Bioprocess development for the cultivation of human T-lymphocytes in a clinical scale

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Abstract

Adoptive transfer of large numbers of donor-derived T-lymphocytes may offer a promising treatment of a variety of viral and malignant diseases. The key step in this approach is the *ex vivo* generation of sufficient quantities of these cells in a short time. We have investigated the influence of several important cultivation parameters on the proliferation of human T-lymphocytes to develop a large-scale fermentation process using different types of stirred bioreactors. Such systems offer many potential advantages over the static culture systems commonly used today. Peripheral blood mononuclear cells of healthy but CMV positive donors were stimulated with monoclonal antibodies (anti-CD3 and anti-CD28) and Interleukin-2. The influence of osmolality, Interleukin-2 concentration, pH, oxygen tension, feeding strategy and temperature on T-cell proliferation was investigated and the optimised conditions were transferred to a novel stirred suspension bioreactor with an especially designed magnetic stirrer to minimize the shear force (working volume 550 ml) and a standard stirred vessel (working volume 1000 ml). Preferable conditions for the cultivation of primary T-lymphocytes were an osmolality of 276–330 mOsmol kg⁻¹, an Interleukin-2 concentration of 100 U ml⁻¹, a pH range of 7.0 to 7.3, an oxygen tension of 5–50% and a temperature of 38.5 °C. After 238 h of cultivation 2.8×10^9 cells in the stirred vessel and 1.5×10^9 cells in the suspension bioreactor were obtained with a percentage of T-cells >94%. The specificity of the cells was maintained during cultivation as proven by IFN- γ secretion after exposure to a hCMV protein.

Introduction

T-lymphocytes are of increasing importance in adoptive immunotherapy of malignant diseases or viral infections as they offer a reduced toxicity compared to commonly used therapies and could provide prolonged protection (Bremers et al., 2000). One important target for immunotherapy is the Cytomegalovirus (CMV) which often causes severe, life threatening complications in immunosuppressed patients (e.g. after chemotherapy or bone marrow transplantation). Such disease might be prevented or treated by transplantation of a large number of autologous hCMV specific T-lymphocytes (Rosenberg et al., 1986; Curti, 1997; Yee et al., 1997). As their number in the patients

blood is originally very low, the *ex vivo* expansion of these cells without a loss of specificity is required.

Currently, in most clinical trial protocols static culture systems like T-flasks, culture trays or culture bags are used (Garland et al., 1999; Lamers et al., 1999; Pandolfino et al., 2001; Knazek et al., 1990), which imply disadvantages in nonuniform culture conditions, low cell densities and uncontrolled process parameters. Thus, static culture systems are not applicable to generate the required number of cells (10^9 to 10^{11}). In contrast stirred systems offer many potential advantages like homogeneity of culture conditions, ease of sampling and implementation of control systems (Carswell et al., 2000a; Hilbert et al., 2001).

But surprisingly little is known about optimal culture conditions of human T-lymphocytes in current

literature. Some aspects about Interleukin-2, oxygen tension and pH effects were examined by Carswell et al. (2000a–c), Krieger et al. (1996) studied the influence of oxygen tension, while Hilbert et al (2001) and Morecki et al. (1997) investigated feeding strategies on long-term cultures of T-lymphocytes. Indeed, there are some studies about a few important cultivation parameters, but there is missing an interrelated investigation.

In this study we have examined the influence of several important cultivation parameters to optimise and setup a fermentation process for primary T-cells in a clinical scale. Thereby osmolality, Interleukin-2 concentration, pH, oxygen tension, feeding strategy and temperature were assessed. Peripheral blood mononuclear cells (PBMC) from healthy, but CMV-positive donors were used. Utilizing the optimised cultivation parameters, T-lymphocytes were cultured in a stirred suspension bioreactor (working volume 50–550 ml) and in a standard stirred vessel (working volume 300–1000 ml). In addition to their number, cytotoxic function and immunogenicity of T-lymphocytes for adoptive immunotherapy are a matter of particular interest (Lamers et al., 1992; Morecki et al., 1997). Therefore specificity of the cells against a known hCMV protein was determined by applying an Interferon- γ (IFN- γ) secretion assay.

