Hybridoma growth in a new generation hollow fibre bioreactor: antibody productivity and consistency

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Received 26 July 1996; accepted 16 November 1996

Key words: antibody consistency, hollow fibre bioreactor, hybridoma, monoclonal antibody

Abstract

This paper analyses the performance of MAbMaxTM/TricentricTM, a new generation hollow fibre bioreactor, for hybridoma growth and antibody productivity, the down stream processing of monoclonal antibody harvests throughout the run and the further control of antibody quality consistency.

Handling and process parameters were optimised using a mouse hybridoma, IgG1_K secretor, and then confirmed with several other hybridomas. Cells were kept at optimal viability during an unusually long period of time and a continuously high production of antibodies was detected over several months. Foetal bovine serum concentration was reduced to 1% and the effects of weaning of cells from serum were monitored in terms of cell metabolism and antibody productivity.

Antibody harvests collected at regular intervals throughout the run (2 to 12 weeks) were purified using affinity chromatography on a recombinant protein A/G matrix and then analysed in terms of antigen binding properties, isoelectric forms and oligosaccharide structures, in order 1) to control antibody quality consistency as a function of time and serum concentration and 2) to compare antibody characteristics as a function of culture conditions, *in vitro* bioreactor cultivation versus *in vivo* mouse ascite cultivation.

Introduction

Since their initial development in the early 70s (Knazek *et al.*, 1972) hollow fibre bioreactors (HFBRs) gained in popularity and several items of commercial equipment have been developed, but none exhibited significant technical advancements. In these HFBRs, cells attach on the outer surface of semi-permeable fibres, growing out in the extracapillary space while medium is circulating in the lumen of the fibres, the so-called intracapillary space. In spite of providing interesting features such as extensive surface area in a small space and tissue culture densities, HFBR technology was associated with several disadvantages including poor cell viability and the formation of large diffusional gradients (Tarakan *et al.*, 1986). In the present investigation, we evaluated the performance of a new generation HFBR for hybridoma growth and monoclonal antibody productivity. The TricentricTM device,

based on a unique, patented fibre within fibre geometry, differs from conventional HFBR systems by offering three different compartments, i.e. an annular cell chamber that keeps cells immobilized within 200 µm of diffusional oxygen and nutrient sources, an intracapillary compartment (the lumen of all inner fibres) and an extracapillary compartment, the latter two dedicated to medium flow. A mouse hybridoma line was employed in the present work 1) to determine the efficacy of the fibre within fibre technology in offering both high cell viability during a long time and high antibody productivity and 2) to demonstrate the consistency of monoclonal antibodies harvested as a continuous process over several months of culture in the bioreactor. Indeed, it is known that proteins can be extensively modified intracellularly by several mechanisms and, in addition, can be further altered in the cultivation degradative environment through several processes involving glycosidases, proteases and amidation/deamidation mod-

ifications (Reisfeld, 1967; Abel *et al.*, 1968; Painter and Freeman, 1971). As a consequence, Regulatory Authorities recommended that particular attention be paid to the integrity of MAbs obtained during continuous cultivation. The analytical methods we used to control the quality of MAbs collected from different points during the production process included lectin analysis of carbohydrate structures, isoelectric focusing and antigen binding properties.

Materials and methods

Cell line and media

The 6C10E4E5H7F10 (6C10) hybridoma line, an IgG1_K secretor was a product from the fusion of P3X63Ag8/653 myeloma cells and mouse spleen cells from a BALB/c mouse immunised with parainfluenza virus type 2.

In static flasks, cells were routinely cultivated in Dulbecco's modified Eagle's medium (DMEM, BioWHITTAKER) containing 4.5 g/l glucose, non essential amino acids, 2 mM glutamine, 20% foetal calf serum (FCS), penicillin and streptomycin, all from BioWHITTAKER.

For cultivation of cells in TricentricTM bioreactor, we used DMEM containing 1 g/l glucose, supplemented with all the above mentioned additives, plus 25 mM Hepes. Decreasing FCS concentrations from 20% to 1% were used throughout the run.

