

Artículos originales cortos

Continuous production of human tissue-type plasminogen activator from Bowes melanoma cells entrapped in alginate beads

M. P. RODRÍGUEZ, R. SERRANO, L. HERNÁNDEZ, P. RODRÍGUEZ, M. CEBRIÁN,
J. DE LA FUENTE and L. HERRERA.

Mammalian Cell Genetics Division, Centro de Ingeniería Genética y Biotecnología,
P.O.Box 6162, Havana, Cuba

Recibido en agosto de 1990

Aprobado en enero de 1991

SUMMARY

Human Bowes melanoma cells were entrapped in calcium alginate beads and cultivated in spinner flasks and in a fluidized-bed reactor. The cell concentration in the gel particles reached more than 10^7 cells/ml gel. The tissue-type plasminogen activator (t-PA) production was studied. By using a fluidized-bed reactor, effective removal of the medium could be achieved and a higher t-PA productivity/cell/day was obtained (0.2 pg/cell/day). The alginate immobilization method was useful for continuous t-PA production from anchorage-dependent Bowes melanoma cells.

RESUMEN

Células de melanoma de Bowes fueron encapsuladas en perlas de alginato de calcio y cultivadas en frascos de rotación y en un reactor de cama fluidizada. En las partículas de gel se alcanzaron concentraciones celulares mayores de 10^7 células/ml de gel. Se estudió la producción del activador tisular del plasminógeno (t-PA). Utilizando el reactor de cama fluidizada se logró un efectivo recambio del medio de cultivo y se alcanzó una mayor productividad de t-PA/célula/día (0,2 pg/célula/día). El método de inmovilización de células en perlas de alginato de calcio fue efectivo para la producción en continuo de t-PA en células de melanoma de Bowes dependientes de anclaje.

INTRODUCTION

Human tissue-type plasminogen activator (t-PA) is a serine protease that converts the proenzyme plasminogen to the active serine protease plasmin. This t-PA has affinity to fibrin and is activated by it (Nieuwenhuizen *et al.*, 1983). The molecule is involved in homeostasis of fibrin and the dissolution of blood clots, making it a useful agent for the treatment of thrombolytic disorders (Bergmann *et al.*, 1983).

The human t-PA has been obtained from Bowes melanoma cells (Collen *et al.*, 1982; Rijken and Collen, 1981; Weimar *et al.*, 1981; Vehar *et al.*, 1984) and the recombinant product has been produced in mammalian cells (Kaufman *et al.*, 1985; Weidle and Buckel, 1987).

Because t-PA is a glycoprotein, only mammalian cells can allow a close approximation of protein synthesis *in vivo*. A method by which mammalian cells can be cultivated for continuous production of t-PA is therefore desirable. Immobilization of animal cells is a useful way to attain high cell concentration (Brunt, 1986; Posillico, 1986; Nilsson, 1987; Shirai *et al.*, 1988;

Smiley *et al.*, 1989). Monoclonal antibodies are continuously produced in high concentration by immobilized hybridoma cells in a fluidized-bed reactor (Iijima *et al.*, 1988)

In the present paper we examined the t-PA production by Bowes melanoma cells cultivated in different systems. A continuous t-PA production was obtained using immobilized cells in a fluidized-bed reactor.

MATERIALS AND METHODS

Cells and culture

Human Bowes melanoma cells were cultivated in Dulbecco's MEM (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate and 2 mM glutamine. Cultured cell stocks were maintained and propagated in roller bottles (1 750 cm³).

Immobilization of cells

Bowes melanoma cells growing logarithmically were collected and suspended in DMEM at a concentration of 10⁶ cells/ml. One ml of the cell suspension was mixed with 2 ml of 2% calcium alginate (Bellco, USA) solution. Calcium alginate was dissolved in 50 mM sodium citrate and filtered

through a 0.2 μm pore membrane filter (Sartorius, FRG). The cell suspension in alginate was then dropped into sterilized 50 mM CaCl₂, 100 mM NaCl solution using a 22 gauge syringe and allowed to stand for 10 min. These gel beads (diameter 3 ± 0.5 mm) were then transferred to the culture vessels (Serrano *et al.*, 1990).

Culture of immobilized cells

For repeated batch cultures, the immobilized cells were cultured in spinner flasks of 6 or 8 L (Bellco, USA) spinning at 20 rpm. The spinner flask contained 2, 4 or 6 L DMEM and 400 ml gel particles and were incubated at 37°C in a 95% air/5% CO₂ atmosphere.