Materials and methods

Culture medium

ALPHA-MEM (Gibco BRL, Eggenstein, Germany) was used as standard culture medium, supplemented with 10% heat inactivated FCS (Gibco BRL, Eggenstein, Germany) and 100 U ml⁻¹ Interleukin-2 (from BHK 21 cells, kindly provided by Dr. Wagner, GBF, Braunschweig, Germany).

Isolation of T-lymphocytes

Mononuclear cells from the peripheral blood (PBMC) of healthy but CMV-positive donors were isolated using a Biocoll (Ficoll separating solution) (Biochrom KG, Berlin, Germany) density gradient. After two hours of plastic-adhesion (separation of monocytes) the non adherent fraction was used for cultivation.

Stimulation of T-lymphocytes

The stimulation of T-lymphocytes were performed in tissue culture flasks (Greiner, Frickenhausen, Germany). First anti-CD3 mAb (produced with the mouse hybridoma cell line OKT3, Forschungszentrum Juelich, Germany) was immobilized (5 μ g ml⁻¹; coated on tissue culture flasks; 2 h). Following the T-lymphocytes were seeded at 5×10^5 cells ml⁻¹ and stimulated for 4 days with immobilized anti-CD3 mAb and 500 ng ml⁻¹ anti-CD28 mAb (BD PharMingen, Heidelberg, Germany). After stimulation the cells were centrifuged and transferred into fresh culture medium at a cell density of 5×10^5 cells ml⁻¹.

Culture systems

Stimulation of PBMC as well as investigation of temperature, osmolality, feeding strategy and Interleukin-2 concentration were carried out in tissue culture flasks (Greiner, Frickenhausen, Germany), while the influence of pH and oxygen tension was investigated using the Cellferm-pro[®] spinner flask culture system (DAS-GIP mbh, Juelich, Germany). The scale-up using the optimized parameters was done in a standard stirred vessel (Applikon Biotek, Knuellwald, Germany) and in a stirred suspension bioreactor developed in our institute.

Cellferm-pro[®]

Cellferm-pro[®] is a modular working cultivation system allowing a parallel fermentation in up to 8 pH- and oxygen-controlled batch-, fedbatch- or continuously operating spinner flasks or bioreactors. All relevant parameters like time, pH, oxygen, temperature and oxygen-uptake-rate (OUR) are measured online.

Standard stirred vessel

A 1 l bioreactor (Applikon Biotek, Knuellwald, Germany) containing pH-, temperature- and oxygen probes (Mettler-Toledo, Steinbach, Germany) was used. Bubbelfree aeration was realised using a membrane (silicon tubings).

Stirred suspension bioreactor

The suspension reactor was a continuously operating stirred vessel with cell retention and a variable culture volume between 50–550 ml. The cell retention was realized by a stainless steel filter with a pore size <9 μ m, which was positioned directly above a specially designed low shear force magnetic stir bar.

Figure 1. Influence of osmolality on viability during batch cultivation of PBMC in T-flasks. Data are shown as mean $\pm$ standard deviation from three experiments from a single donor.

Electrodes for temperature, pH and pO_2 control were integrated (Schmidt et al., 1999).

Analytical methods

Cell counting and viability

Primary T-cells were counted using a CASY 1 particle counting system (Schaefer System, Reutlingen, Germany) and a haemocytometer. Viabilities were quantified applying the trypan blue dye exclusion method.

Flow cytometry

Culture samples of 2×10^5 cells were centrifugated, resuspended in 90 μ l PBS (phosphate-buffered-saline) and incubated with 5 μ l of the corresponding antibodies at 4 °C for 30 min. The cells were then washed with PBS. Three-color flow cytometric analysis was realized using a FACSCalibur cytometer (Becton Dickinson, Heidelberg, Germany) and Cellquest 3.1.

PBMC were phenotyped with the following monoclonal markers: CD3 (CyChrome[®]), CD4 (FITC, fluorescein isothiocyanate), CD8 (PE, phycoerythrin), CD16 (FITC), CD56 (PE), CD19 (FITC), CD14 (PE), CD25 (FITC) (all from BD PharMingen, Heidelberg, Germany). Negative controls consisted of PBMC labeled with mouse IgG1 (CyChrome/PE/FITC; BD PharMingen).

