(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 279 039 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
16.01.2008 Bulletin 2008/03

(21) Application number: **01923411.1**

(22) Date of filing: **20.04.2001**

(51) Int Cl.:
G01N 33/68 *(2006.01)* ***C07K 16/24*** *(2006.01)*

(86) International application number:
PCT/AU2001/000456

(87) International publication number:
WO 2001/081928 (01.11.2001 Gazette 2001/44)

(54) **DIAGNOSTIC ASSAY INVOLVING MACROPHAGE INHIBITORY CYTOKINE -1 (MIC-1)**

DIAGNOSTISCHER ASSAY MIT DEM MAKROPHAGEN-INHIBITORISCHEN-ZYTOKIN-1 (MIC-1)

METHODE DIAGNOSTIQUE IMPLIQUANT LA CYTOKINE-1 INHIBITRICE MACROPHAGE (MIC-1)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **20.04.2000 AU PP703700**
11.05.2000 AU PP746500

(43) Date of publication of application:
29.01.2003 Bulletin 2003/05

(60) Divisional application:
07023699.7

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Description

Field of the Invention:

[0001] This invention relates tp the field of medical diagnostics. In particular, the invention provides methods for diagnosing risk of miscarriage and/or premature birth and foetal abnormalities.

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] The TGF-β superfamily is also thought to perform multiple roles during pregnancy. The ability of the TGF-β isoforms to modulate cell-cell adhesion, cell migration and tissue remodelling has led some authors to suggest that these molecules may control trophoblast invasion and implantation in early pregnancy. Other possible roles include regulation of foetal growth and suppression of the maternal immune system. Placental cells are a major source of TGF-β superfamily molecules and are regulated by at least TGF-β1, TGF-β3, activin and inhibin. For example, activin suppresses the production of inhibin and enhances progesterone, human chorionic gonadotropin (hCG), and gonadotropin-releasing hormone (GnRE) by placental cells (Petraglia et al., 1989). Inhibin suppresses placental hCG, GnRH and activin-induced progesterone release (Petraglia et al., 1989), while TGF-β1 suppresses placental derived human placental lactogen (hPL) production. Activin and TGF-β3 have also been shown to have opposing effects in regulating extravillous trophoblast invasion in early pregnancy (Caniggia et al., 1997; Caniggia et al., 1999). These findings suggest that TGF-β1, TGF-β3, activin and inhibin regulate the growth and differentiation of the placenta in an autocrine manner. TGF-β1, activin and inhibin are also present in the embryo proper where they have been demonstrated to regulate growth and differentiation. In particular, TGF-β superfamily members are well known for their ability to promote mesoderm induction.

[0005] It has also been suggested that TGF-β superfamily proteins promote foetal survival. Experimental evidence suggests that the amniotic fluid concentration of the pro-inflammatory cytokines interleukin-1 (IL-1), IL-6, and tumour necrosis factor-α (TNF-α) rise during labour. Furthermore, pro-inflammatory cytokine production accompanying intrauterine infection has been associated with foetal rejection or preterm labour (Romero et al., 1992; Hillier et al., 1993; Opsjon et al., 1993). TGF-β1 and inhibin have been shown to suppress the production of pro-inflammatory cytokines from macrophages and lymphocytes respectively (Bogdan and Nathan, 1993; Petraglia et al., 1991) while activin has pro-inflammatory effects on macrophages and amnion (Nusing and Barsig, 1997; Petraglia et al., 1993b). This has led to the suggestion that TGF-β1 and inhibin promote foetal survival by suppressing the production of pro-inflammatory cytokines by the maternal immune system.

[0006] WO-A-9906445 suggests the use of GDF-15, which is otherwise known as MIC-1, for preventing premature labour amongst other things. Only murine GDF-15 is disclosed and no actual activity or tissue expression of GDF-15 is shown.

[0007] 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. In order to determine the nature of any role MIC-1 may play in pregnancy, the present applicants have developed a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for MIG-1 quantification and used this to investigate the temporal relationship between human maternal serum MIC-1 concentrations and gestation age, and further, measured its concentration in amniotic fluid and placental extracts. In addition, the present applicants have conducted experimentation to delineate the origins of MIC-1 by assessing the capacity of a placental trophoblastic cell line (BeWo) to synthesise the cytokine. The results presented hereinafter shows that MIC-1 is able to promote foetal

survival by suppressing the production of maternally-derived pro-inflammatory cytokines within the uterus. Consequently, quantitative diagnostic assays of MIC-1 in samples of maternal serum, amniotic fluid and placental extracts offers the possibility of detecting pregnant women with abnormal levels of MIC-1 and which are thereby at risk of miscarriage and/or premature birth.

[0008] 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, the entire contents of which is incorporated herein by reference, 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 forinflammatory disease(s) and/or cancer(s).

Disclosure of the Invention:

[0009] In a preferred embodiment of the invention; the detection of depressed MIC-1 amounts in a body sample, preferably a sample of blood serum, amniotic fluid or placental extracts, from a pregnant test subject would be indicative of a condition wherein there may be an increased risk of miscarriage and/or premature birth.

[0010] Thus, in a first aspect, the present invention provides a method for the diagnosis of miscarriage risk and/or premature birth, said method comprising;

(i) determining the amount of MIC-1 present in a body sample taken from a pregnant test subject having a known gestation age, -and

(ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample (s) taken from normal-pregnant subject(s) of a gestation age which is substantially equivalent to said known gestation age of said test subject.

[0011] As mentioned above, preferred body samples for use in the method of the first aspect are samples of blood serum, amniotic fluid or placental extracts. -However, samples of whole blood plasma, urine and cerebrospinal fluid may also be suitable.

[0012] The amount, or range of amounts, present in body samples of normal pregnant subjects increases with advancing gestation age. It is therefore important that the determined amount of MIC-1 from the test subject sample be compared with the MIC-1 amount(s) present in equivalent sample(s) from normal pregnant subject(s) of substantially equivalent gestation age. Thus, where the body samples used are serum samples, a determined amount of less than or equal to 4 ng/ml from a first trimester test subject, less than or equal to 8 ng/ml from a second trimester test subject, and less than or equal to 12 ng/ml from a third trimester test subject, would be indicative of depressed MIC-1 levels and a consequent increased risk of miscarriage and/or premature birth. Where the body samples are amniotic fluid samples, a determined amount of less than or equal to 10 ng/ml from a second trimester test subject would be indicative of depressed MIC-1 levels and a consequent increased risk of miscarriage and/or premature birth. Finally, where the body samples used are placental extracts, a determined amount of less than or equal to 18 ng/ml, more preferably less than or equal to 10 ng/ml, in a placental extract sample of a third trimester test subject would be indicative of depressed MIC-1 levels and a consequent increased risk of miscarriage and/or premature birth.

[0013] Increased risk of miscarriage and/or premature birth may be the result of abnormal pregnancy and/or placental development associated with depressed MIC-1 levels. That is, where abnormal placental development is determined through detection of depressed MIC-1 levels, this may be indicative of early induction of labour because the foetus may be at risk if the placenta fails to develop and grow normally.

[0014] Successfully assessing the risk of miscarriage and/or premature birth in pregnant women allows for the possibility of preventative therapies and other measures (e.g. rest, improved diet, etc.) to be applied.

[0015] In another preferred embodiment of the invention, the detection of depressed or elevated MIC-1 amounts in a body sample from a pregnant test subject may be indicative of a condition wherein there may be an increased risk of foetal abnormalities.

[0016] Thus, in a second aspect, the present invention provides a method for the diagnosis of foetal abnormalities, said method comprising;

(i) determining the amount of MIC-1 present in a body sample taken from a pregnant test subject having a known gestation age, and

(ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample

(s) from normal pregnant subject(s) with a gestation age which is substantially equivalent to said known gestation age of said test subject

[0017] The amount of MIC-1 present in a body sample may be readily determined by, for example, 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.