MabMaxTM/TricentricTM culture system

The TricentricTM bioreactor integrated in the MabMaxTM pilot unit (SETEC Inc, USA; distribution Boehringer Ingelheim Bioproducts, France), due to the unique fibre within fibre concept of the cartridge, consists of three different chambers: an intracapillary cell chamber in which cells are maintained within 200 μ m of oxygen and nutrient sources and two compartments dedicated to medium flow; the intracapillary chamber (ICC) corresponding to the lumen of all the inner fibres; and the extracapillary chamber (ECC) corresponding to the space around the outer fibres.

The annular cell chamber (4 ml, 200 cm², pore size = 0.2 μ m) was seeded with 3×10^7 6C10 cells harvested from static flasks. One litre DMEM (1 g/l glucose, 20% FCS) was pumped through the lumen of inner fibres as well as through the ECC and flowed across the membranes of inner and outer fibres in

order to deliver nutrients and dissolved oxygen to the cells growing in the annular cell chamber. Medium was continually recirculated through the reactor loop at an increasing rate (10 to 40 ml/min) during the growth phase. At the end of the growth phase, continuous Feed/Bleed was installed at an 800 ml/day rate, with a medium containing decreasing concentration of serum down to 1%. The run was stopped after 3 months.

Nutrient/metabolite assays

Glucose and lactic acid concentrations in the reactor loop were monitored by enzymatic assays, using glucose assay kit and lactate assay kit from Boehringer Mannheim.

Glucose uptake rate (GUR) and lactate production rate (LPR) were calculated on a daily basis, from nutrient/metabolite levels and Feed medium flow rate.

Antibody assays

1) Quantification of MAbs

Mab concentrations in reactor bleed samples were quantified as previously described (Kessler *et al.*, 1993) using an immunocapture ELISA test with a sheep anti-mouse Ig antibody (Boehringer Mannheim) as coating reagent and a peroxidase-labelled goat anti-mouse Ig antibody (Amersham) for antibody detection. A purified mouse IgG1 (Zymed) was used as a standard for determination of antibody concentration in the samples.

2) Antigen binding properties

Antigen binding properties of 6C10 MAbs were evaluated in an ELISA test using purified parainfluenza virus type 2 (PIV2) as coating antigen and peroxidase-labelled goat anti-mouse Ig antibody as a conjugate.

Concentration – purification of MAbs

Bleed harvests covering a one week period were collected at regular intervals. Antibodies were concentrated by ultrafiltration using an Amicon CH2PR system equipped with two H1P30 hollow fibre cartridges (Grace-Amicon) and then submitted to (NH₄)₂SO₄ precipitation. MAbs were further purified from this concentrate by affinity chromatography on immobilised recombinant protein A/G (Immunopure (A/G) IgG purification kit, Pierce) according to the manufacturer's recommendations.

Electrophoresis and immunoblotting techniques

SDS-PAGE of purified MAb samples was performed under reducing conditions with a Mini Protean II system (Biorad) using homogeneous 11% gels.

After SDS-PAGE, proteins were electrically transferred (mini Transblot system, Biorad) to nitrocellulose membrane (0.45 μ m). Detection of mouse MAb chains was performed using a sheep anti-mouse Ig F(ab')₂ fragment linked to horse radish peroxidase, and DAB (3-3'-diaminobenzidine) as substrate.

Isoelectric focusing

Isoelectric focusing of purified MAb samples was performed in 0.8% agarose gels containing an ampholyte mixture 3-10/5-8 in a 1:2 ratio, using a mini IEF cell (Biorad).

Lectin analysis of MAbs

Heavy and light chains of purified MAb samples were separated in SDS-PAGE and then transferred to nitrocellulose membranes as described above. Proteins were then reacted with digoxigenin labelled lectins (DIG glycan differentiation kit, Boehringer Mannheim) according to the manufacturer's recommendations. Lectins and carbohydrate specificities were as follows:

Lectin	Origin	Specificity (terminal sugar)
GNA	Galanthus nivalis	mannose α (1-6) mannose mannose α (1-3) mannose mannose α (1-2) mannose
SNA	Sambucus nigra	sialic acid α (2-6) galactose
MAA	Maackia amurensis	sialic acid α (2-3) galactose
PNA	Peanut	galactose β (1-3) N-acetyl galactosamine
DSA	Datura stramonium	galactose β (1-4) glucosamine
ConA	Canavalia ensiformis	mannose containing structures
AAA	Aleuria aurantia	fucose α (1-6)glucosamine

