RESEARCH

Effects of selection and adaptation of NS0 cells to protein-free medium on the properties, affinity and biological activity of a monoclonal antibody

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ABSTRACT

The extensive and labor-intensive process of selection and adaptation of a cell line to culture medium is the biggest limitation of recombinant protein production in mammalian cell culture, due to a very low frequency of the desired clones within a heterogeneously-distributed population of cells. In this study, the kinetic parameters of different clones of a recombinant NS0 myeloma cell line, obtained by serial cloning and adaptation to protein-free medium and secreting the hR3 IgG1 immunoglobulin, were compared. The physico-chemical parameters, biological activity and antibody affinity constant of the antibody produced was determined for each clone. Two culture systems were used to evaluate the effect of culture conditions on antibody structure and functional heterogeneity: adhesion cell cultures in hollow fiber bioreactors and suspension cultures in spinner flasks. A distinct impact of adaptation and selection processes was evidenced on the microheterogeneity of the cell population, based on kinetic parameters. Some physico-chemical properties of the product such as: molecular weight, specific recognition and isoelectric point remained unaltered, while differences were observed in the biological activity and the affinity constant of different clones and the given clone in different fermentation systems.

Keywords: NS0 myeloma, protein-free medium, physico-chemical properties, MAb, hR3, culture systems

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RESUMEN

Efectos de la selección y adaptación de las células NS0 a medio libre de proteínas sobre las propiedades, afinidad y actividad biológica de un anticuerpo monoclonal. La mayor dificultad en los procesos de producción de proteínas recombinantes en cultivos de células de mamíferos, radica en el extenso y laborioso proceso de selección y adaptación de la línea celular al medio de cultivo, debido a la poca frecuencia de los clones deseados dentro de una población distribuida de forma heterogénea. En este estudio se compararon los parámetros cinéticos de diferentes clones de la línea de mieloma recombinante NSO que secreta una inmunoglobulina IgG1 (hR3), obtenidos mediante clonajes seriados y adaptación a medio libre de proteínas. Además se evaluaron las características físico-químicas, la actividad biológica y la constante de afinidad del anticuerpo monoclonal producido por cada uno de los clones. Se emplearon dos sistemas de cultivo: cultivos adheridos en bioreactores de fibra hueca y cultivos en suspensión en frascos spinners, para evaluar el efecto de las condiciones de cultivo en la estructura y heterogeneidad funcional del anticuerpo. Se evidenció un marcado impacto de los procesos de adaptación y selección en la microheterogeneidad de la población celular, basado en parámetros cinéticos. A pesar de no observarse diferencias en algunas características físico-químicas del producto, como el peso molecular, el reconocimiento específico y el punto isoeléctrico, sí se observaron diferencias en la actividad biológica y la constante de afinidad en los diferentes clones, y en el producto obtenido a partir de un mismo clon en diferentes sistemas de fermentación.

Palabras claves: mieloma NS0, medio libre de proteínas, propiedades físico-químicas, MAb, hR3, sistemas de cultivo

*I*ntroduction

Mammalian cell culture plays an important role in drug discovery and process development for clinical research and diagnosis. The economic relevance of obtaining highly-productive subclones for large scale production in mammalian cells greatly depends on selection of highly-producer cell lines [1]. This is an extremely time-consuming, labor-intensive and expensive process. Its success deals with the staff's skills and experience of the research. Up to date, new technologies are emerging to enable fast screening of large numbers of cells, as well as selection, isolation and collection of the most valuable clones.

On the other hand, propagation of mammalian cells for the production of monoclonal antibodies and recombinant products in serum-free medium has gained interest in the last years in the biotechnological industry [2]. Traditional supplementation of medium with fetal calf serum (FCS) is expensive; commercial sources are sometimes limited too. Moreover, FCS shows other disadvantages such as: batch-to-batch variations, contamination risks and difficulties in subsequent product purification.

During the last decade, a great effort has been made to improve the properties of biopharmaceuticals production processes in mammalian host cells as: increased product titers, complete removal of animal components from transfection to harvest, and shorter developmental processes, among them. Much attention was focused on the impact of the host cell system in product quality.