[0018] Preferred body samples for use in the method of the first aspect are samples of whole blood, serum, plasma and urine. Tissue biopsies may also be suitable.

[0019] 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 1.

[0020] 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.

[0021] The invention will hereinafter be described with reference to the following non-limiting examples and accompanying figures.

Brief description of the accompanying figures:

[0022]

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

Figure 2 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 3 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 4 provides the results of experimentation showing that MIC-1 is present in maternal serum and amniotic fluid during pregnancy in women.

(A) Estimation of MIC-1 concentrations in pooled normal human serum . (NHS), pooled staged maternal serum, and pooled amniotic fluid (AF) as determined by sandwich ELISA.

(B) Immunoprecipitation and western blot analysis of MIG-1 in pooled normal human serum (lane 1), pooled staged maternal serum (lane 2-4), and pooled amniotic fluid (lane 5),

Figure 5 gives the maternal serum MIC-1 concentrations in four pregnant women from 30 weeks of gestation until birth as determined by sandwich ELISA.

Figure 6 gives the results of measurements of MIC-1 concentrations in five different human placental extracts as assessed by sandwich ELISA.

Figure 7 provides the results of experimentation conducted to assess MIC-1 expression and secretion by the human trophoblastic cell line BeWo.

(A) MIC-1 secretion by BeWo cells after 1 and 5 days in culture as determined by sandwich ELISA.

(B) Immunoprecipitation and western blot analysis of secreted MIC-1 by BeWo cells. Lane 1, unconditioned culture media; Lane 2, culture media which had been conditioned by BeWo cells for 5 days.

(C) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of MIC-1 expression by unstimulated BeWo cells. Lane 1, RT-PCR on total RNA from BeWo cells cultured for 24h; Lane 2, Negative control (no total RNA); Lane 3, Positive PCR control.

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

Example 1: Assessment of MIC-1 expression in pregnant women.

METHODS:

Serum and amniotic fluid samples:

[0023] Serum samples were obtained from 22 healthy pregnant women with a normal singleton pregnancy. No medication was being taken by any individual studied. In each case, gestational age was determined by an early pregnancy ultrasound scan. All women subsequently had a normal vaginal delivery at term (37-41 weeks) bf a healthy normally grown infant Serum samples were collected from 6 women between 10-14 weeks of pregnancy, and 8 women between 26-30 weeks and 37-40 weeks of pregnancy. The time periods indicated correspond to the end of each trimester. Samples corresponding to each trimester were pooled prior to measurement of MIC-1 levels. Serial material serum samples were also taken, on a weekly basis approximately, from 4 women from 30 weeks of gestation to delivery. Again, all four women were healthy with a normal singleton pregnancy and had a normal vaginal delivery at term of a normal healthy infant. In addition, amniotic fluid was obtained from 10 women undergoing amniocentesis at 15-17 weeks of gestation for foetal karyotyping. In all cases, the indication for karyotyping was advanced maternal age (>37 years). Amniotic fluid were also pooled prior to measurement of MIC-1 levels.

Placental extracts:

[0024] Between 100-150 mg of placental tissue (rinsed 4-5 times in saline solution and frozen in liquid nitrogen and stored at -80°C) was homogenised in 1 m. of phosphate-buffered saline (PBS). Homogenates were centrifuged at 10,000 rpm for 30 seconds and the supernatant transferred to tubes. Total protein was measured by the BCA total protein assay (Pierce) following the manufacturer's instructions. BSA solutions ranging between 0-1000 μg/ml were used as standard solutions.

BeWo cell culture:

[0025] The human choriocarcinoma trophoblastic cell line (BeWo) was purchased from ATCC (Rockville, MD). Cells were seeded into 96 well tissue culture plates at 5000 cells per well in 250 μl of Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco BRL) containing 4.5 g/1 D-glucose, 110 mg/l sodium pyruvate, 0.584 g/l L-glutamine, 4 mg/l pyridoxine hydrochloride and 1X Nutridoma-SR (Boehringer Mannheim, Germany) and cultured at 37°C in the presence of 5% carbon dioxide for 1-5 days. At this time, the culture plates were spun at 1000 rpm for 10 minutes and the supernatant was removed and stored at -20°C until quantitation of MIC-1.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of MIC-1 mRNA synthesis:

[0026] Total RNA was isolated from BeWo cell monolayers in 96-well plates using Tri-Pure Reagent (Roche) and the method provided by the manufacturer. Reverse transcription (RT) was carried out in a total reaction volume of 20 μl using 1 μg of RNA, a poly$(T)_{15}$ primer and 50 units of Expand Reverse Transcriptase (Roche) using the manufacturer's recommended conditions. A 5 μl aliquot of the RT reaction was amplified in a PCR reaction using Pfu polymerase (Promega) and primers;
MSB-1 (5'-AGGACCTGCTAACCAGGCTGCGGGCCAACCAGAGC-3')
(SEQ ID NO: 5) and
MSB-5 (5'-GGCTAACAAGTCATCATAGGTCTGGAGCGACAC-3')
(SEQ ID NO: 6),
which flank the single intron of MIC-1. PCR conditions were as follows: an initial denaturation step at 95°C for 1 minute, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes. An RT reaction in which the RNA was omitted was used as a negative control, while a plasmid carrying the MIC-1 pre-pro-MIC/FLAG coding sequence (Bootcov et al., 1997) was included as a positive control. PCR products were separated on 0.8% (w/v) agarose gels.

Generation of MIC-1 antibodies:

[0027] 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 des-

ignated 233-P.

[0028] 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, 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. The sensitivity of the PAb and MAb preparations were examined by direct ELISA.

Direct ELISA:

[0029] 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 (0.1M carbonate in distilled H_2O, pH 9.4-9.8) at 40°C for 24hours. 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:

[0030] 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, maternal serum, placental extracts, or amniotic fluid 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 ih this example. All samples were assayed in triplicate on at least two occasions, Results are presented as the mean +/- SD. The sensitivity of the MIG-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:

[0031] 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 (1ml) 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:

[0032] 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 2). 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 rbMIC-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:

[0033] 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 3). 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.

MIC-1 levels in staged maternal pregnancy sera increase during pregnancy:

[0034] 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 (Figure 4A). In pooled maternal serum, the MIC-1 concentration was found to increase dramatically during pregnancy. Material serum samples corresponding to the first trimester contained approximately 6.3 (+/-0.02) ng/ml MIC-1, which rose to 12.24 (+/- 0.54) ng/ml during the second trimester, and peaked at 15.3 (+/- 1.31) ng/ml during the third trimester.

[0035] Immunoprecipitation was used to confirm the presence of MIC-1 in pooled maternal serum samples during pregnancy. MIC-1 was visualised by immunoprecipitation with the anti-MIC-1 MAb followed by immunoblot analysis with PAb 233B3-P. A band corresponding to the disulphide linked mature MIC-1 peptide (approximately 25 kDa) can be observed in the second and third trimester pregnancy serum samples (Figure 4B, lanes 3-4). The highest level of MIC-1 was found in the third trimester sample. No similar band was observed in normal serum or the sample corresponding tb the first trimester due to the lower sensitivity of immunoblot analysis (figure 4B, lanes 1-2);

[0036] Maternal serum MIC-1 concentrations-were also examined in serial samples from four pregnant women. At 30 weeks of gestation, serum from all four women examined contained approximately 4 ng/ml MIC-1 (Figure 5). Maternal serum MIC-1 levels were found to increase from 30 weeks of gestation until birth. Subjects designated MH and JB exhibited a slight decrease in MIC-1 maternal serum levels over the last week of pregnancy.

