EFFECT OF CALCIUM CHLORIDE TREATMENT ON HYBRIDOMA CELL VIABILITY AND GROWTH

Gyun Min Lee*, Bong Kwan Han, Jung Hoe Kim, and Bernhard O. Palsson [1]

Department of Biotechnology, Korea Advanced Institute of Science & Technology,
Kuseong-Dong, Yuseong-Gu, Taejun 305-701, Korea
and
[1]Department of Chemical Engineering, University of Michigan,
Herbert H. Dow Building, Ann Arbor, MI 48109

ABSTRACT

In order to minimize hybridoma cell damage during calcium alginate entrapment, the effect of calcium chloride treatment on hybridoma cell viability and growth was studied in terms of calcium chloride concentration and treatment time. The cell viability as measured by trypan blue exclusion did not decrease rapidly during the first hour of calcium chloride treatment regardless of calcium chloride concentrations used (1.3 and 1.5%). However, 1.3% calcium chloride solution appeared to be more detrimental to the cells than 1.5% calcium chloride solution. The cells in 1.3% calcium chloride solution lost their viability faster than the cells in 1.5% calcium chloride solution. In addition, when the cells treated with calcium chloride were inoculated into spinner flasks containing IMDM with 10% fetal calf serum, the cells treated with 1.3% calcium chloride solution showed a longer lag phase than the cells treated with 1.5% calcium chloride solution.

INTRODUCTION

The entrapment of hybridoma cells either into calcium alginate beads or into poly-l-lysine (PLL) alginate microcapsules has attracted considerable interest because entrapped cell cultures offer several advantages over conventional suspension cultures (Poscillio, 1986; Nilsson, 1987; Shirai *et al.*, 1987; Tyo and Spier, 1987; Lim, 1988; Sinacore *et al.*, 1989; Lee and Palsson 1990; Lee *et al.*, 1991).

In alginate-entrapped cell cultures, much effort has been made to improve volumetric monoclonal antibody (MAb) productivity by either increasing the cell density and/or the cell specific MAb productivity. In contrast, few studies have been aimed at improving the process of hybridoma cell entrapment in calcium alginate beads. This optimization has been overlooked due to the apparent simplicity and gentle nature of the entrapment process. However, when calcium-alginate entrapped hybridoma cells were cultivated, cell viability often decreased at the beginning of culture, resulting in a prolonged lag phase (Lee *et al.*, 1991; Wohlpart *et al.*, 1991). This phenomenon may result from cell damage during the entrapment process and may be avoided by improving the entrapment process.

The entrapment of the cells in calcium alginate beads is achieved by dropping a mixture of sodium alginate and cells into a cold calcium chloride solution. The cells continue to experience calcium chloride

during the bead formation process. Thus, the entrapped cells are exposed to calcium chloride solution for prolonged time periods. However, the effect of calcium chloride treatment which may have an adverse effect on the cells has not been well characterized (Spiekermann et al., 1987). Different concentrations of calcium chloride solution have been used, and different incubation time of the cells in calcium chloride solution have been used (King et al., 1987; Shirai et al., 1987; Lim, 1988; Lee et al., 1991; Wohlpart et al., 1991).

In this article, the effect of calcium chloride treatment on hybridoma cell (S3H5/γ2bA2) viability and growth is characterized in terms of calcium chloride concentration and treatment time. In addition, the effect of calcium chloride treatment on specific MAb productivity is also characterized.

MATERIALS AND METHODS

Cell line, medium, and culture maintenance Two murine hybridoma cell lines were used in this study. One cell line was S3H5/γ2bA2 provided by Dr. Mark Kaminski from the University of the Michigan Medical Center. The antibody produced by this cell line is IgG_{2b} anti-idiotype antibody, directed against a carcinogen-induced B cell tumor (38C13) (Bergman and Haimovich, 1977). The fusion partner used was P3/x63/Ag8.653. The other cell line was S3H5/NP. This cell line lost its antibody productivity, and was originally derived from S3H5/γ2bA2 (Lee and Palsson, 1990).

The medium for culture maintenance was Iscove's modified Dulbecco's medium (IMDM, Gibco Laboratories, Grand Island, NY) supplemented with 10%(v/v) fetal bovine serum (FBS, Gibco Laboratories, Grand). No antibiotics were added to the medium. The cells were maintained in 25 cm^2 T-flasks (Bellco Glass, Inc., Vineland, NJ) in a 5% CO_2/air mixture, humidified at 37°C. The cells were diluted 1:5 with fresh medium every other day.

