(54) Title: DIAGNOSTIC METHOD OF AUTOIMMUNE DISEASES

(57) Abstract: The present invention relates to a method for the diagnosis and/or prognosis of diseases which comprises the use of mesenchymal stem cells.
DESCRIPTION

"Diagnostic method of autoimmune diseases"

Technical background of the invention

The present invention relates to a method for the diagnosis of autoimmune diseases which comprises the use of substrates in place of the animal tissue commonly used.

Description of the prior art

Autoimmune diseases constitute a group of pathologies characterised by an alteration of the immune system which entails the development of cellular and humoral responses directed against components of the organism (self). In a healthy person the immune system distinguishes between the organism's own antigens, denominated self and those which are exogenous (non-self) thanks to a process known as "immunological tolerance". This involves the lymphocytes which activate against the antigens of the self, and which are defined as autoreactive, being eliminated in the primary lymph organs (central tolerance) or eliminated or rendered non-responsive (anergic) in the secondary lymph organs (peripheral tolerance) [Lampropoulou V e coll, 2008]. In an individual suffering from an autoimmune disease however a condition known as "tolerance breakdown" occurs; this
is due to an altered selection and/or regulation of the auto-reactive clones, as well as to anomalies in the presentation of the antigens. On the surface of the cells presenting the antigen (APC) the major histocompatibility complex (MHC II) is found, responsible for the presentation of the antigen to the T lymphocytes. In the case of central tolerance breakdown, the MHC II complex presents the antigen too weakly to activate the autoreactive clones, but sufficiently to actuate positive selection. [Benson R.A e coll, 2010]. The genetic component also plays an important role in autoimmune disease, mainly by the association with the human leukocyte antigen (HLA, the equivalent of MHC for humans). One example of genetic control of tolerance breakdown consists of the Autoimmune Regulator gene (AIRE) found on chromosome 21q22.3. Mutations in the AIRE gene significantly reduce the repertoire of self genes against which the immune system does not react; as a result, as soon as the T lymphocytes are liberated by the thymus into the circulation stream, numerous sensitisation processes of the immune system against self components take place [Matsumoto M., 2009]. As regards classification, autoimmune diseases divide into two groups: organ-specific and systemic. Among
the first are Hashimoto's thyroiditis, primary biliary cirrhosis, celiac disease and diabetes mellitus type I. Among the systemic autoimmune diseases directed against autoantigens of various tissues are rheumatoid arthritis, systemic erythematous lupus, scleroderma and Goodpasture syndrome.

**Significance of autoantibodies**

In the serum of patients suffering from diabetes mellitus type I, the concentration of anti-insulin autoantibodies (IAA) is, at the moment of diagnosis, inversely proportional to the age of the patient. It is believed that during the cell-mediated destruction of the α cells of the islets of Langherans, the endogenous insulin alters its three-dimensional conformation, thereby becoming antigenic and inducing an immune response [Martin S. e coll. 2001]. Moreover, a high level of antibodies in the serum of young patients is correlated with increased aggressiveness of the disease [Patel P., Macerollo A., 2010]. Autoantibodies against the antigens of the pancreatic islets (ICA) are also found in the serum of subjects in the pre-diabetic phase; this therefore represents an effective instrument for predicting diabetes in persons at risk. The age of
onset of the disease and the duration thereof seem to
influence the presence of the antibodies [Levi-
Marchal C. e coll. 1995], which are found most of all
in younger patients [Graham J. e coll. 2002]. This
phenomenon could be the consequence of the reduction
of the antigenic stimulus which occurs following the
destruction of the β cells [Knip M., Siljander H.,
2008]. Their role however is not yet entirely known,
since it has not been possible to ascertain whether
said antibodies participate directly in the
aggression against the β cell, through the bond with
the antigens thereof or if they increase as a result
of the destructive mechanism, following the release
of the antigens liberated by the lysis of the
pancreatic cells.

**Pancreatic islet cell autoantibodies (ICA)**

The ICA are class G organ-specific antibodies,
identified for the first time in 1974. They are
detected by means of an indirect immunofluorescence
test of sections of human pancreatic tissue. The
fluorescence generally affects all the islet cells,
but in some preparations may colour only the β cells.
One of the antigens which these autoantibodies are
directed against is a transmembrane protein of the
islet cells which has a domain very similar to that
of a tyrosine phosphatase present in the cytoplasm of T lymphocytes [Levi-Marchal C. e coll., 1995]. These antibodies may be present in a very high percentage (about 80%) in diabetic patients and their frequency falls over time.

