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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/00 — A1

(11) International Publication Number: **WO 99/21963**

(43) International Publication Date: 6 May 1999 (06.05.99)

(21) International Application Number: PCT/EP98/06715

(22) International Filing Date: 22 October 1998 (22.10.98)

(30) Priority Data:

97118582.2	25 October 1997 (25.10.97)	EP
97120245.2	19 November 1997 (19.11.97)	EP

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: AUTODEGRADABLE MICROCARRIERS AND THEIR USE

(57) Abstract

Autodegradable microcarriers which consist of a mixture comprising, in essentially homogeneous distribution, a proteolytically degradable matrix material crosslinked with a protease having the ability to proteolytically degrade said material are useful as transplantation matrix material, when allogeneic or autologous cells are attached to said microcarriers.

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Autodegradable microcarriers and their use

The invention relates to autodegradable microcarriers and their use, in particular for the production of pharmaceutical agents and for the cultivation of cells. Microspheres which can be used as microcarriers for a large variety of materials, inclusive of materials for the adhesion of cells, are prepared from a variety of materials, e.g., dextran (Levine, D.W., et al., Biotechnol. Bioeng. 21 (1979) 821), polystyrene (Johannson, A., Dept. Biol. Stand. 46 (1980) 125), polyacrylamide (Reuveny, S., et al., Biotechnol. Bioeng. 25 (1983) 469), cellulose (Reuveny, S., et al., In Vitro 18 (1982) 92), and gelatin (Nilsson, K., BioTechnology 4 (1986) 989; and Reuveny, S., Adv. Cell Culture 4 (1985) 213). Microcarriers can be prepared in the form of microspheres of various sizes and porosity and possessing various surface properties (Reuveny, S., Adv. Cell Culture 4 (1985) 213).

Several kinds of microcarriers (MCs) are suitable for immobilization specifically of animal and human cells. However, the MCs which are commercially available were mainly designed to meet certain requirements for large-scale industrial production of biological products. According to Nilsson, K., et al., BioTechnology 4 (1986) 989 and Reuveny, S., Adv. Cell Culture 4 (1985) 213, such microcarriers are not optimized for the cultivation of all kinds of cells.

Altankov, G., et al., J. Biomater. Sci. Polymer Edn. (1991) 81-89, describe microcarriers which are coated and/or derivatized on the surface, with different proteins in order to optimize their properties for better cellular interactions.

When used as pharmaceuticals agents, microcarriers are applied as carriers for pharmaceutically active substances, such as, e.g., therapeutic proteins, or as carriers for cells to be transplanted. In International Patent Application WO 93/23088 there is described a living skin replacement which comprises a plurality of microspheres formed of a material which is biocompatible and resorbable in vivo, and a culture of skin cells coating the microspheres, whereby the microspheres coated with skin cells are applied to the skin injury.

In International Patent Application WO 96/12510 there is described a transplantation material for treating wounds, which material contains epithelial cells attached to microcarriers, whereby the microcarriers are covered to a high degree with cells.

In UK Patent Application GB 2 059 991 there is a described a method of cultivating cells in a culture medium, containing particulate microcarriers, wherein the microcarriers are cross-linked collagen and wherein the cells are released from the carrier with the aid of a proteolytic enzyme. Such a proteolytic enzyme can be, for instance, collagenase when the polypeptide is collagen or gelatin. Subsequent to the termination of the growth of the cells, the cells are released, when desired, from the carrier with such a proteolytic enzyme. However, the combination of such a microcarrier with the subsequent use of a proteolytic enzyme suffers from the drawback that the enzymatic release of the cells from the surface cannot be controlled with respect to time and is not reproducible. Besides, the additional administration of collagenase to, for example, burn wounds could be problematical.

In International Patent Application WO 90/13625 there is described a method of preparing gelatin microcarriers for the cultivation of cells in a stream of air while introducing into the atomizing zone a dry particulate material to form gelatin particles coated with said dry particulate material, and recovering the coated gelatin particles. It is also suggested to treat the coated gelatin particles, in addition, with a medium containing a starch degrading enzyme.

EP-A 0 058 689 discloses a cell culture medium comprising crosslinked gelatin particles which can be totally degraded by proteolytic enzymes, such as collagenase or dispase.

U.S. Patent 4,349,530 describes microcapsules and microbeads containing incorporated therein an inactive form of a protease capable of dissolving albumin. This inactive form of the protease has no effect upon the microcapsules and microbeads when they are stored dry. But when the microbeads and/or microcapsules are injected into an animal's bloodstream, the inactive form of the protease is gradually converted into the active form and the active form of the protease attacks the albumin present in the microcapsules or microbeads, thereby speeding up the release of the active substance therefrom. However, this protease does not degrade the matrix material and encapsulating material of the microbeads.

