

Membrane based cell culture systems - an alternative to in vivo production of monoclonal antibodies.

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Abstract

A new generation of membrane based cell culture devices especially designed for small scale production of monoclonal antibodies (mab's) entered the market in last few years. In contrast to conventional perfusion hollow fibre bioreactors these devices contain two functionally different membranes - one ultrafiltration membrane for nutrient supply and one gas permeable membrane for direct oxygenation of cells. The latest systems of this generation are static culture systems are of moderate costs and either better than, or equal to, the ascites mice in terms of quality and quantity of produced monoclonal antibodies. We have investigated the advantages of the perfused Tecnomouse bioreactor and the static CELLline culture flasks in comparison to ascites production and conventional roller bottle cultures.

Introduction

Following the discovery of hybridoma technology by Köhler and Milstein in 1975 (1), monoclonal antibodies have had profound implications not only on medical research, diagnosis and therapy, but also on biology in general. In the early days of hybridoma technology, the hybridomas developed in vitro were injected into the peritoneal cavity of an animal, so that useful amounts of the desired monoclonal antibody could be harvested from the ascitic fluid. This procedure was considered necessary at the time, since no efficient and cost effective in vitro methods were available. The in vivo method is limited to the production of rat and mice, but no human monoclonal antibody can be produced by this system. Furthermore, the potential risk of product contamination with unspecific immunoglobulins, viruses pathogenic to humans, bioactive cytokines as well as the growing public pressure to replace animal experiments (2,3,4), led to a fast development of a variety of in vitro technologies. Knazek et al. (5) first cultivated cells at tissue-like densities in a hollow fibre bioreactor. Cultivation of cells in conventional hollow fibre cartridges is limited by irregular oxygenation of the growth chamber. Because of an optimal combination of nutrients supply via hollow fibres and supply of oxygen via silicon membranes directly to the cell growth chamber, the hybrid bioreactor system Tecnomouse, developed in the early nineties seems to be well adapted for antibody production. In addition, new membrane based cell culture devices were developed especially for small scale production and entered the market in the last few years. They meet not only the requirements to be scientifically satisfying in terms of antibody quality, concentration and yield but also to be economically in terms of labour, media and disposable costs.

Materials and Methods

Conventional membrane based culture systems, e.g. hollow fibre bioreactors consist of one type of polymeric membranes with a cut off of approximately 10 kD. This type of membrane allows a continuous supply of low molecular weight nutrients, e.g. vitamins, amino acids, salts etc. and a sufficient removal of toxic metabolites such as ammonia and lactate from the cells. Protein components of used additives e.g. fetal calf serum and secreted antibodies will not be diluted through the perfusion membrane. It guarantees a 20 to 50 fold reduction of fetal calf serum compared to suspension culture. Moreover, very high antibody concentrations can be reached. Nevertheless, often these systems are limited in terms of oxygen supply to the cells, which depends on the dissolved oxygen in the perfused culture medium. In the early nineties a second generation of membrane based culture systems entered the market. These systems consist of two functionally different membranes. In addition to the ultrafiltration membrane, a gas permeable membrane directly provides oxygen supply to the cells, as shown in figure 1. Members of this generation are the perfused Tecnomouse hollow fibre bioreactor (6), the rolled flat membrane miniPERM system (7) and the static flat membrane CELLine devices.

The Tecnomouse bioreactor (Integra Biosciences, Germany) allows to cultivate five culture cassettes which can be operated independently at any time. Every cassette consists of about 400 hollow fibres arranged in layers. Silicon membranes realise a direct and homogeneous supply of cells with oxygen and CO₂.

The CELLine culture flask is a single used device, which can be operated in a incubator in the same way as conventional culture flasks.

Media and supplements: A antibiotic-free mixture of Iscoves MEM and Ham's F12 (1:1) supplemented with 2mM glutamine, 1,25ml/l ethanolamine, 2,1g/l sodium bicarbonate, 331mg/l sodium pyruvate and 4,5 g/l glucose without HEPES (Gibco BRL, GB) was used for culture perfusion at the intracapillary space (IC) of the Tecnomouse or in the media reservoir of the CELLine culture flasks. 10% FCS or a special in house formulation of protein free medium were added into the cell growth chamber.

Cell lines: The murine hybridomas CB-41, CB-ap27 and MAX16H5 were developed in our labs. The murine hybridomas 9E10 was kindly provided by Prof. W. Höhne, Charité, Berlin.

Cell culture procedures: For antibody production 5x10⁶ hybridoma cells were inoculated into the EC of each culture cassette of the Tecnomouse in a total amount of 5-10 ml medium. A recirculation mode of perfusion with a speed of 50 ml/h was performed, using 5 l media reservoirs. The reservoirs were renewed when glucose levels dropped to 0,4 g/l. 5 - 10 ml harvests were taken weekly and analysed for cell viability and antibody content. After cultivation the harvests were pooled and the total amounts of produced antibodies detected by ELISA technique. The CELLine CL350 flasks were inoculated with 2x10⁶ cells per ml in a volume of 5ml per flask. The CL1000 flask was inoculated with a total volume of 15ml at a cell density of 2.4x10⁶ cells/ml. The CELLine 350 and 1000 devices were fed twice a week with 350 or 1000 ml respectively.

Roller bottles were inoculated with 10E5 cells per ml and a total volume of 500ml per bottle .