Results and discussion

Cell growth and MAb production kinetic of 6C10 hybridoma line in MAbMaxTM/TricentricTM

A previously established murine hybridoma line, 6C10, whose secreting activity in flask was 20 μ g/ml (twice weekly passages) was used to produce IgG1 κ in MAbMaxTM/TricentricTM bioreactor. Culture was started by inoculation of 3×10^7 hybridoma cells in the annular cell chamber. As shown by daily monitoring of metabolic parameters, both glucose uptake rate (GUR) and lactate production rate (LPR) increased regularly and rapidly during the growth phase (Figure 1); after 5 days they reached values (12–14 mg/h) corresponding to the beginning of the tissue dense stationary phase. On day 5, continuous Feed/Bleed system was started in order to keep media components under limits. The utilisation of both a constant feeding/harvesting rate (800 ml/day) and a low glucose concentration (1 g/l) allowed the maintenance of highly stable and rather low concentrations of nutrient/metabolite during the run (Table 1). Results in Figure 1 showed that the maintenance phase could be divided in two distinct «Plateau» phases. The first one, covering a two month-period, was characterised by very high and constant values of both GUR (14.2 $\pm$ 1 mg/h) and LPR (14.4 $\pm$ 2 mg/h) while the second exhibited constant but lower GUR and LPR values, 8.15 and 8.64 mg/h respectively. It is interesting to note that we regularly decreased FCS concentration from 20% to 1% during the stationary phase and that the abrupt decline we observed in GUR and LPR on day 60 correlated with a drastic reduction in serum; the further maintenance of metabolic parameters under lower but constant values during an additional month gave us arguments favoring the hypothesis of serum starvation as an inducer of the death of a part of cell population rather than a limitation in bioreactor performance. Indeed, cell density was not measured directly because it is not possible to obtain a cell suspension suitable for counting without disturbing the reactor.

Since apparent molar yield of lactate from glucose ($Y_{\text{lac,glc}}$) could be determined without knowledge of the number of viable cells in the reactor, $Y_{\text{lac,glc}}$ values were monitored throughout the run to detect changes in the metabolic pattern of cells. The relatively constant values we found (Table 1) during the two distinct «Plateau» phases provided a good indication of a rather stable culture environment over the three month-cultivation period, without significant change in the use of alternative metabolic pathways. As shown by dai-

Figure 1. Monitoring of glucose consumption and lactate production during the course of 6C10 cell cultivation in Tricentric bioreactor.

Table 1. Cell metabolism during the maintenance phase of 6C10 cells in the TricentricTM bioreactor

Maintenance phase: (days)	mean glucose concentration (mM)	mean lactate concentration (mM)	mean GUR (mg h ⁻¹)	mean LPR (mg h ⁻¹)	Y _{lac,glc} (molar ratio)
6 to 60 (FCS: 15–2%)	2.97±0.32	5.02±1.66	14.20±1	14.40±2	2.02±0.29
61 to 85 (FCS: 1%)	3.79±0.39	2.76±0.59	8.15±1	8.64±1.5	2.13±0.30

ly monitoring, constant pH values were maintained, 7.21 ± 0.07 (mean $\pm$ SD) during the entire run.

Reactor productivity was monitored during both the growth phase and the maintenance phase, using an immunocapture ELISA test for quantification of antibodies. As shown in Figure 2, results were expressed in terms of daily productivity and antibody cumulative amount.

During the growth phase, antibody productivity increased regularly up to 6 mg MAb per day, when the metabolic parameters reached the steady state values.

During the first two weeks of the maintenance phase, MAb productivity continued to increase prior

stabilizing at an 8.5 mg per day plateau value. This value remained stable up to day 60, then decreased abruptly down to 5 mg per day on day 62. From day 62 to day 90 the level of antibody synthesis remained stable or slightly increased.

