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Monoclonal Antibody Production using Free-suspended and Entrapped Hybridoma Cells

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Introduction

Various cell culture methods have been used for the large-scale production of monoclonal antibodies (MAbs). Broadly one can distinguish between the systems in which the cells are entrapped (or immobilized) and those in which the cells are grown in free suspension. Many researchers have recognized the potential for MAb production using entrapped cells. An active discussion has appeared in the literature concerning the relative benefits of entrapped cell culture for large-scale MAb production (Adamson and Schmidli, 1986; Nilsson, 1987; Tyo and Spier, 1987; Heath and Belfort, 1990; Looby and Griffiths, 1990). In fact, MAb production has been carried out successfully using entrapped hybridoma cells (Nilsson *et al.*, 1983, 1986; Shirai *et al.*, 1987; Bugarski *et al.*, 1989; Sinacore, Creswick and Buehler, 1989). The potential advantages that the entrapped cell culture offers over conventional free-suspended cell culture are as follows (Nilsson, 1987; Tyo and Spier, 1987).

First, entrapped cells can be protected from shear stress induced by mechanical agitation and/or air sparging. Hybridoma cells in stationary and decline phases of growth were more sensitive to shear force caused by mechanical agitation than the cells in the growth phase (Lee *et al.*, 1988; Petersen, McIntire and Papoutsakis, 1988). Since a significant portion of MAb is produced during the stationary and decline phases of growth (Boraston *et al.*, 1984; Velez *et al.*, 1986; Brennan, Shevitz and Macmillan, 1987; Hu *et al.*, 1987; Lee *et al.*, 1988; Miller, Blanch and Wilke, 1988; Renard *et al.*, 1988; Dalili and Ollis, 1989; Savinell, Lee and Palsson, 1989), it is important to maintain cell viability during the stationary and decline phases by

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; IL-6, interleukin-6; IMDM, Iscove's modified Dulbecco's medium; MAb, monoclonal antibodies; MWCO, molecular weight cut-off; NP, non-producing hybridoma population; P, producing hybridoma population; PEC, peritoneal exudate cells; SFLP, serum-free, low-protein; $TGF\beta_1$, transforming growth factor β_1.

Biotechnology and Genetic Engineering Reviews — Vol. 12, December 1994
0264–8725/94/12/509–533 $20.00 + $0.00

protecting the cells from shear stress. Furthermore, cells placed in low-serum and serum-free media were more shear-sensitive than cells placed in high-serum media (Murakami, Masui and Salo, 1982; Kunas and Papoutsakis, 1989, 1990; Lee, Savinell and Palsson, 1989; McQueen and Bailey, 1989; Shacter, 1989; van der Pol *et al.*, 1990). More complete reviews on the shear sensitivity of animal cells have been published (Papoutsakis, 1991a,b). The problem of free-suspended hybridoma cells regarding shear sensitivity can be overcome by entrapping cells into hydrogel beads or capsules.

Secondly, entrapped cell particles are about 50–500 times larger than free-suspended cells and are easier to use in perfusion systems because they do not clog conventional filter systems and can be easily isolated during medium changes.

Thirdly, entrapped cells may create a microenvironment that is more favourable for MAb production than the environment found in the surrounding medium. Finally, higher local and overall cell concentrations (of the order of 1×10^7 to 1×10^8 cells ml^{-1}) can be achieved, resulting in higher MAb titre, volumetric MAb productivity and reduced capital cost for a MAb production facility.

Although a few studies on entrapped hybridoma cell culture are available, most of them give very limited information on specific MAb productivity and metabolism (Nilsson *et al.*, 1983, 1986; Shirai *et al.*, 1987; Bugarski *et al.*, 1989; Sinacore, Creswick and Buehler, 1989). Furthermore, since different hybridoma cell lines were employed in different entrapped cell culture systems, it is difficult to determine the advantages of using the entrapped hybridoma cells for MAb production over free-suspended cells.

We have carried out a series of experiments to compare entrapped hybridoma cells with free-suspended hybridoma cells for MAb production. The model cell line used was the murine hybridoma cell line, S3H5/γ2bA2. In this chapter, the advantages of using entrapped cells over free-suspended cells for MAb production are discussed with respect to efficiency of serum utilization for MAb production, specific MAb productivity and stability of MAb productivity. Cells were entrapped into calcium alginate beads (Lee and Palsson, 1990) because calcium alginate can be easily dissolved by treatment with calcium chelating against such as EDTA and citrate buffer (Lim, 1980, 1988).

Efficiency of serum utilization for antibody production

One of the main economic drawbacks in the large-scale cultivation of hybridoma cells is the requirement for expensive serum that is used in growth media (Griffiths, 1986, 1987). Furthermore, animal-derived serum contains high levels of contaminants and unwanted proteins that must be separated from the MAbs during downstream processing, thus increasing production costs (Ratafia, 1989). The serum requirement for growth of a murine hybridoma cell line in batch cultivation has been reduced at high initial cell concentrations (Lee, Huard and Palsson, 1980). Therefore, one would expect high-concentration hybridoma cell cultures to be economically advantageous (Glacken, Fleischaker and Sinskey, 1983). High cell concentrations can be obtained by immobilizing the cells in gel beads. In addition, it has been reported that immobilization enhances the specific productivity of secreted products, such as ethanol by yeast (Okita, Bonham and Gainer, 1985; Doran and Bailey, 1986) and α-

amylse by recombinant yeast (Walls and Gainer, 1989). Immobilization may similarly enhance the specific MAb productivity (q_{MAb}) of hybridoma cells, improving the efficiency of serum utilization for MAb production.

To compare an entrapped cell culture with a free-suspended cell culture with respect to the efficiency of serum utilization for MAb production, free-suspended and calcium alginate-entrapped cell cultures were carried out in a repeated fed-batch mode, stepping down the serum concentration from 10% to 0% v/v (Lee, Varma and Palsson, 1991a). We observed two interesting phenomena in the entrapped cell culture.

First, the q_{MAb}, unlike the specific glucose uptake and lactate production rates (q_{glu} and q_{lac}), was enhanced significantly in the entrapped cell culture, and this enhancement was not a transient phenomenon (*Figure 1*). Intracellular MAb content of the entrapped cells as measured by flow cytometry was also higher than that of free-suspended cells. Secondly, in the entrapped cell culture, the reduction of serum concentration from 10% to 1% did not decrease the volumetric MAb productivity (r_{MAb}). In fact, because the q_{MAb} remained constant, only viable cell concentration directly affected the r_{MAb}; the serum concentration affected r_{MAb} only indirectly. In the free-suspended cell culture, with each decrease in serum concentration, the viable cell concentration fell, thus reducing r_{MAb}. In the entrapped cell culture, with each decrease in the serum concentration from 10% to 1%, the viable cell concentration remained constant, and thereby the r_{MAb} also remained constant. When the serum concentration was reduced further from 1% to 0%, the viable cell concentration fell along with the r_{MAb}. So the limitation to r_{MAb} is probably a direct result of some limit to viable cell concentration. In the entrapped cell culture with the viable cell concentration over 10^7 cells ml^{-1} of gel, there might be some limiting components required for cell growth other than serum. Thereby, the effect of serum concentrations on the viable cell concentrations might be masked by other limitations.

As discussed above, the entrapped cell culture system has a higher r_{MAb} than the free-suspended cell culture system because of both the enhanced q_{MAb} and the maintenance of a high viable cell concentration. In addition, because the reduction of serum concentration from 10% to 1% did not influence the viable cell concentration in the entrapped cell culture, serum concentration in medium could be decreased to 1% without any loss in MAb productivity. Therefore, the entrapped cell culture offers significant advantages in efficiency of serum utilization for MAb production when compared to the free-suspended cell culture. When 1% serum medium was used, the efficiency of serum utilization for MAb production by the entrapped cell culture was about three times higher than that by free-suspended cell culture. By using the entrapped cells with 1% serum medium, MAb yield was approximately 2.2 mg ml^{-1} of serum.