**Anti-insulin autoantibodies (IAA)**

Anti-insulin antibodies do not take part in the immunofluorescence reaction associated with anti-ICA antibodies. They may however be identified in the pre-clinical stage of diabetes, especially in paediatric subjects and have a high predictive value for a rapid development of the disease [Martin S. e coll. 2001, Jaeger C., 2008]. The presence of IAA in the serum is an index of the destruction process of α cells typical of DMT 1. The IAA are fundamental for determining the risk of DMT 1, in that their concentration is very high in patients who develop the disease during infancy and they are the first autoantibodies which are seen before the pathological signs of DMT 1. In addition, the prevalence of IAA is inversely proportional to the age at which the disease is diagnosed [Cambuli V.M., 2010]. Lastly, it is worth noting that the IAA are found in about 90% of patients suffering from DMT 1 who developed the disease before 5 years of age, while determined only
in 20% of subjects suffering from DMT 1 aged over 20
[Instruction Manual MEDIPAN - CentAK® IAA RT, July
20th 2009].

Celiac disease, anti-transglutaminase tissue (anti-
tTG), anti-endomysial (EMA) and anti-smooth muscle
(SMA) autoantibodies

Celiac disease (MC) is an autoimmune disease
caracterised by intestinal malabsorption, by a
caracteristic intestinal lesion and by an intense
lymphocyte infiltration which suggests a priority
role of the immune system in the pathogenesis of the
disease. It mainly affects the intestinal mucosa,
while the sub-mucosa and muscular tunica are not
affected. The lesions may only affect the duodenum
and proximal jejunum, or the entire small intestine,
though more serious in the proximal sections. The
transglutaminase enzyme is the antigenic target of
autoantibodies found in the serum of patients
suffering from MC. The main function of this enzyme
is the deamination of the glutamine residues of the
gliadin peptides and their conversion into glutamic
acid. This modification makes the gliadin negatively
charged, and this causes its bonding with the
antigens HLA-DQ2/DQ8, with the consequent
presentation of the new peptides to the T
lymphocytes. In subjects genetically predisposed to MC, the activation of an immune response of type T and B leads to the synthesis of anti-tTG class A autoantibodies and of pro-inflammatory cytokines, giving rise to a chronic inflammation with consequent destruction of the intestinal mucosa [Lindfors K. E coll. , 2009]. The anti-tTG class A autoantibodies are a highly sensitive and specific marker for the diagnosis of MC, while the type G autoantibodies are used at the moment in which the subject potentially suffering from MC presents a class A immunoglobulin deficit. The anti-tTG antibodies recognise the same antigen as the anti-endomysial (EMA) autoantibodies, from which they differ only in the revelation method used. In fact, while the anti-tTG antibodies are dosed using an immunochemical and thereby quantitative type method, the EMA are evaluated using indirect immunofluorescence (IFA) by means of measurement of the fluorescence with a fluorescent light optic microscope, on cryostatic sections of monkey oesophagus. The EMA test is highly specific, but less sensitive than immunochemical tests for tTG, and is consequently preferably used as a confirmation test [Caglar E. e coll. , 2009]. In healthy subjects, transglutaminase has been found in all the layers of
the wall of the small intestine; the enzyme is expressed in the sub-mucosa; while only a small fraction is located in the epithelium. In subjects suffering from MC, the expression of tTG has been reported in specific districts of the small intestine, such as at the ciliated border and cytoplasm of the enterocytes, as well as in the extra cellular matrix. The autoantibodies anti-tTG and EMA are synthesised by the B lymphocytes at the level of the intestinal mucosa of subjects suffering from MC not following a gluten-free diet. These antibodies may be observed in the site of the inflammatory process, in the serum and in other biological liquids such as saliva and bile [Tonutti E., Bizzaro N. 2007]. The possibility of detecting the tTG response using monoclonal antibodies shows that the target region of the tTG in patients suffering from MC is localised at the centre of the molecule, while in healthy individuals, the anti-tTG autoantibodies, present in any case in a low percentage, recognise molecular fragments not belonging to the central region [Dipper C.R. e coll. 2009]. As a result, the presence of these autoantibodies with various target epitopes for the tTG may clarify the multiple behaviours of some serums during immunochemical and
IFA assays. In fact, in some cases, the anti-tTG IgA may be found to be positive without the EMA being positive. These data suggest that the EMA, detected by means of indirect immunofluorescence recognise conformational epitopes linked to MC [Kagnoff M.F., 2007].

