(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 September 2004 (16.09.2004)

PCT

(10) International Publication Number
WO 2004/078956 A2

(51) International Patent Classification[7]: C12N 5/06

(21) International Application Number:
PCT/IB2004/000930

(22) International Filing Date: 5 March 2004 (05.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/452,601 5 March 2003 (05.03.2003) US

(71) Applicant *(for all designated States except US)*: **CIL-BIOTECH S.A.** [BE/BE]; c/o Faculté Polytechnique de Mons, Rue de l'Epargne 56, B-7000 Mons (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MILLER, Alain** [BE/BE]; 63, boulevard Albert-Elisabeth, B-7000 Mons (BE). **HENRY, Christophe** [BE/BE]; 75, rue Bas-Bonlez, B-1325 Bonlez (BE).

(74) Agents: **MARTIN, Jean-Jacques** et al.; Cabinet Regimbeau, 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/078956 A2

(54) Title: NOVEL PROCESS FOR DETACHING CONFLUENT CELLS FROM TWO DIMENSIONAL MICROSUPPORTS AND ITS APPLICATION FOR PREPARING TRANSPLANTS

(57) Abstract: The present invention relates to a process for detaching confluent anchoragedependent cells (ADCs') from two-dimensional microsupports (2D-MS) onto which these ADCs' are cultivated, particularly ADCs' which can be cultivated in a state suitable for transplantation, such as ADCs' derived from pancreatic cells. The invention further relates to a process for preparing reconstituted organoids from such detached confluent ADCs', which can be immortalized, according to the invention. The invention also relates to the use of organoids obtained by the process according to the invention for screening cell activity modulator or as therapeutic transplant for their administration in patients in need of such treatment. Finally, the present invention relates to a method for preventing or treating diabetes wherein pancreatic cells-derived organoids according to the invention are transplanted in a patient in need of such treatment.

NOVEL PROCESS FOR DETACHING CONFLUENT CELLS FROM TWO DIMENSIONAL MICROSUPPORTS AND ITS APPLICATION FOR PREPARING TRANSPLANTS.

The present invention relates to a process for detaching confluent anchorage-dependent cells (ADCs') from two-dimensional microsupports (2D-MS) onto which these ADCs' are cultivated, particularly ADCs' which can be cultivated in a state suitable for transplantation, such as ADCs' derived from pancreatic cells. The invention further relates to a process for preparing reconstituted organoids from such detached confluent ADCs', which can be immortalized, according to the invention. The invention also relates to the use of organoids obtained by the process according to the invention for screening cell activity modulator or as therapeutic transplant for their administration in patients in need of such treatment. Finally, the present invention relates to a method for preventing or treating diabetes wherein pancreatic cells-derived organoids according to the invention are transplanted in a patient in need of such treatment.

ADCs' are dependent on adhesion to a support for their proliferation and for retaining their cellular functions and viability. The majority of vertebrate cell cultures *in vitro* are grown as monolayers on an artificial substrate bathed in nutrient medium. The nature of the substrate on which the monolayers grow may be solid, such as plastics, or semisolid gels, such as collagen or agar. Now, disposable plastics have become the preferred substrate used in tissue and cell culture. A number of solid microsupports used for the mass cultivation of ADCs' are often characterized by a three-dimensional (3D) geometry such as spherical geometry microbeads (i.e. Cytodex™ Pharmacia (UPPSALA, Sweden)). New microsupports with a two-dimensional geometry (2D-MS) have been since also developed such as those named 2D-MS described in the European patent EP-A-0 579 596, document which is incorporated hereby by reference. Throughout this application, references are made to various publications. The entire disclosures of these references are hereby incorporated by reference into the present application to provide information concerning the state of the art as of the time the invention was made.

Culture to confluence of ADCs' leads to the formation of intercellular bonds or intercellular junctions. These intercellular junctions together with the biological functions of the cells contribute to the properties of the cells.

When attached to their solid microsupport, each single cell spreads into a star-like shape and then divides to finally form a "cobblestone" appearance at confluence at which stages, cells are joined together by intercellular junctions.

The method used to recover the monolayer cells sheet attached to its microsupport, particularly on 2D-MS, while retaining the biological and physiological properties of the cells is one of the limitations of this type of technology on microsupports. Usually, the monolayers are detached from the microsupports onto which they are formed using enzymatic and/or chelating agents treatments, such as for example trypsin and collagenase enzymatic digestion , along with a chelating agent such as ethylene diamine tetraacetate (EDTA), all treatments which can damage the cell functions and particularly the intercellular bonds or intercellular junctions which are necessary to keep their properties.

This limitation is of particular importance when these monolayers are used as inoculum to obtain sufficient amounts of cells in good physiological conditions and adequate three-dimensional organisation for later transplantation.

Indeed, for curing, tissue transplantation has recently been of great interest. For example, for curing diabetes, pancreatic cells such as Langerhans islets-derived cells are transplanted into a patient suffering from diabetes in order to provide the controlled amounts of insulin necessary for the patient.

Diabetes mellitus (usually referred to simply as diabetes) is a complex disease characterized by a grossly abnormal pattern of carbohydrate metabolism (especially a permanent elevated blood glucose concentration) resulting from impaired insulin secretion and/or effectiveness. The incidence of diabetes in industrialized countries is about 10 %. Indeed, diabetes is the most common serious metabolic disease in the world, it affects hundreds of millions of people.

The results reported by the Edmonton group have rekindled interest for the transplantation of islets of Langerhans as a cure for diabetes (Shapiro et al., N. Engl. Med., 2000, 343:230-8; Ryan et al., Diabetes 2002, 51:2148-57 and Diabetes 2001, 50:710-9). However, one of the major obstacles in human islet transplantation is the limited supply of human donor tissue. The pig may be an alternative source of donor organs because porcine and human insulins are very similar, and the metabolic characteristics of pigs are similar to those of humans. Unfortunately, isolation of adult

porcine islets turned out to be difficult. No consistent preparation could be obtained and the isolated islets usually survived poorly when cultured (Brandhorst et al., Transplantation, 1999, 67:1533-1541; Krickhahn et al., Ann Transplant, 2001, 6:48-54 and Nielsen et al., Comp. Med., 2002, 52:127-35). On the other hand, neonatal islets porcine clusters (NIC) are characterized by reproducible successful isolations and high yields, sizeable advantages over adult islets (Vizzardelli et al., Xenotransplantation, 2002, 9:14-24). A procedure was developed for the preparation of NIC (Korbutt et al., J. Clin. Invest., 1996, 97:2119-2129). After being cultivated *in vitro*, the insulin content of these aggregates increased progressively. They were then able to normalize blood glucose concentration when transplanted into diabetic nude mice (Korbutt et al., 1996; Yoon et al., Cell Transplant, 1999, 8:673-689; Trivedi et al., Endocrinology 2001, 142:2115-2122; Lopez-Avalos et al., Transplantation 2001, 71:1154-1162; Tatarkiewicz et al., Transplantation 2001, 71:1518-1526; Vizzardelli et al., 2002). However, this normalisation of blood glucose concentration required 8 to 9 days of culture before transplantation and from 8 to 20 weeks post-transplantation (Korbutt et al., 1996; Trivedi et al., 2001; Yoon et al., 1999). The need for a long *in vivo* functional maturation represents a potential problem in term of monitoring after implant (Vizzardeli et al., 2002). Furthermore, the graft's glucagon content did not increase after transplantation, suggesting that α cells normally present in the islets of Langerhans had an impaired physiology (Korbutt et al., 1996).

Therefore, it is an object of the present invention to provide a novel method for detaching ADCs' from solid microsupports, particularly from 2D-MS onto which these ADCs' are cultivated, whereby cell junctions and viability of these ADCs' are not impaired.

It is also an object of the present invention to provide detached ADCs' flat monolayer sheets from their 2D-MS whereby cell junctions and viability of these detached ADCs' sheets are not impaired and allows one to obtain three dimensional cells culture (such as organoids) in a suitable state for transplantation, particularly for Langerhans islets transplantation.

It is also an object of the present invention to provide a process for preparing Islets of Langerhans transplants, named hereinafter PILS (Pseudo Islets of Langerhans),

containing cells in a suitable state for transplantation and which could differentiate when transplanted into either insulin- or glucagon- producing cells.

The present inventors have discovered that a dispase enzymatic treatment to detach monolayers from solid microsupports onto which they are cultivated allows one to obtain monolayers wherein cell junctions and viability of these proliferative ADCs' are not impaired and wherein said monolayers can be organized in a suitable state for transplantation.

The present inventors have also discovered that monolayers derived from pancreatic cells and detached by a dispase enzymatic treatment from 2D-MS, such as MicroHex™, allows them to obtain spontaneously-formed Pseudo Islets of Langerhans organoid (PILS) containing cells in a suitable state for transplantation and which could differentiate upon transplantation into either insulin- and/or glucagon-synthesizing cells.

So, in a first aspect, the present inventions is directed towards a method to detach a confluent anchorage-dependent cell (ADC) monolayer from the flat microsupport onto which said ADCs' are cultured, characterized in that said method comprises an enzymatic treatment with an enzyme which cleaves fibronectin and type IV collagen.

Among the enzymes which can cleave fibronectin and type IV collagen, proteases such as dispase, collagenase, trypsin or trypsin-EDTA, liberase, elastage, pronase, papain and accutase can be cited. Preferably, said enzyme is a dispase.

In a more preferred embodiment said dispase is a dispase II.

