Co-culture of the 55-6 B cell hybridoma with the EL-4 thymoma cell. Effect on cell growth and monoclonal antibody production

Alicia Martín-López · Lourdes Acosta-López ·
Francisco García-Camacho · Antonio Contreras-Gómez ·
Emilio Molina-Grima

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Abstract The cell growth and monoclonal antibody production of the 55-6 hybridoma cell co-cultured with the murine thymoma cell line EL-4 at different initial 55-6:EL-4 ratios were investigated. Both populations were seeded in co-culture without previous stimulation and therefore with low constitutive CD40 and CD40 ligand (CD154) expression levels, and in the absence of exogenous co-stimuli. Viable cell density and growth rate data seem to suggest a competition for nutrients, which is detrimental for both cells in terms of biomass production and also of growth rate for 55-6. Final concentrations of antibody and specific antibody production rates were affected by the initial 55-6:EL-4 ratio. The 4:1 ratio yielded the highest IgG2a concentration, whereas the highest specific antibody

production rate was obtained at the 2:1 ratio. Changes mainly in CD154 and also in CD40 expression in co-cultures could suggest cross-talk between both populations. In conclusion, different types of interactions are probably present in this co-culture system: competition for nutrients, cognate interaction and/or auto-crine or paracrine interactions that influence the proliferation of both cells and the hybridoma antibody secretion. We are hereby presenting a pre-scale-up process that could speed up the optimization of large-scale monoclonal antibodies production in bioreactors by emulating the *in vivo* cell–cell interaction between B and T cells without previous stimulation or the addition of co-stimulatory molecules.

Keywords Hybridoma · Growth rate · Antibody · Co-culture · CD40 · CD154

A. Martín-López · L. Acosta-López ·
F. García-Camacho · A. Contreras-Gómez (✉) ·
E. Molina-Grima
Department of Chemical Engineering,
University of Almería, Almería, Spain
e-mail: acontre@ual.es

Present Address:
A. Martín-López
MRC-Laboratory of Molecular Biology, Cambridge
Biomedical Campus, Cambridge, UK

Present Address:
L. Acosta-López
Centro Andaluz de Biología Molecular y Medicina
Regenerativa CABIMER, Sevilla, Spain

Introduction

Monoclonal antibodies (mAbs) currently account for a significant proportion of new drugs with diagnostic and therapeutic applications. In response to the increasing demand for large quantities of cost- and time-efficient mAbs, several methods have been established for optimization of *in vitro* mAb production. These methods include (a) the selection of highly productive, genetically stable clones (Meng et al. 2000; Soriano et al. 2002), (b) the extension of cell

survival and the reduction of apoptosis (Figueroa et al. 2004; Figueroa et al. 2007), (c) the development of cell lines expressing recombinant mAbs (i.e., CHO, etc.) (Jones et al. 2003; Deer and Allison 2004), and (d) the use of stimulatory agents modulating the cell metabolism and, perhaps, also gene expression in favour of enhanced protein production and secretion (Franěk et al. 2003; Franěk and Fussenegger 2005).

In this regard, several *in vitro* models have been used to reproduce CD40 activation on B-cell and B-cell hybridomas by using soluble agonists or cellular ligands (Valle et al. 1989; Bergman et al. 1996; Tu et al. 2008; Wiesner et al. 2008). The effects observed in these *in vitro* models after CD40 activation (enhanced cell proliferation, cell survival and antibody production), make CD40 an ideal target for increasing the monoclonal antibody production by B-cell hybridomas, which are supposed to have a component of genes from normal B cells used for cell hybridization.

In previous studies (Martín-López et al. 2007a, b) we have shown that it is possible to activate in B-cell hybridomas the same metabolic routes that take place in B cells to enhance CD40 expression, cell proliferation and mAb production by the addition of lymphocyte mitogens, such as lipopolysaccharide (LPS) and antibodies anti mouse immunoglobulin G (IgG) antibodies. A relationship between cell cycle position, CD40 expression and mAb productivity has also been observed (Martín-López et al. 2007c, 2010).