Metabolic analysis

Osmolality was measured using a freezing-point osmometer Osmomat 030 (Gonotec, Berlin, Germany). Glucose (Ebio compact, Eppendorf, Hamburg, Germany), lactate (YSI 1500L, Yellow Springs Instru-

ments, Yellow Springs, USA), glutamine and glutamate (YSI 2700 select, Yellow Springs Instruments, Yellow Springs, USA) were quantified enzymatically using the indicated automatical analyzer according to the manufacturers instructions.

Amino acids analysis was realized using HPLC (Amino Quant 1090 AX, Hewlett Packard, Waldbronn, Germany). The quantification of Interleukin-2 was carried out using an Interleukin-2 ELISA (OptEIA human IL-2 Set, BD PharMingen, Heidelberg, Germany).

Evaluation of CMV-specific T-lymphocytes

For determination of the portion of CMV-specific T-cells an IFN- γ Cytokine Secretion Assay (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturers instructions (Manz et al., 1995; Brosterhus et al., 1998). For stimulation of the cells the human CMV protein pp65 (AD 169 strain) was used.

Results

After isolation of PBMC of healthy but CMV-positive donors and separation of the adherent fraction the cells were stimulated with anti-CD3 mAb and anti-CD28 mAb for 4 days. This results in proliferation of CD3⁺ cells and finally a population containing more than 95% T-lymphocytes was obtained ($n = 4$). The CD3⁺CD4⁺ and CD3⁺CD8⁺ subpopulation ratio was conserved. The large majority of the cells (>90%) expressed the Interleukin-2 receptor α -chain (CD25).

Figure 2. FACS-analysis of the IL-2 receptor α -chain (CD25) on day 0 (after stimulation) and day 6. PBMC were cultured with 10, 50, 100 and 200 U ml^{-1} IL-2, respectively.

The portion of CD56 + cells (natural killers) was $<2\%$. These cells were used for further experiments.

Osmolality

Especially in batch fermentations the osmolality of the medium changes significantly due to the consumption of substrates and the segregation of metabolites. To set different osmolalities, culture medium was supplemented with 2 M sodium chloride solution and adjusted on 250, 276, 302 and 334 mOsmol kg^{-1} to investigate the influence on T-cell proliferation. Using triplicate T-flasks a batch cultivation of 250 h was done. While no significant influence on the cell density was observed, the growth rate using the medium with the lowest and the highest osmolality was slightly decreased (data not shown). Furthermore the cells cultivated with 334 mOsmol kg^{-1} showed a viability $<90\%$ after 50 h of cultivation (Figure 1). This indicates that an osmolality higher than 330 mOsmol kg^{-1} should be avoided. The culture media (ALPHA-MEM supplemented with 10% heat inactivated FCS) used in this study had an osmolality of 302 mOsmol kg^{-1} .

Interleukin-2 concentration

Proliferation and differentiation of activated T-lymphocytes is controlled by Interleukin-2, which is known as the main growth factor of T-cells. Stimulation with a specific MHC-antigen-complex or a polyclonal anti-CD3 mAb and a costimulatory signal (anti-CD28 mAb) induces the synthesis of the α -chain (CD25) resulting in the high affinity Interleukin-2 receptor consisting of an α -, β - and a γ -chain. To determine the necessary IL-2 concentration for maximum proliferation 10, 50, 100 and 200 U ml^{-1} were used in batch cultures in triplicate T-flasks. For 100 and 200 U ml^{-1} nearly identical cell densities (3.60 and $3.75 \times 10^6 \text{ cells ml}^{-1}$, respectively) were obtained after 10 days of culture, while the maximum cell densities for 10 and 50 U ml^{-1} were about 15% lower. The growth rate in media supplemented with 10 and 50 U ml^{-1} also decreased faster. The IL-2 concentration was determined several times during the cultivation. After 2 days of cultivation the samples for the 10 and 50 U ml^{-1} cultures were IL-2 limited, resulting in a reduced expression of the Interleukin-2 receptor α -chain (Figures 2 and 3).