Specific MAb productivity

A high volumetric MAb productivity (r_{MAb}) which can be achieved by increasing the cell concentration and/or increasing the specific MAb productivity (q_{MAb}), is desired for economical production of MAbs. Accordingly, the enhanced q_{MAb} of calcium alginate-entrapped S3H5/γ2bA2 hybridoma cells could improve the economics of *in vitro* production of MAbs (Lee, Varma and Palsson, 1991a).

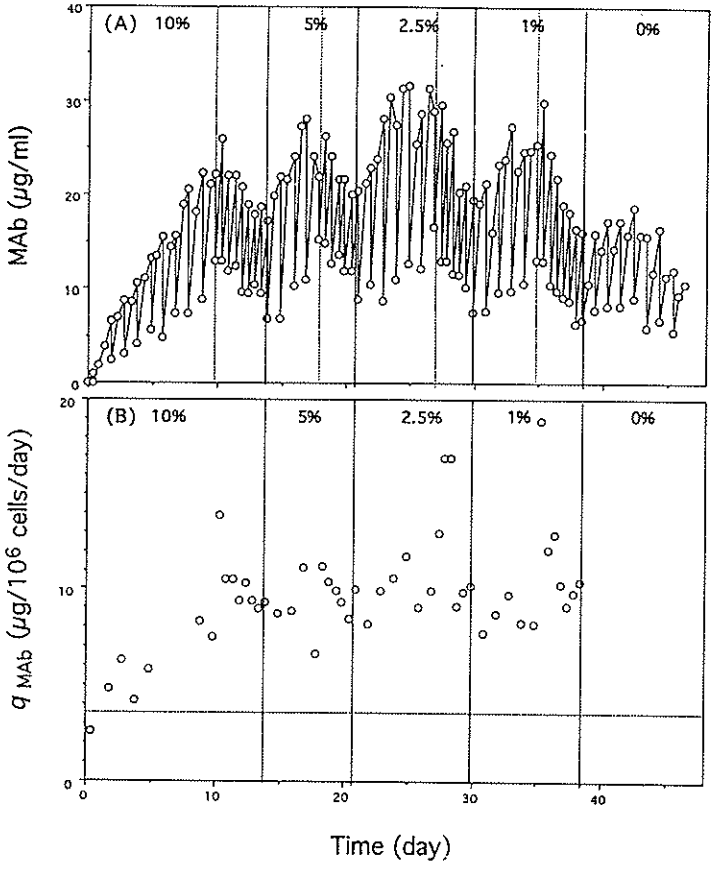

Figure 1. MAb concentration and specific MAb productivity (q_{MAb}) in the immobilized cell culture. Viable cell concentration at the beginning of this experiment was 1.8×10^6 cells ml^{-1}, and decreased to 1.0×10^6 cells ml^{-1} after 2 days of cultivation in 10% serum medium. This decrease in viable cell concentration may have occurred because of cell damage during the immobilization process. Viable cell concentration increased to 1.8×10^6 cells ml^{-1} after 8 days of cultivation in 10% serum medium. Viable cell concentration in 1% serum medium on cultivation day 38 was 1.7×10^6 cells ml^{-1} with a cell viability of 50%. The percentages in the figure represent the serum concentrations used in the media. (A) MAb concentration. Dotted and solid lines indicate times when the feeding schedule was changed. After the dotted line, 50 ml of spent medium was replaced by fresh medium twice a day. After the solid line, 60–65 ml of spent medium was replaced once a day. (B) q_{MAb} of immobilized cells. Dotted line represents an average q_{MAb} of free-suspended cells in a control experiment.

Contrary to our observation of the enhanced q_{MAb} of entrapped hybridoma cells, other researchers have reported that the q_{MAb} of entrapped hybridoma cells was comparable with that of hybridoma cells grown in suspension (Shirai *et al.*, 1987; Sinacore, Creswick and Buehler, 1989; Wohlpart, Gainer and Kirwan, 1991). These results were obtained using several hybridoma cell lines immobilized in calcium alginate beads. The differences between the results we reported and those found by others (Shirai *et al.*, 1987; Sinacore, Creswick and Buehler, 1989; Wohlpart, Gainer

and Kirwan, 1991) are possibly due to the different culture conditions under which the alginate beads were carried, or due to differences in the cell lines used.

In our work, the entrapped S3H5/γ2bA2 hybridoma cells were cultivated in a spinner flask (Lee, Varma and Palsson, 1991a). A threefold increase in q_{MAb} was obtained only after viable cell concentrations in 1 mm diameter calcium alginate beads reached the maximum. Culture conditions under which other researchers repported that the q_{MAb} of the entrapped cells was comparable with that of free-suspended cells are summarized as follows. Shirai *et al.* (1987) cultivated 4H11 human–mouse hybridoma cells immobilized in 2 mm diameter calcium alginate beads in a six-well plate. Wohlpart and co-workers cultivated 63D hybridoma cells immobilized in 1 mm and 3.9 mm diameter calcium alginate beads in a spinner flask, but they did not measure the q_{MAb} of entrapped cells in the spinner flask (Wohlpart, Gainer and Kirwan, 1991). The q_{MAb} of entrapped cells was measured by incubating washed beads (removed from the spinner flask) in fresh medium in a sterile culture tube for 4 hours. Sinacore and co-workers cultivated KTI16A hybridoma cells in 1–1.5 mm diameter calcium alginate beads in a spinner vessel (Sinacore, Creswick and Buehler, 1989). The q_{MAb} was measured during the growth phase of entrapped cells in a batch culture.

Considering the importance of q_{MAb}, it is necessary to determine whether the enhanced q_{MAb} of entrapped hybridoma cells is, in fact, cell-line specific or whether the differences in the results reported are due to cell culture conditions. Thus, we cultivated S3H5/γ2bA2 hybridoma cells entrapped into calcium alginate beads with different bead sizes in both T- and spinner flasks in order to determine whether cultivation methods and bead sizes, as employed by other researchers (Shirai *et al.*, 1987; Wohlpart, Gainer and Kirwan, 1991), influence the q_{MAb}. We also carried out free-suspended cell cultures with the cells recovered from calcium alginate beads to determine whether changes in the q_{MAb} are reversible.

The cultivation method was found to influence significantly the q_{MAb} of the entrapped cells (*Table 1*). When the entrapped cells in 3 mm diameter alginate beads were cultivated in T-flasks, q_{MAb} was not enhanced by 200% as previously observed in an entrapped cell culture using 1 mm diameter alginate beads in spinner flasks. The q_{MAb} of the entrapped cells was approximately 72% higher than that of the free-suspended cells in a control experiment. Therefore, the conclusion that the enhanced q_{MAb} of entrapped cells is cell-line specific cannot be drawn. Because the degree of q_{MAb} enhancement varies with cultivation method, the q_{MAb} of entrapped 4H11 hybridoma cells may be improved by cultivating them in spinner flasks. Unlike the cultivation methods, the size of alginate beads in the range 1–3 mm did not influence significantly the q_{MAb} of the entrapped cells in T-flasks. These results are consistent with the results of Wohlpart, Gainer and Kirwan (1991), that the q_{MAb} of entrapped cells was little affected by bead size in the range of 1–3.9 mm.