The SMA (Smooth Muscle Autoantibodies) are autoantibodies present in 85% and more of patients with chronic active hepatitis and in about 50% of patients with primary biliary cirrhosis. Their presence may sometimes be found in celiac patients too.

**Anti-nuclear antibodies (ANA)**

represent an ample and heterogeneous population of anomalous antibodies directed against intracellular components of human cells, in particular nuclear (DNA, RNA, ribonucleoproteins, histones, centromeres etc.). The ANA have an important role in the medical sphere - despite being present in minimal amounts and at low intensity in many healthy individuals, they tend to increase considerably in the blood of subjects suffering from systemic autoimmune diseases.

Currently, the diagnosis of autoimmune diseases uses two main techniques by measuring:
- ELISA;
- IFA (indirect immunofluorescence).

In particular, in the field of indirect immunofluorescence, cryostatic sections of animals are used for the anti-tissue antibodies. For some determinations, as of now primate tissue is indispensable. For the anti-endomysial antibodies used to diagnose celiac disease, the third distal of the oesophagus of the monkey is used, while for example for the paraneoplastic syndromes of the central nervous system, the cerebellum of the monkey is used.

The need for a diagnostic method using alternative materials while at the same time assuring reliability of the diagnostic results obtained is therefore recognised.

Summary of the invention

The present invention exploits the potential of stem cells, differentiated and manipulated ad hoc if necessary, in place of animal tissues and cryostatic section technologies. Compared to sacrificing animals so as to freeze the tissue thereof and preparing cryostatic sections to use as diagnostic substrates, the culture of cells appears a less cruel, more
flexible method easily applicable to the preparation of diagnostic kits.

Brief description of the drawings

Figure 1 show the results of chondrocyte differentiation (A: enlargement 4X; B: enlargement 10X); Figure 2 show the results of osteoblast differentiation (A: enlargement 4X; B: enlargement 10X); Figure 3 show the results of myogenic differentiation (A: positive for myogenin with nuclear contrast DAPI; B: myogenin only; C: DAPI only); Figure 4 shows the results of maturation of the β cells after culture of HI-MSC cells in suspension; Figure 5 shows the results of the immunochemical assay on differentiated pancreas cells from pancreas MSC (A: negative control, B: after incubation with the pool of positive serums for IAA, C: after incubation with the pool of positive serums for ICA); Figure 6 shows the results of the immunochemical assay on differentiated pancreas cells from the bone marrow (A: after incubation with the pool of positive serums for IAA, B: after incubation with the pool of positive serums for ICA); Figure 7 shows the results of the assay for SMA; Figures 8 and 9 show the results of the assay for ANA; Figure 10 shows the results of the assay on tumour cells of neuroglioblastoma (A:}
negative control; B: untreated cells; C: cells treated with quercetin).

Object of the invention
A first object of the invention is represented by an in vitro method for the diagnosis of autoimmune diseases which comprises the use of stem cells. According to one particular aspect, embryonic, tumour, mesenchymal, etc. stem cells are used and preferably are mesenchymal stem cells. According to one aspect of the invention, such stem cells are differentiated into other types of cells. In a second object of the invention, the method may also be used for the prognosis of autoimmune diseases.

A further object of the invention is represented by a kit for the diagnosis and/or prognosis of autoimmune diseases.

Detailed description of the invention
Stem cells are defined as such on account of their plastic capacity to differentiate into different lines or to identically regenerate themselves. In particular, embryonic stem cells (ES), inasmuch as pluripotent, may originate all the different tissues of the organism. Their use is limited by ethical issues, although cells of embryonic derivation but
partially commissioned, can be derived from amniotic liquid and from the umbilical cord.

The study of stem cells has shown that the propagation and dissemination of tumours in the organism depends on sub-populations of tumour cells with stem properties, in other words capable of auto-renewal and favouring the recurrence of the tumour.

As regards the sources of tumour stem cells, literature has reported their presence in almost all types of leukaemia and solid human tumours (Dalerba et al, Ann. Rev. Med, 2007).

The neuroglioblastoma is an embryonic tumour of the central nervous system which appears in children and only rarely in adults. The presence of tumour stem cells in this tumour has been demonstrated and described in literature (Raso A, Negri F, Neutopath Appl Neurobiol 2008 Jun; 34(3): 306-15).

