Growth of MRC-5 diploid cells on three types of microcarriers

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Summary. In order to investigate the potential of the microcarrier homogeneous submerse culture technique for mass production of human diploid cells, 3 types of microcarriers were selected as substrate for the growth of MRC-5 human diploid line; comparative data using different microcarrier and cell concentrations are presented and discussed.

The increasing need for cell products and viral vaccines for human use requires the mass production of suitable cell substrates for their economic production. Continuous cell lines ('transformed' lines) can be selected to be grown in large fermentors as homogeneous suspension cultures, offering a potentially attractive approach; the major disadvantage lies in the possibility that biologicals for human use produced from these cells may prove unsafe. Human diploid cell lines such as WI-38 and MRC-5 are widely used and accepted as safe substrates for viral vaccine production[1], but they cannot grow in suspension. This anchorage-dependence is the principal limiting factor for a large-scale production. A promising system for overcoming this problem is offered by the microcarrier culture technique developed by Van Wezel[2], in which the cells are grown on small beads suspended in the culture medium, thus acquiring all the advantages of the suspension culture method. However, the microcarriers initially used caused a certain degree of inhibition of cell growth at concentrations over 1 g/l, and this toxic effect was particularly evident in the case of human diploid cells[3], leading to serious limitations to the development of this new technique.

Recent improvements in microcarrier manufacture[4,5] have led to the availability of different types of beads that are able to support the growth of many cell types[6]. In order to investigate the potential of this technique using human diploid cell lines, a program was set up to study the scaling-up possibilities using the MRC-5 line. As many substrates may be used as microcarrier, the aim of this study was to identify the most suitable carriers of those currently available, which come closest to the established theoretical parameters[3], and compare their performances under the same culture conditions. On this basis, 3 different types of microcarriers were selected for testing:

a) Cytodex™1 (Pharmacia Fine Chemicals, Uppsala, Sweden);

b) Superbeads™ (Flow Laboratories Inc., McLean, Virginia, USA), both composed of Sephadex® with low DEAE substitution, and

c) Biocarriers™ (Bio-Rad Laboratories, Richmond, California, USA) composed of polyacrylamide/DMAP substituted.

All the microcarriers were prepared according to the manufacturers' recommendations and were then suspended in