Our approach was to determine the possible changes in the physico-chemical properties, biological

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activity and affinity constant of the hR_3 monoclonal antibody (MAb), produced in different subclones of the parental NS0 cell line in culture, following a process of selection and adaptation to protein-free media, and by culturing them in different culture systems. The hR_3 is an IgG1 (kappa) humanized MAb developed to recognize with high affinity the hEGFR and able to compete with hEGF for its binding in a competition assay [3]. Increased cell growth appeared in some types of human tumor cells, where the activity of the human epidermal growth factor (hEGF) and its receptor (hEGFR) increased; due to an overexpression of the hEGFR, with possible binding of the hEGF molecule and subsequent induction of malignant cellular growth [4]. Thus, optimize production of this MAb would be a considerable impact in this pathology, as well as characterizing its molecular properties after selection and adaptation of the producer cell line to different culture conditions.

Materials and methods

Cell line Generation and Media

The NS0 myeloma cell line was engineered to express the hR_3, a human IgG1 MAb humanized by inserting the complementary determining regions of the murine MAb ior egf/r3 to a human framework [5]. The T16 clone was obtained by transfection and single cell cloning by the serial dilution method, and cultured in Dulbecco-modified Eagle's medium (DMEM, Gibco, Invitrogen Corporation, UK) supplemented to 26 mM sodium bicarbonate, 15 mM HEPES, 2 mM glutamine, $2x10^{-5}$ M mercaptoethanol (2-ME), 1mM sodium pyruvate and 5% (v/v) FCS (Hyclone, Utah, USA).

Adaptation and selection

The A7 clone was previously obtained in our lab by single cell cloning of the T16 transfectant in 96-well plates (Nunclon, Brand Products, Denmark) containing RPMI-1640 at 5% FCS. This clone grew in RPMI-1640 medium supplemented with 1% FCS in a week; and re-adapted to grow in two protein-free media: PFHM-II (Gibco, UK) and Turbodoma THP formulated by Dr. F. Messi (Cell Culture Technologies), both supplemented with 0.1% pluronic-F68 after 100 days of culture.

The A7 clone adapted to grow in PFHM-II medium was cloned by the method previously mentioned and the 3B6 and 2E7 clones being subsequently selected and cultured in PFHM-II and THP media.

Another cell line, E7-45, was obtained by single cell cloning of T16 cells and cultured in selective DMEM/F12 medium supplemented to 26 mM bicarbonate sodium, 15 mM HEPES, 2 mM glutamine, $2x10^{-5}$ M 2-ME, 1 mM sodium pyruvate and 5% FCS. Further selection of cells in the same medium, containing an increased concentration of mycophenolic acid and xanthine was made.

Cells were routinely passaged twice a week at a ratio of 1:3 and 1:6 according to cell density, to allow the formation of a fresh monolayer prior to passage. Cells were cultured in T-75 static flasks at 37 °C and 5% CO_2 under a humidified atmosphere.

Culture in spinner flasks and hollow-fiber cartridges

Suspension-adapted cells were obtained from T-flask cultures, counted and seeded at $2\pm0.5 \times 10^5$ cells/mL in the different culture media. They were evaluated in 1 L-spinner flasks (IBS Integra Bioscience, California, USA) rotated at 60 rpm under a humidified atmosphere containing 5% CO_2.

An Acucell 10 000 MW cartridge (Cellex Inc.) was used for attached cells in Acusyst-R bioreactors (Endotronics, Inc. Minneapolis, MN) with DMEM/F12 at 5% FCS in the extracapilary space (ECS) and the same medium without serum in the intracapilary space (ICS). The medium flow and the intracapilary recirculation ranged from 30-400 mL/min to 100-500 mL/h, respectively, and the ECS flow was maintained at 0-20 mL/h. The culture pH ranged 6.9-7.2 and temperature was automatically maintained at 37±0.1 °C.

Product purification

Cell suspensions were centrifuged at 1000 rpm, 10 min and 4 °C, to eliminate cells and debris before purification. Centrifuged supernatants were purified through a HiTrap Protein A Sepharose Fast Flow column (Amersham Pharmacia Biotech AB, Uppsala), being previously equilibrated with phosphate-buffered saline (PBS), pH 7.2. The absorbed fraction was eluted with 0.1 M sodium citrate, pH 3.5, and passed through a gel filtration column PD-10 (Amersham Pharmacia Biotech AB, Uppsala) previously equilibrated with 15 mM sodium phosphate and 0.15 M sodium chloride buffer, pH 7.0.

