# Characterization of a New Miniaturized Hollow-Fiber Bioreactor for Cultivation of Cell Lines and Primary Cells to Improve Cytostatic Drug Testing *in vitro.*

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Hiermit erkläre ich, daß die vorliegende Arbeit von mir selbständig, und nur unter Verwendung der angegebenen Quellen und Hilfsmittel, angefertigt wurde.
Weiterhin habe ich noch keinen Promotionsversuch unternommen oder diese Dissertation in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.

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# Chapter 1 Introduction

## *1.1 Limitations of conventional cell culture systems as model systems for the in vivo situation*

Isolation of mammalian cells and their culture in monolayer systems is a basic but valuable procedure for investigations in cell biological research and on the pathogenesis of many diseases. Nevertheless, there is a need for more sophisticated model systems, as isolated cells can only simulate a small part of the body´s tremendous complexicity. Insufficient nutrient supply at increasing cell mass, e.g. observed during the cultivation of clinically useful cartilage (Freed et al., 1993) and in cultivation of hematopoietic stem cells (Koller et al., 1993b) or the lack of an appropriate extracellular matrix also contribute to model failure (Naughton et al., 1990; Makrynikola and Bradstock, 1993).

### 1.1.1 *In vitro* tumor models

Experimental systems used to study carcinogenesis, for example, often fail as models for human cancers, because conventional cell cultures show several limitations, e.g. cell growth in two-dimensions only or cell adhesion to unsuitable or incompatible substrates. Also, *in vitro* models often do not reflect the marked differences in cell morphology, vascularity, and stromal involvement of tumor cells *in vivo* (Jain, 1994; Melzig et al., 1995). Thus, the behaviour of monolayer cultures often differs markedly from the behaviour of tumors in patients (Vendrik et al., 1992). For solid tumors, three-dimensional culture systems like spheroid culture or histoculture are of improved prognostic value since they more closely mimic the tumor in individual patients compared to monolayer cultures (Sutherland, 1988; Hoffman, 1991; Melzig et al., 1995; Mueller-Klieser, 1997). High density culture of solid tumor cells resemble more the original tumor, e.g. in terms of tumor antigen expression (Rutzky et al., 1979) and tumor spheroids represent better the heterogeneity found in tumors *in vivo* not only in terms of morphology (Desoize et al., 1998) and tumor micromilieu (Kunz-Schughart et al., 1998), but also in terms of drug resistance (Wartenberg et al., 1998; O´Connor, 1999).

### 1.1.2 Alternatives to conventional cell culture systems

To overcome the drawbacks mentioned above, animal models are used with the hope to more closely mimic the *in vivo* situation (Mattern et al., 1988; Mulvin et al., 1992; Hollingshead et al., 1995). However, animal models do not take into account some important interspecies differences, e.g. regarding involvement of stroma cells, tumor vascularisation and pharmacokinetic parameters (Mattern et al., 1988). This difficulty shows that alternative *in vitro* culture methods and systems are clearly needed (Freeman and Hoffman, 1986).

While tissue engineering has grown into a field of intense research in recent years, it has the potential to bridge this gap. Focusing on, first, the *in vitro* construction of transplantable vital tissues (Brown et al., 2000; Heath, 2000; Sodian et al., 2000) and, second, on the development of *in vitro* models that are superior to conventional monolayer cell cultures, tissue engineering may ultimately lead to true three-dimensional cultures which more closely resemble the human *in vivo* situation (O´Connor, 1999; Vescio et al., 1991). As tissue culture needs more sophisticated culture methods than conventional culture in monolayer or suspension culture, specialized culture techniques may contribute to realize cultivation of tissue *in vitro* (Minuth et al., 1992; Sittinger et al., 1996).

## *1.2 Hollow-fiber bioreactor technology*

### 1.2.1 The hollow-fiber bioreactor concept makes use of semi-permeable membranes

*Principle of semi-permeable membranes*

Semi-permeable membranes are selectively permeable for molecules of different size, permeability being dependent on pore-size. Membranes are generally used to separate two independent compartments. Thus, delivery to, removal from and retention of substances within a compartment can be realized. Fig. 1 illustrates the use of a semi-permeable membrane in a hollow-fiber bioreactor.

*Use of semi-permeable membranes in medical applications*

Semi-permeable membranes find broad application in medical applications, e.g. in artificial kidneys to treat acute and chronic renal failure (Klinkmann and Vienken, 1995; Clark, 2000) or in artificial livers, to gap the time after organ failure until transplantation can be performed (Gerlach et al., 1994; Flendrig et al., 1997b).

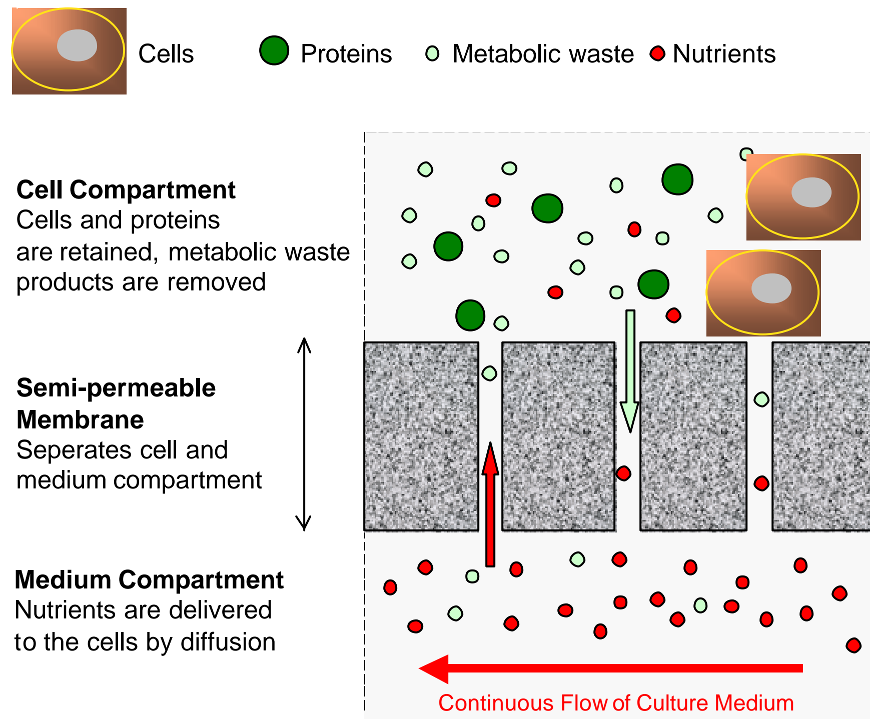


**Fig. 1:** Principle of the use of semi-permeable membranes in hollow-fiber bioreactors. The membranes allow passage or retention of molecules (e.g. proteins) depending on their pore-size.

During open heart surgery, oxygenators containing oxygenation membranes ensure optimal supply with oxygen of the patient (Wegner, 1997; Mueller et al., 2000). Cells can be encapsulated in membrane tubes and are thus isolated from the immune system of the host after transplantation. Immunoisolated cells allow controlled release of therapeutics, e.g. aiming at new therapeutic options for chronic pain patients, patients with endocrine disorders, diseases of the central nervous system and other diseases (Aebischer et al., 1991; Lysaght et al., 1994; Lee and Bae, 2000; Uludag et al., 2000).

*Use of semi-permeable membranes in cell culture*

For *in vitro* studies with cell cultures, semi-permeable membranes are used for various purposes and are commercially available in different designs (Tab. 1). For example, in Transwells$^{TM}$, different cell populations can be separated to perform migration studies (Bradstock et al., 2000; Heissig et al., 2000). Additionally, membrane-supported culture systems can contribute to increase product yields in production processes, as shown for the miniPERM system (Falkenberg et al., 1995; Schutt et al., 1997) or the CELLine products (Trebak et al., 1999). Moreover other dialysis-membrane based systems (Mathiot et al., 1993) or hollow-fiber bioreactors,

like the Tecnomouse system (Marx et al., 1993) can be used for cell cultures (see 1.2.2).

**Tab. 1** Commercially available membrane based cell culture devices (selection).

| | Transwells™ | miniPERM | CELLine | Tecnomouse |
|---|---|---|---|---|
| Oxygenation | No oxygenation | Silicone flat membranes | Oxygenation flat membranes | Silicone flat membranes |
| Perfusion | No perfusion possible | No perfusion possible | No perfusion possible | Necessary |
| Culture Volumes | Inserts for conventional culture well plates | 40 ml | 0.75, 5 and 15 ml | 8 ml |
| Microscopy | Depends on membrane used | Not possible | Not possible | Visual control but no microscopy |
| Taking samples | Sampling by a pipet | Sampling port for a syringe | Sampling port for a syringe | Sampling port for a syringe |

### 1.2.2 Principle and advantages of hollow-fiber bioreactor technology

In batch culture, depletion of key nutrients, accumulation of toxic by-products such as ammonia and lactate or changes of pH and osmolarity can lead to cell death, thus limiting the theoretical maximum cell density (Hassel et al., 1991). This limitation can be overcome by maintaining a steady-state level of nutrients and metabolites by the use of a hollow-fiber bioreactor system, that can, in principle, support the cultivation of large numbers of mammalian cells.

The principle of a hollow-fiber bioreactor based on hemodialyzer modules was first described by Knazek (Knazek et al., 1972). Today, also flat-bed hollow-fiber cell culture systems are described (Nagel et al., 1999). Hollow-fiber membranes are potted in a shell, thus creating a medium and a cell compartment which are separated by the membranes [Fig. 2]. Cells are typically inoculated outside the hollow-fibers in the extracapillary space. Culture medium is recirculated through the capillaries resulting in a continuous delivery of nutrients to the cells and removal of metabolic by-products.

In addition, sufficient oxygenation of the cell culture has to be ensured. Hollow-fiber bioreactor technology enables increased cell densities compared to conventional cell culture procedures, leading to three-dimensional structures (Callies et al., 1994), increased concentrations of secreted effector molecules, thus offering a unique cell culture environment not available in traditional low density or monolayer culture.

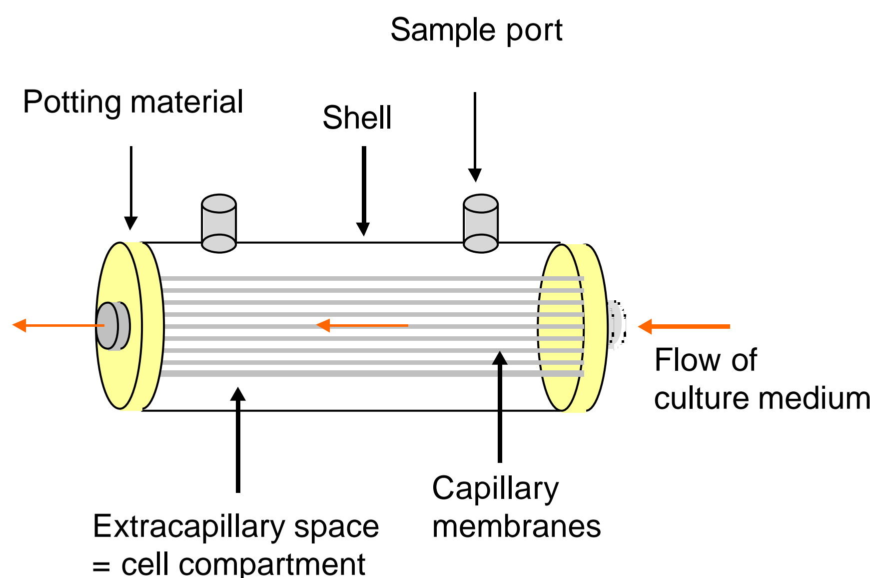


**Fig. 2:** Principle of a hollow-fiber bioreactor.

### 1.2.3 Need for monitoring of cells cultivated in hollow-fiber bioreactors

Hollow-fiber bioreactor technology has evolved as an alternative to traditional cell culture methods for the production of cells (Knazek et al., 1990; Lewko et al., 1994) and cell-derived products (Kurkela et al., 1993; Lamers et al., 1999). Furthermore, it is used more frequently in pharmacology and toxicology (Jauregui et al., 1994), as part of artificial organs and in tissue engineering (Gerlach et al., 1995; Flendrig et al., 1997a; Humes et al., 1999). As cell viability and proliferation are crucial factors indicating the success of the cultivation procedure, these parameters should be monitored during cultivation. Morphological changes are often used to check viability of the cell culture. Therefore, microscopy of the cell culture is highly desirable, which is currently not possible in commercially available hollow-fiber bioreactors. As cell harvest in hollow-fiber bioreactors is often inconvenient (Sardonini and Wu, 1993) and cell harvest is often not representative for the whole cell culture, alternatives to off-line procedures are in demand.

### 1.2.4 Advantages of hollow-fiber bioreactors for testing drug candidates *in vitro*

*Need for improved test systems*

Current *in vitro* test systems used for testing of compounds with potential in chemotherapy, suffer from the same limitations of cell cultures mentioned above (see chapter 1.1). Solid tumors *in vivo* are complex systems, consisting of many heterogenous cell populations (Konemann et al., 2000). Therefore, tumor cells

cultivated in two-dimensional cultures are at best only a relatively simple model of the *in vivo* situation. It has been shown, for example, that multilayer cultures (Padron et al., 2000) and also cells in spheroids (Sutherland, 1988; Kunz-Schughart et al., 1998) are more resistant to cytostatic drugs compared to cells in monolayers (Vescio et al., 1991), which can be due to altered penetration of cytostatic drugs in spheroid culture (Erlanson et al., 1992). On the other hand there is a tremendous need for simple, but realistic *in vitro* test systems for the development of new anticancer drugs (Weisenthal, 1981; O´Connor, 1999) as well as for rational, non-empiric approaches in selecting the most appropriate chemotherapeutic regimen for an individual patient (Von Hoff et al., 1990; Kern and Weisenthal, 1990; Pieters et al., 1994; Bosanquet and Bell, 1996).

*Need for co-culture in drug testing*

Co-culture systems, e.g. in collagen gels (Nakashiro et al., 2000), are often used to investigate the interaction of different cell types *in vitro*. For primary leukemic cells it has been reported that co-culture with stromal cells prevents spontaneous apoptosis of B-CLL (Lagneaux et al., 1998) as well as of ALL cells (Campana et al., 1993) *in vitro*. Stroma co-culture also contributes to a better prediction of treatment outcome for childhood B-ALL (Kumagai et al., 1996). Also, co-culture of cells with hepatocytes adhering to membranes (Gerlach et al., 1990b; Qiang et al., 1997) would be interesting to enable testing of drugs that need to be converted into an active form *in vivo* (Donato et al., 1990).

*Autocrine factors and cell density*

As autocrine growth factors play an important role in proliferation of e.g. AML blasts *in vitro* (Murohashi et al., 1989), retention or even concentration of these factors in the culture medium might stimulate the growth of the cells depending on the cell density, in particular, as sensitivity of leukemic cells has been reported to be cell density dependent (Takemura et al., 1991; Kobayashi et al., 1992). Culture systems that support culture of cells in high cell densities or co-cultures thus offer advantages not only for drug testing on solid tumors but also on leukemic cells.

*Pharmacokinetic considerations*

Due to up-take, distribution within body compartments, metabolic processes and clearance mechanisms, drugs display characteristic pharmacokinetics *in vivo*. Pharmacokinetics are determined by measuring (plasma) concentration of the drug and its metabolites over time. Conventional test systems in monolayers cannot reflect

the pharmacokinetic process. First attempts to model pharmacokinetic profiles *in vitro* have been made by Gimmel et al. using a microperfusion system (Gimmel et al., 1993; Gimmel and Maurer, 1994) and also a hollow-fiber bioreactor was used to simulate pharmacokinetic profiles of anti-retroviral drugs (Bilello et al., 1994).

### *1.3 Objectives*

Task of the present work was to overcome the limitations of conventional culture systems with regard to

(1) continuous supply of the cells with nutrients and oxygen,
(2) continuous detoxification of the culture,
(3) cultivation of cells in cell densities $>10^7$ cells/ml,
(4) support of three dimensional growth of cells,
(5) creation of an *in vivo* like microenvironment,
(6) controlled delivery of substances to the cells realizing time dependent profiles
(7) microscopy,
(8) monitoring of the viability of the cultured cells without disturbance of the culture at any time point,
(9) concentration of soluble factors, pH, oxygen content and gas composition, and
(10) a small culture volume to improve toxicity testing *in vitro*.

Although hollow-fiber bioreactor systems are known since 1972, these systems are characterized by large culture volumes, do not support microscopy of the cell culture due to their geometry and taking samples of the culture is often inconvenient. In chapter 2 a new miniaturized hollow-fiber bioreactor is described in which a small culture volume, microscopic observation of the cells and easy sampling is realized. This new bioreactor has been characterized with regard to transport characteristics and growth of leukemic cell lines. The system offers the possibility of culturing higher cell densities compared to standard culture, thus creating a more *in vivo* like environment for the cultured cells.

In chapter 3 a newly developed method for monitoring of cell viability and cell growth in a hollow-fiber bioreactor system is described. Metabolic activity of the cultured cells can thus be monitored without disturbance of the cell culture by use of the fluorescent dye Alamar Blue™.

In chapter 4 the suitability of the new *in vitro* culture system described in chapter 1 is evaluated as a tool for toxicity testing. In particular, the use of hollow-fiber membranes supports the generation of phamacokinetic profiles.

# Chapter 2

# New miniaturized hollow-fiber bioreactor for in vivo like cell culture and production of cell derived products

## *2.1 Abstract*

A new miniaturized hollow-fiber bioreactor system for mammalian cell culture in volumes between 0.5 and 3 ml was developed. The cell and medium compartments are separated by semi-permeable membranes. Oxygenation of the cell compartment is accomplished using oxygenation membranes. Due to a transparent housing, cells can be observed by microscopy during culture. The leukemic cell lines CCRF-CEM, HL-60 and REH were cultivated up to densities of 3.5 x $10^7$/ml without medium change or manipulation of the cells. The growth and viability of the cells in the bioreactor were the same or better, and the viable cell count was always higher compared to culture in Transwell® plates. As shown using CCRF-CEM cells, growth in the bioreactor was strongly influenced and could be controlled by the medium flow rate. As a consequence, consumption of glucose and generation of lactate varied with flow rate. Influx of low molecular weight substances in the cell compartment could be regulated by variation of the concentration in the medium compartment. Thus, time dependent concentration profiles (e.g. pharmacokinetic profiles of drugs) can be realized as illustrated using glucose as a model compound. Depending on the molecular size cut-off of the membranes used, added growth factors like GM-CSF and IL-3 as well as factors secreted from the cells are retained in the cell compartment for up to one week. This new miniaturized hollow-fiber bioreactor offers advantages in tissue engineering by continuous nutrient supply of high density cell culture, retention of added or autocrine produced factors and undisturbed long-term culture in a closed system.