Calcium chloride treatment, cell viability, and cell growth Two different concentrations of calcium chloride solution (Sigma, St. Louis, MO) were prepared. A 1.3% calcium chloride solution buffered with 13mM HEPES (Sigma) was prepared as described by Lim (1988), and a 1.5% calcium chloride solution was prepared by King et al. (1987). Osmolarities of 1.3% and 1.5% calcium chloride solutions measured using an Osmometer (Osmette, model 2007, Precision systems, Inc., Natick, MA) were 322 and 346 mOsm, respectively.

The effect of calcium chloride on cell viability was studied as follows: Exponentially growing cells (cell density $\approx 2.0 \times 10^6$ cells/ml) were centrifuged at 1000 rpm for 10 min. After discarding the supernatant, cells were resuspended in two different concentrations of calcium chloride solution (4°C), respectively, and then were kept in an ice bath. Viable cell concentration was monitored as a function of calcium chloride treatment time by the trypan blue dye exclusion method. When the trypan blue in phosphate buffer was used, precipitation of calcium ions with phosphate ions made it difficult to distinguish viable cells from dead cells (Shirai and Hashimoto, 1989). Thus, a 4%(w/v) trypan blue in an 0.85% NaCl solution (pH 7.4) was used to avoid precipitation.

The effect of calcium chloride treatment on cell growth was studied as follows: After incubating the cells in calcium chloride solution at 4°C as previously described, the cells were centrifuged at 1000 rpm for 10 min. The time required for centrifugation was also included for calculating the incubation time of the cells in calcium chloride solution. After discarding the supernatant, the cells were resuspended in IMDM supplemented with 10% FBS, and then were centrifuged again. After discarding the supernatant, the cells were resuspended in IMDM with 10% FBS. The cells were inoculated into the spinner flasks (Bellco Glass, Inc.) containing 50 ml of IMDM with 10% FBS at various initial cell concentrations. The agitation speed used in the spinner flasks was 100 rpm. The cultures were kept at 37°C in a humidified CO_2 incubator (VWR Scientific, San Fransisco, CA).

Analytical methods The cell culture suspension was centrifuged. The supernatant was aliquoted and kept frozen at -80°C. The total secreted antibody, IgG_{2b}, was quantified using an enzyme linked immunosorbent assay (ELISA) as described previously (Lee et al., 1989).
The specific growth rate and specific MAb production rate were evaluated as described earlier (Lee et al., 1989).

Viability

1.0
0.8
0.6
0.4
0.2
0.0

O   1.5% calcium chloride
●   1.3% calcium chloride

(A)

0   20   40   60
Time (min)

Viability

1.0
0.8
0.6
0.4
0.2
0.0

O   1.5% calcium chloride
●   1.3% calcium chloride

(B)
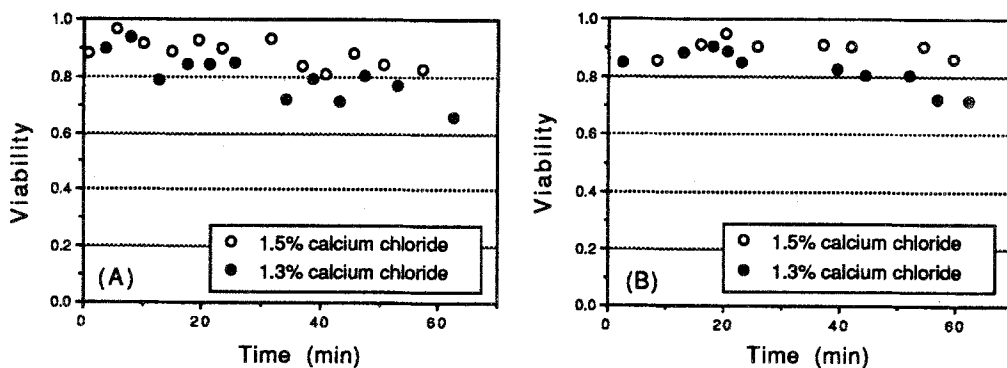
0   20   40   60
Time (min)

Figure 1. Cell viability versus calcium chloride treatment time: (a) S3H5/γ2bA2 (b) S3H5/NP

