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(54) **Diagnostic assay involving macrophage inhibitory cytokine-1 (MIC-1)**

Diagnostischer Assay mit makrophagenhemmendem Zytokin-1 (MIC-1)

Analyseur de diagnostic impliquant la cytokine-1 (MIC-1) inhibitrice des macrophages

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(73) Proprietor: **ST VINCENT'S HOSPITAL SYDNEY LIMITED**
Darlinghurst, NSW 2010 (AU)

(72) Inventors:
- **Breit, Samuel Norbert**
Gordon, NSW 2072 (AU)
- **Brown, David Alexander**
Bondi Beach, NSW 2026 (AU)

(74) Representative: **Harding, Charles Thomas**
D Young & Co LLP
120 Holborn
London
EC1N 2DY (GB)

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- **FAIRLIE W D ET AL: "MIC-1 IS A NOVEL TFG-BETA SUPERFAMILY CYTOKINE ASSOCIATED WITH MACROPHAGE ACTIVATION" JOURNAL OF LEUKOCYTE BIOLOGY, FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL, US, vol. 65, no. 1, January 1999 (1999-01), pages 2-5, XP000937688 ISSN: 0741-5400**

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Description

Field of the Invention:

[0001] This invention relates to the field of medical diagnostics. In particular, the invention provides methods for diagnosing rheumatoid arthritis.

Background to the Invention:

[0002] The Transforming growth factor-β (TGF-β) superfamily consists of an increasing number of molecules that regulate a variety of cellular processes such as growth, differentiation and oncogenesis. Members of the TGF-β superfamily have been classified into major family groupings which include TGF-β, bone morphogenic protein (BMP), growth and differentiation factor (GDF), inhibin/activin, mullerian inhibitory substance (MIS), glial derived neurotrophic factor (GDNF) and, more recently, macrophage inhibitory cytokine-1 (Bootcov et al., 1997). The involvement of the TGF-β superfamily in human pregnancy is indicated by the detection of TGF-β1, TGF-β2, TGF-β3, activin and inhibin in amniotic fluid and the localisation of TGF-β1, activin and inhibin to the placental villi (Graham et al., 1992; Petraglia et al., 1993a; Petraglia et al., 1992; Minami et al., 1992; Lang and Searle, 1994; Qu and Thomas, 1992; Altman et al., 1990; Canniggia et al., 1999; Wallace et al., 1997).

[0003] The TGF-β superfamily has been studied intensively because of their biological importance and therapeutic potential. Their biology and functions are well known and have been extensively reviewed (e.g. Miyazono et al., 1993; Wahl, 1992; and Roberts et al., 1993). They are potent chemotactic factors for macrophages and fibroblasts and generally inhibit cell proliferation, perhaps because of their role in differentiation. In the context of inflammation, TGF-β is a potent stimulator of fibroblasts, collagen and matrix protein synthesis, promotes angiogenesis, modulates expression of adhesion molecules and inhibits lymphocyte proliferation, production of some lymphokines and NK cell function. TGF-β proteins have also been heavily implicated in the pathogenesis of chronic inflammatory processes and mechanisms.

[0004] Rheumatoid arthritis is an autoimmune disease associated with chronic inflammatory processes. Methods of diagnosing and detecting autoimmune disorders typically rely on the onset of symptoms which characterise the disorder. More recently, studies have been performed to diagnose rheumatoid arthritis by measuring the levels of certain factors in patient samples. For example, WO 99/21011 discloses that in autoimmune disease such as rheumatoid arthritis, collagen types IX and XI, as well as collagen type II are expressed. Detecting and measuring the changes in distribution and levels of type IX collagen in a patient sample such as serum or synovial fluid, as well as type II collagen in some embodiments, may provide for a diagnostic assessment of the pathological states of autoimmune disorders (such as rheumatoid arthritis) and connective tissue disorders associated with Type IX collagen.

[0005] The present applicants have recently cloned and characterised a divergent member of the TGF-β superfamily, macrophage inhibitory cytokine-1 (MIC-1) (Bootcov *et al.*, 1997), whose expression is associated with macrophage activation. The present applicants have also developed a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for MIC-1 quantification and used this to investigate the relationship between human serum MIC-1 concentrations and inflammatory disease, such as rheumatoid arthritis.

[0006] In addition, the present applicants have found that a number of allelic variants of MIC-1 exist, all of which show minor amino acid sequence differences at positions 9, 48 and 202 (see International patent publication No. WO 97/00958 wherein MIC-1 is referred to as CL13). The most significant of these positions is amino acid position 202 since this corresponds to position 6 of the mature form of MIC-1 (i.e. with the leader sequence having been removed through cleavage). In some of the identified variants, the normal histidine (H) residue at position 202 (or "H6") is substituted with aspartic acid (D). This is due to a single nucleotide substitution within the MIC-1 gene such that a cytosine (C) at position 604 is substituted by a guanosine (G). The present applicants have now recognised that subjects which are either heterozygous or homozygous for the Asp^{202} -MIC-1 (or "D6") allelic variant may have an altered predisposition and disease course for inflammatory disease(s) and/or cancer(s).

Disclosure of the Invention:

[0007] The present applicants have found that elevated MIC-1 levels in certain body samples can be associated with rheumatoid arthritis (see Figure 8D). For example, examination of biopsies of subjects after treatment with a high dose of intravenously administered corticosteroid, showed a marked decrease in MIC-1 expression in infiltrating cells (see Figure 1).

[0008] Thus, in a first aspect, the present invention provides a method for the diagnosis of rheumatoid arthritis, said method comprising;

(i) determining the amount of MIC-1 present in a body sample from a test subject, and

(ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample (s) from normal subject(s) wherein elevated MIC-1 levels are associated with rheumatoid arthritis.

[0009] The body sample used in the method of the first aspect may be a sample of urine, cerebrospinal fluid, seminal fluid or tissue biopsy. However, preferably, the body sample is a sample of blood serum or plasma, or synovial fluid.

[0010] The amount of MIC-1 present in a body sample may be readily determined by immunoassays or immunohistochemistry (e.g. with sections from tissue biopsies) using antibodies (monoclonal or polyclonal) or fragments thereof against MIC-1. Anti-MIC-1 antibodies and fragments thereof can be produced by any of the methods known to the art.

[0011] In a second aspect, the present invention provides a method for the diagnosis or assessment of rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

[0012] In a third aspect, the present invention provides a method for assessing a predisposition to rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

[0013] The detection of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature wild type human MIC-1 is indicative of rheumatoid arthritis or a predisposition to rheumatoid arthritis.

[0014] The presence of the variant MIC-1 protein may be readily determined by immunoassay using antibodies, or fragments thereof, capable of discriminating between normal human or "wild type" MIC-1 or variants which have histidine at position 202 and MIC-1 variants which have aspartic acid at position 202. Such antibodies, or fragments thereof, may be raised with MIC-1 or Asp^{202}-MIC-1 using any of the methods commonly known in the art. Alternatively, suitably discriminating antibodies, or fragments thereof, may be raised using immunogenic peptides, optionally conjugated to a carrier protein such as bovine serum albumin, which include an epitope spanning position 202 of immature human wild type MIC-1 protein or, in a variant protein, spanning a position corresponding to position 202 of immature human wild type MIC-1. For instance, an antibody which specifically binds to Asp^{202}-MIC-1 may be raised using an immunogenic peptide comprising the amino acid sequence; Ala-Arg-Asn-Gly-Asp-Asp-Cys-Pro-Leu (SEQ ID NO: 7).

[0015] Preferably, the presence of a MIC-1 protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 is determined by immunoassay using an antibody which specifically binds to such a protein. However, where an antibody, or fragment thereof, is used which is specific for wild type MIC-1 and/or variants which have histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-1, the absence of any detectable binding, or a reduced level of binding, can be taken as being determinative of the presence of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1. For such assays, it is preferred that a positive control be conducted to ensure the presence of a MIC-1 protein in the sample (e.g. by immunoassay with a non-discriminatory antibody, or fragment thereof, which binds to both wild type and variant MIC-1 proteins).

[0016] It will be understood that subjects which are heterozygous or homozygous for a MIC-1 protein having the normal histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 may, conversely, show a reduced predisposition to inflammatory disease such as rheumatoid arthritis.

[0017] Thus, in a fourth aspect, the present invention provides a method for the diagnosis or assessment of rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

[0018] Assessment of rheumatoid arthritis includes assessment of disease course. For example, it has been found that of subjects suffering from rheumatoid arthritis, those which are heterozygous or homozygous for Asp^{202} -MIC-1 variant are likely to experience a worse degree of disease than subjects which are homozygous for wild type MIC-1 (and/or variants which have histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-1).

[0019] In a fifth aspect, the present invention provides a method for assessing a predisposition to rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-I in a suitable sample from said subject.

[0020] The DNA and amino acid sequences of human MIC-1 (i.e. "wild type") and the variant, Asp^{202}-MIC-1 are shown at Figure 2.

[0021] As used herein, "immature human wild type MIC-1" refers to MIC-1 protein having the amino acid sequence shown in Figure 2 as "MIC-1/H6", and "wild type MIC-1" refers to the mature form (i.e. with the leader sequence having been removed through cleavage) of that protein.

[0022] The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

[0023] Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

[0024] The invention will hereinafter be described with reference to the following non-limiting examples and accompanying figures.

Brief description of the accompanying figures:

[0025]

Figure 1 provides a graph showing serum MIC-1 levels in 14 unselected subjects with rheumatoid arthritis.

Figure 2 provides the amino acid sequences (A) and DNA sequences (B) of human MIC-1 and the variant, Asp^{202}-MIC-1.

Figure 3 provides a graph showing the sensitivity of sheep and mouse anti-MIC-1 antisera. Plates were coated with 1.8 ng rhMIC-1, 2-ng rhTGF-β1, or coating buffer alone. Culture supernatant containing an anti-MIC-1 mouse monoclonal antibody (MAb), culture media conditioned by the mouse myeloma cell line SP2/0, unconditioned culture media (DMEM+Nutridoma), and antibody diluant (Ab dil) were assessed undiluted while IgG enriched normal sheep serum and the sheep polyclonal antibody 233-P were diluted 1:500,000 in Ab dil. Mouse IgG1 was assessed at 20 ng/ml.

Figure 4 provides a recombinant human MIC-1 standard curve generated by sandwich ELISA utilising the anti-MIC-1 MAb for capture and the sheep polyclonal antibody 233B3-P for detection.

Figure 5 provides a typical standard curve from MIC-1 sandwich ELISA (rhMIC-1, 1000-7.8 pg/ml, i.e. 8 doubling dilutions).