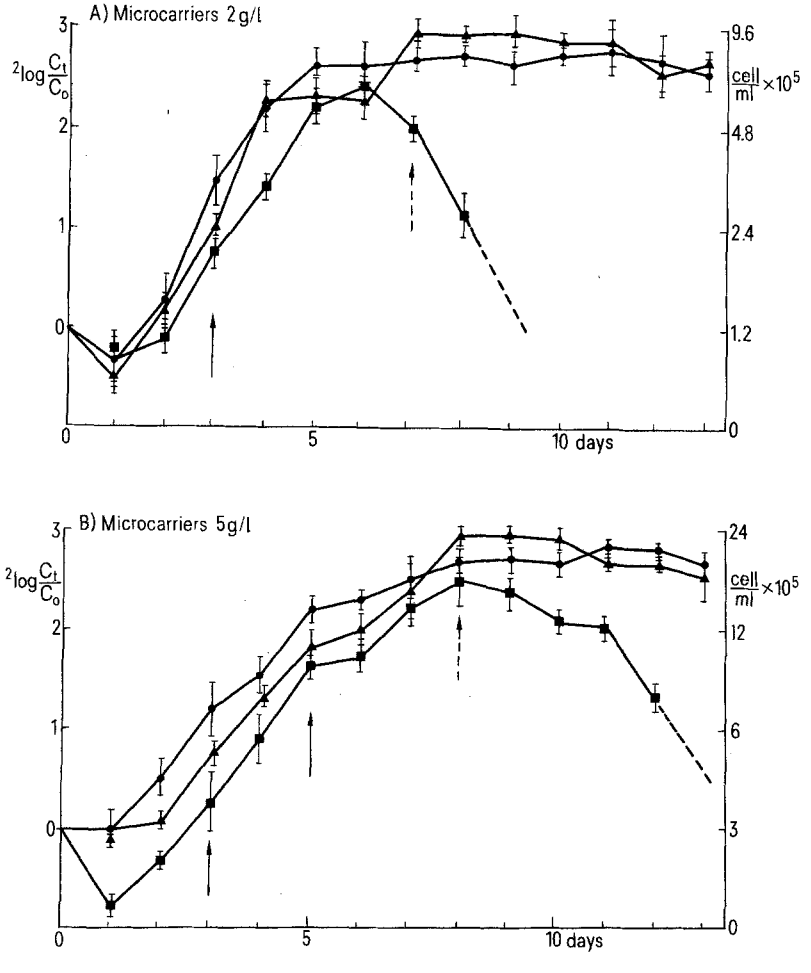

Figure 1. Growth curves of human MRC-5 diploid cells on 3 types of microcarriers: Cytodex™1 (●), Superbeads™ (▲), and Biocarriers™ (■). Part A: cell input of 1.2×10^5 cells/ml of medium. Part B: cell input of 3×10^5 cells/ml of medium. Dotted lines indicate irreversible cell detachment from the microcarriers; cell counting was stopped at the end of the solid line. Solid arrows indicate growth-medium replacements: 600 ml of spent medium were replaced with the same volume of fresh growth medium. Dotted arrows indicate complete replacement of the growth medium with maintenance medium. Average results of at least 3 experiments. SE for the time points of the growth curves are determined.

$\frac{C_t}{C_0}$ = cells per ml at time t/cells per ml at time 0.

growth medium at concentrations of 2 and 5 g/l (1 g of microcarrier can be considered equivalent to a growth surface of about 0.5–0.6 m^2). All cultures were grown in fermentors (working volumes of 1200 ml) equipped with automatic controls of pH, pO_2 and temperature (Biolafitte, Poissy, France); a growth medium composed of equal parts of Dulbecco Modified Eagle (DME) and HAM F12 supplemented with 10% fetal calf serum (FCS) was used. The fermentor cultures were initiated by adding the cell suspensions to the medium containing the microcarriers; the cells were obtained by trypsinization of static cultures and were counted using a Burker chamber, after trypan blue staining. As the cells attach themselves to the substrate in a dynamic system and therefore in critical conditions, in most experiments the level of inoculum (6×10^7 cells/g of beads) was selected in order to guarantee an excess of cells with respect to the number of beads present (1 g of microcarrier contains approximately $5–6\times 10^6$ beads).

These cultures were kept at 37 °C, pH 7.3, with 40% air saturation and stirring at 50–75 rpm. Cellular morphology was monitored by direct microscopic examination, with or without staining of the microcarrier-attached cells, and the cells counted at regular intervals by the nuclei count method as described by Van Wezel[7].

Results and discussion. Figure 1 shows growth curves of the MRC-5 diploid cell line at the 30th population doubling level (PDL) with microcarrier concentrations of 2 and 5 g. At the lower concentration (part A), all 3 microcarriers show basically the same behaviour and do not evidence any toxic effects during attachment of the cells, which reach saturation density levels (SDL) around 7.5×10^5 cells/ml after 5 days of culture, but while the 2 Sephadex substrates are able to maintain SDL of 1×10^6, the polyacrylamide substrate shows a premature cell detachment. At the concentration of 5 g/l (part B), because of the increased growth surface, a SDL of about 2×10^6 cells/ml can be reached with all microcarriers; however, at this concentration a toxic effect on the cells grown on the polyacrylamide substrate is manifest, evidenced by a less efficient attachment by the cells to the beads; 44% of the cells (41–47%) are not attached to the support after 24 h, and die in the culture medium. The remaining cells do grow normally but at confluence, when the growth medium is substituted with maintenance medium (DME with 2% FCS), a gradual detachment and death of the cells are again found. Under the same conditions, the other 2 microcarriers give a percentage of cell attachment in the range of 95–98% and were able to maintain the SDL until at least the 14th day of culture, when the cultures themselves were generally stopped. In figure 2, the growth level of the cells is analyzed in relation to the number of cells seeded, with a microcarrier concentration of 5 g/l. In all cases, the cells showed a good level of anchorage to the carriers on day 1 and, as found with static cultures using greater cell input, shorter times are required to reach the same SDL. For instance, the highest cell input used (6×10^5 cells/ml of medium) gives a final SDL 1–2 days earlier, requiring only a single change of medium. Moreover, the final SDL appears to be only slightly affected by decreasing the cell input; there is, however, a limit below which the cells present a notably prolonged lag phase (from the normal 1–2 days to 4 days) even though they show normal anchorage to the microcarriers, and they are unable to reach SDL comparable to those obtained using a larger inoculum. In the conditions adopted in this experiment, this limit is represented by seeding levels of about 7.5×10^4 cells/ml, and the microscopic examination effected after 9 days of culture showed prevalently empty microcarriers.