MIC-1 can be detected in amniotic fluid:

[0037] In addition to maternal serum, amniotic fluid collected from 10 women during the second trimester for karyotyping purposes was pooled prior to quantification of MIC-1 levels by sandwich ELISA. It was determined that the pooled amniotic fluid sample contained approximately 13.68 (+/- 0.16) ng/ml MIC-1 (Figure 4A). Immunoprecipitation and western blot analysis of pooled amniotic fluid revealed a band of approximately 25 kDa, which corresponds to the disulphide linked mature MIC-1 peptide (Figure 4B, lane 5).

MIC-1 can be detected in human placental extracts:

[0038] In order to test whether the placenta is-a major source of circulating MIC-1 in the serum of pregnant women, 5 human placenta extracts were examined for the presence of MIC-1 by sandwich ELISA. All-five samples were found to be positive for MIC-1 (Figure 6), ranging in concentration from 5:04 - 54 ng/ml. Significantly the sample designated PL2, which was the only one derived from a premature birth, contained much lower levels of MIC-1 than the other samples.

Cultured BeWo cells constitutively express MIC-1 RNA and secrete mature MIC-1:

[0039] As high levels of MIC-1 were detected in placental extracts it seemed likely that the placental trophoblastic cell line, BeWo, also produces this cytokine. An examination of tissue culture media conditioned by BeWo cells under resting conditions for the presence of secreted MIC-1 by sandwich ELISA was therefore conducted. It was determined that media used to culture BeWo cells for 24 hours contained approximately 21.6 (+/- 2.95) ng/ml MIC-1 (Figure 7A). The

concentration of MIC-1 in the culture media after a five day incubation increased to approximately 117(+/-7.2)ng/ml The ability of unstimulated BeWo cells to secrete MIC-1 was also examined by immunoprecipitation and western blot-analysis. High levels of secreted mature-MIC-1, as indicated by a band-at approximately 25-kDa were observed in media condition by Be Wo cells for 5 days (Figure 7B). Additional bands migrating at 55 kDa and 12.5 kDa bands were observed, which may represent incompletely processed MIC-1 hemidimer and monomer respectively. Culture media which had not been exposed to BeWo cells contained no detectable MIC-1 when examined by sandwich ELISA or by immunoprecipitation.

[0040] RT-PCR was used to investigate the presence of the MIC-1 transcript in unstimulated BeWo cells. Total RNA was extracted from BeWo cells cultured for 24 hours and subjected to RT-PCR as described. A single product of 0.4 kbp was observed, indicating that the MIC-1 transcript was present in BeWo cells (Figure 7C). No product was detected in the absence of BeWo or plasmid DNA.

DISCUSSION:

[0041] The results of example 1 indicate that MIC-1 is present in large amounts in maternal sera and that levels rise substantially with advancing gestation.

[0042] Whilst elevated levels of MIC-1 occur in maternal serum during pregnancy, this does not necessarily mean that tha developing foetus is exposed to this cytokine. However, the detection of MIC-1 in amniotic fluid represents direct evidence for foetal exposure. The level of MIC-1 in amniotic fluid was comparable to that present in second and third trimester maternal serum and well in excess of that present in normal human serum. During pregnancy the foetus ingests large amounts of amniotic fluid and may also absorb-amniotic fluid via the thin foetal epidermis. These findings therefore provide strong evidence that the developing foetus is exposed to high concentrations of MIC-1.

[0043] In order to investigate whether maternal serum and amniotic fluid MIC-1 originates from a foetal or maternal squrce, MIC-1 in human placental extracts was measured and this demonstrated that they contain large amounts of MIC-1 protein. Interestingly, the quantity of MIC-1 present in 4 of the 5 placental extracts (>18 ng/ml) was higher than that defected in pooled maternal sera and amniotic fluid. Using immunohistochemistry and *in situ* hybridisation it has been demonstrated that the MIC-1 transcript and protein is present in the terminal villi of the placenta (Paralkar et al., 1998), a structure-rich in syncitiotrophoblasts. It is therefore reasoned that the BeWo human trophoblastic cell line may synthesise and secrete this cytokine. The BeWo cells constitutively express the MIC-1 transcript and secrete large amounts of MIC-1 under resting conditions. Those-findings suggest that the trophoblastic cells within the placenta are a major source of the MIC-1 present in maternal serum and amniotic fluid. However, the localisation of the MIC-1 transcript and protein to the developing epidermis in day 18 rat embryos (Paralkar et al., 1998) suggests the embryo may also contribute to the MIC-1 levels observed.

[0044] The precise role of MIC-1 during pregnancy is unknown. However, based upon the results described above and experimentation reported elsewhere, it appears that MIC-1 has an immunomodulatory role during pregnancy. For example, it has been reported previously that rhMIC-1 inhibits the release of pro-inflammatory cytokines from LPS-activated macrophages (Bootcov et al., 1997). Further, MIC-1 is known to suppress the formation of erythrocyte and granulocyte/macrophage cell lineages from normal human non-adherent T-cell depleted marrow cells (Hromas et al., 1997). These findings indicate that MIC-1 is a broad inhibitor of inflammation, suppressing both the development of the monocyte/macrophage lineage and their ability to produce pro-inflammatory , mediators.

[0045] Intrauterine inflammation accompanying pro-inflammatory cytokine production has been associated with foetal rejection or preterm labour (Romero et al., 1992; Hillier et al., 1993; Opsjon et al., 1993). In this context, the present applicants consider that MIC-1 present in the placenta and amniotic fluid acts to maintain pregnancy by suppressing the production of pro-inflammatory cytokines within the uterus. The finding in the present example that placental extracts derived from a premature labour contained depressed concentrations of MIC-1 when compared to normal pregnancies provides strong support for this.

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

[0046] 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:

[0047] 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.

[0048] 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).

[0049] 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.

[0050] 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.

[0051] 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.

[0052] 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 26G6H6:

[0053] 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).

[0054] Assays were conducted as follows:

[0055] 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.

[0056] 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.

[0057] 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:

[0058] 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 IgG1, 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.

[0059] 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-β superfamily. A typical assay standard curve is shown in Figure 10 with error bars representing one standard deviation.

Determination of MIC-1 genotype using 13C4H4:

[0060] 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 13C4H4. 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.

[0061] 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.

[0062] 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.

[0063] 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).

[0064] The assays were conducted as follows:

[0065] 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 100 μl/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.

[0066] 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.

[0067] 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:

[0068] 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 26G6H6 assay. The cut-off ratios for the various alleles were deter-

mined by homozygous H6/H6 and D as well as heterozygous (HD) controls used in both assays. Validation data was included as set out below,

[0069] 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.

[0070] Data derived from 38 healthy ambulatory laboratory workers is shown below in tabulated form Of these, is 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.

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Sequence Listing:

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Claims

1. A method of diagnosis of miscarriage risk and/or premature birth, said method comprising;

   (i) determining the amount of MIC-1 present in a body sample taken from a pregnant test subject having a known gestation age, and
   (ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample(s) taken from normal pregnant subject(s) of a gestation age which is equivalent to said known gestation age of said test subject.

2. A method according to claim 1, wherein said body sample is selected from whole blood, blood serum, plasma, amniotic fluid, placental extracts, urine and cerebrospinal fluid.

3. A method according to claim 2, wherein the body sample is selected from blood serum, amniotic fluid and placental extracts.

4. A method according to any one of claims 1 to 3, wherein the body sample is a blood serum sample.

5. A method according to claim 4, wherein the test subject is in the first trimester of pregnancy and wherein a determined amount of MIC-1 present in the blood serum of said test subject that is less than or equal to 4 ng/ml is indicative of increased risk of miscarriage and/or premature birth.

6. A method according to claim 4, wherein the test subject is in the second trimester of pregnancy and wherein a determined amount of MIC-1 present in the blood serum sample of said test subject that is less than or equal to 8 ng/ml is indicative of increased risk of miscarriage and/or premature birth.