## *2.2 Introduction*

Culture of mammalian cells in monolayers on tissue culture plates or in suspension culture is fundamental in cell biology and for the investigation of pathogenesis of human disease. An alternative cell culture technology is the hollow-fiber bioreactor, first described by Knazek et al. (Knazek et al., 1972). With this technology, in principle, it is possible to overcome some limitations of monolayer cultures and to realize increased cell densities, increased concentrations of secreted effector molecules (Kyung et al., 1994; Lewko et al., 1994; Nagel et al., 1999) and to permit a continuous cell cultivation process approaching tissue-like cell densities (Gerlach et al., 1995). Most hollow-fiber bioreactors are similar in design and size to modules used in hemodialysis of uremic patients (Lowrey et al., 1994). For culture, cells are placed in the extraluminal compartment of the module and continuously fed by an intraluminal stream of nutrients which passes through the hollow-fiber membrane by diffusion. Hollow-fiber bioreactors are used for production of cell-derived products such as antibodies (Gorter et al., 1993), cytokines (Lamers et al., 1999), specific tumor-associated antigens (Quarles et al., 1980) and for the expansion of activated lymphocytes (Knazek et al., 1990; Hillman et al., 1994). More sophisticated applications of the hollow-fiber bioreactor technology are in tissue engineering to realize a biohybrid kidney (Humes et al., 1999) or hybrid liver support systems (Busse and Gerlach, 1999). However, most commercially available hollow-fiber bioreactors represent a large scale approach. This report describes a newly developed, miniaturized hollow-fiber bioreactor system combining the advantages and flexibility of a hollow-fiber bioreactor in a small scale system. Moreover, in contrast to a conventional hollow-fiber bioreactor (Kaesehagen et al., 1991), visual examination of the cells during the cultivation process is possible.

## *2.3 Materials and Methods*

### 2.3.1 Culture medium, cell lines and analytical methods.

RPMI1640 supplemented with 25 mM HEPES, 2 mM Glutamine and 10 % FCS (all from Sigma, Deisenhofen, Germany) was used as culture medium throughout the experiments. The leukemic cell lines CCRF-CEM, HL-60 and REH were routinely cultivated in culture flasks (Falcon®, Becton Dickenson, Heidelberg, Germany) at 37°C, 5% $CO_2$, in an humidified incubator (Heraeus, Hanau, Germany) and subcultivated after 3 or 4 days. For the experiments, cells were harvested during the

exponential growth phase. Viable and total cell counts were determined using an automatic cell counter (CASY®1 TTC, Schärfe System GmbH, Reutlingen, Germany) (Glauner, 1991; Winkelmeier et al., 1993) after validation against trypan blue exclusion (data not shown). Glucose and lactate concentrations in the culture medium were measured using a blood gas analyser (Omni, AVL, Bad Homburg, Germany).

### 2.3.2 Miniaturized hollow-fiber bioreactor system for cell culture

The bioreactor [Fig. 3] (built in the workshops at Akzo Nobel Central Research, Obernburg, Germany) contains two independent membrane bundles embedded into a transparent polycarbonate housing [1] enabling microscopy of the cell culture in the cell compartment. The dialysis membranes (25 fbers Cuprophan® D2, Membrana GmbH, Wuppertal, Germany, ultrafiltration rate 3 ml / h x m² x mmHg, wall thickness 16 µm, inner diameter 215 µm, 3.6 cm²) [2] separate the medium [3] from the cell compartment [4] with a volume of 1 ml. Oxygenation membranes (17 fibers Oxyphan®, Membrana GmbH, Wuppertal, Germany, maximum pore size < 0.2 µm, wall thickness 50 µm, inner diameter 280 µm, 3.9 cm²) [5] are used for passive gas exchange e.g. in an incubator (37 °C, 5% $CO_2$). Diffusive nutrient supply and removal of metabolic waste products across the membrane is realized by a peristaltic pump (IPC, Ismatec Laboriumstechnik GmbH, Wertheim-Mondfeld, Germany) operated in a recirculation mode [Fig. 4].

### 2.3.3 Transport kinetics of the hollow-fiber bioreactor

One ml of PBS (Sigma, Deisenhofen, Germany) was placed in the cell compartment of the bioreactor. One hundred ml of glucose (concentrations: 10 mM, 20 mM to 40 mM) in RPMI 1640 without FCS was recirculated with 50 ml/h at room temperature. Samples were taken after 1, 2, 6, 16 and 24 hours. To realize the pharmacokinetic profile a 10:1 model of the bioreactor was used. 10 ml of PBS were placed on the extraluminar side of the membranes and 100 ml of 1 M glucose in PBS was recirculated for 30 min in the lumen of the membranes. Then the glucose containing PBS was replaced by PBS without glucose. Samples were taken at the times given in Fig. 7 and glucose concentration was measured. To investigate retention of cytokines, RPMI 1640 containing 10 ng/ml Interleukin-3 (IL-3, Stratmann Biotech GmbH, Hannover, Germany) and 100 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Leucomax, Essex Pharma, München, Germany,

measured as 12.6 ng/ml by ELISA, see below) was placed in the cell compartment of the bioreactor at the start of the experiment. Ten ml of cell culture medium without cytokines was recirculated with a flow rate of 14 ml/h in the medium compartment. After 1, 6, 8, 24, 48 and 168 hours samples were taken from medium and cell compartment and concentration of the cytokines was measured by ELISA (Cytoscreen®, BioSource, Ratingen, Germany). Controls were run in a polystyrene tube (Sarstedt, Nürnbrecht, Germany) and another bioreactor control was run with cytokines in the medium compartment to study adsorption of the cytokines to medium reservoir, tubing system and hollow-fiber lumen surface.

### 2.3.4 Growth curves for cell lines in the hollow-fiber bioreactor

For experiments in 24-well-plates (Falcon, Becton Dickinson, Heidelberg, Germany) CCRF-CEM cells were seeded in each well (1 ml, 2 x $10^5$/ml). Up to day 7, a 1 ml sample was taken daily (one well per timepoint), the cells were counted and the supernatant was removed by centrifugation (400 x g, 5 min) to allow determination of glucose and lactate concentrations. Experiments with Transwell® inserts (containing a polycarbonate membrane, pore size: 0.4 µm, Costar, Bodenheim, Germany) were performed by using 33 ml RPMI 1640 in polypropylene vessels (Merck Eurolab, 216G3180, Darmstadt, Germany) after steam sterilization at 121 °C for 20 minutes. One ml of CCRF-CEM, HL-60 and REH cell suspension (2 x $10^6$/ml) was placed into the Transwell® inserts, the vessels were covered allowing gas exchange, and placed into an incubator. After 24, 48 and 72 hours cells were counted and viability calculated, employing one Transwell® culture per day.

For hollow-fiber bioreactor experiments, the complete system was rinsed with pyrogen-free water (Braun, Melsungen, Germany) and sterilized by autoclaving (121°C, 20 min). In the humified incubator (37°C, 5% $CO_2$) equilibration of the system was achieved by overnight recirculation of 10 ml RPMI 1640 in the medium compartment and incubation with 1 ml in the cell compartment. Culture medium in the medium compartment was exchanged at the start of the experiment and 2 x $10^6$ cells/ml were inoculated. Routinely a medium flow rate of 14 ml/h was used. To study the influence of different flow rates on cell growth in the bioreactor system, CCRF-CEM cells were inoculated into the bioreactor at a density of 2 x $10^6$/ml and flow rates of 0.7, 14 and 50 ml/h were used. A bioreactor system with no medium flow (0 ml/h) served as control. Harvesting of cells was performed subsequently by rinsing the cell compartment three times with 1 ml of RPMI 1640. Cells were counted and viability

determined. The total medium volume employed was 20, 50 and 100 ml for 3, 5 and 7 days of culture, respectively.

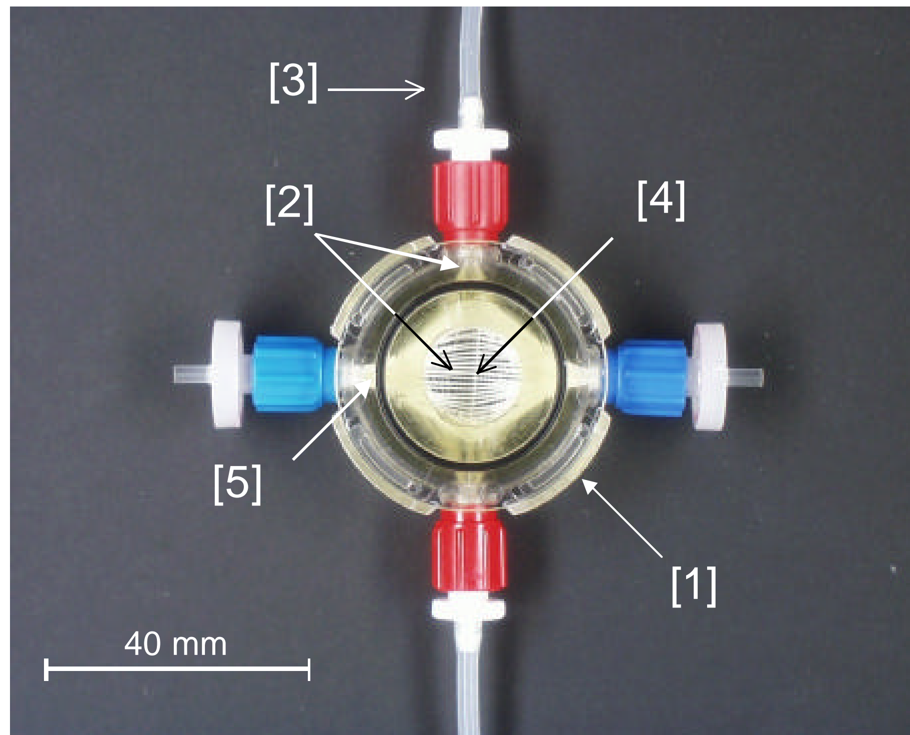


**Fig. 3** New miniaturized hollow-fiber bioreactor. The bioreactor contains two independent membrane systems embedded into a transparent polycarbonate housing [1] enabling microscopy of the cell culture in the cell compartment. Hollow-fiber dialysis membranes [2] separate medium [3] from cell compartment [4] (red line). Passive gas exchange is realized by hollow-fiber oxygenation membranes [5] (blue line).

## *2.4 Results*

### 2.4.1 Growth of cell lines in the bioreactor compared to conventional cultures

CCRF-CEM cells grown in conventional 24-well-plates reached a plateau after 4 days at about 2.5 x $10^6$ cells/ml. Decreasing glucose and increasing lactate concentration in the supernatant correlated with cell growth which demonstrated growth inhibition due to exhaustion of nutrients and accumulation of metabolic waste [Fig. 5a]. Starting with a 10-fold higher cell density of 2 x $10^6$/ml in conventional cultures, glucose was exhausted and maximum lactate generation was reached already after 1 day (data not shown). In contrast, in the bioreactor starting with 2 x $10^6$ cells/ml cell densities of 1.16 ± 0.39 x $10^7$/ml for CCRF-CEM, 8.4 ± 0.5 x $10^6$/ml for HL-60 and 1.01 ± 0.77 x $10^7$/ml for REH cells were reached after 3 days, respectively [Fig. 5b]. After one week of continuous, undisturbed culture CCRF-CEM cells reached a density of 1.58 ± 0.39 x $10^7$/ml and 3.74 ± 0.18 x $10^7$/ml when

inoculated with 2 x $10^5$/ml and 2 x $10^6$/ml, respectively. Growth and viability of the cell lines in the bioreactor were the same or better and the viable cell count was always higher compared to Transwell® cultures (see methods) [Tab. 2].

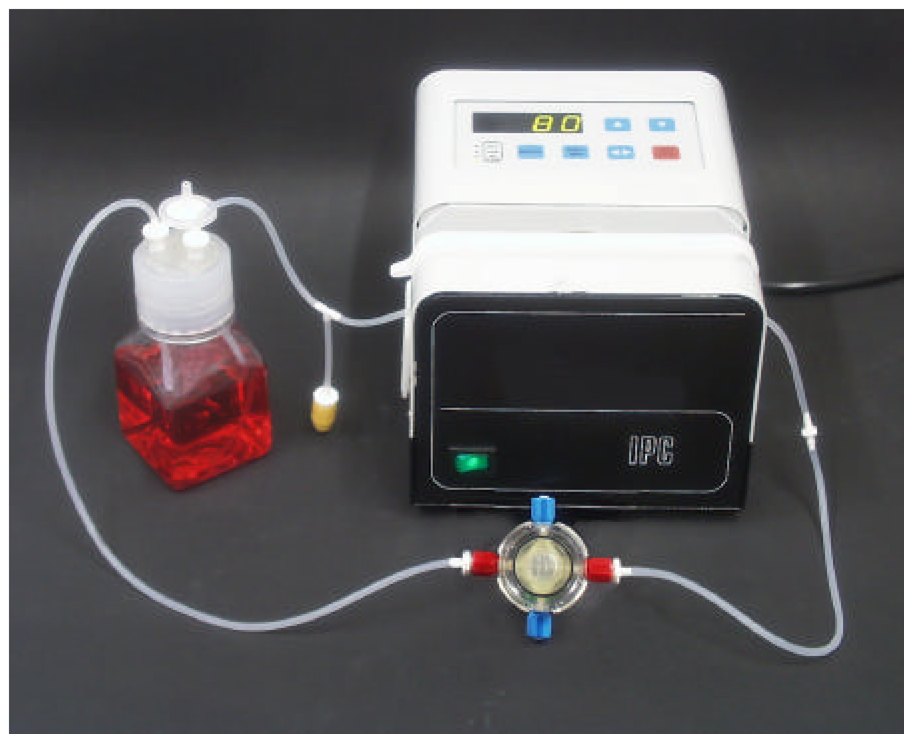


**Fig. 4** New miniaturized hollow-fiber bioreactor system in recirculation mode. Diffusive nutrient supply and removal of metabolic waste products across the membrane is realized by constantly recirculating culture medium through the system using a peristaltic pump.

**Tab. 2** Growth and viability of cells cultivated in bioreactors compared to Transwell® after 3 days of culture (mean of n = 3 experiments ± SD).

| | Bioreactor Culture 20 ml Medium Volume | | Transwell® Culture 34 ml Medium Volume | |
|---|---|---|---|---|
| | Viable Cells [x$10^6$/ml] | Viability [%] | Viable Cells [x$10^6$/ml] | Viability [%] |
| CCRF-CEM | 11.6 ± 0.4 | 94 ± 0.6 | 10.1 ± 0.2 | 92 ± 2.9 |
| HL-60 | 8.4 ± 0.5 | 89 ± 1.0 | 6.9 ± 0.8 | 86 ± 2.6 |
| REH | 10.1 ± 0.8 | 95 ± 0.6 | 9.9 ± 0.4 | 95 ± 1.0 |

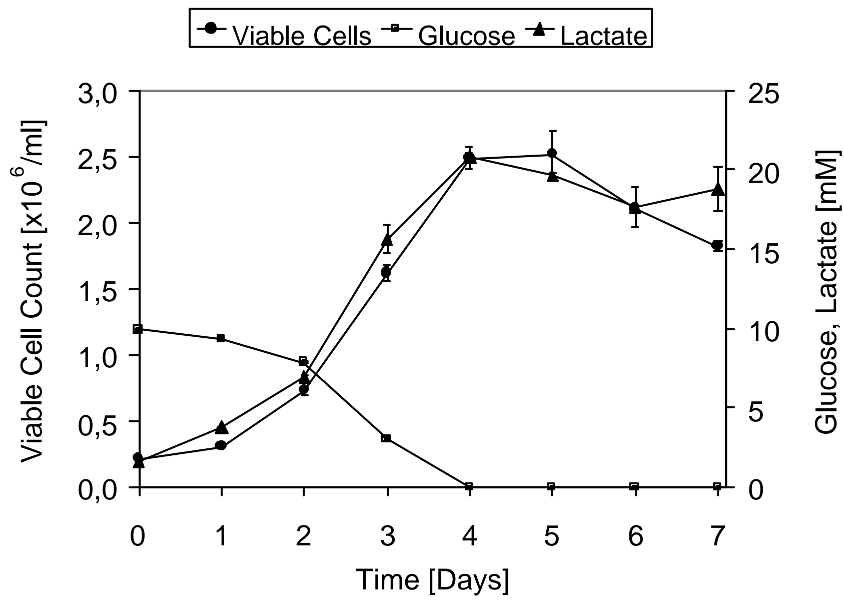


**Fig. 5a** Growth curve of the leukemic cell line CCRF-CEM in 24-well-plates, starting with 2 x $10^5$/ml cells, correlates with glucose and lactate concentrations in the supernatant (mean of n = 3 experiments ± SD).

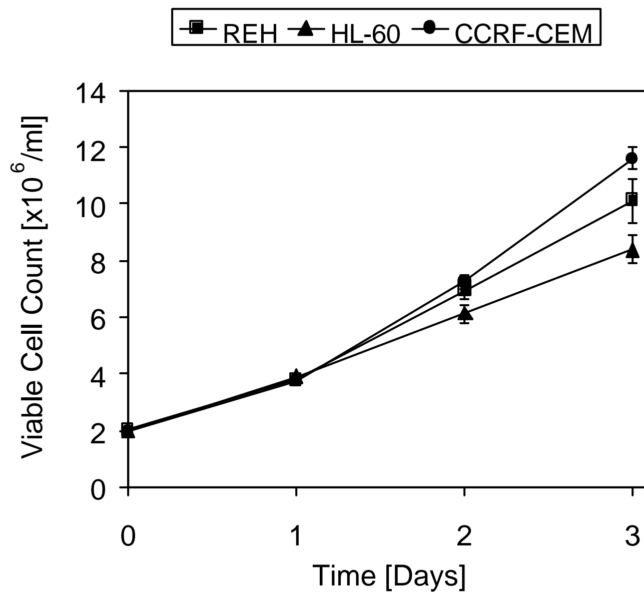


**Fig. 5b** Growth of the leukemic cell lines CCRF-CEM, HL-60 and REH in hollow fiber bioreactors during 3 days of culture, starting with 2 x $10^6$/ml (mean of n = 3 experiments ± SD).

2.4.2 Evaluation of membrane transport characteristics

One striking difference between conventional and hollow-fiber bioreactor cultures is the nutrient supply to the cells. By variation of the glucose concentration in the medium compartment, influx into and thus concentration of glucose within the cell compartment of the bioreactor could be controlled as shown in a cell free experiment [Fig. 6]. Within 24 hours equilibrium was almost reached between the two compartments.

