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Fortsættes ...

Description

The invention relates to an extremely simple method for in vitro production of a cell aggregate of three-dimensional, vital and mechanically stable cartilage or bone tissue and to the use thereof as a transplantation material for treating cartilage or bone defects and degenerative diseases such as e.g. rheumatism or arthrosis, and to the use thereof in testing active substances and physical factors. According to the invention, the most varied cartilage and bone tissues can be produced, thus e.g. hyaline, elastic, fibrous, or connective tissue cartilages such as for example joint cartilage, nose cartilage, meniscus cartilage, or intervertebral cartilage.

In the field of tissue engineering, solutions to build up endogenous tissue have been sought for a long time. To this end, endogenous cells with and without support material are used on the one hand, and, on the other hand, support materials exclusively are incorporated into the defect where, depending on the indication, it is possible to use absorbable or non-absorbable materials.

In the use of support materials it is disadvantageous that decomposition products thereof may damage other tissues, and that when using non-autogenous support materials, i.e. those not derived from the patient, immune reactions or infections with animal or human pathogens may arise.

A known method using endogenous cells is transplantation of cartilage and bone cells, which is used for the treatment of cartilage and bone defects. Here, the potential of cartilage and bone cells is utilized to build up new tissue *in vivo*. Thus, for example, cartilage or bone biopsies are taken from the patient, cartilage or bone cells are isolated therefrom, multiplied by means of cell cultivation, and the cells are subsequently transplanted into the patient in the region of the tissue defect, e.g. by injecting. There, they form new

tissue and thus completely fill the defect.

Through the mentioned methods it is achieved that tissue is built up in the body after application of the cell transplants or incorporating of the support materials.

However, a further aim in tissue engineering consists in already prefabricating endogenous tissue *in vitro*. For this, a variety of methods are known from the literature (cf. DE 195 40 487, WO 97/46665, DE 197 52 900, US 5.932.459), which either require special apparatus or supports, contain numerous process steps, or necessitate the addition of growth-stimulating compounds which represent foreign substances to the body. It was therefore the object of the present invention to provide as simple a method as possible for producing typical cartilage or bone tissue, by which vital, three-dimensional and mechanically stable tissue can be produced, which is suitable for transplantation and ensures a rapid adhesion in the body or respectively a rapid filling of the cartilage or bone defect. Furthermore, the tissue produced in vitro should as far as possible not trigger any immunological reactions in the organism which receives the transplant.

US 5,723,331 discloses cylindrical cell aggregates which are formed in differently preformed wells, according to requirements. The preformed wells consist of flat bases. Accordingly, flat cartilage transplants are formed, which can be integrated macrosurgically into the wound base. According to US 5,723,331 cultivation is carried out exclusively with xenogenic serums.

In Franchimont et al. "Effects of hormones and drugs on cartilage repair" from the Journal of Rheumatology, 1989, Vol. 16, pages 5-9, the suspension cultivation of chondrocytes is described.

Fritsch et al. "Advances in autologous chondrocyte transplantation (ACT)" Techvest's first annual conference on

tissue repair, replacement and regeneration, October 27-28, 1999, New York describes the formation of cartilage clusters after 4 days' culture and that chondrocytes multiply within the first 15 days in culture.

Löhnert et al. "Autologe Chondozytentransplantation (ACT) im Kniegelenk; erste klinische Ergebnisse" from Arthroskopie, Vol. 12, 1999, pages 34-42 describes the application of chondrocytes produced in vitro by means of a very thin cannula into the defect region.

Gruber et al. "Untersuchung zur In-vitro Kultivierung von Humanchondrozyten bei Einsatz von autologem Humanserum als Mediumzusatz: Minimierung der möglichen Risikos einer Infektion mit Erregern von Prionen-Erkrankungen"; Laryngo-Rhino-Otol. 75 (1996) 105-108, observes an improved proliferation behaviour in human chondrocytes, grown with human serum, with minimizing of the risk of an infection through prions.

Surprisingly, it has been found that this object can be accomplished by the simple method indicated in Claim 1.