7. A method according to claim 4, wherein the test subject is in the third trimester of pregnancy and wherein a determined amount of MIC-1 present in the blood serum of said test subject that is less than or equal to 12 ng/ml is indicative of increased risk of miscarriage and/or premature birth.

8. A method according to any one of claims 1 to 3, wherein the body sample is an amniotic fluid sample.

9. A method according to claim 8, wherein said test subject is in the second trimester of pregnancy and wherein a determined amount of MIC-1 present in the amniotic fluid sample of said test subject that is less than or equal to 10 ng/ml is indicative of an increased risk of miscarriage and/or premature birth.

10. A method according to any one of claims 1 to 3, wherein the body sample is a placental extract sample.

11. A method according to claim 10, wherein said test subject is in the third trimester of pregnancy and wherein a determined amount of MIC-1 present in the placental extract sample that is less than or equal to 18 ng/ml is indicative of an increased risk of miscarriage and/or premature birth.

12. A method according to claim 10, wherein said test subject is in the third trimester of pregnancy and wherein a determined amount of MIC-1 present in the placental extract sample that is less than or equal to 10 ng/ml is indicative of an increased risk of miscarriage and/or premature birth.

13. A method of diagnosis of foetal abnormalities, said method comprising;

(i) determining the amount of MIC-1 present in a body sample taken from a pregnant test subject having a known gestation age, and
(ii) comparing said determined amount against the amount, or range of amounts, present in equivalent body sample(s) from normal pregnant subject(s) with a gestation age which is equivalent to said known gestation age of said test subject.

14. A method according to any one of claims 1 to 13, 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.

Patentansprüche

1. Verfahren zur Diagnose des Fehlgeburtsrisikos und/oder einer Frühgeburt, wobei das Verfahren umfaßt:

(i) die Bestimmung der Menge an MIC-1, die in einer Körperprobe vorliegt, welche aus einem schwangeren Testsubjekt mit einem bekannten Gestationsalter entnommen wurde; und
(ii) den Vergleich der bestimmten Menge gegenüber der Menge oder dem Mengenbereich, der in (einer) äquivalenten Körperprobe(n) vorliegt, welche aus (einem) normalen schwangeren Subjekt(en) mit einem Gestationsalter, welches äquivalent zu dem bekannten Gestationsalter des Testsubjekts ist, entnommen wurde(n).

2. Verfahren nach Anspruch 1, wobei die Körperprobe ausgewählt ist unter Vollblut, Blutserum, Plasma, Fruchtwasser, Placentraextrakt, Urin und Zerebrospinalflüssigkeit.

3. Verfahren nach Anspruch 2, wobei die Körperprobe ausgewählt ist unter Blutserum, Fruchtwasser und Placentaextrakten.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei die Körperprobe eine Blutserumprobe ist.

5. Verfahren nach Anspruch 4, wobei das Testsubjekt im ersten Trimester der Schwangerschaft ist, und wobei eine bestimmte Menge an MIC-1, die in dem Blutserum des Testsubjektes vorliegt, die weniger als oder gleich 4 ng/ml ist, indikativ für ein erhöhtes Risiko einer Fehlgeburt und/oder einer Frühgeburt ist.

6. Verfahren nach Anspruch 4, wobei das Testsubjekt im zweiten Trimester der Schwangerschaft ist, und wobei eine bestimmte Menge an MIC-1, die in dem Blutserum des Testsubjektes vorliegt, die weniger als oder gleich 8 ng/ml ist, indikativ für ein erhöhtes Risiko einer Fehlgeburt und/oder einer Frühgeburt ist.

7. Verfahren nach Anspruch 4, wobei das Testsubjekt im dritten Trimester der Schwangerschaft ist, und wobei eine bestimmte Menge an MIC-1, die in dem Blutserum des Testsubjektes vorliegt, die weniger als oder gleich 12 ng/ml ist, indikativ für ein erhöhtes Risiko einer Fehlgeburt und/oder einer Frühgeburt ist.

8. Verfahren nach einem der Ansprüche 1 bis 3, wobei die Körperprobe eine Fruchtwasserprobe ist.

9. Verfahren nach Anspruch 8, wobei das Testsubjekt im zweiten Trimester der Schwangerschaft ist, und wobei eine bestimmte Menge an MIC-1, die in der Fruchtwasserprobe des Testsubjekts vorliegt, die weniger als oder gleich 10 ng/ml ist, indikativ für ein erhöhtes Risiko einer Fehlgeburt und/oder einer Frühgeburt ist.

10. Verfahren nach einem der Ansprüche 1 bis 3, wobei die Körperprobe eine Placentaextraktprobe ist.

11. Verfahren nach Anspruch 10, wobei das Testsubjekt im dritten Trimester der Schwangerschaft ist, und wobei eine bestimmte Menge an MIC-1, die in der Placentaextraktprobe vorliegt, die weniger als oder gleich 18 ng/ml ist, indikativ für ein erhöhtes Risiko einer Fehlgeburt und/oder einer Frühgeburt ist.

12. Verfahren nach Anspruch 10, wobei das Testsubjekt im dritten Trimester der Schwangerschaft ist, und wobei eine bestimmte Menge an MIC-1, die in der Placentaextraktprobe vorliegt, die weniger als oder gleich 10 ng/ml ist, indikativ für ein erhöhtes Risiko einer Fehlgeburt und/oder einer Frühgeburt ist.

13. Verfahren zur Diagnose von fötalen Anomalien, wobei das Verfahren umfaßt:

(i) die Bestimmung der Menge an MIC-1, die in einer Körperprobe vorliegt, welche aus einem schwangeren Testsubjekt mit einem bekannten Gestationsalter entnommen wurde, und
(ii) den Vergleich der bestimmten Menge gegen die Menge oder den Mengenbereich, der in (einer) äquivalenten Körperprobe(n) aus (einem) normalen schwangeren - Testsubjekt(en) mit einem Gestationsalter, welches äquivalent zu dem bekannten Gestationsalter des Testsubjekts ist, vorliegt.

14. Verfahren nach einem der Ansprüche 1 bis 13, wobei die Menge an MIC-1, die in der Körperprobe vorliegt, bestimmt wird durch ein Immunoassay oder Immunohistochemie unter Verwendung von Antikörpern oder von Fragmenten davon gegen MIC-1.

Revendications

1. Méthode pour le diagnostic d'un risque de fausse couche et/ou de naissance prématurée, ladite méthode comprenant :

(i) la détermination de la quantité de MIC-1 présente dans un échantillon corporel prélevé chez un sujet d'essai gravide ayant un âge de gestation connu, et
(ii) la comparaison de ladite quantité déterminée à la quantité, ou à l'intervalle de quantités, existant dans un ou plusieurs échantillons corporels équivalents prélevés chez un ou plusieurs sujets gravides normaux ayant un âge de gestation qui est équivalent audit âge de gestation connu dudit sujet d'essai.

2. Méthode suivant la revendication 1, dans laquelle l'échantillon corporel est choisi entre le sang entier, le sérum sanguin, le plasma, le liquide amniotique, des extraits placentaires, l'urine et le liquide céphalo-rachidien.

3. Méthode suivant la revendication 2, dans laquelle l'échantillon corporel est choisi entre le sérum sanguin, le liquide amniotique et des extraits placentaires.

4. Méthode suivant l'une quelconque des revendications 1 à 3, dans laquelle l'échantillon corporel est un échantillon de sérum sanguin.

5. Méthode suivant la revendication 4, dans laquelle le sujet d'essai est au premier trimestre de la gestation, et dans laquelle une quantité déterminée de MIC-1 présente dans le sérum sanguin dudit sujet d'essai qui est inférieure ou égale à 4 ng/ml est une indication d'un risque accru de fausse couche et/ou de naissance prématurée.