When free-suspended cell cultures with the cells recovered from calcium alginate beads were carried out, the enhanced q_{MAb} as well as q_{glu} and q_{lac} decreased to the value of the free-suspended cells in a control free-suspended cell culture (*Figure 2*). Thus, the changes in the entrapped cells were reversible. In addition, the changes in the entrapped cells more rapidly reverted in spinner flasks than in T-flasks. In spinner flasks, the q_{MAb} of free-suspended cells recovered from the beads dropped significantly at the first passage (*Figure 2*) and the enhanced intracellular MAb contents

Table 1. The q_{MAb}, q_{glu}, q_{lac}, and $Y_{lac/glu}$ of S3H5/γ2bA2 hybridoma cells under various culture conditions

Cultivation method	Bead size (mm)	q_{MAb} (μg 10^6 cell^{-1} day^{-1})	q_{glu} (mmol 10^9 cells^{-1} day^{-1})	q_{lac} (mmol 10^9 cells^{-1} day^{-1})	$Y_{lac/glu}$ (mmol mmol^{-1})
Free cell culture in T-flasks[a]		3.88±0.53 (3.88±0.98)	3.32±0.42 (3.26±0.43)	5.57±0.82 (5.37±0.70)	1.69±0.18 (1.65±0.14)
Free cell culture in spinner flasks		3.70±0.25	3.83±0.46	6.85±1.06	1.78±0.07
Entrapped cell culture in T-flasks	1.1	6.13±1.27	6.90±1.07	12.49±1.64	1.83±0.26
	1.6	6.16±1.03	7.00±0.79	13.57±1.45	1.95±0.19
	3	6.66±1.78	7.14±1.36	13.76±1.38	1.98±0.42
Entrapped cell culture in spinner flasks	1	10.93±1.57	5.24±1.24	9.97±1.57	1.95±0.30
	3	15.91±1.39	8.18±1.34	14.84±1.50	1.83±0.16
Recovered free cell culture in T-flasks[b]	1.1	3.51±0.45	3.92±0.29	6.97±0.18	1.79±0.10
	1.6	2.76±0.16	4.08±0.44	6.76±0.16	1.66±0.03
	3	3.32±0.80	3.21±0.04	5.44±0.41	1.70±0.15
Recovered free cell culture in spinner flasks[b]	3	5.89±1.26	4.46±0.56	7.16±0.06	1.62±0.19

[a] For free-suspended cell culture in T-flasks, duplicate cultures (flasks A and B) were carried out in a repeated-fed batch mode.
[b] The values are average data from the last three batches.

decreased to the value of the free-suspended cells used for entrapment at the second passage. Hybridoma cells are often described as a heterogeneous population with regard to distribution of intracellular MAb content (Heath, Dilwith and Belford, 1990), surface MAb content (McKinney, Dilwith and Belfort, 1991), and chromosome number (Goding, 1980). The microenvironment in alginate beads may favour the population of a high MAb producer. We could hypothesize that the population of a high MAb producer became dominant during the entrapped cel culture, resulting in the enhanced q_{MAb} of entrapped cells. However, the results obtained here do not support this hypothesis, since the reversion of changes in the entrapped cells occurs quickly, within two passages. Thus, the enhanced q_{MAb} of entrapped cells is more likely to be due to changes in cellular behaviour caused by entrapment.

As discussed above, the cultivation method (T- or spinner flasks) was found to influence significantly the q_{MAb} of the calcium alginate-entrapped S3H5/γ2bA2 hybridoma cells, indicating that the enhanced q_{MAb} may not be cell-line specific. Thus, the remaining question to be addressed is to determine whether the enhanced q_{MAb} of entrapped hybridomas is cell-line specific. We cultivated calcium alginate-entrapped

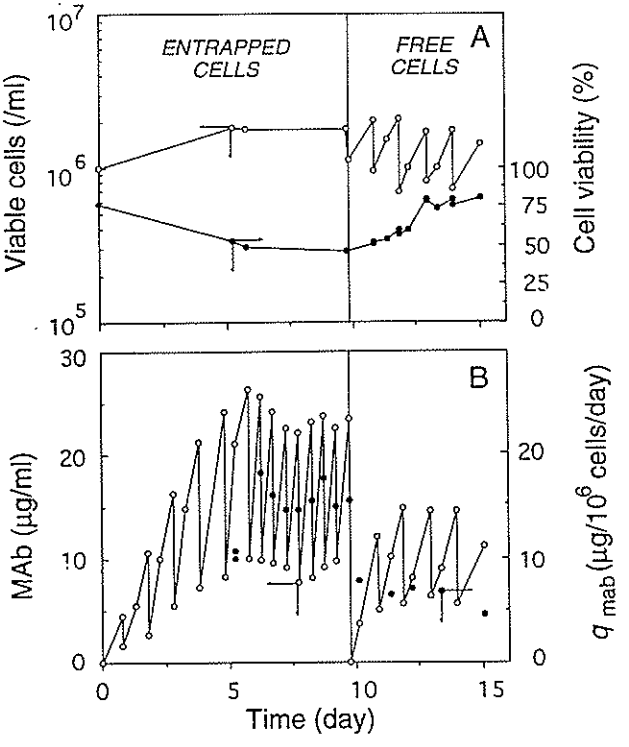

Figure 2. Alginate-entrapped (3 mm diameter) S3H5/γ2bA2 hybridoma cell culture followed by free-suspended cell culture in spinner flasks: (A) viable cell concentration and cell viability. (B) MAb concentration and q_{MAb}. The entrapped cells were cultivated in a repeated fed-batch mode. After 10 days, the alginate beads containing the cells were recovered from the spinner flasks and were dissolved in an isotonic citrate solution for 10 minutes. The cells were then centrifuged at 1500 r.p.m. for 10 minutes and then washed with a fresh medium. The recovered, free suspended cells were re-inoculated into a spinner flask. The cells were diluted 1:2 with fresh medium every day.

murine hybridomas under the conditions where we observed the significantly enhanced q_{MAb} of calcium alginate-entrapped S3H5/γ2bA2 hybridoma (Lee, Varma and Palsson, 1991) and, thereby, determined whether the enhanced q_{MAb} of entrapped hybridomas is indeed cell line-specific.

The two murine hybridomas used were 4A2 and DB9G8 hybridomas. Both hybridomas have been extensively studied in the literature (Heath, 1988; Mancuso *et al.*, 1990; Hiller *et al.*, 1991; McKinney, Dilwith and Belfort, 1991; Hiller, Clark and Blanch, 1993; Lee and Palsson, 1993). Since the physiological properties of hybridomas can change during passage, there might be some difficulty in comparing the results obtained here directly with those in the literature. Some of the features of hybridomas used here are as follows. The q_{MAb} of a slow-growing, high-concentration culture of 4A2 hybridoma in a hollow fibre reactor with high porosity and large pore size was almost identical to that of free-suspended cells in continuous suspension culture at low dilution rate (Mancuso *et al.*, 1990). Accordingly, we may be able to compare the q_{MAb} of calcium alginate-entrapped cells indirectly with that of the cells in the hollow fibre reactor. It has been suggested that the DB9G8 hybridoma has an upper limit for MAb concentration in the culture medium as the q_{MAb} dropped to zero at around 27–36 μg ml^{-1} antibody (McKinney, Dilwith and Belfort, 1991). It may thus be informative to monitor the changes in q_{MAb} of the calcium alginate-entrapped DB9G8 hybridoma during the culture.