For continuous culture, a fluidized-bed reactor (2.8 cm diameter x 13 cm length) was used (see figure 1). The DMEM was continuously supplied at a rate of 20-40 ml/h. The reactor contained 50 ml alginate beads and 40 ml medium. A 95% air/5% CO₂ mixture was supplied from the bottom of the reactor at a flow rate of 30 L/h.

t-PA assay

The t-PA in the culture supernatant was estimated by a specific ELISA (Pérez *et al.*, 1990; Hernández *et al.*, 1990) and by fibrin plate assay (Astrup and Müllertz, 1952). The number of cells was counted using a hemocytometer after the alginate gels were solubilized in 50 mM sodium citrate.

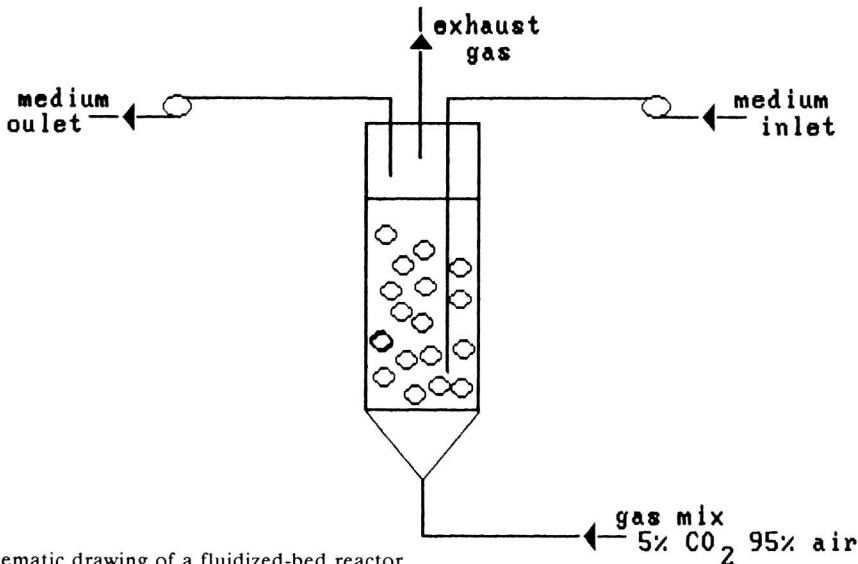


FIG. 1. Schematic drawing of a fluidized-bed reactor

RESULTS AND DISCUSSION

Cultivation of Bowes melanoma cells in alginate particles

The Bowes melanoma cells are anchorage-dependent cells. In order to allow the cell growth in suspension, the cells were immobilized in calcium alginate particles and cultivated in spinner flasks. Immobilization in alginate gel beads have been used before for cultivation of anchorage-dependent animal cells (Shirai *et al.*, 1988).

Figure 2 shows Bowes melanoma cells immobilized in a calcium alginate gel particle (a) just after immobilization; (b) 10 days after immobilization. The cells in the alginate gel beads grew three-dimensional in channel-like structures as it was reported before (Shirai *et al.*, 1988).

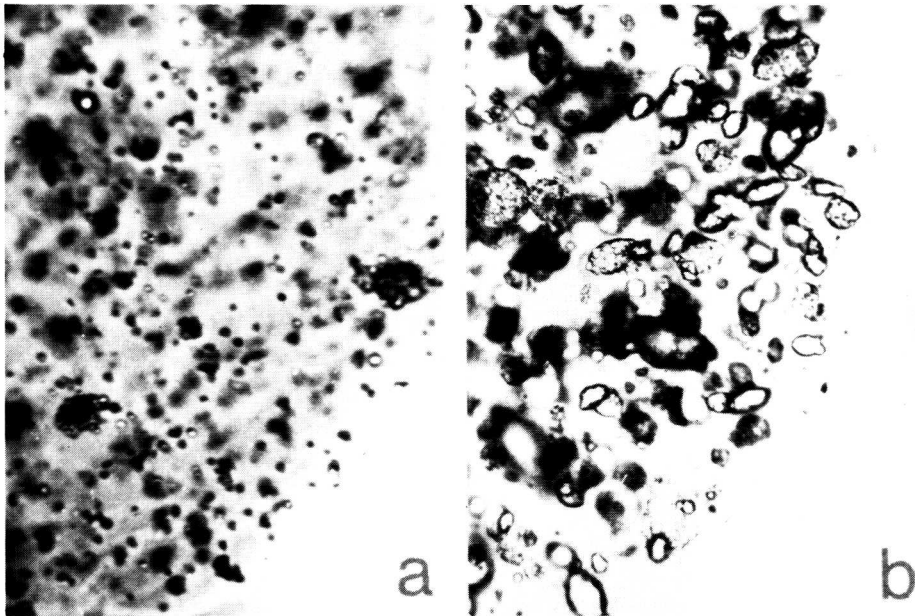


FIG. 2. Photograph of sodium alginate particles of immobilized Bowes melanoma cells under a microscope (Olympus IMT-2, Tokyo, Japan) with magnification of 60 X immediately after (a) and 10 days after (b) immobilization.

