Production of Monoclonal Antibody Using Free-Suspended and Immobilized Hybridoma Cells: Effect of Serum

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The effect of serum on cell growth and monoclonal antibody (MAb) productivity was studied in a repeated fed-batch mode using both free-suspended and immobilized S3H5/γ2bA2 hybridoma cells. In the suspension culture, serum influenced the cell growth rate but not the specific MAb productivity. The average specific growth rate of the suspension culture in medium containing 10% serum was approximately 0.99 ±0.18 day^{-1} (±standard deviation), while that in medium containing 1% serum was approximately 0.73 ±0.12 day^{-1}. The specific MAb productivity was almost constant at 3.69 ±0.57 $\mu g/10^6$ cells/day irrespective of serum concentration. In the immobilized cell culture, cell concentration reached a maximum at ca. 1.8×10^6 cells/mL of medium in 10% serum medium, and the cell concentration remained constant even when the serum concentration was gradually reduced to 1%. The specific MAb productivity of the immobilized cells was more than three times higher than that of the free-suspended cells. The amount of serum in the medium did not influence the specific MAb production rate of the immobilized cells. The maintenance of high cell concentration and the enhanced specific MAb productivity of the immobilized cell culture resulted in a higher volumetric MAb productivity. In addition, MAb yield in the immobilized cell culture with medium containing 1% serum was 2.2 mg/mL of serum, which was approximately three times higher than that in the suspension culture.
Key words: hybridoma • immobilization • serum • flow cytometry • antibody productivity

INTRODUCTION

Currently, large-scale cultivation of hybridoma cells requires expensive serum to supplement the growth medium.[9,10] The serum requirement for growth of a murine hybridoma cell line (S3H5/γ2bA2) in a batch cultivation has been reduced using high initial cell concentrations.[15] Therefore, one would expect high-concentration hybridoma cell cultures to be economically advantageous.[7]

High cell concentrations can be obtained by immobilizing the cells in gel particles. Immobilized cell cultures have several advantages when compared with free cell suspension cultures.[25] (1) Immobilized cells can be protected from mechanical stress. Since cells in low-serum or serum-free medium are more shear sensitive than cells in high-serum medium,[17,24,33] it is advantageous to protect cells from mechanical damage by immobilizing them in gel beads. (2) Immobilized cell particles are about 50–1,000 times larger than free cells and are easier to use in perfusion systems; they do not clog conventional filter systems, and they can be easily separated during medium changes. (3) It has been reported that immobilization enhances the specific productivity of secreted products such as ethanol by yeast[5,26] and α-amylase by recombinant yeast.[40] Immobilization may similarly enhance the specific MAb productivity of hybridoma cells. The potential of using immobilized hybridoma cells for MAb production has been widely recognized but not sufficiently substantiated.[34,35]

In this study, we compare an immobilized cell culture with a suspension culture with regard to efficiency of serum utilization for MAb production. The effect of serum on cell growth and metabolism is also investigated.

MATERIALS AND METHODS

Cell Line and Culture Maintenance

The murine hybridoma used was S3H5/γ2bA2, provided by Dr. Mark Kaminski of the University of the Michigan Medical Center. The antibody produced by this cell line is γ2b anti-idiotype antibody, directed against a carcinogen-induced B cell tumor (38C13).[2] The fusion partner used was P3/x63/Ag8.653. The cell culture medium for stock cells was Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY) and 100 U of penicillin and 100 μg/mL of streptomycin (Sigma). The cells were maintained in T25 cm^2 plastic cell culture flasks (Bellco Glass, Inc., Vineland, NJ) at 37°C in a humidified CO_2 incubator (VWR Scientific, San Francisco, CA). The cells were diluted 1:5 with fresh medium every other day.

Immobilization

Hybridoma cells are sensitive to toxic compounds and changes in the environment caused by chemical reaction. Alginate was used as a gel matrix because it is

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biocompatible and it undergoes a mild gelation reaction.[34–36] Cells were entrapped in gel beads of calcium alginate (Keltone LV, Kelco, Chicago, IL) as described previously.[16] The initial cell concentration was approximately 6×10^6 cells/mL in 1.5% sodium alginate. The size of the gel beads was in the range of 0.8–1.0 mm.

Cell Culture

For the free cell suspension culture, exponentially growing cells in IMDM containing 10% FBS were inoculated into spinner flasks (Bellco) containing 50 mL of IMDM with 10% FBS at an initial concentration of 1×10^5 cells/mL. The cells were cultivated in a repeated fed-batch mode, stepping down the serum concentration from 10% to 0% over 80 days. The cells were always passed in the late exponential phase. The cells were diluted 1:5 with fresh medium with two exceptions. (1) To study the effect of dilution ratios on cell growth and MAb productivity, the cells cultivated in 1% serum medium were passed at a dilution of 1:5 for the first six passages and at a dilution of 3:10 for the next seven passages. (2) To maintain the high cell concentration, the cells in plain IMDM were diluted 1:2 (see Fig. 1).

For the immobilized cell culture, cells immobilized in alginate beads were inoculated into spinner flasks containing IMDM with 10% serum. The working volume of the spinner flask was 100 mL. Like the free-suspended cells, the immobilized cells were cultivated in a repeated-fed-batch mode. The serum concentration was stepped down from 10% to 0% over 45 days. To exchange the medium, the beads were first allowed to settle, and then the supernatant was removed, and the fresh medium was added to the spinner flask. At each serum concentration, the medium was exchanged once a day for the first several days and twice a day for the next several days (refer to Fig. 6).