In this study we have established a model of B-cell hybridoma stimulation to increase mAb production based on the *in vitro* interaction between hybridoma and T cells. This model involves the co-culture of the B-cell hybridoma line 55-6 with the murine T thymoma cell line EL-4 in the absence of exogenous co-stimuli. Unlike other methodologies for mAbs production, the production of mAbs by this method consists of a short process, which eliminates the time-consuming steps of transfection of the gene of interest into the cells, the selection of clones or the addition of stimulatory agents, which all contribute to increased cost of the final product.

Materials and methods

Cell lines and cell maintenance

The cell lines used were a mouse–mouse B-cell hybridoma designated 55-6 (ATCC: CRL-2156) and a

T thymoma, designated EL-4 (ATCC: TIB-39). Hybridoma 55-6 produces IgG2a monoclonal antibodies to human immunodeficiency virus type 1 (HIV-1) glycoprotein 120 (gp120). The cells were grown in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 2.1 mM L-glutamine, 100 U/mL penicillin–streptomycin, 0.125 µg/mL amphotericin B, hypoxanthine-thymidine (HT) media supplement (50X) Hybri-Max*Gamm, and incubated at 37 °C in a 5 % CO₂ atmosphere. All chemicals were from Sigma-Aldrich, Inc., St. Louis, MO, USA.

Co-cultures of 55-6 and EL-4 cells

Co-cultures were carried out in batch mode in 175 cm² static T-flasks (Nunc, Roskilde, Denmark) with 200 mL of RPMI 1640, supplemented as described above, and were prepared by mixing 55-6 and EL-4 cells at different ratios. Four initial 55-6 to EL-4 ratios were used: 4:1, 3:1, 2:1 and 1:1. These ratios were obtained by varying the initial cell number of both populations while keeping a constant initial total cell density at 5×10^4 cells/mL. Separated cultures of both cell lines 55-6 and EL-4 with an initial cell density of 5×10^4 cells/mL were used as controls. Antibody anti-CD90.2, a pan T cell marker also known as anti-Thy-1.2, was used to distinguish EL-4 cells from 55-6 cells in cultures containing both cell types, as described in the next section.

Dual-platform protocol

The number of viable cells for each population in the co-culture was determined using a ‘dual-platform’ method, which derives the absolute viable cell count for each population from a percentage obtained by flow cytometry (by staining with a cell marker plus the exclusion of dead cells using propidium iodide (PI)), and an absolute total count obtained by manual cell counts on a hemocytometer. The relative EL-4 and 55-6 viable cell number in the co-culture was determined by the staining with antibodies anti-CD90.2 and PI. Only EL-4 cells showed a surface CD90.2 expression. Moreover, the percentage of EL-4^{CD90.2+} cells was almost 100 % of the EL-4 population. As CD90.2 is not expressed on B-cells, this approach is a robust method to distinguish both populations in the co-culture. The numbers of total and viable 55-6 and EL-4 cells in the control cultures were determined by counts

using trypan blue (0.4 %) exclusion. Specific growth rates (μ) were calculated as described elsewhere (Acosta et al., 2007) using the following equation:

$$\mu = \frac{1}{X_V} \frac{dX_T}{dt}$$

where X_T and X_V are total and viable cell densities and t is time.

Flow cytometry analysis

The surface CD90.2, CD40 and CD40 ligand (CD154) expressions for both 55-6 and EL-4 cells, alone or in co-culture, were determined by direct immunofluorescence staining using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). For these analyses, 100 μ L aliquots containing approximately 1×10^6 cells were incubated with 10 μ L of mAbs fluorescein isothiocyanate (FITC) conjugated to defined antigens (eBioscience, San Jose, CA, USA) at 4 °C for 30 min. After two washing steps with 2 mL of PBS/BSA, the cells were analyzed by cytofluorometry. Dead cells were excluded both by PI staining shortly before cell analysis, and by gating alive cells in regards to their different forward and side scatter signals. In order to achieve that, data acquired until 10,000 events were collected from the live gate using forward/side scatter plots. The live gate was set to exclude events with low forward scatter signals (cell debris and dead cells). The data analysis was performed using FLOWJO software (Tree Star, Inc., Ashland, OR, USA).