Figure 6 shows restriction enzyme cleavage points for AvaII in wild type MIC-1 and Asp^{202}-MIC-1 DNA sequences.

Figure 7 shows a digest of a genomic PCR of six individuals labelled with indicated genotypes, confirmed by DNA sequencing. Run on 3% agarose gel with ethidium bromide. The 45 base pair product of the PCR can be seen in the homozygote H6 marked by an arrow.

Figure 8 shows immunohistochemistry for MIC-1 with 233-P. A. Prostate carcinoma; B. Bowel carcinoma; C. Breast carcinoma; D. Rheumatoid synovium. Arrows represent areas of MIC-1 staining. Panels on right are the respective controls stained with IgG enriched normal sheep serum.

Figure 9 shows MIC-1 levels in normal subjects compared with RA patients, as measured by MIC-1 sandwich ELISA.

Figure 10 provides a graph showing the proportion of erosive (black) versus non-erosive (white) RA among the two most common genotypes, homozygous (H6/H6) and heterozygous (H6/D6).

Figure 11 shows a graph of CRP compared to the presence or absence of erosive RA.

Figure 12 provides a graph showing CRP compared to the homozygous (H6/H6) and heterozygous (H6/D6) genotypes.

Example 1: Assessment of MIC-1 expression

METHODS:

Generation of MIC-1 antibodies:

[0026] A sheep anti-MIC-1 polyclonal antibody (PAb) 233B3 was generated by immunisation with recombinant human MIC-1 (rhMIC-1), which was synthesised in accordance with the method described in International patent publication No. WO 97/00958, in Complete Freunds Adjuvant. Additional boosts were given over a period of 6 months and the sheep were bled 10 days after the final injection. An enriched IgG fraction of normal sheep serum and 233B3 were prepared by caprylic acid precipitation followed by ammonium sulphate precipitation. The IgG enriched 233B3 fraction was designated 233-P.

[0027] A mouse anti-MIC-1 monoclonal antibody (MAb) secreting hybridoma was generated from mice immunised with rhMIC-1. Hybridomas were cultured in DMEM (Gibco BRL) containing 4.5 g/l D-glucose, 10 mg/l sodium pyruvate, 0.584 g/l L-glutamine, 4 mg/l pyridoxine hydrochloride supplemented with 20% FCS (CSL, Melbourne). For MAb collection, the hybridomas were transferred into fresh DMEM-hi glucose supplemented with Nutridoma-SR (Boehringer Mannheim) for 7 days. The culture supernatants were spun at 2000 rpm for 10 minutes to remove cell debris and frozen until

used. The sensitivity of the PAb and MAb preparations were examined by direct ELISA. Direct ELISA:

[0028] Ninety-six well Maxisorp ELISA plates (Nunc) were coated (100 μl/well) with either 18 ng/ml rhMIC-1 or 20 ng/ml rhTGF-β1 (R&D Systems) in coating buffer (0.1 M carbonate in distilled H_2O, pH 9.4-9.8) at 40°C for 24 hours. Plates were then washed three times with 300 μl of wash buffer (PBS containing 0.05% (v/v) Tween-20 (Sigma)) and non-specific binding was blocked with 250 μl of 1% (w/v) BSA (Boehringer Mannheim) in PBS for 2 hours at 37°C. Hybridoma serum-free media containing the anti-MIC-1 MAb, sheep PAb 233B3-P diluted 1:500,000 in antibody diluant (PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween-20), culture media conditioned by the mouse myeloma cell line SP2/0, DMEM+Nutridoma, immunoglobin G enriched normal sheep serum diluted 1:500,000 in antibody diluant, 200 ng/ml mouse IgG1 (R&D Systems) in DMEM+Nutridoma, or antibody diluant alone were then added to the plates (100 μl/well) and incubated for 1 hour at 37°C. The plates were washed three times followed by the addition of 100 μl/well of biotinylated donkey anti-sheep IgG (Jackson Immunoresearch) or biotinylated goat anti-mouse IgG (Jackson Immunoresearch) diluted 1:10,000 in antibody diluant and incubated for 1 hour at 37°C. The plates were washed three times and 100 μl/well of horseradish peroxidase-conjugated streptavidin (Genzyme) diluted 1:2000 in antibody diluant was added to the plates and incubated for 30 minutes at 37°C. Plates were washed four times followed by the addition of 100 μl/well of peroxidase substrate (1 mg/ml o-phenylenediamine dihydrochloride (Sigma) in 0.05M phosphate-citrate buffer containing 0.014% H_2O_2, pH5.0 (Sigma)). Colour development was allowed to proceed for 5-15 minutes and was terminated by the addition of 100 μl/well of 4N H_2SO_4. The absorbance was measured at 490 nm in a microplate reader (Pasteur Diagnostics).

MIC-1 sandwich ELISA:

[0029] A MIC-1 sandwich ELISA was established utilising the anti-MIC-1 mouse MAb for antigen capture and the sheep PAb 233-P for detection. The optimum concentration of both antibodies was determined empirically then used for all subsequent studies. Ninety-six well Maxisorp ELISA plates were coated with anti-MIC-1 MAb supernatant diluted 1:5 (final immunoglobin concentration was approximately 20 ng/ml) in coating buffer at 40°C for 24 hours. Plates were then washed three times with 300 μl of wash buffer and non-specific binding was blocked with 250 μl of 1% (w/v) BSA in PBS for 2 hours at 37°C. rhMIC-1 standards, tissue culture supernatant, or serum diluted in antibody diluant, were then added to the plates (100 μl/well) and incubated for 1 hour at 37°C. The plates were washed three times followed by the addition of 100 μl/well of the sheep PAb 233-P diluted 1:5000 in antibody diluant and incubated for 1 hour at 37°C. The plates were then washed three times and 100 μl/well of biotinylated donkey anti-sheep IgG diluted to 1:5000 in antibody diluant was added and incubated for 1 hour at 37°C. The plates were then developed as for the direct ELISA. The concentration of hMIC-1 in the samples was determined by comparison with the rhMIC-1 standard curve. The level of rhMIC-1 in this standard curve was determined on the basis of total protein content and thus in terms of absolute amount is subject to significant error. However, as the same standards were used throughout, this makes no difference to the relative values estimated in this example. All samples were assayed in triplicate on at least two occasions. Results are presented as the mean +/- SD. The sensitivity of the MIC-1 sandwich assay was assessed by testing with up to 500 pg/ml amounts of TGF-β1 and inhibin-A (which are both members of the TGF-β superfamily).

Immunoprecipitation:

[0030] Immunoprecipitation was carried out using 0.2 ml hybridoma serum-free media containing the anti-MIC-1 MAb adsorbed to protein-A Sepharose. Serum and medium samples (1 ml) were incubated with these antibodies overnight at 40°C then washed 5 times with PBS containing 1% (v/v) Triton X-100. Bound proteins were eluted using non-reducing sodium dodecyl sulphate (SDS)-sample buffer and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by immunoblot analysis with the sheep polyclonal antibody 233-P. Immunoblot analysis was performed essentially as described by Bootcov *et al.,* (1997) except that polyclonal antibody 233-P was used as primary antibody at a dilution of 1:7000 and the secondary antibody was donkey anti-sheep IgG-biotin at a dilution of 1:5000.

RESULTS:

Sensitivity of anti-MIC-1 PAb and MAb:

[0031] The ability of the sheep PAb 233-P and the mouse MAb to bind to rhMIC-1 was examined by direct ELISA. It was found that both undiluted tissue culture supernatant containing the MAb and the sheep PAb 233-P at a dilution of 1:500,000 in antibody diluant bound strongly to 1.8 ng immobilised rhMIC-1 (Figure 3). Neither culture media conditioned by the mouse myeloma cell line SP2/0, unconditioned culture media, mouse IgG1, immunoglobin enriched normal sheep serum, or antibody diluant reacted with rhMIC-1. Minimal background binding to uncoated wells was observed for all samples examined. No reactivity was detected when either the anti-MIC-1 MAb or polyclonal antibody 233-P were

incubated with immobilised rhTGF-β1.

MIC-1 sandwich ELISA:

[0032] A sandwich ELISA employing the anti-MIC-1 MAb and the PAb 233-P was established which could accurately quantify rhMIC-1 in the range of 10-500 pg/ml (Figure 4). To examine the effect of factors present in human serum and culture media on estimation of this cytokine, 500 pg/ml of rhMIC-1 was added to antibody diluant containing either 10%, (v/v) normal human serum or 10% (v/v) DMEM+Nutridoma and then quantified. It was found that the sandwich ELISA was accurate to within 5% of the correct value. Run to run variation was less than 5%. In sandwich ELISA with TGF-β1 and inhibin-A, no cross-reaction with these structurally related cytokines was observed.

[0033] Pooled serum samples were diluted between 1:5-1:20 in antibody diluant prior to MIC-1 quantitation by sandwich ELISA. It was determined that pooled normal human sera contained approximately 0.36 (+/-0.04) ng/ml MIC-1.

Example 2: MIC-1 variant detection and genotyping by immunoassay.

[0034] In the process of cloning MIC-1 it was realised that there were at least two alleles of this TGF-β superfamily cytokine. In subsequent investigation of human material it was confirmed that the 2 alleles were represented in the general community. These alleles differ by a point mutation yielding a change from histidine at position 6 of the amino acid sequence of mature normal or "wild type" MIC-1 (H6), to an aspartic acid at position 6 (D6). This represents a non-conservative substitution of a weakly basic, aromatic amino acid to a strongly acidic, acyclic amino acid.

METHODS AND RESULTS:

Generation of anti-MIC-1 antibodies:

[0035] Anti-MIC-1 monoclonal antibody (MAb) secreting hybridomas were generated from mice immunised with recombinant human MIC-1 (rhMIC-1), which was produced in yeast (*Pichia pastoris*) in accordance with the method described in International patent publication No. WO 97/00958. Hybridomas were cultured in DMEM (Gibco BRL) containing 4.5 g/l D-glucose, 110 mg/l sodium pyruvate, 0.584 g/l L-glutamine, 4 mg/l pyridoxine hydrochloride supplemented with 20% FCS (CSL, Melbourne). For MAb collection, the hybridomas were transferred into fresh DMEM-hi glucose supplemented with Nutridoma-SR (Boehringer Mannheim) for 7 days. The culture supernatant's were spun at 2000 rpm for 10 minutes to remove cell debris and frozen until used.