Mesenchymal stem cells (MSCs) are also pluripotent cells; in fact, grown in vitro with special culture mediums they differentiate into various cytotypes acquiring characteristics typical of the various tissues (bone, fat, muscle, endothelium, skin etc.)

As of today, the main source of MSCs is bone marrow (BM), however, the collection of a sample of bone marrow is a somewhat invasive procedure and in
addition it has been shown that the number and differentiating potential of the MSCs from bone marrow decreases proportionally with age. Precisely for such reason, other sources of MSCs have been sought and among the various possibilities the greatest success has been obtained by isolating MSCs from blood coming from the umbilical cord (UC-MSC), from the pancreatic islets (HI-MSC—human islet mesenchymal stem cell) and dental pulp (DP-MSC). The mesenchymal stem cells obtained from BM-MSC, dental pulp PD-MSC and umbilical cord tissue have been isolated according to one of the methods reported in Chapter 1, Mesenchymal Stem Cell Assays and Applications, Methods in Molecular Biology, vol. 698, Springer-Verlag, 2011; or in Salvadè et al., TISSUE ENGINEERING: Part C, Volume 15, Number 00, 2009; or in Rodriguez-Lozano.

Other "secondary" sources of MSCs may comprise peripheral blood, adipose tissue, amniotic liquid, renal glomerulus, liver and stratified human epithelium.

The autoimmune diseases for which the present method may be used comprise: celiac disease, paraneoplastic syndromes, diabetes mellitus I, Hashimoto's thyroiditis, rheumatoid arthritis, systemic
erythematous lupus, scleroderma, Goodpasture syndrome, autoimmune hepatopathy, anti-phospholipid antibody syndrome, anticoagulant lupus.

According to a preferred aspect, the method according to the invention is aimed at the diagnosis of diabetes mellitus type I and celiac disease.

It has been found, in addition, that the method described herein is also useful for the prognosis of said diseases, meaning that the method provides useful information for assessing and predicting whether the subject will develop the disease over time.

More in detail, the method comprises the determination of the presence of specific antibodies in a sample of serum of a subject on whom one wishes to perform diagnosis or prognosis.

In particular, the method proceeds by placing the serum sample in contact with a preparation of mesenchymal stem cells for a sufficient length of time to permit the formation of specific antigen-antibody complexes.

To such purpose, the incubation may be protracted by 10-60 minutes, preferably 20-40 minutes and generally 30 minutes, after which washing is performed to remove any non-bonded components.
The presence of antibodies is determined by indirect immunofluorescence.

In fact, after washing, a secondary antibody is added (anti-IgG or anti IgA) marked with fluorescein which forms an immunocomplex with the antigen-antibody complex.

In turn the quantity of secondary antibody bonded will be assessed and determined.

For the purposes of the present invention the MSCs are used in the form of a preparation.

In particular, such preparation is obtained according to known methods of the prior art (see for example: Suva et al 2004, Lokesh Batulla et al 2007; Isolation of multipotent mesenchymal stem cells from umbilical cord. Blood 2004; 103: 1669-1675).

More preferably, the cells are mesenchymal stem cells on a solid support and to such purpose a normal laboratory slide may be used.

In addition, to increase the adhesion of the cells to the support, methylcellulose or fibronectin may be used.

In a first preferred embodiment, the mesenchymal stem cells used in the diagnostic/prognostic method are obtained from one of the sources mentioned above or sourced commercially.
Alternatively, it has been found, surprisingly, that the diagnostic method according to the present invention may use stem cells obtained by differentiating mesenchymal stem cells. In fact, thanks to appropriate cultivation techniques, MSC cells of the peripheral blood, adipose tissue, amniotic liquid, renal glomerulus, liver, stratified human epithelium, pancreatic islets and dental pulp can be differentiated into various cytotypes: osteoblasts, chondrocytes, myocytes, adipocytes, pancreatic β cells, etc. In a preferred aspect of the invention, pancreatic β cells are obtained from MSCs isolated from pancreas islets, from bone marrow or from dental pulp. According to a second object of the invention, a kit to be used for the diagnosis or prognosis of autoimmune diseases which comprises a preparation of mesenchymal stem cells is described. Preferably, the autoimmune diseases diagnosed/prognosticated are those mentioned above. For the purposes of the present invention the cells are immobilised on a solid support, for example consisting of a normal laboratory slide. In particular, in a preferred aspect, the adhesion of the cells to the support is promoted by the use of
methylcellulose or fibronectin, as for example performed when operating with pancreatic cells. As well as the MSCs preparation, the kit according to the invention comprises anti-IgG antibodies appropriately marked for their determination. In a preferred aspect, these are fluorescein-marked antibodies.

