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하이브리도마 세포배양에서 암모늄 이온의 영향 및 고정화 흡착제에 의한 암모늄 이온의 동시제거

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Ammonium Ion Effects and Its *In Situ* Removal by Using Immobilized Adsorbent in Hybridoma Cell Culture

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ABSTRACT

The effects of ammonium ion on cell growth kinetics, monoclonal antibody productivity, and cell metabolism of hybridoma cells were investigated. The mouse-mouse hybridoma cell line VIIIH-8 producing mouse IgG2a was used as a model system. Ammonium ion showed an inhibitory effect on cell growth and monoclonal antibody production. New immobilized adsorbents were developed for the reduction of the inhibitory effect of ammonium ion. The ammonium ion selective zeolite, Phillipsite-Gismondine was entrapped in calcium alginate bead or in dialysis membrane and applied to the hybridoma cell culture system for the *in situ* removal of ammonium ion from culture media. The effects of ammonium the both serum supplemented and serum free media on the cell growth were studied by applying immobilized adsorbents of calcium alginate bead type. The results demonstrated a substantial enhancement in cell growth. Applying immobilized adsorbents of dialysis membrane type to serum supplemented media also resulted in the stimulation of cell growth, cell viability and monoclonal antibody production.

Introduction

The accumulation of ammonium ion in an animal cell culture media is inevitable because ammonium ion is excreted as a byproduct of glutamine metabolism (1-9), which is essential to provide mammalian cells with major energy and precursors for proteins, nucleotide, and lipids (1-8). Ammonium ion is also generated in cell culture medium during storage by spontaneous first

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order deamination of glutamine (2, 3, 5, 9-12). The final ammonium ion concentration in batch culture is normally between 2 and 5mM (2), and it increases with glutamine concentration of media (2, 4, 5, 13-16).

Ammonium ion toxicity on animal cells and its several mechanisms have been reported. However following two mechanisms concerning the intercompartmental pH change were generally well accepted because of the existence of sufficient experimental evidence (8). The first mechanism involves the uptake of the weak base NH_3 into lysosomes causing increase in intralysosomal pH. The increase of intralysosomal pH inhibits one of the steps, mostly receptor-recyling step, of the receptor-mediated endocytosis involving various material from simple compound such as iron (17) or mannose-glycoconjugates (18) to complex material such as alpha-2-macroglobulin (19) and EGF (20). The second mechanism involves the uptake of weak acid NH_4^+ into the cytoplasm causing cytoplasmic acidification which leads to the inhibition of cell growth by low cytoplasmic pH (6, 8, 21). No matter which mechanism involves ammonium ion toxicity of animal cells, it has been well known that the accumulated ammonium ion inhibits cell growth (2, 3, 10, 13-15, 22-26) and/or product formation (3-5, 7, 24, 26-30).

Therefore, it is very important to reduce the concentration of ammonium ion in a cell culture media in order to increase cell growth and productivity (4, 5, 31, 32). An investigation of the effects of ammonium ion on the cell growth and metabolism is a prerequisite for the development of rational strategy for the reduction of the accumulation of ammonium ion in animal cell culture media. The effects of ammonium ion on the cell growth kinetics, monoclonal antibody productivity, and cell metabolism of hybridoma cells were investigated in this paper. We intended to find out a general trends of ammonium ion effects prior to develop a rigorous quantitative model of ammonium ion inhibition which enables to estimate the appropriate level of ammonium ion removal for the desired cell growth and productivity.

Adsorption method might be one of the promising alternatives for the *in situ* removal of ammonium ion from the animal cell culture media. Only few group, however, has been involved in the trial to apply adsorption method to *in situ* removal of ammonium ion from the culture media. Iio *et al.* (31) tried to apply synthetic zeolite ZCP-50 to the human hybridoma culture in serum free media and accomplished 45% reduction in ammonium ion concentration and 31% increase in cell number. However they were unable to enhance cell number in case of culture with serum-supplemented mdia because it seemed that serum contaild a factor which suppressed the ammonium ion toxity. Recently, Jeong and Wang applied another synthetic zeolite Phillipsite-Gismondine, which has a high selectivity and high capacity of ammonium ion adsorption, to the serum-supplemented culture of mouse hybridoma cells and demonstrated the enhancement in both cell growth and antibody production (33). This zeolite can be regenerated by using mixed salt regenerant containing sodium and calcium ions (34).