Analytical Methods

Cell growth was monitored by counting viable cells with a hemocytometer. Viable cells were distinguished from dead cells by the trypan blue dye exclusion method. The cell culture suspension was centrifuged and the supernatant was aliquoted and kept frozen at −80°C.

Glucose and lactate were measured using a glucose/lactate analyzer (Yellow Spring Instruments, Model 2000, Yellow Spring, OH); ammonia was measured using an ammonia electrode (Orion Research Inc., Model 95-12, Cambridge, MA). Glutamine was determined by reverse-phase HPLC column (Rainin Instrument, Inc., Microsorb, short-one C18, Woburn, MA) as described by Seaver.[32]

The total antibody, γ2b, was quantified using an enzyme-linked immunosorbent assay (ELISA) as described previously.[15] The amounts of intracellular antibody and DNA were quantified simultaneously using flow cytometry as described previously.[16]

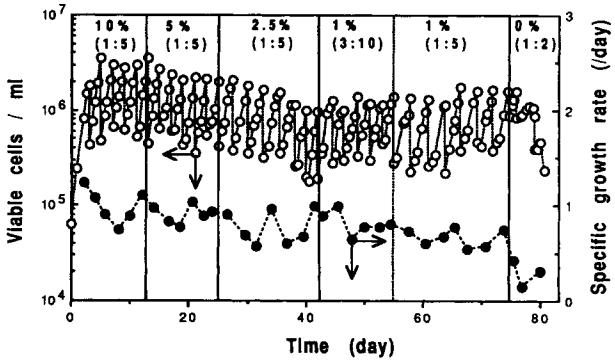

Figure 1. Cell growth and specific growth rate in the free cell culture. The figure indicates how serum concentration and dilution ratio were changed with time. Percentages represent the serum concentration, and ratios in brackets represent the dilution ratio. The specific growth rates were calculated using linear regression of data in each harvest/feeding cycle.

To count the cells and prepare the flow cytometric sample from the immobilized cell culture, 40–100 gel beads containing the cells were dissolved in isotonic citrate solution (3% aqueous sodium citrate diluted 1:1 in 0.9% NaCl and adjusted to pH 7.4) for 5 min.[18]

Determination of Specific Growth Rates and Metabolic Quotients

The specific growth rate, μ, was calculated from data collected during the exponential growth phase and is defined as follows:

$$\mu = \frac{1}{X_v}\frac{dX_v}{dt} \tag{1}$$

where X_v denotes the concentration of viable cells and t denotes the cultivation time. The specific metabolic quotient calculations for glucose consumption and lactate formation (q_{glu} and q_{lac}, respectively) were also based on data collected during the exponential phase of growth. They are defined as follows:

$$-q_{Glu} = \frac{1}{X_v}\frac{d[Glu]}{dt} \tag{2}$$

$$q_{Lac} = \frac{1}{X_v}\frac{d[Lac]}{dt} \tag{3}$$

where $[Glu]$ and $[Lac]$ are glucose and lactate concentrations, respectively.

Glutamine spontaneously decomposes following first-order kinetics to pyrrolidone carboxylate and ammonia.[38] The specific glutamine consumption and ammonia production rates (q_{Gln} and q_{NH_4}, respectively) were determined by accounting for the degradation of glutamine at 37°C:[8,29]

$$-\frac{d[Gln]}{dt} = k[Gln] + q_{Gln}X_v \tag{4}$$

$$\frac{d[NH_4^+]}{dt} = k[Gln] + q_{NH_4^+}X_v \tag{5}$$

where $[Gln]$ is the glutamine concentration (mM); $[NH_4^+]$ is the ammonium ion concentration (mM); k is the first-order rate constant for glutamine decomposition (day^{-1}). Since the first-order decomposition rate varies with serum and medium components[23,29] we measured experimentally the values of k. The values of k were 0.0552–0.0720 day^{-1} in IMDM medium, at 37°C and pH 7.4 under 5% CO_2 atmosphere, for the different serum concentrations used in this experiment.

The yield coefficients of glucose consumed to lactate produced and of glutamine consumed to ammonia produced ($Y_{Lac/Glu}$ and $Y_{NH_4^+/Gln}$, respectively) are defined as follows:

$$Y_{Lac/Glu} = \frac{q_{Lac}}{q_{Glu}} \tag{6}$$

$$Y_{NH_4^+/Gln} = \frac{q_{NH_4^+}}{q_{Gln}} \tag{7}$$

The specific MAb productivity, q_{MAb}, was based on the data obtained from each batch cycle:

$$q_{MAb} = \frac{[MAb] - [MAb]_o}{\int_0^t X_v\,dt} \tag{8}$$

where $[MAb]$ and $[MAb]_o$ denote the concentration of MAb and the initial concentration of MAb for each batch cycle, respectively. In the free cell suspension culture, q_{MAb} was constant, and was therefore calculated from the plot of $[MAb]$ versus $\int_0^t X_v\,dt$. In the immobilized cell culture, q_{MAb} was not constant when the medium was changed once a day. We thus define an overall specific MAb productivity, q'_{MAb}, as:

$$q'_{MAb} = \frac{[MAb]_{t_c} - [MAb]_o}{\int_0^{t_c} X_v\,dt} \tag{9}$$

where t_c denotes the total cell culture time of each batch cycle, and $[MAb]_{t_c}$ denotes the final MAb concentration of each batch cycle. In the free cell suspension culture, q'_{MAb} is essentially identical to q_{MAb}, because q_{MAb} is constant.