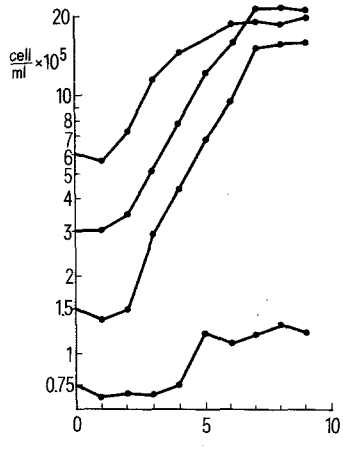

Figure 2. Growth of the human MRC-5 diploid cells on microcarriers at different levels of cell input. Medium replacement (600 ml of spent medium with 600 ml of fresh growth medium) were generally accomplished on day 3 and 5, except for the highest cell input with only 1 change on day 3. Cytodex™1 and Superbeads™ microcarriers were used separately in 2 different series of experiments with no significant differences in the performance during cell growth. For clarity, the results reported here only refer to the Cytodex™1 microcarrier.

Few data are available on the use of the MRC-5 cell line in microcarrier cultures, and deal with cultures under non-optimized conditions[8] or with substrates and techniques which are not easily expandable to a large scale[9]. As the substrates used in these studies were carefully chosen on the basis of precise theoretical parameters, our results indicate that the microcarriers chosen differ only in their secondary characteristics, since it is possible to grow MRC-5 cells on all 3 types; however, these observed differences were highly reproducible as documented by the very small values of the standard errors determined for the time points of the growth curves. In the case of the Biocarriers™, a degree of toxicity was found particularly at the highest concentration used, both during the phase of cell anchorage to the carriers and at confluence where a tendency was noted towards detachment of the cells from the substrate. This fact could cause some difficulties when the monolayers need to be maintained for some time or subjected to repeated washings. The Superbeads™ appeared to be the least homogeneous with regard to the bead size distribution, causing a slight lack of balance in the cultures since the cells on the smaller microcarriers reached confluence earlier.

Using suitable microcarriers and with the diploid cell line adopted, it proved to be of paramount importance to operate with cell inputs which guarantee at least 5–6 vital cells per bead, with an optimal level of about 10 cells/bead (i.e. 3×10^5 cells/ml with 5 g/l of microcarrier). It is thus possible to make use of all the available growth surface, reducing to a minimum the number of empty microcarriers and obtaining normal levels of cell growth with optimal final saturation density levels, generally corresponding to 60–80 cells/bead. On the contrary, it is not possible to attain a satisfactory final SDL with even slightly lower cell inputs (1–2 cells/bead), because most of the microcarriers remain unused and the cells are probably unable to create the microenvironment necessary to initiate and maintain their multiplication.

In conclusion, our results indicate that it is possible to obtain high concentrations of human MRC-5 diploid cells in submerse cultures using the microcarrier technique, especially using dextran beads with low DEAE substitution (charges of 1.5–2 meq/g); with currently available microcarriers and fermentor technology, this system seems suit-

able for a real industrial expansion. From a purely economic point of view (an important factor for scale-up), it is worth mentioning that on the basis of current distribution prices, microcarriers Nos 1 and 3 or our list, available as dry powder, are cheaper than No. 2 which is supplied, however, ready for use resuspended in the appropriate buffer.

1 J.P. Jacobs, Devs biol. Standard. *42*, 13 (1979).
2 A.L. Van Wezel, Nature, Lond. *216*, 64 (1967).
3 A.L. Van Wezel, Devs biol. Standard. *37*, 143 (1977).
4 D.W. Levine, J.S. Wong, D.I.C. Wang and W.G. Thilly, Somatic Cell Genet. *3*, 149 (1977).
5 D.W. Levine, D.I.C. Wang and W.G. Thilly, Biotechnol. Bioeng. *21*, 821 (1979).
6 M. Hirtenstein, J. Clark, G. Lindgren and P. Vretblod, Devs biol. Standard. *46*, 109 (1980).
7 A.L. Van Wezel, in: Tissue culture methods and application, p. 372. Eds P.F. Kruse and M.K. Patterson, Jr. Academic Press, New York and London 1973.
8 D.W. Levine, W.G. Thilly and D.I.C. Wang, Devs biol. Standard. *42*, 159 (1979).
9 V. Nielsen and A. Johansson, Devs biol. Standard. *46*, 131 (1980).