Experimental part

Adipocyte differentiation

1x10^5 mesenchymal stem cells (MSCs) were expanded with a serum-free expansion medium (Euromed Mesenchymal medium MSC) and brought into confluence over a period of 1-2 weeks. They are then placed in a medium comprising suitable differentiation factors (such as for example, the Euromed Human Adipogenic differentiation kit). The differentiation into adipocytes is observed after 2-3 weeks (Concise Review: Adipose Tissue-Derived Stromal Cells—Basic and Clinical Implications for Novel Cell-Based Therapies. Stem Cells. 2007;25;818-827).

Chondrocyte differentiation

The MSC cells are induced to chondrogenic differentiation in pellets. 2.5x10^5 of MSC are placed in a 15 ml bottle in 1 ml of serum-free expansion medium (Euromed Mesenchymal medium MSC) and grown for
1-2 weeks. They are subsequently centrifuged at 150 x g for 5 min. at room temperature, aspirating and eliminating the supernatant (the operation should be repeated twice). 5 ml of complete medium comprising suitable differentiation factors (such as the Chondrogenic differentiation kit) is added to the pellet thus formed according to the methods reported in literature (Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315-7). Every two/three days the medium is aspirated with a sterile pipette and replaced with fresh medium. Complete differentiation takes 14-28 days. The pellet is fixed in formaldehyde or alcohol and enclosed in paraffin for the preparation of histological sections. The sections are then coloured with safranin O to highlight the glycosaminoglycans. The mucopolysaccharides of the cartilage are coloured a bright blue (Figure 1).

Osteoblast differentiation

The MSC cells are differentiated into osteoblasts using a medium comprising suitable differentiation factors (such as for example EUROMED Human
Osteogenic) placing 1x10^5 MSC cells in a T25 for about 2-3 weeks (Barker E, Shiga A, Davies JE: Growing human bone in vitro. In: Bone Engineering. Emsquared Inc. Toronto, pp 63-77, 1999). The cells are then coloured with Alzarin Red to identify the calcium deposits present in the extracellular matrix during mineralisation of the bone (Figure 2).

Myogenic differentiation

For the differentiation in a myogenic sense 5x10^5 cells of umbilical cord were grown in DMEM with 10% of serum knockout and 0.1% β-mercaptoethanol and 40 ng/ml of Fibroblast Growth Factor (FGF) (Research Article Minced Umbilical Cord Fragments as a Source of Cells for Orthopaedic Tissue Engineering: An In Vitro Study A. Marmotti et al 2012). After 3 weeks the cells were marked with a specific antibody for human myogenin (Abcam, Cambridge, UK) (Figure 3).

Differentiation into pancreatic β-cells

For the differentiation into pancreatic β cells, 10^{15} MSC cells isolated from the pancreas, bone marrow (BM-MSC) and dental pulp, were put on plates in a T75 bottle and placed in a serum-free culture medium called EuroMed Human Mesenchymal Stem Cell Kit (ECM0888K) for 1-2 weeks; once 60% confluence was reached, the medium was replaced with another
containing DMEM, glucose in a variable concentration from 4.5 to 25 mM, platelet lysate 5%, retinoic acid, activin, glucagon-like peptide I (GLP-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), β-cellulin, nicotinamide and glutamine 1X, to assess the differentiation capacity and neogenesis of the β cells.

Results
The IFA analysis of serums containing EMA, IAA and ICA, SMA and ANA were performed at a referral laboratory using highly automated methods and, subsequently, the same samples were manually measured at the EuroClone laboratory (Molecular Biotechnology Center, Turin).

The two methods of analysis were compared paying attention to the concordance and discordance between the results obtained with the automated method and those obtained using a manual method which envisaged the use of a substrate consisting of MSC, MSC differentiated into pancreatic cells and MB cells.

IFA for serums containing ICA and IAA
The serums containing ICA and anti-insulin IAA are diluted in PBS at a proportion of 1:2. 100 µL of diluted serum are placed in each well of every slide; these are left to incubate for 30
minutes, after which the slide is washed with PBS to remove the aspecific bonds, immersed for 5 minutes in a Coplin tray containing PBS, and incubated for 30 minutes with 100 μL of secondary antibody fluorescein-marked anti-IgG. After the incubation time is up, the slide is washed in PBS, mounted and observed under the fluorescence microscope with the filter regulated to the wavelength of the fluorescein.