RESULTS

Free Cell Suspension Culture

Specific growth rates and viable cell concentrations decreased as the serum concentration in medium decreased (Fig. 1). The average specific growth rate in medium containing 10% serum was approximately 0.99 ±0.18 day^{-1} (±standard deviation), while that in medium containing 1% serum was approximately 0.73 ±0.12 day^{-1}. Cells cultivated in 1% serum medium were passed to medium without serum at the dilution ratio of 1 to 2 in order to test how long the cells can grow or maintain viability in 0% serum medium by starting with a cell concentration of over 5×10^5 cells/mL. Cell death occurred after two passages in 0% serum medium. In 1% serum concentration, there was no significant effect of dilution ratio on the specific growth rate.

Figure 2 shows MAb concentrations and specific MAb productivities for the culture conditions shown in Figure 1. The specific MAb productivity was almost constant at 3.69 ±0.57 $\mu g/10^6$ cell/day irrespective of the serum concentrations. Even the cells in medium without serum produced MAb at the same rate. This observation suggests that one can use serum-free medium for MAb production as long as the cells maintain viability. The MAb concentration in 0% serum medium was as high as that in 10% serum medium because the dilution ratio in 0% serum medium was lower than that in 10% serum medium.

Figure 3 shows intracellular MAb and DNA data obtained by flow cytometry. Intracellular MAb contents of the cells in the late exponential phase of growth are shown in Figure 3(A). The X-axis represents the culture condition and passage number and the Y-axis represents the log green fluorescence intensity that indicates the level of intracellular MAb within the cell population.[12,16] As shown in Figure 3(A), the intracellular MAb content, like the specific MAb productivity shown in Figure 2, was almost constant throughout the reduction of serum concentrations in the medium. Figure 3(B) shows the distribution of cells through the different stages of the growth cycle. The cells assayed here at dif-

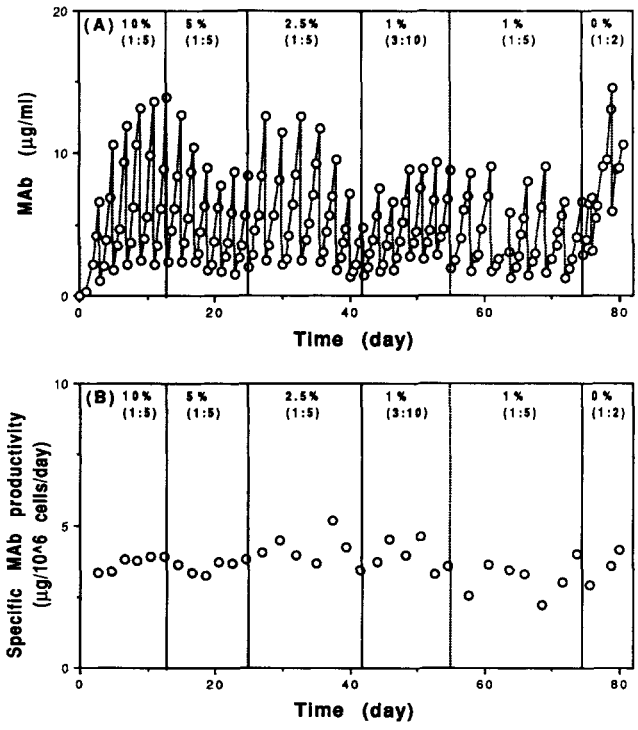

Figure 2. MAb concentration and specific MAb productivity in the free cell culture condition shown in Figure 1. The specific MAb productivities were calculated using linear regression of data in each harvest/feeding cycle.

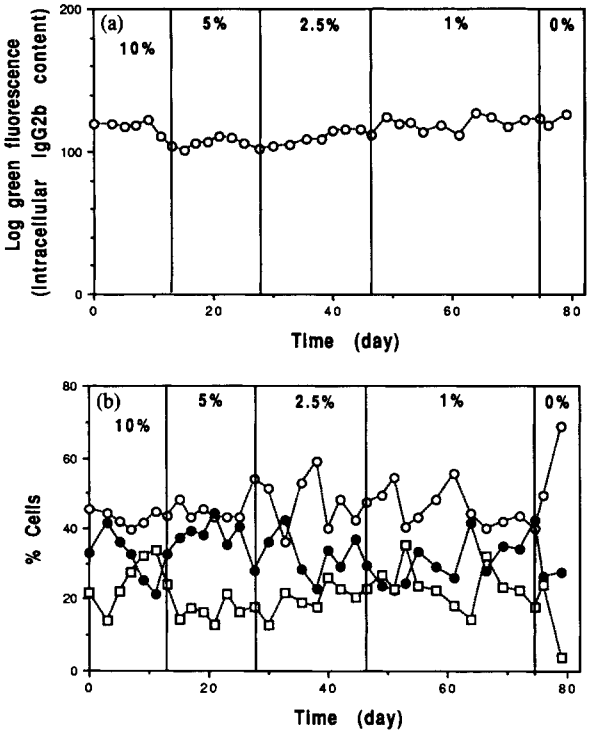

Figure 3. (A) Intracellular MAb (IgG2b) content of the free-suspended cells in the late exponential phase. Samples accumulated were stained and analyzed at the same time and conditions in order to avoid day-to-day variation in flow cytometer measurements. (B) Cell cycle analysis of the free-suspended cells in the late exponential phase at the same condition as (A). Open circles represent the percentage of cells in G0 + G1 phase. Closed circles represent the percentage of cells in S phase. Squares represent the percentage of cells in G2 + M phase.