6. Méthode suivant la revendication 4, dans laquelle le sujet d'essai est au deuxième trimestre de la gestation, et dans laquelle une quantité déterminée de MIC-1 présente dans l'échantillon de sérum sanguin dudit sujet d'essai qui est inférieure ou égale à 8 ng/ml est une indication d'un risque accru de fausse couche et/ou de naissance prématurée.

7. Méthode suivant la revendication 4, dans laquelle le sujet d'essai est au troisième trimestre de la gestation, et dans laquelle une quantité déterminée de MIC-1 présente dans le sérum sanguin dudit sujet d'essai qui est inférieure ou égale à 12 ng/ml est une indication d'un risque accru de fausse couche et/ou de naissance prématurée.

8. Méthode suivant l'une quelconque des revendications 1 à 3, dans laquelle l'échantillon corporel est un échantillon de liquide amniotique.

9. Méthode suivant la revendication 8, dans laquelle ledit sujet d'essai est au deuxième trimestre de la gestation et dans laquelle une quantité déterminée de MIC-1 présente dans l'échantillon de liquide amniotique dudit sujet d'essai

qui est inférieure ou égale à 10 ng/ml est une indication d'un risque accru de fausse couche et/ou de naissance prématurée.

10. Méthode suivant l'une quelconque des revendications 1 à 3, dans laquelle l'échantillon corporel est un échantillon d'extrait placentaire.

11. Méthode suivant la revendication 10, dans laquelle ledit sujet d'essai est au troisième trimestre de la gestation, et dans laquelle une quantité déterminée de MIC-1 présente dans l'échantillon d'extrait placentaire qui est inférieure ou égale à 18 ng/ml est une indication d'un risque accru de fausse couche et/ou de naissance prématurée.

12. Méthode suivant la revendication 10, dans laquelle ledit sujet d'essai est au troisième trimestre de la gestation, et dans laquelle une quantité déterminée de MIC-1 présente dans l'échantillon d'extrait placentaire qui est inférieure ou égale à 10 ng/ml est une indication d'un risque accru de fausse couche et/ou de naissance prématurée.

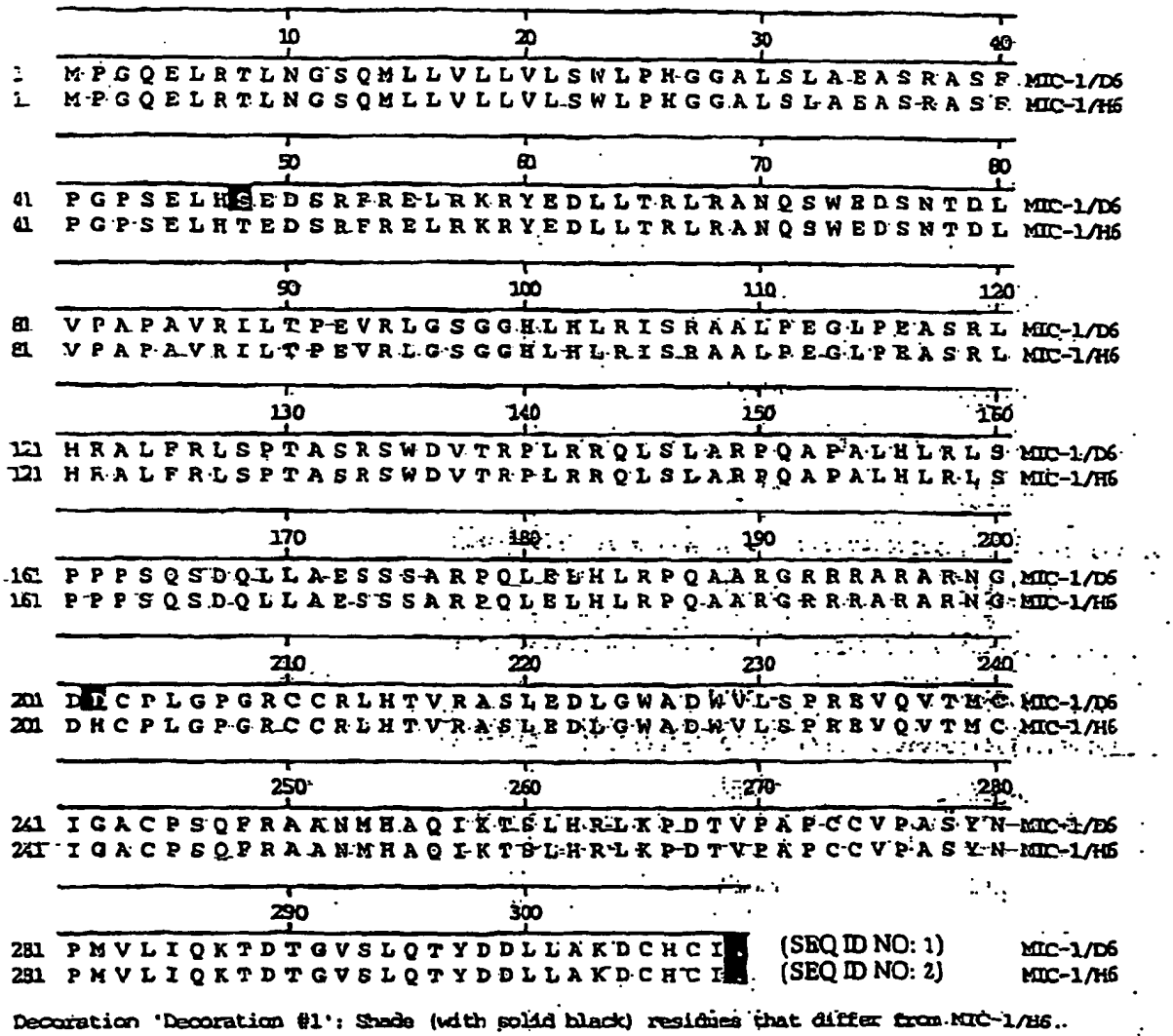
13. Méthode de diagnostic d'anomalies foetales, ladite méthode comprenant :

(i) la détermination de la quantité de MIC-1 présente dans un échantillon corporel prélevé chez un sujet d'essai gravide ayant un âge de gestation connu, et
(ii) la comparaison de ladite quantité déterminée avec la quantité, ou l'intervalle de quantités, existant dans un ou plusieurs échantillons corporels équivalents provenant d'un ou plusieurs sujets gravides normaux ayant un âge de gestation qui est équivalent audit âge de gestation connu dudit sujet d'essai.

14. Méthode suivant l'une quelconque des revendications 1 à 13, dans laquelle la quantité de MIC-1 présente dans l'échantillon corporel est déterminée par analyse immunologique ou immunohistochimie en utilisant des anticorps ou leurs fragments contre MIC-1.