Dispase, particularly Dispase II, has proven to be an effective but gentle neutral protease for separating intact epidermis from the dermis (Reenstra et al., Anat. Rec., 232(3):340-8, 1992) or to separate porcine keratinocytes primary culture in logarithmic growth phase from the floor of the petri dishes onto which they are cultivated (Cao et al., Zhonghua Wai Ke Za Zhi 2002, 40:24-26). Dispase II cleaves fibronectin and type IV collagen whereas it degrades type I collagen only minimally (Stenn et al., J. Invest. Dermatol., 1989, 93(2):287-290).

In a more preferred embodiment, the invention is directed towards a method for detaching monolayers from their flat microsupport according to the present invention wherein the dispase II is added to a flask containing the microsupport suspension onto which the anchorage-dependent cells have reached confluence.

In a further more preferred embodiment, the invention is directed towards a method for detaching monolayers from their flat microsupport according to the present invention wherein about 5 mg/ml (5 ± 1 mg/ml, preferred is 5 ± 0.5 mg/ml, most preferred is 5 ± 0.1 mg/ml) of dispase II is added to the flask. In a further more preferred embodiment, the invention is directed towards a method for detaching monolayers from their microsupport according to the present invention wherein the dispase II treatment is carried out during at least about 30 min (30 ± 5min), preferably during a time comprised between about 35-45 min (30 ± 5min - 45 ± 5min), more preferably at about 37°C (37 ± 5°C).

In a particularly more preferred embodiment, the invention is directed towards a method for detaching monolayers from their flat microsupport according to the present invention wherein said solid microsupport is a two-dimensional geometry microsupport (2D-MS).

In the present specification, the term "two-dimensional geometry" means that the thickness of these microsupports tends to become infinitesimal and negligible compared with the dimensions of the cultivated cells. This reduction in thickness is such that there is no possibility of cell growth on the microsupport's edges, but only on the two apposed faces of the 2D-MS. Such 2D-MS offer the principal advantage of an anchorage surface per unit volume that is higher that of 3D microsupports.

These 2D-MS are particularly well described in the European patent EP-A-0 579 596, the entire disclosures of this document which provide the characteristics of these two-dimensional geometry microsupports (2D-MS) is incorporated hereby by reference.

In the most preferred embodiment, the invention is directed towards a method for detaching monolayers from their 2D-MS according to the present invention wherein said microsupport is the two-dimensional geometry microsupports MicroHex™ (provided by Nunc, Roskilde, Denmark).

In another preferred embodiment, the invention encompasses a method for detaching monolayers from their 2D-MS according to the present invention wherein said ADCs' contain one type cell, such as ADCs' derived from one cell clone, or wherein said ADCs' contain at least two type cells such as derived from a co-culture of least two cell clones or derived from a mix of two distinct cell cultures or derived from

a tissue containing two distinct cell type (e.g. pancreatic cells containing α- and β-cells).

The invention is directed to a method for detaching monolayers from their microsupports according to the present invention wherein said ADCs' can be derived from mammalian tissues, non-human or human mammal.

Any mammalian cell may be used in the present invention.

It is preferred among these mammals cells those which may be grown in the three dimensional culture systems. The most preferred are brain and other neural tissue, pancreatic, bone marrow, skin, liver, pancreas, kidney, heart, mucosal epithelium, neuroepithelial haemopoietic, endothelial, adenocarcinoma or melanoma tissue, or stem cells.

By "stem cell", it is intented here to designate a pluripotent progenitor cell that has been characterized as a cell which can self-replicate and has multilineage potential, optionally that is transplantable.

In a more preferred embodiment, the invention is directed towards a method for detaching monolayers from their microsupports according to the present invention wherein said ADCs' are derived from foetal or neonatal tissue, preferably from human foetal or neonatal tissue.

In a further more preferred embodiment, the invention is directed towards a method for detaching monolayers from their microsupports according to the present invention wherein said ADCs' are derived from pancreas.

In the most preferred embodiment, the invention pertains to a method for detaching monolayers from their solid microsupports according to the present invention wherein said ADCs' are derived from pancreatic cells, preferably from human or pig pancreas.

In another most preferred embodiment, the invention pertains to a method for detaching monolayers from their solid microsupports according to the present invention wherein said ADCs' are derived from foetal cells, preferably neural foetal cells.

In another most preferred embodiment, the invention encompasses a method for detaching monolayers from their solid microsupports according to the present invention wherein said ADCs' are immortalized, preferably by a method comprising the incorporation of a myc gene (here named "myc-immortalized").

Methods for preparing cells which are immortal, preferably which are suitable for transplantation therapy and, so, which are immortal up to the time of their transplantation, are well known by the skilled man.

For example, it can be cited the international PCT patent application document published on September 13, 2001 under the number WO 01/66781 (Sinden et al., Reneuron LTD, GB) which discloses immortal cells including an exogenous polynucleotide introduced into the cell that encodes a member of the myc oncogene family and a conditionally-inducible oncogene which can be regulated under certain conditions. The oncogene will undergo expression when permissive conditions are applied. For example, the oncogene that can be used could be a non-DNA binding, temperature-sensitive, mutant of the SV40 large T-antigen gene, e.g. U19tsA58 (Almazon and McKay, Brain Res., 1992; 579:234-245). Suitable alternatives are also known and include the oncogene of the polyoma Tantigen adenovirus EIA and HPV16 or 18E7. Suitable cDNA variants may also be used.

Among the myc proto-oncogene family, C-myc, N-myc, L-myc, B-myc, V-myc and Gag-myc can be suitable for usein the present method for preparing the so-called myc-immortalized cells, although C-myc is preferred. A review of the myc family is provided by Alt et al., Cold Spring Harbour Symp. Quant. Biol., 1986, 51:931-941. At least C-myc, N-myc, Lmyc and B-myc have a similar gene structure and encode nuclear phosphoproteins with homologous amino acid sequences (Legouy et al., EMBO J, 1987, 6:3359-3366; and Ingvarsson et al., Mol. Cell Biol., 1988, 8:3168-3174).

For therapeutic use of myc-immortalised cells, a preferred embodiment would permit control over the function of the Myc protein, such that the immortalising protein was not functional after transplanting the cells. This would reduce the risk of overgrowth or tumour growth by the transplanted cells. A preferred conditional form of Myc is a fusion between Myc and the hormone binding domain of a modified estrogen receptor (Littlewood, T., Hancock, D., Danielian, P., Parker, M., and Evan, G., 1995. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nuc. Acids. Res., 23, 1686-1690), a variant of an earlier idea from Eilers and Bishop (Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, M. J., 1989. Chimaeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature, 340, 66-68). In the preferred form

of this immortalising fusion gene described by Littlewood et al. above, the functional Myc protein is activated by adding the synthetic steroid 4-hydroxytamoxifen to the cell growth media.

The conditionally-inducible oncogene and the polynucleotide encoding the myc gene may be comprised in a recombinant DNA or retroviral vector or construct to transduce/infect the cells. The two components may be incorporated into one vector or each may be comprised in a separate vector which may further comprise a suitable promoter region to initiate transcription of DNA and a selectable marker which may be used to identify those cells that have undergone transduction/infection. Regulation of expression may be carried out by methods known to the skilled person.

For example, regulation may be effected using the long terminal repeat (LTR) promoter. Alternative promoters will be apparent to the skilled person. For example, regulation may be effected using the cytomegalovirus (CMV) promoter. The CMV promoter is a very strong promoter, and may be preferred when the cells are neural cells, e.g. neuroepithelial stem cells.

Methods for constructing myc constructs for transduction into cells can be found in Bernard et al., supra. Retroviral vectors, including the Zen vectors (Harilaran et al., Oncogene Research, 1988, 3:387-399) may be utilised.

Preferably, the oncogene and the myc gene are incorporated into the cell during the early culture phase, usually within the first 10 cell divisions. The order of incorporating the oncogene and myc gene is not critical, although it is preferred that the myc gene is introduced first because introducing the myc gene first provides better assurance for achieving a diploid cell line.

The transduced or infected cells may be cultured under conditions known to those skilled in the art. It is preferable that the cells are cultured under non-stressed conditions. A skilled person will appreciate the conditions suitable for each particular cell type, based on conventional culture techniques.

Constructs including an oncogene and the myc gene may be used to transduce suitable cells to produce conditionally-immortalised recombinant cells that have improved stability during passaging. The myc gene may activate directly the catalytic subunit of telomerase (Wu et al., Nature Genetics, 1999, 21:220-224). This may maintain the chromosomes during cell replication.

In a further aspect, the present invention is directed towards a method for preparing isolated organoids characterized in that said method comprises the following steps of:

a) detaching a confluent monolayers made out of ADCs' from the solid microsupport onto which said ADCs' are cultivated by the method according to the invention;

b) separating the spontaneously formed organoids from the solid microsupports, said organoids being spontaneously formed from the monolayer detached from their solid microsupports.

Two-dimensional geometry microsupports (2D-MS) such as MicroHex™ have been developed in order to dispose of a new microcarrier for the culture of anchorage-dependent cells at high cell concentration. The production of isolated organoids according to the present invention consists in attaching cells onto, for example, MicroHex™ until they form a monolayer. When the confluence is reached, the cells are then detached by an enzymatic dispase or enzyme having a dispase type activity treatment according to the present invention which preserves, particularly, the intercellular cell junctions. The cell sheet, free from the microcarrier, then re-organises itself spontaneously into an organoid. This technology is thus of great help to provide both a culture system for the scale-up of precursor cells as well as means to sustain the cell differentiation and functionality by providing a 3D organisation close to that of the real organ.

When the organoids are derived from proliferative anchorage-dependent pancreatic cells, in a first approximation, these small spheres can be compared with the small endocrine organoids dispersed in the pancreas, called islets of Langerhans. If pancreatic cells are cultivated onto MicroHex™ the organoids which consequently form, are called PILS (Pseudo Islets of Langerhans).