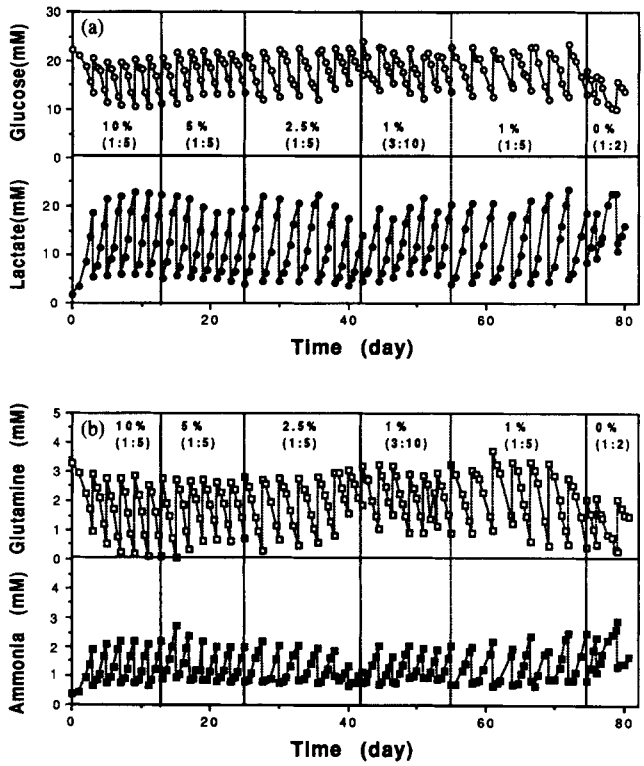

Figure 4. Metabolite concentration profiles in the free cell culture: (A) glucose and lactate (B) glutamine and ammonia.

ferent serum concentrations were primarily in the late exponential phase of growth.

The cell cycle analysis was based on data from DNA histograms obtained using flow cytometry. There were no noticeable differences in the cell cycle between cultures in 10% to 1% serum medium. However, in 0% serum medium the population of the cells in G2 + M phases was very small.

Glucose and glutamine, which are the major carbon and energy sources in most cell culture media and which are required for cell growth, were measured during the cultivation. The major byproducts, lactate and ammonia, were also measured. The glucose and lactate concentrations during the cultivation are shown in Figure 4(A). The glucose levels decreased markedly during the exponential growth, and glucose utilization was accompanied by a corresponding accumulation of lactate. Glucose was not a limiting nutrient for cell growth in all culture conditions tested.

With high-serum medium, the uptake rate of glucose decreased (Fig. 5[A]), although the specific growth rate was higher (Fig. 1). This result indicates that the cells utilize glucose more efficiently in high-serum medium. An average glucose uptake rate of the cells in 10% serum medium was 3.84 mmol/10^9 cells/day, while the average glucose uptake rate increased to 5.36 mmol/10^9 cells/day in 1% serum medium. Although the glucose uptake rate did depend on the serum concentrations, the ratio of lactate produced to glucose consumed, $Y_{Lac/Glu}$, was almost constant at ca. 1.8 mmol/mmol throughout the reduction of serum in the culture (refer to Fig. 7[A]). Glutamine was not completely depleted throughout the culture, although the concentration of glutamine in 10% serum medium decreased to 0.1 mmol (Fig. 4([B]). The effect of serum on the glutamine uptake rate was similar to that of the glucose uptake rate, although the difference between different serum concentrations was not as significant as with the glucose uptake rate (Fig. 5[B]). On average, the glutamine uptake rates in 10% and 1% serum medium were 0.91 mmol/10^9 cells/day and 1.11 mmol/10^9 cells/day, respectively. The ratio of ammonia produced to glutamine consumed ($Y_{NH_4^+/Gln}$), like $Y_{Lac/Glu}$, was not significantly influenced by the reduction of serum in the medium. $Y_{NH_4^+/Gln}$ remained constant at ca. 0.47 mmol/mmol. However, there was greater variation in $Y_{NH_4^+/Gln}$ than in $Y_{Lac/Glu}$ (refer to Fig. 7[B]). Dalili and Ollis[3] showed a similar trend for the effect of serum on specific glucose and glutamine uptake rates.

Immobilized Cell Culture

Unlike the free cell suspension culture, the immobilized cell culture had a limit in the sampling frequency of alginate beads for cell counts. Because some alginate

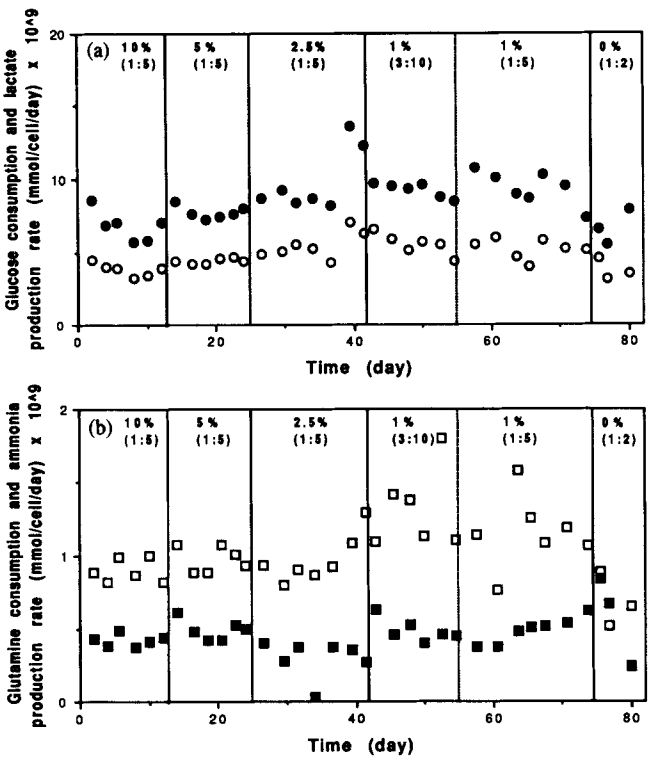

Figure 5. Specific metabolic quotients in the free cell culture: (A) glucose (○) and lactate (●) (B) glutamine (□) and ammonia (■). The specific metabolic quotients were calculated using linear regression of data in each cycle.