RESULTS AND DISCUSSION

In order to investigate the effect of calcium chloride treatment on cell viability, the cells were incubated in different concentrations of calcium chloride solution. The calcium alginate concentrations used were 1.3% and 1.5%, since both have been widely used for entrapping hybridoma cells into calcium alginate beads. The cell viability was plotted as a function of the calcium chloride treatment time (Fig. 1). Since total cell concentration did not decrease during calcium chloride treatment, the cell viability directly indicates the changes in viable cell concentration. Although the cell viability did not decrease rapidly during calcium chloride treatment regardless of calcium chloride concentrations, the cells in 1.3% calcium chloride solution appeared to lose their viability faster than the cells in 1.5% calcium chloride solution. The reason for this result might be due to the difference in osmolarities of calcium chloride solutions. Assuming that the decrease in the cell viability follows the first order kinetics with respect to calcium chloride treatment time, the viability decay rate constant (min^{-1}), k, was determined as described by Aiba et al. (1973). For S3H5/γ2bA2 cells, the values of k in 1.3% and 1.5% calcium chloride solution were approximately 0.0043 and 0.0020 min^{-1}, respectively. For S3H5/NP cells, the values of k in 1.3% and 1.5% calcium chloride solution were approximately 0.0034 and 0.0003 min^{-1}, respectively. Accordingly, if the cells (S3H5/γ2bA2) are exposed to 1.5% calcium chloride solution for less than an hour during the immobilization process, the loss of cell viability by detrimental effect of calcium chloride solution would be less than 12% of an initial viable cell concentration.

However, cell viability was estimated by trypan blue dye exclusion method which relies on a breakdown in membrane integrity determined by the uptake of trypan blue. Dye exclusion method tends to overestimate viability (Freshney, 1987). Thus, the observation that the cells in calcium chloride solution did not lose their viability significantly does not necessarily mean that the cells were not damaged with respect to their growth ability and that the cells in 1.5% calcium chloride were less damaged than the cells in 1.3% calcium chloride solution. Therefore, the effect of calcium chloride solution on cell growth ability (S3H5/γ2bA2) was investigated in terms of calcium chloride treatment time and concentration.
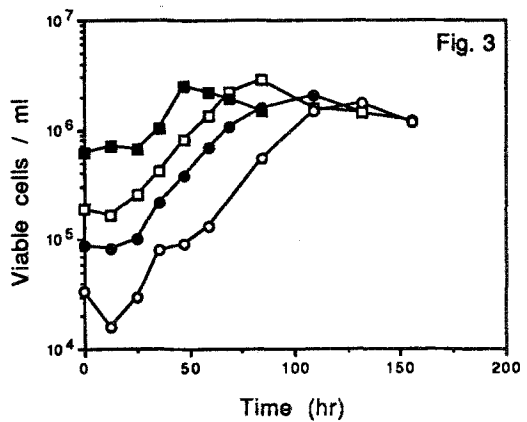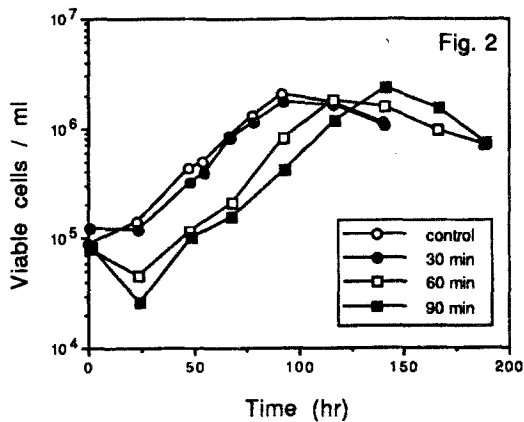
Figure 2. Growth curves of S3H5/γ2bA2 cells after varying exposure to 1.5% calcium chloride. Cells without calcium chloride treatment were used as a control. Different symbols indicate various calcium chloride treatment time.

Figure 3. Effect of initial cell concentration on growth of S3H5/γ2bA2 cells following 60 min exposure to 1.5% calcium chloride solution.