WO 95/34328 describes a gel matrix containing a matrix protein selected from the group consisting of gelatin and albumin, a polymeric stabilizer and/or an external crosslinker and an

enzyme capable of degrading said protein. However, the matrix protein is not crosslinked with said enzyme.

All of the known microcarriers suffer from the drawback, however, of being not completely, or only very slowly, biodegraded on, or in, the human body.

The object of the invention is to provide microcarriers which are autodegradable, especially when being used as carriers for transplantation materials, and thus do not remain within the treated patient for a prolonged period of time.

This object is accomplished by autodegradable microcarriers which consist essentially of a mixture comprising, in essentially homogeneous distribution, a proteolytically degradable matrix material crosslinked with a protease having the ability to proteolytically degrade said matrix material. The protease in these microcarriers is inactive first. When the medium surrounding the microcarriers is changed in such a way that physiological conditions will result, the activation of the protease is effected.

It has surprisingly been found that such autodegradable microcarriers according to the invention are degraded reliably in vivo within a predetermined period of time, which is essentially dependent upon the ratio of protease and proteolytically degradable matrix material.

According to the invention, a two-component microcarrier containing enzyme and substrate is synthesized under conditions in which the enzyme is in an inactive state (caused by an inhibitor), and said inactive enzyme is chemically crosslinked with its substrate, the latter being the main matrix material of the microcarrier, at least 50%, preferably at least 80% (of the microcarrier). Switching to normal cell or tissue culture conditions, the enzyme is activated, which results in microcarrier degradation.

Therefore, according to the invention, the proteolytic enzyme is present before and during the synthesis of the microcarrier as well as before the cells are attached to the microcarrier.

"Proteolytically degradable matrix material" is to be understood to include material useful for the manufacture of microcarriers, which generally are proteins, e.g. gelatin, albumin or collagen, or mixtures of such materials. Said matrix material is proteolytically degradable and therefore inactivated by the protease used according to the invention.

„Inactivation of the matrix material" as used herein is understood as an essentially occurring destruction of the biological function and of the structure of the monomeric form of the matrix material, for example, and as a proteolytic degradation into short-chain polypeptide fragments and/or single amino acids. The microcarriers of the invention are thus being degraded in the human body in rapid and reliable fashion, while no undesired immunoreactions against the microcarriers or parts thereof are observed.

A protease having the ability to proteolytically degrade said matrix material ("associated protease") is to be understood to mean protease which is capable of proteolytically degrading the basic proteolytically degradable matrix material of the microcarrier. Examples of such combinations of basic matrix material and protease suitable in accordance with the invention are collagen or gelatin and collagenase or also albumin and trypsin.

The mixture of proteins and corresponding proteases can be used to improve the properties of the microcarriers. For example, different ratios of collagen and gelatin can be applied to regulate the biodegradability when using the same amount of collagenases.

In order to be suitable as a microcarrier in accordance with the invention, it is essential that the proteolytically degradable matrix material and the protease are homogeneously distributed within the microcarrier and the proteolytic enzyme is in an inactive state. This can be accomplished, preferably, by preparing the microcarriers from the proteolytically degradable matrix material in the presence of a protease and an inhibitor, wherein both the proteolytically degradable matrix material, the protease and the inhibitor are present in homogeneous suspension or, preferably, in solution, and are subsequently crosslinked, thereby forming the microcarriers.

In a preferred embodiment of the invention, the microcarriers according to the invention, as well as the starting solution from which the microcarriers are prepared, contain, in addition, an inhibitor of the protease applied. This inhibitor may be, for example, a chelating agent for divalent cations (e.g., EDTA) (for collagenase) or trypsin inhibitor for trypsin and is added together with the proteolytic enzyme, or beforehand, but not later. Since the inhibitor is not crosslinked with the other components of the microcarrier, said inhibitor can be released from the microcarriers under aqueous buffer conditions (preferably between pH 6 and 8) or under cell culture/tissue culture conditions. In this manner the protease is reactivated, the microcarriers are degraded, and the cells are released in a controlled manner from said microcarriers during said degradation.