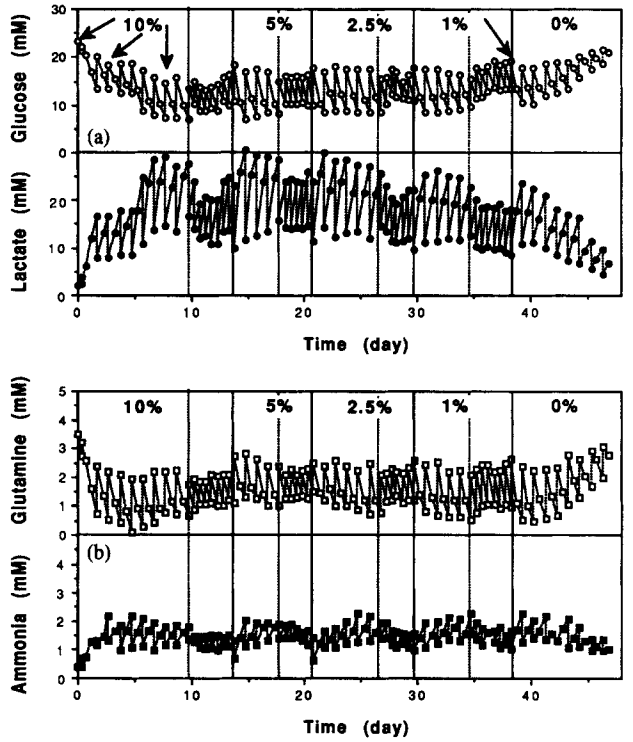

Figure 6. Metabolite concentration profiles in the immobilized cell culture: (A) glucose and lactate (B) glutamine and ammonia. Arrows indicate the time when cells were counted. The figure indicates how serum concentration and dilution ratio were changed with time. Dotted lines indicate times when feeding 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 by fresh medium once a day.

beads need to be dissolved to count the cells, we lose approximately 1% of the initial number of alginate beads for each sampling, thus lowering the cell concentration in the spinner flasks. To prevent a significant loss of beads in the culture, we ran a duplicate culture to provide additional data. We measured glucose, glutamine, lactate, and ammonia concentrations at each sampling. These metabolite concentrations may indirectly indicate the changes in the cell concentrations (Fig. 6).

The cells were grown in 10% serum medium. After 2 weeks we switched the medium to 5% serum medium, followed by further decreases of serum concentrations in the medium. The arrows indicate the times when the cell concentration was determined. The viable cell concentration in the beginning of this experiment was 1.8×10^6 cells/mL of medium, and decreased to 1.0×10^6 cells/mL after 2 days of cultivation. This decrease in viable cell concentration may have occurred because of cell damage during the immobilization process.

The viable cell concentration increased to 1.8×10^6/mL after 8 days of cultivation in 10% serum medium. The viable cell concentration in 1% serum medium on cultivation day 38 was 1.7×10^6 cells/mL with a cell viability of 50%. Viable cell concentrations on cultivation day 18 and 26 measured from the duplicate experiment were 1.7×10^6 cells/mL and 1.8×10^6 cells/mL, respectively. Therefore, it seems that the viable cell concentration remained constant after reaching the maximal concentration, regardless of the reduction in serum concentration. This result is supported by the observation that there were no significant variations in glucose consumption and lactate production profiles for this period. Glucose consumption and lactate production profiles quickly stabilized after changes in the feeding schedule (Fig. 6[A]). The free-suspended cells in the culture medium were less than 2.5×10^5 cells/mL, which is about 14% of the cell concentration achieved in the immobilized cell culture.

Throughout the culture, glucose was not limiting. When 0% serum medium was used, glucose consumption decreased gradually over a week, indicating that the cells were dying. Although the immobilized cells could maintain viability longer than the free-suspended cells, the long-term cultivation of cells using plain medium could not be achieved, even by starting with a cell concentration of greater than 10^6 cells/mL.

The glucose uptake rate of the immobilized cells was calculated assuming that the cell concentration was 1.8×10^6 cells/mL of medium from day 8 to day 48. Free-suspended cells in the medium were neglected in the calculation of metabolite uptake and production rates. Average glucose uptake rates in 10% serum and 1% serum medium, during those periods were 5.12 and 5.08 mmol/10^9 cells/day, respectively. These values of

glucose uptake rates as well as the yield coefficients, $Y_{Lac/Glu}$, are similar to those of the free-suspended cells (Fig. 7[A]). Glutamine, like glucose, was not limiting throughout the culture (Fig. 6[B]). Glutamine uptake rates and the yield coefficient, $Y_{NH_4^+/Gln}$ for the immobilized cells were similar to those for the free-suspended cells (Fig. 7[B]). The average glutamine uptake rates in 10% and 1% serum medium were 0.82 and 1.17 mmol/10^9 cells/day, respectively.