First, the cells which had been incubated in 1.5% calcium chloride solution with varying incubation times were inoculated into spinner flasks. As shown in Fig. 2, cell growth ability was affected by calcium chloride treatment. While the cells were incubated in calcium chloride solution, the cell growth ability deteriorated. Unlike the cells without calcium chloride treatment, the cells incubated in calcium chloride solution longer than an hour experienced one or two days of lag phase. Since the growth of S3H5/γ2bA2 hybridoma cell was reported to be cell concentration-dependent (Lee et $al.$, 1989), the lag phase induced by cell damage during calcium chloride treatment may be reduced by increasing the initial cell concentration. Thus, the cells which had been incubated in calcium chloride solution for an hour were inoculated into spinner flasks at various initial cell concentrations. As shown in Fig. 3, it was found that the duration of lag phase could not be shortened noticeably by increasing the initial cell density up to 6×10^5 cells/ml. Second, in order to investigate the effect of calcium chloride concentration on cell growth ability, the cells treated with 1.3% and 1.5% calcium chloride for an hour were inoculated into spinner flasks, respectively. The cells treated with 1.3% calcium chloride solution showed a longer lag phase than the cells treated with 1.5% calcium chloride solution, confirming the previous results that the cells in 1.3% calcium chloride solution lost their viability faster than the cells in 1.5% calcium chloride solution during calcium chloride treatment (Fig. 4). Accordingly, in order to minimize cell (S3H5/γ2bA2 hybridoma) damage during calcium alginate entrapment, it is desirable to use a 1.5% calcium chloride solution rather than a 1.3% calcium chloride solution. In addition, an exposure time of the cells to a calcium chloride solution should be minimized. When a commercially available alginate, Keltone LV (Kelco, Chicago, IL), was used, an exposure time of the cells to a calcium chloride solution could be shortened to less than 30 min without

weakening the mechanical strength of calcium alginate beads. The calcium alginate-entrapped cells did not show any significant lag phase and could be cultivated in a spinner flask for more than two weeks by keeping the integrity of calcium alginate beads (Lee *et al.*, 1992).
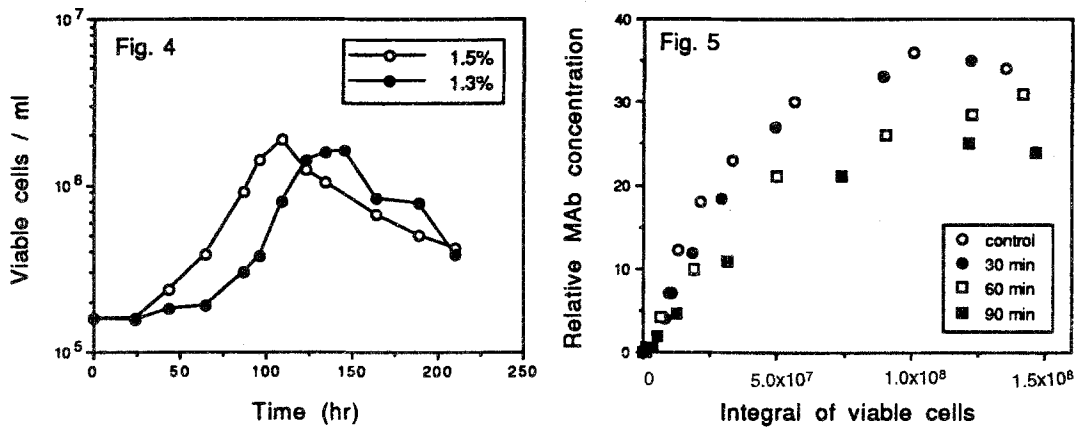

Figure 4. Effect of calcium chloride concentration on S3H5/γ2bA2 cell growth ability.

Figure 5. MAb concentrations versus integral of viable cell concentrations with respect to culture time. These data originated from the culture shown in Figure 2.

Specific MAb productivity of hybridoma cells often increased by environmental stresses (e.g., hyperosmolarity, pH shocks, and thawing and freezing of hybridoma cells) (Miller *et al.*, 1988; Miller *et al.*, 1988a; Berg *et al.*, 1990; Hiller *et al.*, 1991). Entrapped S3H5/γ2bA2 hybridoma cells in calcium alginate beads were observed to have enhanced specific MAb productivity for which calcium chloride treatment might be responsible (Lee *et al.*, 1991). In order to test this hypothesis, specific MAb production rates of the cells with varying time of calcium chloride treatment were obtained by plotting MAb concentration versus the integral of viable cell concentration with respect to time during the cultures which correspond to Fig. 2. The slopes of curves indicate specific MAb production rates. As shown in Fig. 5, the specific MAb production rate of cells treated with calcium chloride did not increase, and in fact deteriorated. Thus, the enhanced specific MAb production rate of calcium alginate entrapped cells was not due to calcium chloride treatment.

In conclusion, the cell viability did not decrease rapidly during one hour calcium chloride treatment, but the cell growth ability deteriorated. In addition, 1.3% calcium chloride solution appeared to be more detrimental to the cells than 1.5% calcium chloride solution. Thus, it is desirable to use 1.5% calcium chloride solution and to minimize the exposure time of the cells to calcium chloride solution.

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