Calcium alginate-entrapped 4A2 and DB9G8 hybridomas showed very similar changes in q_{MAb} during culture. For calcium alginate-entrapped S3H5/γ2bA2 hybridoma, the q_{MAb} as well as the intracellular MAb content was significantly enhanced since the maximum cell concentration was achieved. Unlike S3H5/γ2bA2 hybridoma, neither 4A2 nor DB9G8 hybridomas showed the persistently enhanced q_{MAb} when they were entrapped in calcium alginate beads (*Figures 3* and *4*). A similar observation has been reported for the 63D3 hybridoma (Wohlpart, Gainer and Kirwan, 1991). Thus, the enhanced q_{MAb} of calcium alginate-entrapped hybridomas appears to be cell-line specific. The enhanced q_{MAb} of entrapped 4A2 and DB9G8 hybridomas, which was 2–3 times higher than the q_{MAb} of free-suspended cells in a control experiment, was observed only during the early stage of the culture. As the cells resumed growth, this enhanced q_{MAb} decreased to the level of q_{MAb} of free-suspended cells in a control experiment. The intracellular MAb content of entrapped cells at the end of entrapped cell culture was also comparable to that of free-suspended cells in a control experiment. During the early stage of the culture, viable cell concentration was decreasing, probably due to cell damage during the entrapment process. However, the enhanced q_{MAb} of entrapped cells during the early stage of the culture was not just due to a low cell concentration, as indicated by the results from experiments with varying initial cell concentrations (*Figure 4*). On day 5, the viable cell concentration in the experiments with different initial cell concentrations was similar. However, the entrapped cells with an initial cell concentration of 1.0×10^6 cells ml^{-1} were actively growing while the entrapped cells with an initial cell concentration of 2.0×10^6 cells ml^{-1} were still in the lag phase. The q_{MAb} of entrapped cells with an initial cell concentration of 1.0×10^6 cells ml^{-1} was 28.29 μg 10^6 cells^{-1} day^{-1} while the q_{MAb} of entrapped cells with an initial cell concentration of 2.0×10^6cells ml^{-1} was 64.24 μg 10^6 cells^{-1} day^{-1}. Thus, the enhanced q_{MAb} of entrapped cells at the beginning of

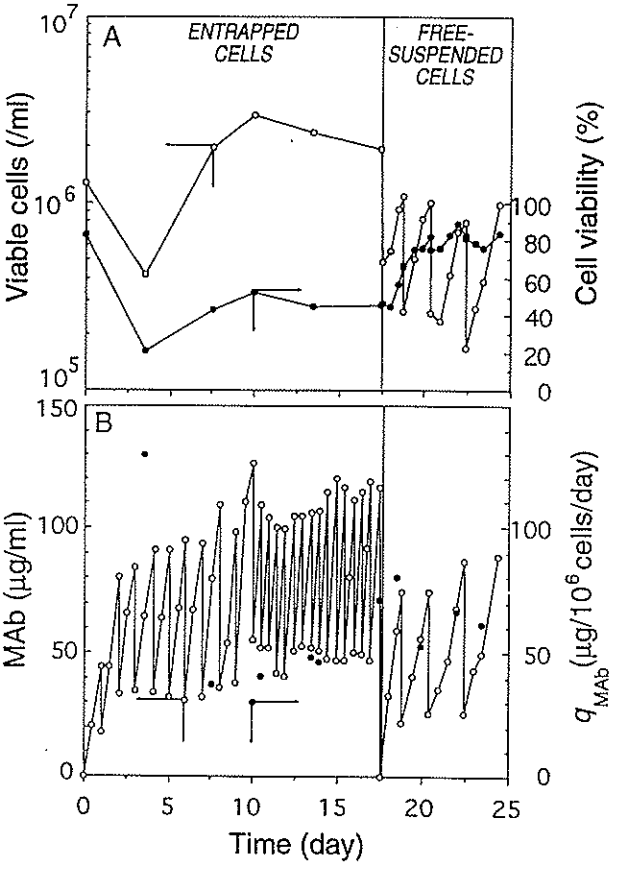

Figure 3. Entrapped 4A2 hybridoma cell culture followed by free-suspended cell culture in spinner flasks: (A) viable cell concentration and cell viability. (B) MAb concentration and q_{MAb}.

the culture was not just due to the low cell concentration. Furthermore, released antibody from dead cells did not account for the enhanced q_{MAb} because the antibody contained in viable and dead cells together accounted for less than 10% of the total extracellular antibody (Meilhoc, Wittrup and Bailey, 1989; Passini and Goochee, 1989; Reddy, Bauer and Miller, 1992). Accordingly, the enhanced q_{MAb} of entrapped cells was likely to be due to the stressful culture conditions induced by entrapment. After the cells adapted to a new microenvironment in the beads, the entrapment might not provide the stressful culture condition to the cells any longer. Thus, when the cells resumed growth, the enhanced q_{MAb} of entrapped cells might disappear.

The enhancement of q_{MAb} in immobilized cell culture could be attributed to a number of causes: slowed growth rate, stressful culture conditions and presence of autocrine growth factors. The specific growth rate (μ) of immobilized cells was almost zero after the cell concentration reached saturation. Many reports show that

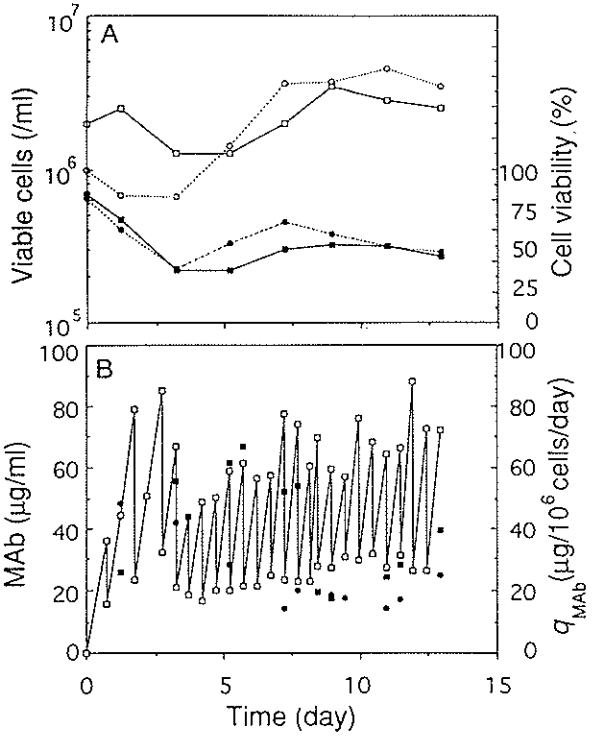

Figure 4. Entrapped DB9G8 hybridoma cell culture: effect of initial cell concentration. (A) Initial cell concentration of 1×10^6 cells ml^{-1}, viable cell concentration (O) and cell viability (●); initial cell concentration of 2×10^6 cells ml^{-1}, viable cell concentration (□) and cell viability (■). (B) Initial cell concentration of 1×10^6 cells ml^{-1}, q_{MAb} (●); initial cell concentration of 2×10^6 cells ml^{-1}, MAb concentration (□) and q_{MAb} (■). To avoid an overly complex figure, only q_{MAb} for the entrapped cells with an initial cell concentration of 1.0×10^6 cells ml^{-1} has been plotted.

q_{MAb} is inversely related to the μ of hybridoma cells (Reuveny *et al.*, 1986; Dean *et al.*, 1987; Low, Harbour and Barford, 1987; Heath, 1988; Miller, Blanch and Wilke, 1988; Hagedorn and Kargi, 1990; van der Pol *et al.*, 1990), although there are some exceptions (Shirai *et al.*, 1987; Glacken, Adema and Sinskey, 1988; Dalili and Ollis, 1989). One structured model, proposed by Suzuki and Ollis (1990), shows that the q_{MAb} can be increased by 3.5 times by reducing the μ to zero. Stressful culture conditions would also result in slowed cell growth rate. The q_{MAb} of the S3H5/γ2bA2 hybridoma was elevated at slowed growth rates in free-suspended cell cultures (Lee, Huard and Palsson, 1989). However, when the entrapped S3H5/γ2bA2 hybridoma showed slowed growth rates and was under stressful conditions, the q_{MAb} was not always enhanced (Lee, Chuck and Palsson, 1993). Cultivation methods (T-flask and spinner flask) determined the extent of enhancement of q_{MAb} of entrapped S3H5/γ2bA2 hybridoma. Thus, slowed growth rates and stressful culture conditions of the entrapped S3H5/γ2bA2 hybridoma do not directly account for its enhanced q_{MAb}.