Ca^{2+}-dependence and metabolic status of an obligate thermophile, *Thermoactinomyces vulgaris*, under shake culture conditions

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Summary. Ca^{2+} stimulates germination of *T. vulgaris* spores. There is a higher mycelial yield as well as higher protein, DNA, RNA and free Pi content in cultures grown in the presence of Ca^{2+} as compared to those grown in the absence of this divalent cation.

The association of Ca^{2+} with thermophilic growth[3,4] as well as with the catalytic activity and stability of thermophilic enzymes[5–8] has been well documented in a variety of microbial systems. However, information concerning the status of germination, growth yield, soluble protein, DNA, RNA and free Pi in response to Ca^{2+} has not been obtained for thermophilic actinomycetes under liquid shake culture conditions. Therefore the present investigation was undertaken using the obligate thermophile *Thermoactinomyces vulgaris*.

A wild-type strain (stock No. 1227) of *T. vulgaris*, which was kindly supplied by Professor D.A. Hopwood, John Innes Institute, Norwich, U.K., was used in the present investigation. The media described by Hopwood & Wright[9] were used with certain modifications[8,10]. Liquid medium was prepared without adding agar and pH was adjusted to 6.8.

Approximately 10^9 spores/30 ml of the medium, in 250-ml conical flasks, were incubated for 6 h (as at this stage the logarithmic phase of growth continues) in a Gyrotory Shaker (New Brunswick, Model G 25) at 50–52 °C at 150 rpm. The mycelium was harvested on Whatman No. 1 filter paper. A homogenate was prepared in glass distilled water with the help of a mortar and pestle, using acid-washed sea sand as an abrasive. The slurry was centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was retained and stored at 4 °C until used. Germination of spores was examined under a compound microscope and its

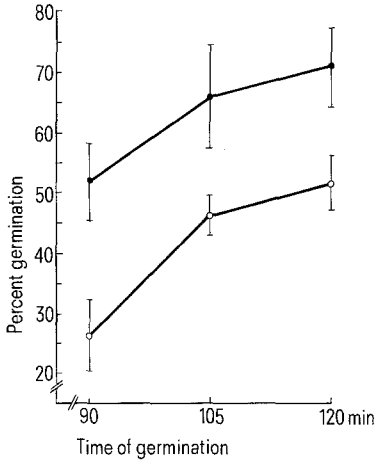

Figure 1. Percentage germination of *Thermoactinomyces vulgaris* spores at different time intervals in the absence (open circles) and presence (closed circles) of Ca^{2+} (0.6 mM) under shake culture conditions. Mean values are based on duplicate readings of 3 independent determinations.

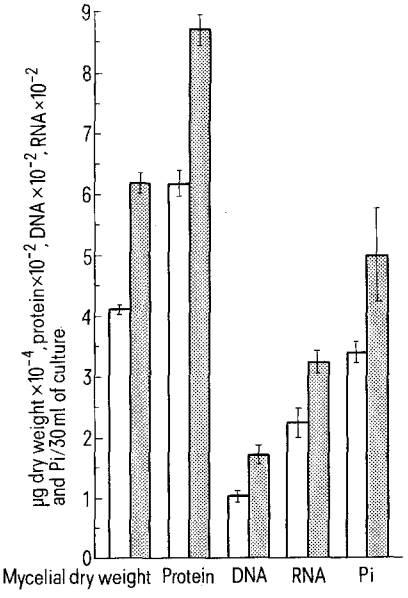

Figure 2. Mycelial dry weight, protein, DNA, RNA and Pi in the liquid shake cultures of *Thermoactinomyces vulgaris* grown in the absence (open bars) or presence (solid bars) of Ca^{2+} (0.6 mM). Mean values are based on duplicate readings of 3 independent determinations.