Figure 3 shows the growth and t-PA production by Bowes melanoma cells entrapped in alginate beads and cultivated in a 6 L spinner flask. The total volume of medium was increased by day 18 from 2 L to 4 L. The cell concentration reached 1.5×10^7 cells/ml gel 21 days after the start of cultivation. However, the t-PA production/day was constant during the first 18 days ($120 \mu\text{g}$ t-PA/day). When the volume was increased to 4 L, the total t-PA production reached a value of $260 \mu\text{g}/\text{day}$ and again it was constant for the next three days. This result suggests that despite the continuous cell growth observed, the t-PA production is "inhibited" probably due to culture state because of metabolic product release and/or nutrient or oxygen limitation. A similar result was obtained when the entrapped cells were cultivated in an 8 L spinner flask with culture volumes of 2 L, 4 L and 6 L during 10 days (data not shown).

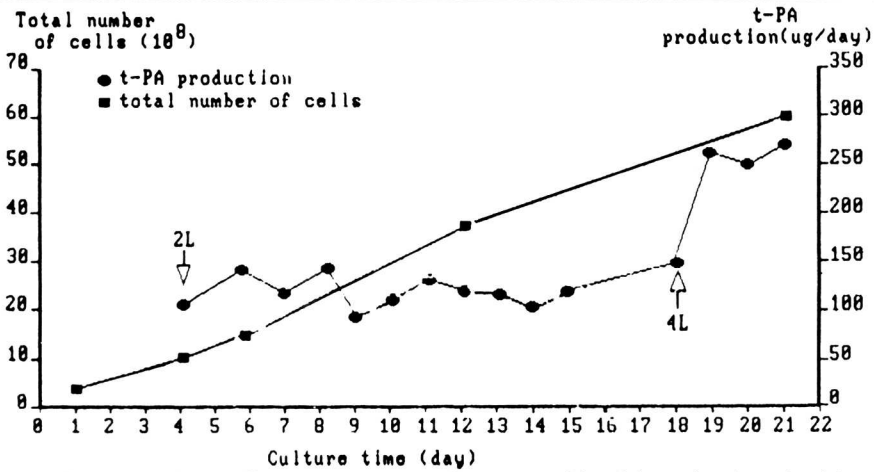


FIG. 3. Batch t-PA production by Bowes melanoma cells entrapped in alginate beads and cultivated in a 6 L spinner flask. The total culture volumes are indicated (2 L and 4 L). The medium was collected every 24 hours, assayed for t-PA activity, the protein corresponding to t-PA measured by ELISA and an equal volume of fresh medium added. This experiment was repeated twice.

Continuous t-PA production in a fluidized-bed reactor by Bowes melanoma cells entrapped in alginate beads

In order to avoid the "inhibitory" effect on t-PA production, a continuous culture system was assayed. Using a fluidized-bed reactor (see figure 1) a higher t-PA productivity was obtained (0.2 pg/cell/day)

compared to that obtained in roller bottles or spinner flasks batch cultures (see figure 4). In this system the production of t-PA by immobilized cells is higher compared to intact cells (see figure 4). Although no additional data is available, it is probably the result of a non-nutrient limited physiological stage of the cell, due to the continuous flow of medium through the reactor.

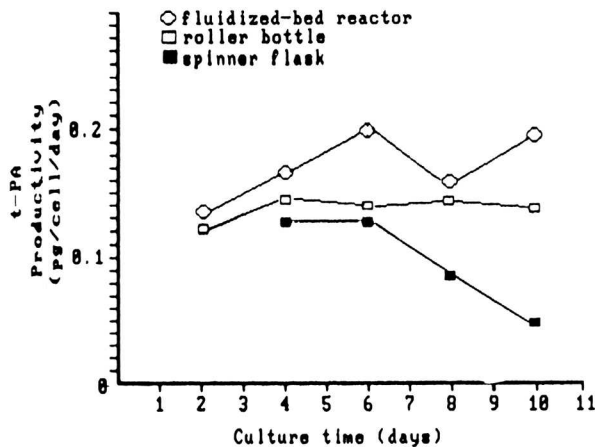


FIG. 4. t-PA productivity by Bowes melanoma cells immobilized in calcium alginate beads. The experiment was carried out in the conditions described in Figure 3 legend.