According to the invention, patient-derived tissue biopsies or samples or mesenchymal stem cells, e.g. from peripheral blood or bone marrow are used as starting material. The tissue-building cells are isolated from the biopsies by conventional methods, using enzymatic digestion of the tissue, by migration, or by reagents which recognize target cells. According to the invention, these cells are then cultivated in a stationary manner in suspension in a simple fashion, using conventional culture medium in cell culture vessels with hydrophobic surfaces and tapered base, until a three-dimensional cell aggregate results which contains at least 40% by volume, preferably at least 60% by volume up to a maximum of 95% by volume of extracellular matrix (ECM) in which differentiated cells are embedded. The cell aggregate having formed has an outer area in which proliferation- and

migration-capable cells are present. This structure of the cell aggregates obtained according to the invention is illustrated by the microscopic photographs in Figs. 1 and 1a, wherein Fig. 1 shows the detail enlargement of the cross-section of a cell aggregate according to the invention, with vP as the zone where the first tissue-specific matrix proteins occur, and M as the zone where tissue-specific matrix proteins are formed, and Fig. 1a shows the entire cell aggregate including the outer zone of cells P capable of proliferation and migration (zone of protein S 100 expression).

It is surprising that all the cells integrated in the spheroids produced according to this invention survive, and that the cells inside do not die off even after a progressive period of cultivation. With a progressive period of cultivation, the cells inside the aggregates undergo differentiation and spheroids form consisting of ECM, differentiated cells and a peripheral proliferation zone. The process of formation of this tissue-specific matrix with embedded cells is very similar to the process of tissue formation or respectively regeneration and reorganization in the body. During differentiation in cell culture, the spacing of the aggregated cells becomes ever greater due to the formation of the tissue-specific matrix. A tissue histology develops inside the spheroids which is very similar to the natural tissue. As in the natural cartilage, the cells inside the spheroids are supplied with nutrients by way of diffusion only. During the further production of the spheroids, the zone of cells capable of proliferation and migration is formed at the boundary of the spheroids. This zone has the invaluable advantage that following incorporation of the spheroids into defects, the cells situated in this peripheral zone are capable of migrating and of making active contact with the surrounding tissue or respectively enable an integration of the tissue formed in vitro into the environment thereof. Thus, the tissue-specific cell aggregates produced are excellently suitable for the treatment of tissue defects and for the in vitro and *in vivo* rebuilding of tissue.

Depending on the size of the tissue defect to be treated, it can be advantageous to already transplant larger pieces of tissue so as to achieve more rapid filling of the defect. In this event, at least two, but preferably more, of the cell aggregates obtained are fused by being further cultivated together under the same conditions and in the same culture vessels as described above until the desired size is reached.

Fig. 1b shows two spheroids which are to be fused, after one day.

Fig. 1c shows that already some hours later, the boundary between the two spheroids cannot be recognized any longer. After a further week, the spheroids are completely fused, and a larger in vitro tissue piece has formed (Fig. 1d). The structure of the larger cell aggregates thus obtained is identical to that of the spheroids obtained initially. They may contain ECM up to a maximum of 95%, and all of the cells contained in the piece of tissue obtained are vital.

The cartilage or bone tissue obtained is extraordinarily stable. The cell aggregates can be compressed to ¾ of their diameter without breaking or falling apart for example when being injected into the body by means of a cannula. It is possible to remove these small pieces of tissue from the cell culture vessel using pincers or a pipette.

In an advantageous embodiment of the invention, the cells derived from the patient are initially multiplied in monolayer culture in a manner known per se in order to have sufficient cartilage or bone cells available for the suspension cultivation according to the invention. The passage of the cells in monolayer culture is kept as low as possible. After reaching the confluent stage, the cells grown in monolayer are harvested and cultivated according to the inventive method in suspension as described above.

A medium usual both for suspension and monolayer culture, e.g. *Dulbecco's MEM*, with addition of serum, can be used as cell culture medium. Preferably DMEM and Hams is used at a ratio of 1:1. However, to avoid immunological reactions of the patient to the tissue produced in vitro, preferably autogenous serum from the patient is used as serum. It is also possible to use xenogenic or allogenic serum.

According to the invention, no antibiotics, fungistatics or other auxiliary substances are added to the culture medium. It has been found that only the autogenous, xenogenic or allogenic cultivation of the cells and cell aggregates and the cultivation without antibiotics and fungistatics enable an unaffected morphology and differentiation of the cells in the monolayer culture and an undisturbed formation of the specific matrix in the cell aggregates. Furthermore, by dispensing with all additives during the production, all immunological reactions are excluded after incorporating the tissue produced in vitro into the human and also animal organism.