Time dependent concentration profiles in the cell compartment could be achieved by variation of the glucose concentration in the medium compartment using a 10:1 bioreactor model system [Fig. 7].

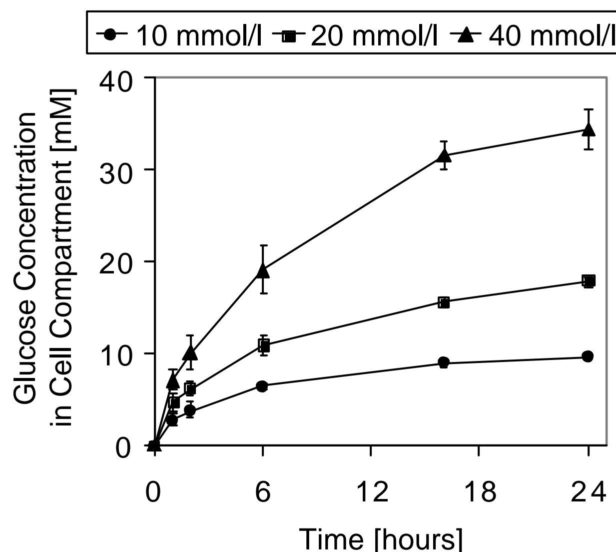


**Fig. 6** Influx of low-molecular weight substances into the cell compartment can be regulated by variation of the concentration in the medium compartment as shown for glucose (mean of n = 3 experiments ± SD).

In contrast to the efficient transport of small molecules such as glucose, the cytokines GM-CSF (Mr 22 000) and IL-3 (Mr 15 000) were selectively retained in the cell compartment [Fig. 8a + 8b]. About 50% of cytokine antigen could be detected within the cell compartment after 7d and no loss into the medium compartment was observed.

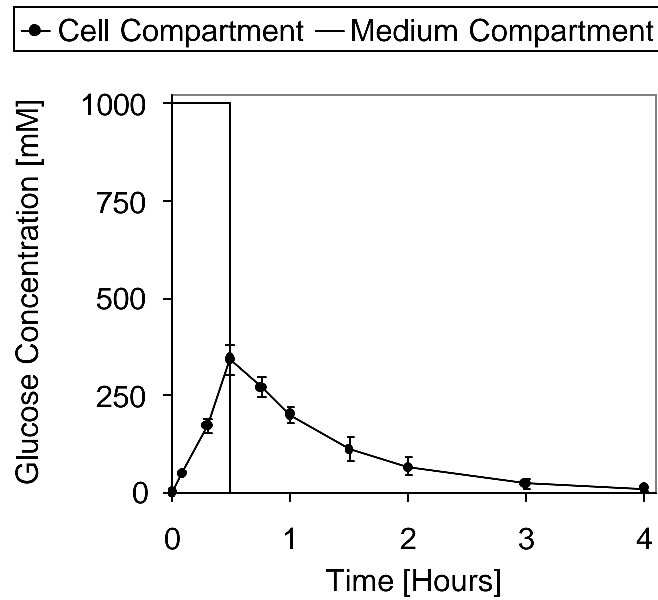


**Fig. 7** Time depending profiles of low molecular weight substances can be realized in the cell compartment by altering the concentration in the medium compartment, as shown for glucose (mean of n = 3 experiments ± SD).

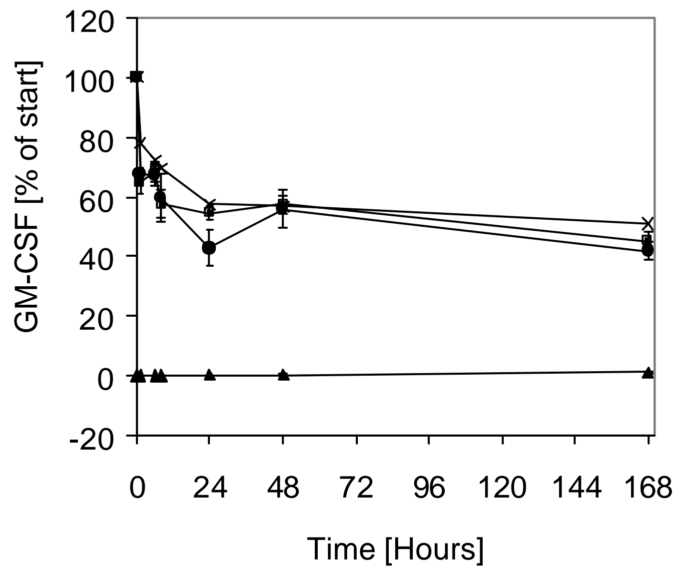


**Fig. 8a** Retention of GM-CSF in the cell compartment after 7 days of recirculation of culture medium in the medium compartment (● concentration of GM-CSF in the cell compartment with recirculation of culture medium in the medium compartment, ■ control with recirculation of culture medium in the medium compartment, X control in polystyrene tube, ▲ concentration of GM-CSF in the medium compartment; mean of n = 3 experiments ± SD).

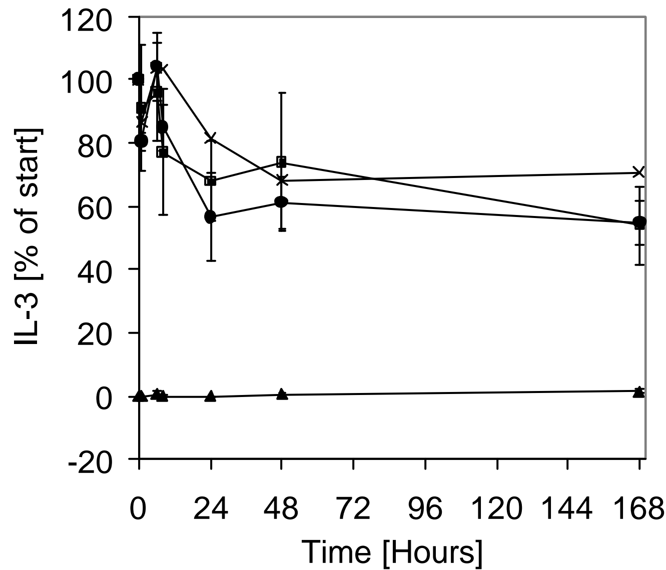


**Fig. 8b** Retention of IL-3 in the cell compartment after 7 days of recirculation of culture medium in the medium compartment (● concentration of IL-3 in the cell compartment with recirculation of culture medium in the medium compartment, ■ control recirculation of culture medium in the medium compartment, X control in polystyrene tube, ▲ concentration of IL-3 in the medium compartment; mean of n = 3 experiments ± SD).

### 2.4.3 Cell growth in the bioreactor depends on flow rates

If cell growth depends on the availability of nutrients, a static culture in tissue culture plates should resemble closely a bioreactor operated with zero flow of medium. In Fig. 9a it is shown that this was indeed the case.

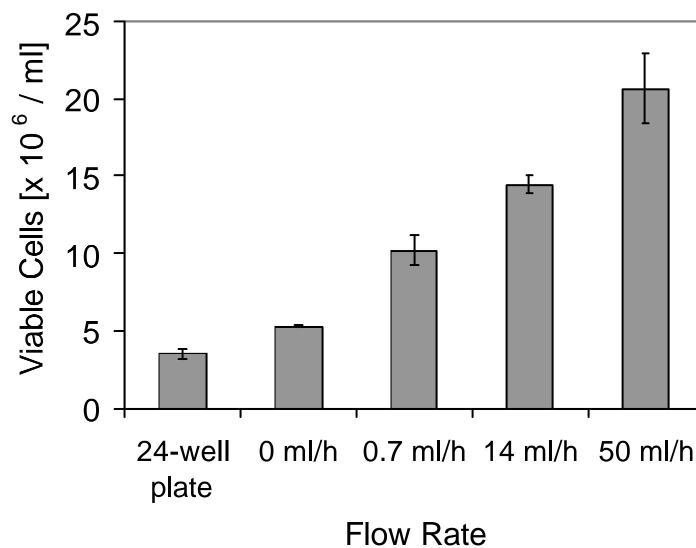


**Fig. 9a** Growth of CCRF-CEM depends on different flow rates in the hollow fiber bioreactor after 5 days recirculation of 100 ml RPMI starting with 2 x $10^6$ cells in 1 ml (mean of n = 4 experiments ± SD).

Moreover, growth of CCRF-CEM in the bioreactor was strongly influenced and thus, can be controlled by the medium flow rate. As a consequence, consumption of glucose and generation of lactate varied with the flow rate [Fig. 9b].

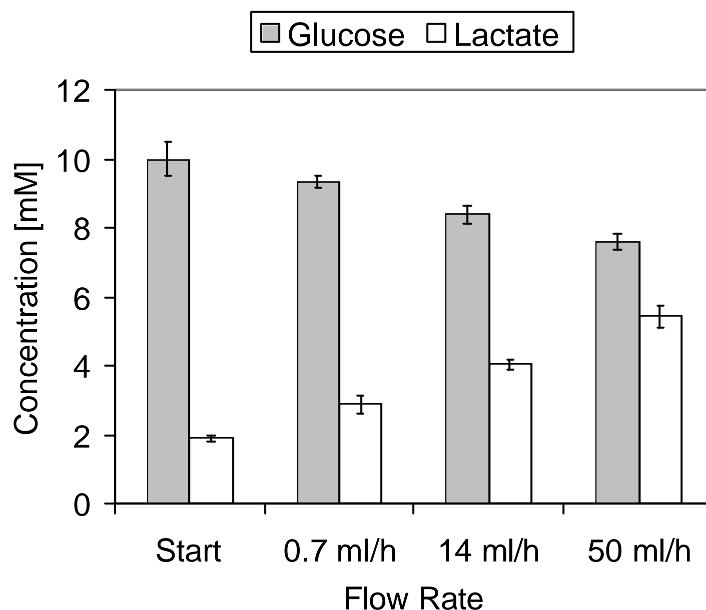


**Fig. 9b** Glucose and lactate concentrations in the medium compartment of the hollow fiber bioreactor system after 5 days depend on the flow rate (mean of n = 4 experiments ± SD).

## *2.5 Discussion*

### 2.5.1 Design of the hollow-fiber bioreactor

Often, fermentation processes require large culture volumes to achieve high product yields. Hollow-fiber bioreactors have been developed with extracapillary volumes between 2.6 and 50 ml (Quarles et al., 1980; Knazek et al., 1990; Jauregui et al., 1994; Jackson et al., 1996). Since most hollow-fiber bioreactors are conceptually based on conventional hemodialysis modules, retrieving the cells is inefficient and awkward (Sardonini and Wu, 1993) often resulting in damage to the cells (Quarles et al., 1980). The hollow-fiber bioreactor described in this report has a volume of the cell compartment between 0.5 and 3 ml which makes it ideal for the small scale production of cell-derived products or the cultivation of rare cell populations at high cell densities (see below). It offers direct access to the cell compartment and a more convenient sampling procedure that also reduces the risk of contamination compared to hollow-fiber bioreactors originally designed as hemodialyzers. So far the bioreactor described is the only small-scale hollow-fiber bioreactor allowing microscopy of the cells during culture.

2.5.2 Growth of leukemic cell lines in the bioreactor compared to conventional culture

Three different leukemic cell lines representing the different phenotypes found in leukemic patients were used to characterize cell growth in the bioreactor. CCRF-CEM (Foley et al., 1965) and REH (Rosenfeld et al., 1977) cells are T- and B-lymphocytic cell lines, respectively, and HL-60 (Collins et al., 1977) is a promyeloic cell line. In conventional batch culture without a change of medium, leukemic cell lines reach a maximum cell density of 1.5 – 2.3 x $10^6$ cells/ml as was shown for CCRF-CEM cells and HL-60 cells (Horakova et al., 1989; Schwarze et al., 1999; Valkov et al., 2000) and reconfirmed in Fig. 5a. In the hollow-fiber bioreactor a 4 - 5-fold increase in cell density after 3 days and a 10-fold increase after 7 days was observed compared to conventional cultures. That HL-60 cells can reach cell densities of 7 x $10^7$/ml when cultured under optimized conditions in dialysis tube membranes was shown previously (Schumpp and Schlaeger, 1990). The behaviour of the cell lines in this system for longer time periods (> 7d) remains to be investigated. Since HL-60 and CCRF-CEM cells cultivated in the hollow-fiber bioreactor reach such high densities, it can be hypothesized that this offers a distinct advantage for the cultivation of cells, e.g. primary AML cells, because high cell density (Maruyama et al., 1990) and also better cell-cell contact (Reilly et al., 1989) yielded both in improved autonomous colony formation of AML blasts from leukemic patients.

2.5.3 Concentration Profiles in the Bioreactor

There is a need for systems resembling the clinical situation with regard to drug responses and toxicity. *In vivo* drug concentrations change continuously over time according to the individual pharmacokinetic profile of each drug. Animal models suffer from the disadvantage of species specific pharmacokinetics (Mattern et al., 1988) and are not directly transferable to the human situation. Currently, drug responses are evaluated in microtiterplates where each drug concentration is tested in a separate well (Campling et al., 1991; Martin and Clynes, 1993; Wientjes et al., 1995). The use of semi-permeable membranes in the bioreactor allows continuous and controlled delivery and also removal of substances from the cell compartment as shown in Fig. 6 and Fig. 7. In addition, human cells can be exposed to drug concentration profiles simulating the *in vivo* pharmacokinetic situation *in vitro*.

Moreover, this system can help to optimize therapeutic regimens with regard to mode of drug application (e.g. oral versus bolus injection) and combination therapies.

### 2.5.4 Mass Transfer Considerations

In this study three different culture systems were compared with regard to cell growth. Conventional cell culture plates do not have a membrane separating cell and medium compartment and are static with regard to medium flux. In both Transwell® cultures and in the bioreactor, membranes separate cells from medium. However, compared to the bioreactor, in Transwell® culture, no flow of medium is realized. All three systems have a static cell compartment in common. Moreover, in the three systems studied, medium volume differs between 1 ml, 34 ml and 20 ml, respectively. In conventional culture, growth of cells is limited by exhaustion of nutrients, e.g. glucose. In Transwell® culture as well as in the bioreactor set-up a larger medium volume can explain the higher cell densities observed. However, in the bioreactor compared to Transwell® culture a higher cell density was reached despite a lower medium volume. Since the hypothesis was that this was due to the medium flux and consequently better nutrient supply, the experiment shown in Fig. 9a was designed. This experiment demonstrated that in the range between 0.7 - 50 ml/h, cell growth increased with medium flow rate. Medium flow rates above 50 ml/h were not examined since in cell-free experiments showed that glucose transport across the membrane, while dependent on medium flux below 50 ml/h, was in saturation above this value (data not shown). For the period of 5 days and a cell density up to $2 \times 10^7$ / ml cells can be maintained at a viability > 89% and glucose concentration is maintained at > 75% of the initial concentration [Fig. 9b].

### 2.5.5 Conclusions

Perfusion cell culture is an advantage e.g. for antibody production (Gorter et al., 1993), for the cultivation of hematopoietic stem cells (Koller et al., 1993a; Sandstrom et al., 1995) and a prerequisite for tissue engineering, e.g. of cartilage (Sittinger et al., 1994). As cell growth is correlated with decrease of glucose and glutamine and an increase of lactate and ammonium (Schumpp and Schlaeger, 1992), the achievable maximum of cell density in conventional culture is limited. By realizing a continuous supply of fresh medium and removal of toxic metabolic by-products, an increased perfusion rate also leads to an increased cell and antibody yield (Velez et al., 1987). This is in accordance with our finding that a higher cell density of leukemic

cell lines growing in the hollow-fiber bioreactor system can be reached with a higher flow rate. It is remarkable, that in contrast to static cultures where the growth rate depends mainly on cell count at the start of the culture (Kaesehagen et al., 1991), in the system described the growth rate of the cell line CCRF-CEM can be regulated by flow rate.

An additional advantage of this hollow-fiber bioreactor system is the retention of both cells and also of soluble factors in the cell compartment, as shown for GM-CSF and IL-3 (provided the membrane cut-off is low enough). In conventional culture, feeding procedures may lead to the dilution or even complete removal of autocrine growth factors, e.g. of GM-CSF important for the autonomous growth of the blast cell population in AML samples (Bradbury et al., 1990), or also of added factors needed in e.g. cultivation of stem cells (Palsson et al., 1995). Retention of cytokines translates to economy of cost since less cytokines can be used combined with the growth advantages of a perfusion system.

# Chapter 3

# Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue$^{TM}$

## *3.1 Abstract*

A method for monitoring cell proliferation in a small scale hollow-fiber bioreactor (culture volume: 1 ml) by use of the Alamar Blue$^{TM}$ dye is described. Alamar Blue$^{TM}$ is a non-fluorescent compound which yields a fluorescent product after reduction e.g. by living cells. In contrast to the MTT-assay, the Alamar Blue$^{TM}$-assay does not lead to cell death. However, when not removed from the cells, the Alamar Blue$^{TM}$ dye shows a reversible, time- and concentration-dependent growth inhibition as observed for the leukemic cell lines CCRF-CEM, HL-60 and REH. When applied in the medium compartment of a hollow-fiber bioreactor system, the dye is delivered to the cells across the hollow-fiber membrane, reduced by the cells and released from the cell into the medium compartment back again. Thus, fluorescence intensity can be measured in medium samples reflecting growth of the cells in the cell compartment. This procedure offers several advantages. First, exposure of the cells to the dye can be reduced compared to conventional culture in plates. Second, handling steps are minimized since no sample of the cells needs to be taken for readout. Moreover, for the exchange of medium, a centrifugation step can be avoided and the cells can be cultivated further. Third, the method allows to discriminate between cell densities of $10^5$, $10^6$ and $10^7$ of proliferating HL-60 cells cultivated in the cell compartment of the bioreactor. Measurement of fluorescence in the medium compartment is more sensitive compared to glucose or lactate measurement for cell counts below $10^6$ cells/ml, in particular. In conclusion, the Alamar Blue$^{TM}$-assay combined with a hollow-fiber bioreactor offers distinct advantages for the non-invasive monitoring of cell viability and proliferation in a closed system.

## 3.2 Introduction

For most investigations in cell biology the determination of cell count and the evaluation of cell viability is essential. For this purpose, a variety of assays have been developed, e.g. assays that rely on membrane integrity, metabolic activity, proliferation rate or protein content of the cell population. One popular assay is the MTT-assay measuring the activity of mitochondrial dehydrogenases colorimetrically (Mosmann, 1983). However, the assay procedure leads to cell death. An alternative indicator of the cellular redox state is the Alamar Blue$^{TM}$ dye, that is reduced from a non-fluorescent form to a fluorescent product due to metabolic activity of the cultured cells (Fields and Lancaster, 1993).