Determination of antibody concentration

Immunoglobulin concentrations in purified products and supernatants were determined by absorbance at 280 nm in an Ultrospec III spectrophotometer (Pharmacia LKB, Uppsala, Sweden) and an enzyme-linked immunosorbent assay (ELISA) respectively. For ELISA determinations, Costar plates (Corning, N.Y, USA) were coated overnight at 37 °C with an anti-human IgG antibody (Sigma Aldrich Chemie Gmbh, Germany). After washing with PBS 1X-0.2% Tween 20 solution (TBS), plates were blocked for 1 h with the previously diluted samples in PBS 1X-Tween 20 and 5% inactivated FCS. Plates were washed and incubated with an anti-human IgG-phosphatase conjugate (Sigma Aldrich, Germany) (1/1600) for 60 min at 37 °C. The reaction was developed by adding 1 mg/mL of 4-nitrofenil phosphate (Sigma Aldrich, Germany) in dietanolamine buffer, pH 9.8, for 30 min and the assay was read at 405 nm in an ELISA reader (Organon Teknika N.V, Veedijk, Belgium).

Physico-chemical characterization

SDS-PAGE

SDS-PAGE was performed in a Mini Protean 3 (Bio-Rad) on 7.5% or 12.5% polyacrylamide gel at reducing and non- reducing conditions according to Laemmli´s method [6].

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Inmunoblotting

Separated proteins were transferred to 0.45 mm nitrocellulose membranes (Whatman International Ltd. Maldstone, England) in Hoefer Semi-phor System (Amersham Pharmacia Biotech, AB) within 90 min at 250 mA. Membranes were blocked with 5% w/v skim milk in TBS for 2 h, washed and incubated with a phosphatase-anti-human IgG kappa chain conjugate (Sigma Aldrich) in PBS 1X. After 2 h, the membrane was washed three times at 15 min each with PBS 1X and 0.05% Tween 20, and the proteins were detected by adding Naphtol AS-MX (Sigma Chemical CO. St Louis, USA) as substrate.

Isoelectrofocusing

Isoelectrofocusing (IEF) was carried out in a Multiphor II electrophoresis system (Amersham Pharmacia Biotech, AB). Polyacrylamide IEF gels were previously incubated 1 h in ampholine solution, pH 3.5-10 (Amersham Pharmacia Biotech, AB).

HPLC gel filtration

Purified products were previously prepared at 0.5 mg/mL, and 5 mL were loaded onto a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, AB) equilibrated with sodium phosphate 0.05 M and sodium chloride 0.15 M buffer, pH 7.0. Samples were eluted in the same buffer at a flow rate of 1 mL/min. The column was calibrated with a molecular weight standard. An AKTA Explorer System was used with the aid of the Unicorn software.

Biological activity

The biological activity was assessed to express a percent control of antibody specific recognition with respect to the ligand. This assay was carried out in a FacScan System flow cytometer (Becton Dickinson, San Jose, California) coupled to the FACSComp software of the Macquintosh system. First, 250 mL of cell suspension (1 x 10^6 cells/mL) were added in each tube, centrifuged at 1000 rpm for 6 min and the supernatant was removed. The pellet was resuspended in PBS 1X and 20 µL of each sample was added and incubated for 30 min at 4 °C in different tubes. Later on, 1 mL of the anti- human IgG-FITC conjugate was added to each tube and incubated for another 30 min at 4 °C. Finally, the tubes were washed with 2 mL of PBS buffer, centrifuged and the supernatant removed, and 250 mL of PBS 1X were added for readings. An irrelevant MAb was used at 0.02 mg/mL in PBS 1X as control.

Assessment of Affinity constant

It was necessary a displacement assay to assess the affinity constant by measuring the mean fluorescence intensity displacement for the product of interest, when it is incubated at different dilutions with a fixed concentration of the biotinylated antibody (MAb*). This assay is very similar to the previous presented in the specific recognition; but a step was added, the samples were incubated with 20 µL of MAb* following the non-biotinylated MAb to allow the displacement of the fluorescence signal when increasing the MAb concentration. Washing, centrifugation and separation steps were repeated. The samples were incubated with the streptavidin-FITC complex (1:40) for 30 min, followed by another round of separation steps. Finally, 250 µL of PBS were added to all the tubes and readings were carried out in a cytometer. The curves of MAb concentration *vs* the fluorescence intensity and calculations of the dissociation constant were established for the products analyzed with the aid of the Graph Pad Prism program. The affinity constant was considered as the inverse of the dissociation constant.