The difference in the q_{MAb} of entrapped S3H5/γ2bA2 hybridoma might be ex-

plained by different mass transfer characteristics of autocrine growth factors. Increasing evidence exists to show that hybridoma cells produce autocrine growth factors which either stimulate or inhibit MAb production. Kidwell and co-workers observed that NOT human–human hybridoma cells and human normal lymphocytes grew better in hollow-fibre cartridges with low molecular weight cut-offs (3000 MWCO) (Kidwell, Knazek and Wu, 1990). Direct evidence for the presence of growth-stimulating factor accumulation in the hollow-fibre reactor with 3000 MWCO fibres was obtained by recovering growth-stimulating activity from the extracapillary space of the bioreactor cartridge (Kidwell, Knazeck and Wu, 1990). Interleukin-6 (IL-6, molecular weight approximately 26 kDa) is an autocrine growth factor known to stimulate immonoglobulin synthesis in lymphoid cells (Hirano *et al.*, 1986; Kawano *et al.*, 1988; Aarden, 1989). Makishima and co-workers found high IL-6 levels in active culture supernatants of peritoneal exudate cells (PEC), which may be responsible for the enhanced q_{MAb} *in vivo* (Makishima, Mikami and Terada, 1990). They also found that recombinant human IL-6 significantly enhanced the q_{MAb} of 2E3 hybridoma cells *in vitro* and it did not augment proliferation of the cells (Makishima, Mikami and Terada, 1990; Terada, Makishima and Takamatsu, 1992).

On the other hand, END and Col12 hybridomas are found to produce inhibitory factors, one of which has been identified as transforming growth factor β_1 (TGFβ_1). TGFβ_1 inhibits MAb production as well as cell growth (Kidwell, 1989). Its specific productivity was also found to be cell-line dependent (Kidwell, 1989). Thus, when compared with ultrafiltration membrane-based hollow-fibre cartridges with low molecular cut-offs (4000 MWCO), yield of hybridoma cells and MAb production were significantly higher in bioreactors based on high MWCO hollow fibres (0.5 μm pore) that allow exchange of very large macromolecules across their walls (Kidwell, 1989).

Hagedorn and Kargi (1990) made a similar observation in flat-sheet membrane bioreactors with several different MWCO membranes. When HDP1 hybridoma cells were cultivated in a flat sheet membrane bioreactor, the q_{MAb} was significantly enhanced to different degrees depending on the MWCO of the membrane used. The q_{MAb} in a 50 000 MWCO membrane system was 3.5 times greater than the q_{MAb} in a batch reactor without a membrane, whereas the q_{MAb} in a 100 000 MWCO membrane system was five times greater than the q_{MAb} in a batch reactor without a membrane. Accordingly, inhibitors such as TGFβ_1 may not be removed rapidly enough or may be unable to diffuse out of the 50 000 MWCO membrane system, resulting in the lower q_{MAb}. TGFβ_1 has a molecular weight of approximately 25 000, but is often recovered from cell-conditioned media as a high molecular weight complex (Kidwell, Mohanam and Salomon, 1987).

The results from the experiment employing varying bead size in both T- and spinner flasks indicate that, in the bead size in the range of 1–3 mm, external mass-transfer resistance is more influential on the q_{MAb} of S3H5/γ2bA2 hybridoma than internal mass-transfer resistance. Thus, the accumulation of inhibitors around the beads in T-flasks may hamper the inhibitors diffusing out of the beads and, thereby, reduce the q_{MAb}.

The enhanced q_{MAb} of the entrapped cells was not unique to calcium alginate-entrapped S3H5/γ2bA2 hybridoma cells in spinner flasks. A significant enhancement of q_{MAb} has been observed for SPO1 hybridoma cells entrapped in alginate/agarose

composite gel in a packed bed perfusion reactor (Shen, Reid and Greenfield, 1992). When the medium was exchanged three to four times a day, the q_{MAb} of the entrapped cells was three to eight times greater than that of free-suspended cells in a control suspension culture. The q_{MAb} of hybridoma cells entrapped in collagen microspheres in a fluidized bed perfusion bioreactor was higher than that of free-suspended cells in a continuous stirred tank bioreactor (Ray *et al.*, 1990). Because the medium was recirculated very rapidly in both packed bed perfusion and fluidized bed perfusion bioreactors (Ray *et al.*, 1990; Shen, Reid and Greenfield, 1992), the external mass-transfer resistance around the beads may have been insignificant.

In contrast, the enhancement in q_{MAb} of free-suspended 9.2.27 hybridoma cells was not observed in the total cell recycling suspension reactor where a high cell concentration with a very slow hybridoma growth was achieved (Flickinger, Goebel and Bohn, 1990). In addition, the q_{MAb} of a slow-growing, high-concentration culture of X-D hybridoma cells in a hollow fibre with a high porosity and large pore size, was almost identical to that of free-suspended cells in continuous suspension culture at a low dilution rate (Mancuso *et al.*, 1990). When S3H5/γ2bA2 hybridoma cells used in this study were cultivated in a hollow-fibre bioreactor, the intracellular MAb content of the cells, unlike the calcium alginate-entrapped cells, did not increase (Halberstadt, 1991). Accordingly, mass transfer of possible autocrine growth factors, as well as high cell concentration with slowed growth rate, may be an important factor to enhance q_{MAb}.

As discussed earlier, the transiently enhanced q_{MAb} of 4A2 and DB9G8 hybridomas is likely due to stressful conditions. Thus, even when the q_{MAb} is enhanced by entrapment, the reason for the enhanced q_{MAb} of entrapped cells appears to be different among cell lines. Different responses of the cells to entrapment are probably because of the nature of hybridoma cells. Since hybridomas are the randomly fused cells between immortalized myeloma and antibody-producing lymphocytes, their properties are expected to be very different. For example, 4A2 and DB9G8 hybridomas showed similar responses to entrapment with regard to their MAb production. However, they showed different responses to stress induced by freezing and thawing concerning their MAb production (Lee, Kim and Palsson, in press). Reddy and Miller (1994) showed that the effect of hyperosmotic stress on q_{MAb} is cell-line dependent. Furthermore, the production of autocrine growth factors and inhibitory factors was also found to be cell-line dependent (Kidwell, 1989). Accordingly, as shown in this study, it is unlikely for all the hybridomas to show a similar response to cell entrapment with respect to their MAb production.

The results reported here may be compared with those in the literature (Mancuso *et al.*, 1990; McKinney, Dilwith and Belfort, 1991). For 4A2 hybridoma cells, like the q_{MAb} of a slow-growing, high-concentration culture of 4A2 hybridoma cells in a hollow fibre reactor with high porosity and large pore size, the q_{MAb} of calcium alginate-entrapped cells was similar to that of free-suspended cells in a control experiment. Thus, calcium alginate beads do not provide 4A2 hybridomas with a unique environment to improve the q_{MAb}. For DB9G8 hybridoma cells, we did not find any MAb feedback inhibition. However, as we mentioned earlier, direct comparison may be difficult because culture history was different. In addition, a different medium was used here.

In conclusion, unlike the S3H5/γ2bA2 hybridoma, neither 4A2 nor DB9G8

hybridomas showed the persistently enhanced q_{MAb} when they were entrapped in calcium alginate beads. Thus, the enhanced q_{MAb} of calcium alginate-entrapped hybridomas appears to be cell-line specific. Although calcium alginate entrapment did not enhance the q_{MAb} of 4A2 and DB9G8 hybridomas, it did enhance the r_{MAb} because of high cell concentration. For both cell lines, the r_{MAb} in entrapped cell culture was approximately 3–4 times higher than that in free-suspended cell culture. As long as entrapment does not deteriorate the q_{MAb}, MAb production using entrapped cells may be feasible.