[0036] The collected Mabs were subjected to epitope mapping studies using Western blot analysis an extensive panel of MIC-1 relatives, mutants and chimaeras. None of the Mabs was able to cross-react with either the murine homologue of MIC-1 or with hTGF-β1, and all of the Mab epitopes were conformation-dependent A distinct cross-reactivity pattern with the various antigens was observed for each of the Mabs suggesting the presence of at least five immunogenic regions on the MIC-1 surface. Two of the Mabs (13C4H3 and 26G6H6) were selected for further study on the basis of their high affinities (each having ED50's in the range of 1.3-2.5 x 10^{-9} M).

[0037] Mab 13C4H3 was found to bind to the amino terminus (positions 1-13) of mature human wild type MIC-1 (i.e. with histidine at position 6) with significantly greater affinity than that of the corresponding epitope of Asp^{202}-MIC-1, and is therefore able to discriminate between human wild type MIC-1 and Asp^{202}-MIC-1. As Mab 13C4H3 was unable to recognise a murine-human MIC-1 chimaera (wherein all of the amino acids of the amino terminus (1-13) which are dissimilar to the human sequence, were replaced with the corresponding amino acids of human MIC-1), it was concluded that additional residues outside of the amino terminus which differ between the human and mouse proteins are possibly also involved.

[0038] Mab 26G6H6 was found to be directed against an epitope (comprising amino acids in the region of positions 24-37, 56-68 and 91-98 of mature human wild type MIC-1) located near the tips of the so-called "fingers" of MIC-1. Mab 26G6H6 did not discriminate between MIC-1 proteins having histidine or aspartic acid at position 6.

[0039] These antibodies therefore enable the detection of heterozygote and homozygote individuals by measuring bound MIC-1 levels in immunoassays.
That is, with Mab 13C4H3 it would be expected that maximal binding would be observed with H6/H6 homozygotes and zero binding with D6/D6 homozygotes, while an intermediate (e.g. 50%) level of binding would be expected with H6/D6 heterozygotes.

[0040] The epitope binding specificities of the above anti-MIC-1 antibodies are described in detail in Fairlie et al., 2001.

Total MIC-1 determination using_26G8H6:

[0041] ELISA plates (Maxisorb, Nunc) were coated for 24 hours at 4°C with 80μl, 1:500 of 26G6H6 in bicarbonate

buffer pH 9.4-9.8 with care taken to prevent significant evaporation, samples were diluted 1:3-1:100, depending on estimated MIC-1 concentration, in Sample buffer (1% w/v BSA (Progen), 0.05% v/v Tween (Sigma) in PBS, pH 7.2, and a MIC-1 "Standard" prepared by diluting 1μg/ml rhMIC-1 (in 1% BSA w/v, 3mM HCl) 1:1000 in sample buffer followed by eight doubling dilutions (1000 pg/ml- 7.8 pg/ml).

Assays were conducted as follows:

[0042] Coated plates were washed three times with wash buffer (0.05% v/v Tween in PBS) 300μl/well. Blocking was performed by incubation with 250 μl 1% BSA w/v at 21 DegC for 1 hour. Blocking buffer was then removed and 100μl/well of standards or samples added without intervening washing for 1 hour at 21°C. Subsequently, the detection antibody, 233-P, 1:25000, in sample buffer v/v, was added, 100 μl/well and incubated for 16 hours at 4°C. Donkey, anti-sheep, biotinylated IgG (Jackson's Laboratories) 1:5000 in sample buffer v/v, 100μl/well, was then added and incubated for 1 hour at 21°C followed by incubation with Streptaviden-HRP conjugate (Genzyme) 1:2000 in sample buffer v/v, 100 μl/well, for 30 minutes at 21°C. OPD (Sigma) 0.4 mg/ml, in the manufacturer's recommended buffer, was incubated at 100 μl/well until a clear difference was seen between the 7.8 pg/ml standard and the zero standard. The 1000 pg/ml standard should have an OD of at least greater than one. Finally, the reaction was stopped with 100μl/well of 2N H_2SO_4.

[0043] Plates can be read at 490 nm and a standard curve constructed using a two binding site hyperbole. Sample values are extrapolated from this curve.

[0044] The plates were washed with 300 μl/well of wash buffer after each step from before the addition of the detection antibody 233-P till the addition of OPD.

Sensitivity and Specificity of anti-MIC-1 PAb and Mab:

[0045] The ability of the sheep PAb 233-P and the mouse MAb 13C4H3 to bind to rhMIC-1 was examined by direct ELISA. It was found that both undiluted tissue culture supernatant containing the MAb 13C4H3 and the sheep PAb 233-P at a dilution of 1:500,000 in antibody diluent bound strongly to 1.8 ng immobilised rhMIC-1. No reaction was observed between rhMIC-1 and culture media conditioned by the mouse myeloma cell line SP2/0, unconditioned culture media, mouse IgGI, immunoglobin enriched normal sheep serum, or antibody diluent. Minimal background binding to uncoated wells was observed for all samples examined. No reactivity was detected when either 13C4H3 or 233-P were incubated with immobilised rhTGF-β1.

[0046] Specificity of the antibodies was determined by immunoprecipitation of purified rhMIC-1 with MAb 13C4H4 and 26G6H6, followed by immunoblot analysis with various MIC-1 specific antibodies. All the MIC-1 antibodies specifically recognised the 25 kD dimeric MIC-1. Additionally, blocking of the antibodies was performed by pre-incubating the antibody with purified rhMIC-1, prior to Western Blot analysis. This greatly reduced the interaction of the antibody with the MIC-1 specific 25 kD band, confirming specificity of the antibodies Mab 13C4H4, 26G6H6 and 233-P. Furthermore, those antibodies tested failed to recognise inhibin, another member of the TGF-13 superfamily. A typical assay standard curve is shown in Figure 5 with error bars representing one standard deviation.

Determination of MIC-1 genotype using 13C4H4:

[0047] The higher affinity of the detection antibody 233-P, to a multitude of MIC-1 epitopes, compared to 13C4H4 led to a greater difference in the detected MIC-1 between the H6 and D6 alleles. This difference is a function of the differing affinities of the H6 and D6 epitopes to 13C4H3. The presence of 233-P, in a long incubation, leads to progressively less D6 being bound to the capture antibody, 13C4H4. These, now unbound, molecules become progressively bound to the higher affinity components of the polyclonal antibody that are specific for the 13C4H4 binding site. These molecules are now excluded from measurable MIC-1.

[0048] Another effect is also observed. That is, each molecule of MIC-1 that is excluded from binding the capture antibody excludes a multiple of 233-P antibodies. This occurs as 233-P is polyclonal and binds to multiple parts of the MIC-1 molecule. The result is that these immune complexes, between MIC-1 and 233-P, are excluded from the assay. As the 233-P antibody is the major contributor to background, the observed difference in MIC-1 concentration is further magnified. In the case of a homozygous D6/D6 genotype, the background staining is reduced to the point that a reading below the zero is obtained over wide concentration differences. In the case of the H6 allele, the rate of MIC-1 becoming free to bind the polyclonal antibody, solely, is much less, creating a wider difference in observed MIC-1 concentration.

[0049] The two sandwich enzyme linked immunosorbant assays involved in the determination of the MIC-1 concentration and MIC-1 allele in a particular sample, use 26G6H6 and 13C4H4 as the capture antibodies, respectively.
The samples analysed may be from tissue culture (tissue culture medium or cell extract), human serum or plasma, or any human sample that is in fluid phase or may be processed into fluid phase by any process.

[0050] The assays used ELISA plates (Maxisorb, Nunc) coated for 24 hours at 4°C with 80μl, 1:500 of 13C4H4 in

bicarbonate buffer pH 9.4-9.8 (care should be taken to prevent significant evaporation). Samples were diluted 1:3-1: 100, depending on estimated MIC-1, determined in 13C4H4 assay concentration, in Sample buffer (1% w/v BSA (Progen), 0.05% v/v Tween (Sigma) in PBS, pH 7.2. The sample concentration should be between 50 and 150 pg/ml. The MIC-1 Standard (1μg/ml recombinant MIC-1 in 1% BSA w/v, 3mM HCL) was diluted 1:1000 in sample buffer and eight doubling dilutions then performed (1000 pg/ml- 7.8 pg/ml).

[0051] The assays were conducted as follows:

Coated plates were washed three times with wash buffer (0.05% v/v Tween in PBS) 300μl/well. Blocking was performed by incubation with 250 μl 1% BSA w/v at 21°C for 1 hour. Blocking buffer was then removed and 100μl/well of standards or samples added without intervening washing for 1 hour at 21°C. The detection antibody, 233-P, 1:10000, in sample buffer v/v, was added, 100 μl/well and incubated for 16 hours at 4°C. Donkey, anti-sheep, biotinolated IgG (Jackson's Laboratories) 1:5000 in sample buffer v/v, 100μl/well, was then added and incubated for 1 hour at 21°C followed by incubation with Streptaviden-HRP conjugate (Genzyme) 1:2000 in sample buffer v/v, 100 μl/well, for 30 minutes at 21°C. OPD (Sigma) 0.4 mg/ml, in the manufacturer's recommended buffer, was incubated at 100 μl/well until a clear difference was seen between the 7.8 pg/ml standard and the zero standard. The 1000 pg/ml standard should have an OD of at least greater than one. The reaction is stopped with 100μl/well of 2N H_2SO_4.

Plates were read at 490 nm and a standard curve constructed using a two binding site hyperbole model. Sample values can be extrapolated from this curve.

The plates were washed with 300 μl/well of wash buffer after each step from before the addition of the detection antibody 233-P till the addition of OPD.

DISCUSSION:

[0052] To determine the MIC-1 allele, the observed MIC-1 concentration, obtained from the 13C4H6 assay was divided by the total MIC-1 concentration, determined in the 26G8H6 assay. The cut-off ratios for the various alleles were determined by homozygous H6/H6 and D as well as heterozygous (HD) controls used in both assays. Validation data was included as set out below.

[0053] A ratio of less than 0 indicates a homozygous D6/D6 genotype, 0-0.6 is heterozygous and greater than 0. 7 is homozygous H6/H6. It is noted that there are ratios greater than 1. Because of the dynamics of the assay, with regard to homozygous D6/D6 protein, higher concentrations lead to an OD further below zero.

[0054] Data derived from 38 healthy ambulatory laboratory workers is shown below in tabulated form. Of these, 18 had their MIC-1 genotype determined by DNA sequencing. There was a 100% agreement between the 18 subjects' DNA sequence and genotype determined by the ELISA method. A further 95 samples were analysed from healthy blood donors with 48 males and 47 females, with an age range of 20-69 and 17-71 years respectively. There were five subjects with a homozygous D6/D6 genotype, 45 with a heterozygous genotype and 45 with a homozygous H6/H6 genotype.