Results

Culture parameters

Although screening and selection processes were fairly well established, they were very tedious, lengthy and labour intensive. Our process of serial cloning and adaptation to serum-free and protein-free medium allowed selecting the 3B6 and 2E7 clones, with the highest titers of product**.** The culture parameters for the clones were analyzed in suspension, from kinetic analysis of the different cultures in the spinner flasks. The maximal cellular concentration (Xv máx), specific growth rate (m), specific production rate (qAb) and the maximal IgG concentration for selected cell clones (A7, E7-45, 3B6 and 2E7) are shown in table 1. Total protein and IgG concentrations and the specific activity of each supernatant were assessed and compared (Table 2).

Antibody characterization

The SDS-PAGE analyses of sample supernatants clearly showed the absence of bovine serum albumin (BSA) in the protein-free medium samples but its presence in samples corresponding to media supplemented with FCS (1 or 5%).

Previous gels including molecular weight standards and the control product enabled to identify the first band of approximately 160 kDa band as the hR3 MAb. Denaturing SDS-PAGE gel analysis with samples of purified products only showed two bands of

Table 1. Kinetic parameters of the different culture clones in spinner flasks

Cell clones and culture medium	µ[a] (1/h)	qAb[b] (pg/cel h)	Xv max[c] (*10^6 cel/ml)	IgG max[d] (µg/mL)
A7 / RPMI + 1%	0.073	0.13	1.26	10.7
A7 / PFHM-II	0.076	0.25	1.51	41.5
A7 / THP	0.036	0.197	0.94	32.0
3B6 / THP	0.096	0.28	1.75	60.7
2E7 / THP	0.123	0.18	1.55	68.6

[a] µ-specific growth rate
[b] qAb- specific production rate
[c] Xv max-maximal cell density
[d] IgG max- maximal product concentration

Table 2. Supernatant concentrations of the different clones and culture time

Cell line/medum /culture system	Conc. Total Proteins (mg/mL)	Conc. IgG (µg/mL)	Esp. Act. (%)	Culture time (d)
A7/THP/spinner flasks	1.06	20.5	1.90	10
A7/THP/hollow fiber	5.71	18.8	0.33	50
3B6/THP/spinner flasks	1.5	41.8	2.7	10
2E7/THP/spinner flasks	2.14	62.0	2.9	10
A7/PFHM/spinner flasks	1.93	38.8	2.02	10
A7/RPMI+1%FCS/ spinner flasks	4.28	10.7	0.25	10
E7-45/DMEM/F12+5% FCS/spinner flasks	3.71	15.5	0.42	10

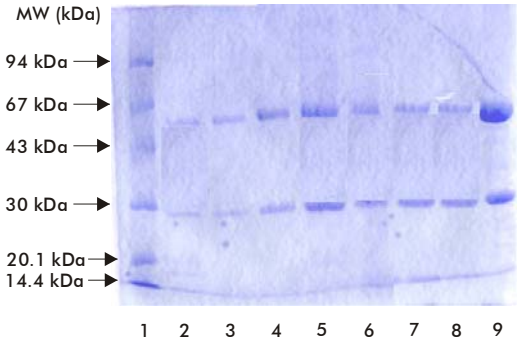

Figure 1. SDS-PAGE on a 12.5 % polyacrylamide using Coomassie Blue staining of purified products under reduced conditions. Lane 1: Protein low molecular weight standard; lane 2: clone A7 in THP cultured in spinner flasks; lane 3: clone A7 in PFHM culture in spinner flasks; lane 4: clone 3B6 in THP cultured in spinner flasks; lane 5: clone 2E7 in THP culture in spinner flasks; lane 6: clone A7 in THP in hollow fiber bioreactor; lane 7: clone E7-45 in DMEM/F12 + 5% FCS in spinner flasks; lane 8: clone A7 in RPMI + 1% FCS in spinner flasks; and lane 9: E7-45 clone in DMEM/F12 + 5% FCS in hollow fiber bioreactor.

