide) more strongly than the respective arginine peptide.
We found that the peptide CCl and, to a lesser extent, the peptide CC2 specifically bound autoantibodies against citrullinated proteins. These results indicate that the arginine near the carboxyterminal end susceptible to the action of PAD enzyme.

EXAMPLE 2

Chemiluminescence assay

The measurements were done with two-site chemiluminescence immunoassays, which detect IgG antibodies against the synthetic C-telopeptides of the α1 chains of human type I and II collagens (EKAHDGGRYYRA (SEQ ID NO:4), and EKGPDPLQYMRA (SEQ ID NO:5), or EKAHDGGRYYXA (SEQ ID NO:1), and EKGPDPLQYMXA (SEQ ID NO:3), respectively, where X stands for citrulline, from NeoMPS, Strasbour, France). The serum samples were first diluted 1:10 in a buffer containing 10mM Tris-HCl, 350mM NaCl, 1% BSA, 1% [vol/vol] Triton X-100, 0.5% [wt/vol] Na-deoxycholate, 0.1% SDS; pH 7.6 and incubated with suitable concentrations of one of the above peptides (in biotinylated form) and streptavidin-coated magnetic particles for 10 minutes at 37°C. The unbound biotinylated antigen and antibodies were separated from the complex bound to the magnetic particles by aspiration of the reaction mixture and subsequent washing. Thereafter, acridinium-labeled anti-human IgG antibodies were added to the reaction mixture followed by another 10-minute incubation to produce the sandwich complex. The unbound label was separated by aspiration of the reaction mixture and subsequent washing. To the washed magnetic particles with the complex we injected the triggers 1 and 2, initiating the chemiluminescence reaction. The trigger 1 solution contained hydrogen peroxide in diluted acid, and the trigger 2 solution contained diluted sodium hydroxide.

Serum samples from 120 patients with early RA were obtained from the Division of Rheumatology of Oulu University Hospital. The controls consisted of 81 sera from age- and sex-matched healthy persons.

In 44 RA patients out of 120, the sera showed increased binding of citrullinated synthetic C-telopeptide derived from the α1 chain of type I collagen (p = 0.003 compared to controls). For a corresponding C-telopeptide pair from the α1 chain of type II collagen, 35 patients’ sera bound the citrullinated peptide more strongly than the arginine peptide, but
the difference compared to the controls was not significant \( p = 0.074 \). The correlation between the two carboxytelopeptides was \( r = 0.473 \) \( p < 0.001 \).

EXAMPLE 3

The specificity of autoantibodies in sera of rheumatoid arthritis patients

For detecting the antibody specificity we tested both type I and II collagen telopeptide assays and an anti-CCP Mark2 assay kit. Patient sera were diluted in assay buffer so that in each assay the initial binding could be noticeably inhibited with the respective peptides. Serial dilutions of competing peptides (arginine and citrulline forms of collagen C-telopeptides of \( \alpha 1(1) \) and \( \alpha 1(II) \)) were added (Figure 2). Inhibition by the soluble form of the same peptide as that coupled to the plate served as a specificity control in both telopeptide assays. For anti-CCP assay no such peptide was available for specificity testing. The percentage inhibition was plotted against the soluble peptide concentration. The signal (wavelength of 450 nm) obtained with the human serum only (initial binding) was defined as 0 % inhibition, and the signal of the blank (no serum sample) was defined as 100 % inhibition. We inhibited the binding with either the normal or the citrullinated antigen in soluble form. The soluble telopeptide antigens inhibited the binding in both type I and type II collagen telopeptide assays (Figure 2A and 2B).

Figure 2. Competition assays. One serum sample was tested in three different ELISAs: citrullinated carboxytelopeptide assays of the \( \alpha 1 \) -chain of type I (A) and II collagens (B) and anti-CCP assays (C). The inhibitors were EKAHDGGRYYRA (SEQ ID NO:4), (open triangles), EKAHDGGRYYXA (SEQ ID NO: 1), (closed triangles, corresponding to the immobilized antigen in the type I collagen assay), EKGPDPLQYMRA (SEQ ID NO:5), (open square) and EKGPDPLQYMXA(SEQ ID NO:3), (closed square, corresponding to the immobilized antigen in the type II collagen assay).

Thus it seems that identical or similar antibodies are involved in the binding. However, the collagen telopeptides bind a different antibody species than does the anti-CCP assay, since the anti-CCP binding cannot be inhibited with these peptides (Figure 2C).
Although the autoantibodies against collagen are different from those detected with the anti-CCP assay, both autoantibodies are increased in patients with rheumatoid arthritis. As shown in table 2, there was a significant correlation between these autoantibodies.