Stability of MAb production

Loss of MAb productivity of hybridoma cells in long-term cultivation has become a major concern to those interested in large-scale production of MAbs. This loss of MAb productivity is often attributed to the appearance of a non-producing population of hybridomas (NP) in the culture which has a growth advantage over a producing population of hybridomas (P) (Frame and Hu, 1990, 1991a,b; Lee and Palsson, 1990; Ozturk and Palsson, 1990; Chuck and Palsson, 1992; Merritt and Palsson, 1993). When an NP appears in the culture, it does not always take over the whole culture (Westwoudt, Naipal and Harrisson, 1984; Gardner *et al.*, 1985; Chuck and Palsson, 1992). Populations of P and NP may be balanced under certain culture conditions, depending on the relative growth rate of producing and non-producing cells and the rate of loss of MAb productivity (Lee, Varma and Palsson, 1991b). The appearance of an NP is thought to be due to mutations or loss of genes associated with antibody regulation as well as antibody synthesis ability because the losses in MAb productivity are usually reversible (Galfré *et al.*, 1980; Wilde and Milstein, 1980; Gardner *et al.*, 1985). Several hybridoma cell lines have been reported to lose their MAb productivity at a rate of 10^{-2}–10^{-5} cells/generation (Cotton, Secher and Milstein, 1973; Galfré *et al.*, 1980; Clark *et al.*, 1983; Gardner *et al.*, 1985). Although the tendency to lose MAb productivity is particular to each cell line, cells in low-serum or serum-free medium are often found to be more prone to lose MAb productivity than cells in high-serum medium (Lee and Palsson, 1990; Ozturk and Palsson, 1990).

Immobilization has been suggested to improve plasmid stability in genetically modified bacteria in bioreactors (Kumar *et al.*, 1991; Flickinger and Rouse, 1993). The observation that plasmid copy number can be stabilized in cells immobilized in gel beads and biofilms has been made and can be predicted mathematically (de Taxis du Poët *et al.*, 1986, 1987; Nasri *et al.*, 1987; Sayadi *et al.*, 1988, 1989; Barbotin, Nava Saucedo and Thomasset, 1990; Kumar and Schügerl, 1990), although the mechanism for this is unknown (Huang, Peretti and Bryers, 1993). Immobilization may similarly improve the stability of the hybridoma cells' MAb productivity.

We examined the hypothesis that the instability of S3H5/γ2bA2 hybridoma cells' MAb productivity in low-serum or serum-free medium may be overcome by entrapping the cells in gel beads (Lee and Palsson, 1990). First, free-suspended cell cultures with low-serum and serum-free media were carried out for several months to assess cell growth and loss of MAb productivity. Cell culture was performed using spinner flasks in a repeated fed-batch mode. Cells were cultivated in 1% serum media and then passed to serum-free media followed by serum-free, low-protein (SFLP) media. The specific growth rates of the cells in 1% serum media were in the range 0.6–1.2

day^{-1}. Cells in serum-free and SFLP media grew as fast as in 1% serum media for 2 months. MAb productivity was maintained in 1% serum media. However, cells in serum-free media showed a decrease in MAb productivity, and it completely disappeared in IMDM-based SFLP media (*Figure 5*). Flow cytometric study showed that the loss of MAb productivity was due to the occurrence of an NP which lost its MAb productivity. A single peak of P was found in 1% serum medium. However, in serum-free media, a second peak of NP appeared. As the passage number increased, the NP increased, and after 15–20 passages, the NP took over the whole culture, resulting in a complete loss of MAb productivity. Next, cells immobilized in alginate beads were also cultivated in serum-free media to test whether immobilization can improve the stability of the cell's MAb productivity. As shown in *Figure 6*, calcium alginate-entrapped hybridoma cells in SFLP medium were producing MAb over a month, indicating that immobilization can improve the stability of the cell's MAb-producing ability. The intracellular MAb content of free-suspended cells is compared with immobilized cells in *Figure 7*. The fluorescent intensity of immobilized cells in SFLP, which indicates the amount of intracellular MAb, was approximately three times higher than that of free-suspended cells in 10% serum medium. The q_{MAb} of immobilized cells in SFLP medium was determined to be approximately 9.6 μg 10^6 cells^{-1} day^{-1}, which was also approximately three times higher than that of free-suspended cells in 10% serum medium. Consequently, we found that immobilization not only improved the stability of S3H5/γ2bA2 hybridoma cells, but also enhanced their q_{MAb}.

Several lines of reasoning argue for this improved stability of MAb productivity of calcium alginate-entraped S3H5/γ2bA2 hybridoma cells. First, a microenvironment, created by a high local cell concentration in the calcium alginate beads, may keep the cells from a genetic drift or instability. Secondly, entrapped cells may have limited

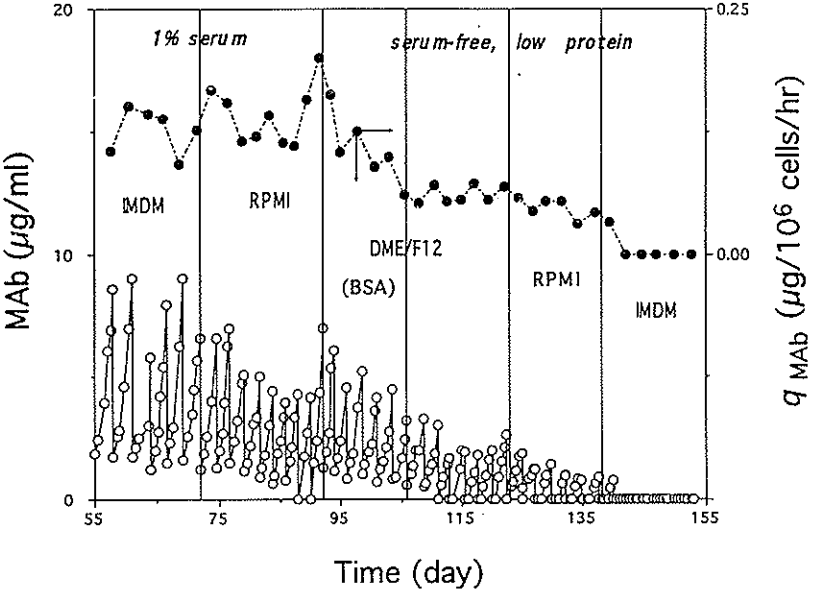

Figure 5. MAb production of free-suspended S3H5/γ2bA2 hybridoma cells in low-serum and serum-free media. The figure indicates how the media composition was changed with time. BSA, bovine serum albumin; DME/F12, IMDM and RPMI are the basal media used.

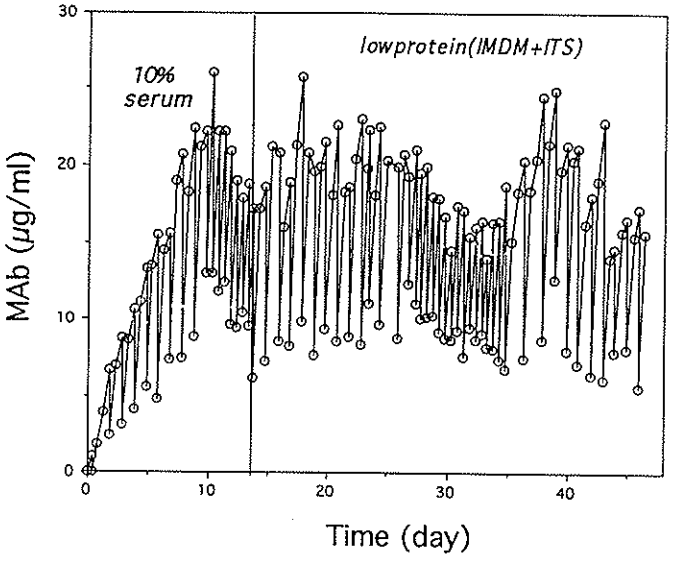

Figure 6. MAb concentrations in entrapped S3H5/γ2bA2 hybridoma cell culture. The feeding schedule was as follows: days 0–10, 60 ml/24 h; days 10–14, 50 ml/12 h; days 14–26, 60 ml/24 h; days 26–34, 50 ml/12 h; day 34 onwards, 60 ml/24 h.