It is, indeed, surprising that growth factors or other growth-stimulating additives are required neither in suspension cultivation nor in monolayer cultivation. Despite the absence of these additives, three-dimensional cell aggregates with tissue-specific properties are already obtained after two days of suspension cultivation according to the invention. Of course, the size depends on the number of introduced cells per volume of culture medium. For example, when 1 x 10^7 cells are incorporated in 300 µl of culture medium, three-dimensional spheroids about 500-700 µm in diameter are formed within 1 week. For a tissue defect of 1 cm^2, it would be necessary to transplant about 100 of such spheroids, e.g. by injection. The other possibility is the in vitro fusion of the small cell aggregates to form larger ones - as described above - and incorporation of the latter into the defect. According to the invention, preferably between 1 x 10^4 and 1 x 10^7 cells are used in 300 µl of culture medium to produce the small cell aggregates, particularly preferably 1 x 10^5 cells. Depending on

the cell type and the patient-specific characteristics, the spheroids formed after a few days are then cultivated in the suitable culture medium for at least 2-4 weeks to induce the formation of the tissue-specific matrix. In the particular case, from about one week of cultivation onwards, individual spheroids can then be fused, so as to increase the size of the tissue piece.

As cell culture vessels, for the inventive cultivation in suspension, those having a hydrophobic, i.e. adhesion-preventing surface, such as e.g. polystyrene or Teflon must be used. Cell culture vessels with non-hydrophobic surface can be made hydrophobic by coating with agar or agarose. Further additives are not required. Preferably, well plates serve as cell culture vessels. For example, 96-well plates can be used here to produce the small cell aggregates, and 24-well plates to produce the fused aggregates.

According to the invention, the cell culture vessels must have a tapered, preferably curved base. It has been found that the tissue according to the invention does not form in vessels with a flat base. Obviously, the depression serves for finding the cells.

The present invention also discloses the cartilage or bone tissue produced according to the above-described method, which tissue can be used as an autogenous, xenogenic or allogenic transplantation material in the treatment of cartilage or bone defects and degenerative diseases such as e.g. arthrosis or rheumatism. To this end, the cell aggregates according to the invention, produced in vitro, are injected into the diseased or degraded tissue by means of injection. To do so, the injection needle or another suitable application system must have at least the diameter of the spheroids. The cells in the zone of cells capable of proliferation and migration at the boundary of the spheroids rapidly grow into the surrounding tissue and enable a rapid integration of the tissue produced in vitro, and represent a potential for the regeneration of

tissue in the environment of the spheroids, because these cells still exhibit proliferation and form matrix. Representing an advancement compared to previous methods, this treatment can take place by means of arthroscopy in tissue engineering.

The present invention also discloses therapeutic formulations comprising the cartilage or bone tissue according to the invention, e.g. injection solutions.

The present invention also discloses the use of the cartilage or bone tissue according to the invention for the testing of various factors, such as e.g. active substances and physical factors which e.g. influence the formation and differentiation of matrix and cells, wherein the physical factors can be e.g. pressure or electric fields. To this end, the cell spheroids are produced according to the invention, and the medicaments to be tested are added at various stages of maturity, and the most varied parameters of spheroid formation and maturing are characterized. Compared to conventional medicament tests on animals or tumour systems, these tests are highly patient-specific and enable individual diagnosis as a result of using autologous material only.

The invention is to be explained in more detail below with reference to example embodiments, without restricting it thereto.

Example embodiments

Example 1: In vitro production of cartilage tissue

A biopsy is taken from the patient from a region of hyaline, healthy cartilage. The chondrocytes are isolated from this biopsy, using enzymatic digestion by incubation with collagenase solution. Following separation of the isolated cells from the undigested cartilage tissue, these cells are transferred in cell culture flasks and, with the addition of

DMEM/Hams F12 culture medium (1/1) and 10% autologous serum from the patient, are incubated at 37°C and 5% CO_2. The medium is exchanged twice a week. After reaching the confluent stage, the cell layer is washed with physiological saline solution and harvested from the cell culture surface using trypsin. Following another washing, 1 x 10^5 cells each time are transferred into a cell culture vessel which is coated with agarose. After one day, the first cells have arranged themselves into aggregates. These aggregates are supplied with fresh medium every 2 days and cultivated for at least 2 weeks. Already after one week, type II collagen and proteoglycans were detected in the aggregates. To this end, a specific antibody to type II collagen was used. The primary antibody bound to type II collagen was detected using a second antibody and an ABC system coupled thereto. That is, the enzyme alkaline phosphatase is coupled via avidin-biotin to the 2nd antibody, which enzyme effects reaction of the substrate fuchsin, with a red dye being formed.