Figure 3. Influence of the Interleukin-2 concentration on the IL-2R α -chain during cultivation of activated T-lymphocytes.

Feeding strategy

The *in vitro* proliferation of T-lymphocytes is not only influenced by growth factors like cytokines but also by substrate and metabolite concentrations. This is of particular interest as the metabolic activity of growing T-cells is very high. To investigate this aspect different frequencies of feeding have been compared. Daily, bi-daily or every 3rd day 50% of the culture medium were replaced. A batch fermentation was done as a control. The fed cultures proliferate much better than the batch cultures (Figure 4). However, in all experiments, except in the daily fed batch culture, substrate limitations (especially of glucose and glutamine) occurred and even in the daily fed culture a further supplementation of glucose seems to be recommendable although no acute limitation was observed (Data not shown).

Temperature

The initial idea to investigate the influence of temperature on the cultivation of human T-cells originates from a well known phenomenon of the *in vivo* defense against infections: fever. This systemic effect is caused by different cytokines like the endogen pyrogenes Interleukin-1, Interleukin-6 and tumor necrosis factor- α (TNF- α). With increasing temperature the human body supports the cellular and humoral response to the infections and tries to weaken the (microbial) intruder.

A number of experiments comparing different fermentation temperatures between 34.0 and 40.0 °C

showed a remarkable increase in the maximum growth rate at 38.5 °C compared to the commonly used temperature of 37.0 °C (Figure 5). A further increase in the temperature to 40.0 °C had an adverse effect (comparable to an *in vivo* life-threatening situation). At 34.0 and 35.5 °C a decrease in the proliferation was observed. These results were produced in triplicate T-flasks using three different donors.

pH

The fermentation system cellferm-pro[®] was used to compare different pH values (6.7, 7.0, 7.3 and 7.5, respectively) in four parallel batch cultures to investigate the influence of the pH on the proliferation of stimulated T-cells. After stimulation the PBMC were cultivated for 72 h to gain the necessary number of cells from a single donor. After that the spinner flasks were inoculated. The oxygen tension was controlled at 50% of air saturation.

Figure 6 pictures the run of the cell density curve during the cultivation. Within the exponential growth phase no significant differences have been observed between pH = 7.0 and pH = 7.5, while a pH of 6.7 resulted in a slower proliferation. A lower final cell number could be noticed at pH = 6.7 and pH = 7.5 after 207 h in the spinner flasks. At a pH = 7.0 the highest cell density of 4.72×10^6 cells ml⁻¹ was obtained (Figure 6).

Figure 4. Effect of different feeding strategies on the proliferation of T-cells. Cells were stimulated with α -CD3 mAb and α -CD28 mAb for 98 h. Daily, bi-daily or every 3rd day 50% of the culture medium were replaced. A batch fermentation was done as a control. Data are shown as mean $\pm$ standard deviation from three experiments from a single donor.

Oxygen tension

In an experimental setup, similar to that described above, oxygen tension of 5, 25, 50 and 75% of air saturation have been investigated in four parallel spinner flasks. The pH was controlled at 7.2. The maximum growth rates observed for 5, 25 and 50% of air saturation were rather similar (between 0.0375 and 0.0362 h^{-1}), however, the best expansion (394-fold) was obtained for the lowest oxygen tension (342-fold and 351-fold for 25 and 50% oxygen tension, respectively) after 210 h of cultivation (data not shown). The T-cells cultivated at an oxygen tension of 75% showed the lowest growth rates (0.0292 h^{-1}).

Cultivation in stirred bioreactors

Based on the optimized process parameters (Table 1) large-scale fed-batch fermentations were done using a standard stirred vessel (working volume 470 to 1000 ml) and our recently developed stirred suspension bioreactor (working volume 50 to 550 ml) (Schmidt et al., 1999).

PBMC were activated for 4 days as described before. The standard stirred vessel was inoculated with a cell density of 1.35×10^5 cells ml^{-1} in 470 ml medium and the stirred suspension bioreactor with a cell density of 5×10^5 cells ml^{-1} in 50 ml medium. A nutristatally process was realized with glucose as reference substrate. Furthermore triplicate T-flasks were utilized for comparison and were inoculated with a

cell density of 5×10^5 cells ml^{-1} . 50% of the medium were replaced daily. To quantify the portion of specific cells before and after cultivation an IFN- γ secretion assay was used.