Example 3: Ratiometric PCR RFLP assay for determination of MIC-1 genotype.

[0055] Restriction fragment length polymorphism (RFLP) assays have been a mainstay of DNA mutational analysis for many years. Some of these assays have been superseded by more sensitive, less labour intensive polymerase chain reaction (PCR) assays. In other, mutation detection, assays the two methods have been combined to detect different DNA polymorphisms. In the case of MIC-1, the area of point mutation for the H6-D6 allele is approximately 90% GC rich. This makes it very difficult to use strategies such as competitive PCR to determine allelic, or genotypic differences. This necessitated the use of a RFLP analysis of PCR amplified DNA segments.

[0056] The RFLP assay depends on differences in DNA restriction enzyme sites conferred by differences in the DNA sequence. These sites are usually unique or give a distinct difference in the pattern of bands seen when restriction enzyme digests are separated, according to molecular weight. Typically, this is done using agarose gel DNA electrophoresis. In the region of the allelic differences in MIC-1 there are no useful unique restriction sites conferred by the point mutation C to G. This necessitated a novel modification to the RFLP assay exploiting the properties of DNA agarose gel electrophoresis with ethidium bromide detection. A high agarose concentration (e.g3%) has been employed to give better resolution for small molecular DNA bands. When irradiated with UV light, differences in ethidium bromide staining are proportional to differences in DNA concentration.

[0057] PCR primers (5p, 5'GCCGCCGCCGTCGCAGTCGGA3' SEQ ID NO: 8; 3p, 5'CAGGCGGTGCAGGCTCGTCTTGAT3' SEQ ID NO: 9) were designed to give a product such that the common AVRII sites in the D6 allele gave a major product, upon digestion, of 147 bp. In the case of the H6 allele, the extra AVRII site gave a major product of 102 bp, close to the detection limit of DNA agarose gel electrophoresis. The remaining fragment is a smaller 45 bp product that is difficult to see on agarose gel (see restriction maps in Figure 6).

METHODS:

PCR from genomic DNA:

[0058] A standard master mix for pfu DNA polymerase (Stratagene) was made up as per the manufacturer's recommendations with 1 μl each of 10 pM 5p and 3p primers to a volume of 20 μl per reaction. 100ng of genomic DNA from each test subject was used as template.

PCR:

Denaturation	94°C	1 min
Annealing	65°C	1 min
Extension	72°C	2 min

[0059] Performed for 40 cycles in MJ Research PTC-200 Peltier thermal cycler. Digest PCR products at 3°C overnight with AVA II (New England Biolabs), as per the manufacturer's instructions.

[0060] Run on 3% agarose gel, 0.02% w/v ethidium bromide, at 80 V until separate bands were observable. Genotypes were then determined as per controls (DD, HD, HH).

RESULTS AND DISCUSSION.

[0061] As shown in Figure 7, in the case of the homozygous D6/D6 allele only products of 147 base pairs are visible. The heterozygotes gave two products, 147 and 102 bp in a ratio of 3:1, while the homozygous H6/H6 gave equal amounts of the 147 and 102 bp fragment. The exaggerated differences in the ratio of digestion products between the homozygous H6/H6 and the heterozygous allele is easily observable by the eye, and requires no specialised analysis. There are slight differences in intensity observed between the 147 and 102 bp products. This is due to differences in the amount of ethidium bromide intercalation. This effect further enhances differences in intensity of staining at different ratios of small products of DNA digestion. In the case of larger DNA fragments this effect is far less pronounced.

[0062] Of the 38 healthy ambulatory laboratory workers, 18 had their MIC-1 genotype determined by sequencing. Products from the above PCR were purified from agarose gel and sequenced using the manufacturer's recommended protocol for the Perkins-Elmer ABI prism DNA sequencer. Each subject had forward and reverse sequencing using the 5p and 3p primers respectively.

[0063] Results from ELISA, ratiometric PCR RFLP and DNA sequencing were tabulated (Table 1).

There was 100% concordance between these methods for the 18 subjects that had DNA sequencing performed. A further 21 subjects had their genotype determined by ELISA to determine the range of 13/26 ratios for a range of concentrations of MIC-1 of various genotypes.

TABLE 1:

SPEC ID	R26	R13	13/26	MIC-1 μg/ml	Allele/Genotype	DNA SEQUENCE
SH	54	15	0.3	270	HD	HD
TL	66	74	1.1	328	HH	HH
AB	50	12	0.2	250	HD	HD
DX	41	9	0.2	205	HD	HD
DF	3B	45	1.2	192	HH	HH
AC	124	118	0.9	620	HH	HH
JL	36	6	0.2	179	HD	HD
NX	182	123	0.7	912	HH	HH
TK	37	7	0.2	185	HD	HD
DJ	48	44	0.9	238	HH	HH
GG	45	<0	<0	227	DD	DD
JK	83	21	0.3	414	HD	HD

(continued)

SPEC ID	R26	R13	13/26	MIC-1 μg/ml	Allele/Genotype	DNA SEQUENCE
KW	46	7	0.1	228	HD	HD
WW	58	66	1.1	291	HH	HH
KS	74	71	1	369	HH	HH
RO	838	216	0.3	4190	HD	HD
DB	33	38	1.2	162	HH	HH
GL	40	50	1.2	199	HH	
KM	80	98	1.2	400	HH	
AsB	719	384	0.5	3594	HD	
NR	112	90	0.8	559	HH	
MS	49	69	1.4	243	HH	
CS	49	57	1.2	243	HH	
RL	271	131	0.5	1355	HD	
KiW	44	7	0.2	218	HD	
BS	130	48	0.4	651	HD	
MM	65	21	0.3	324	HD	
JZ	66	27	0.4	332	HD	
ML	209	108	0.5	1046	HD	
MN	44	51	1.2	220	HH	
CH	39	8	0.2	197	HD	
IS	39	47	1.2	196	HH	
LP	42	<0	<0	209	DD	
HL	90	46	0.5	450	DH	
GH	40	14	0.3	201	HD	
LS	37	38	1.1	182	HH	
DS	503	521	1	2516	HH	
PF	113	47	0.4	565	HD	

Example 4: ELISA assays performed with samples from rheumatoid arthritis (RA) patients.

[0064] BSA assays according to the following methods were performed on serum samples taken from an unselected population of 21 individuals with RA, and a further 9 individuals having very severe RA which had failed to respond to traditional therapies. The results are presented in Table 2 below.

METHODS

MIC-1 sandwich ELISA:

[0065] A MIC-1 sandwich ELISA was established utilising the anti-MIC-1 mouse Mabs (13C4H3 and 26G6H6) for antigen capture and the labelled sheep polyclonal antibody (PAb 233-P) for detection. The optimum concentration of the antibodies was determined empirically then used for all subsequent studies. Ninety-six well Maxisorp ELISA plates were coated with anti LUC-1 MAb supernatant diluted 1:5 (final immunoglobin concentration was approximately 20 ng (ml) in coating buffer at 40°C for 24 hours. Plates were then washed three times with 300 μl of wash buffer and non-specific binding was blocked with 250 μl of 1% (w/v) BSA in PBS for 2 hours at 37°C. rhMIC-1 standards, tissue culture

supernatant and serum were then added to the plates (100 μl/well) and incubated for 1 hour at 37°C. The plates were washed three times followed by the addition of 100 μl/well of the sheep PAb 233B3-P diluted 1:5000 in antibody diluant and incubated for 1 hour at 37°C.

The plates were then washed three times and 100 μl/well of biotinylated donkey anti-sheep IgG diluted to 1:5000 in antibody diluant was added and incubated for 1 hour at 37°C. The plates were then developed as for the direct ELISA. The concentration of hMIC-1 in the samples was determined by comparison with the rhMIC-1 standard curve.

TABLE 2:

Rheumatoid Arthritis	No.	(No.)/% of at least one D6 allele
Unselected RA	21	(1)/5%
Severe RA*	9	(4)/44%
*RA severe enough to need experimental treatment (e.g. bone marrow transplant).		

[0066] The results suggest that D6/D6 homozygote and H6/D6 heterozygote individuals have an increased predisposition to severe rheumatoid arthritis.

Example 5: Changes in MIC-1 levels in rheumatoid arthritis.

[0067] The present applicants also looked at two groups of individuals with rheumatoid arthritis (RA). One was an unselected group of 20 patients with RA who had had previous treatment and were presenting with a flare of disease. These individuals were treated with 1 gram of intravenous methyl prednisolone, an anti-inflammatory drug. Individuals were assessed pre-treatment and 4 and 24 hours post-treatment. Serum C-reactive protein (CRP) was determined for these time points using standard laboratory techniques.

[0068] The second group consisted of 23 individuals who underwent autologous stem cell transplant for severe, active RA. These individuals had previously failed treatment with five disease modifying drugs. Stem cells were harvested after pre-treatment with granulocyte-colony stimulating factor. Individuals were then treated with high doses of cyclophosphamide, a chemotherapeutic agent. The autologous, previously harvested, stem cells, were then infused. This effectively "rescued" bone marrow function. Blood samples were taken 6 days before treatment and at 1.5 months post-treatment. The CRP and tumour necrosis factor (TNF) serum levels were determined.

[0069] All individuals had a joint swollen score and a joint tender score determined, by standard methods, as well as a health assessment questionnaire (HAQ) performed. These measurements were determined for each time point.

[0070] Serum samples were analysed for MIC-1 genotype and serum level by our standard ELISA method for each time point. These results were compared with the above variables and the MIC-1 serum levels of a normal population of 100-normal blood donors.

RESULTS:

[0071] Using an unpaired t-test MIC-1 serum levels were significantly higher in RA patients (n=43) compared with a normal population (n=100) (RA: mean = 893 pg/ml: SD=614: normal: mean=406 pg/ml: SD=253 $p<0.0001$) (Figure 9).

[0072] In the transplant population, MIC-1 serum levels were higher 1.5 months post-transplant compared to pre-transplant serum levels (using paired t-test analysis; $p=0.021$). Also, it is notable that the joint swollen and tender scores and HAQ also fell significantly ($p<0.003$) 1.5 months post-transplant. There were no significant changes in CRP and TNF serum levels (paired t-test). The degree of change of MIC-1 levels between pre- and 1.5 months post-transplant was positively correlated with the change in joint score at 1.5 months ($p=0.006$; correlation Z-test). An abnormally high MIC-1 serum level (>1050 pg/ml) post-transplant is negatively correlated with changes in TNF levels ($p<0.03$; Mann-Whitney-u test). The MIC-1 serum levels pre-transplant were related to TNF serum levels pre-transplant, but this just failed to reach significance ($p=0.058$; Kendall correlation test). There were no other significant relationships.

