Special Issue

Changes in monoclonal antibody productivity of recombinant BHK cells immobilized in collagen gel particles

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Key words: monoclonal antibody, immobilization, collagen gel, BHK, productivity, recombinant, high density culture

Abstract

Animal cell perfusion high density culture is often adopted for the production of biologicals in industry. In high density culture sometimes the productivity of biologicals has been found to be enhanced. Especially in immobilized animal cell culture, significant increase in the productivity has been reported. We have found that the specific monoclonal antibody (MAb) productivity of an immobilized hybridoma cell is enhanced more than double. Several examples of enhancing productivities have been also shown by collagen immobilized cells. Immobilized cells involve some different points from non-immobilized cells in high density culture: In immobilized culture, some cells are contacted together, resulting in locally much higher cell concentration more than 10^8 cells/ml. Information originating from a cell can be easily transduced to the others in immobilized culture because the distance between cells is much nearer. Here we have performed collagen gel immobilized culture of recombinant BHK cells which produce a human IgG monoclonal antibody in a protein-free medium for more than three months. In this high density culture a stabilized monoclonal antibody production was found with around 8 times higher specific monoclonal antibody productivity compared with that in a batch serum containing culture. No higher MAb productivity was observed using a conditioned medium which was obtained from the high density culture, indicating that no components secreted from the immobilized cells work for enhancing monoclonal antibody production. The MAb productivity by the non-immobilized cells obtained by dissolving collagen using a collagenase gradually decreased and returned to the original level in the batch culture using a fresh medium. This suggests that the direct contact of the cells or a very close distance between the cells has something to do with the enhancement of the MAb productivity, and the higher productivity is kept for a while in each cell after they are drawn apart.

Introduction

Development of genetic engineering using cultured animal cells enables us to synthesize desired biologicals which can be used in the field of clinical diagnostics and therapeutics (McCabe *et al.*, 1988; Khazeli *et al.*, 1988). The productivity of biologicals by cultured animal cells is focused on, relating to the culture conditions. The volumetric productivity of biologicals earlier was examined, resulting in the development of high density culture (Brennan *et al.*, 1987; Tokashiki

et al., 1987; 1990; Tolbert *et al.*, 1981; Velez *et al.*, 1989). In some high density animal cell culture, not only a volumetric productivity but also specific productivity per cell were found to be enhanced. Especially immobilized animal cells contribute to increase in the specific productivity (Lee *et al.*, 1991; 1993; Ray *et al.*, 1990; Shen *et al.*, 1992). We have also found that the specific monoclonal antibody (MAb) productivity of an immobilized mouse-human hybridoma cell, 4H11, is enhanced more than double under a low DO condition (Shirai *et al.*, 1992), although no enhancement

in MAb productivity was found under an atmospheric oxygen pressure (Shirai *et al.*, 1987). Several examples of enhancing productivities have been also shown by collagen immobilized cells (Ray *et al.*, 1990). However, in some cases, no enhancement in the productivity of biologicals have found in the immobilized animal cell culture (De la Broise *et al.*, 1992; Sinacore *et al.*, 1989; Wohlpert *et al.*, 1991). The reasons why the productivity is either sometimes enhanced or not remains still obscure.

A great interest in the specific productivity in the field animal cell culture has been stimulated by many researchers in this decade: The relationship between specific productivities and cell growth rates has been investigated to find mainly a negative association (Al-Rubeai and Emery, 1990; Merten, Miller *et al.*, 1988; Reuveny *et al.*, 1986; Suzuki and Ollis, 1990), indicating that the lower the cell growth rates, the higher the specific productivities they obtain. Higher productivities can be achieved by adjusting the culture conditions so that they can be maintained at the optimum conditions. Kyung and Hu (1995) reported enhanced productivity of protein C by recombinant human cells in automated fed-batch cultures. Productivities often depends on components of culture media including autocrine factors and some components which are derived from cells after their lyses. Mochizuki *et al.* (1993) found that fructose replacing glucose in serum-free culture media for the production of human monoclonal antibodies enhances specific MAb productivity. Especially immunoglobulin production stimulating factors (IPSF) which were derived from a Namalwa cells to accelerate MAb productivity have been investigated by many researchers (Shinmoto *et al.*, 1988; Sugahara *et al.*, 1991; Toyoda *et al.*, 1990; Yamada *et al.*, 1989). Farrell *et al.* (1994) recognized that endogenous proteins derived from hybridoma cells contributes to increase MAB productivities. Autocrine factors such as cytokines including interleukin-6 (IL-6) can stimulate MAB production (Makishima *et al.*, 1990). However, some cytokines such as TGF β 1 affect hybridoma growth and MAB production (Kidwell, 1989; Pendse and Bailey, 1990; Makishima *et al.*, 1990).