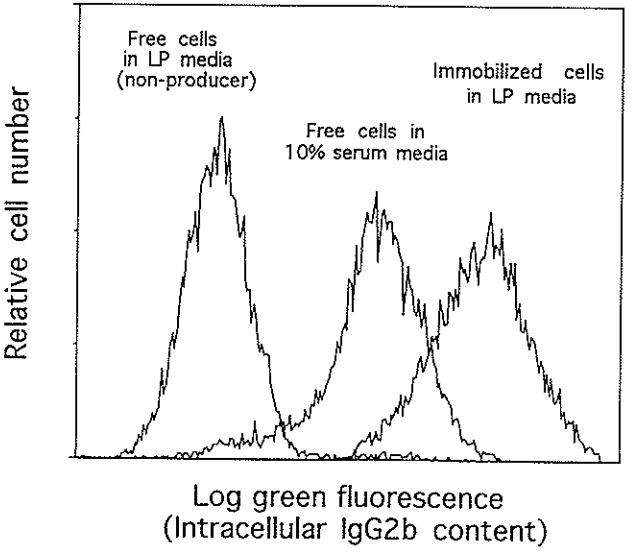

Figure 7. Comparison of intracellular MAb contents between free-suspended and entrapped S3H5/γ2bA2 hybridoma cells.

division, and thus genetic drift or instability is less likely to occur. Thirdly, growth characteristics of entrapped cells may account for this improved stability. The growth rates of entrapped cells are reported to be almost zero or negative after the cell concentration reaches its maximum (Nilsson *et al.*, 1986; Shirai *et al.*, 1987; Lee and Palsson, 1990; Lee, Varma and Palsson, 1991; Overgaard *et al.*, 1991; Wohlpart, Gainer and Kirwan, 1991; de la Broise *et al.*, 1992; Ogata *et al.*, 1992; Shirai,

Hashimoto and Steele, 1992). Accordingly, when an NP outgrowing a P appears in the culture, a rapid loss of MAb productivity of entrapped cells may not occur due to the slow growth rate of entrapped cells.

We examined the hypothesis that entrapped cells can have improved stability of MAb productivity due to limited cell growth. During maintenance of hybridoma cell line 4A2 in IMDM supplemented with 10% FBS, the cells lost MAb productivity and were used as an NP in this study. An NP outgrowing a P was co-entrapped with a P in calcium alginate beads, then cultivated in spinner flasks in a repeated fed-batch mode. As a control, a free-suspended cell culture with the same population of a P and an NP was also carried out in spinner flasks in a repeated fed-batch mode. As shown in *Figure 8*, the cell growth characteristics in the free-suspended cell culture did not change significantly throughout the culture. Unlike cell growth, MAb production deteriorated very rapidly and completely disappeared after the third batch. The q_{MAb} in the first batch was approximately 11.23 μg 106 cells^{-1} day^{-1}, which is less than one-fifth of the q_{MAb} of a pure P in serum-free medium. Thus, a significant portion of an inoculum is thought to be NP. An NP present in an inoculum appeared to take over the whole culture completely within three batches, resulting in a rapid decrease in the q_{MAb}.

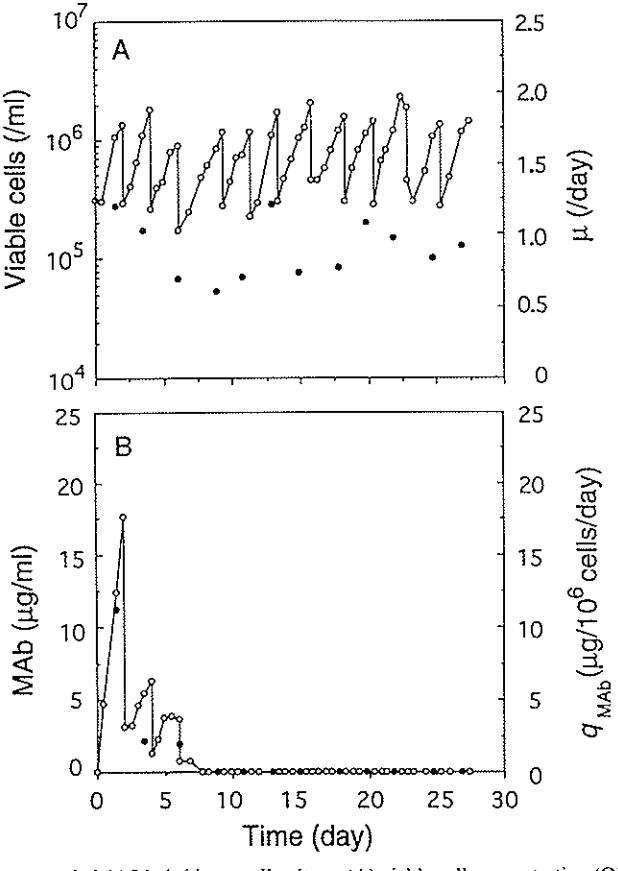

Figure 8. Free-suspended 4A2 hybridoma cell culture: (A) viable cell concentration (O) and μ (●). (B) MAb concentration (O) and q_{MAb} (●).

To examine whether entrapped cells can have improved stability of MAb productivity due to limited cell growth, two sets of entrapped cell cultures were carried out in a repeated fed-batch mode. Since the same inoculum used for free-suspended cell culture was also used for cell entrapment, a significant portion of the calcium alginate-entrapped cells at the beginning of culture belonged to an NP. Unlike the free-suspended cell culture, MAb production in the entrapped cell culture was maintained until the end of the culture, though a little deteriorated (*Figure 9*). The MAb concentration dropped significantly on day 8 because medium was exchanged twice a day after 8 days of cultivation. Accordingly, the r_{MAb} did not drop significantly on day 8. The r_{MAb} initially increased and gradually decreased in accordance with the viable cell concentration. However, the changes in q_{MAb} differed considerably from those in r_{MAb}. The q_{MAb} of entrapped cells at the beginning of the culture was approximately 11.58 μg 106 cells^{-1} day^{-1}, which is close to that of the first batch of free-suspended cell culture (*Figure 8*). The q_{MAb} dropped significantly during the growth phase of the entrapped cells and thereafter remained almost constant at 2.76±0.45 μg 10^6 cells^{-1} day^{-1}. Thus, like free-suspended cell culture, an NP appeared to outgrow a P in entrapped cell culture during the growth phase. Because the estimation of q_{MAb} is based on total viable cell concentration including an NP, the rapid outgrowth of an NP in entrapped cell culture results in a significant decrease in q_{MAb}. However, when the cell growth is limited, an NP cannot outgrow a P in a short period of time, as indicated by the stable value of q_{MAb} after 8 days of cultivation. Assuming that the q_{MAb} of the entrapped cells was equal to that of the free-suspended cells in serum-free medium, less than 5% of total viable cell concentration in entrapped cells at the end of the culture belonged to a P.