The Alamar Blue$^{TM}$ assay has been widely used for determination of cytotoxicity and drug effects (Sawabe et al., 1996; Suggs et al., 1999; Lee et al., 2000) , for biocompatibility testing (Benghuzzi, 1995) and for quantitative measurement of lymphocyte proliferation (Ahmed et al., 1994; de Fries and Mitsuhashi, 1995). The assay has been compared to other viability assays showing a good correlation, e.g. with the trypan blue exclusion- (Gazzano-Santoro et al., 1997), the MTT- (Back et al., 1999), and the XTT-assay (Pagé et al., 1993).

In chapter 2 a new small-scale bioreactor for cultivation of cells in a volume of 1 ml and densities up to 3 x $10^7$/ml, consisting of a medium and a cell compartment separated by hollow-fiber membranes is described. Due to the small culture volume it was of paramount importance to estimate the cell count in the system without taking samples from the cell compartment in order to prevent loss of cells and to realize undisturbed culture of the cells. Since the Alamar Blue$^{TM}$ method allows further cultivation of the cells after assay readout, the present study was performed to investigate the application of the method to monitor cell proliferation and cell count in this novel miniaturized hollow-fiber bioreactor system.

## 3.3 Materials and Methods

### 3.3.1 Culture medium, cell lines and analytical methods

RPMI1640 supplemented with 25 mM HEPES, 2 mM glutamine and 10 % FCS (all from Sigma, Deisenhofen, Germany) was used as culture medium throughout the experiments. The leukemic cell lines CCRF-CEM, HL-60 and REH were routinely cultivated in culture flasks (Falcon®, BD Biosciences, Heidelberg, Germany) at 37°C, 5% $CO_2$, in an humidified incubator (Heraeus, Hanau, Germany) and subcultivated

after 3 or 4 days. For the experiments, cells were taken from the exponential growth phase. Viable and total cell counts were determined routinely using an automatic cell counter (CASY®1 TTC, Schärfe System GmbH, Reutlingen, Germany) (Glauner, 1991; Winkelmeier et al., 1993) after validation against trypan blue exclusion (data not shown). Glucose and lactate concentration of the culture medium was measured with a blood gas analyser in aqueous solution mode (Omni, AVL, Bad Homburg, Germany) either on the same day or after storage of the samples at -20°C. Fluorescence (excitation wavelength: 544 nm and emission wavelength: 590 nm) of the reduced Alamar Blue™ dye was measured with an automatic microplate fluorometer (Fluoroscan II, Labsystems, Frankfurt, Germany).

### 3.3.2 Miniaturized hollow-fiber bioreactor system for cell culture

The bioreactor with a volume of 1 ml (built in the workshops at Akzo Nobel Central Research, Obernburg, Germany) contains a hollow-fiber dialysis membrane (Cuprophan® D2, Membrana GmbH, Wuppertal, Germany, ultrafiltration rate 3 ml / h x m² x mmHg, wall thickness 16 µm, inner diameter 215 µm) through which culture medium is constantly recirculated by use of a peristaltic pump (IPC, Ismatec Laboriumstechnik GmbH, Wertheim-Mondfeld, Germany) in an incubator (37°C, 5% $CO_2$). In addition, the bioreactor is equipped with hollow-fiber oxygenation membranes (Oxyphan®, Membrana GmbH, Wuppertal, Germany, maximum pore size < 0.2 µm, wall thickness 50 µm, inner diameter 280 µm) which are used for passive gas exchange [Fig. 3 and 4, see chapter 2].

### 3.3.3 Growth inhibition of leukemic cell lines by the Alamar Blue™ dye in standard culture

CCRF-CEM, HL-60 and REH cells (5 ml) were added into culture flasks (Falcon®, BD Biosciences, Heidelberg, Germany) at a density of 4 x $10^5$/ml. Alamar Blue™ (Biozol Diagnostica Vertrieb GmbH, Eching, Germany) containing medium was used to dilute the cells to a density of 2 x $10^5$/ml. Final concentrations of Alamar Blue™ were 1, 2.5, 5, 10 and 20 % v/v. Controls did not contain Alamar Blue™ dye. Cells were cultivated for 3 days, then counted and percentage of viable cells calculated: [Viable cells] / [Viable $cells_{control}$] x 100. To optimize the Alamar Blue™ method with regard to growth inhibition and reversibility of inhibition, HL-60 cells (15 ml) were added into culture flasks at a density of 4 x $10^5$/ml. The cells were diluted with Alamar Blue™ containing medium (5%, v/v) to achieve a density of 2 x $10^5$/ml. At the times given in Fig. 2d 5 ml

of cell suspension was removed from the flasks, centrifuged at 400 x g (Heraeus Digifuge, Hanau, Germany) and fluorescence measured in the supernatant. Five ml of fresh culture medium was added to the culture and cells were further cultivated until day 3. Cells were counted and percentage of viable cells was calculated.

### 3.3.4 Growth curve, glucose consumption and lactate generation of CCRF-CEM cells in the hollow-fiber bioreactor system

To correlate growth of the cell line CCRF-CEM in the bioreactor with consumption of glucose and generation of lactate, 2 x $10^6$ cells / ml were inoculated into the cell compartment of the bioreactor. Cell harvest was performed at times given in the figure by removing the cells completely and rinsing the cell compartment 3 times with 1 ml of culture medium. At the same time, samples for glucose and lactate measurement were taken from the medium compartment. Consumption of glucose and generation of lactate were calculated as follows:

Consumption of glucose = (concentration of glucose $_{start}$ – concentration of glucose $_{day\ X}$) x volume

Generation of lactate = (Concentration of lactate $_{day\ X}$ – concentration of lactate $_{start}$) x volume.

### 3.3.5 Application of the Alamar Blue™ dye in the hollow-fiber bioreactor system

CCRF-CEM cells (2 x $10^6$/ml) in culture medium were inoculated into the cell compartment of the bioreactor and culture medium was recirculated in the medium compartment (volume: 10 ml, flow-rate: 14 ml/h). After 24 hours of culture, the Alamar Blue™ dye was added (2.5 %, v/v) to the medium compartment and recirculated for 24 hours. Samples were taken from the medium compartment and fluorescence, glucose and lactate determined. Glucose consumption and lactate generation was calculated. To investigate the possibility of a further cultivation of the cells, culture medium in the medium compartment was exchanged at day 2. At day 3, the medium volume in the medium compartment was increased to 20 ml by adding fresh culture medium and cells were cultivated further for 4 days. To discriminate between different cell densities, $10^5$, $10^6$ and $10^7$ HL-60 cells in 1 ml of culture medium were inoculated into the cell compartment of the bioreactor and culture medium containing Alamar Blue™ was recirculated for 24 hours.

## *3.4 Results*

### 3.4.1 Optimization of cell growth in the presence of Alamar Blue™ in standard culture

When the Alamar Blue™ dye was added to conventional cell culture, fluorescence increased in the supernatant over time and already after 2 hours of culture, cell proliferation could be detected [Fig. 10a]. However, as compared to untreated controls, growth of the leukemic cell lines CCRF-CEM, HL-60 and REH was inhibited by Alamar Blue™, depending on the concentration used [Fig. 10b]. Growth inhibition was also time dependent, as shown for HL-60 cells [Fig. 10c]. HL-60 cells appeared to be more sensitive to the growth inhibitory effects of Alamar Blue™ compared to other cell lines. To investigate the reversibility of growth inhibition by Alamar Blue™, HL-60 cells were cultured for different time intervals in the presence of the dye, centrifuged and resuspended in fresh culture medium. Within the first two hours almost no inhibition of growth was observed which increased for longer incubation times [Fig. 10d].

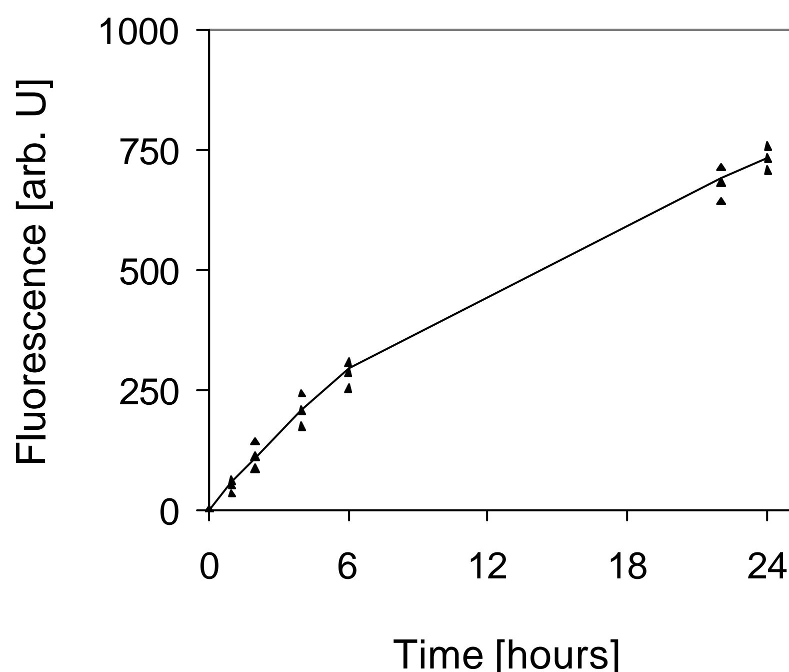


**Fig. 10a** Development of fluorescence over time for HL-60 cells incubated with 2.5 % (v/v) Alamar Blue™, starting with 2 x $10^5$ cells/ml (mean of n = 3 experiments).

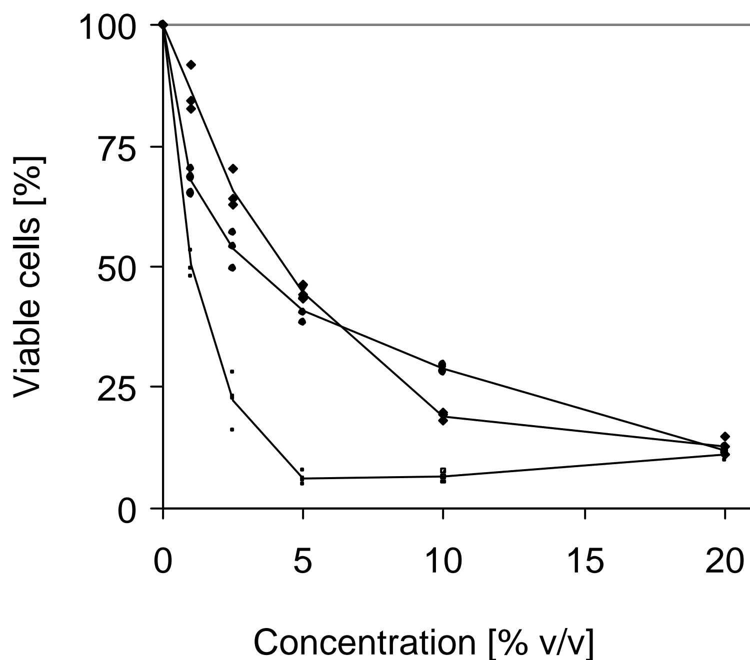


**Fig. 10b** Growth inhibition of different cell lines depends on the concentration of the Alamar Blue™ dye in the supernatant (● CCRF-CEM cells, ■ HL-60 cells, ◆ REH cells; mean of n = 3 experiments).
Starting with 2 x $10^5$ cells/ml cells were exposed to the Alamar Blue™ dye for 3 days. Viable cell count was calculated against untreated controls.

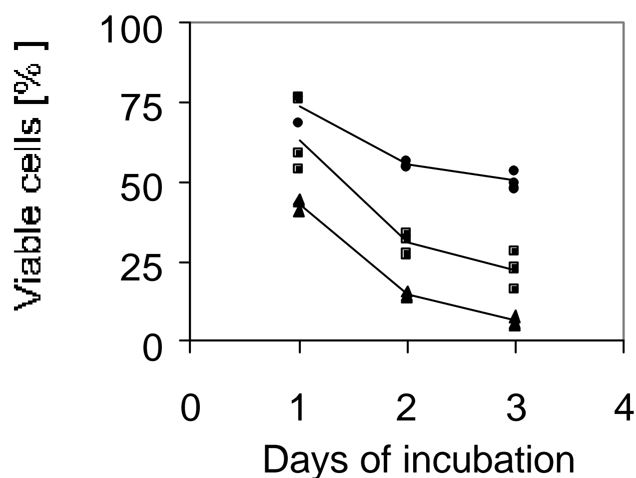


**Fig. 10c** Growth inhibition of HL-60 cells depends on Alamar Blue™ concentration and the duration of incubation with the Alamar Blue™ dye (● 1 % v/v, ■ 2.5 % v/v, ▲ 5 % v/v; mean of n = 3 experiments).
Starting with 2 x $10^5$ cells/ml cells were exposed to the Alamar Blue™ dye and viable cell count was calculated against untreated controls.

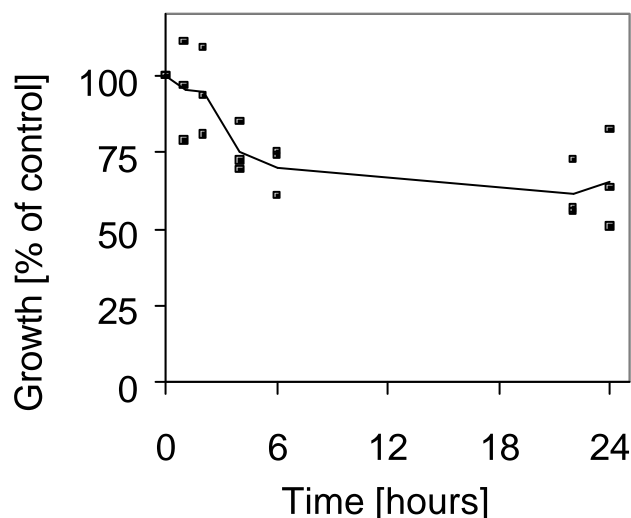


**Fig. 10d** Growth inhibition of HL-60 cells can be reversed by removing the Alamar Blue$^{TM}$ dye by centrifugation of the cells (mean of n = 3 experiments).
Starting with 2 x $10^5$ cells/ml in one culture flask, samples were removed at each time point, centrifuged and supplied with fresh medium. All samples were cultivated in total for 3 days but with different times of exposure to the dye.

### 3.4.2 The Alamar Blue$^{TM}$ method provides a convenient and advantageous method to quantify proliferating cells in a hollow-fiber bioreactor system

Starting with 2 x $10^6$ cells / ml, the leukemic cell line CCRF-CEM reached a cell density of 11.59 ± 0.39 x $10^6$ / ml after 3 days of culture in the bioreactor system, showing a viability of 94 ± 0.58 % at day 3. Consumption of glucose and accumulation of lactate, which was calculated from glucose and lactate concentrations in the medium compartment, correlated with the growth curve of the cells [Fig. 11]. When CCRF-CEM cells were cultivated in the bioreactor system and Alamar Blue$^{TM}$ containing medium was recirculated in the medium compartment, the dye was delivered to the cells across the perfusion membrane of the bioreactor, reduced by the cells to a fluorescent product which was released back into the medium compartment where fluorescence increased over time. Thus, proliferation of cells could be determined by taking samples from the medium compartment without impeding the culture process [Fig. 12].

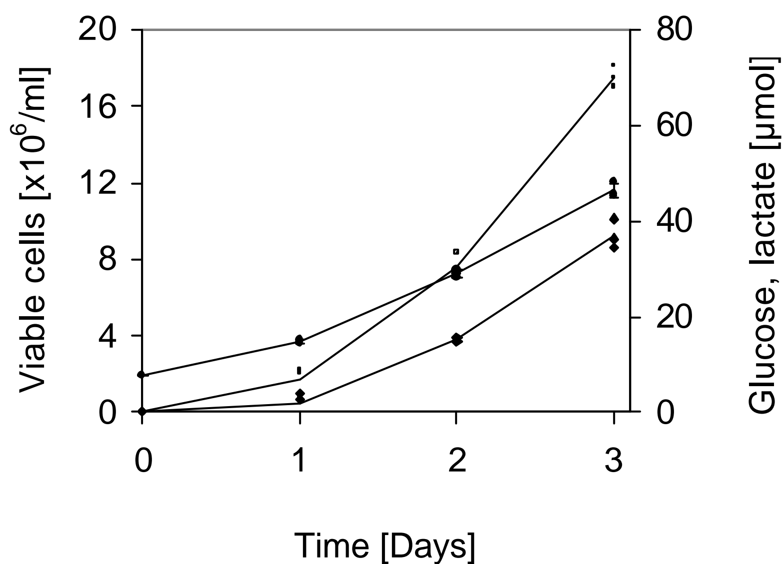


**Fig. 11** Cell growth of the leukemic cell line CCRF-CEM starting with 2 x $10^6$ cells/ml in the hollow-fiber bioreactor correlates with the metabolic activity of the cells indicated by consumption of glucose and generation of lactate (● cell count in the bioreactor, ■ generation of lactate, ◆ consumption of glucose; mean of n = 3 experiments).

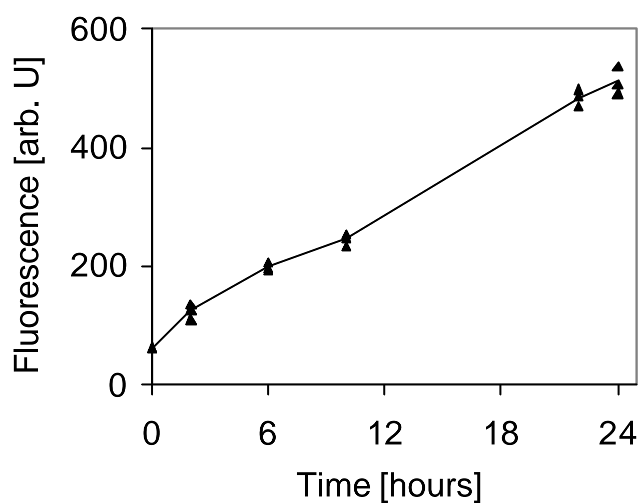


**Fig. 12** Development of fluorescence in the medium compartment of the bioreactor when Alamar Blue™ is added into recirculation 24 hours after inoculation of 2 x $10^6$ cells/ml (mean of n = 3 experiments).