**IFA on differentiated pancreatic cells starting from MSC from the pancreas and MSC from the bone marrow.**

**IFA performed on positive serums for EMA, IAA, ICA**

An immunochemical assay was conducted in differentiated pancreas cells starting in the first experiment from pancreas MSC and in the second from bone marrow MSC. Both cell populations were then fixed to the slide using fibronectin.

Both pancreatic cell populations were incubated with a negative control belonging to the kit marketed by INOVA diagnostic Inc., with a pool of positive serums for IAA and with a pool of positive serums for ICA. The differentiated pancreatic cells starting from pancreas MSC and incubated with the negative control INOVA diagnostic Inc., did not show fluorescence (Figure 5A) despite showing a slightly positive
background probably on account of molecular interactions with the fibronectin.

The pancreatic cells incubated respectively with the pool of positive serums for IAA (figure 5B) and with the pool of positive serums for ICA (figure 5C) showed instead, some fluorescence.

In the second experiment too, the differentiated pancreatic cells starting from bone marrow MSC and incubated with the negative control INOVA diagnostic Inc., did not show positivity, despite a slight fluorescence of the background. The pancreas cells incubated instead with the pool of positive serums for IAA and ICA, showed a good fluorescence (Figures 6A and 6B respectively).

SMA on umbilical cord mesenchymal cells

Umbilical cord mesenchymal cells (UC-MSCs) were sown in concentrations of 2x10⁴ in each well of the slides Lab-Tek® Chamber-Slide™ 2 well glass slide (code 177380, NUNC). After 48 hours in the incubator at 37°C at 5% CO₂, the cells achieved confluence, the culture medium was aspirated and washing performed with PBS. Subsequently the UC-MSCs on the slide were fixed with cold acetone for 5 minutes.

The serums of patients with celiac disease were diluted 1:5.
100 µL of diluted serum are placed in each well of every slide; these are left to incubate for 30 minutes, after which the slide is washed with PBS to remove the aspecific bonds, immersed for 5 minutes in a Coplin tray containing PBS, and incubated for 30 minutes with 100 µL of secondary antibody fluorescein-marked anti-IgA. After the incubation time is up, the slide is washed in PBS, mounted and observed under the fluorescence microscope with the filter regulated to the wavelength of the fluorescein.

Figure 1 shows positivity of fibrils referable to filaments of actin and rod cells corresponding to the anti-endomysial antibodies.

**ANA on umbilical cord mesenchymal cells**

Serums of patients with autoimmunity of various origin were diluted 1:40 to verify anti-nuclear positivity. 100 µL of diluted serum are placed in each well of every slide; these are left to incubate for 30 minutes, after which the slide is washed with PBS to remove the aspecific bonds, immersed for 5 minutes in a Coplin tray containing PBS, and incubated for 30 minutes with 100 µL of secondary antibody fluorescein-treated total anti-IgG.
Figure 2 shows the positivity with aspects of nuclear mitotic apparatus (NUMA) and high granular positivity (APG), while Figure 3 shows anti-nuclear positivity with high positivity of the SPECKLED type.

**IFA for EMA on tumour stem cells from neuroglioblastoma**

The serum containing EMA is diluted 1: 2.5 with PBS and subsequently a volume of 100 µL is placed in the well of the slide made previously. The serum is left to incubate for 30 minutes and after such time the slide is washed with PBS to remove the fraction of antibodies which have not bonded, immersed for 5 minutes in a Coplin tray containing PBS and subsequently incubated with 100 µL of secondary fluorescein-marked secondary antibody (anti-IgA 1:40 for EMA) for 30 minutes in a humidity chamber in the dark. After incubation, the slide is washed following the procedure described in the washing step above, a slide cover applied to it and fixed with glycerol and subsequently read under the fluorescence microscope with the filter regulated to the wavelength of the fluorescein.

To assay the capacity to induce a considerable hyper-expression of anti-transglutaminase (tTG), both MB cells not treated with any type of drug and cells
treated with quercetin 10 \( \mu \text{M} \), a molecule able to significantly increase the expression of tTG, were placed on each slide. The positive serum for the EM, with a tTG value of 119 U/mL, showed a good level of fluorescence of the MB cells (see Figure 10B), while the negative serum, with a value of 0 U/mL of tTG, gave no fluorescence (see figure 10A).