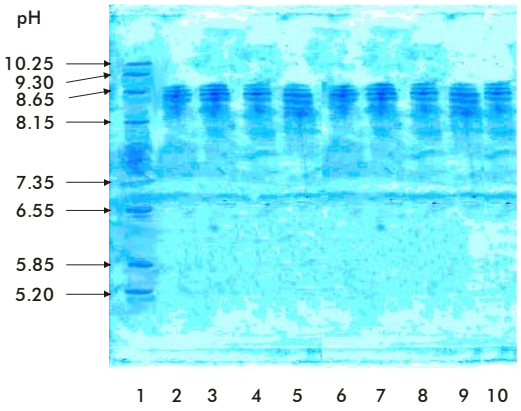

Figure 3: Isoelectrofocusing of purified products. Lane 1: High PI standard, lane 2: A7 clone in THP cultured in spinner flask, lane 3: A7 clone in PFHM cultured in spinner flask, lane 4: 3B6 clone in THP cultured in spinner flask, lane 5: 2E7 clone in THP cultured in spinner flask, lane 6: A7 clone in THP in hollow fiber bioreactor , lane 7: E7-45 clone in DMEM/F12 + 5% FCS cultured in spinner flask, lane 8: A7 clone in RPMI + 1% FBS cultured in spinner flask, lane 9 and 10: E7-45 clone in DMEM/F12 + 5% FBS cultured in hollow fiber bioreactor.

approximately 50 and 25 kDa each, corresponding to the heavy and light chains of the MAb, respectively (Figure 1). MAb expression was also confirmed by Western blot analyses, corresponding to the protein bands previously mentioned (Figure 2). The cascade of bands that appeared just below the main MAb bands could be glycosilation isoforms forms of the molecule, as reported by R. Van Erp [7]. However, they would also be IgG immunoglobulin degradation fragments. A similar pattern was observed by IEF in all the samples, with four more intense bands and two lighter ones in the 8.1-8.7 units (Figure 3).

Gel filtration analysis

A standard chromatogram of a gel filtration column, as shown in figure 4, representing the elution pattern of a control sample (hR3) and protein standards was analyzed. A retention time of 12.7 min for the hR3 MAb was established under the tested conditions. The retention times were calculated for all the culture conditions tested summarized in table 3. All the products were obtained with more than 90% of purity, as expected from protein A affinity chromatography procedures. The purification procedure used was efficient for this operational scale, since protein contaminants were removed from samples at least to SDS-PAGE and HPLC detection levels.

Specific binding and affinity constant

The optimal hR3 MAb and MAb* concentrations were determined as 10 and 12 µg/mL, respectively, by plotting the logarithms of MAb and MAb* concentrations against the percent of specific recognition (Figure 5). These values were used for both displacement and biological activity assays, corresponding to the maximum fluorescence intensities.

The displacement assays were carried out at product concentrations of 10, 20, 40, 80 and 160 nmol (1.5, 3, 6, 12 and 24 µg/mL, respectively) to evaluate affinity constants, while MAb* concentration was fixed to 80

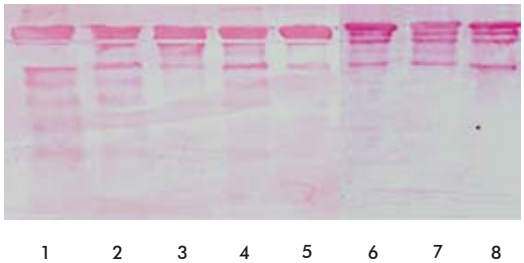

Figure 2. Western blot analysis demonstrated specific recognition of the product hR_3 obtained from different clones. Lane 1: clone A7 in THP cultured in spinner flasks; lane 2: clone A7 in PFHM culture in spinner flasks; lane 3: clone 3B6 in THP cultured in spinner flasks; lane 4: clone 2E7 in THP culture in spinner flasks; lane 5: clone A7 in THP in hollow fiber bioreactor; lane 6: clone E7-45 in DMEM/F12 + 5% FCS in spinner flasks; lane 7: clone A7 in RPMI + 1% FCS in spinner flasks; and lane 8: clone E7-45 in DMEM/F12 + 5% FCS in hollow fiber bioreactor.