[0073] Taken together these trends may indicate that the MIC-1 serum level is a predictor of synovial joint dysfunction 1.5 months post-transplant. The data also indicates that MIC-1 serum levels, and changes in those levels, may be related to the TNF serum level. TNF is a cytokine known to contribute to RA pathogenesis. Alternatively, this may represent increased cytokine secretion from reconstituting bone marrow.

[0074] In the unselected RA population, there was a relationship between MIC-1 serum levels and age, but this just failed to reach significance ($p=0.064$) using the correlation Z test.

[0075] In the transplant group homozygous H6/H6 genotype, individuals had higher TNF serum levels and higher joint swollen scores post-transplant ($p<0.05$; ANOVA). This was also true for the pre-transplant TNF level, but this fell just

short of statistical significance (p=0.058; ANOVA).

[0076] In the unselected RA group, the HD genotype was 2 times more likely to have erosive disease ($p<0.02$) (Figure 10). These individuals also had significantly lower levels of C-reactive protein (CRP) pre-treatment and at 4 and 24 hours after treatment ($p<0.02$; Mann-Whitney-u test). Individuals with erosive disease also had lower levels of CRP at all three time points ($p<0.05$; Mann-Whitney-u test) (Figures 11 and 12). This suggests that the genotype of MIC-1 has a functional role in determining the manifestations of RA.

DISCUSSION

[0077] There are clear relationships between MIC-1 genotype and erosive disease. MIC-1 genotype is also related to variations in CRP serum levels in RA. CRP is one of the major measurements of inflammatory activity in RA. Additionally, the MIC-1 serum level is significantly raised in RA compared to a normal group. Changes in MIC-1 serum levels are likely to be related to TNF serum level changes. These are MIC-1 genotype dependant TNF is another cytokine that plays a major role in RA pathogenesis. The combined analysis of these correlations is that MIC-1 is likely to play a role in the pathogenesis of RA and that a given individual's MIC-1 genotype can predict the course of disease.

References:

[0078]

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SUMMARY PARAGRAPHS

[0079] The present invention is defined in the claims and the accompanying description.

[0080] For convenience, other aspects of the present invention are presented herein by way of numbered paragraphs (para.s).

1. A method of diagnosis of rheumatoid arthritis, said method comprising;

(i) determining the amount of MIC-1 present in a body sample from a test subject, and
(ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample(s) from normal subject(s).

2. A method according to para. 1, wherein the body sample is selected from blood serum, plasma and synovial fluid.

3. A method according to any one of para.s 1 or 2, wherein the amount of MIC-1 present in the body sample is determined by immunoassay or immunohistochemistry using antibodies or fragments thereof against MIC-1.

4. A method of treating rheumatoid arthritis in the subject, said method comprising administering to said subject an effective amount of MIC-1 optionally in admixture with a pharmacalogically-acceptable carrier and/or excipient.

5. A method of diagnosis or assessment of rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

6. A method of assessing a predisposition to rheumatoid arthritis in a human subject, said method comprising determining the presence of a MICK1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

7. A method of diagnosis or assessment of rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

8. A method for assessing a predisposition to rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having histidine at position 202 or a position corresponding to position 202 or immature human wild type MIC-1 in a suitable sample from said subject.

9. A method according to any one of para.s 5 to 8, wherein the presence of a MIC-1 variant protein in the said sample is determined by immunoassay using antibodies or fragments thereof which preferentially bind to either human wild type MIC-1 and/or variants which have histidine at position 202, or MIC-1 variant proteins which have aspartic acid at position 202.

10. A method according to any one of para.s 5 to 8, wherein said sample is selected from whole blood, blood serum, plasma, urine or tissue biopsy.

11. A method for diagnosing or assessing rheumatoid arthritis in a human subject or, otherwise, assessing a predisposition to rheumatoid arthritis in a human subject, said method comprising genotyping said subject in relation to MIC-1 by determining whether said subject is homozygous or heterozygous for a MIC-1 protein having histidine at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 or a MIC-1 variant protein which has aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1.

Sequence Listing:

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ctgcggcgtc agctcagcct tgcaagaccc caggcgcccg cgctgcacct gcgactgtcg 480
ccgccgccgt cgcagtcgga ccaactgctg gcagaatctt cgtccgcacg gccccagctg 540
gagttgcact tgcggccgca agccgccagg gggcgccgca gagcgcgtgc gcgcaacggg 600
gacgactgtc cgctcgggcc cgggcgttgc tgccgtctgc acacggtccg cgcgtcgctg 660
gaagacctgg gctgggccga ttgggtgctg tcgccacggg aggtgcaagt gaccatgtgc 720
atcggcgcgt gcccgagcca gttccgggcg gcaaacatgc acgcgcagat caagacgagc 780
ctgcaccgcc tgaagcccga cacggtgcca gcgccctgct gcgtgcccgc cagctacaat 840
cccatggtgc tcattcaaaa gaccgacacc ggggtgtcgc tccagaccta tgatgacttg 900
ttagccaaag actgccactg catatga                                      927
```

<210> 5
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 5
aggacctgct aaccaggctg cgggccaacc agagc 35

<210> 6
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

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ggctaacaag tcatcatagg tctggagcga cac 33

<210> 7
<211> 9
<212> PRT
<213> Homo sapiens

<400> 7

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Ala Arg Asn Gly Asp Asp Cys Pro Leu
 1               5
```

<210> 8
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 8
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<210> 9
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 9
caggcggtgc aggctcgtct tgat 24

Claims

1. A method of diagnosis of rheumatoid arthritis, said method comprising;

   (i) determining the amount of MIC-1 present in a body sample from a test subject, and
   (ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample(s) from normal subject(s); wherein elevated MIC-1 levels are associated with rheumatoid arthritis.

2. A method according to claim 1, wherein the amount of MIC-1 present in the body sample is determined by immunoassay or immunohistochemistry using antibodies or fragments thereof against MIC-1.

3. methods of diagnosis or assessment of rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

4. A method of assessing a predisposition to rheumatoid arthritis in a human subject, said method comprising determining the presence of a MIC-1 variant protein having aspartic acid at position 202 or a position corresponding to position 202 of immature human wild type MIC-1 in a suitable sample from said subject.

5. A method according to claim 3 or 4, wherein the presence of a Mix-1 variant protein in the said sample is determined by immunoassay.

6. A method according to any one of claims 1 to 5, wherein said sample is selected from whole blood, blood serum, plasma, urine or tissue biopsy.

Patentansprüche

1. Verfahren zum Diagnostizieren von rheumatoider Arthritis, wobei das Verfahren umfasst:

   (i) die Bestimmung der Menge von in einer Körperprobe eines Testsubjekts vorliegendem MIC-1 und
   (ii) Vergleich der bestimmten Menge mit der Menge oder dem Mengenbereich, der in (einer) äquivalenten Körperprobe(n) aus (einem) normalen Subjekt(en) vorliegt, wobei erhöhte MIC-1-Spiegel mit rheumatoider Arthritis assoziiert sind.

2. Verfahren nach Anspruch 1, wobei die Menge an in der Körperprobe vorliegendem MIC-1 durch ein Immunoassay oder durch Immunhistochemie unter Verwendung von Antikörpern gegen MIC-1 oder von Fragmenten davon bestimmt wird.

3. Verfahren zum Diagnostizieren oder zum Beurteilen von rheumatoider Arthritis in einem humanen Subjekt, wobei

das Verfahren umfasst, dass man die Gegenwart einer MIC-1-Proteinvariante mit Asparaginsäure in Position 202 oder einer mit Position 202 korrespondierenden Position von unreifem humanen Wildtyp-MIC-1 in einer geeigneten Probe aus dem Subjekt bestimmt.

4. Verfahren zur Beurteilung einer Prädisposition gegenüber rheumatoider Arthritis in einem humanen Subjekt, wobei das Verfahren umfasst, dass man die Gegenwart einer MIC-1-Proteinvariante mit Asparaginsäure in Position 202 oder einer mit Position 202 korrespondierenden Position von unreifem humanen Wildtyp-MIC-1 in einer geeigneten Probe aus dem Subjekt bestimmt.

5. Verfahren nach Anspruch 3 oder 4, wobei die Gegenwart einer MIC-1-Proteinvariante in der Probe durch ein Immunoassay bestimmt wird.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei die Probe ausgewählt ist unter Vollblut, Blutserum, Plasma, Urin oder Gewebebiopsie.

Revendications

1. Procédé de diagnostic de la polyarthrite rhumatoïde, ledit procédé comprenant :

   (i) la détermination de la quantité de MIC-1 présente dans un échantillon du corps d'un sujet à tester, et
   (ii) la comparaison de ladite quantité déterminée contre la quantité, ou plage de quantités, présente dans un/des échantillon(s) équivalent(s) du corps de sujet(s) normal/normaux ; où des taux élevés de MIC-1 sont associés à une polyarthrite rhumatoïde.

2. Procédé selon la revendication 1, dans lequel la quantité de MIC-1 présente dans l'échantillon du corps est déterminée par un test immunologique ou par immunohistochimie en utilisant des anticorps ou des fragments de ceux-ci dirigés contre la MIC-1.

3. Procédé de diagnostic ou d'estimation de la polyarthrite rhumatoïde chez un sujet humain, ledit procédé comprenant la détermination de la présence d'une protéine variante de la MIC-1 présentant un acide aspartique au niveau de la position 202 ou d'une position correspondant à la position 202 de la MIC-1 de type sauvage humaine immature dans un échantillon approprié provenant dudit sujet.

4. Procédé d'estimation d'une prédisposition à la polyarthrite rhumatoïde chez un sujet humain, ledit procédé comprenant la détermination de la présence d'une protéine variante de la MIC-1 présentant un acide aspartique au niveau de la position 202 ou d'une position correspondant à la position 202 de la MIC-1 de type sauvage humaine immature dans un échantillon approprié provenant dudit sujet.

5. Procédé selon la revendication 3 ou 4, dans lequel la présence d'une protéine variante de la MIC-1 dans ledit échantillon est déterminée par un test immunologique.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel ledit échantillon est choisi parmi le sang total, le sérum sanguin, le plasma, l'urine ou une biopsie tissulaire.