Determination of antibody concentration

IgG2a concentration was measured by sandwich-type ELISA using goat anti-mouse IgG2a-coated 96-well plates. The bound antibody from culture supernatants was detected by incubation with peroxidase-conjugated goat anti-mouse IgG2a. All antibodies were obtained from Bethyl Laboratories, Inc. (Montgomery, TX, USA). Tetramethyl-benzidine (Pierce, Rockford, IL, USA) was used as substrate and the absorbance measured at 450 nm. Specific antibody production rates (q_{mAb}) were calculated as described elsewhere (Acosta et al. 2007) using the following equation:

$$q_{mAb} = \frac{1}{X_V} \frac{d[mAb]}{dt}$$

where $[mAb]$ is the concentration of IgG2a antibody.

Fig. 1 Viable cell concentration of 55-6 cells (a) and EL-4 cells (b). The data are from one experiment, which is representative of three independent experiments

Results

Cell growth

Figure 1 shows the progress of 55-6 and EL-4 viable cell concentrations in co-cultures at the different initial 55-6:EL-4 ratios. Figure 1a shows that for 55-6 control, the viable cell concentration increased continuously during the cultivation, reaching maximum values of about 1.15×10^6 cell/mL. After reaching the maximum, the concentration of viable cells declined immediately. A similar pattern was observed for 55-6 cells in co-cultures with peaks for viable cells between 70 and 80 h. Nonetheless, the maximum viable cell concentration was decreasing as the initial 55-6:EL-4 ratio was reduced. A lag phase was not observed at any 55-6:EL-4 ratio.

Similar behaviour was observed for EL-4 cells (Fig. 1b). For the control of EL-4, the viable cell

Fig. 2 Mean specific growth rates during exponential growth phase (μ_m) for 55-6 and EL-4 cell lines as a function of the initial 55-6:EL-4 ratio. Values are shown as the average for triplicate cultures $\pm$ SD

concentration increased continuously during the cultivation. Since EL-4 has a more efficient use of glucose and glutamine (data not shown), maximum cell concentration values of almost 2×10^6 cell/mL were obtained at around 100 h. When EL-4 cells were in co-cultures, the maximum viable cell density decreased with the increase of the 55-6:EL-4 ratio.

Figure 2 shows the effect of the initial 55-6:EL-4 ratio on the mean growth rate during the exponential growth phase, μ_m , of both 55-6 and EL-4 cell lines. Although no significant differences in μ_m were observed between the control cultures of 55-6 (0.052 h^{-1}) and EL-4 (0.048 h^{-1}), when they were co-cultured the μ_m of both cell lines was significantly affected. 55-6 cells were negatively affected by EL-4 cells, since their growth rate decreased as the initial 55-6:EL-4 ratio was reduced. For the 1:1 ratio, the μ_m (0.042 h^{-1}) was 20 % lower than that of the 55-6 control culture. On the other hand, EL-4 cells were positively affected by 55-6 cells, since their growth rate increased when the initial 55-6:EL-4 ratio was increased. For the 4:1 ratio, the μ_m (0.058 h^{-1}) was 20 % higher than that of the EL-4 control culture.

Monoclonal antibody production

As shown in Fig. 3, the total IgG2a concentration increased with the culture time for all of the experiments. Final concentrations were affected by the initial 55-6:EL-4 ratio. The 4:1 ratio yielded the highest IgG2a concentration, even though the 55-6

Fig. 3 Time course of IgG2a concentration for the 55-6 control culture and co-cultures carried out at different initial 55-6:EL-4 ratios. The data are from one experiment, which is representative of three independent experiments

control culture presented a higher initial cell density. For the rest of initial 55-6:EL-4 ratios, the final IgG2a concentration diminished as the initial ratio was reduced.