The proteoglycans were detected by means of Goldner staining. Type II collagen and proteoglycans are components of the cartilage matrix *in vivo* and represent the most important structural proteins which are of crucial significance for the function of the cartilage.

At the same time, the protein S 100 specific for cartilage cells was detected in the outer layer of the aggregates. S 100 is not expressed in bone tissue and connective tissue. It is only these latter tissues which also could have formed here. Consequently, the tissue having developed was unambiguously proven to be cartilage tissue.

After cultivation for 1-2 weeks, the cells still lie close together. With increasing cultivation time, the proportion of extracellular matrix increases and the proportion of cells decreases. After one week, at least 40% ECM can be detected, and after 3 weeks about 60% ECM had already developed. After 3 months' cultivation of the cartilage tissue, the proportion of

ECM has increased to 80-90%. That is, cartilage-like tissue has been built up inside the produced aggregates, which tissue in its structure corresponds to the *in vivo* cartilage and can also assume the function of cartilage tissue.

Example 2: Transplantation of cartilage tissue

The tissue produced in Example 1 (about 200 spheroids, each one comprised of $1*10^5$ cells) was taken up in physiological saline solution and injected into a cartilage defect of the subject about 1 cm^2 in size, covered with periosteum. It was found that the cartilage defect is already filled with cartilage tissue within 1 to 2 months, while the filling of a defect of such a size is only to be observed after 6-12 months through the previous transplantation of juvenile cartilage cells merely multiplied in vitro. Thus, in addition to fulfilling the mechanical function of the produced tissue, the cartilage tissue according to the invention produced in vitro ensures the rapid integration of the produced tissue pieces by the cells capable of proliferation and migration in the outer layer of the aggregates. Consequently, the structure and function of the tissue pieces also permits the rapid repair of defects and degenerate cartilage tissue.

Example 3: In vitro production of bone tissue

A bone biopsy is taken from the patient from the region of the cancellous bone. The osteoblasts are isolated from this biopsy, using enzymatic digestion by incubation with collagenase solution. Following separation of the isolated cells from the undigested bone tissue, these cells are transferred in cell culture flasks and, with the addition of DMEM/Hams F12 culture medium (1/1) and 10% autologous serum of the patient, are incubated at 37°C and 5% CO_2. The medium is exchanged twice a week. After reaching the confluent stage, the cell layer is washed with physiological saline solution and harvested from the cell culture surface using trypsin. Following another washing, 1 x 10^5 cells each time are

transferred into a cell culture vessel which is coated with agarose. After one day, the first cells have arranged themselves into aggregates. These aggregates are supplied with fresh medium every 2 days and are cultivated for at least 2 weeks.

Already after one week, type I collagen and proteoglycans were detected in the aggregates. To this end, a specific antibody to type I collagen was used. By detecting collagen I, unambiguous proof was provided that this is not cartilage tissue. The primary antibody bound to type I collagen was detected using a second antibody and an ABC system coupled thereto. That is, the enzyme alkaline phosphatase is coupled via avidin-biotin to the 2nd antibody, which enzyme effects reaction of the substrate fuchsin, with a red dye being formed.

As in Example 1, the proteoglycans were detected by means of Goldner staining. Type I collagen and proteoglycans are components of the bone matrix *in vivo* and represent the most important structural proteins which are of crucial significance for the function of the bone.

At the same time, bone cells capable of proliferation were detected in the outer layer of the aggregates.

After cultivation for 2 weeks, the cells still lie close together. With increasing cultivation time, the proportion of extracellular matrix increases and the proportion of cells decreases. After one week, at least 40% ECM can be detected, and after 3 weeks about 60% ECM had already developed. That is, bone-like tissue was built up inside the produced aggregates, which tissue corresponds in composition to the *in vivo* bone and is also capable of assuming the function of bone tissue.