After removing the Alamar Blue™ dye from the medium compartment by complete exchange of medium, cells grew further as demonstrated by consumption of glucose and generation of lactate [Fig. 13]. However, when compared on day 3 to a cell culture in the absence of Alamar Blue™, glucose consumption and lactate generation was reduced (glucose: 32.0 ± 2.1 µmol vs. 37.0 ± 3.2 µmol, lactate: 54.6 ± 4.7 µmol vs. 70.1 ± 2.2 µmol) [compare Fig. 11 + Fig. 13]. This shows a distinct advantage of the hollow-fiber bioreactor system in so far as, although growth inhibition caused by the dye was observed, it was reversible to a large extend without using a centrifugation step and exposure of the cells to the dye was transient.

In addition, compared to measurement of glucose and lactate concentration the three different densities $10^5$, $10^6$ and $10^7$ of proliferating HL-60 cells could be discriminated by use of the Alamar Blue™ method [Fig. 14]. When measuring glucose and lactate concentration in the medium compartment the presence of either $10^5$ or $10^6$ cells in the cell compartment could not be distinguished [Fig. 15].

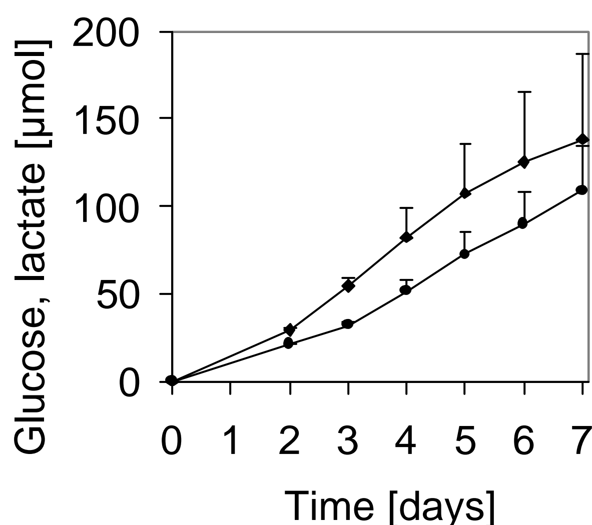


**Fig. 13** Consumption of glucose and generation of lactate after application of Alamar Blue™ dye (for 24 hours after day 1) in the medium compartment of the bioreactor system (● glucose, ◆ lactate; mean of n = 3 experiments ± SD). After a preincubation period of one day Alamar Blue™ was added to the medium and recirculated for one day. After 48 hours medium containing Alamar Blue™ was replaced by fresh culture medium and the cells were cultivated for another 5 days.

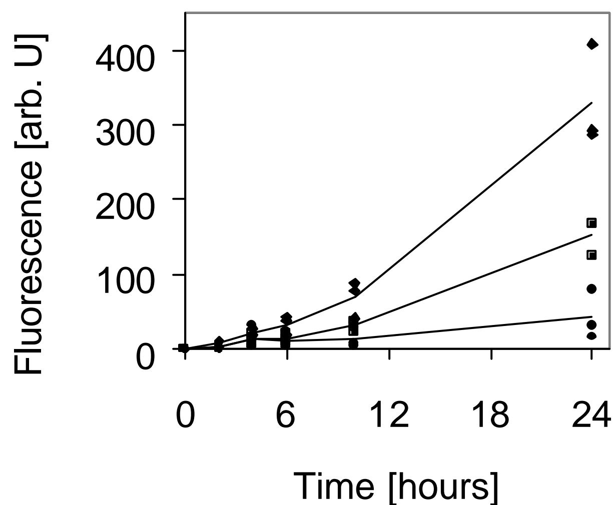


**Fig. 14** Discrimination of 3 different cell densities of HL-60 cells cultivated in the bioreactor recirculating Alamar Blue™ in the medium compartment (cell count at start: ● $10^5$, ■ $10^6$ and ◆ $10^7$ cells; mean of n = 3 experiments).

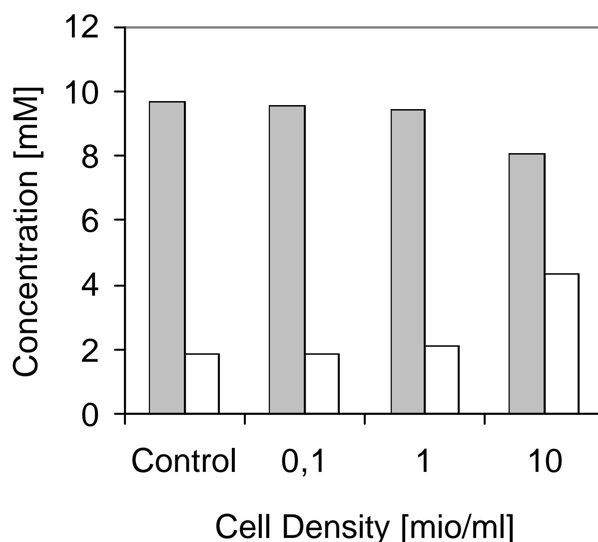


**Fig. 15** Discrimination of 3 different cell densities by glucose and lactate after 24 hours of recirculation of Alamar Blue™ in the medium compartment (■ glucose concentration, □ lactate concentration, measured in the medium; mean of n = 3 experiments).

## *3.5 Discussion*

### 3.5.1 Need for bioprocess monitoring in hollow-fiber bioreactor systems

Today, continuously perfused cell culture systems, especially hollow-fiber bioreactors, find broad application in the production of cell-derived products like antibodies (Gorter et al., 1993) and cytokines (Lamers et al., 1999), for the expansion

of activated lymphocytes (Knazek et al., 1990; Hillman et al., 1994) and other cell types (Marx et al., 1993; Jauregui et al., 1994). For bioprocess control biologically meaningful parameters have to be defined to monitor cell proliferation, density and viability. Measuring metabolic parameters as indicators for cell growth, e.g. consumption of glucose and generation of lactate is a common practice (Knazek et al., 1990; Jackson et al., 1996).

### 3.5.2 Advantages of the Alamar Blue™ dye

The Alamar Blue™ dye provides a new method to determine cell proliferation in biotechnological processes, e.g. in hollow-fiber bioreactor systems. In principle, cell proliferation leads to reduction of the dye resulting in a change of color from blue to red and from non-fluorescent to fluorescent (Pagé and Pagé, 1995), thus offering the possibility of either colorimetric or fluorimetric evaluation. The Alamar Blue™ method is not biased by medium components (Pagé et al., 1993), changes in fluorescence are directly related to mitochondrial function (Springer et al., 1998) and a linear relationship between cell count and fluorescence has been reported (Gazzano-Santoro et al., 1997; Larson et al., 1997; Nakayama et al., 1997).

### 3.5.3 Use of the Alamar Blue dye in hollow-fiber bioreactors

The Alamar Blue™ dye is regarded as non-toxic for lymphocytes (Ahmed et al., 1994), for the B-lymphoblastoid cell line WILL-2 (Gazzano-Santoro et al., 1997), and corneal endothelial cells (Larson et al., 1997). However, for epithelial ovarian carcinoma cells cytotoxicity of the Alamar Blue™ dye has been documented, depending on the dye concentration and the exposure time (Squatrito et al., 1995). This is in accordance with the findings for leukemic cell lines shown in Fig. 10b. Besides a different sensitivity of the cell types against Alamar Blue™, assay protocols varying in the concentration of Alamar Blue™ and the incubation time have to be considered to explain these differences. Alamar Blue™ concentrations in the literature range from 5 % (v/v) (Pagé et al., 1993; Lee et al., 2000) to 25 % (v/v) (Gazzano-Santoro et al., 1997) and incubation times from 2 to 72 hours (Ahmed et al., 1994; Nociari et al., 1998; Back et al., 1999). For HL-60 and other leukemic cell lines a Alamar Blue™ concentration of 10 % v/v for 6 hours was recommended (Nakayama et al., 1997). In contrast, in our experiment with the same cell line in conventional culture a concentration of 5 % v/v for 4 hours caused a markedly reduced growth of cells after 3 days. Based on these data it is obvious that the

application of Alamar Blue™ must be optimized for each cell line and experimental set-up.

To use the Alamar Blue™ dye in the hollow-fiber bioreactor system described, was motivated by the small volume of the cell compartment of just 1 ml since in a small-scale system sampling of cells and medium is very limited. In contrast, in larger systems e.g. with a volume up to 10 l, sampling of cells and medium is much more convenient. However, in hollow-fiber bioreactor systems, in particular, cell samples taken from the extracapillary space may not be representative for the whole cell culture. In large scale systems costs of the Alamar Blue™ dye have to be taken into account and balanced against the value of the culture or of the culture products.

### 3.5.4 The Alamar Blue™ method is more sensitive than glucose and lactate measurement

In conventional culture, growth inhibition caused by Alamar Blue™ was reversible by centrifugation of the cells for exposure times up to 2 hours [Fig. 10d]. In the hollow-fiber bioreactor system recirculation of the culture medium leads to accumulation of fluorescence in the medium compartment [Fig. 12]. This method can be used with as low as $10^5$ cells and offers the possibility to discriminate cell density over at least 3 logs [Fig. 14]. Also, with the hollow-fiber bioreactor the dye can be completely removed without centrifugation, simply by changing the culture medium in the medium reservoir. This procedure offers advantages in terms of undisturbed culture, a reduced risk of contamination and is less labour intensive. After removal of the dye the cells in the cell compartment of the bioreactor can be cultivated further as demonstrated for CCRF-CEM cells [Fig. 13]. Therefore, application of the Alamar Blue™ dye in perfused systems is an advantage for monitoring cell proliferation, in particular, since we have shown that the Alamar Blue™ method is more sensitive compared to measurement of glucose and lactate for low cell numbers below $10^6$ [Fig. 14].

### 3.5.5 Possible applications of the described method

Possible applications for this new method are optimization of culture conditions for expansion of cells, e.g. for cell therapy, for cytotoxicity testing and pharmacological screening of substances of therapeutic value. The suitability of the method for other perfused culture systems and in tissue engineering remains to be shown, an example being engineering of cartilage (Minuth et al., 1992; Sittinger et al., 1994).

In summary, a non-invasive method to monitor proliferation of cells and to quantify cells grown in a hollow-fiber bioreactor culture by use of the Alamar Blue$^{TM}$ dye is proposed. Further development may combine on-line sensor technology with Alamar Blue$^{TM}$ fluorescence measurement, offering bioprocess control by integration of a fluorescence sensor either into the medium or the cell compartment of the system.

# Chapter 4

## New miniaturized hollow-fiber bioreactor as a tool for *in vitro* toxicity testing and drug screening

### *4.1 Abstract*

Models for toxicity as well as efficacy testing of drugs *in vitro* allow only limited conclusions with regard to the *in vivo* situation. Examples of restrictions are the lack of realistic *in vitro* tumor models and difficulties to model drug pharmacokinetics. A new hollow-fiber bioreactor was evaluated as a tool for, in particular, more *in vivo*-like toxicity testing e.g. during the development of cytostatic drugs or drug combinations. The bioreactor has a volume in the cell compartment of only 1-2 ml, proved to be pyrogen free and is steam-sterilizable. Leukemic cell lines like HL-60 and primary cells such as PHA-stimulated lymphocytes can be grown up to high densities of 1-3 x $10^7$ and analyzed during growth in the bioreactor by light-microscopy. The cytostatic drug Ara-C shows a dose-dependent growth inhibition of HL-60 cells and a dose-response curve similar to controls in culture plates. The bioreactor system is highly flexible since several systems can be run in parallel, soluble drugs can be delivered continuously via a perfusion membrane and gaseous compounds via an oxygenation membrane which also allows to control $pO_2$ and pH (via $pCO_2$) during culture in the cell compartment. These properties allow a more *in vivo*-like growth of (tumor) cells and tissue and the realization of *in vivo*-like pharmacokinetics. Thus the bioreactor system could serve as an alternative to existing *in vitro* and animal drug screening models.

## *4.2 Introduction*

During drug development as well as in toxicity testing, model systems are required to screen possible effects of new compounds on the human organism. Beside animals, cell lines as well as primary cell cultures (Grisham and Smith, 1984) are extensively used for *in vitro* studies. Today, chemotherapy with cytostatic drugs is a common treatment in anticancer therapy both for solid and leukemic tumors. However, despite some progress in the treatment of cancer, drug resistance is still a major problem in cancer chemotherapy (Dietel, 1991; Vendrik et al., 1992; Dietel, 1993; Hannun, 1997). One attempt to overcome this limitation is to predict the response to chemotherapy by assessing the chemosensitivity of patients cells *in vitro* (Bosanquet and Bell, 1996; Hoffman, 1997) with the aim to individualize treatment with regard to specific resistance profiles. Various model systems have been used so far to evaluate drug effects on human cancers *in vitro*, using either clonogenic assays (Hamburger and Salmon, 1977; Von Hoff et al., 1986) or suspension and monolayer cultures with different end-points (Pieters et al., 1989; Dietel et al., 1993; Hoffman, 1993a). However, current tumor models (Dietel et al., 1993; Hoffman, 1993b) are often limited with regard to morphology, histology, vascularity, stromal involvement and cell-matrix interaction. For evaluation of drug effects on leukemic cells, most test systems do not take into consideration the influence of cell density. It is known that higher cell densities may lead to an increased resistance to cytostatic drugs *in vitro* (Ohnuma et al., 1986; Takemura et al., 1991; Kobayashi et al.,1992). All models have in common that pharmacokinetic and pharmacodynamic aspects in drug testing can hardly be modelled *in vitro*. In conclusion, there is a lack of systems to model human cancer *in vitro* (Gura, 1997). Better systems will improve the development of new cytostatic drugs, but also the prediction of individual patients response to certain chemotherapeutic regimes. The application of more sophisticated cell culture methods *in vitro* should thus lead to more *in vivo* like toxicity testing resembling better the situation in humans. To this end the suitability of a miniaturized hollow-fiber bioreactor for toxicity testing and the possibility to test cytostatic drugs on human leukemia cell lines and on human lymphocytes cultured in high cell densities was evaluated.

## 4.3 Materials and methods

### 4.3.1 Miniaturized hollow-fiber bioreactor system for cell culture

The hollow-fiber bioreactor (built in the workshops at Akzo Nobel Central Research, Obernburg, Germany) was steam-sterilized (121°C, 20 min) before use. Cell and medium compartment are separated by a dialysis membrane. In addition, an oxygenation membrane ensures efficient oxygenation of the cell culture. Cells are placed into the cell compartment with a volume of 1-2 ml. Culture medium is constantly recirculated from the medium reservoir through the lumen of the hollow-fiber membranes by use of a peristaltic pump (Gloeckner et al., 2001). Bioreactors containing Cuprophan® hollow-fibers were employed for the experiments with HL-60 cells and bioreactors equipped with DIAPES® hollow-fibers (Membrana GmbH, Wuppertal, Germany) were used for culture of lymphocytes. To enable parallel screening of many compounds and to provide a system with high flexibility, the hollow-fiber bioreactor system is based on a modular concept. Up to 6 systems can be operated on a single platform [Fig. 16] with dimensions allowing to be placed in an commercially available incubator.

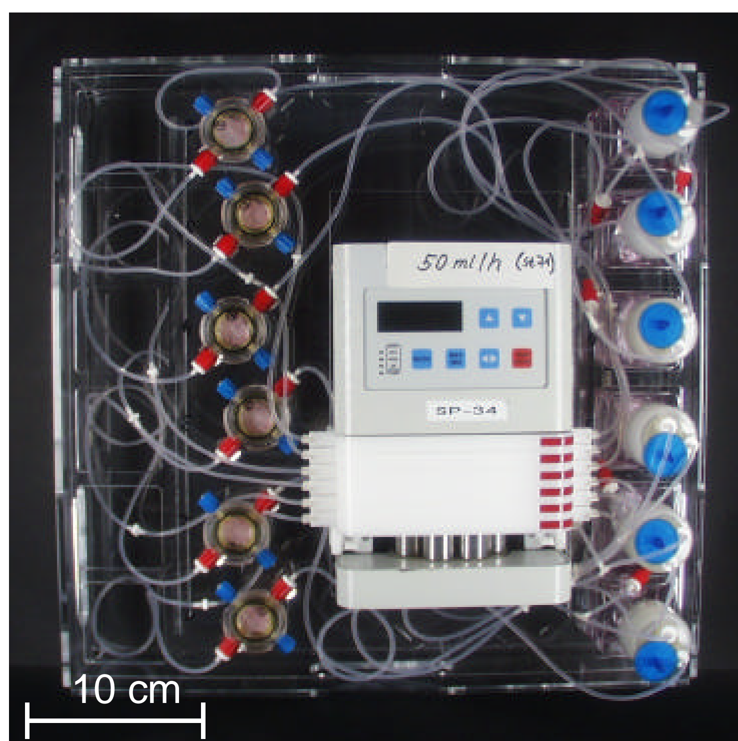


**Fig. 16** Up to 6 systems with 6 bioreactors can be placed on one platform. The hollow-fiber bioreactor system in recirculation mode is assembled on a transparent platform.

### 4.3.2 Cells, culture medium and analytical methods

RPMI1640 supplemented with 25 mM HEPES, 2 mM Glutamine and 10 % FCS (all from Sigma, Deisenhofen, Germany) was used as culture medium throughout the experiments.

The promyeloic cell line HL-60 was routinely cultivated in culture flasks (Falcon®, BD Biosciences, Heidelberg, Germany) at 37°C, 5% $CO_2$ in an humidified incubator (Heraeus, Hanau, Germany) and subcultivated after 3 or 4 days. For all experiments, cells were taken from the exponential growth phase.

Whole blood was obtained from healthy volunteers and anticoagulated with heparin (5 U/ml, Sigma, H-7005). Mononuclear cells (MNC) were isolated in polypropylene tubes (50 ml, Falcon®, BD Biosciences, Heidelberg, Germany) by layering 10 ml of heparinized whole blood which was diluted 1+1 with PBS (Sigma) on top of a step gradient of 10 ml Histopaque®-1119 and 10 ml of Histopaque®-1077 (Sigma). Tubes were centrifuged at 700 x g for 30 min at room temperature (Digifuge, Heraeus, Hanau, Germany). The MNC layer was removed and cells washed 3 times with PBS / EDTA (2 mM EDTA in PBS, Sigma) by centrifugation (5 min at 300 x g). Erythrocytes were removed by lysis with ammonium chloride (8,29 g $NH_4Cl$ + 1 g $KHCO_3$ + 0,0372 g EDTA in 1 l pyrogen-free water, pH 7,29) for 5 min at 5°C. Monocytes were partly removed by adherence to culture flasks (1 h at 37°C in culture medium).