Figure 8(A) shows the MAb concentration profile for the immobilized cell culture. Decreasing serum concentration from 10% to 1% did not significantly affect the MAb production, probably because the cell concentration remained constant. However, MAb production gradually decreased after serum was completely removed. Maximum MAb concentration obtained was in the range of 25–30 μg/mL. MAb production ceased after the MAb concentration reached 25–30 μg/mL. It is not clear whether the essential precursors for MAb synthesis were limiting or whether MAb synthesis was prevented by unknown accumulated inhibitors or feedback regulation.[20]

Figure 8(B) shows specific MAb productivities of immobilized cells. The specific MAb productivity, unlike the glucose and glutamine uptake rates, was enhanced significantly in the immobilized cell culture when compared to the free cell suspension culture. The specific

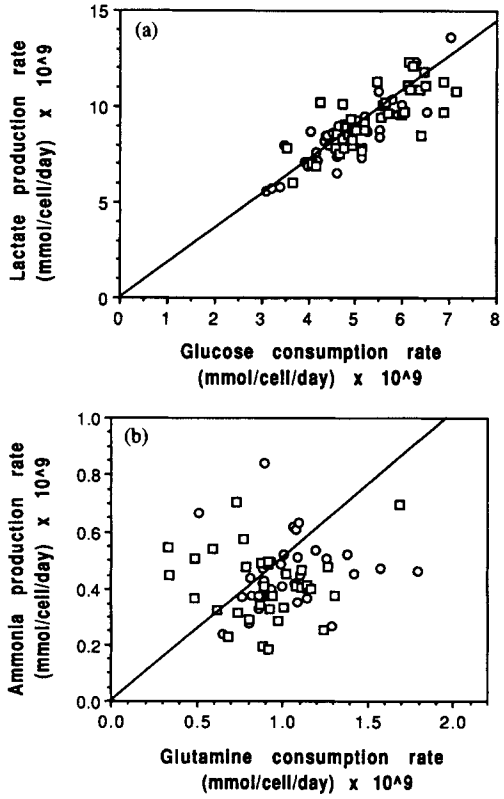

Figure 7. Metabolic yields: (A) lactate production rate versus glucose consumption rate, (B) ammonia production rate vs. glutamine consumption rate. Data obtained throughout the culture of the free-suspended (○) and the immobilized cells (□) are plotted.

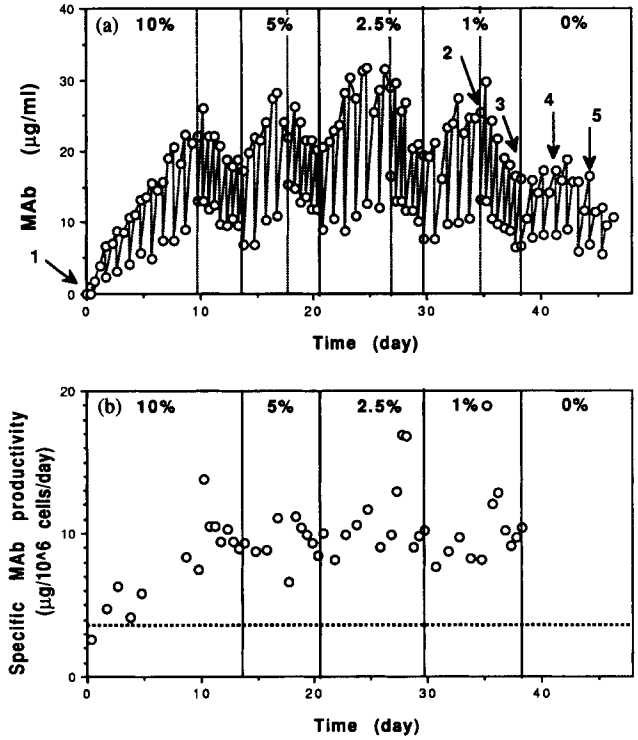

Figure 8. MAb concentration and specific MAb productivity in the immobilized cell culture conditions shown in Figure 6. (A) MAb concentration. Arrows indicate the time when samples for flow cytometry were prepared. (B) Specific MAb productivity of immobilized cells. The overall specific MAb productivity was calculated. The dotted line represents an average specific MAb productivity of the free-suspended cells shown in Figure 2.

MAb productivities increased gradually until cell concentration reached the maximum. Thereafter, there were no noticeable differences in the specific MAb productivity regardless of serum concentrations in the medium, as long as serum was present. Therefore, the specific MAb productivity of S3H5/γ2bA2 hybridoma appeared to increase after the maximum viable cell concentrations were reached or during the period of slow growth.

Intracellular IgG2b contents of the immobilized cells were measured. To compare the intracellular MAb contents of the immobilized cells in media with different serum concentrations, histograms obtained from different samples were plotted together (Fig. 9). These samples were prepared and analyzed at the same time. Intracellular MAb (IgG2b) content of the immobilized cells in 1% serum medium as well as the specific MAb productivity was enhanced by about 2.5 times when compared with that of cells at the beginning of the culture. When 0% serum medium was used, intracellular MAb content of the immobilized cells decreased, but it was still about 1.7 times higher than that of cells in the beginning of the culture.