Example 4: Transplantation of bone tissue

The tissues produced in Example 3 (about 50 pieces) are taken up in physiological saline solution and injected into the region of a difficultly healing bone fracture. It was determined that the process of bone healing was able to be induced and new bone tissue was already formed after 3 weeks, whereas healing of the fracture would have taken place only after months if the defect had remained untreated. Therefore, the bone tissue grown in vitro ensures the rapid integration of the tissue into the surrounding tissue and the filling of bone defects. Consequently, the structure and function of the tissue pieces permits the healing of bone defects, the induction of osteosynthesis and the treatment of degenerative bone diseases.

Patentkrav

1. Fremgangsmåde til in vitro-fremstilling af et celleaggregat af tredimensionelt, vitalt brusk- eller knoglevæv, som er en sfæroid, kendetegnet ved, at bruskceller, knogleceller eller mesenchymale stamceller, der er udvundet af en menneskelig eller animalsk organisme, i cellekulturskåle med hydrofob overflade og bund, som bliver tyndere, som suspensionskultur under tilsætning af autogent serum eller autologt serum og uden vækstfaktorer og uden antibiotika og uden fungistatika stationært kultiveres, indtil der opstår et celleaggregat, som er en sfæroid, og som indeholder i det mindste 40 vol.-% extracellulær matrix (ECM), hvori differentierede celler er indlejret, og som har et ydre område, hvori der er proliferations- og migrationsegnede celler til stede.

2. Fremgangsmåde ifølge krav 1, kendetegnet ved, at de af eksplantatet udvundne brusk- eller knogleceller i første omgang formeres i monolayerkultur, og kultiveringen efterfølgende foretages i suspension ifølge krav 1.

3. Fremgangsmåde ifølge et af kravene 1 til 2, kendetegnet ved, at der alt efter ønsket vævsstørrelse fusioneres i det mindste to af de opståede celleaggregater, som er sfæroider, idet de i fællesskab viderekultiveres i cellekulturskåle med hydrofob overflade og bund, som bliver tyndere, i suspension.

4. Fremgangsmåde ifølge et af kravene 1 til 3, kendetegnet ved, at der som cellekulturskål anvendes hulplader.

5. Fremgangsmåde ifølge et af kravene 1 til 4, kendetegnet ved, at cellekulturskåle med ikke-hydrofob overflade hydrofoberes ved at belægge dem med agar eller agarose.

6. Fremgangsmåde ifølge et af kravene 1 til 5, kendetegnet ved, at kultiveringen i suspensionskultur gennemføres, indtil det opnåede celleaggregat har i det mindste 60% ECM.