Table 2.
Contingency coefficients between antibody binding to citrullinated C-telopeptides from type I (α1 and α2 chains) and type II (α1 chain) collagens and the anti-CCP assay results (based on sera from 120 rheumatoid arthritis patients). *** p < 0.001

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EXAMPLE 4

Inhibition assay

Since there are also antibodies against normal type I and type II collagens in rheumatoid arthritis, for detecting specific antibodies against citrullinated forms two assays should be performed using both arginine and citrulline containing peptides of the type I or type II carboxytelopeptides. The absorbance of the reaction with arginine containing peptides is then subtracted from that of the reaction with the respective citrulline containing peptides, or the binding ratio of citrulline and arginine containing peptides is calculated.
Preferentially it is also possible to use only citrullinated peptide assay version, where this assay is performed with standard condition and with adding the same or similar soluble peptide in assay solution (200 µg/ml).

Sera from RA patients (n = 120) and controls (n = 80) were diluted 1:100 in assay buffer and/or inhibiting buffer (with soluble citrulline containing peptide (200 µg/ml)). The inhibition time was 30 minutes. After the inhibition reaction, the sera were moved to the wells of streptavidin-coated 96-well assay plates to which the biotinylated citrulline containing peptides had been coupled. After washing (3 times with PBS/0.05% [vol/vol] Tween-20), 100 µl of anti-human IgG conjugated to peroxidase (Product # 31412, Pierce, Rockford, IL, USA) diluted 1:7500 in EIA buffer (2OmM Tris-HCl, 15OmM NaCl, 0.1% BSA, 0.05% Tween-20, pH 7.5) was added. After incubation for an hour at room temperature, the plates were washed (3 times with PBS/Tween-20). The bound antibodies were detected with 3,3'-5,5'-tetramethyl-benzidine (Sigma-Aldrich, St.Louis, MN, USA) as a substrate (0.01 mg/100 µl per well in 100 mM sodium acetate trihydrate, 1.5 mM citric acid monohydrate, 0.0015 % H₂O₂). After 30 minutes, the reaction was stopped by adding 100 µl 2M sulphuric acid/well. The plates were read at a wavelength of 450 nm in a Victor² instrument (Wallac, Turku, Finland). All the sera were tested in duplicate. The coefficients of variation were generally less than 10 %.

With type II collagen carboxytelopeptide in an ELISA experiment, the inhibition-% with the respective soluble antigen in controls was 5.4 % ± 3.8 (mean ± SD), and in RA patients 38 out of 120 showed increased inhibition (more inhibition than 13.0%).

Thus it is sufficient to make the assays with citrullinated forms of telopeptides, if in addition is confirmed that these autoantibodies can be inhibited with corresponding soluble antigens. This modification reduced the variance, since only one assay is used, and surprisingly more positives were found than with test using two ELISA assays.
What is claimed is:

1. A method for detecting autoantibodies formed in relation to rheumatoid arthritis, which comprises the steps:
   - contacting a peptide or peptides with a biological fluid sample under suitable conditions for the formation of an immunological complex or complexes; and
   - detecting and optionally measuring the amount of autoantibodies formed, wherein the peptide is selected from the group comprising:
     - a peptide which comprises a sequence derived from a carboxyterminal telopeptide of α1 chain of type I collagen,
     - a peptide which comprises a sequence derived from a carboxyterminal telopeptide of α2 chain of type I collagen,
     - a peptide which comprises a sequence derived from a carboxyterminal telopeptide of α1 chain of type II collagen,
   and wherein at least the last arginine residue in the peptide sequence is converted to citrulline.

2. The method according to in claim 1, wherein the peptide(s) ends to a sequence selected from the group comprising -YYXA, -FYXA and -YMXA, wherein X is citrulline.

3. The method according to in claim 1 or 2, wherein the peptide(s) is/are isolated from tissue collagens and the arginine(s) is/are converted to citrulline(s) in vitro.

4. The method according to claim 1 or 2, wherein the peptide(s) is partly or fully a synthetic peptide.

5. The method according to any one of the preceding claims, wherein the length of the peptide(s) is 10 to 50, preferably 12 to 30 amino acids.

6. The method according to any one of the preceding claims, wherein the peptide(s) comprises a sequence selected from the group comprising EKAHDGGRYYXA (SEQ ID NO:1), YDFGYDGDFYXA (SEQ ID NO:2), and EKGPDPLQYMXA (SEQ ID NO:3), where X is citrulline.
7. The method according to any one of the preceding claims, wherein the biological fluid sample is a serum sample or a synovial fluid sample.

8. The method according to any one of the preceding claims, wherein the peptide(s) comprises a label selected from the group comprising a radioactive label, a luminescent label, a fluorescent label, a lanthanide, and an enzyme.

9. The method according to any one of the preceding claims, wherein the immunologic reaction between the peptide and the biological fluid sample is carried out in a solid phase and the peptide is bound directly or indirectly to the test tube or plate or a corresponding solid phase.

10. The method according to any one of the preceding claims, wherein the immunologic reaction between the peptide and the biological fluid sample is carried out in a liquid phase and the peptide sequence is bound directly or indirectly to solid particles in the liquid.

11. The method according to any one of the preceding claims, wherein the autoantibodies are detected or their amount measured as the intensity of the absorbance in an ELISA assay.

12. The method according to any one of the preceding claims, wherein the autoantibodies are detected or their amount measured as the emitted light in a chemiluminescence assay.