In a more preferred embodiment, the invention is directed towards a method for preparing isolated organoids according to the present invention, wherein the step b) of separating the organoids from the microsupports is carried out by filtration.

In a further more preferred embodiment, the invention is directed towards a method for preparing isolated organoids according to the present invention, wherein the step of filtration occurs through a nylon screen.

In a further more preferred embodiment, the invention is directed towards a method for preparing isolated organoids according to the present invention, wherein the step of filtration occurs through a nylon screen of 100 μm average porosity.

In a further aspect, the present invention is directed towards a method for preparing islets of Langerhans transplants containing cells in a suitable state for transplantation and capable of differentiating, upon transplantation, into either insulin- and/or glucagon-synthesizing cells, characterized in that said method comprises a step of preparing pancreas-derived organoid, preferably islets of Langerhans-derived organoid, more preferably all pancreas-derived cells from neonatal (3 days-old) piglets organoid, said step of preparing pancreas-derived organoid comprising the following steps of:

a) detaching a confluent monolayers made out of ADCs' pancreas-derived cells from the solid microsupport onto which said ADCs' are cultivated, preferably by the method for detaching monolayers from their solid microsupports according to the present invention and wherein said ADCs' are derived from pancreatic cells, preferably from human or pig pancreas, more preferably from piglet pancreatic neonatal tissue; and

b) separating the spontaneously formed organoids from the solid microsupports, said organoids being spontaneously formed from the monolayer detached from their solid microsupports,

wherein the solid microsupport used is a 2D-MS, particularly the MicroHex™ microsupport.

In addition, the resulting detached monolayers from their microsupport according to the present invention or the resulting isolated organoids according to the present invention, may be used as model systems for the study of physiologic or pathologic conditions. For example, in a specific embodiment of the invention, these detached monolayers or isolated organoids derived therefrom may be used as a model to study the protein secretion of these ADCs', their receptors ligands, the signal induced by the ligand/receptor interaction, their proliferation or differentiation, notably in presence of compounds of interest. These resulting detached ADCs' or isolated organoids may have a variety of applications ranging from transplantation or implantation *in vivo*, cytotoxicity testing and screening compounds *in vitro*, and as micro-bioreactors for the production of biological materials *in vitro*.

So, the present invention is also directed to a method for preparing islets of Langerhans transplants containing cells in a suitable state for transplantation and capable of differentiating when transplanted into either insulin or glucagon cells, characterized in that said method comprises the step of:

a) preparing pancreas-derived organoid by a method according to the invention claims, wherein the solid microsupport used is a two-dimensional geometry microsupport (2D-MS);

b) selecting the separated spontaneously formed organoids obtained as Langerhans transplants.

In a preferred embodiment said detached monolayer cells cultured on the microsupport are pancreas cells from neonatal piglets or/and the solid microsupport used is the MicroHex™ microsupport.

The present invention also pertains to the use of detached monolayer or organoids obtained by the method according to the invention as an *in vitro* model system for the study of physiologic or pathologic conditions of cells which grown in a three dimensional system.

The present invention also relates to an *in vitro* use of detached monolayer or organoids obtained by the method according to the invention for the screening of compounds that can modulate the activity of cells which grown in a three dimensional system, comprising a step of contacting said detached cell monolayer or organoids with the compound to be tested and selecting the compound if the activity of the cell which is desired to modulate is modulated by the tested compound.

The present invention also relates to an *in vitro* use of detached monolayer or organoids obtained by the method according to the invention as bioreactors for the production of biological materials *in vitro*.

In a preferred embodiment, the *in vitro* method for the production of biological material comprises the following steps of:

a) culturing in appropriate conditions and medium the detached monolayer or organoids obtained by the method according to the invention; and

b) recuperating from the cultured cells or from the cultured organoid obtained in step a), or from culture medium thereof, the biological material which is desired to produce.

In a more preferred embodiment, said biological material is a protein and said cells of said cultured cells or of said organoid in step a) produce naturally or by recombinant route the protein which is desired to produce.

In another object, the present invention concerns an *in vitro* method for the production of a detached cells monolayer or organoid proportionally enriched in β-cells from anchorage-dependent cells (ADC) which are cultured onto a flat microsupport, said ADCs' being derived from pancreatic cells, containing an initial proportion of α- and β-cells, said method comprising the following steps of detaching the cells monolayer obtained by the methods according to the present invention or preparing pancreas-derived organoid by a method according to the present invention.

In a further aspect, the present invention is directed towards the use of the organoids obtained by the methods according to the present invention as a transplant, preferably a therapeutic transplant, preferably for the treatment of disease requiring the transplantation of tissue capable of proliferating *in vivo*.

In case the cells are differentiated cells, detached monolayers from their microsupport according to the present invention or the resulting isolated organoids according to the present invention may be used for the preparation of a therapeutic transplant for the prevention or the treatment of organ defect such as cancer, or a genetic disease associated to a disease causing mutation wherein the mutation of said cells is prior corrected by an *ex vivo* homologous recombination method well known by the skilled person. For example in case the cells are detached Islets of Langerhans cell monolayer or the resulting isolated organoids according to the present invention, these cells may be used for the prevention or the treatment of diabetes or for the treatment of pancreatic cancer, or liver cancer in case the cells are hepatocytes, etc. Endothelial cells may be used for the revascularisation of the leg, heart and other organs. Human neuroepithelial stem cell may be used in neural transplantation to repair cell loss or damage and correct behavioural or psychological deficits. Neuroepithelial cells may be used in the treatment of Alzheimer's disease, Parkinson's disease, stroke and other forms of cerebral ischaemia, cerebral palsy, multiple sclerosis, Huntingdon's disease and Creuzfeld-Jacob's disease.

This detached cell monolayer, recombinant cell monolayer or organoids spontaneously formed by this detached (recombinant) cell monolayer according to the

present invention may have use in therapy. Methods for the preparation of formulations for delivery to a patient will be apparent to the skilled person. Suitable excipients, diluents etc., will again be apparent based on current practice in preparing cell-based therapies. The amount of cells or organoids required for delivery will vary depending on the form of treatment, the severity of the disease/damage, and the need for applying multiple doses over a treatment period. The skilled person can readily determine the appropriate treatment based on existing cell transplantation therapies. The cells may be administered using conventional techniques.

In another aspect, the present invention is directed towards a method for the treatment of disease requiring the transplantation of tissue wherein said tissue is an organoid obtained by the method according to the present invention and wherein said organoid is transplanted in the patient in need of such treatment.

In a final aspect, the present invention is directed to a method for the treatment of diabetes wherein an organoid obtained by the method according to the present invention is transplanted in a patient in need of such treatment.

The following examples and the figures are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

DESCRIPTION OF THE FIGURES

Figure 1: Cells isolated from the pancreas of 3 days-old piglets incubated (20.10^6 cells/0.14 g MicroHex™) with MicroHex™ (R&D grade-950 cm^2/g) at a ratio of 256:1. The extent of colonisation was followed during 7 days; number of independent experiments = 3.

Figure 2: 3 days-old piglets' pancreatic cells are incubated with MicroHex™ (R&D grade) at a ratio 256:1. The mean number of cells attached per MicroHex™ was followed during 7 days; number of independent experiments = 3.

Figures 3A and 3B:

Figure 3A: 3 days-old piglet pancreatic cells on MicroHex™ 7 days after inoculation in a Techne™ spinner flask (inoculation at 256 cells/microsupport); cells are stained with DAPI and picture is taken under UV-fluorescence.

Figure 3B: PILS next to MicroHex™, 7 days after inoculation of pancreatic cells in a Techne™ spinner flask (256 cells/microsupports) after detachment of the monolayer with Dispase II.

Figure 4: Size distribution histogram of PILS; number of independent experiments n=3. The yield of the culture is 17,200 ± 13,400 PILS/g of pancreas giving about 40,000 PILS per piglet.

Figures 5A to 5D: Light micrographs of native neonatal (1-3d old) porcine pancreas (Figure 5A), fresh Neonatal Islet Cluster (NIC) (Figure 5B), cells isolated following dissociation of NIC (Figure 5C) and PILS (Figure 5D). Sections (5 microns apart) were counterstained with hematoxylin then immunohistochemically stained for insulin.

Figure 6: Electron micrograph of PILS after 7 days of culture. Cells show zymogen-like granules (arrowhead) and microvilli at their membrane (arrow).

Figure 7: Blood glucose levels of transplanted diabetic nude mice with 20,000 PILS under the kidney capsule, non-transplanted control and diabetic non-transplanted control.

Figure 8: Body weight of transplanted diabetic nude mice with 20,000 PILS under the kidney capsule, non-transplanted control and diabetic non-transplanted control.

Figures 9A and 9B: Light micrographs of PILS 21 days after transplantation under the kidney capsule of streptozotocined diabetic nude mice. Sections (5 microns apart) were counterstained with hematoxylin then immunohistochemically stained for insulin (A) or glucagon (B).

Figures 10A to 10F: Figure 10A shows the majority of the MicroHex™ to be confluent with some aggregation of cell-laden microsupport beginning already to appear. Addition of Dispase II solution to the 60 ml culture and incubation during 35 min. (Figure 10B) or 55 min. (Figure 10D) at 37°C makes the cells to quantitatively detach from the microsupport as films which reorganize themselves into aggregates. Aggregation is somewhat better when after 35 min. incubation at 37°C, the aggregates-naked microsupport suspension is left to stand without agitation (Figure 10C). Cells in such aggregates are bound together firmly enough to resist a 20 min. exposure to PBS-EDTA (05 mM) at 37°C (Figure 10E). This is to compare with the quantitative detachment already after 10 min. of these cell cultivated as monolayers in T flasks. In presence of r-

Protease, detachment from the microsupport and dissociation of the aggregates into isolated cells occur as quickly (5 min. at 37°C) as in monolayer (Figure 10F).