The effect of the co-culture on IgG2a productivity can be seen in Fig. 4, which shows the mean specific IgG2a productivity of the 55-6 cells during the exponential growth phase for the control and co-cultures. IgG2a productivity increased as the initial 55-6:EL-4 ratio was reduced from 4:1 to 2:1, reaching a maximum value of $1.1 \times 10^{-6} \text{ μg/cell h}$, twice the value obtained in the control culture ($0.54 \times 10^{-6} \text{ μg/cell h}$). A further reduction to 1:1 produced a decrease in IgG2a productivity.

Regulation of surface CD40 and CD154 expression on 55-6/EL-4 cell interaction

In order to study the regulation of CD40 and CD154 expression on 55-6 and EL-4 cells, CD40 and CD154 were determined on the cell surface of 55-6 and EL-4 cells in control cultures and in co-cultures at a 2:1 ratio (in which the highest IgG2a productivity was reached).

As evident from Fig. 5, the expression of CD40 in the 55-6 control culture was low. No significant changes were observed in the co-culture, in which a similar or slightly reduced CD40 expression compared to that of the control culture was obtained. Figure 6 indicates that the CD154 expression by EL-4 cells increased over 100 % in the presence of 55-6 cells in a

Fig. 4 Mean specific IgG2a productivity during the exponential growth phase for the 55-6 control culture and co-cultures carried out at different initial 55-6:EL-4 ratios. Values are shown as the average for triplicate cultures $\pm$ SD

Fig. 5 CD40 + MFI relative to the 55-6 control culture at time 0 h for the control culture and co-culture with an initial 55-6:EL-4 ratio of 2:1. Values are shown as the average for triplicate cultures $\pm$ SD

time-dependent manner, compared to the CD154 expression by EL-4 control cells.

Discussion

In this study we have analyzed the proliferation and IgG2a production of a B-cell hybridoma, 55-6, co-cultured with the EL-4 thymoma cell line. Data of viable cell numbers and growth rates shown in Figs. 1 and 2 suggest a competition for nutrients that is detrimental to both cells in terms of biomass production, since the maximum viable cell concentrations in

Fig. 6 CD154 + MFI relative to the EL-4 control culture at time 0 h for the control culture and co-culture with an initial 55-6:EL-4 ratio of 2:1. Values are shown as the average for triplicate cultures $\pm$ SD

co-cultures are always lower than the maximum viable cell concentrations in the control cultures; and also in terms of growth rate for 55-6 cell line, since its growth rate in co-cultures is always lower than the growth rate of the control culture. Nonetheless, Fig. 2 also suggests another type of interaction between both cell lines, which counteracts the negative effect of competition and leads to an increase in the growth rate of EL-4 cells. This will be further discussed below.

Clearly, the bidirectional mixed cell culture system established in this study did not arrest the proliferation of any of the two cells. Traditionally, most mixed cell cultures are unidirectional, involving the cell cycle arrest of one population, known as feeder cells. Because feeder cells need to provide active signals to the target cells, it is important to maintain them viable and in a metabolically active state, allowing the continued expression of specific ligands or cytokines. The most commonly used techniques to inactivate the feeder cells in this manner are the exposure of cells to γ -irradiation or to mitomycin C (Kasakura and Lowenstein 1967). Although these procedures are powerful and an efficient means to support cell growth, in some cases they have the disadvantage of altering cell surface antigen expression, loss of viability, loss of function, biochemical changes and alterations in biophysical structure and subcellular components of the cells (Kasakura and Lowenstein 1967; Malinowski et al. 1992). The bidirectional, in vivo, cell–cell interaction system used in this study avoids these disadvantages. However, bidirectional

systems require the optimization of the culture conditions and the initial ratio of two populations, in order to avoid domination of one of the two populations in co-culture. Furthermore, consequences of metabolic regulation in these processes could be unpredictable, like in other complex systems in which mixed cultures of cells or microorganisms are involved, and potentially including metabolic shifts and transient behaviour.