After 238 h of culture 2.8×10^9 cells in the stirred vessel and 1.5×10^9 cells in the suspension bioreactor were obtained. The observed maximum growth rates were $\mu_{\max} = 0.0383 \text{ h}^{-1}$ (stirred vessel), $\mu_{\max} = 0.0512 \text{ h}^{-1}$ (suspension bioreactor) and $\mu_{\max} = 0.0463 \text{ h}^{-1}$ (T-flasks).

The flow cytometric analysis showed a nearly pure population of CD3⁺ T-lymphocytes at the end of cultivation (>94%). The CD4:CD8 ratio was inverted from 2.4:1 before the cultivation to 1:5 (stirred vessel), 1:2.7 (suspension bioreactor) and 1:4.8 (T-flask) after 238 h. More than 90% of the T-cells expressed the IL-2R α -chain after the polyclonal stimulation. During fermentation in both bioreactors the IL-2R was downregulated earlier than in the static T-flask culture (Figure 7), a finding also reported by Carswell et al. (2000a).

The portion of cells specific for the tested hCMV protein was not only maintained but even slightly increased, a finding that was unexpected due to the polyclonal stimulation of the cells (Figure 8).

Figure 5. The influence of different temperatures (34.0, 35.5, 37.0, 38.5, 40.0 °C) on proliferation of T-cells. These results were produced in triplicate T-flasks using three different donors.

Figure 6. Influence of pH on cell density during batch cultivations in spinner flasks.

Table 1. Optimized process parameters used for the cultivations in stirred bioreactors

Osmolality	IL-2 concentration	Temperature	pH	Oxygen tension
302 mOsmol kg ⁻¹	100 U ml ⁻¹	38.5 °C	7.0 to 7.3	5 to 50%

Figure 7. The influence on the IL-2R α -chain during the fermentation in different culture systems.

Figure 8. Specificity of the cells against hCMV protein AD169 before and after cultivation detected with IFN- γ secretion assay.

Discussion

The aim of this study was to determine optimal conditions for the large-scale expansion of primary T-lymphocytes by investigating the influence of several important cultivation parameters. Our results demonstrate that primary human T-lymphocytes can effectively be stimulated using immobilized anti-CD3 mAb ($5 \mu\text{g ml}^{-1}$), soluble anti-CD28 mAb (500 ng ml^{-1}) and IL-2 (100 U ml^{-1}). The addition of anti-CD28 mAb instead of components like PHA is known to augment lymphokine secretion, to upregulate IL-2R and additionally to increase the total number of cells (Morecki et al., 1997; Cerdan et al., 1995). The costimulatory effect of CD28 on both IL-2 and IL-2R α -chain expression results from a coordinated transcriptional activation of their genes as well as from transcript stabilization (Cerdan et al., 1995). Our procedure of PBMC activation gives rise to phenotypically heterogeneous cell populations with reversed ratios of CD4⁺ and CD8⁺ cells (1:2) compared with fresh PBMC. A similar finding has been described earlier (Morecki et al., 1997), but the reasons for this require additional investigation.

Although the osmolality of the culture medium has been described to be an important parameter in hematopoietic cell culture (Noll et al., 2001), we found a wide range of 276 to 330 mOsmol kg^{-1} which did not affect the cells. The physiological value of blood is 300 mOsmol kg^{-1} (Waymouth et al., 1970).

IL-2 is the main growth factor for primary T-cells (Carswell et al., 2000). Proliferation and differentiation are driven by this cytokine (Cerdan et al., 1995). Cultivation of T-lymphocytes with 100 U ml^{-1} IL-2 results in higher cell density and growth rate compared to a concentration of 10 and 50 U ml^{-1} . This concentration was also used by Carswell et al. (2000) and Hilbert et al. (2001). A further increase does not result in higher proliferation.