EXAMPLES

Materials and Methods

Media and products: The isolation and culture of porcine pancreatic cells was carried out in Ham's F10 tissue culture medium (Gibco, Grand Island, N.Y., U.S.A., Grand Island, N.Y., U.S.A.) supplemented with 10 % fetal bovine serum (Gibco, Grand Island, N.Y., U.S.A.), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, Grand Island, N.Y., U.S.A.), 2.5 µg/ml Fungizone (Gibco, Grand Island, N.Y., U.S.A.), 50 µl/liter IBMX (isobutylmethylxanthine) (ICN Biomedicals Inc., Aurora, Ohio, U.S.A.), 10 mmol/liter nicotinamide (Sigma, St. Louis, Mo., U.S.A., St. Louis, Mo., U.S.A.). Washing of cell material was carried out with Earle-HEPES buffer Ca++ free: NaCl (124 mmol/liter), KCl (5.4 mmol/liter), $MgSO_4.7H_2O$ (0.8 mmol/liter), NaH_2PO_4 (1 mmol/liter), $NaHCO_3$ (14.3 mmol/liter), HEPES (10 mmol/liter). Collagenase (Type V) was obtained from Sigma, and Dnase I and Dispase II from Roche (Indianapolis, U.S.A.). MicroHex™ R&D grade were kindly provided by Nunc (Roskilde, Denmark). DAPI (diamidinophenylindole), a fluorescent nucleic acid stain was purchased from Sigma. Mowiol used to fix the cells on MicroHex™ under DAPI staining is composed of: Tris pH 8.5 (12 ml), Glycerol (6 grams), H_2O (6ml), Polyvinylalcohol (2.4 grams) and after warming at 50°C, 2.5 % of DABCO (1,4-diazobicyclo-(2,2,2)-octane). Primary antibodies mouse anti-insulin and anti-glucagon were from Novo Nordisk (Copenhagen, Denmark), secondary sheep antibodies anti-mouse IgG biotinylated were from Amersham (Bergrand, Nederland). Streptavidin-Peroxidase was from Roche (Indianapolis, U.S.A.).

Animals: All procedures were carried out with the approval of the animal ethics committees of the Catholic University of Louvain. Donor pancreases were obtained from 1 to 3d-old Landrace neonatal pigs (1.5-2.0 kg body wt) of either sex. Piglets were anesthetized by intramuscular injection of 2 mg/kg Rompun 2 % (Bayer, Leverkusen, Germany) and 6mg/kg Zoletil 100 (Vibrac, Carros, France) and subjected to laparotomy and complete exsanguination. The pancreas was then carefully dissected from surrounding tissue and placed in cooled (4°C) Ham's F10 (supplemented as above).

Female, inbred, athymic nude swiss mice (age 6-8 wk) were used as recipients of the PILS (Iffa Credo, Lyon, France). Mice were made diabetic by intravenous injection of (250 mg/kg) body wt steptozotocine (Sigma, St. Louis, U.S.A.; freshly dissolved in citrate buffer pH 6.4) 4-5 d before transplantation. Normoglycemic, age-matched mice served as normal controls. All animals entering this study exhibited blood glucose levels above 300 mg/dl. Blood samples were obtained from the tail vein for glucose assay (Glucotouch, Lifescan, Neckargemünd, Germany). Animals were maintained under virus-antibody-free conditions in climatized rooms with free access to sterilized tap water and granulated food.

Preparation and culture of porcine pancreatic cells: Individual glands were cut with a pair of scissors into fragments of ~ 1-2 mm^3, then transferred to sterile tubes containing Ham's F10 with 1 mg/ml collagenase, and gently agitated for 15 minutes in a water bath at 37°C. The digest was filtered through a nylon screen (500 µm), washed three times with Earle-hepes. The aggregates were then transferred to sterile tubes containing 0.2 mg/ml Dnase I and 1 mg/ml Dispase II and aspirated during 6 minutes through a 19G needle at 37°C. The isolated cells were then centrifuged on a percoll gradient (density 1.06 g/l) at 450 g during 15 minutes. The cell pellet was washed three times with Earle-HEPES and the cells then being suspended in Ham's F10 culture medium (supplemented as described above). 20 millions cells were mixed together with 0.140 grams MicroHex™ R&D grade in 125 ml Techne™ flasks with pendulum stirring at 37°C; gassed with 95%/5% O_2/CO_2 mixture incubator. During the first 24 hours, stirring (25 rpm) was discontinuous (5 min. on / 120 min. off) and the culture volume was 60 ml. Twenty four hours later, stirring (25 rpm) became continuous and the volume culture was brought to 120 ml. Growth was followed by taking 500 µl the suspension. MicroHex™ were fixed on a glass slide with Mowiol containing 50 µg/ml DAPI (Sigma). Cells on MicroHex™ were counted under UV fluorescence microscope. At 7th day of culture, when confluence was reached, the cell monolayer was detached from MicroHex™. Stirring was interrupted, MicroHex™ allowed to fall on the bottom of the flask and 60 ml of the medium was removed. 300 mg Dispase II (Roche) were then dissolved into 10 ml of medium and filtered directly into the flask through a 0.22 µm filter and stirring was resumed at 25 rpm. Cells were maintained together with the enzyme at 37°C for 35-45 minutes. PILS were then separated from MicroHex™ by

filtration through a 100 µm nylon screen. Before transplantation, PILS were washed three times with Earle-HEPES (450 g; 5 min.).

Immunohistochemistry: Tissue, isolated cells or cell cluster were fixed overnight in Bouin's solution then washed 24 H in H_2O. Fixed cells and tissues were then dehydrated, embedded in paraffin and tissue sections were cut (5 µm). Immunohistochemistry was performed to localise glucagon and insulin using a modified avidin-biotin peroxidase method (Petrik et al., Endocrinology 1998, 139:2994-3004). Slides were incubated overnight at 4°C in a humidified chamber with either mouse anti-insulin (1:6000 dilution) or mouse anti-glucagon (1:6000 dilution) (Novo Nordisk, Copenhagen, Denmark). All antisera were diluted in 0.01 mol/l PBS (pH 7.5) containing 5 % (w/v) FCS and 0.01 % (w/v) sodium azide (1 ml per slide). Biotinylated-sheep anti-mouse IgG (1:1500 dilution) (Amersham, Bergrand, Nederland) were used as secondary antibodies. The immunocytochemical staining has been validated in previous studies (Petrik et al., 1998; Petrik et al., Endocrinology, 1999, 140:4861-4873). Tissue sections were counter-stained with Carrazi's hematoxylin.

Measurement of insulin: Five hundred microliters samples were sonicated in ethanol acid (356 ml ethanol, 136 ml H_2O, 7.5 ml HCl 37 %), centrifuged (800 g, 15 minutes), then supernatants were collected and stored at –20°C until assayed for insulin content. Insulin content was measured by ELISA using a commercial kit (Mercodia, Uppsala, Sweden).

Electronic Microscopy: Samples were fixed in glutaraldehyde, postfixed in osmium tetroxide and embedded in epoxy resin as described elsewhere (Remacle et al., Cell Tissue Res., 1980, 207:429-448).

Transplantation, follow-up and graft removal: Normoglycemic and diabetic male Swiss nude mice (Iffa Credo, Lyon, France), aged 6-8 weeks, were used as graft recipients. Diabetes was induced by ip injection of Streptozotocin (250 mg/kg; Sigma). PILS (20,000) were transplanted beneath the left kidney capsule under Pentobarbital anesthesia (Nembutal, Sanofi, Bruxelles, Belgium). After transplantation, body weight and blood glucose levels i fed state were determined weekly. For one animal, kidney wearing the graft was removed under Pentobarbital anesthesia and fixed, Bouin's alumn and processed for histology.

Example 1: Culture of the isolated pancreatic cells on MicroHex™

After being isolated from the pancreas of 3 days-old piglets, pancreatic cells were cultivated in Techne™ spinner flasks. Following the first 24 hours of culture (interrupted stirring mode) approximately 5 % of the cells attached to the microsupport. For an optimised ratio of 256 cells/microsupports, the extent of colonisation (% of MicroHex™ bearing a minimum of one cell) of the culture is around 75 %; this proportion did not change during the culture even when the stirring became continuous 48 hours after the inoculation (Figure 1).

Pancreatic cells proliferated with a population doubling time of 2 days and confluence was reached after 7 days of culture. At that time the monolayer contained a mean number of 29 ± 2 cells (Figure 2).

Example 2: Formation and caracterisation of the PILS

Dispase II-mediated detachment of the pancreatic cell monolayer from the microsupport, makes it to spontaneously organise into an organoid (PILS) (Figure 3).

The diameter of these organoids is heterogenous ranging from 40- to 100 μm (Figure 4) with a mean diameter of 69 ± 8 μm.

The neonate pancreas can not be considered as a mature organ: its endocrine cells are dispersed as small clusters in the pancreatic tissue; no islet-like structure is observed at this stage (Figure 5A). Collagenase digestion produces pieces (Neonatal Islet Cluster – NIC) simultaneously preserving the insulin cell content of the whole organ (Figure 5B). Upon enzymatic digestion, these NICs' are dissociated and isolated cells appear. These cells are a mixture of all pancreatic cells types including insulin-and glucagon synthesizing beta cells and alpha cells respectively (Figure 5C).