The cells treated instead with quercetin 10 \( \mu \text{M} \) for 24 hours, and incubated with the positive serum for EMA, showed more marked fluorescence compared to the non-treated cells; such effect was not however found in the MB cells treated with doxorubicin 10 nM for 24 hours, showing a practically identical pattern to the cells not treated with any drug (Figure 10C).

From the description given above of the method for diagnosing/prognosticating an autoimmune disease, the advantages provided compared to the methods used up to now will be evident to a person skilled in the art.

In particular, the innovation described in this patent application permits the replacement of the use of human and animal tissue with a set of adequately manipulated stem cells. This technology therefore makes it possible to avoid sacrificing animals,
thereby offering numerous advantages both of an economic and ethical nature. In addition, it makes it possible to simplify the procedures and operations for preparing diagnostic substrates. In addition, it makes the reading of the results simpler in that advanced skills in the morphological field no longer need to be availed of. Another considerable advantage is related to the possibility of automating the diagnostic procedure thus developed.
CLAIMS

1. *In vitro* method for the diagnosis and/or prognosis of diseases in a subject comprising the use of stem cells.

2. *In vitro* method for the diagnosis and/or prognosis of diseases according to the previous claim, wherein said stem cells are of tumoral, embryonic or mesenchymal origin.

3. *In vitro* method for the diagnosis and/or prognosis of diseases according to claim 2, where the tumour stem cells are obtained from a neuroglioblastoma.

4. Method according to the previous claim for the diagnosis and/or prognosis of celiac disease.

5. *In vitro* method for the diagnosis and/or prognosis of diseases according to claim 2, wherein the embryonic stem cells are obtained from amniotic liquid or from the umbilical cord.

6. *In vitro* method for the diagnosis and/or prognosis of diseases according to claim 1 or 2, wherein said stem cells are mesenchymal stem cells.

7. *In vitro* method according to the previous claim wherein said mesenchymal stem cells are isolated from the pancreas, bone marrow, blood coming from the umbilical cord, dental pulp, adipose tissue, amniotic liquid, renal glomerulus, liver and stratified human epithelium.

8. *In vitro* method according to the previous claim wherein said mesenchymal stem cells are isolated from the pancreas.

9. *In vitro* method according to the previous claim wherein said mesenchymal stem cells are
mesenchymal stem cells isolated from the pancreas islets.

10. *In vitro* method according to any of the claims from 7 to 9, wherein said mesenchymal stem cells have been differentiated into pancreatic β cells, adipocytes, chondrocytes, osteoblasts or myocytes.

11. *In vitro* method according to the previous claim, wherein mesenchymal stem cells isolated from the pancreas islets or bone marrow have been differentiated into pancreatic β cells.

12. *In vitro* method according to any of the previous claims, wherein said diseases are autoimmune diseases.

13. *In vitro* method according to the previous claim wherein said diseases comprise: celiac disease, paraneoplastic syndromes, diabetes mellitus I, Hashimoto's thyroiditis, rheumatoid arthritis, systemic erythematosus lupus, scleroderma, Goodpasture syndrome, autoimmune hepatitis, anti-phospholipid antibody syndrome, anticoagulant lupus.

14. *In vitro* method according to any of the previous claims, comprising the step of placing the serum sample of said subject in contact with a preparation of said stem cells.

15. *In vitro* method according to the previous claim wherein said stem cells are mesenchymal stem cells.

16. *In vitro* method according to claim 14 or 15, wherein the serum sample and the preparation of stem cells are incubated for a sufficient
length of time to permit the formation
immunocomplexes.

17. In vitro method according to any of the
claims from 14 to 16, further comprising the
step of determining and/or quantifying the
presence of antibodies against such disease by
means of immunofluorescence.

18. In vitro method according to the previous
claim wherein said antibodies are IAA, ICA, EMA,
SMA or ANA antibodies.

19. In vitro method according to any of the
claims from 14 to 18, wherein said determination
and/or quantification is conducted by the
addition of a secondary antibody.

20. In vitro method according to the previous
claim wherein said secondary antibody may be
determined/quantified by fluorescence.

21. In vitro method for the diagnosis of
diabetes mellitus I in a subject, comprising the
step of placing the serum sample of said subject
in contact with a preparation of pancreatic β
cells obtained from the differentiation of
mesenchymal stem cells.