In previous works we have reported that the same metabolic routes that take place in B cells can be triggered in the B-cell hybridoma line 55-6, in order to increase cell proliferation and mAb production using B-cell mitogens (Martín-López et al. 2007a, b). Furthermore, the results in Figs. 3 and 4 clearly show that hybridoma cell-T cell interaction can also be used to increase mAb production. In terms of antibody concentration, the highest concentration of IgG2a was obtained in the co-culture with the 4:1 ratio, thus making this condition the most ideal for use in batch bioreactors. When continuous perfusion bioreactors are used, specific antibody productivity per cell becomes an important economic variable (Cohen et al. 1992), thus co-cultures with a 2:1 ratio would be a candidate for perfusion bioreactor configuration.

The data on antibody production show that, as pointed out above, another type of interaction apart from competition exists between 55-6 and EL-4. This interaction may include cognate interaction and/or interaction between T-cell-derived cytokines and hybridoma cells. Although many cell interactions or triggering molecules exist and may well contribute to T-cell-dependent hybridoma-cell stimulation, CD40:CD154 interaction plays a critical role (Bergman et al. 1996). We therefore decided to analyze CD40 and CD154 expression in 55-6 and EL-4 cells, respectively, in the control cultures and in the co-culture with the highest specific production of antibody (initial 55-6:EL-4 ratio 2:1).

The low expression of CD40 in 55-6 cells (Fig. 5) is in line with other results reported in literature (Tone et al. 2001, 2002). The signal-transducing CD40 isoform is constitutively expressed in most non-stimulated cells at low levels (Tone et al. 2001). It is thought that the CD40 gene expression occurs in a range of processes that operate at three levels of gene expression: transcriptional, post-transcriptional and post-translational regulation (Tone et al. 2002). Post-transcriptional regulation and post-translational regulation have

become involved in the form of alternative splicing to reduce the amount of CD40 functional isoform, and block signalling through this receptor (Tone et al. 2001, 2002).

The observed increase in CD154 expression in a time-dependent manner by EL-4 cells (Fig. 6) is in accordance with the CD154 expression found in mouse CD4 T cells, which demonstrably occurs in two phases (Lee et al. 2002). The early phase of CD154 expression occurs within hours of the T-cell activation and is regulated independently of the local cytokine milieu (Lee et al. 2002). In this phase, the CD154 expression diminishes rapidly and is undetectable within 12–24 h after activation. However, the second phase of CD154 expression occurs between 24 and 72 h and is highly dependent on cytokines (Skov et al. 2000; Lee et al. 2002; Snyder et al. 2007) and co-stimulatory molecules (Jenkins and Johnson 1993; Kaminski et al. 2009), which can sustain CD154 expression for several days.

These changes in CD40 and mainly in CD154 expression on the surface of 55-6 and EL-4 cells could suggest a cross-talk between both populations. The CD40 down-regulation may be due to an increase of other CD40 isoforms by a mechanism of post-transcriptional regulation, which reduces and blocks the signal-transducing CD40 isoform. CD154 up-regulation induced by the presence of 55-6 cells probably corresponds to the second phase of CD154 expression sustained by cytokines and co-stimulatory molecules released to the medium during 55-6/EL-4 cell interaction.

In conclusion, the results observed for proliferation of both cell lines, antibody production by 55-6, and CD40-CD154 expression suggest a complex relationship between the two cells, where all types of interactions described above are probably present: competition for nutrients, cognate interaction and/or autocrine or paracrine interactions that influence the proliferation of both cells and the hybridoma antibody secretion.

The production of mAb in mammalian cells consists of a long process that involves pre- and scale-up processes for the optimization of large-scale mAb production in bioreactors. The existing pre-scale-up processes, such as the transfection of the gene of interest into the cells, the selection of the most appropriate clone, and the adaptation to different culture conditions, are very time-consuming and therefore responsible for the delays observed in mAb

becoming commercially available. We herewith present an efficient pre-scale-up process, which could speed up the optimization of large-scale mAb production in bioreactors by emulating the *in vivo* cell–cell interaction between B and T cells without previous stimulation or the addition of co-stimulatory molecules. The next step will be the scaling of the process to stirred bioreactors and the optimization of the operational conditions (agitation rate, oxygen saturation, pH, and so on). Nonetheless, more work is needed to explore the applicability of this type of mixed cultures to other hybridomas and stimulatory cells, including activated normal T cells expressing CD154.

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