Considering mean daily MAb production as a function of FCS concentration (Figure 3a), it appeared that MAb specific productivity was significantly increased with FCS reduction. The optimal reactor productivity was observed with equivalent or lower than 5% FCS concentrations. The analysis of $Y_{\text{MAb,glc}}$, the apparent yield of product from substrate (Figure 3b) confirmed 1) the consistency of specific MAb productivity when using $\leq 5\%$ FCS concentrations in the culture medium

Figure 2. Monitoring of MAb production during the course of 6C10 cell cultivation in Tricentric bioreactor.

Figure 3. (a) Mean daily MAb production as a function of FCS concentration, (b) apparent yield of product from substrate as a function of FCS concentration.

Table 2. Degree of correlation (r^2) between metabolic parameters and antibody production rates

	r^2			
	Growth phase		Maintenance phase	
	MAb	LPR	MAb	LPR
GUR	0.978	0.926	0.660	0.807
LPR	0.971	–	0.558	–

and 2) the death of a part of reactor cell population in response to an overly drastic reduction in serum concentration.

Additional informations on the relationships between antibody production and metabolic parameters were obtained when calculating the degree of correlation between antibody production rate distribution and either GUR and LPR value distribution. Conversion from glucose to lactate was also controlled using the same test. As shown in Table 2, GUR and LPR were both closely and directly related to the rate of antibody production during the growth phase with r^2 values ≥ 0.97 . A similarly close correlation was found between GUR and LPR values during the same period of time. As previously mentioned by Davis et al (1995) it seems likely that such a close relationship was largely due to the increase in biomass under no limiting growth conditions. Lower but interesting r^2 values ($0.56 \leq r^2 \leq 0.66$) were obtained from the analysis of data collected throughout the three-month maintenance phase. These results, evidencing a looser but continuing relationship between glucose/lactate metabolism and antibody productivity under conditions usually considered as limiting due to tissue density in the cellular space, are in favour of excellent nutrient delivery during the whole course of the long-term culture of hybridoma cells in the TricentricTM bioreactor.

The TricentricTM hollow fibre system, which was fed and harvested continuously for three months, gave an average antibody production rate of 6.76 mg per day when considering the total length of the run, and a 7.27 mg per day production rate when considering the two-month period with optimal cell viability and optimal steady state metabolic parameters. These production rates led to a cumulative amount of 550 mg MAbs.

A comparison was made between our results and those published by Kurkela *et al.* (1993) when using a conventional HFBR system, due to the very similar characteristics of the hybridoma line these authors used and our own cell line, i.e. a similar IgG1 isotype, and a

similar secreting activity: 20 $\mu\text{g/ml}$ in static flasks. For such a comparison, we used, as mentioned by Kurkela *et al.*, the results obtained from a two-month cultivation period and expressed as the reactor productivity (mg/l/day) based on the size of the cell chamber.

The results we found with the fibre within fibre system were significantly higher than those obtained with the conventional system, $1817 \pm 265 \text{ mg/l/day}$ and $307 \pm 142 \text{ mg/l/day}$ respectively; in addition, due to the consistency of our system the standard error was only 14% as against 46%.

Monitoring of MAb quality and functionality as a function of time and FCS concentration

The quality of products obtained from HFBR technology is becoming a major concern for people interested in producing proteins for *in vitro* diagnostic or therapeutic use.

We have shown that, due to the unique concept of the annular cell chamber, TricentricTM bioreactor offers extremely high cell viability throughout the run and minimizes the residency time of products in the potentially degradative environment of the bioreactor. In order to verify the consequences of such a design on MAb structural integrity and functionality maintenance, we periodically checked MAb quality. To do this, MAbs derived from harvests collected at different times during the bioreactor life time (2, 6, 9 and 12 weeks respectively) as well as ascite derived MAbs were purified for subsequent analysis of oligosaccharide structures, change in charge heterogeneity and antigen-binding properties.

Immunoblotting analyses performed after SDS-PAGE of purified MAbs (Figure 4) did not show any difference between the samples as a function of 1) the bioreactor harvest, and 2) the *in vitro* or *in vivo* origin of MAbs. We only noted in 2W and 6W bioreactor harvests the presence, in addition to heavy and light chains, of a further component due possibly to non reduced aggregates.