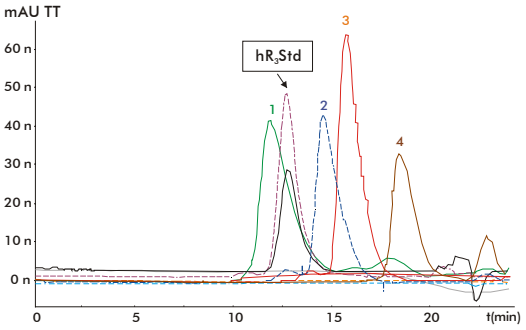

Figure 4. Molecular exclusion chromatograms of the hR3 standard (hR_3Std), produced from clone E7-45 in a hollow fiber system (at two concentrations) and some proteins used as molecular weight standard :1- Tyroglobulin, 669 kDa; 2- Ferritin, 440 kDa; 3- Bovine Sera Albumin (BSA), 67 kDa; 4- Ovalbumin: 43 kDa.

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Table 3: Purification results of clones

Cell line and culture condition	Retention time (min)	Purity (%)
A7 clone in THP cultured in spinner flask	12.76	100
A7 clone in PFHM cultured in spinner flask	12.79	100
3B6 clone in THP cultured in spinner flask	12.69	94.92
2E7 clone in THP cultured in spinner flask	12.70	96.23
A7 clone in THP cultured in hollow fiber	12.70	100

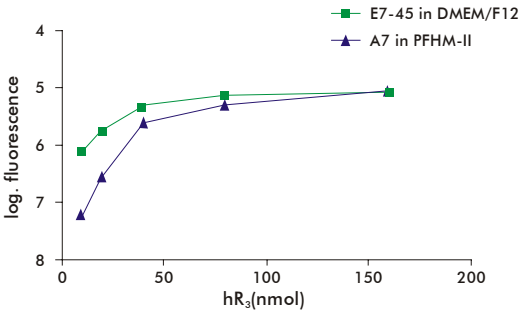

Figure 6. Displacement curves of the antibody produced from clones E7-45 and A7 cultured in DMEM/F12 and PFHM, respectively, *vs* the biotinilated antibody.

nmol (12 µg/mL), as mentioned above. Two representative displacement curves, as shown in figure 6, corresponding to the products of clone A7 cultured in PFHM medium in spinner flasks, and clone E7-45 cultured in DMEM/F12 supplemented with FCS in a hollow fiber bioreactor, respectively were assessed.

All the products, except for those obtained from the A7 clone cultured in THP medium in spinner flasks, showed biological activity values higher than 98% and an affinity constant one order of magnitude lower (Table 4).

Discussion

Adaptation to protein free media

Taking into account, adaptation of mammalian cells to grow in protein-free media is a labor-intensive and time-consuming process, where the stability of foreign genes during cell cultivation and the quality of the desired molecule can be affected, we successfully adapted the NS0 clone A7 to grow in protein-free medium, by reducing serum supplementation in the RPMI 1640 medium and extensive adaptation to PFHM and THP media in mixtures of RPMI 1640 and PFHM or THP, by increasingly adding protein-free medium.

The specific growth rate of this clone was decreased by adapting it to THP media while the specific production rate remained steady; nevertheless, the maximal IgG value was higher than that obtained by culturing the A7 clone in serum-supplemented medium. It could be plausible since growth factors, hormones, vitamins and proteases inhibitors present in serum can drastically influence the specific growth rate (m)[8], with clones showing increased m and slower specific production rates (qAb) in the presence of serum. Thus, lower IgG secretion rates to the culture medium, obtained by decreasing serum content, have been described for cells grown in batch cultures [9].

Specific activity values were distinctive between clones cultured with and without serum, for the same reason. In general, supernatants from cultures in protein-free medium showed a specific activity higher than 2%, whereas serum-supplemented media showed values below 0.5%. In agreement with the above mentioned parameters, the effect of serum on favouring protein production did not counteract the balance between both parameters. In fact, this is one of the advantages of protein-free media for industrial production.

All the resulting MAbs showed similar properties of molecular weight, specific binding and isoelectric point, as evidenced in the characterization assays. On the contrary, there were marked differences in the biological activity and the affinity constant for MAbs obtained by culturing the A7 clone in THP medium, when compared to those from the other clones cultured in PFHM and RPMI/1% FCS media (Table 4).