The ratio of the amounts of proteolytically degradable matrix material and protease and protease inhibitor in the microcarriers is essentially dependent upon the desired dissolving time of the microcarriers. If cell-coated microcarriers are used as a transplantation material for wound healing (cf. International Patent Application WO 96/12510, European Patent Application EP-A 0 267 015, International Patent Application WO 81/00260, European Patent Application EP-A 0 242 305, International Patent Application WO 89/03228, Canadian Patent No. 1,215,922 or according to Boyce, S.T., et al., Surgery 4 (1988) 421-431) it is preferred that the microcarriers should degrade within 1 to 5 days, thus releasing the attached epithelial cells.

In a preferred embodiment of the invention, type I clostridial collagenase, type II clostridial collagenase and/or neutral protease are used as collagenase.

In order to stabilize the microcarriers prepared according to the invention, it is further preferred to treat them with a cross-linking agent such as aldehyde (e.g., glutaraldehyde, formaldehyde, glioxal, acetaldehyde and propiolic aldehyde). This can be accomplished, for instance, by adding to a suspension of the microcarriers prepared according to the invention the aldehyde in an amount of from 0.001 to 10%, preferably from 0.2 to 0.4%, or by adding the said amount of aldehyde to the mixture or suspension of proteolytically degradable matrix material and protease already during the production of the microcarriers.

In another preferred embodiment, the free aldehyde groups may also be saturated with an appropriate adhesive protein in order to improve the surface properties of the microcarriers. It is preferred to use fibronectin, collagen, vitronectin or laminin as adhesive proteins. It is further preferred to reduce, after the said treatment with adhesive proteins, any remaining free aldehyde groups by an oxidating agent, such as, e.g., sodium borohydride, which may be added to the aqueous phase of sedimented microcarriers. It is preferred to add sodium borohydride at a concentration of 20 mg/ml suspension which preferably contains approximately 10^5 microcarriers/ml. The treatment of the microcarriers with the adhesive protein, preferably with fibronectin, can be done preferably after the modification and/or saturation step. In a particularly preferred embodiment of the invention, the microcarrier can be incubated with human plasma and then washed, e.g. with buffer solution.

According to the invention it is preferred to use microcarriers with a size in the range between 10 and 2,000 µm, more preferably between 50 and 250 µm.

In order to avoid autodegradation of the microcarriers during storage, the microcarriers according to the invention are preferably kept in an inactivation solution, preferably in an inactivation buffer. Such an inactivation solution contains a protease inhibitor, e.g., a chelating agent, when a protease, such as collagenase, is used.

For activation, the microcarriers according to the invention only need to be washed in order to remove the protease inhibitor from the solution. Thereafter, the microcarriers can be coated with the desirable cells for transplantation, optionally stored once more in the presence of a protease inhibitor, under frozen conditions, or be used directly as a transplantation material.

The proteolytic enzyme may be added before the emulsification step for synthesizing the microcarriers (mixing together with the proteolytically degradable matrix material and the inhibitors, or during the modification step for crosslinking). In the latter case it is preferred to saturate free aldehyde groups with a proteolytic enzyme, preferably with the enzyme contained in the microcarriers (saturation step). In a further step, remaining aldehyde groups are removed, preferably by a strongly reducing agent, such as borohydride.

A further embodiment of the invention is a method of producing autodegradable microcarriers, wherein a homogeneous solution or suspension is prepared from a proteolytically degradable matrix material and a protease having the ability to proteolytically degrade said matrix material, so as to form microparticles having a size of from 10 to 2,000 μm.

A further embodiment of the invention is a method of producing a pharmaceutical agent for transplantation of cells to a patient in need of the transplantation, when said cells are attached to autodegradable microcarriers which consist of a proteolytically degradable material crosslinked with a protease, said protease being in a reversible inactive state, whereby said microcarriers are autodegraded during the pharmaceutical application, whereby said cells are released from said microcarriers.

A further embodiment of the invention is a pharmaceutical composition consisting of cells which are allogeneic or autologous to a patient, attached to autodegradable microcarriers which consist of a mixture comprising, in essentially homogeneous distribution, a proteolytically degradable matrix material crosslinked with a protease having the ability to proteolytically degrade said matrix material.

In a further preferred embodiment, the microcarriers according to the invention are used as carriers for pharmaceutically active substances such as, e.g., therapeutic proteins, or as carriers for animal or human cells to be transplanted. Coating of the microcarriers with cells can be carried out, for example, as described in WO 93/23088 and WO 96/12510.

The following examples, references and drawing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Fig. 1 illustrates the microcarrier degradation in dependence upon the time and amount of protease (Collagenase; A: 1 µg/ml, B: 10 µg/ml, C: 100 µg/ml, D: 250 µg/ml) contained in the microcarrier.