Oligosaccharide structures of 6C10 MAb samples were analysed using lectin reactivity patterns of heavy and light chains, after PAGE and nitrocellulose transfer of purified MAbs.

The results we obtained using several lectins specific for different terminal sugar residues are shown in Table 3.

Heavy chains were identified as containing «high mannose»/«hybrid type» carbohydrate chains due to the reactivity of terminal mannose residues with GNA.

Figure 4. Immunoblot of 6C10 MAb derived from ascite and TricentricTM harvests. Nitrocellulose sheet was reacted with a peroxidase labelled goat anti mouse Ig antibody and DAB (di-amino benzidine) was used as substrate. 1 = Tricentric harvest MAb, + 12 Weeks; 2 = Tricentric harvest MAb, + 9 Weeks; 3 = Tricentric harvest MAb, + 6 Weeks; 4 = Tricentric harvest MAb, + 2 Weeks; 5 = Ascite derived MAb.

The occurrence of «high mannose» carbohydrate structures was reported previously for a mouse IgG2_a using lectins in dot blot experiments (Coco-Martin *et al.*, 1992) and for a human IgG by using lectin-ELISA test (Leibiger and Marx, 1995). In addition, 6C10 heavy chains were shown to contain «complex type» sialylated carbohydrate chains due to the reactivity of terminal $\alpha(2-6)$ linked sialic acid and terminal $\alpha(2-3)$ linked sialic acid with SNA and MAA respectively. The presence of terminal fucose was demonstrated using AAA. The absence of reactivity of 6C10 heavy chains with PNA and DSA suggests the absence of O-glycans and/or the substitution of galactose $\beta(1-3)$ N-acetylgalactosamine and gal $\beta(1-4)$ N-acetylglucosamine by sialic acid.

Interestingly, 6C10 light chains were found glycosylated (mannose, sialic acid) when using either ConA or MAA. Such a glycosylation of Ig light chains has been previously mentioned for human antibodies (Tachibana *et al.*, 1992; Leibiger and Marx, 1995).

Regarding the influence of source (*in vivo/in vitro* production) and/or cultivation conditions (FCS concentration, time of harvest) we did not found any significant difference between MAbs, with the exception of the reactivity of heavy chains with SNA. Indeed, as shown in Figure 5, a very low reactivity with SNA was

observed with MAbs purified from either ascite or 2W-harvest while, on the contrary, a strong response was characteristic of MAbs derived from harvests collected after 6, 9 and 12 weeks respectively. These results show a difference in the amount of sialic acid $\alpha(2-6)$ galactose residues in heavy chains as a function of the sample. Previous studies on murine IgG1 derived respectively from ascite, from serum-supplemented or serum-free cultures (Patel *et al.*, 1992) showed that differences in glycosylation pattern of MAbs occurred exclusively with respect to the content of sialic acid. Ascite-derived IgG had no detectable sialylated oligosaccharides, IgG derived after culture in serum-free medium had a significant content of sialylated oligosaccharides and IgG derived after culture in serum-containing medium had an intermediate content. From these results it is reasonable to conclude that the differences we detected between the glycosylation pattern of MAbs from the early harvest (2W) and those from latter harvests were due to big differences in the FCS concentration of the culture medium $\geq 10\%$ versus $\leq 3\%$, and not to a lack of glycosylation consistency over time.

Isoelectric focusing was performed on the different purified MAb samples in order to search for potential changes in isoelectric point (pI) and charge heterogeneity as a function of MAb source and culture con-

Table 3. Lectin reactivity of 6C10 MAbs derived from ascite or bioreactor harvests

Lectins	6C10 MAb source									
	Ascite		Tricentric TM harvests							
			2 weeks (15% FCS)		6 weeks (3% FCS)		9 weeks (1% FCS)		12 weeks (1% FCS)	
	H*	L*	H	L	H	L	H	L	H	L
SNA α (2-6) linked sialic acid	±	–	±	–	+	–	+	–	+	–
MAA α (2-3) linked sialic acid	+	+	+	+	+	+	+	+	+	+
GNA terminal mannose	+	–	+	–	+	–	+	–	+	–
DSA Gal α (1–4) Glc	–	–	–	–	–	–	–	–	–	–
PNA Gal α (1–3)GalNAc	–	–	–	–	–	–	–	–	–	–
ConA mannose containing structures	+	+	+	+	+	+	+	+	+	+
AAA Fuc α (1–6)GlcNAc	+	–	+	–	+	–	+	–	+	–

H* = heavy chains, L* = light chains.