Immobilized cells involve some different points from non-immobilized cells in high density culture: In an immobilization system, some cells are contacted together, resulting in locally much higher cell concentration more than 10^8 cells/ml. Information originating from a cell can be easily transduced to the others in immobilized cells because the distance cells in much nearer. Therefore, concentrations of autocrine factors

in the immobilized gel particles would be locally higher than those in a non-immobilized culture medium because of locally high production rates of autocrine factors by the immobilized cells and low diffusivities of proteins from the immobilized gel particles. Cell proliferation rates would be slowed down due to a physical limitation of the space where the cell further proliferates. In immobilized gel, therefore, productivities would be improved due to suppressive cell growth rate and high concentrations of some factors derived from the cells which contribute to enhance the productivities. Moreover, stresses given by immobilization such as compression, low dissolved oxygen concentration would provide a favorable circumstance to produce biologicals (Ray *et al.*, 1990).

Here we have performed collagen gel immobilized culture of a recombinant BHK cell which produces a human IgG monoclonal antibody in a protein-free medium for more than three months. The change in the specific monoclonal antibody productivity during the culture duration was examined. Moreover, the effect of a conditioned medium obtained from immobilization culture on the MAB productivity was investigated. On the other hand, it was also examined how the MAB productivity of the cells which had been cultivated in the collagen gel beads was changed when they were cultured under a non-immobilized condition with a fresh medium.

Materials and methods

Materials

A baby hamster kidney cell which possesses AE6F4 human IgG gene (BHK-AE6) was used in this experiment (Kawamoto *et al.*, 1995).

Serum-containing (10% FCS), serum-free and protein-free eRDF media (Kyokuto Pharm. Co., Japan) were used for the cultivation of BHK-AE6 cells. Insulin, transferrin, mono-ethanolamine and selenite were included as serum-free components. Ferric citrate was replaced by transferrin and insulin in the protein-free medium. Table 1 indicates the media compositions used.

Immobilization and cultivation of the cells

Cells were immobilized in special collagen gel particles supplied by Cellex Bioscience Inc. (USA), which

Table 1. Media compositions

Serum medium	e-RDF	17.7 g
	sodium hydrogen carbonate	1.1 g
	FCS	100 ml
	in distilled water	1000 ml
Serum-free medium	e-RDF	17.7 g
	sodium hydrogen carbonate	1.1 g
	insulin	10 mg
	transferrin	20 mg
	monoethanolamine	20 μ mol
	sodium selenite	1 nmol
	in distilled water	1000 ml
Protein-free medium	e-RDF	17.7 g
	sodium hydrogen carbonate	1.1 g
	ferric citrate	12.2 mg
	linoleic acid	1 μ l
	taurine	1.25 mg
	monoethanolamine	20 μ mol
	sodium selenite	1 nmol
	in distilled water	1000 ml

have a porous structure for entrapping the cells with tiny stainless steel balls for adjusting the density.

For immobilization and cultivation of the cells, a Verax System One bioreactor system automatically controlled (Cellex Bioscience Inc. USA) was used, containing a tapered fluidized reactor with an external gas and heat exchanger through which the culture medium flows. Oxygen was supplied through a silicon tube spiraling in the external gas and heat exchanger. The working volume of the bioreactor is 100 ml. The detailed scheme of the experimental apparatus was shown in the previous paper (Shirai *et al.*, 1994).

In the cultivation, the medium was circulated at the rate of 100 ml/min. Fresh medium was supplied to the medium inlet spot locating at the tubing, while the spent medium overflowed from the top of the reactor and was recovered. Adequate perfusion rates were chosen elapsed culture time. The DO level was kept constant at the 60% of air saturated level under an atmospheric pressure by a computer programmed system. The culture pH was adjusted at 7.0 by introducing carbon dioxide gas and a higher pH medium. The culture temperature was 36.5 °C. Under the operating condition, the volume of the expanded gel bed was initially 40 ml.

For immobilization, 20 ml of wet collagen gels were loaded into the tapered reactor. Around 7×10^7

cells in total were gathered for immobilization from a preculture in the middle of their exponential growth. The cells were mixed with 5 ml of wet collagen gels and centrifuged at a rotation of 500 rpm for 5 min. to facilitate the cells being entrapped by the gels (Shirai *et al.*, 1994). Then the cell-gel mixture was loaded into the reactor. The medium was circulating for one night with neither feed nor withdrawal of the medium to complete cell entrapment in the collagen pores.