The intracellular MAb content of the cells whose MAb secretion had almost ceased (histogram #2) was identical to that of the cells secreting MAb actively (histogram #3). If the cells in histogram #2 had contin-

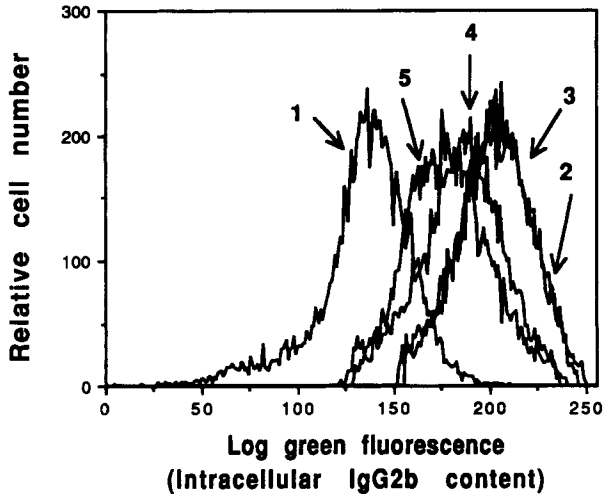

Figure 9. Intracellular MAb contents of the immobilized cells at different culture conditions. Numbers in the histogram correspond to the arrows in Figure 8(A). Histogram #3 overlaps histogram #2.

ued to synthesize MAb, one would expect the intracellular MAb content to increase above that shown in histogram #3. However, the intracellular MAb contents from histograms #2 and #3 are similar, indicating that both MAb synthesis and secretion by the cells in histogram #2 had ceased.

Volumetric MAb productivities in the free cell suspension and the immobilized cell culture were compared. As shown in Figure 10(A), the volumetric MAb productivity in the free cell suspension culture decreased as the serum concentration decreased, probably because of the reduced cell concentration in low-serum medium. The average volumetric productivities in 10% and 1% serum medium at the dilution ratio of 1:5 are 4.84 ±1.36 and 2.19 ±0.55 μg/day/mL, respectively. In the immobilized cell culture, the volumetric MAb productivity did not decrease throughout the reduction of serum concentration in medium because of constant cell concentration. The volumetric MAb productivity in the immobilized cell culture system was about 3–7 times higher than in the free cell suspension culture because of both the enhanced specific MAb productivity and the high cell concentration. Since serum is the most expensive component in the medium, MAb yield based on serum used was plotted to show how efficiently serum was used for MAb production in the free and the immobilized cell culture.

As shown in Figure 11, there are no significant differences for the efficiency of serum utilization for MAb production in 10% serum medium. However, in 1% serum medium, the efficiency of serum utilization for MAb production by the immobilized cell culture was about three times higher than that by the free cell culture system. By using the immobilized cells with 1% serum medium, MAb yield was about 2.2 mg/mL of serum.

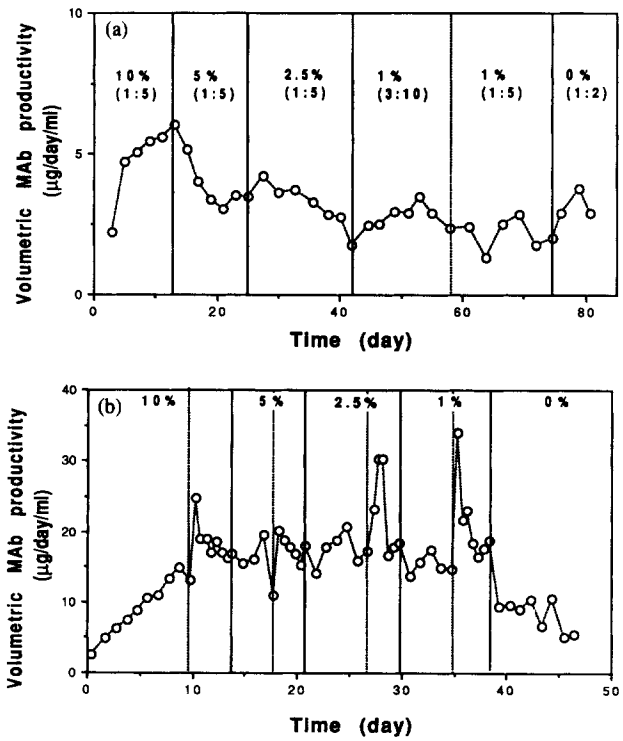

Figure 10. Comparison of the volumetric MAb productivity based on the working volume of the spinner flasks between the free and the immobilized cell cultures: (A) free-suspended cells, (B) immobilized cells.

DISCUSSION

Free cell suspension and immobilized cell culture systems were compared with respect to the efficiency of serum utilization for MAb production. We have observed three interesting phenomena of the immobilized cell culture.

First, the specific MAb productivity, unlike the glucose and glutamine uptake rates, was enhanced significantly in the immobilized cell culture, and this

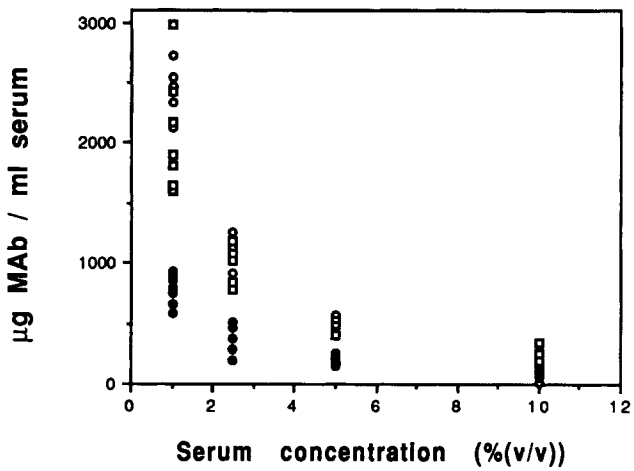

Figure 11. Comparison of overall MAb yield based on serum used between the free and the immobilized cell cultures: (A) free-suspended cells; ● (B) immobilized cells; □, medium was replaced twice a day; ○, medium was replaced once a day.

enhancement was not a transient phenomenon. Intracellular MAb content of the immobilized cells as measured by flow cytometry was also higher than that of free-suspended cells. The enhancement of the specific MAb production in the immobilized cell culture could be attributed to a number of possibilities.