FIGURE 1

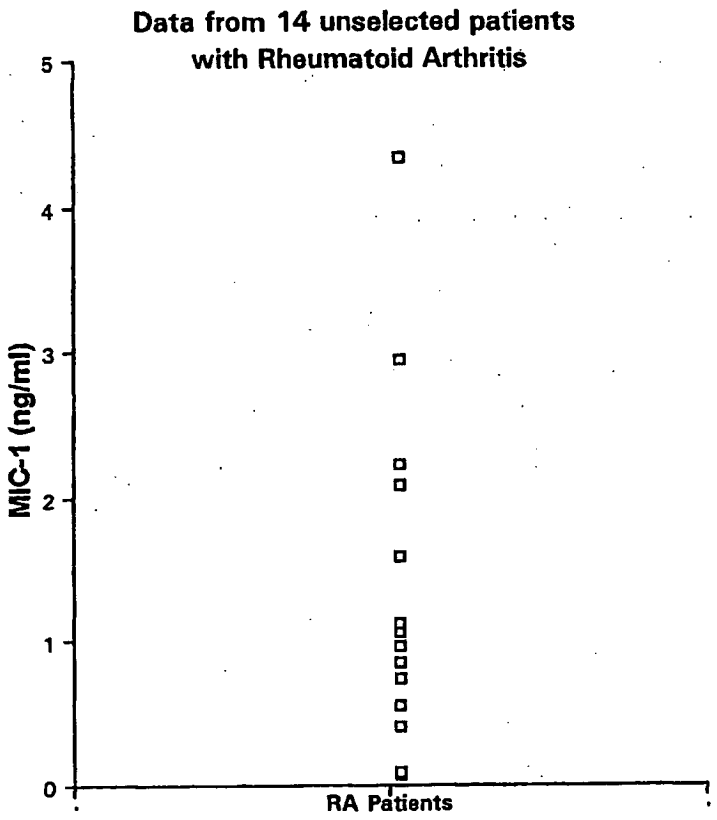

Data from 14 unselected patients
with Rheumatoid Arthritis
MIC-1 (ng/ml)
5
4
3
2
1
0
RA Patients

FIGURE 2 A

Alignment of MIC-1 protein from cDNA clones bearing H6 or D6 Substitutions at position 202 of full length MIC-1 (position 6 of mature MIC-1)

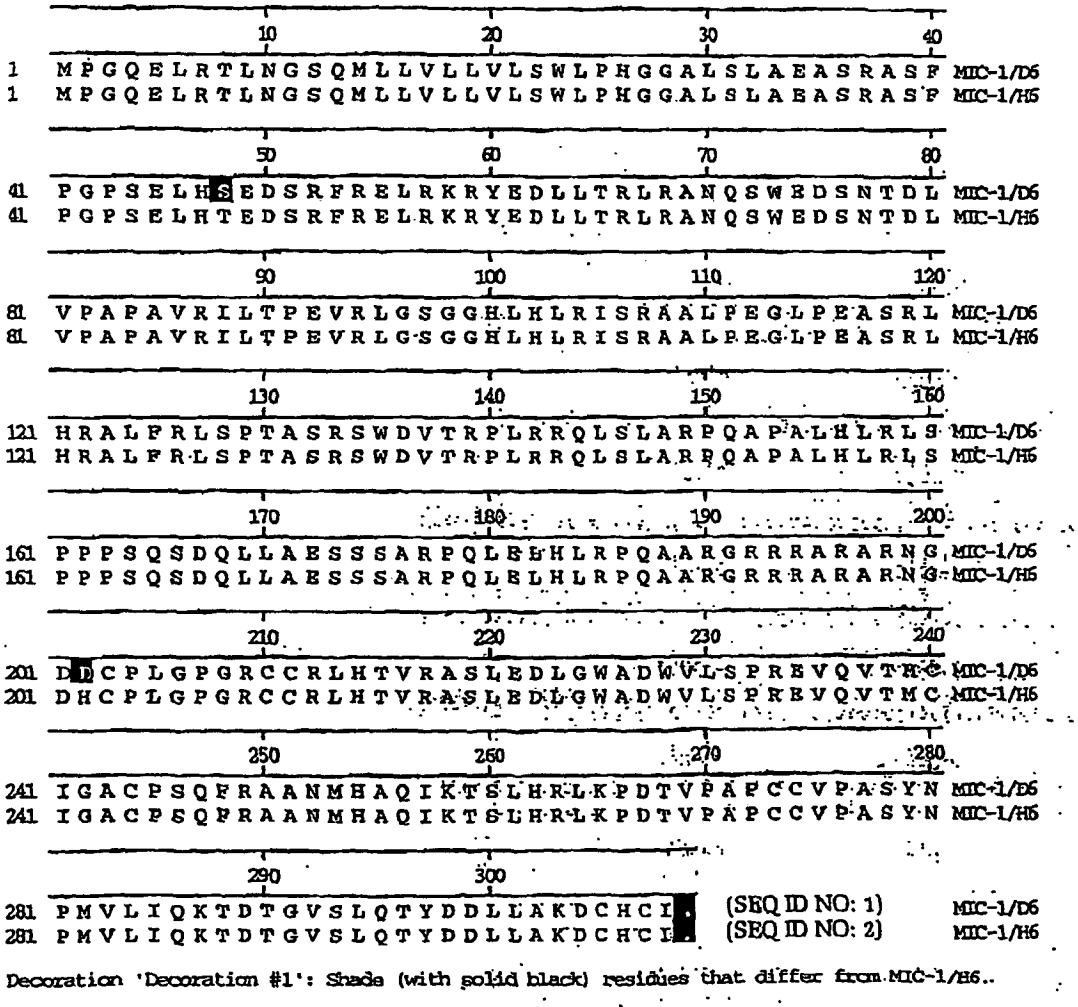
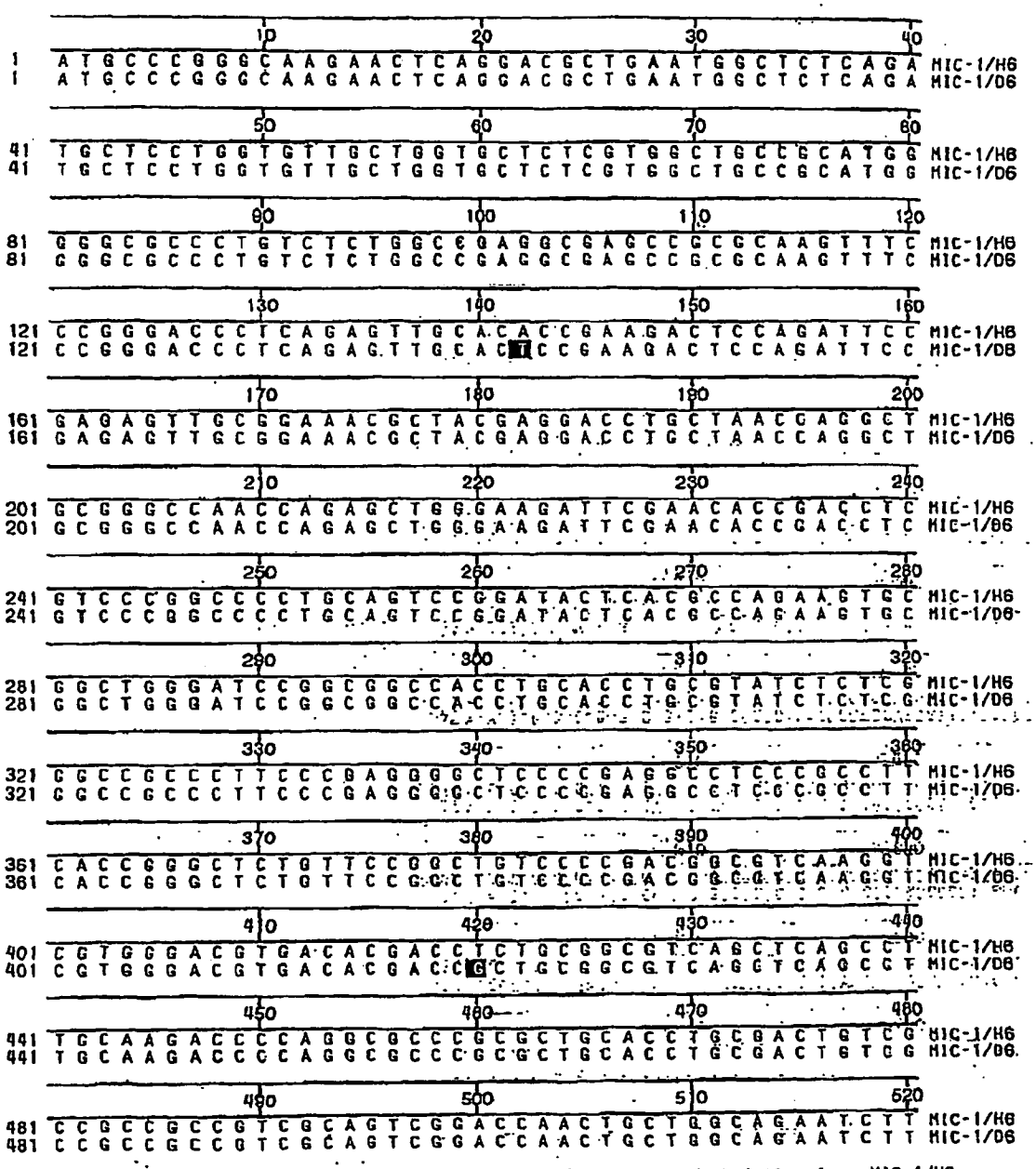