Viable and total cell counts were determined by using an automatic cell counter (CASY® 1 TTC, Schärfe System GmbH, Reutlingen, Germany) (Glauner, 1991; Winkelmeier et al., 1993) or by trypan blue exclusion. The Alamar Blue™ assay for determination of viability was performed by adding 10 µl of Alamar Blue™ dye (Biozol Diagnostica VertriebsGmbH, Eching, Germany) to 200 µl of cell suspension. After 6 hours of incubation (Nakayama et al., 1997) 100 µl of cell suspension was transferred into a black 96-well microtitre plate (Cliniplate, Merlin GmbH, Bornheim-Hersel, Germany) and fluorescence of the reduced Alamar Blue™ dye was measured with an automatic microplate fluorometer (excitation wavelength: 544 nm and emission wavelength: 590 nm; Flouoroscan II, Labsystems, Frankfurt, Germany). Viability of the cells was calculated: $Fluorescence_{sample} / Fluorescence_{control} \times 100$.

For detection of apoptosis 1 x $10^6$ cells were washed in PBS by centrifugation (350 x g, 5 min) and fixed in 1 ml of ice cold 70% ethanol. Cells were stored at -20°C until propidium iodide staining was performed: cells were centrifuged at

200 x g for 5 min and incubated (30 min, room temperature, in the dark) with 500 µl of PBS containing 50 µg/ml propidium iodide (Sigma, P-4170), 1 mg/ml RNase (Sigma, R-5000) and 1% glucose (Sigma, G-7021). Samples were analysed by flow cytometry using a FACScan equipped with a 488 nm laser, a Power Macintosh 7300/200 computer and CellQuest software, version 3.1 (BD Biosciences).

### 4.3.3 Pyrogen assay

2 ml of heparinized whole blood was incubated in the cell compartment of the hollow-fiber bioreactor and in 6-well-plates (control) and IL-1β release into plasma was investigated. To correlate IL-1β release with a defined pyrogen stimulus, whole blood was incubated in the presence of 0, 1, 10 and 100 ng/ml LPS (Salmonella minnesota Re595, List Biological Laboratories Inc, Campbell, CA, USA) in polystyrene tubes (Falcon®, BD Biosciences). All blood samples were incubated for 3 hours at 37°C and shaken with 70 rpm. After 3 hours EDTA solution (220 µl, 100 mM, Merck KgaA, Darmstadt, Germany) was added to tubes and bioreactors. All samples were centrifuged at 2000 x g for 10 min at 5°C. Supernatant was stored at –80°C until the IL-1β ELISA assay was performed in plasma (Immunotech, Beckman Coulter GmbH, Krefeld, Germany; ELISA reader: Biorad Laboratories GmbH, München, Germany). The detection limit of the IL-1β assay was 3.9 pg/ml.

### 4.3.4 Growth of PHA stimulated lymphocytes in the hollow-fiber bioreactor

2 x $10^6$ isolated cells were inoculated into the cell compartment of the hollow-fiber bioreactor and in parallel into 6-well-plates. 10 ml of culture medium was recirculated in the medium compartment (flow rate: 50 ml/h) of the hollow-fiber bioreactor system by use of a peristaltic pump (IPC, Ismatec Laboriumstechnik GmbH, Wertheim-Mondfeld, Germany). Lymphocytes were stimulated by phytohaemagglutinin (PHA, 0.05 % in culture medium, Sigma, L-9132). After 24 hours IL-2 (5 U/ml in culture medium, Strathmann Biotech GmbH, Hannover, Germany) was added to cell and medium compartment of the bioreactor and into the 6-well-plates. In the bioreactor system, fresh culture medium containing IL-2 was added into the medium compartment at day 3 and day 5. Controls in 6-well-plates were grown in wells by adding fresh medium containing IL-2 at day 3 and day 5. Cells were harvested at day 3, 6 and 7 by rinsing the cell compartment and the 6-well-plates 3 times with culture medium and counted by using the CASY® cell counter.

4.3.5 Dose response curve of HL-60 cells using the Alamar Blue™ assay

The cytostatic drug cytosine arabinoside (Ara-C, ARA-cell, cell pharm GmbH, Hannover, Germany) was serially diluted from 200 µM to 0.8 µM. 100 µl of HL-60 cells at a concentration of 4 x $10^5$ / ml were added to each well of a 96-well microtitre plate (Falcon®, BD Biosciences) to reach a final cell concentration of 2 x $10^5$ / ml and Ara-C concentrations ranging from 100 µM to 0.4 µM. Cells were continuously exposed to the drug for 3 days before the Alamar Blue™ assay was performed.

4.3.6 Permeability of the perfusion membrane for Ara-C and stability of the drug

Culture medium was placed in the cell compartment of the bioreactor (1 ml) and culture medium containing Ara-C (19 ml, 50 µM) was recirculated in the medium compartment of the bioreactor system with a flow rate of 50 ml/h. Ara-C in a polypropylene tube (Eppendorf, Hamburg, Germany) was placed in an incubator (10 ml, 50 µM). After 24 hours 500 µl samples were taken from the control tube, the cell and the medium compartment. The samples were mixed with 500 µl of 4 x $10^5$ cells / ml of HL-60 cells in 24-well-plates (Falcon®, BD Biosciences). Cells were incubated for 3 days, then 200 µl of the cell suspension were transferred into a 96-well microtitre plate and the Alamar Blue™ assay was performed.
To analyze the stability of the cytostatic drug Ara-C, culture medium containing different concentrations of the drug (1, 10 and 100 µM, 1 ml each) was stored at room temperature and at 37°C in 1.5 ml polypropylene micro-tubes (Eppendorf, Hamburg, Germany) for 3 days. To investigate instability or adsorption of the drug to the materials (Wagner et al., 1999) of the bioreactor system culture medium with Ara-C (10 ml, 10 µM) was recirculated for 3 days in the medium compartment with a flow rate of 14 ml/h. After 3 days, Ara-C was freshly diluted in culture medium to match the concentrations of the stored control samples. 100 µl of each sample were added to 100 µl of HL-60 cells (4 x $10^5$ / ml) in flat-bottom 96-well microtitre plates (Falcon®, BD Biosciences) and incubated for another 3 days. After 3 days the Alamar Blue™ assay was performed.

4.3.7 Application of the cytostatic drug Ara-C in the bioreactor system

HL-60 cells were seeded at a density of 2 x $10^6$ cells / ml in the cell compartment of the bioreactor and cultivated for 3 days. Ara-C containing culture medium was recirculated in the medium compartment (19 ml, 0.5, 5 and 50 µM for 3 days, flow

rate: 14 ml/h). Cells were harvested at day 3, counted and viability was determined by the trypan blue assay, CASY® analysis or propidium iodide staining. For analysis with the Alamar Blue™ assay, 1 ml of cell harvest was incubated with 50 µl of Alamar Blue™ dye and 2 h later fluorescence was measured.

## *4.4 Results*

### 4.4.1 No release of IL-1β in the bioreactor after incubation with whole blood

To investigate pyrogen contamination of the hollow-fiber bioreactor e.g. during the manufacturing process, release of IL-1β into plasma after incubation of the cell compartment with whole blood was measured by ELISA [Fig. 17]. Data were compared to the release of IL-1β from whole blood incubated with increasing concentrations of LPS. The variability of IL-1β release between the experiments was due to donor variability. IL-1β release in the cell compartment was 5.9 ± 2.7 pg/ml which is very close to the detection limit of the ELISA and far below the release caused by a LPS concentration of 1 ng/ml.

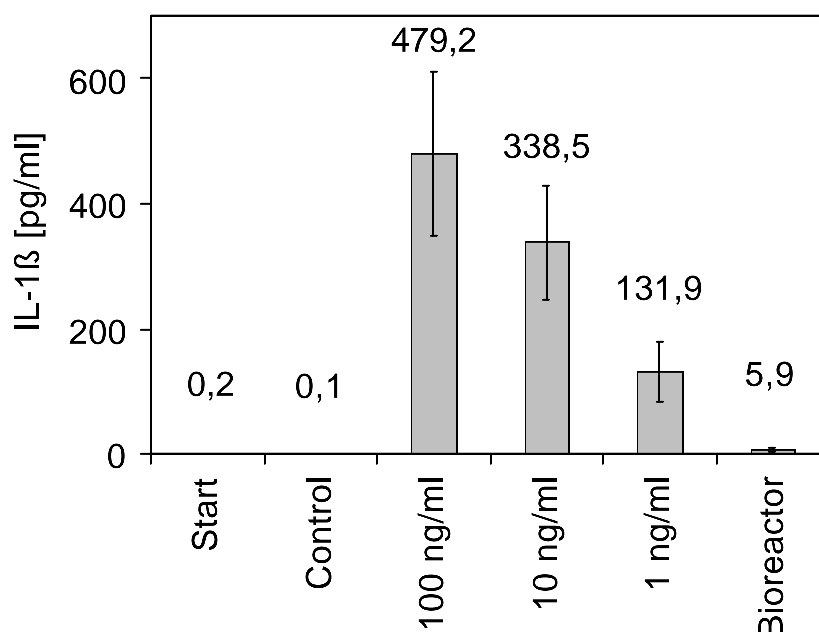


**Fig. 17** Release of IL-1ß into plasma after incubation of the hollow-fiber bioreactor with 2 ml of whole blood for 3 hours (mean of n = 6 experiments ± SD). Concentration of IL-1ß in plasma was close to the limit of detection. Blood incubated in 6-well-plates served as controls. Incubation of whole blood with increasing amounts of LPS resulted in a concentration dependent release of IL-1ß into plasma.

### 4.4.2 Growth of PHA stimulated lymphocytes in the hollow-fiber bioreactor is comparable with growth in culture plates

Growth of lymphocytes in the hollow-fiber bioreactor was compared to growth in 6-well-plates throughout the culture process by microscopy. Cells were harvested and analyzed at day 3, 6 and 7 [Fig. 18a]. Variability in cell growth between experiments was largely due to donor variability and the cell count obtained in the hollow-fiber bioreactor at each time point was comparable to the 6-well-plate. After 7 days of culture, proliferation of large cell clusters between the perfusion membranes was observed in the cell compartment of the bioreactor [Fig. 18b]. This demonstrates the possibility to analyze the cells in the cell compartment by microscopy.

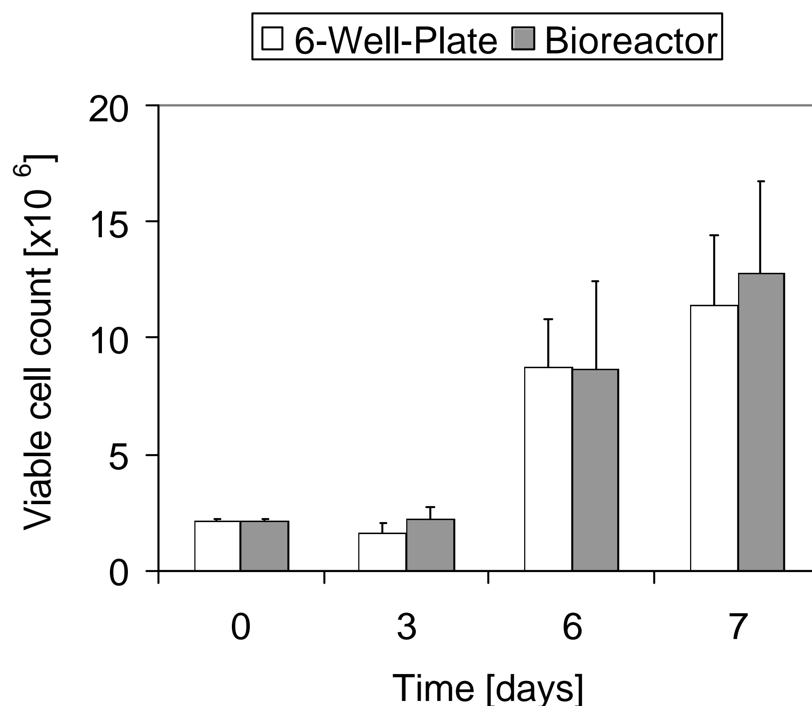


**Fig. 18a** Growth of PHA-stimulated lymphocytes in the hollow-fiber bioreactor compared to culture in 6-well-plates. 2 x $10^6$ cell were inoculated at the start of the experiment. Hardly any growth was observed until day 3 (mean of n = 3 experiments ± SD). On day 6 and day 7 (mean of n = 4 experiments ± SD) cell count was similar for bioreactor and 6-well-plates.

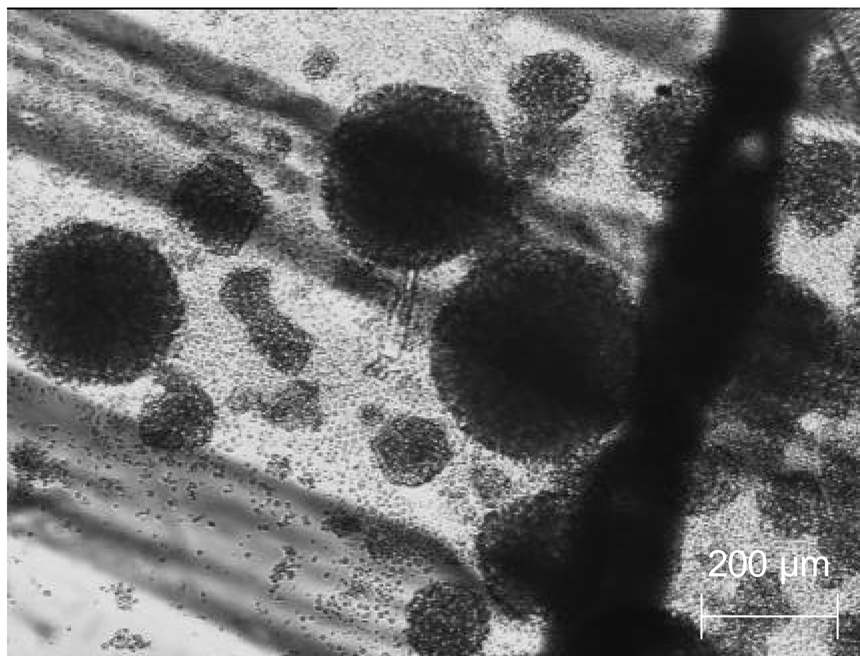


**Fig. 18b** Microscopy of PHA stimulated lymphocytes after 7 days of culture starting with 2 x $10^6$ cells in the hollow-fiber bioreactor.

## 4.4.3 The perfusion membrane is permeable for the cytostatic drug Ara-C

When stored in tubes at 23°C (i.e. room temperature) and at 37°C Ara-C showed no loss of activity after 3 days. In the bioreactor system, no loss of activity was observed compared to fresh Ara-C solution, thus indicating that Ara-C also did not adsorb e.g. to the tubing materials in the medium compartment (Tab. 3). After the cytostatic drug Ara-C had been put into the medium compartment of the bioreactor system, samples were taken from the cell and the medium compartment of the bioreactor system to determine if equilibrium between the compartments was reached after 24 hours. The samples were incubated with HL-60 cells for 3 days and compared to controls. Viability, as determined with the Alamar Blue™ assay, was around 15% - 20% for all samples proving equilibrium between medium and cell compartment [Fig. 19]. Since no growth inhibition occurred when 10 ml of culture medium was recirculated in the bioreactor system we can conclude that no growth inhibitory substances were eluted into our system within 24 hours of recirculation at 37°C (data not shown).

**Tab. 3** Viability of HL-60 cells after incubation with stored or freshly diluted Ara-C solutions measured by Alamar Blue™ assay, indicating stability of different concentrations of Ara-C after 3 days. (mean of 3 experiments ± SD)

| | 1 µM | 10 µM | 100 µM |
|---|---|---|---|
| Fresh | 78.8 ± 3.4 | 55.8 ± 3.6 | 7.2 ± 0.5 |
| 23° C | 73.3 ± 1.3 | 53.9 ± 2.5 | 6.6 ± 0.4 |
| 37° C | 76.6 ± 3.9 | 54.7 ± 1.9 | 6.4 ± 0.4 |
| Tubing system | | 61.6 ± 1.4 | |

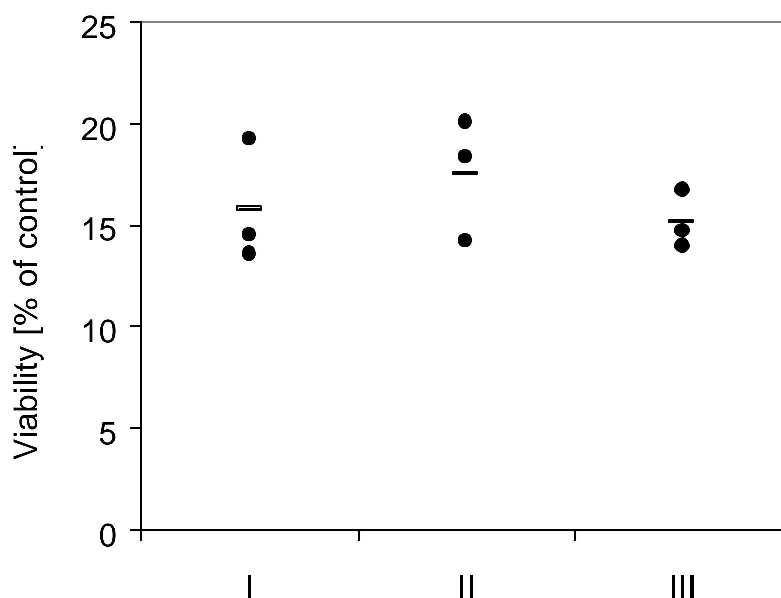


**Fig. 19** Effect of Ara-C on viability of HL-60 after incubation of samples obtained from the cell and the medium compartment of the hollow-fiber bioreactor. Samples were taken after recirculation of Ara-C-containing cell culture medium (50 µM) for 24 hours at 37°C. Viability was measured with the Alamar Blue™ assay. No Ara-C was present in the cell compartment at the start of the experiment. Controls (cell culture medium with 50 µM Ara-C) were samples stored in tubes for 24 hours at 37°C. (I = medium compartment, II = Cell compartment, III = control; ● single experiment, - mean of n = 3 experiments).

### 4.4.4 Growth inhibition of HL-60 cells by Ara-C following continuous exposure in the bioreactor system is dose dependent and correlates with conventional culture

The effect on HL-60 cells was dose dependent over a range of 100 µM to 0.4 µM Ara-C as determined by the Alamar Blue™ assay. The dose-response curve obtained in 96-well-plates is shown in Fig. 20.

When 3 different concentrations of Ara-C were recirculated for 3 days in the medium compartment with no Ara-C in the cell compartment at the start of the experiment, growth of HL-60 cells is dependent on the concentration of Ara-C present in the medium compartment. Cell count of surviving cells correlates with the amount of apoptotic cells found in the cell compartment [Fig. 21a]. The dose-response in the hollow-fiber bioreactor is comparable to the dose-response curve obtained in 96-well microtitre plates. Different end-points were used to confirm these conclusions [Fig. 21b].