After 7 days of culture, the ratio of cells stained for insulin in the PILS is 5.2 ± 1.8 % (Figure 5D). This proportion is twice that observed with whole neonate pancreas, that of fresh NIC and that of the isolated cell mixture used to inoculate the culture medium (Figure 5). The mean insulin content of PILS is 0.034 ± 0.022 (ng insulin/ng ADN).

Cells within the PILS were also characterised by electron microscopy (Figure 6). Many cells exhibit microvilli at their cell membrane and zymogen-like granules in their

cytoplasm. Taken together, these observations suggest the presence of epithelial duct cells.

All the characteristics of the PILS are summarized in Table 1.

Table 1: Characterisation of PILS before transplantation

Yield (PILS/gram of pancreas) (n=6)	Mean size (µm) (n=4)	Mean number of cells per MicroHex™ (n=3)	Proportion of β cells (% of the totality) (n=4)	Insulin content (ng insulin/ng DNA) (n=3)
17,233 ± 13,481	70.2 ± 6.9	29 ± 2	5.2 ± 1.8	0.034 ± 0.022

Example 3: Functionality of PILS *in vivo*

PILSs' functionality was assessed in an *in vivo* model using streptozotocined nude mice. The animals made diabetic by injection of streptozotocine were transplanted with 20,000 PILS under the left kidney capsule. Their blood glycemia (Figure 7) and their weight (Figure 8) were followed weekly.

All the transplanted mice have shown their glycemia return to normal value (Figure 7). However, the time spent before reaching the normality is probably dependent upon the degree of diabetes and may also vary from one animal to another. Furthermore, all these transplanted animals have shown their body weight decreased directly after the transplantation due probably to the operative shock (Figure 8). Yet, after normalisation of their glycemia transplanted mice recovered their weight. Only the diabetic control mice lost weight irreversibly.

For the transplanted mice 2, the kidney bearing the graft was removed 21 days after the transplantation. This organ was then fixed and cut into several sections. It was possible to localise by this mean PILS under the kidney capsule (Figures 9A and 9B). Immunohistochemical staining was realised for insulin (Figure 9A) and glucagons (Figure 9B) on serial slices (5 microns apart).

PILS appear to be well conserved in terms of size and structure. They are surrounded by a fibroblastic tissue and neo-vascularisation can be observed in the graft area. Staining for insulin even if it cannot be quantified, appears to be much more intense than in the PILS before transplantation. Some cells are simultaneously stained for insulin and glucagon but the majority of them are stained either for insulin or for glucagon (Figure 9).

Example 4: Culture of myc-immortalized human fetal neural stem cells derived from cortex on laminin-coated MicroHex™

Cells from a myc-immortalized cloned human fetal cortex cell line (EM/CTX, from ReNeuron, Guildford-Surrey-UK) were cultivated as following:

- inoculation: 25,000 EM/CTX cells/cm² microsupport MicroHex™ in Techne™ spinner flask on laminin-coated (0.25 µg laminin/cm² MicroHex™ - 10 cm² microsupport/ml) in 100 ml culture volume of serum-free DMEM/F12-based growth medium (Gibco cat. 21331-020) enriched with Gibco B27 supplement (cat. 17504-044), EGF (20 ng/ml), bFGF (10 ng/ml), heparin (10 Units/ml) and glutamine (2 mM).

- culturation: continuously agitated at 30 rpm in a 5 % CO2 in an air gas mixture humidified cabinet at 37°C with its medium completely renewed with fresh medium every day. After 4 days, the laminin-coated MicroHex™ were confluent (Figure 10A).

Example 5: Formation of aggregates from confluent EM/CTX monolayers. Stability of the aggregates

One day after confluence is reached, the cell-laden microsupport are washed with growth medium and resuspended in 60 ml growth medium. Ten milliliters of a Dispase II solution in growth medium is added and the culture then incubated for 35-45 min. at 37°C.

Dispase II-mediated detachment (35 min at 37°C with continuous agitation at 30 rpm) of EM/CTX cell monolayer from the laminin-coated microsupport made it to spontaneously organize into an aggregate (Figure 10 B). Extending the incubation to 55 minutes did not improve the process (Figure 10 D). However leaving the 35 min sample during 20 min without agitation at room temperature made the aggregate to appear more compact (Figure 10C). Ten minutes exposure to a buffered-EDTA (0.5 mM) solution of an EM/CTX confluent monolayer in T-flask sufficed to detach the cells. Figure 10E shows that 20 minutes agitation in the same solution made the cells aggregates to only start disaggregation. Monolayers and aggregates dissociate equally well following a 5 min incubation at 37°C with rProtease (Gibco cat. 12563-029) (Figure 10 F).

CLAIMS

1. Method to detach a confluent anchorage-dependent cell (ADC) monolayer from the flat microsupport onto which said ADCs' are cultured, characterized in that said method comprises an enzymatic treatment with an enzyme which cleaves fibronectin and type IV collagen.

2. Method according to claim 1, wherein said enzyme is a dispase.

3. Method according to claim 2, wherein said dispase is a dispase II.

4. Method according to claims 1 to 3, wherein said enzyme is added to a flask containing the microsupport suspension onto which the ADCs' culture has reached the confluence.

5. Method according to claims 1 to 4, wherein said enzyme is the dispase II and is added to the flask at a concentration of about 4.5 to 5.5 mg/ml.

6. Method according to claim 5, wherein the dispase II treatment is carried out during at least about 30 min.

7. Method according to claims 1 to 6, wherein said flat microsupport is a two-dimensional geometry microsupport (2D-MS).

8. Method according to claims 1 to 7, wherein said microsupport is the two-dimensional geometry microsupports MicroHex™.

9. Method according to claims 1 to 8, wherein said ADCs' are derived from one cell clone or from a co-culture of least two cell clones.

10. Method according to claims 1 to 9, wherein said ADCs' are derived from foetal, neonatal or adult, non-human mammal or human, tissue which may be grown in a three dimensional culture system.

11. Method according to claims 1 to 10, wherein said ADCs' are derived from human neonatal tissue or from human foetal tissue.

12. Method according to claim 10 or 11, wherein said ADCs' are derived from a tissue which is selected from the group consisting of brain or other neural tissue, pancreas, liver, kidney, bone marrow, skin, mucosal epithelium, adenocarcinoma or melanoma tissue, or stem cells.

13. Method according to claims 10 to 12, wherein said ADCs' are derived from pancreatic cells, preferably from human or pig pancreas.

14. Method according to claims 10 to 12, wherein said ADCs' are derived from human foetal cells.

15. Method according to claim 14, wherein said foetal cells are neural foetal cells or pancreatic foetal cells.

16. Method according to claim 14 or 15, wherein said foetal cells are myc-immortalized.

17. Method for preparing isolated organoid characterized in that said method comprises the following steps of:

a) detaching a monolayer from the microsupport onto which said ADCs' are cultivated by the method according to claims 1 to 16; and

b) separating the spontaneously formed organoids from the solid microsupports, said organoids being spontaneously formed from the monolayer detached from their microsupports.

18. Method for preparing isolated organoid according to claim 17, wherein the step b) of separating the organoids from the microsupports is carried out by a step of filtration.

19. Method for preparing isolated organoid according to claim 18, wherein the step of filtration is a filtration through a nylon screen.

20. Method for preparing isolated organoid according to claims 18 and 19, wherein the step of filtration is a filtration through a nylon screen having a 100 μm average porosity.

21. Method for preparing islets of Langerhans transplants containing cells in a suitable state for transplantation and capable of differentiating when transplanted into either insulin or glucagon cells, characterized in that said method comprises the step of:

a) preparing pancreas-derived organoid by a method according to claims 17 to 20, wherein the solid microsupport used is a two-dimensional geometry microsupport (2D-MS);

b) selecting the separated spontaneously formed organoids obtained as Langerhans transplants.

22. Method for preparing islets of Langerhans transplants according to claim 21, wherein said detached monolayer cells cultured on the microsupport are pancreas

cells from neonatal piglets and wherein the solid microsupport used is the MicroHex™ microsupport.

23. Use of detached monolayer obtained by the methods according to claims 1 to 16 or organoids obtained by the methods according to claims 17 to 22 as an *in vitro* model system for the study of physiologic or pathologic conditions of cells which grown in a three dimensional system, or for the cytotoxicity study of compounds on cells which grown in a three dimensional system, or for the screening of compounds that can modulate the activity of cells which grown in a three dimensional system.

24. Use of detached cells monolayer obtained by the methods according to claims 1 to 16 or organoids obtained by the methods according to claims 17 to 22 as bioreactors for the production of biological materials *in vitro*.

25. An *in vitro* method for the production of biological material, said method comprising the following steps of:

a) culturing in appropriate conditions the detached cells monolayer obtained by the methods according to claims 1 to 16 or the organoids obtained by the methods according to claims 17 to 22; and

b) recuperating from the cultured cells or from the cultured organoid obtained in step a), or from culture medium thereof, the biological material which is desired to produce.

26. An *in vitro* method according to claim 25 wherein said biological material is a protein and wherein cells of said cultured cells or of said organoid in step a) produce naturally or by recombinant route the protein which is desired to produce.

27. An *in vitro* method for the production of a detached cells monolayer or organoid proportionally enriched in β-cells from anchorage-dependent cells (ADC) which are cultured onto a flat microsupport, said ADCs' being derived from pancreatic cells containing an initial proportion of α- and β-cells, said method comprising the following steps of detaching the cells monolayer obtained by the methods according to claims 1 to 16 or preparing pancreas-derived organoid by a method according to claims 17 to 20.