+ = High reactivity; ± = weak reactivity; – = no reactivity.

ditions, since this test can be considered as the most sensitive indicator of antibody consistency (Hamilton *et al.*, 1987b). As shown in Figure 6, MAbs displayed specific banding patterns with narrow pI range, 0.35 for ascite derived antibody and 0.50 for reactor derived antibodies. This narrow pI range is characteristic of monoclonal antibodies, unlike polyclonal IgG (Hamilton *et al.*, 1987a). The analysis of the banding pattern of the different samples showed that reactor derived MAbs exhibited two additional minor cathodic bands when compared to ascite produced MAbs. Identical IEF pattern deviations have been observed by Moellering *et al.* (1990) when comparing antibodies produced by serum-free and ascite media and they have been related to a deamidation/amidation phenomenon as a function of the environment; indeed, ascitic fluid and serum-rich culture media can be considered as a degradative environment in comparison with low serum and serum-free media.

It is likely that the differences we observed previously in sialic acid amount in 6C10 heavy chains

as a function of culture conditions did not affect IEF banding of MAbs since Painter and Freedman (1971) demonstrated that the release of sialic acid from antibodies using neuraminidase treatment altered only the density of IEF band(s) at a given pI and not the number of bands observed.

The monitoring of IEF profile of MAbs produced in TricentricTM brought additional arguments regarding the consistency of product over time.

The last marker we chose for studying MAb quality is the analysis of the binding properties of antibodies to homologous antigen. From the results in Figure 7 establishing the specific binding of MAbs to PIV2 (anti-PIV2 ELISA titre/mg MAb) it appeared that serum concentration, as previously mentioned, is the only factor influencing the marker. When FCS concentration is low, we observed a remarkable consistency over time, of binding properties of MAbs to the specific antigen.

Figure 5. SNA reactivity of 6C10 MAb derived from ascite and TricentricTM harvests. 1 = Ascite derived MAb; 2 = Tricentric harvest MAb, + 2 Weeks; 3 = Tricentric harvest MAb, + 6 Weeks; 4 = Tricentric harvest MAb, + 9 Weeks; 5 = Tricentric harvest MAb, + 12 Weeks; 6 = Positive control = Transferrin.

Figure 6. Isoelectric focusing of 6C10 MAb derived from ascite and TricentricTM harvests. 1 = Standard pI range 4.45–9.6; 2 = Ascite derived MAb; 3 = Tricentric harvest MAb, + 6 Weeks; 4 = Tricentric harvest MAb, + 9 Weeks; 5 = Tricentric harvest MAb, + 12 Weeks.

Conclusion

Using 6C10 hybridoma line, whose growth characteristics and antibody secreting activity in static flasks were representative of a large majority of mouse hybrido-

mas, we demonstrated the excellent performance of TricentricTM bioreactor in terms of cell growth, persistence of high cell viability for a long time, and antibody productivity. Several other investigations we performed using hybridoma lines derived from

Figure 7. PIV2 binding activity of 6C10 MABs as a function of source and reactor harvest.

different myeloma fusion partners (P3X63Ag8/653, SP2O/Ag 14) and/or secreting various IgG isotypes (IgG1_k, IgG2a_k, IgG3_k) have confirmed the efficiency of this new generation HFBR for production of monoclonal antibodies. The different controls we applied to MABs taken from different points during the production process confirmed the consistency of antibodies over time in terms of sub-unit structure, glycosylation, charge homogeneity and antigen-binding properties. All these data support the use of MAbMaxTM/TricentricTM for production of monoclonal antibodies for various applications.

Acknowledgements

This work was supported in part by Boehringer Ingelheim Bioproducts, France.

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