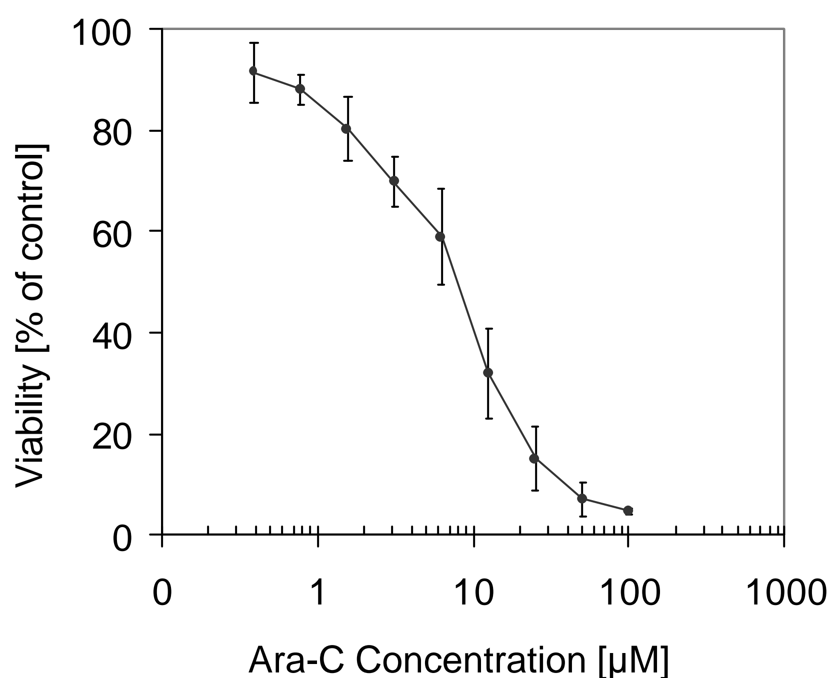


**Fig. 20** Dose-response curve for HL-60 cells obtained after continuous incubation with different concentrations of Ara-C in 96-well-plates for 3 days. Viability of the cells was measured by the Alamar Blue$^{TM}$ assay (mean of n = 3 experiments ± SD) .

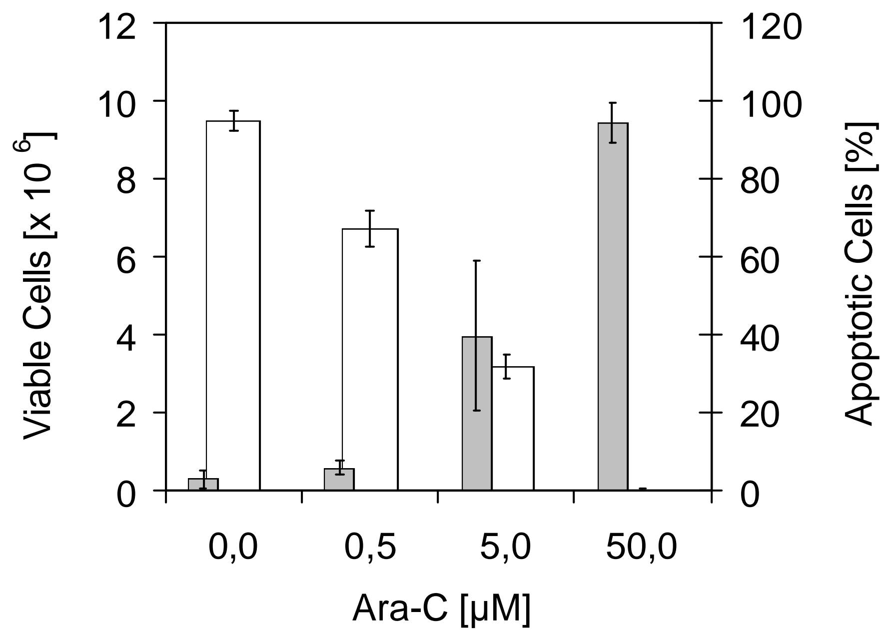


**Fig. 21a** Viability of HL-60 cells after 3 days of recirculation of 3 different concentrations of Ara-C (0.5, 5.0 and 50 µM) in the medium compartment of the hollow-fiber bioreactor system (mean of n = 3 experiments ± SD). Absolute viable cell count (□) versus percentage of apoptotic cells measured by PI staining (■).

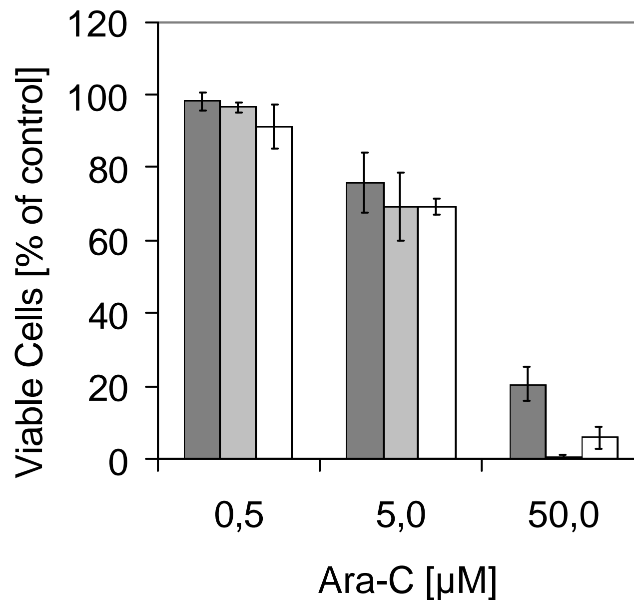


**Fig. 21b** Viability of HL-60 cells after 3 days of recirculation of 3 different concentrations of Ara-C (0.5, 5.0 and 50 µM) in the medium compartment of the hollow-fiber bioreactor system (mean of n = 3 experiments ± SD). Cells were analyzed by three independent methods for determinations of viability against cells cultivated in a bioreactor system without Ara-C.

## 4.5 Discussion

### 4.5.1 Cells can be cultured pyrogen-free in the bioreactor

IL-1β is a proinflammatory cytokine that is released by activated monocytes into plasma after stimulation with e.g. endotoxin and other pyrogens (Dinarello, 1984). Thus, IL-1β release has been proposed as an alternative, highly sensitive pyrogen test in the biocompatibility evaluation of medical devices in contact with blood (Herbelin et al., 1992; Fennrich et al., 1999). Many human primary cell types are sensitive to pyrogens and thus pyrogen contamination may disturb the culture process. In addition, the bioreactor is a more complex device compared to a culture plate with several different materials and manufacturing steps which carry the risk of contaminating the device. By incubation of the cell compartment with human whole blood, no pyrogen contamination was detectable which excludes non-specific stimulation of cells in our system. Thus, the hollow-fiber bioreactor system could not only be maintained sterile for at least 4 weeks (data not shown) but can also be regarded as pyrogen-free during operation.

### 4.5.2 Growth of PHA-stimulated lymphocytes in the hollow-fiber bioreactor

To investigate the hollow-fiber bioreactor system with regard to growth of primary cells, isolated human lymphocytes were stimulated with PHA and cultivated for 7 days in the bioreactor system. Culture of human lymphocytes in hollow-fiber bioreactors have been described before (Knazek et al., 1990; Hillman et al., 1994; Lewko et al., 1994; Lamers et al., 1999). Plant mitogens can be used *in vitro* as well as clinically as activators of lymphocytes (Wimer, 1997). Following stimulation by PHA, IL-2 is produced by T-cells, which is an essential growth factor for T-lymphocytes. IL-2 promotes entry of quiescent cells into the cell cycle, thus advancing clonal proliferation of activated T-lymphocytes *in vivo* and *in vitro*. To support proliferation of cells in conventional culture, IL-2 has to be added to the cells in the culture plates, which results in disturbance of the culture. In addition, at higher cell densities medium has to be changed more often. In the hollow-fiber bioreactor system, high density culture of cells is supported since IL-2 and medium components are delivered continuously to the cells across the perfusion membrane. This offers a clear advantage in handling and makes possible further automation of culture procedures in the future.

### 4.5.3 Validation of the bioreactor system for toxicity studies

Since a hollow-fiber system is a more complex system compared to microtiter plates or similar devices we wanted to demonstrate the functionality of our device. In particular, membrane transport and adsorption properties were examined. First, stability of the drug for 3 day at either room temperature or 37°C and second, permeability of the perfusion membrane for the cytostatic drug Ara-C reaching equilibrium after 1 day of recirculation was shown [Fig. 19a]. Regarding stability we reconfirmed earlier *in vitro* data of (Bosanquet, 1989) in our system. Permeability of the Cuprophan® membrane for Ara-C, which was also used in the bioreactor, has been demonstrated in detoxification studies of patients on hemodialysis, also for other cytostatic drugs (Sauer et al., 1990). No loss of drug activity e.g. by adsorption was observed in the medium compartment reconfirming earlier investigations with an infusion set containing silicone, PVC and PVC/PE material (Chevrier et al., 1995). Thus, drugs can be delivered from the medium compartment into the cell compartment as demonstrated for the cytostatic drug Ara-C.

### 4.5.4 Dose-response curve of Ara-C obtained in the bioreactor is comparable with conventional culture plates

Ara-C is an antimetabolite that is incorporated into DNA thus inhibiting DNA polymerase (Wiley et al., 1982; Zittoun et al., 1989). To obtain a dose-response curve for Ara-C, the Alamar Blue™ assay was used which has been described previously to determine drug effects on cancer and other diseases (Seki et al., 1998). The Alamar Blue™ dye is an indicator of the cellular redox state. The dye is reduced from a non-fluorescent form to a fluorescent product due to metabolic activity of the cultured cells (Fields and Lancaster, 1993). Thus, it can be used for drug sensitivity tests *in vitro* (Pagé et al., 1993). In our experiments HL-60 cells showed, as expected, a decrease of viability depending on the Ara-C concentration used. Continuous exposure of HL-60 to Ara-C for 3 days in the hollow-fiber bioreactor resulted in a dose-response curve similar to conventional culture. Cell count of viable cells correlated inversely with the count of apoptotic cells after 3 days. Determination of viability with 3 different methods gave comparable results demonstrating that many different readouts can be performed with the hollow-fiber bioreactor system e.g., after drug testing.

### 4.5.5 The bioreactor concept is modular and flexible

The modular concept of a bioreactors system allows realization of a variety of different design properties. For example, with the use of membranes with different cut-offs, culture conditions in the cell compartment can be controlled either by selective delivery or retention of substances e.g. for chemosensitivity testing in the presence of cytokines (te Boekhorst et al., 1993). As shown in chapter 2 [Fig. 7] time-dependent concentration profiles can be realized with the hollow-fiber bioreactor described. Thus, pharmacokinetic aspects can be incorporated into *in vitro* drug testing. The integration of an oxygenation membrane offers several additional advantages. First, pH can be controlled via $pCO_2$, second oxygenation status of the cell culture in the cell compartment can be varied. Thus, the investigation of the influence of $pO_2$ on cell growth and inhibition of cell growth will be easily possible. Third, toxicity testing of gaseous substances can be performed since such molecules can be delivered to the cells across the oxygenation membrane by controlled perfusion of the cell compartment. Moreover the bioreactor can be run in recirculation mode as well as in single-pass and based on the modular concept of the system, parallel testing of samples is feasible. The combination of these features may lead to an improved *in vitro* system for toxicity testing by more closely resembling the *in vivo* situation. Whereas several distinct advantages of the new system have been demonstrated, more work has to be done to promote *in vitro* systems in toxicity testing and drug development further and to reduce the need for animal tests.

# Chapter 5 Discussion

## *5.1 The bioreactor meets the requirements for advanced cell culture*

### 5.1.1 Hollow-fiber bioreactor technology improves culture conditions for cells

Perfusion cell culture is an advantage e.g. for antibody production (Gorter et al., 1993), for the cultivation of hematopoietic stem cells (Koller et al., 1993b; Sandstrom et al., 1995) and a prerequisite for tissue engineering, e.g. of cartilage (Sittinger et al., 1994). As cell growth is correlated with a decrease of e.g. glucose and glutamine and an increase of lactate and ammonium (Schumpp and Schlaeger, 1992), the achievable maximum of cell density in conventional culture is limited. By realizing a continuous supply of fresh medium and removal of toxic metabolic by-products, an increased perfusion rate also leads to an increased cell and antibody yield (Velez et al., 1987). As can be read from Fig. 6, a continuous supply of the cultured cells with nutrients is realized in the bioreactor described. This allows culture of cell suspensions in higher densities compared to conventional culture [Fig. 5a + 5b]. Moreover, retention of cytokines [Fig. 8a + 8b] contributes to the generation of a culture environment more closely resembling the *in vivo* situation. Oxygenation of the culture realized by integration of oxygenation membranes into the hollow-fiber bioreactor [Fig. 3] additionally supports high density culture of cells (Gerlach et al., 1990a). The system is thus „simulating an *in vivo* capillary system for *in vitro* growth of cells“ (Lipman and Jackson, 1998).

### 5.1.2 Variation of cell growth by flow-rate

In static cultures cell growth rate depends mainly on cell count at the start of the culture (Kaesehagen et al., 1991). Cells in a hollow-fiber bioreactor operated at zero flow should grow similar to cells in a static system. Since a perfused hollow-fiber bioreactor system allows the cultivation of cells at higher densities compared to a static system it was interesting to study the influence of flow-rate on growth. In the system described the growth rate of the cell line CCRF-CEM depends on flow rate from 0 up to 50 ml/h [Fig. 9a] and glucose concentration decreases only from 10 mM to about 8 mM [Fig. 9b]. A similar observation has not been described before. In (Velez et al., 1987) just two feeding flow rates were studied but the information is to

scarce to draw a general conclusion. It is interesting to speculate what limits the static compared to the perfusion system. Based on the slightly higher cell density of the bioreactor operated with flow zero compared to the 24-well-plate one would favour the better oxygenation due to the presence of the oxygenation membrane in the bioreactor as a cause for the improved growth also at higher flow-rates in the bioreactor.

### 5.1.3 *In vitro* tumor models

Higher cell densities compared to conventional culture can be achieved for leukemic cell lines by using the hollow-fiber bioreactor described. This may offer an advantage, as for some primary leukemic cells, like AML cells, high cell density (Maruyama et al., 1990) and also better cell-cell contact (Reilly et al., 1989) improve colony formation. Also, for solid tumors three dimensional tumor models more closely mimic the *in vivo* situation. Moreover, as it has been shown that histotypic association of tumor cells of human colon adenocarcinoma cells is supported by hollow-fiber bioreactor technology (Rutzky et al., 1979), hollow-fiber bioreactor technology seems to be of advantage in tumor modelling.

Three-dimensional growth of adherent cells can be enhanced by using biodegradable polymer matrices, as shown in the immunoencapsulation technique and in tissue engineering of cartilage or smooth muscle tissue (Freed et al., 1993; Baldwin and Saltzman, 1996; Kim and Mooney, 1998). Synthetic polymer foams can serve as an internal matrix, offering an appropriate microenvironment to the cells (Li et al., 1998). Polyester fibers incorporated into an artificial liver support system (Flendrig et al., 1997a) or polymer sheets in a perfusion culture device (Minuth et al., 1992) have been shown to provide a suitable adhesion matrix for cells.

Therefore, a fibrous adhesion support for adherent cells was also incorporated into the hollow-fiber bioreactor supporting three dimensional growth of anchorage dependent cells (Gloeckner et al., 2000a). Further development of the hollow-fiber bioreactor and further cell culture experiments are needed to prove the suitability of these fibers for the growth of solid tumors, which should ultimately lead to the creation of improved three-dimensional tumor models.

## *5.2. Bioprocess optimization, monitoring, and GMP*

### 5.2.1 The bioreactor as a tool in process optimization

The miniaturized bioreactor can be used for the evaluation of optimal culture conditions of various cell types. Nutrient composition can be varied stepwise or protocols can be developed for repeated and automated feeding of cell cultures aiming at higher product yields (Hu et al., 1987). Optimization of the oxygen content in the culture can be performed easily in the system described by using the oxygenation membranes to deliver a controlled gas stream.
Also, using two or more types of perfusion membranes with different cut-offs in the same bioreactor, which would of course require constructive changes, one could achieve selective delivery, removal and retention of different e.g. growth factors in the same housing. A miniaturized system could safe costs and materials in the process optimization phase. Once optimized culture conditions for the desired cell type are known, the device should be up-scaled (Gramer and Poeschl, 1998).

### 5.2.2 Bioprocess monitoring

In high density cultures, monitoring of cell count and growth is essential for optimal bioprocess engineering (Margis and Borojevic, 1989; Konstantinov et al., 1996). Besides microscopy of the cells which can be performed at any desired time-point of culture (see chapter 2), viability of the cells can be evaluated by taking samples of the medium compartment, which minimizes disturbance of the culture in the cell compartment. Glucose consumption and lactate generation are often used in hollow-fiber bioreactor systems to monitor cell growth (Knazek et al., 1990; Nayak and Herman, 1997). In chapter 3 a more sensitive method using the dye Alamar Blue$^{TM}$ is described, especially for systems with low cell numbers. However, with the Alamar Blue$^{TM}$ assay cell death cannot be accurately detected, probably due to the fact that the color is stable once color changed (Ahmed et al., 1994; Squatrito et al., 1995). A monitoring alternative could be a highly specific sensor system incorporated into the hollow-fiber bioreactor since sensor technology is also a developing field for bioprocess control (Scheper, 1992; Blankenstein et al., 1994; Konstantinov et al., 1994; Sonnleitner, 2000).

### 5.2.3 Good manufacturing practice (GMP)

Hollow-fiber bioreactors can be incorporated into closed systems, which offer decise advantages in terms of the maintenance of sterility in the clinical and therapeutical transplantation of cells or cell-derived products. Hollow-fiber bioreactors might contribute to optimal quality control especially when combined with bioprocess monitoring (see 5.3.2) and control for GMP-production processes (Bosse et al., 1997; Cao et al., 2000; Doblhoff-Dier and Bliem, 1999). Also, bioreactor technology can replace in an economic way manual and labor intensive procedures through automated cell culture in closed systems required for GMP.