28. Use of the organoid obtained by the methods according to claims 17 to 22 as a transplant, preferably a therapeutic transplant.

29. Use of an organoid obtained by the method according to claims 17 to 22 in the manufacture of a therapeutic transplant for the treatment of disease requiring the transplantation of tissue capable of proliferating *in vivo*.

30. Use of an organoid obtained by the method according to claims 21 or 22 in the manufacture of a therapeutic transplant for the treatment of diabetes.

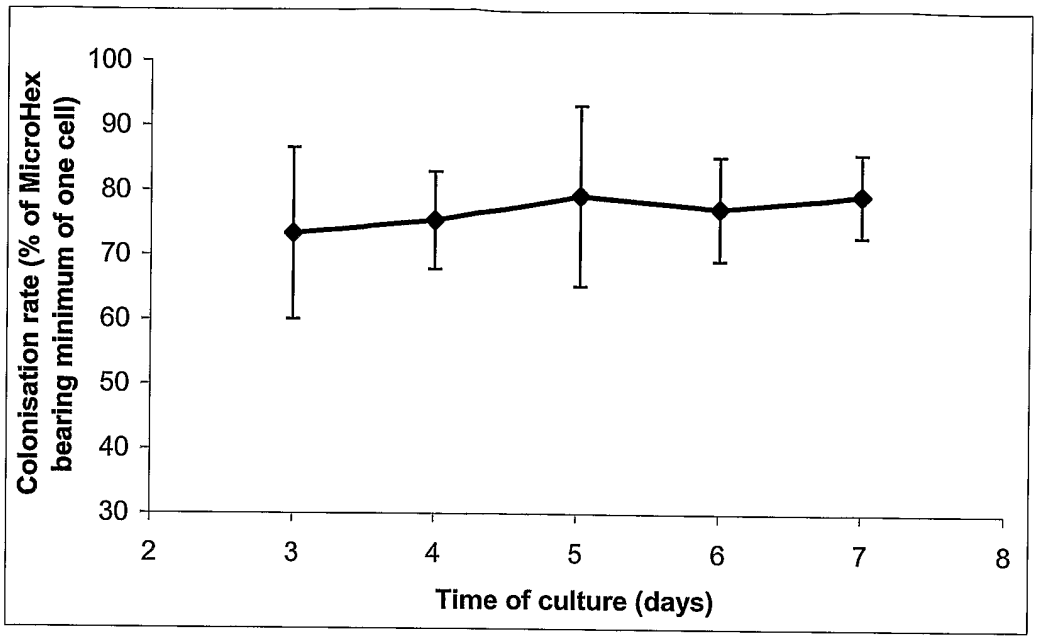

FIGURE 1

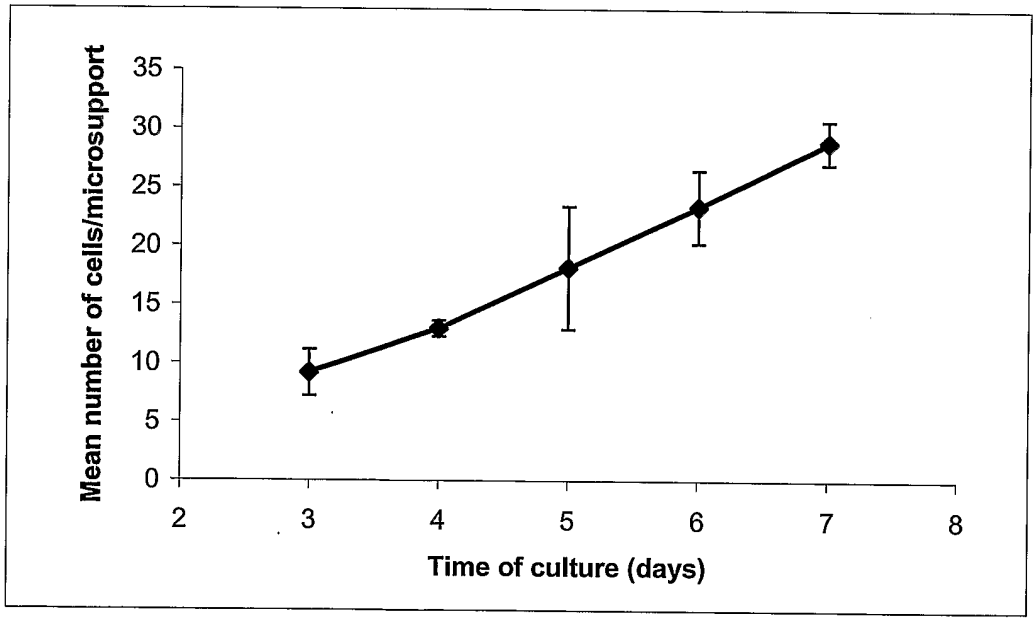

FIGURE 2

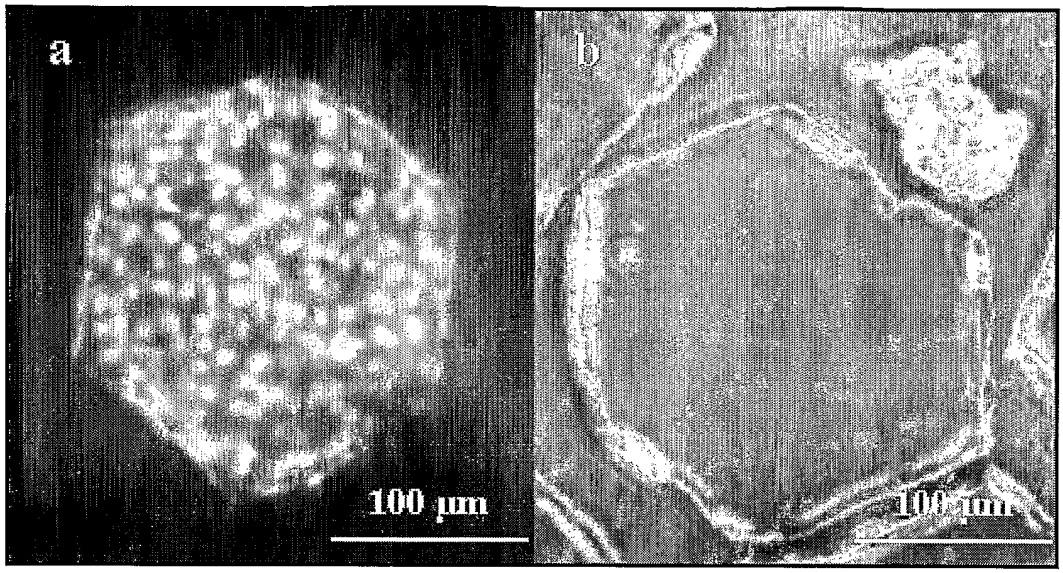

FIGURE 3A

FIGURE 3B

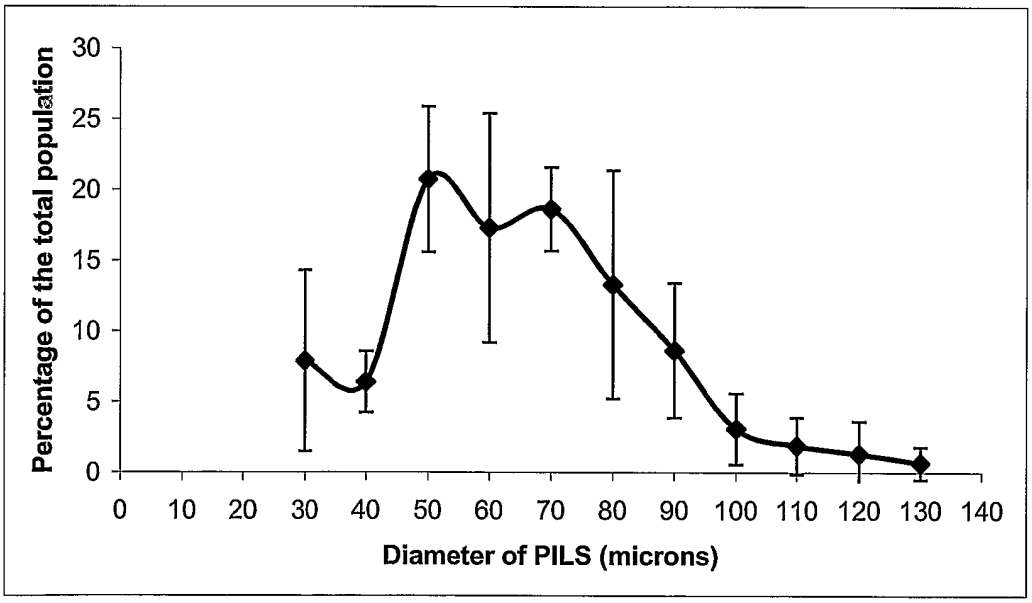

FIGURE 4

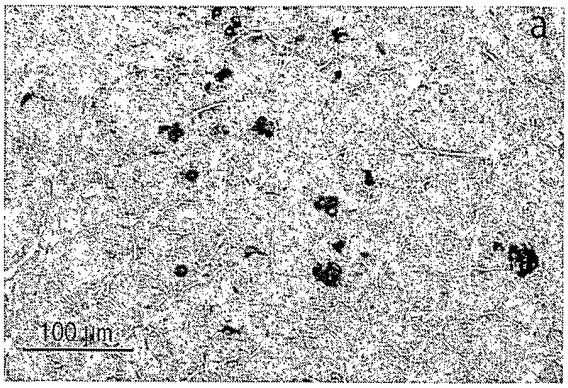

FIGURE 5A

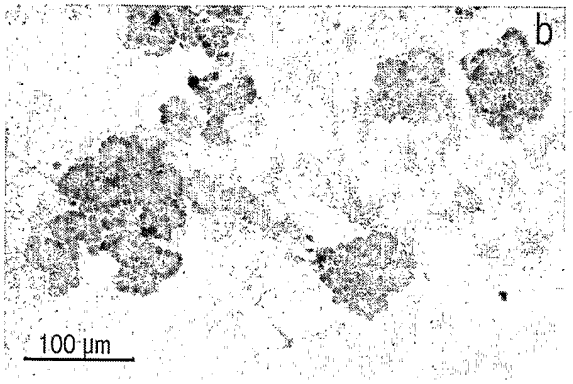

FIGURE 5B

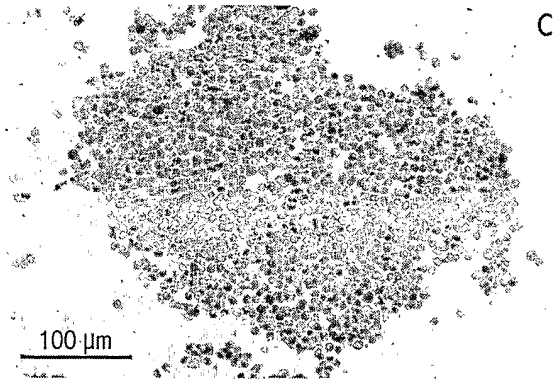

FIGURE 5C

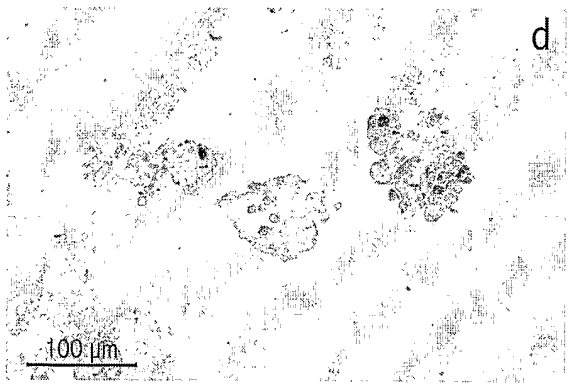

FIGURE 5D

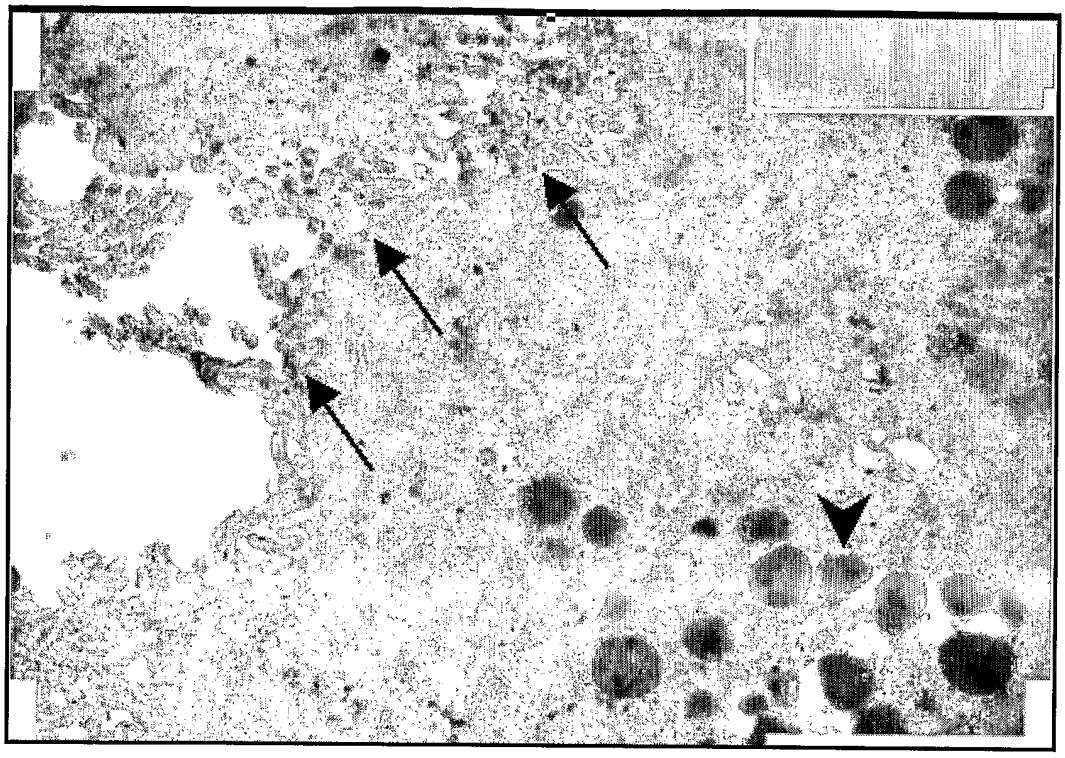

FIGURE 6

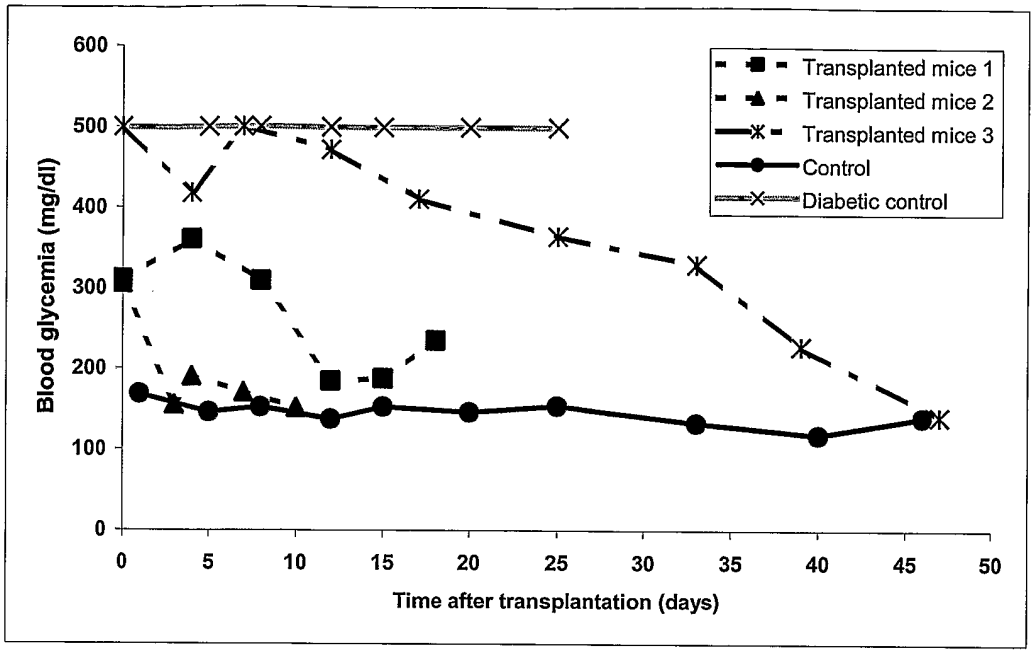