Next, to examine whether maintenance of a P in the entrapped cells is due to the limited cell growth, the second set of entrapped cell culture was carried out in a repeated fed-batch mode. After 19 days of cultivation, the alginate bead-entrapped cells were recovered aseptically from the spinner flask and were dissolved in a sterile isotonic citrate solution. The recovered, free-suspended cells were cultivated in a repeated fed-batch mode so that the NP could freely compete against the P. As shown in *Figure 10*, the cell growth, viability and MAb production characteristics of the entrapped cells were similar to those in *Figure 9*. Thus, we assumed that the conditions of the entrapped cells in two sets of entrapped cell cultures were almost identical. When the free-suspended cells recovered from the alginate beads were subcultured, MAb production deteriorated very rapidly and completely disappeared within two batches. Thus, a P present at a small fraction of viable cell concentration in the beginning of the free-suspended cell culture appeared to be outgrown rapidly by an NP and diluted out from the spinner flask. Accordingly, these results support the hypothesis that an NP cannot outgrow a P rapidly in the entrapped cell culture due to limited cell growth, resulting in improved stability of MAb productivity.

As mentioned earlier, the improved stability of MAb productivity of entrapped cells may be due to either the microenvironment in the gel beads, where genetic drift or instability of the cells is less likely to occur, or the limited cell growth of entrapped cells. It is still not known whether the microenvironment in the gel beads keeps the cells from a genetic drift or instability. However, the data obtained here suggest that even if an NP occurs in the long-term culture of entrapped cells, it cannot take over the whole culture in a short period of time due to the limited growth of these cells.

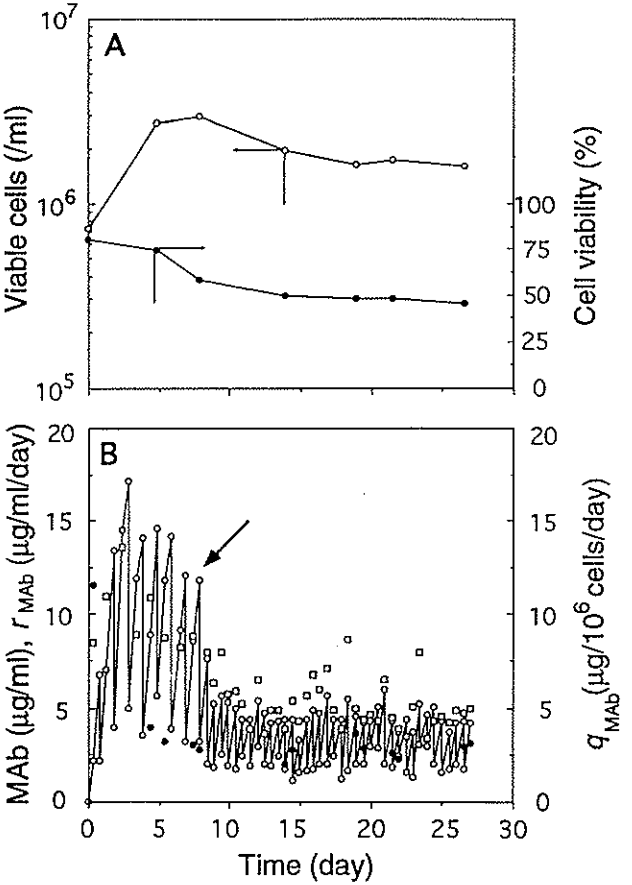

Figure 9. Entrapped 4A2 hybridoma cell culture: (A) viable cell concentration (O) and cell viability (●); (B) MAb concentration (O), r_{MAb} (□), and q_{MAb} (●). The arrow indicates changes in the feeding schedule.

Furthermore, even if an NP occurs in the exponential phase of growth in entrapped cell culture, the q_{MAb} can be decreased, but not significantly, due to the relatively short duration of the exponential growth phase. When an initial viable cell concentration in entrapped cell culture is approximately $1–2 \times 10^6$ cells ml^{-1} of medium, a maximum cell concentration is often achieved in less than 10 days (Lee and Palsson, 1990; Lee, Varma and Palsson, 1991a). In conclusion, due to the characteristics of growth of entrapped cells, the stability of MAb productivity of the cells can be improved using cell entrapment in gel beads.

Conclusion

Taken together, the results from questions addressed in this chapter show that immobilized cell culture is more efficient for MAb production than free-suspended cell culture with respect to the efficiency of serum utilization for MAb production, volumetric MAb productivity and stability of MAb production.

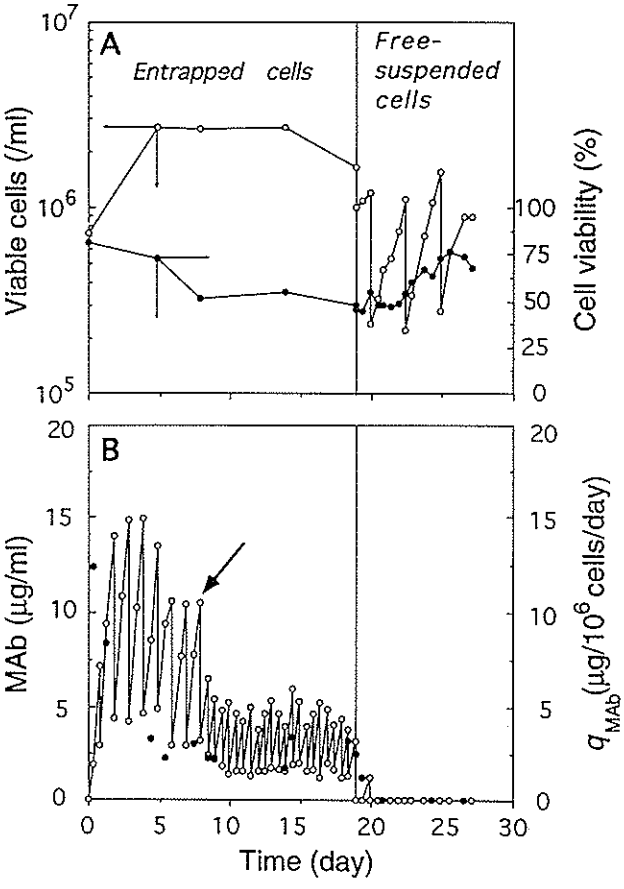

Figure 10. Entrapped 4A2 hybridoma cell culture followed by free-suspended cell culture: (A) viable cell concentration (O) and cell viability (●); (B) MAb concentration (O) and q_{MAb} (●). The arrow indicates changes in the feeding schedule.

The possible disadvantages of an entrapped cell culture system are the material cost, the potential for contamination during the immobilization process, the lack of compatibility of some cell types with the matrix components, and the potential for oxygen limitation within beads or microcapsules. However, some of these potential disadvantages can be overcome by choosing the proper immobilization method. Sodium alginate, which was used here for a gel material, is inexpensive and biocompatible with most hybridoma cell lines. The risk of contamination during immobilization is not significant because the process of making calcium alginate beads is a one-step process. The typical radius of alginate beads is in the range 400–500 μm, so that oxygen transport may not be limited. Typical penetration depth of oxygen for immobilized animal cells is approximately 500–1000 μm (Chang and Moo-Young, 1988). The main drawback of alginate beads is the possible diffusional limitation with respect to a large protein molecule like IgM and the difficulty of using media containing high phosphate concentrations. However, it was observed that IgM produced by the entrapped cells could diffuse out of the calcium alginate beads

regardless of alginate concentrations tested (0.8–2.5%) (Kim, Han and Lee, in press). When a medium with high phosphate concentration, such as RPMI1640 must be used, agarose (Nilsson *et al.*, 1983; 1986) or porous microcarrier (Runstadler and Cernek, 1988; Cahn, 1990; Griffiths, 1990; Looby and Griffiths, 1990) is recommended as a matrix for immobilization.

In conclusion, immobilization technology holds the potential of both increasing MAb productivity and improving the stability of hybridoma cells. Future developments will see an integrated development of immobilization methods, the bioreactor and the physiological requirements of the hybridoma cells.

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