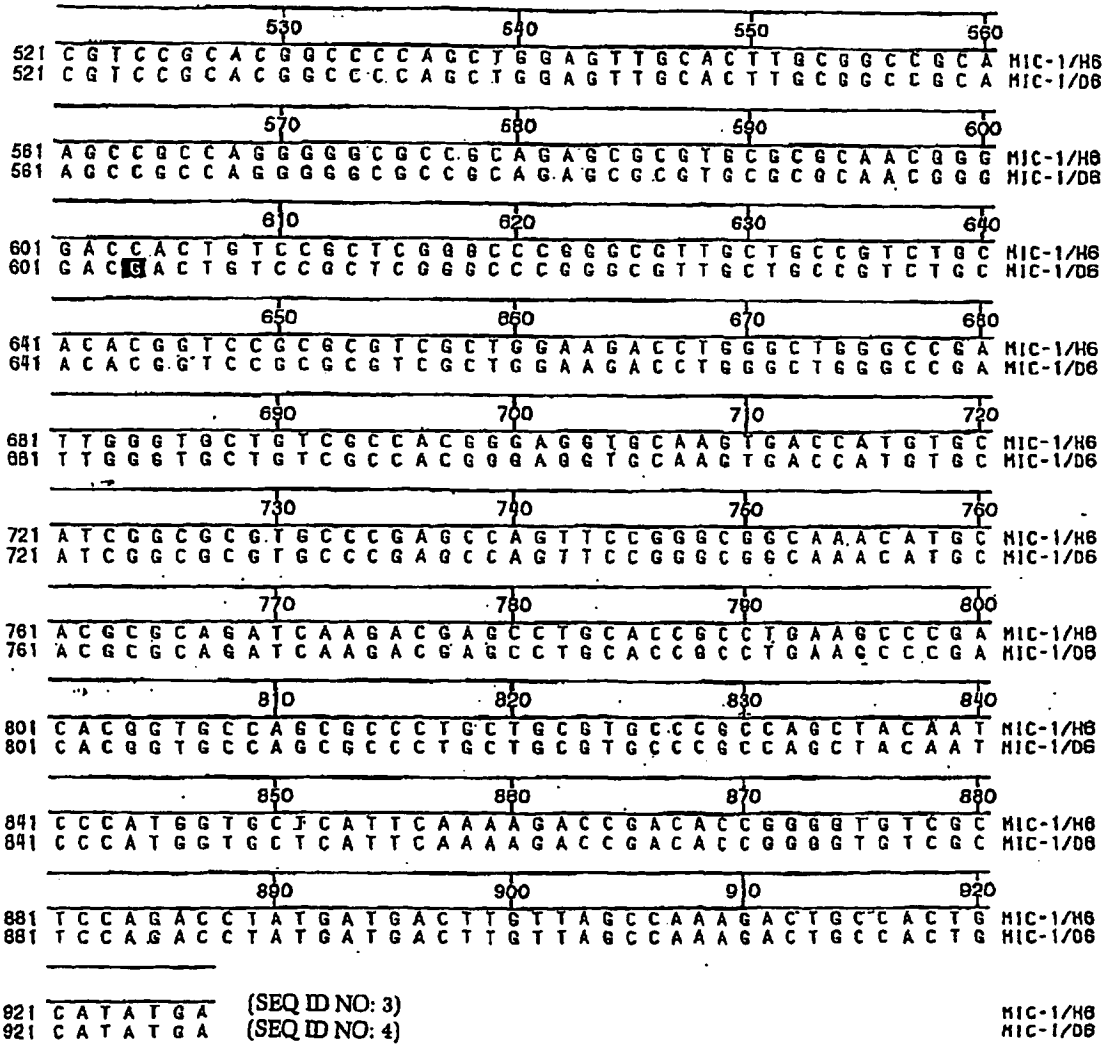
FIGURE 2B

Alignment of MIC-1 coding region DNA from cDNA clones bearing C to G substitution at position 604 (H6 to D6 amino acid substitutions



Decoration 'Decoration #1': Shade (with solid black) residues that differ from MIC-1/H6.

FIGURE 2B continued



Decoration 'Decoration #1': Shade (with solid black) residues that differ from MIC-1/H6.

FIGURE 3

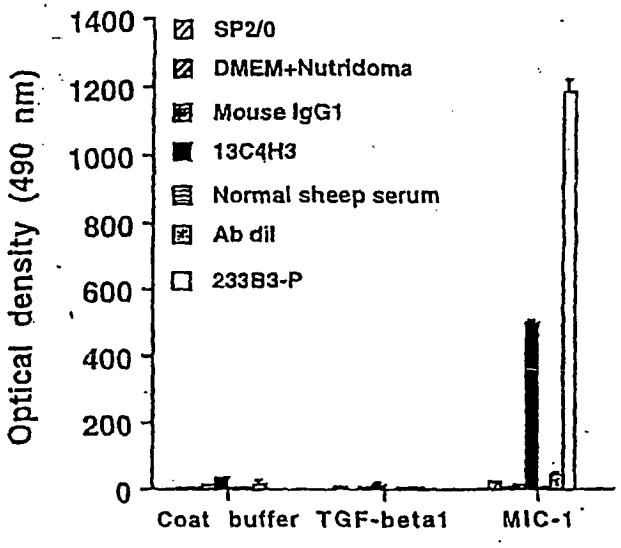

Optical density (490 nm)
1400
1200
1000
800
600
400
200
0
SP2/0
DMEM+Nutridoma
Mouse IgG1
13C4H3
Normal sheep serum
Ab dil
233B3-P
Coat buffer
TGF-beta1
MIC-1

FIGURE 4

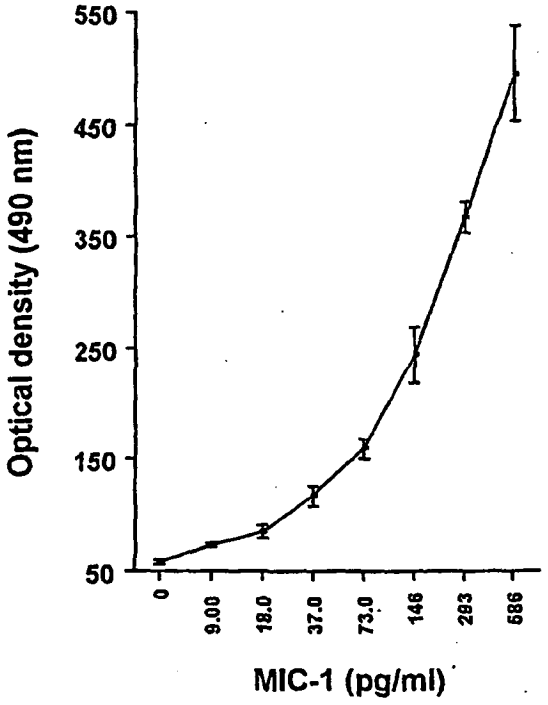

550
450
350
250
150
50
Optical density (490 nm)
0
9.00
18.0
37.0
73.0
146
293
586
MIC-1 (pg/ml)

FIGURE 5

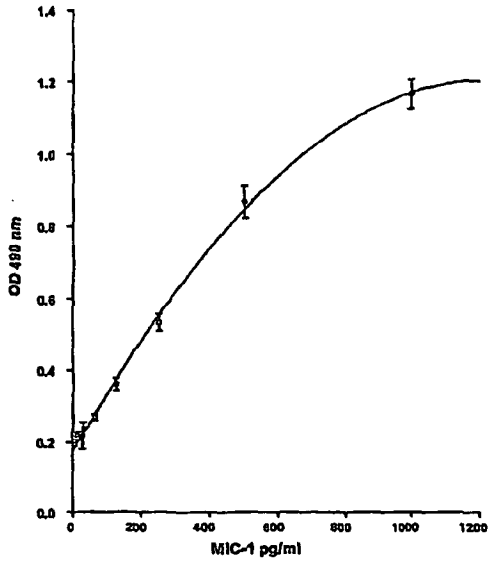
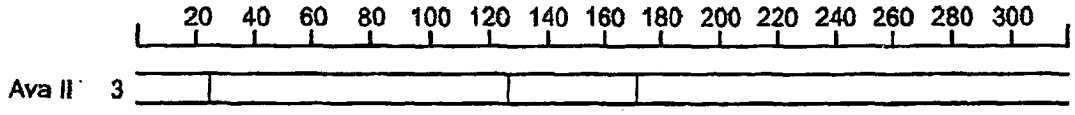

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 490 nm
0
200
400
600
800
1000
1200
MIC-1 pg/ml