FIGURE 7

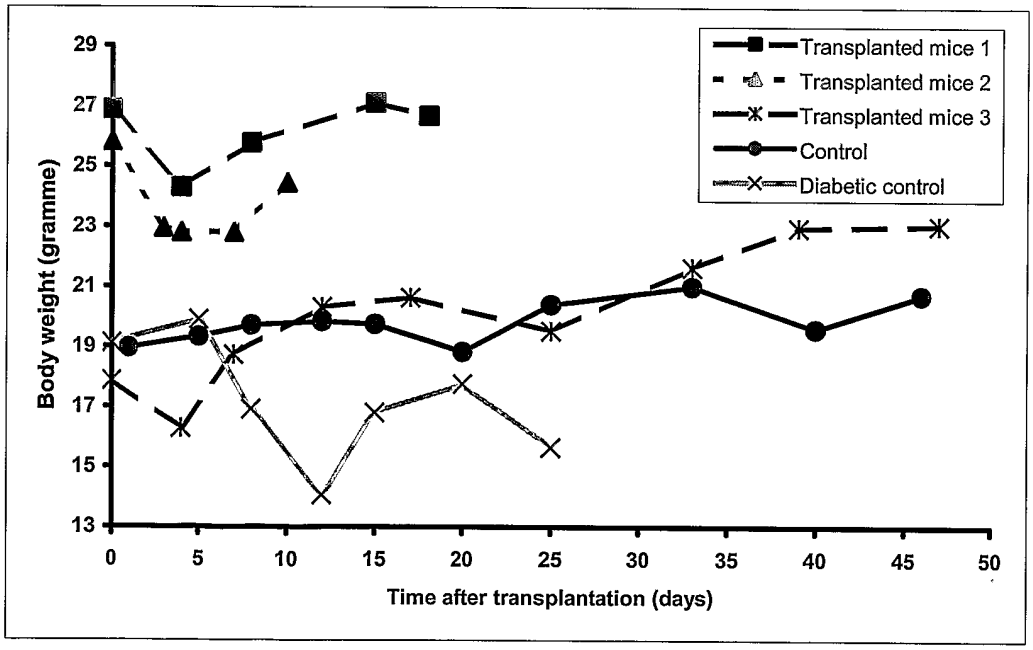

FIGURE 8

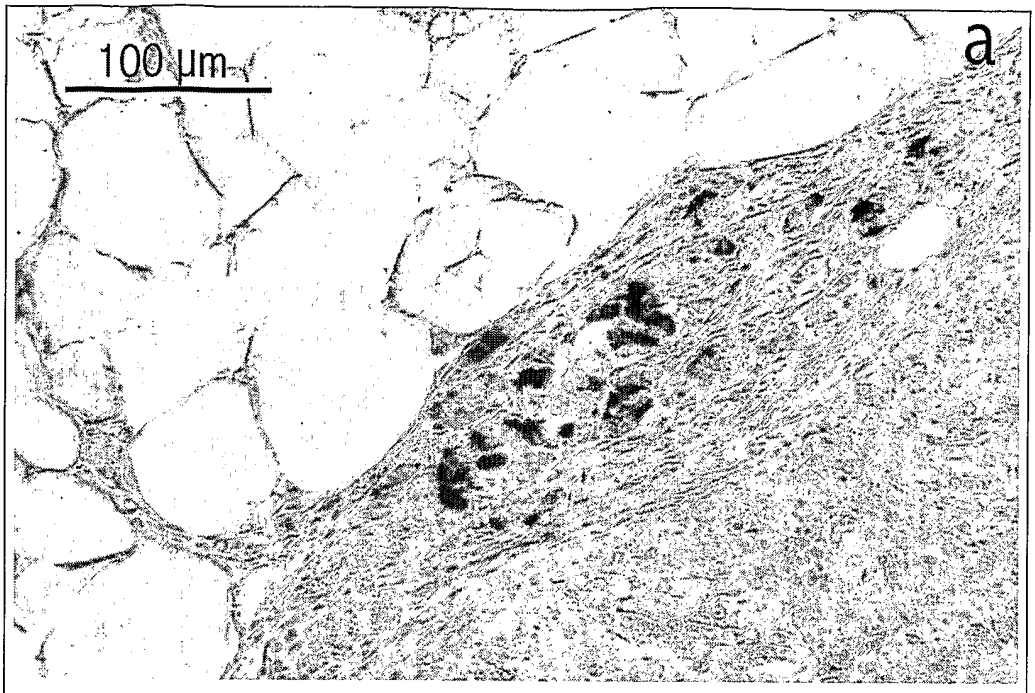

FIGURE 9A

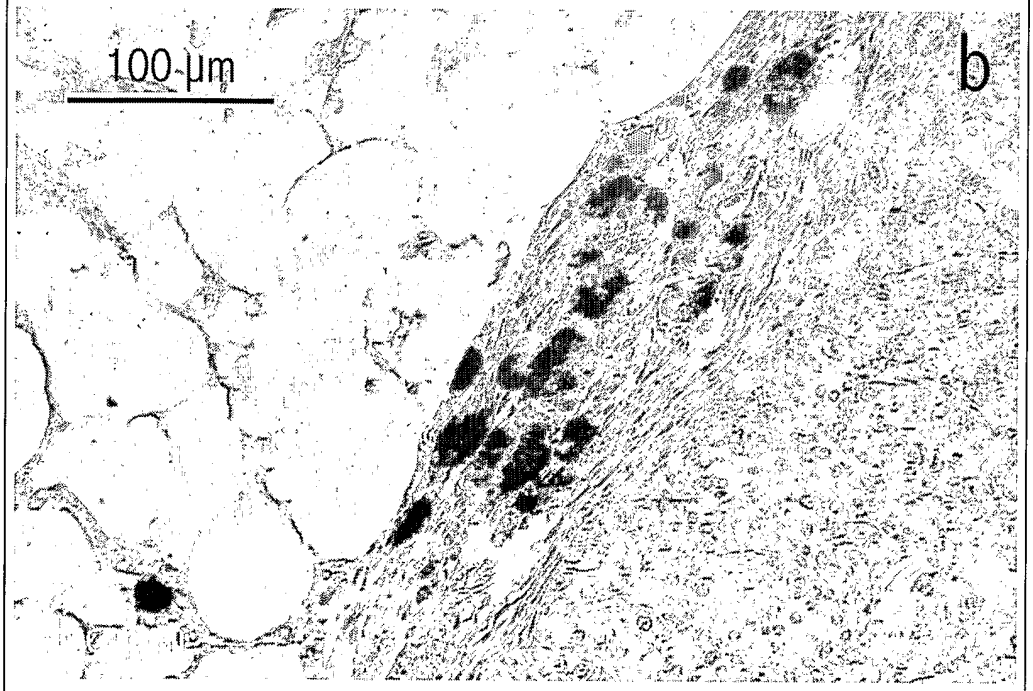

FIGURE 9B

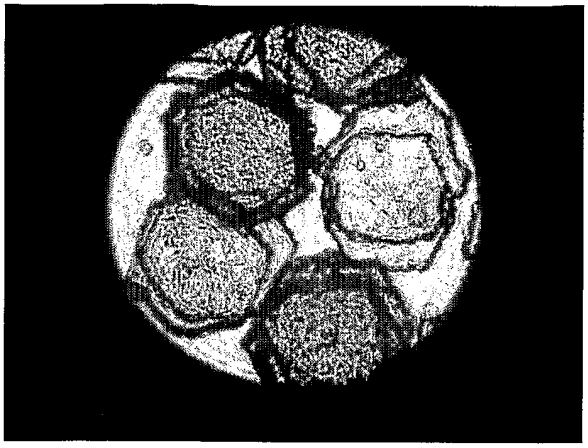

FIGURE 10A

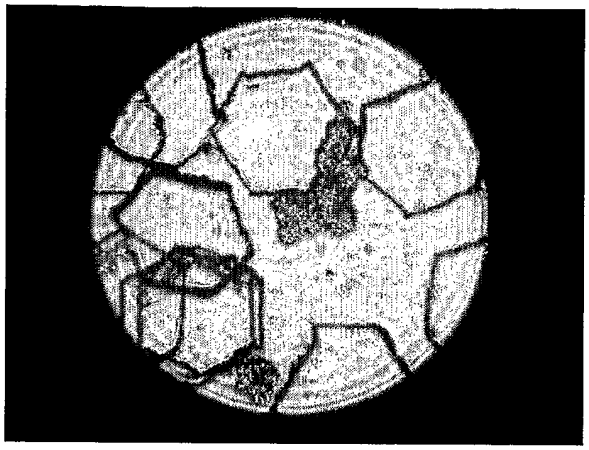

FIGURE 10B

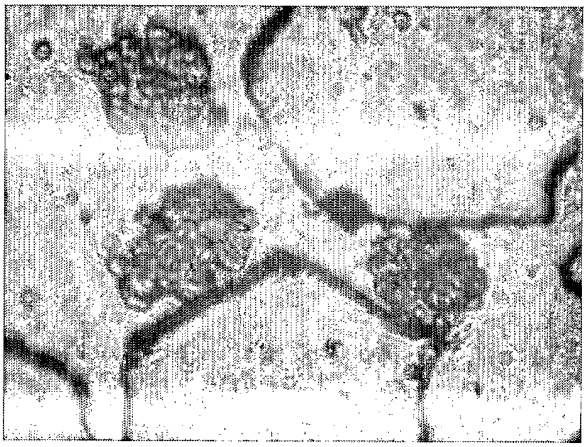

FIGURE 10C

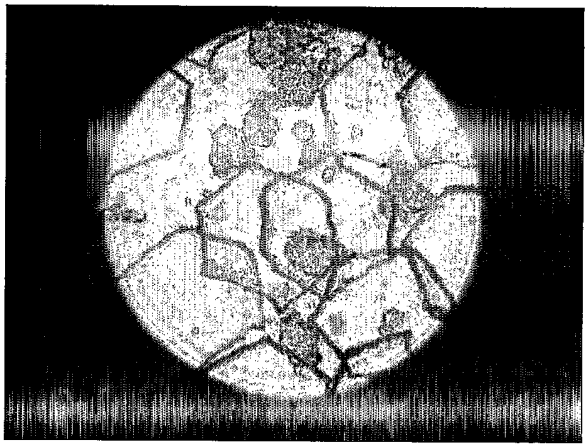

FIGURE 10D

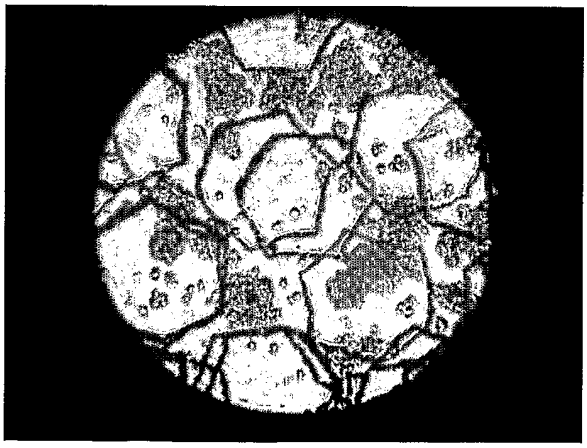

FIGURE 10E

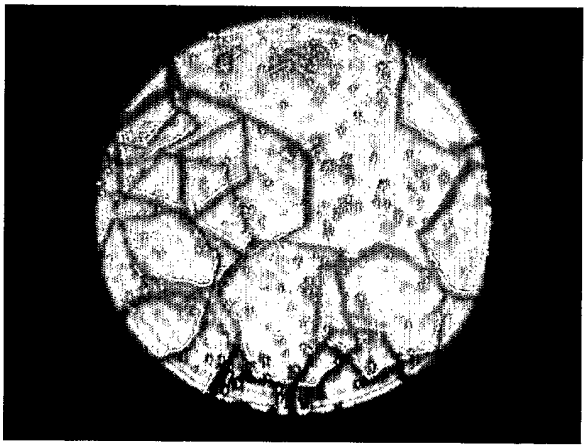

FIGURE 10F