FIGURE 1A

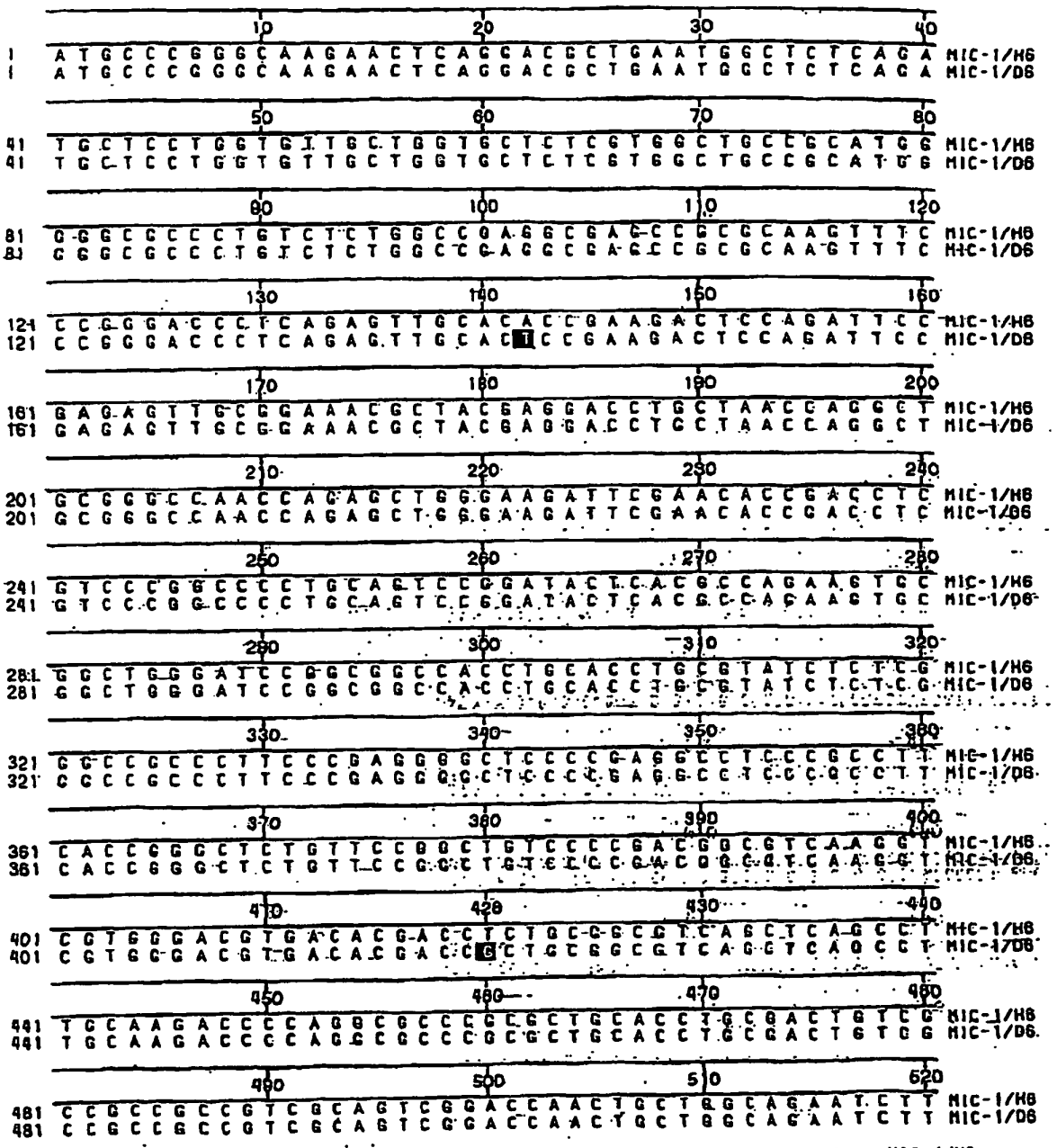
Alignment of MIC-1 protein from cDNA clones bearing H6 or D6 Substitutions at position 202 of full length MIC-1 (position 6 of mature MIC-1)



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

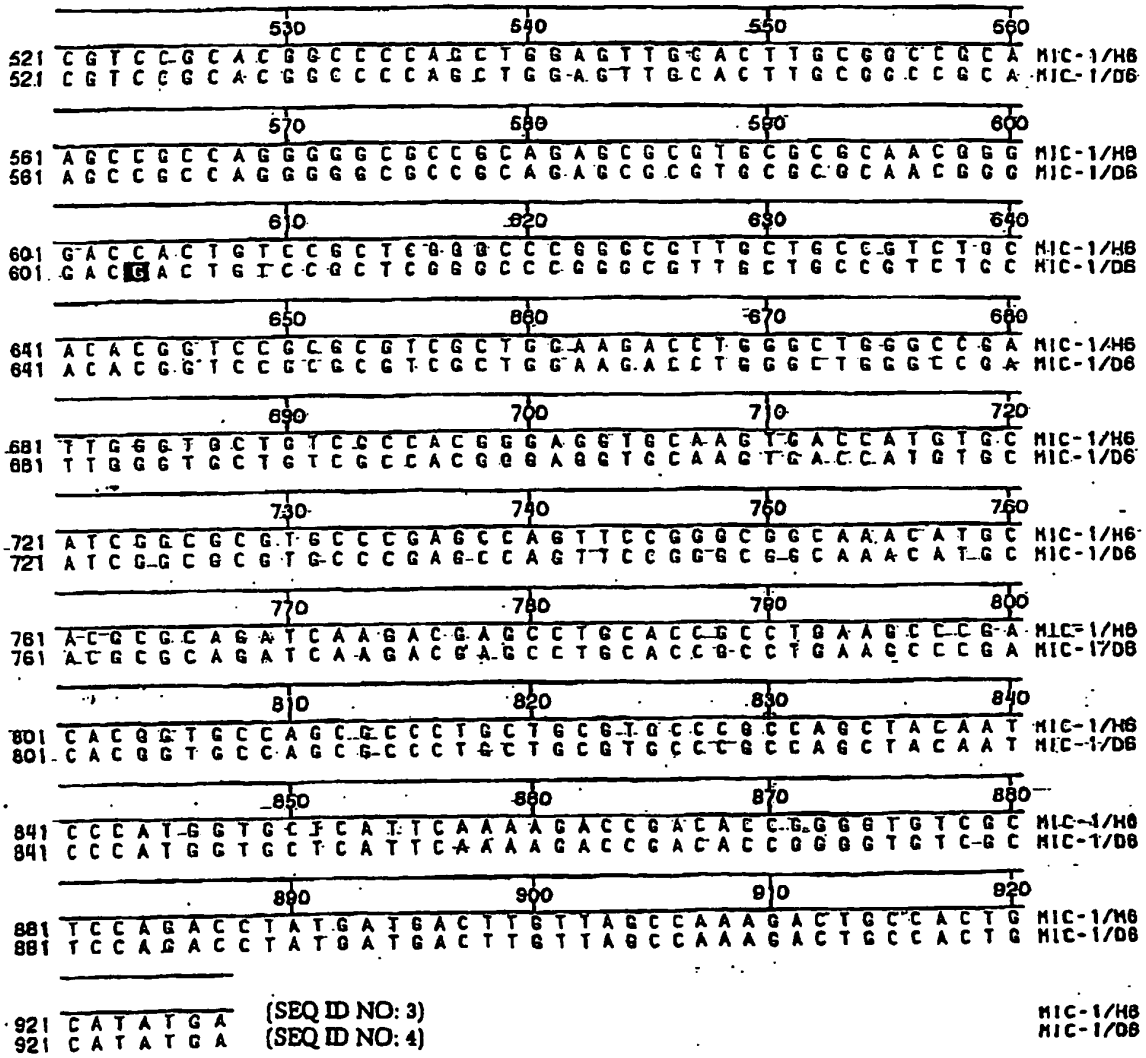
FIGURE 1B

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



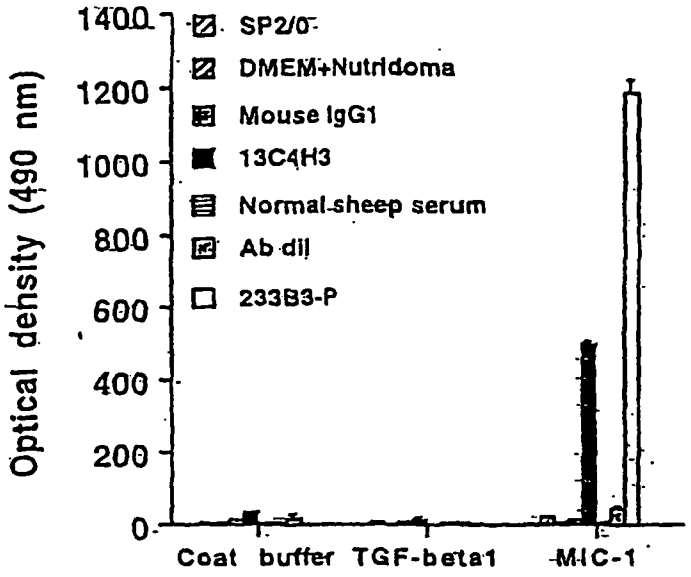
Decoration 'Decoration #1': Shade (with solid black) residues that differ from MIC-1/H6.