The feeding strategy showed that a daily half change of culture medium during cultivation resulted in a better proliferation and cell viability as compared to a medium exchange every 2nd or 3rd day. A complete change of the culture medium would result in a loss of autocrine factors like Interferon- γ (IFN- γ), IL-1 β and tumor necrosis factor- α (TNF- α), all of which might contribute to additional activation of long-term proliferating cells and their cytotoxic activity (Morecki et al., 1997; Nomura et al., 1993). Furthermore a daily half exchange of medium replaces

substrates like Glucose or Glutamin and prevents limitations.

For the examination of temperature effects, *in vivo* observations of immune response were transferred to *in vitro* cultivation of T-lymphocytes. The remarkable increase of the maximum growth rate at 38.5°C compared to the commonly used temperature of 37.0°C showed the immune stimulatory effect of an increased temperature.

The pH is known as a potent modulator of cellular proliferation and differentiation and can also influence cytokine secretion (McAdams et al., 1997b). Metabolic products like lactate inhibit proliferation by accumulation and thereby production of an acidic environment (Patel et al., 2000). We used the fermentation system cellferm-pro[®] with pH- and oxygen-control to investigate the influence of pH on the proliferation of human T-lymphocytes and determined an optimal pH range of 7.0 to 7.3. Carswell et al. (2000b) reported of a more than three-fold increase of proliferation capacity of T-cells for the pH 7.0 and 7.2 compared with pH 7.4. We did not observe such dramatic effects, but this might be caused by the different experimental setup. While Carswell et al. (2000b) preequilibrated the media using NaOH or HCl and measured pH after taking samples, we used an integrated online control method.

Oxygen tension is a critical parameter for the *in vitro* culture of T-cells (Krieger et al., 1996). Our results show the distinct effects of oxygen tension on growth rates and cell densities of cultivated T-lymphocytes. A low oxygen tension of 5% of air saturation led to higher cell densities in comparison to 25, 50 or 75% of air saturation. PBMC proliferation and overall viability may be altered by pO_2 , therefore it might be anticipated that a significant increase in IFN- γ , IL-2 and IL-4 levels between high and low O_2 groups is responsible for this observed effect (Krieger et al., 1996). Carswell et al. (2000c) found in a similar study that T-cells cultivated with low oxygen tension (5%) exhibit higher proliferation compared to 20% oxygen. However, regulation of low oxygen tension like 5% demands an automated fermentation systems to prevent oxygen limitations.

We demonstrate that the expansion of T-lymphocytes in several types of agitated bioreactors was possible. Using optimized parameters after a culture duration of 238 h 2.8 and 1.5×10^9 cells with a proportion of more than 94% of T-cells were obtained in the stirred vessel and in the suspension bioreactor, respectively. The down regulation of the IL-2R α -chain was increased in

both stirred systems in comparison to the static T-flask and resulted in a reduced expansion, which was also observed by Carswell et al. (2000a). It has to be shown in the future if this effect can be diminished or avoided by the use of shear protective additives.

The maintenance of immunological specificity after cultivation was tested and confirmed using an IFN- γ secretion assay against a hCMV protein. This was of particular importance as the stimulation of the cells prior to cultivation was antibody-mediated and therefore polyclonal. The slight increase in the portion of CMV-specific cells after cultivation might be caused by a higher proliferative potential of these cells than the average, but this remains to be investigated.

For immunotherapy of a CMV infection a number of 10^9 specific cells is proposed for the treatment of one patient and considering this number of cells based on our results a total volume of 120 l per patient would be necessary. Although not impossible this is difficult to handle in a clinical setting. An alternative is the specific stimulation using antigen-presenting cells loaded with the antigen of interest (e.g. a hCMV peptide or protein). Thus, a CMV-specific stimulation and following large-scale fermentation would be able to generate the sufficient number of T-cells. Investigations to setup a large-scale bioprocess based on this approach are currently underway.

In conclusion, we have shown that it is possible to set-up a large-scale fermentation process for *ex vivo* expansion of human T-lymphocytes, which can give access to a cell number sufficient for adoptive immunotherapy. The cellular proliferation in such processes is strongly influenced by a number of fermentation parameters which ought to be controlled carefully during cultivation to ensure maximum expansion.

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