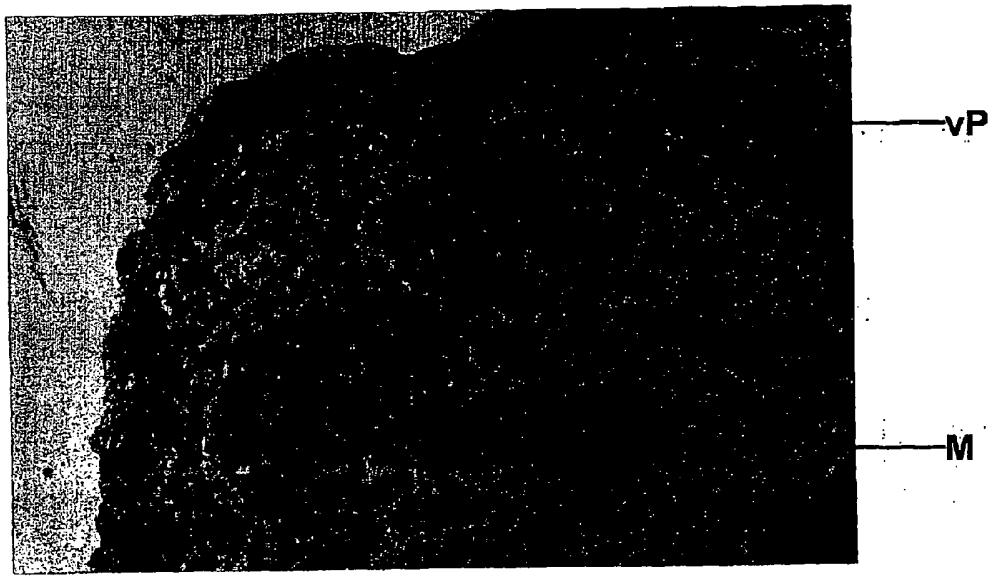

Fig. 1

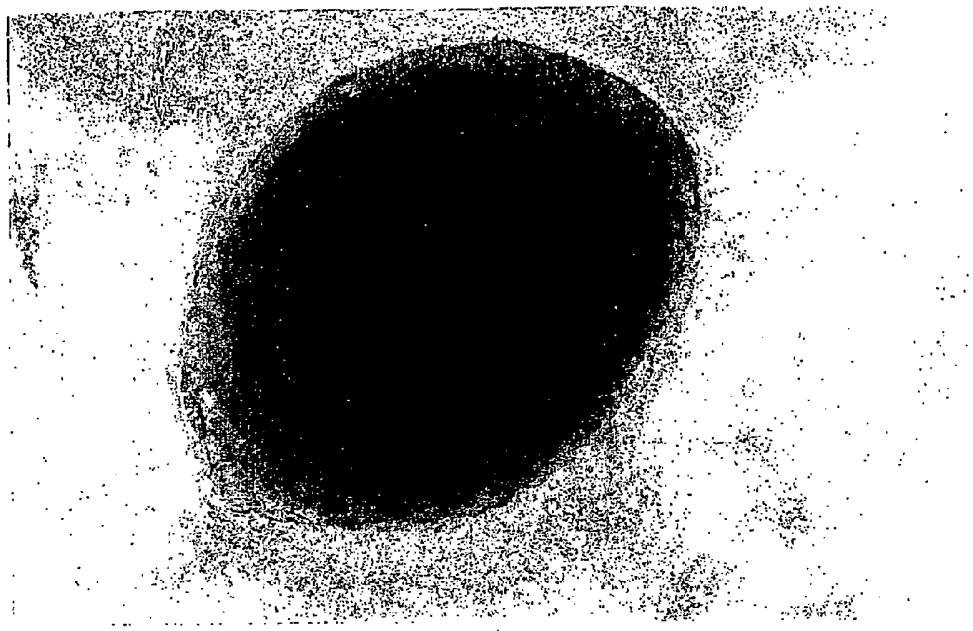

Fig. 1a

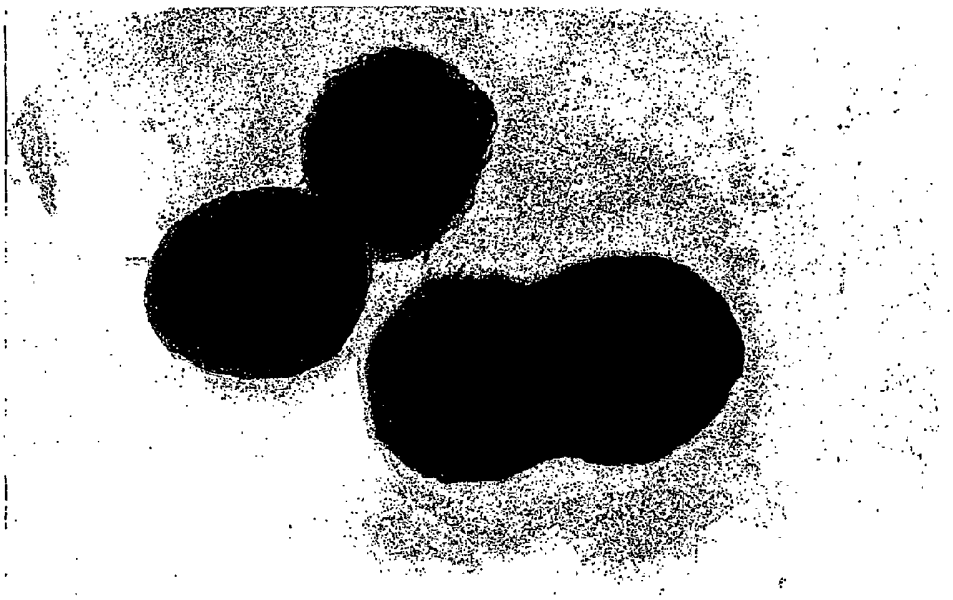