FIGURE 1B continued



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

FIGURE 2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1000
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600
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200
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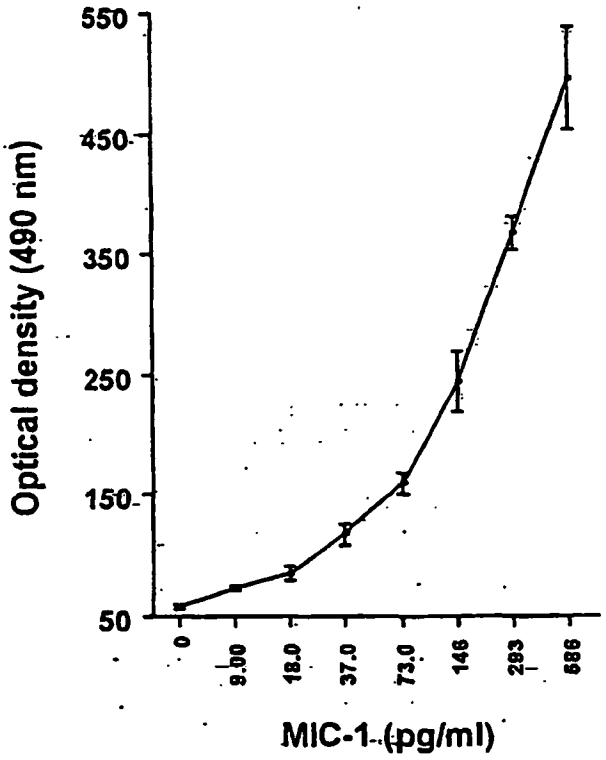
Normal sheep serum
Ab dil
233B3-P
Coat buffer
TGF-beta1
MIC-1

FIGURE 3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250
150
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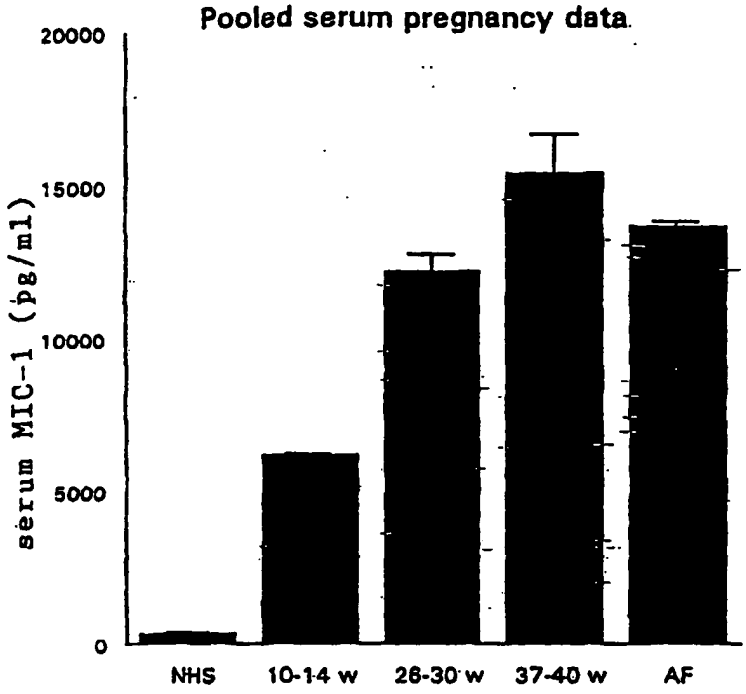
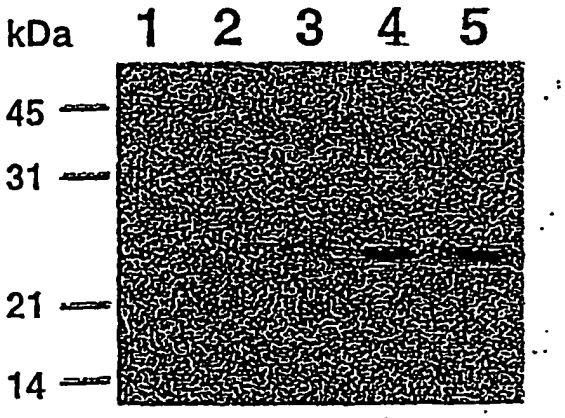
146
293
586
MIC-1 (pg/ml)

FIGURE 4A



4

FIGURE 4B



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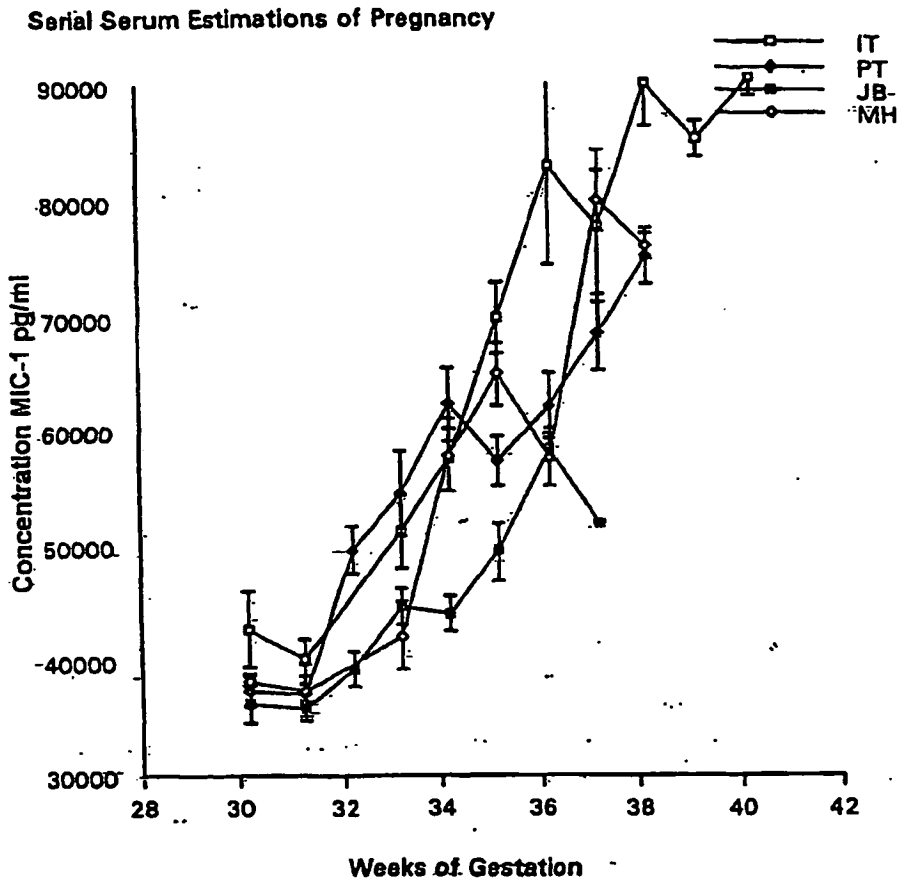

Serial Serum Estimations of Pregnancy
IT
PT
JB-
MH
90000
80000
70000
60000
50000
40000
30000
Concentration MIC-1 pg/ml
28
30
32
34
36
38
40
42
Weeks of Gestation