Examination of increase in MAb productivity

To examine the effect of factors derived from immobilized cells on the MAb production, a conditioned medium was sampled from the bioreactor. Since less nutrients and much toxic by-products such as lactate and ammonium were included in the conditioned medium, a fresh medium was mixed with the conditioned medium half by half to cultivate non-immobilized cells statically. The specific MAb productivity of the cells cultured in a medium which includes the conditioned medium was compared with that obtained from the immobilized cells.

The monoclonal antibody (MAb) productivity of the cells in a static batch culture, which had been cultivated in the collagen gel beads, was compared with that in the immobilization culture. Figure 1 shows a

Figure 1. A procedure to remove cells from collagen gels.

procedure to remove cells from the immobilized gel particles. The cells were removed from the collagen gel beads treated by a collagenase after the stainless steel balls had been settled down at the bottom of a test tube. The supernatant with living cells was transferred to a centrifugal tube and then centrifuged. After removing the waste medium, the cells were washed several times with a fresh medium. The static batch culture was renewed at every 100 hours by diluting the culture medium with a fresh medium.

The assay methods including MAb concentration and cell number measurements were shown in the previous papers (Shirai *et al.*, 1994). The MAb productivity was calculated based on the kinetic determination shown in the previous paper (Shirai *et al.*, 1994).

Results

Growth of immobilized cells

Figure 2 shows the cell growth curves during a continuous operation by an immobilization culture. The operation was successfully stabilized for three months. The cell concentration averaged by a reactor working volume is shown because the cells were locally concentrated in a collagen gel beads. The cell concentration

Figure 2. Growth of BHK-AE6 cells immobilized in collagen gels: up to the first arrow; serum containing culture, up to the second arrow; serumfree culture, after the second arrow; protein free culture.

Figure 3. Cell concentration in collagen gel beads.

was stabilized at around 7×10^6 cells/ml-reactor even when the serum-free medium (after the first arrow) and the protein-free medium (after the second arrow) were used. The increase in the cell concentration was ceased after the medium was changed to the serum-free medium. Figure 3 shows the change in the local cell concentration in the collagen gel bead. The local cell concentration was reached 4×10^8 cells/ml-bead which would be the limit of the capacity of cell in the porous collagen gel bead, and stabilized for the rest of the elapsed culture time, indicating that the increase in the cell concentration was ceased not because of the replacement of the medium but because of the full capacity of the cell.

Figure 4. Total cell concentration proliferating from the start of culture: up to the first arrow; serum containing culture, up to the second arrow; serumfree culture, after the second arrow; protein free culture.

Figure 4 shows the total number of the cells proliferating in the bioreactor including immobilized cells and leaked cells which were washed out from the bioreactor. The slopes in the figure indicate the cell growth rates during the elapsed culture time. Figure 4 indicates that the very slow cell growth rate can be found after the cells were fully packed in the gel beads. A part of the cells was leaked from the gels. That is why the cell growth continued for compensating the cell loss by cell leakage. The cell growth was unchanged even when the serum-free medium was replaced by the protein-free medium.

In the static batch culture, the specific growth rates of the cells in a serum containing medium and serum-free medium were 0.030 h^{-1} and 0.015 h^{-1} , respectively. Much slower specific growth rate was observed in the serum-free culture. A big drop of the cell concentration found after a medium change from a serum containing to serum-free one would be ascribed to the low specific growth rate in the serum-free medium (Figure 2). However, the number of cells in the collagen gels was maintained as shown in Figure 3, indicating that serum-free as well as protein-free media can maintain the cell number in the gel beads in spite of the low growth rate.

MAB productivity in immobilization culture

Figure 5 shows the change in the volumetric MAB productivity. In the early stage of the culture, the significant increase in the MAB productivity is found. This is ascribed to the increase in the cell concentration in the

Figure 5. Change in volumetric monoclonal antibody productivity: up to the first arrow; serum containing culture, up to the second arrow; serumfree culture, after the second arrow; protein free culture.

Figure 6. Change in specific monoclonal antibody productivity: up to the first arrow; serum containing culture, up to the second arrow; serumfree culture, after the second arrow; protein free culture.

bioreactor. After a rapid drop in the MAB productivity, it again started to increase gradually in spite of the constant cell number. This indicates the increase in the specific MAB productivity per cell. Figure 6 shows the change in the specific MAB production rate, indicating that the productivity increases at first drastically and then gradually in the elapsed culture time.