Fig . 1b

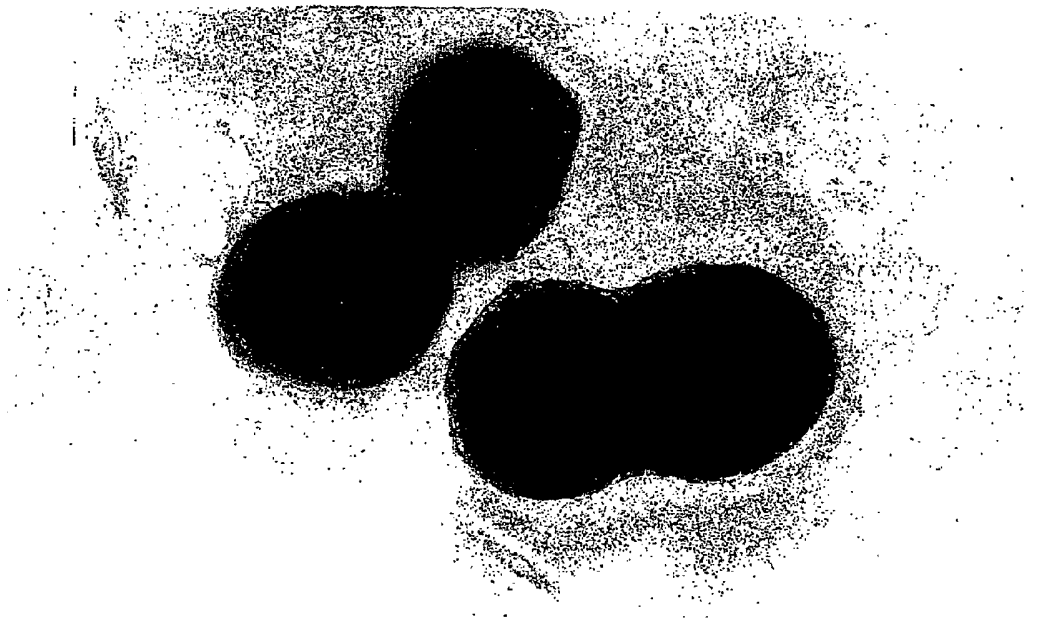

Fig. 1c

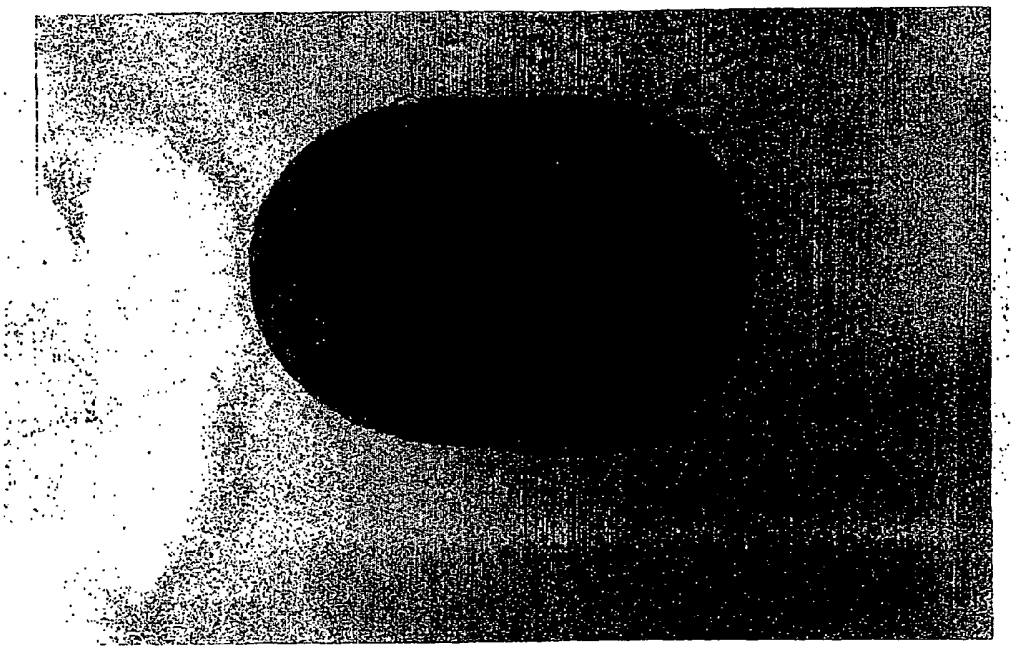

Fig. 1d