FIGURE 6

H6 restriction map



D6 restriction map

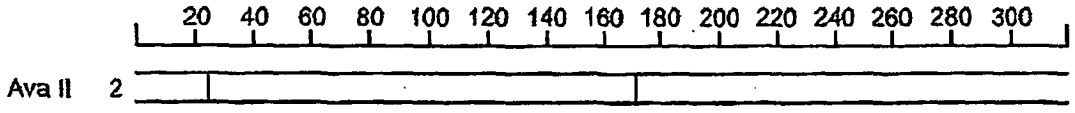


FIGURE 7

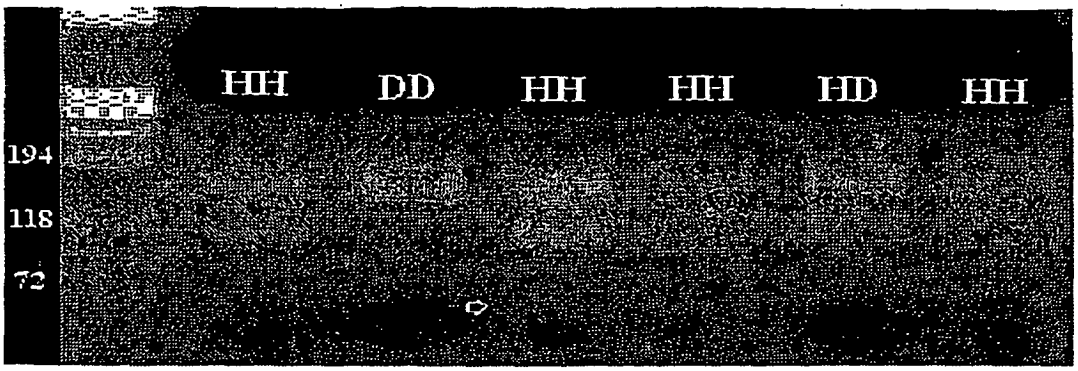


FIGURE 8

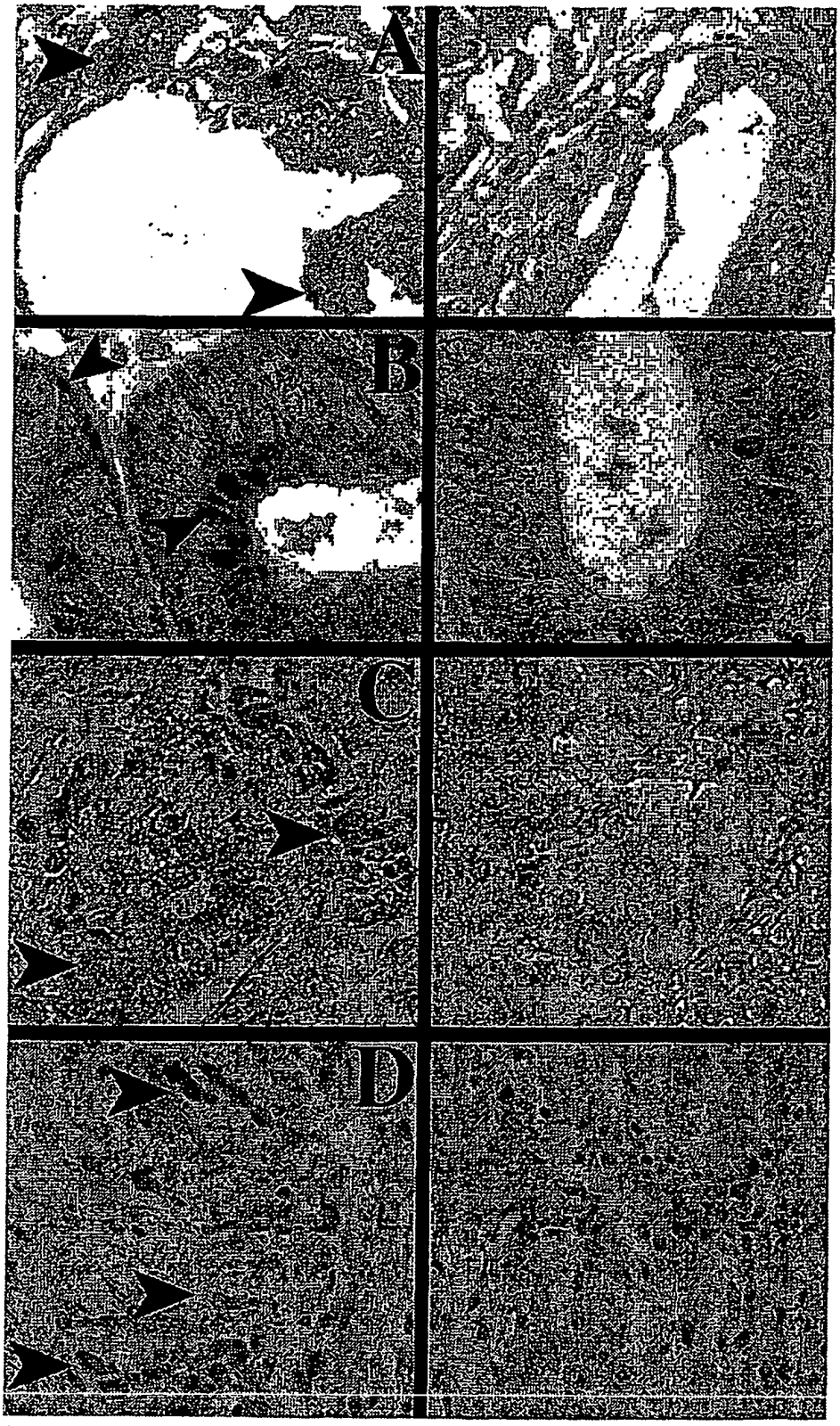


FIGURE 9

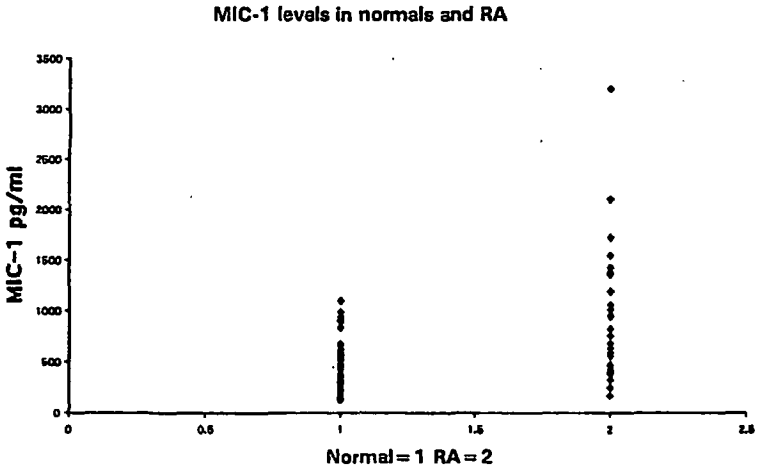

MIC-1 levels in normals and RA
MIC-1 pg/ml
3500
3000
2500
2000
1500
1000
500
0
0
0.5
1
1.5
2
2.5
Normal = 1 RA = 2

FIGURE 10

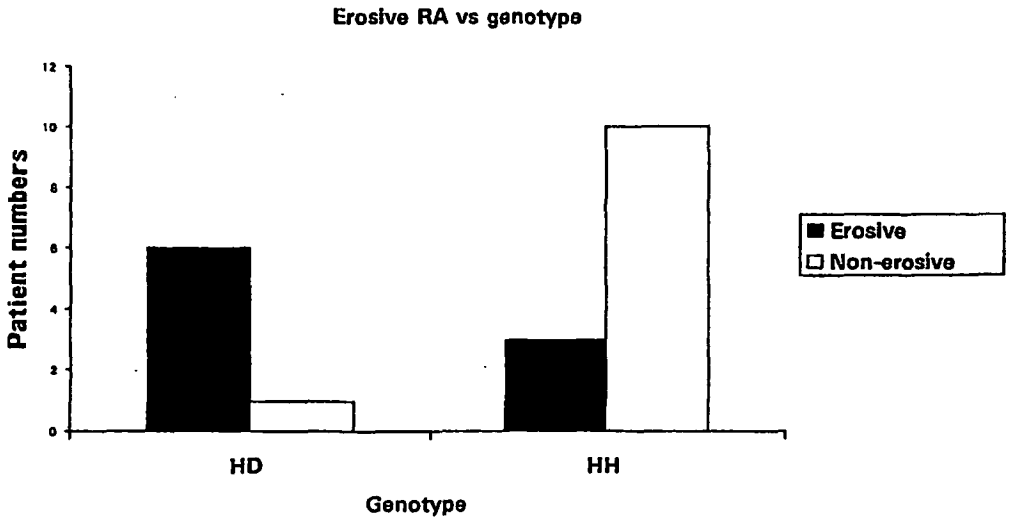

Erosive RA vs genotype
Patient numbers
12
10
8
6
4
2
0
HD
HH
Genotype
Erosive
Non-erosive

FIGURE 11

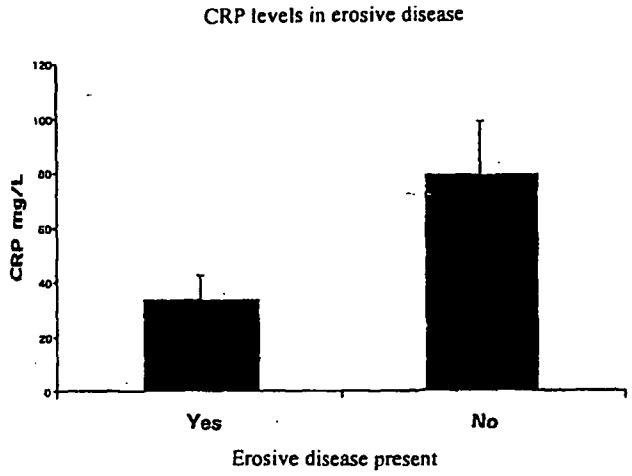
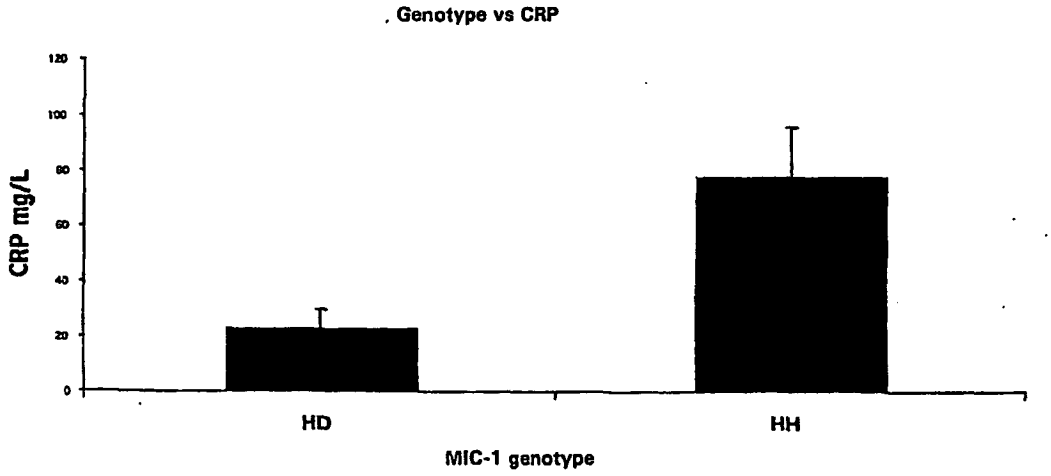

CRP levels in erosive disease
CRP mg/L
120
100
80
60
40
20
0
Yes
No
Erosive disease present

FIGURE 12


Genotype vs CRP
CRP mg/L
120
100
80
60
40
20
0
HD
HH
MIC-1 genotype

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

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- WO 9700958 A **[0006] [0026] [0035]**

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专利名称(译)	涉及巨噬细胞抑制性细胞因子-1（MIC-1）的诊断分析		
公开(公告)号	EP1914554B1	公开(公告)日	2010-06-09
申请号	EP2007023699	申请日	2001-04-20
申请(专利权)人(译)	圣文森特医院SYDNEY LIMITED		
当前申请(专利权)人(译)	圣文森特医院SYDNEY LIMITED		
[标]发明人	BREIT SAMUEL NORBERT BROWN DAVID ALEXANDER		
发明人	BREIT, SAMUEL NORBERT BROWN, DAVID ALEXANDER		
IPC分类号	G01N33/68 G01N33/53 A61K38/00 A61K38/19 A61K48/00 A61P15/06 A61P29/00 A61P35/00 A61P43/00 C07K16/24 C12N15/09 C12N15/19 C12Q1/68 G01N33/574		
CPC分类号	A61K38/195 A61P15/06 A61P29/00 A61P35/00 A61P43/00 C07K16/24 C07K2317/34 C12Q1/6883 C12Q1/6886 C12Q2600/156 C12Q2600/158 G01N33/6893 G01N2800/102		
代理机构(译)	HARDING，CHARLES THOMAS		
优先权	2000PQ7037 2000-04-20 AU 2000PQ7465 2000-05-11 AU		
其他公开文献	EP1914554A3 EP1914554A2		
外部链接	Espacenet		

摘要(译)

公开了用于诊断流产和/或早产，胎儿异常，癌症（例如前列腺癌）和炎性疾病（例如类风湿性关节炎）的风险的方法，其涉及确定体内巨噬细胞抑制性细胞因子-1（MIC-1）的异常水平。样品或，否则，确定MIC-1变体蛋白的存在。还公开了降低妊娠受试者中流产和/或早产风险的方法，以及治疗炎性疾病和/或癌症的方法。