Ascitic fluid was produced according to a conventional protocol as described in Marx et al. (8). Briefly, 2x10⁷ cells per mouse and cell line were inoculated in primed balb/c mice. Ascitic fluid was collected until day 28.

Analytical methods: Cell count and viability were determined by trypan blue exclusion method and acridine orange / ethidium bromid fluorescence staining. Yields of monoclonal antibodies were detected using a sandwich ELISA. SDS-PAGE of the harvests were performed on a PhastSystem (Pharmacia, Sweden) according to the user instructions.

Results

Over a period of 28 days the three cell lines CB-41, CB-ap27 and 9E10 were cultured successfully in cassettes of the Tecnomouse bioreactor, in ascitic fluid and in roller bottles. Harvests were pooled and analysed. Table 1 shows the total amount and the concentration of mab's generated in the Tecnomouse bioreactor system. As expected each hybridoma cell line is characterised by its specific behaviour and productivity. That leads to a 3.4 fold higher yield of the 9E10 antibody (286 mg) in comparison to the CB-41 antibody (83 mg). Remarkably for all three production procedures an average antibody concentration higher than 1mg/ml could be obtained (2.4 mg/ml, 1.1 mg/ml and 4.1 mg/ml for CB-41, CB-ap27 and 9E10 respectively). This relates to a 133 fold, 44 fold and 82 fold increase of mab concentrations compared to the respective roller bottle suspension. In ascitic fluids product concentrations of all three antibodies were a bit higher than these of the compared Tecnomouse cultures. Stained SDS-Gels (Electrophoresis) of the harvests of all three production systems are shown in figure 2 a, b and c. It becomes obvious, that only molecules with a molecular weight of

approximately 145 kD corresponding to the monoclonal antibody and unspecific murine immunoglobulins could be found in the ascitic fluid (fig 2 a; lane 2). In contrast, the harvests of both the Tecnomouse culture (fig 2 b; lane 2) and the suspension roller bottle culture (fig 2 c; lane 2) contain significant contamination of fetal calf serum proteins, mainly serum albumin, in addition to the mab. In order to investigate the advantages of protein-free culture conditions, a murine hybridoma was adapted to a protein-free medium and cultured in the Tecnomouse bioreactor. As shown in figure 2 d, an electrophoretical pure mab could be harvested. We used the high producer cell line MAX16H5 to evaluate the characteristics of the novel static membrane based culture devices CELLine CL1000 and CELLine CL350 concerning antibody yields and quality. As shown in figure 3 131 mg of the particular antibody could be produced in the CL1000 culture device over 29 days. In this case 10% FCS were supplemented to the culture medium. The cell line was adapted to protein-free medium and the productivity was compared to the serum containing culture using CL350 devices. Figure 4 shows the results of this two cultures over a period of 15 days, leading to a total amount of 13 mg antibody in the serum containing culture and 10,4 mg in the protein-free culture.

Discussion

The following criteria were responsible for the extensive use of mice as "ideal" living production systems over the last 23 years:

- * optimal supply of nutrients to the cells,
- * regulated removal of metabolic products,
- * supply of essential composition eg. proteins and other additives,
- * removal of cell debris by macrophages,
- * guarantee of sterility according to microbial infections,
- * constant physical parameters, e.g. pH, pO₂, temperature etc,
- * tissue compatibility of the host animal to murine hybridomas.

Moreover the mouse is a cheap and easy to handle "culture system". Already in the early eighties it became clear, that there are also a number of disadvantages of the in vivo method, namely the contamination of the product with unspecific mouse Ig, the potential risk of human pathogenic virus infections of the production strains and the tissue incompatibility to human or heterohybridomas. Nevertheless, there were substantial economical advantages of this method in comparison to in vitro suspension culture in systems for small scale antibody production. Not even the introduction of conventional hollow fibre bioreactors in the eighties, which were scientifically satisfying in terms of product concentration and even better in terms of antibody quality (9,10), did solve the problem of extensive use of mice. High investments were necessary to purchase hollow fibre systems. Users were limited by propagating only one cell line per bioreactor run and system. A basically new approach was done in the early nineties with the development and marketing of the Tecnomouse bioreactor. In addition to the continuous perfusion of the cell culture with nutrients the direct oxygen supply to the cells via a silicon membrane was realised in this bioreactor (11). Moreover, five different cell lines could be propagated simultaneously within one bioreactor system. Investment costs were reduced in comparison to other hollow fibre systems of this scale.

Corresponding with the results of other groups we could show that this system fulfil all above mentioned criteria except the ability of active cell debris removal from the culture. Since 1993 we have been using the Tecnomouse routinely for mab production replacing approximately 10 mice per run. Nevertheless, special culture know-how, initial investments and substantial running costs related to the use of the Tecnomouse system, making the system mainly attractive for laboratories, which require 200 to 1000 mg of monoclonal antibodies from a large number of different cell lines simultaneously. With the entry of the two disposable culture devices miniPERM and CELLine into the market, investment costs for cell culture laboratories could be cut dramatically to the roller apparatus in case of the miniPERM system and can totally avoided in case of CELLine.

Conclusion

The results presented here demonstrate that membrane based cell culture technologies are scientifically and economically convincing alternatives to malignant ascites production for monoclonal antibodies.

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