Cell culture conditions such as: pH, ammonium concentration, depletion of the carbon source and animal serum could significantly influence the extent of glycosylation, the degree of branching and completeness of sialylation [10-14]. Moreover, other components in the medium could affect the glycosylation pattern by unknown mechanisms [15]. Glycosylation profoundly could change the biology activity of antibody molecules, the physico-chemical environment of the cell affecting glycosylation, all these leading to some specific MAb glycoforms with potentially different biological properties [16, 17].

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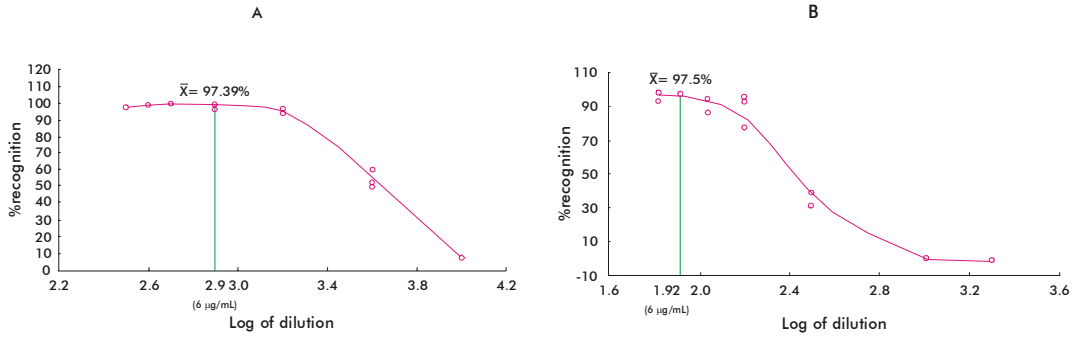

Figure 5: Recognition curve of A) non-biotinylated hR_3 and B) biotinylated hR_3, cultured in a suspension system

Table 4: Biological activity and affinity constant of products obtained from different cultured clones

Clone	Culture media	Fermentation system			
		Hollow fiber		Spinner flasks	
		Biol. Act. (%)	Ka($nmol^{-1}$)	Biol Act (%)	Ka($nmol^{-1}$)
A7	RPMI + FCS 1%	-	-	99.48	0.27×10^9
	PFHM-II	-	-	98.67	0.33×10^9
	THP	99.87	0.11×10^9	94.07	0.8×10^8
3B6	THP	-	-	99.71	0.16×10^9
2E7	THP	-	-	99.77	0.28×10^9
E7-45	DMEM/F12 + FCS 5%	99.63 %	0.35×10^9	99.37	0.92×10^8

If we consider that there were no differences between the physico-chemical properties mentioned before, we could assume that changes in the glycosylation pattern of hR_3 molecule could arise. On the other hand, some authors have stated that mutations or the loss of chromosomes which increase heterogeneity in the cell population could be responsible for this effect [18, 19].

Selection of clones

Clones 3B6 and 2E7 were selected by serial dilution of clone A7 in PFHM medium, and further re-adapted to grow in THP medium. Although their maximal cellular concentrations (Xv_{max}) and specific production rates (q_{Ab}) were very similar to the parental A7 in PFHM, their specific growth rates (μ) and maximal IgG concentrations were higher, mainly in the 2E7 clone (Table 1). Besides, the specific activities of both clones were the highest among all the clones tested (Table 2). Otherwise, their biological activities and affinities resembled those of the A7 clone cultured in PFHM and RPMI 1640 at 1% FCS (Table 3).

Once again, we stated that changes in the glycosylation pattern and not point mutations could be responsible for the decreased biological activity and affinity of the hR3 MAb from clone A7 obtained in THP medium, since point mutations could also induce changes during cell line manipulation (*e.g.*, cloning, adaptation to serum- or protein-free medium, etc.)[20, 21]. Additionally, clonal variations could be a source of glycosylation changes, promoting the expression of a distinct subset of potential structures by each cell within a given population.

It is well known that the glycoform profile of the antibody varies according to the culture conditions and during the culture stages. Minor populations of abnormal glycoforms as aglycosylated and high mannose variants are very common [22-24].