In conclusion, t-PA was produced continuously by anchorage-dependent Bowes melanoma cells immobilized in calcium alginate beads. They were cultivated in the gel particles to a cell concentration of more than 10^7 cells/ml gel. The use of these immobilized Bowes melanoma cells in a fluidized-bed reactor improved the t-PA production.

ACKNOWLEDGEMENTS

We would like to thank Luis Sorel for his excellent technical assistance.

REFERENCES

- ASTRUP, T. and S. MÜLLERTZ (1952). The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* **40**: 346-351.
- BERGMANN, S.; K. FOX; M. TER-POGOSSIAN; B. SOBEL and D. COLLEN (1983). Clot-selective coronary thrombolysis with tissue-type plasminogen activator. *Science* **220**: 1181-1183.
- BRUNT, J. VAN (1986). Immobilized mammalian cells: the gentle way to productivity. *Bio-technology* **4**: 505-510.
- COLLEN, D.; D. RIJKEN; I. VAN DAMM and A. BILLIAN (1982). Purification of human tissue-type plasminogen activator in centigram quantities from human melanoma cell culture fluid and its conditions for use *in vivo*. *Thromb. Haemost.* **48**: 294-296.
- HERNANDEZ, L.; L. SORELL; P. RODRIGUEZ; M. RODRIGUEZ; M.E. PEREZ and J. DE LA FUENTE (1990). Characterization of three monoclonal antibodies against human tissue-type plasminogen activator. International Symposium on Plasminogen Activators: from Cloning to Therapy (Italy). Ed. by R. Abbate and T. Barni. *Serono Symposia Review. Supplement 3*: 22.
- IJIMA, S.; T. MANO; M. TANIGUCHI and T. KOBAYASHI (1988). Immobilization of hybridoma cells with alginate and urethane polymer and improved monoclonal antibody production. *Appl. Microbiol. Biotechnol.* **28**: 572-576.
- KAUFMAN, R.; L. WASLEY; A. SPILIOUES; S. GOSSALS; S. LATT; G. LARSEN and R. KAY (1985). Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydro-folate reductase sequences in Chinese hamster ovary cells. *Mol. Cell. Biol.* **5**: 1750-1759.
- NIEUWENHUIZEN, W.; A. VERMOND; M. VOSKVILEN; D. TRAAS; and J. VERHEINJEN (1983). Identification of a site in fibrin(ogen) which is involved in the acceleration of plasminogen activation by a tissue-type plasminogen activator. *Biochem. Biophys. Acta* **748**: 86-92.
- NILSSON, K. (1987). Methods for immobilizing animal cells. *Tibtech* **5**: 73-78.
- PEREZ, M.E.; L. SORELL; P. RODRIGUEZ; L. HERNANDEZ; M. RODRIGUEZ; M. AGUILAR y J. GAVILONDO (1990). Obtención y empleo de anticuerpos monoclonales contra el activador tisular del plasminógeno. *Biotechnología Aplicada* **7**: 182-187.
- POSILLICO, E.G. (1986). Microencapsulation technology for large scale antibody production. *Bio-Technology* **4**: 114-117.
- RIJKEN, D. and D. COLLEN (1981). Purification and characterization of the plasminogen activator secreted from human melanoma cells. *J. Biol. Chem.* **256**: 7035-7041.
- SERRANO, R.; P. RODRIGUEZ; L. SORELL; A. AGUILAR; L. HERRERA and J. DE LA FUENTE (1990). Cell entrapment in calcium alginate beads: possible applications. *Biotechnología Aplicada* **7**: 341-345.
- SHIRAI, Y.; R. SASAKI; K. HASHIMOTO; H. KAWAHARA; K. HITOMI and H. CHIBA (1988). Continuous production of erythropoietin with immobilized animal cells. *Appl. Microbiol. Biotechnol.* **29**: 544-549.
- SMILEY, A.; W-S. HU and D. WANG (1989). Production of human immune interferon by recombinant mammalian cells cultivated on microcarriers. *Biotechnol. Bioeng.* **33**: 1182-1190.
- VEHAR, G.; W. KOHR; W. BENNETT; D. PENNICA; C. WARD; R. HARKINS and D. COLLEN (1984). Characterization studies on human melanoma cell tissue-type plasminogen activator. *Bio-Technology* **12**: 1051-1057.
- WEIDLE, U.H. and P. BUCKEL (1987). Establishment of stable mouse myeloma cells constitutively secreting tissue-type plasminogen activator. *Gene* **57**: 131-141.
- WEIMAR, W.; J. STIBBE; A. VAN-SEYEN; A. BILLIAU; P. DESOMER and D. COLLEN (1981). Specific lysis of an iliofemoral thrombus by administration of extrinsic (tissue-type) plasminogen activator. *The Lancet* **2**: 1018-1020.