Example

Emulsification (emulsification step):

Oil phase (e.g., 300 ml sunflower oil), heated to an appropriate temperature, principally below 45°C (because of denaturation of collagenase, e.g., 46°C), was stirred (example: with rotating paddle at 200 r.p.m.). Aqueous phase containing gelatin, collagenase and inhibitor of collagenase (e.g., divalent cation chelating agent EDTA) at different molecular ratios (collagenase with concentration between 10^{-9} and 10^{-1} g per liter, gelatin between 1 and 400 g per liter and EDTA between 10^{-6} and 10^{3} g per liter) was added to the oil and stirred (example: approximately 5 min).

Example: Solution of 12 g gelatin dissolved in 60 ml water containing clostridial collagenase at a concentration of 1-250 µg/ml, 140 mM NaCl and 0.02% EDTA was added to the oil phase and stirred for 5 min.

Separation:

Excess of water containing inhibitor of collagenase, such as divalent cation chelating agent EDTA (example: 500 ml inactivating buffer containing 20 mM Hepes buffer, 140 mM NaCl and 0.02% EDTA, pH 7), with a temperature between 0 and 20°C was added to the emulsion and stirred additionally (e.g., 3 times, 5 min each, with 100 r.p.m.). Then the gelatin beads

were allowed to settle down out of the oil phase (for about 1 h), then washed with the same inhibiting buffer until the oil is removed.

Stabilization step:

The beads in inactivation conditions as above, at pH ranging between 3 and 10, were treated with a cross-linking agent, such as aldehyde (e.g., glutaraldehyde at a final concentration of 0.2% was added and stirred additionally for approximately 1 h). Instead of glutaraldehyde, formaldehyde, glioxal, acetaldehyde and propiolic alden could be used.

Saturation step:

Free aldehyde groups on that step of synthesis can be saturated with appropriate adhesive protein in order to improve the surface properties of microcarriers. Fibronectin, collagen, vitronectin, laminin and other adhesive proteins, at different concentrations could be used by adding them to the inhibiting buffer. At the end of this procedure, any remaining free aldehyde groups were reduced by adding sodium borohydride which may be added to the aqueous phase of sedimented microcarriers at a concentration between 10^{-9} and 10^{2} g per liter (in example, final concentration of 20 µg/ml in 10 mM carbonate buffer pH 9 was used) under stirring or static conditions, and for an appropriate time (in example, 1 h).

This method converts approximately 95% of the initial gelatin solution into stable pale yellow-colored microspheres with a size in the range between 50 and 250 µm.

Sterilization:

With 70% ethanol (for 1 h) followed by 3 times washing with sterile inhibiting buffer as above. Ethylene oxide and UV or gamma radiation can also be used without producing significant damage of microcarriers.

Storage:

Collagenase containing microcarriers were stored at 4°C in inactivation buffer (e.g., buffer containing 20 mM Hepes, 0.02% EDTA, 140 mM NaCl, pH 7).

Activation:

Wash with an excess of tissue culture medium, or with any aqueous solution containing divalent cations in physiological ranges, e.g., buffer containing 20 mM Hepes, 140 mM NaCl, 3 mM $CaCl_2$ and 0.5 mM $MgCl_2$ pH 7.4.

Degradation criteria: Macroscopic diminish up to disappearance of sedimented microcarriers.

Results:

Typical results were shown on Fig. 1 where different amounts of collagenase (A: 1 µg/ml, B: 10 µg/ml, C: 100 µg/ml, D: 250 µg/ml) have been added to the gelatin suspension. Resulting microcarriers were washed and incubated at 37°C in activation buffer as follows: 20 mM Hepes, 140 mM NaCl, 3 mM $CaCl_2$, 0.5 mM $MgCl_2$ at pH 7.4.

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WO 81/00260
WO 89/03228
WO 90/13625
WO 93/23088
WO 95/34328
WO 96/12510

Patent Claims

1. Autodegradable microcarriers which consist of a mixture comprising, in essentially homogeneous distribution, a proteolytically degradable matrix material crosslinked with a protease having the ability to proteolytically degrade said matrix material.

2. Autodegradable microcarriers as claimed in claim 1, wherein said proteolytically degradable matrix material is gelatin or collagen and said protease is collagenase.

3. Autodegradable microcarriers as claimed in claim 1 or 2, with a size in the range between 50 and 250 μm.

4. A storable composition of autodegradable microcarriers as claimed in claims 1 to 3, wherein the composition contains, in addition, a protease inhibitor which can be released from the microcarrier by treatment with an aqueous buffer solution pH 6 to 8.