In fact, changes in the biological activities of MAbs due to altered glycosylation have been observed when they were expressed in different cell lines and under different culture conditions [25, 26]. In some cases, IgG proteins could also be glycosylated within variable regions, so could significantly influence its binding affinity [27, 28]. As Fc glycans are essential for the structural integrity of the antibody [29-31]; alterations of these oligosaccharides have been reported affecting MAb susceptibility to proteolytic degradation, clearance rate, Fc receptor binding, antibody-dependent cellular cytotoxicity (ADCC), monocyte binding, protein G binding and C1q component binding/C1 whole complement activation [32-36]. Fab N-linked glycosylation in the hypervariable regions, although infrequent, has been reported to influence the affinity for antigen binding. The availability of various processing enzymes, together with their kinetic properties and compartmentalization within the endoplasmatic reticulum and Golgi complex, are known to affect N-linked glycosylation. All these factors depend on the age, health, specie and cell type [37].

Studies by Mateo *et al*. [5] showed that the affinity for full binding of an antigen can be recovered by inserting three aminoacids of the murine ior egf/r3 into the hR3 human frameworks. We inferred from these results that the MAb obtained by culturing the A7 clone in THP medium may carry conformational changes generated by point mutations in the regions responsible for hEGFR recognition, subsequently decreasing the MAb affinity, as reported by other authors [38-40]. Such changes remain to be precisely identified. Additionally, all these do not argue against selection of clones exhibiting optimal target binding representative of properly-folded structures, such as the 3B6 and 2E7 clones, which were selected from a heterogeneous population of the clone A7 grown in THP medium.

Fermentation system

The MAb from clone A7 cultured in THP medium and clone E7-45 cultured in DMEM/F12 medium supplemented with 5% FCS were compared, obtained both in suspension in spinner flasks or a hollow fiber bioreactor. The products of clone A7 in THP medium from both culture platforms showed similar specific production rates but rather different specific activities, because of progressive decrease in the total protein content due to the increasing proteolytic activity in the hollow fiber ECS as culture progressed.

Moreover, both A7 and E7-45 affinity constants decreased when clones were cultured in suspension (Table 3). As it was stated, different cell culture systems and conditions could affect glycosylation of Mabs and other recombinant proteins [41]. It was previously demonstrated by Andersen *et al*. that the culture system was one of the parameters influencing protein glycosylation [42]. For example, an IgG_1 MAb produced in murine cells in feed-batch culture increased in high-mannose and truncated complex glycans while the culture aged [43].

Patel *et al.* [44] and Maiorella *et al.* [25], demonstrated that culture strategy or methodology was one of the two parameters highly influencing the appearance or variation of glycosylation sites in the variable region of the MAb. As we showed previously, the isoelectric point profile did not change in any product, irrespective of possible changes that could have occurred in the IgG molecule.

Certainly, some small glycosylation sites in the variable region that could be affected by changing the

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culture system, not varying the isoelectric point profile of the antibody but could affect its affinity if those residues were implied in the recognition site. For example, several human IgG MAbs produced in virus-transformed B-cell lines in serum-free media were far more galactosylated when obtained in low-density batch culture than in high-density hollow fiber bioreactor [45].

Conclusions

The impact of adaptation and selection processes to protein-free media (PFHM-II - THP), and the effect of using different fermentation systems in the physico-chemical characteristics, biological activity and the affinity constant of a humanized MAb (hR_3) was evaluated.

Molecular weight, specific recognition and isoelectric point of the same MAb obtained from clones cultured in spinner flasks (suspension system) and in the mentioned protein free media remained unaltered, compared to the standard product from clone E7-45 when cultured in DMEM/F12 plus 5% FCS in a hollow fiber bioreactor. The use of pluronic F-68 did not change any of the properties evaluated.

Nevertheless, some differences appeared in MAb biological activity and affinity, during adaptation to protein-free media and selection, due to changes in culture methodology and systems employed. The changes in MAb glycosylation, proposed as responsible for the variations detected, could require further mass spectrometry determinations to confirm its role; also verify the primary aminoacid sequences of MAbs´ variable regions. This study additionally demonstrated that the displacement assay by FACS was a simple and quick method to determine the affinity constant and to select from a given group those products bearing the properties required,therefore, very useful to compare studies of biotechnological processes although it was not the main purpose of this experimental work.

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