FIGURE 6

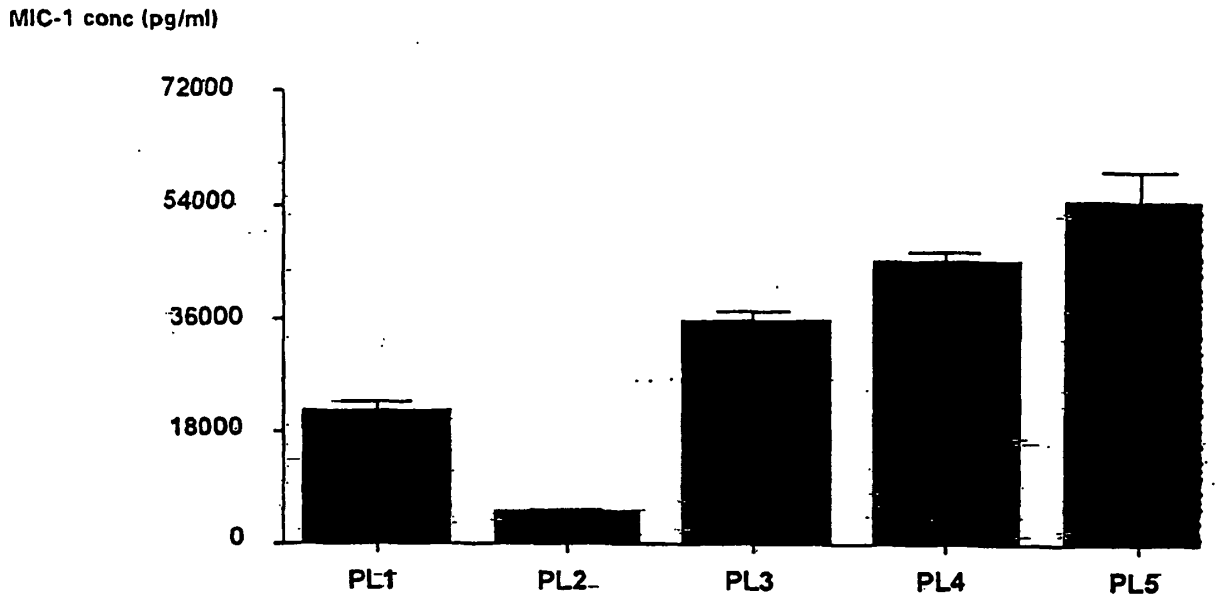


FIGURE 7A

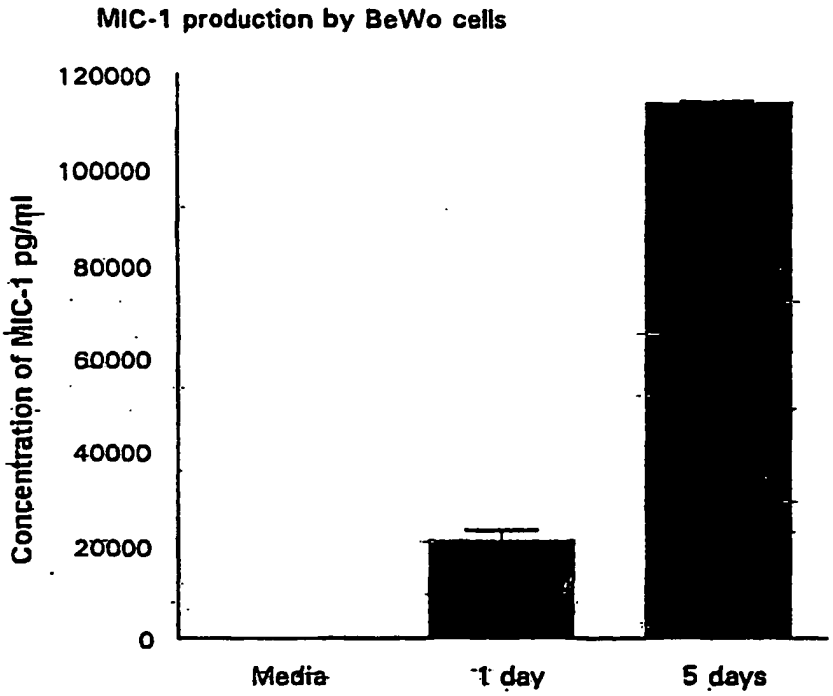


FIGURE 7

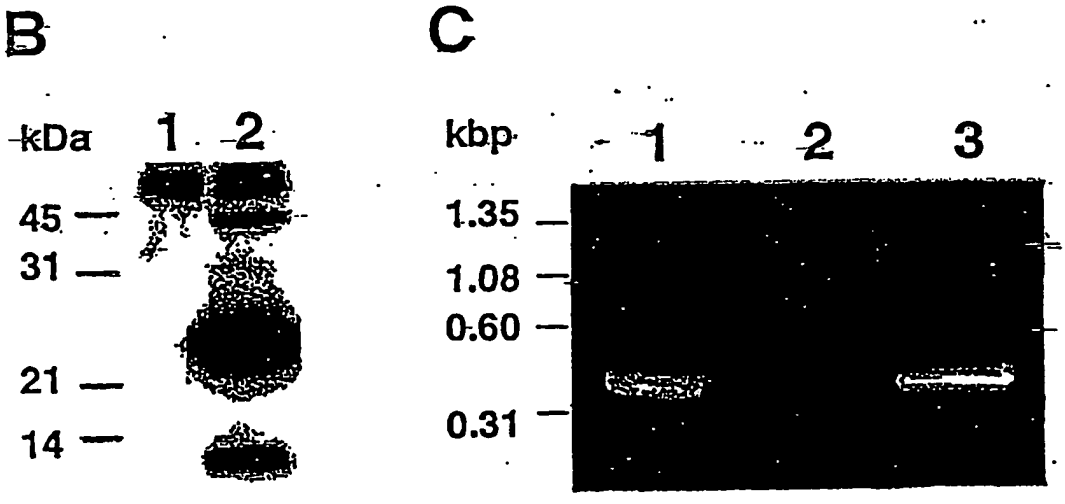
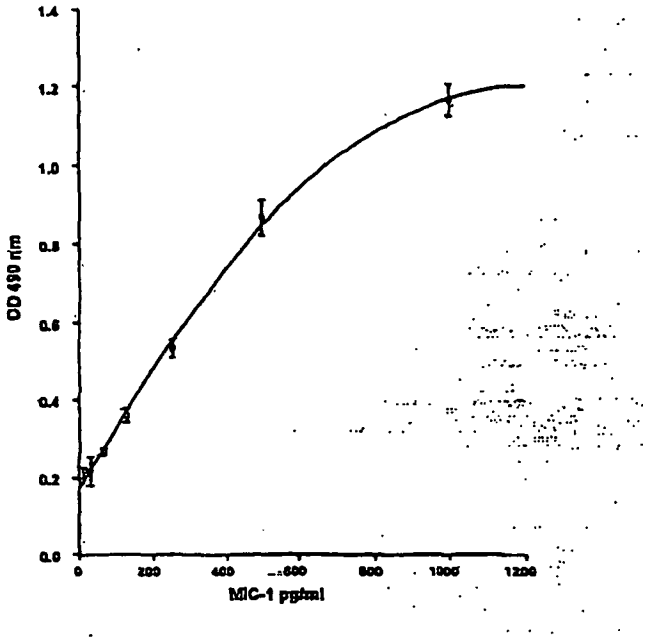


FIGURE 8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1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 490 nm
0
200
400
600
800
1000
1200
MIC-1 pg/ml

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	巨噬细胞抑制性细胞因子-1（mic-1）的诊断分析		
公开(公告)号	EP1279039B1	公开(公告)日	2008-01-16
申请号	EP2001923411	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 C07K16/24 G01N33/53 A61K38/00 A61K38/19 A61K48/00 A61P15/06 A61P29/00 A61P35/00 A61P43/00 C12N15/09 C12N15/19 C12Q1/68 G01N33/574		
CPC分类号	A61K38/195 A61P15/06 A61P29/00 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	EP1279039A4 EP1279039A1		
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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。