Table 2 summarizes the MAB productivities obtained from immobilized cells cultured with a protein free medium and from non-immobilized cells in batch cultures with 10% FCS and the serum-free components. From 30 to 60 times of the volumetric MAB productivity are found from the immobilized cells, compared with those from the batch culture. On the

Table 2. Monoclonal antibody productivity

	Specific MAb productivity [ng/(cell h)]	Volumetric MAb productivity [ng/(ml d)]
Immobilized cell (protein-free)	4.7×10^{-5}	8.4×10^3
Batch culture (10% FCS)	6.1×10^{-6}	1.4×10^2
Batch culture (serum-free)	1.2×10^{-5}	2.9×10^2

Figure 7. Effect of conditioned medium on the MAb production.

other hand, the specific MAb productivities obtained from the immobilized cells were also enhanced from 4 to 8 times compared with those from the batch culture. The enhancement of the specific MAb productivity in the collagen gel bioreactor was confirmed.

MAb productivity change by immobilization

The effect of a conditioned medium on the MAb productivity was shown in Figure 7. In the conditioned medium, monoclonal antibody has been already included. Therefore, the initial MAb concentration in the conditioned medium is much higher than that in a fresh medium. Figure 7 shows almost the same increases in MAb concentration both in a fresh medium and a medium containing the conditioned medium, indicating that no effect on the enhancement of MAb productivity is found in the conditioned medium. In our experiments it was confirmed that factors derived from

Figure 8. Change in specific MAb productivity of the cells once cultured in immobilized gels in the elapsed static batch culture time.

the immobilized cells show no effects on improving MAb productivities.

In Table 3 MAb productivities are compared which were obtained from a serum-free culture by the cells which have been cultivated in the collagen gel beads and from static batch cultures using a serum containing and serum-free media. The MAb productivity at time zero was obtained from the early 24 hours serum-free culture after the cells were washed by a fresh medium. The MAb productivity even in a suspension culture by the cells which had been once in the collagen gel beads was much higher than that from static batch culture. Figure 8 shows the change in the MAb productivity after the cells were transferred to the static culture from the immobilized gels. The productivity obtained at each passage is averaged value between each two passages. Even after the first pas-

Table 3. MAb productivity in static batch culture [ng/cell h]

	10% FCS culture	Serum-free culture
Cells not immobilized	6.1×10^{-6}	1.2×10^{-5}
Cells once immobilized in immobilized gels	4.4×10^{-5}	3.0×10^{-5}

sage of the batch culture, the MAb productivity still higher. However, it decreased gradually as the number of the passage increased and finally returned to the previous level which was obtained in the non-immobilized batch culture in which the cells had not experienced to be cultured in the collagen gel beads. This would indicate that the high MAb productivity is ascribed to the high density immobilization culture in the collagen gel beads. Moreover, the capability to produce monoclonal antibody was memorized in the cell because the MAb productivity by the cells which had been experienced to be in the gel beads was still high even when they were cultivated in static batch culture.

Discussion

The enhancement of the productivity of biologicals by animal cells is ascribed to the followings (Lee *et al.*, 1993); 1) low growth rate of the cells, 2) communication between the cells locally packed in the gel particle, 3) stress affecting the cell physiology, 4) autocrine factors produced by the cells. Every factor would be available in our immobilization system. In Figure 4, a slow down of the specific growth rate of the cells immobilized in collagen gel beads is found. Mentioned in the previous section, this cell line produces more monoclonal antibody with a lower growth rate in a serum-free medium than in a serum containing medium in which the cells grow faster. The increase in the specific MAb productivity in the late stage of the culture partly ascribes to the slow growth rate, as suggested by Suzuki and Ollis (1990). However, the MAb productivity also enhanced even with a higher growth rate in the early stage of the culture. As shown in Figure 8, a conditioned medium obtained from the immobilization culture had no effect on the enhancement of the MAb productivity. Therefore, the increase in MAb productivity would be ascribed to communication between the cells locally packed in the gel particle rather than the effect of autocrine factors derived from the immobilized cells.

The important finding in our experiment is that the capability enhancing MAb productivity was memorized in the cell for a while because their productivity was enhanced even in the static batch culture. In the static batch culture, a higher growth rate was confirmed. Less communication and less stress would be expected because of low concentration. Autocrine factors produced by the cells should have been removed at the washing step. Therefore, the capability of the enhancement triggered by some factors in the high density immobilization culture is not lost quickly after the factors are removed. A gradual decrease in the MAb productivity would suggest that the above factors are not direct ones which control the MAb productivity, but indirect ones which change cell physiology to produce monoclonal antibody shifting up to the status of the high production stage of monoclonal antibody. The MAb productivity strongly depends on mRNA stabilities (Savinell *et al.*, 1989; Suzuki, 1990; Takahashi, 1994). The mRNA of the monoclonal antibody might be enough stable to maintain high productivity for a while. This would be one explanation why the MAb productivity was enhanced. However, no explanation is given to clarify why the factors including enhancement of the mRNA's stability were triggered in the immobilization culture. These should be further examined at the next steps.

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