## *5.3 Possible applications of the miniaturized hollow-fiber bioreactor*

### 5.3.1 Improved culture system for growth and expansion of cells

Besides culture of hybridoma cells for small scale antibody production (Lipman and Jackson, 1998) as a "classical" application of hollow-fiber bioreactor technology (Knazek et al., 1972), the expansion of rare cell populations warrants future investigations. Culture of genetically modified cells, e.g. transduced lymphocytes (Stroncek et al., 1999) is supported by hollow-fiber bioreactor technology, and should be possible also in the miniaturized hollow-fiber bioreactor described [Fig. 18a + 18b]. In conventional culture, feeding procedures may lead to the dilution or even complete removal of autocrine growth factors, e.g. of GM-CSF important for the autonomous growth of the blast cell population in AML samples (Bradbury et al., 1990), or also of added factors needed in cultivation of stem cells (Koller et al., 1993a; Palsson et al., 1995). Retention of both, cells and also of soluble factors, in the cell compartment has been shown for GM-CSF and IL-3 [Fig. 8a + 8b]. In addition, retention of cytokines translates into cost savings since less cytokines can be used combined with the other advantages of a perfusion system.

### 5.3.2. Test system for toxicity testing

*General toxicity testing and replacement of animal testing*

There is an ongoing attempt to replace animal testing, e.g. in toxicity testing (Balls, 1994; Festing, 1999) based on the concept of the three R´s (reduction, refinement and replacement) (Russel and Burch, 1959). This effort is not only driven by ethical considerations, but also to avoid interspecies variation (Mattern et al., 1988), to provide superior *in vitro* models and last but not least, to save costs. High cell

densities, supporting three-dimensional growth of cells, approaching tissue organisation, should be an advantage overcoming the limitation of conventional culture systems also for drug and toxicity testing.

The hollow-fiber bioreactor allows parallel testing of different substances [Fig. 16]. Due to the small culture volume, which can be minimized to at least 500 µl, also cell material can be saved. Pharmacokinetic parameters (Gimmel and Maurer, 1994) can be taken into consideration and different application schemes (e.g. oral, intravenous) can be studied *in vitro* in order to optimize or rationalize therapeutic regimes. In hollow-fiber bioreactor systems drug combination testing (Aapro, 1984) can be easily performed because the active components can be delivered to and removed from the cells automatically by manipulation of the perfusion path (see chapter 4). To realize time-dependent profiles of test substances resembling the *in vivo* plasma concentration of drugs, perfusion systems provide the best solution *in vitro* (Unowsky et al., 1989). Additionally, combination therapies can be tested easily in perfusion systems, as drugs can be sequentially delivered and removed from the cell culture to mimic *in vivo* clearance of the drug.

Application and testing of gaseous substances is feasible using oxygenation membranes. Combined with undisturbed, long-term culture the miniaturized hollow-fiber bioreactor should be a device for optimized toxicity testing. Monitoring can be performed e.g. by use of the Alamar Blue$^{TM}$ assay (see chapter 3).

High density culture should also lead to improved co-culture systems of different cell types. Toxicity testing will be faciliated, as e.g. stroma-supported culture of childhood B-lineage ALL cells predicts treatment outcome (Kumagai et al., 1996). Co-culture systems can also contribute to evaluate the therapeutic window of a new drug by mimicking the *in vivo* situation of tumor and extratumoral tissue exposed to cytostatic chemotherapy (El Mir et al., 1998). However, these prospects as well as complex tissue models have to be evaluated in further studies.

*Evaluation of chemotherapeutics for cancer therapy*

Tumor cells encapsulated into hollow-fibers have been used as a screening system for chemotherapeutic drugs, either *in vitro* (Casciari et al., 1994) or *in vivo* (Hollingshead et al., 1995). By implanting the encapsulated cancer cells, pharmacokinetic profiles are realized and parallel testing of substances within the same host becomes feasible. But again interspecies variability has to be considered as a serious drawback. Based on the benefits discussed above, the hollow-fiber

bioreactor should have its place as a better alternative to current test systems, not only for the development of new drugs, but also as a test system for patient specific therapies.

*Individualized chemotherapy in the treatment of malignancies*

Resistance of tumors to chemotherapeutic agents is an important factor limiting the successful treatment of a wide range of malignant diseases. Therefore, different attempts have been made to individualize chemotherapy for leukemias as well as for solid tumors, by using various *in vitro* culture systems and end-points (Finlay et al., 1986; Kern and Weisenthal, 1990; Von Hoff et al., 1990; Von Hoff et al., 1991; Dietel et al., 1993; Bosanquet and Bell, 1996). Other approaches monitor active drug levels in the patients serum to include individual clearance rates into the therapy (Evans et al., 1998). *In vitro* testing of sensitivity aims at stratifying patients into the appropriate risk groups, e.g. according to their individual resistance profiles to different drugs (Pieters et al., 1994) and identifying high-risk patients early in the clinical course (Kumagai et al., 1996). The use of prognostic markers on a cellular basis (Vendrik et al., 1992) like phenotyping of the patients MDR status is also feasible (Sauerbrey et al., 1994; Del Poeta et al., 1996; Leith, 1998). Also genetic criteria (Mandelli et al., 1998) may help in the stratification, e.g. to identify individual resistance profiles by microarray technology (Kononen et al., 1998). Many studies have correlated the *in vitro* results of sensitivity testing with chemotherapy outcome either in prospective trials (Sanfilippo et al., 1989) or retrospectively (Kern and Weisenthal, 1990; Bosanquet, 1994). In summary, the results show a better prognosis of resistance than of sensitivity (Bertelsen et al., 1984; Dietel et al., 1993; Furukawa et al., 1995).
Since cell harvest can be performed easily in the new hollow-fiber bioreactor (chapter 4) any analysis of the cells can, in principle, be performed after harvest. Alternatively, the fluorescent dye Alamar Blue™ can be applied in the medium compartment (see chapter 3). Further investigations will attempt to cultivate primary patient cells and to remove drugs from the system and cultivate resistant cell clones further. Combined with the advantages mentioned in chapter 5.3 the miniaturized hollow-fiber bioreactor represents an improved tool for toxicological and pharmacological investigations (Gloeckner et al., 2000b).

# Summaries

***Summary***

Monolayer or suspension cell cultures are of only limited value as experimental models for human cancer. Therefore, more sophisticated, three-dimensional culture systems like spheroid cultures or histocultures are used, which more closely mimic the tumor in individual patients compared to monolayer or suspension cultures. As tissue culture or tissue engineering requires more sophisticated culture, specialized *in vitro* techniques may also improve experimental tumor models.

In the present work, a new miniaturized hollow-fiber bioreactor system for mammalian cell culture in small volumes (up to 3 ml) is characterized with regard to transport characteristics and growth of leukemic cell lines (chapter 2). Cell and medium compartment are separated by dialysis membranes and oxygenation is accomplished using oxygenation membranes. Due to a transparent housing, cells can be observed by microscopy during culture. The leukemic cell lines CCRF-CEM, HL-60 and REH were cultivated up to densities of 3.5 x $10^7$/ml without medium change or manipulation of the cells. Growth and viability of the cells in the bioreactor were the same or better, and the viable cell count was always higher compared to culture in Transwell® plates. As shown using CCRF-CEM cells, growth in the bioreactor was strongly influenced and could be controlled by the medium flow rate. As a consequence, consumption of glucose and generation of lactate varied with the flow rate. Influx of low molecular weight substances in the cell compartment could be regulated by variation of the concentration in the medium compartment. Thus, time dependent concentration profiles (e.g. pharmacokinetic profiles of drugs) can be realized as illustrated using glucose as a model compound. Depending on the molecular size cut-off of the membranes used, added growth factors like GM-CSF and IL-3 as well as factors secreted from the cells are retained in the cell compartment for up to one week.

Second, a method for monitoring cell proliferation the hollow-fiber bioreactor by use of the Alamar Blue™ dye was developed (chapter 3). Alamar Blue™ is a non-fluorescent compound which yields a fluorescent product after reduction e.g. by living

cells. In contrast to the MTT-assay, the Alamar Blue$^{TM}$-assay does not lead to cell death. However, when not removed from the cells, the Alamar Blue$^{TM}$ dye shows a reversible, time- and concentration-dependent growth inhibition as observed for leukemic cell lines. When applied in the medium compartment of a hollow-fiber bioreactor system, the dye is delivered to the cells across the hollow-fiber membrane, reduced by the cells and released from the cell into the medium compartment back again. Thus, fluorescence intensity can be measured in medium samples reflecting growth of the cells in the cell compartment. This procedure offers several advantages. First, exposure of the cells to the dye can be reduced compared to conventional culture in plates. Second, handling steps are minimized since no sample of the cells needs to be taken for readout. Moreover, for the exchange of medium, a centrifugation step can be avoided and the cells can be cultivated further. Third, the method allows to discriminate between cell densities of $10^5$, $10^6$ and $10^7$ of proliferating HL-60 cells cultivated in the cell compartment of the bioreactor. Measurement of fluorescence in the medium compartment is more sensitive compared to glucose or lactate measurement for cell counts below $10^6$ cells/ml, in particular. In conclusion, the Alamar Blue$^{TM}$-assay combined with the hollow-fiber bioreactor offers distinct advantages for the non-invasive monitoring of cell viability and proliferation in a closed system.

In chapter 4 the use of the hollow-fiber bioreactor as a tool for toxicity testing was investigated, as current models for toxicity as well as efficacy testing of drugs *in vitro* allow only limited conclusions with regard to the *in vivo* situation. Examples of the drawbacks of current test systems are the lack of realistic *in vitro* tumor models and difficulties to model drug pharmacokinetics. The bioreactor proved to be pyrogen free and is steam-sterilizable. Leukemic cell lines like HL-60 and primary cells such as PHA-stimulated lymphocytes can be grown up to high densities of 1-3 x $10^7$ and analyzed during growth in the bioreactor by light-microscopy. The cytostatic drug Ara-C shows a dose-dependent growth inhibition of HL-60 cells and a dose-response curve similar to controls in culture plates. The bioreactor system is highly flexible since several systems can be run in parallel, soluble drugs can be delivered continuously via a perfusion membrane and gaseous compounds via an oxygenation membrane which also allows to control $pO_2$ and pH (via $pCO_2$) during culture in the cell compartment.

The modular concept of the bioreactor system allows realization of a variety of different design properties, which may lead to an improved *in vitro* system for toxicity testing by more closely resembling the *in vivo* situation. Whereas several distinct advantages of the new system have been demonstrated, more work has to be done to promote *in vitro* systems in toxicity testing and drug development further and to reduce the need for animal tests.

***Zusammenfassung***

Konventionelle Zellkulturmethoden, wie Monolayer- oder Suspensionskulturen weisen im Vergleich zu dreidimensionalen Kultursystemen (z.B. Sphäroid- oder Gewebekultur) wesentliche Limitationen auf. So sind *in vitro* Systeme als Modelle für humane Tumore häufig ungeeignet, besonders im Hinblick auf die Wirkstofftestung von Zytostatika. Dreidimensionale Kulturmodelle, die dem Verhalten von Tumoren *in vivo* besser entsprechen, erfordern technisch ausgereiftere Kulturtechniken als die konventionelle Zellkultur. Diese könnten dazu beitragen, eine dreidimensionale Kultur von Gewebe und dadurch *in vivo* ähnliche Bedingungen zu realisieren.

In der vorliegenden Arbeit wurde ein neuentwickelter, miniaturisierter Hohlfaserbioreakor hinsichtlich seiner Transportcharakteristik, sowie bezüglich des Wachstums von leukämischen Zellinien untersucht (Kapitel 2). Der Zellkulturraum, mit einem Volumen von bis zu 3 ml, ist durch Dialysemembranen vom Mediumkompartiment getrennt. Eine zusätzliche Oxygenierung der Zellkultur erfolgt über Oxygenationsmembranen. Aufgrund der Verwendung eines transparenten Gehäuses können die Zellen während der Kultur mikroskopisch beobachtet werden. Die leukämischen Zellinien CCRF-CEM, HL-60 und REH konnten in dem neuen Hohlfaserbioreaktor in Zelldichten bis 3.5 x $10^7$/ml kultiviert werden, ohne daß ein Mediumwechsel oder eine andere Manipulation der Zellkultur notwendig war. Das Wachstum und die Vitalität der Zellkulturen war vergleichbar oder besser als von Kontrollen in Transwell® Kulturen. Wie für die Zellinie CCRF-CEM gezeigt werden konnte, war das Wachstum der Zellen abhängig von der Mediumflußrate und konnte durch deren Variation kontrolliert werden. Daraus resultierte auch ein veränderter Glukoseverbrauch und eine veränderte Laktatproduktion der Zellen. Der Eintrag von niedermolekularen Substanzen in den Zellkulturraum konnte durch die Variation der Konzentration der Substanz im Mediumkompartiment reguliert werden. Auf diese

Weise können zeitabhängige Konzentrationsprofile, z. B. pharmakokinetische Profile von Wirkstoffen, realisiert werden, wie mit der Modellsubstanz Glukose gezeigt wurde. Abhängig vom molekularen Cut-off der verwendeten Membranen, werden im Zellkulturraum sowohl zugegebene, als auch autokrine Faktoren für bis zu einer Woche zurückgehalten, wie für GM-CSF oder IL-3 gezeigt wurde.

Weiterhin wurde eine Methode entwickelt, um in dem miniaturisierten Hohlfaserbioreaktor die Zellproliferation mittels des Farbstoffes Alamar Blue™ zu ermitteln (Kapitel 3). Alamar Blue™ ist ein nicht-fluoreszierender Farbstoff, der nach Reduktion durch z.B. lebende Zellen in ein fluoreszierendes Produkt umgewandelt wird. Im Gegensatz zum MTT-Assay, führt der Alamar Blue™-Assay jedoch nicht zum Zelltod. Wird der Farbstoff nicht aus der Zellkultur entfernt, zeigt sich eine reversible, zeit- und konzentrationsabhängige Wachstumsinhibiton der Zellen, wie für leukämische Zellinien gezeigt werden konnte. Verwendet man den Farbstoff im Mediumkompartiment eines Hohlfaserbioreaktor-System, wird er über die Hohlfasermembran zu den Zellen angeliefert, von den Zellen reduziert, und über die Membran wieder in das Mediumkompartiment abgeführt. Auf diese Weise reflektiert die Zunahme der Fluoreszenz im Mediumkompartiment das Wachstum der Zellen im Zellkulturraum. Das Verfahren bietet mehrere Vorteile: Erstens kann der Kontakt der Zellen mit dem Farbstoff im Vergleich zur konventionellen Zellkultur reduziert werden und das notwendige Handling wird minimiert, da keine Probennahme aus der Zellkultur zur Auswertung erforderlich ist. Zweitens ist zum Austauschen des Mediums kein Zentrifugationsschritt notwendig, so daß die Zellen ohne Störung weiterkultiviert werden können. Drittens erlaubt diese Methode eine Diskriminierung von Zelldichten von $10^5$, $10^6$ und $10^7$ proliferierenden HL-60 Zellen im Zellkulturraum des Bioreaktors. Es konnte gezeigt werden, daß die Fluoreszenzmessung im Mediumkompartment im Vergleich zur Messung von Glukose oder Laktat besonders für Zellzahlen unterhalb $10^6$ Zellen/ml sensitiver ist. Zusammenfassend bietet der Alamar Blue™-Assay in Verbindung mit dem Hohlfaserbioreaktor klare Vorteile für ein nicht-invasives Monitoring der Zellvitalität und Proliferation in einem geschlossenen System.

In Kapitel 4 wird die Verwendung des miniturisierten Hohlfaserbioreaktors als Modellsystem für toxikologische Untersuchungen beschrieben. Gegenwärtig fehlen realisitische *in vitro* Modelle, vor allem zur Modellierung von pharmakokinetischen

Profilen. Der Bioreaktor erwies sich als pyrogenfrei und dampfsterilisierbar. Leukämische Zellinien, z. B. HL-60 Zellen sowie Primärzellen, wie z. B. PHA-stimulierte Lymphozyten konnten in Zelldichten bis zu 1-3 x $10^7$ Zellen/ml kultiviert werden. Das Zytostatikum Ara-C wies eine dosisabhängige Wachstumsinhibition im Hohlfaserbioreaktor auf, wie für HL-60 Zellen gezeigt wurde. Die Dosis-Wirkungs-Kurve war vergleichbar dem Ergebnis in 96-Well-Platten. Das Bioreaktor System bietet eine hohe Flexibilität, da mehrere Systeme parallel untersucht werden können. Lösliche Substanzen können kontinuierlich über die Perfusionsmembran angeliefert werden und gasförmige Komponenten über die Oxygenationsmembran. Diese ermöglicht zudem eine Kontrolle des $pO_2$ und des pH-Wertes (via $pCO_2$) im Zellkompartiment während der Kultur.

Das modulare Konzept des Bioreaktor Systems ermöglicht die Realisierung unterschiedlicher Designs. Obgleich einige deutliche Vorteile des neuen Bioreaktorsystems gezeigt wurden, müssen weitere Untersuchungen durchgeführt werden, um den Einsatz von *in vitro* Systemen in der Entwicklung neuer Wirkstoffe voranzutreiben und die Notwendigkeit von Tierexperimenten zu verringern.

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# Appendix

## *Abbreviations*

| | |
|---|---|
| AML | Acute myeloid leukemia |
| Ara-C | Cytosine arabinoside |
| BrdU | 5-bromo-2´-deoxyuridine |
| CLL | Chronic lymphocytic leukemia |
| ELISA | Enzyme linked immunosorbent assay |
| FACScan | Fluorescence activated cell sorter |
| FCS | Fetal calf serum |
| GLP | Good laboratory practice |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HEPES | N-[2-Hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid] |
| IL-3 | Interleukin-3 |
| IL-3 | Interleukin-3 |
| LPS | Lipopolysaccharides |
| MNC | Mononuclear cells |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide |
| PBS | Phosphate buffered saline |
| PE | Polyethylene |
| PHA | phytohemagglutinin |
| PI | Propidium iodide |
| PS | Polystyrene |
| PVC | Polyvinylchloride |
| XTT | sodium3´-[(1-phenylamino)-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzene-sulfonic acid hydrate |

## Curriculum vitae

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### *Education*

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### *Profession*

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| 1987 – 1994 | Study of biology at the Technische Hochschule Darmstadt | |
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## Publications

### *Research Articles*

Gloeckner, H., T. Jonuleit, and H.D. Lemke. 2001. Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue™. *J. Immunol. Methods* 252:131-138.

***Gloeckner, H., and H.D. Lemke. 2001. New miniaturized hollow-fiber bioreactor for* in vivo *like cell culture, cell expansion and production of cell derived products.* Biotechnol Prog *(accepted for publication in April 2001)***

Gloeckner H., and H.D. Lemke. New Miniaturized Hollow-Fiber Bioreactor as a tool for *in vitro* toxicity testing and drug screening (manuscript in preparation).

### *Patent applications*

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