- The enhanced MAb productivity may be due to the slowed growth rate. The growth rate of the immobilized cells was almost zero after the cell concentration reached the maximum. The specific MAb productivity of the cells was almost constant at approximately 3.69 $\mu g/10^6$ cells/day with the specific growth rates greater than 0.7 day^{-1}, which were observed in the free cell suspension culture. Then, at a lower specific growth rate of 0.5 day^{-1}, which was observed in the free cell suspension culture with low initial cell concentrations, the specific MAb productivity increased approximately 20% over 3.69 $\mu g/10^6$ cells/day to 4.43 $\mu g/10^6$ cells/day.[14] Finally, at the specific growth rate of almost zero, which was observed in the immobilized cell culture, the specific MAb productivity significantly increased approximately 200% over 3.69 $\mu g/10^6$ cells/day to 11 $\mu g/10^6$ cells/day. There are many reports that show that the specific MAb productivity is enhanced with the slowed growth rate of hybridoma cells,[4,11,12,19,21,30,31] although there are some exceptions.[3,6,34] One structured model, proposed by Suzuki and Ollis,[37] shows that the specific MAb productivity can be increased by 3.5 times by reducing the specific growth rate to zero.
- The enhanced MAb productivity may be due to stressful conditions. The cell viability of the immobilized cells was about 50%, while the cell viability in free cell suspension culture was maintained over 90%. The stressful condition, indicated by the low cell viability may have been induced by changes in pH,[21,28] dissolved oxygen,[22] and osmolarity.[27,28] During the cultures, the changes in pH and osmolarity in the medium in the immobilized cell culture were similar to those in the free cell suspension culture. The initial pH of 7.2–7.4 dropped to 6.8–7.0 at the end of each batch cycle. The changes in osmolarity of the medium were found to be less than 5% during the culture (data not shown). However, the immobilized cells are in a microenvironment where the pH, osmolarity, and dissolved oxygen concentration differ from the environment of the surrounding medium.
- The enhanced MAb productivity may be due to autocrine growth factors. The concentration of autocrine growth factors inside the gel beads may be high because of the high local cell concentration. These factors may stimulate MAb synthesis. For example, interleukin 6, one of the autocrine growth factors, is known to stimulate immunoglobulin synthesis in lymphoid cells.[1,13]

Second, in the immobilized cell culture, the reduction of serum concentration from 10% to 1% did not decrease the volumetric MAb productivity. In fact, because the specific MAb productivity remained constant, only viable cell concentration directly affected the volumetric MAb productivity; the serum concentration affected volumetric MAb productivity only indirectly. In the free cell suspension culture, with each decrease in serum concentration, the viable cell concentration fell, thus reducing volumetric MAb productivity. In the immobilized cell culture, with each decrease in the serum concentration from 10% to 1%, the viable cell concentration remained constant, and thereby the volumetric MAb productivity also remained constant. And then, when the serum concentration was reduced from 1% to 0%, the viable cell concentration fell along with the volumetric MAb productivity. So the limitation to volumetric MAb productivity is probably a direct result of some limit to viable cell concentration. In the immobilized cell culture, the viable cell concentration of 10^7 cells/mL 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.

Third, MAb production in the immobilized cell culture ceased after the MAb concentration reached 25–30 $\mu g/mL$. It has been often mentioned that high cell concentration will result in a high MAb concentration.[7,39] By using the immobilized cell culture, we were able to enhance volumetric MAb productivity by frequently replacing medium, but we could not enhance the maximum concentration of MAb in the culture solution.

When the MAb concentration in the immobilized cell culture reached the range of 25–30 $\mu g/mL$, which could be also obtained in a batch cultivation using the free cell suspension culture,[15] further MAb production did not occur. Serum concentration in the medium did not influence this maximum concentration significantly. Also, there was no limitation of glucose, glutamine, or other amino acids tested (aspartate, glutamate, asparagine, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, lysine) during the culture. Some unknown precursors required for MAb synthesis might have been limited, or unknown inhibitors might have been accumulated to levels high enough to inhibit MAb synthesis as well as MAb secretion. Feedback inhibition in MAb synthesis, as observed by Merten et al.,[20] is also possible. Because MAb in the cell culture solution can be concentrated 10 to 100 times by one step ultrafiltration,[4] high-volumetric MAb productivity obtained from the immobilized cell culture seems to be more important than MAb concentration for the economical production of MAb.

In conclusion, we have shown the feasibility of using immobilized cells for MAb production. The immobilized cell culture system has a higher volumetric MAb productivity than the free cell suspension culture system because of both the enhanced specific MAb productivity and the maintenance of high viable cell concen-

tration in the spinner flasks. In addition, because the reduction of serum concentration from 10% to 1% did not influence the viable cell concentration in the immobilized cell culture, serum concentration in medium could be decreased to 1% without any loss in MAb productivity. Therefore, the immobilized cell culture offers significant advantages in both cost and efficiency of MAb production when compared to the free cell suspension culture.

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