22. In vitro method for the diagnosis of
diabetes mellitus I according to the previous
claim wherein said pancreatic β cells are
obtained from the differentiation of mesenchymal
stem cells isolated from the pancreas, from bone
marrow or from dental pulp.

23. In vitro method for the diagnosis of
diabetes mellitus I according to the previous
claim wherein said mesenchymal cells have been
differentiated by means of use of the culture medium Euromed Human Mesenchymal Stem Cell Kit.

24. In vitro method for the diagnosis of diabetes mellitus I according to the previous claim further comprising the step of determining the presence of ICA and/or IAA antibodies.

25. In vitro method for the diagnosis of celiac disease in a subject comprising the step of placing the serum sample of said subject in contact with a preparation of mesenchymal cells from the umbilical cord.

26. In vitro method for the diagnosis of celiac disease according to the previous claim further comprising the step of determining the presence of SMA antibodies in said serum sample.

27. In vitro method according to any of the claims from 21 to 26, wherein said preparation of mesenchymal stem cells is stuck to a solid support.

28. A diagnostic/prognostic kit for the diagnosis and/or prognosis of diseases comprising a solid support comprising:
   a) a preparation of mesenchymal stem cells, or
   b) a preparation of differentiated mesenchymal stem cells;
      adhering thereto.

29. Kit according to the previous claim, wherein said diseases are autoimmune diseases.

30. Kit according to claim 28 or 29, wherein said diseases comprise celiac disease, paraneoplastic syndromes, diabetes mellitus I, Hashimoto's thyroiditis, rheumatoid arthritis,
systemic erythematous lupus, scleroderma, Goodpasture syndrome.

31. Kit according to any of the claims from 28 to 30, further comprising secondary antibodies anti-IgG or anti IgA.

32. Kit according to the previous claim, wherein said secondary antibodies anti-IgG may be determined by fluorescence.

33. Kit according to any of the claims from 28 to 32, wherein said preparation a) comprises mesenchymal stem cells isolated from the pancreas, bone marrow, blood coming from the umbilical cord, dental pulp, adipose tissue, amniotic liquid, renal glomerulus, liver and stratified human epithelium.

34. Kit according to any of the claims from 28 to 32, wherein said preparation b) comprises mesenchymal stem cells differentiated into pancreatic β cells.

35. Kit according to the previous claim, wherein said pancreatic β cells are obtained starting from mesenchymal stem cells obtained from the pancreas islets or from bone marrow.

36. Use of stem cells for the diagnosis and/or prognosis of diseases.

37. Use of stem cells according to the previous claim for the diagnosis and/or prognosis of autoimmune diseases.

38. Use of stem cells according to claim 36 or 37, wherein said stem cells are embryonic, tumour or mesenchymal stem cells.
39. Use of stem cells according to any of the claims from 36 to 38, wherein said cells are mesenchymal stem cells.

40. Use of stem cells according to claim 36 or 37 for the diagnosis and/or prognosis of celiac disease.

41. Use of mesenchymal stem cells for the diagnosis and/or prognosis of celiac disease, paraneoplastic syndromes, diabetes mellitus I, Hashimoto's thyroiditis, rheumatoid arthritis, systemic erythematous lupus, scleroderma, Goodpasture syndrome.

42. Use of stem cells according to the previous claim, wherein said mesenchymal stem cells are isolated from the pancreas, bone marrow, blood coming from the umbilical cord, dental pulp, adipose tissue, amniotic liquid, renal glomerulus, liver and stratified human epithelium.

43. Use of mesenchymal stem cells according to any of the claims 41 or 42, wherein said mesenchymal stem cells are differentiated into pancreatic cells, adipocytes, chondrocytes, osteoblasts or myocytes.
Fig. 2

A

B
Fig. 3

A  Merge
B  Myogenin
C  Dapi
Fig. 5

A  
Negative control

B  
IAA

C  
ICA
Fig. 6

A

B
7/10

Fig. 7
10/10

Fig. 10

A  B  C
A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Relevant to claim No.</th>
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<td>WO 2012/152717 A1 (EUROCLONE SPA [IT]; LEO IZZI [IT]; CRISTINA ZANINI [IT]) 15 November 2012 (2012-11-15) p. 5-6 and 13-14</td>
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Further documents are listed in the continuation of Box C.

See patent family annex.

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Date of the actual completion of the international search: 3 July 2013
Date of mailing of the international search report: 11/07/2013

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