13. The method according to any one of the preceding claims, wherein the autoantibodies are detected or their amount measured so that bound citrulline peptide is compared to bound arginine peptide.

14. The method according to any one of the preceding claims, wherein the autoantibodies are detected or their amount measured in assay solution comprising soluble citrulline peptides.

15. A citrullinated peptide selected from the group comprising
   - a peptide which comprises a sequence derived from a carboxyterminal telopeptide of \( \alpha_1 \) chain of type I collagen,
21. A peptide which comprises a sequence derived from a carboxyterminal telopeptide of α2 chain of type I collagen,
-a peptide which comprises a sequence derived from a carboxyterminal telopeptide of α1 chain of type II collagen,
and wherein at least the last arginine residue in the peptide sequence is converted to citrulline.

16. The citrullinated peptide according to claim 15, wherein the peptide ends to a sequence selected from the group comprising -YYXA, - FYXA and -YMXA, wherein X is citrulline.

17. The citrullinated peptide according to claim 15 or 16, wherein the length of the peptide peptide(s) is 10 to 50, preferably 12 to 30 amino acids.

18. The citrullinated peptide according to any one of claims 15 to 17, wherein the peptide sequence comprises a sequence selected from the group comprising EKAHDGGRYYXA (SEQ ID NO:1), YDFGYDGDFYXA (SEQ ID NO:2), and EKGPDPLQYMXA (SEQ ID NO:3).

19. A kit comprising at least one citrullinated peptide according to any one of claims 15 to 18.

20. A pharmaceutical composition comprising an effective amount of at least one of the peptides according to any one of claims 15 to 18.

21. The pharmaceutical composition according to claim 20, wherein the composition is suitable for oral administration or injection.

22. The pharmaceutical composition according to claim 20 or 21, wherein the composition is administrated to a patient in the amount of 1 - 50 µg/day, preferably 1 - 25 µg/day.

23. A method for treating rheumatoid arthritis, which comprises that to a person in need of such treatment is administrated an effective amount of at least one of the peptides according to any one of claims 15 to 18.
24. A method for inducing immunological tolerance against type I and/or type II collagen, which comprises that to a person in need of such treatment is administrated an effective amount of at least one of the peptides according to any one of claims 15 to 18.
Fig. 1

A. Collagenase cleavage-site

- GPPGPQG LAGQGGE -

B. 

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<td>CC4</td>
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X = arginine / citrulline

C. C-telopeptide of type II collagen

D. 

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X = arginine / citrulline
Procollagen Oy

Method for detecting autoantibodies formed in rheumatoid arthritis

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20050814
2005-08-11

PatentIn version 3.3

1
12
PRT

Homo sapiens

misc_feature
Xaa is citrulline

1

Glu Lys Ala His Asp Gly Gly Arg Tyr Tyr Xaa Ala
1 5 10

2

Tyr Asp Phe Gly Tyr Asp Gly Asp Phe Tyr Xaa Ala
1 5 10

3

Glu Lys Gly Pro Asp Pro Leu Gin Tyr Met Xaa Ala
1 5 10

4

Glu Lys Ala His Asp Gly Gly Arg Tyr Arg Ala
1 5 10

5
Homo sapiens

Glu Lys Gly Pro Leu Gin Tyr Met Arg Ala
1 5 10

Glu Lys Gly Pro Asp Pro Leu Gin Tyr Met Arg Ala
1 5 10

MISC_FEATURE
Xaa is arginine/citrulline

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1 5 10

Leu Ala Gly Gin Xaa Gly lile Val Gly Leu Pro
1 5 10

Gly Pro Met Gly pro Xaa Gly pro pro Gly Pro Ala
1 5 10

Gly Pro Gly pro Gin Gin Xaa Gin Arg Gly Gin
1 5 10

Gly Pro Gly pro Gin Gin Xaa Gin Arg Gly Gin Met
1 5 10

Gly Pro Gly pro Gin Gin Xaa Gin Arg Gly Gin Leu Gly
1 5 10

Xaa is arginine/citrulline
Pro Arg Glu Lys Gly Pro Asp Pro Leu Gin Tyr Met xaa Ala

20 25 30
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N 33/564

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data, EMBASE, BIOSIS, INSPEC, FSTA, COMPENDEX, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents

'A' document defining the general state of the art which is not considered to bifurcate

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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'Y' document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

'&' document member of the same patent family

Date of the actual completion of the international search 12 December 2006

Date of mailing of the international search report 21/12/2006

Name and mailing address of the ISA/Authorized officer

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NL - 2280 HV Rijswijk
TW (+31-70) 340-2040, Tx 31651 epo nl,
Fax (+31-70) 340-3016

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This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claims Nos.:**
   - because they relate to subject matter not required to be searched by this Authority, namely:
     
     Although claims 23-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. **Claims Nos.:**
   - because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. **Claims Nos.:**
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

1. **As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.**

2. **As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.**

3. **As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:**

4. **No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:**

**Remark on Protest**

- [ ] The additional search fees were accompanied by the applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.
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