5. The composition as claimed in claim 4, wherein the protease inhibitor is a chelating agent.

6. A method of producing autodegradable microcarriers, wherein a homogeneous solution or suspension is prepared from a proteolytically degradable matrix material and a protease having the ability to proteolytically degrade said matrix material, said proteolytically degradable matrix material and said protease are crosslinked under conditions resulting in the formation of microparticles having a size of from 10 to 2,000 μm.

7. A method as claimed in claim 6, wherein the solution or suspension contains, in addition, an inhibitor of said protease.

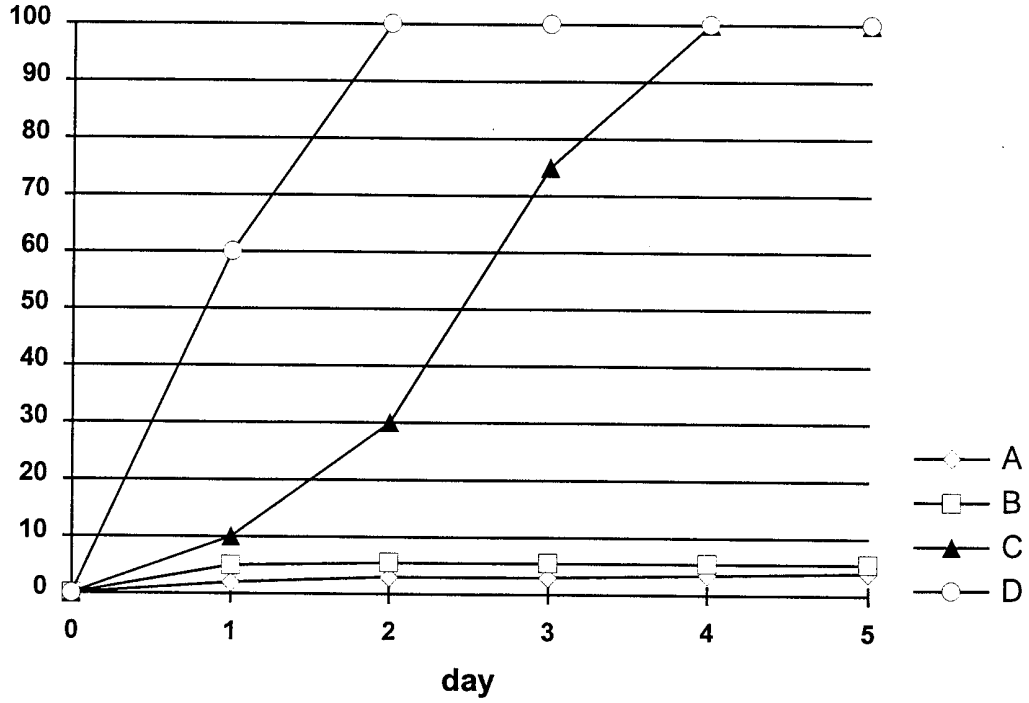
8. A method as claimed in claim 6 or 7, wherein subsequent to the formation of the microparticles, free aldehyde groups on said microparticles are saturated with an adhesive protein.

9. A method as claimed in claim 8, wherein said adhesive protein is fibronectin, collagen, vitronectin, or laminin.

10. A method of producing a pharmaceutical agent for transplantation of cells to a patient in need of the transplantation, when said cells are attached to autodegradable microcarriers which consist of a proteolytically degradable matrix material crosslinked with a protease, said protease being in a reversible inactive state, whereby said microcarriers are autodegraded during the pharmaceutical application, whereby said cells are released from said microcarriers.

11. A pharmaceutical composition consisting of cells which are allogeneic or autologous to a patient, attached to autodegradable microcarriers which consist of a mixture comprising, in essentially homogeneous distribution, a proteolytically degradable matrix material crosslinked with a protease having the ability to proteolytically degrade said matrix material.

Fig. 1



INTERNATIONAL SEARCH REPORT

Int ıtional Application No
PCT/EP 98/06715

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00

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

B. FIELDS SEARCHED

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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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 349 530 A (ROYER GARFIELD P) 14 September 1982 see column 3, line 50 - column 4, line 4 see column 5, line 1 - line 26; claims 1,16,49,50,63,64	1,6,10, 11
Y		2,3

Y	WO 95 34328 A (ROYER GARFIELD P) 21 December 1995 see page 6, line 18 - page 8, line 11 see page 14, line 1 - page 16, line 19	2,3

A	WO 93 23088 A (NATIONAL RESEARCH COUNCIL OF CANADA) 25 November 1993 cited in the application see page 21, line 19 - line 25; claim 7	1-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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17 March 1999	24/03/1999

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06715

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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