Although the application of Phillipsite-Gismondine powder to spinner reactor culture showed successful increase in cell density and cell viability (33), the direct application of powder form has the following problems. The first problem is the difficulty in separating absorbent particles from media and cells. This separation of absorbents from media and cells is needed for cell harvest and absorbents recycle after regeneration and for preventing from clogging in perfusion application. The second problem is the product loss by adsorption to the surface of particles. These problems can be resolved by immobilizing zeolite into calcium alginate bead or dialysis membrane. In this report, immobilized adsorbent in alginate beads were developed and the results of application them to the in-situ removal of ammonium ion from the both serum supplemented and serum free media will be discussed in terms of cell growth and cell viability because an important and effective way of increasing the productivity

of monoclonal antibodies by hybridoma cells is to keep a high cell density and a high cell viability for as long as possible (16, 35, 36).

Materials and Methods

Cell line

The mouse-mouse hybridoma cells (VIII H-8) used in this study were kindly given by New Brunswick Scientific Co. (Edison, NJ). These cells produce immunoglobulin, IgG2a which is specific for the whole cell of bacteria *Rhizobium japonicum* NR-7. These cells were obtained by fusion of mouse myeloma cells and spleen lymphocytes from BALB/C mice immunized with whole cells of *Rhizobium japonicum* NR-7. The antibody reacted specifically with a lipopolysacchride fraction isolated from broth cultures of *Rhizobium japonicum* NR-7.

Cell culture

For maintenance and ammonium ion effect study

The cells were maintained in DMEM (GIBCO 430-1600) containing 4.5g/L of glucose, supplemented with NCTC 135 (0.94g/L, GIBCO), oxaloacetate (150mg/L), insulin (75.5μg/L), mercaptoethanol (3.5mg/L), sodium bicarbonate (40mM), streptomycin (100μg/mL), penicillin (100U/mL), and 5% (v/v) calf bovine serum (GIBCO). The medium was filtered with 0.22μm membrane. Cells were maintained in 75cm T-flask at 37℃ in 7% CO_2 atmosphere. For studying ammonium ion effect, a specific amount of membrane sterilized ammonium chloride corresponding to each experimental plan was added to prepare the experimental medium. The maintained cells were centrifuged and washed with experimental medium and cultivated in 150cm^2 T-flask at 37℃ in 7% CO_2 atmosphere.

For *in situ* removal of ammonium ion

The same medium as maintenance medium was used for serum supplemented medium. For serum free medium, a protein free media PHHM-ll (Cat. No. 430-3600EB) in powder form were purchased from GIBCO. The powder were dissolved in deionized water and 2.0g of $NaHCO_3$ and 1.5g of glucose were added to one liter of media. Four mM of glutamine was also added and pH was adjusted to pH 7.0. Prepared medium was filtered through 0.22μm membrane.

Immobilized adsorbents of calcium alginate bead type were prepared as follows. The alginate of 1.4% in water solution was prepared. Thirty grams of Phillipsite-Gismondine were well mixed with 100mL of 1.4% alginate solution in the mixer. While Phillipsite-Gismondine particles were suspended in the mixer, some part of adborbent slurry was withdrawn by pumps and injected into a 50mM calcium chloride solution tank through the nozzle (diameter 0.4mm). Droplets (average diameter of 2.64mm) were formed after nozzle and alginate matrix were cured with aid of calcium ions. 7.5 grams of the prepared immobilized beads were weighed, autoclaved, and aseptically transfered into each 150cm^2 T-flask which contains 75mL of serum supplemented or serum free medium. Cells were cultivated at 37℃ in 7% CO_2 atmosphere.

For immobilized adsorbent of dialysis membrane type, four grams of Phillipsite-Gismondine was packed in cellulose membrane and attached to the impeller of spinner. Cells were cultivated in the spinner of 150mL working volume at 65rpm, 37℃ in 7% CO_2 atmosphere.

Analyses

Two independently taken samples were mixed to average the cell number in each sample. The mixed cell sample was diluted 1:1 with 0.4% trypan blue (GIBCO, Grand Island, NY) in normal saline. A small amount of sample was vortexed and injected under the coverslip of a hemocytometer and examined under the microscope. The trypan blue dye is taken up only by the nonviable cells, so it is possible to differentiate between viable cells (transparent) and dead cells (blue stained). An average of two count was used to determine the viable cell concentra-

tion and percent viability.

Glucose was assayed by a Beckman Glucose Analyzer 2 which measures the rate of change in oxygen consumption when a sample is injected into an enzyme solution. When sample is injected into the enzyme solution containing glucose oxidase, β-D-glucose from the sapce conbines with dissolued oxygen from the solution, generaty gluconic acid and peroxid. At all times during the reaction, the rate of oxygen consumption is directly proportional to the concentration of glucose.

The ammonium ion concentration was measured using a 9512 ammonium ion electrode and a digital pH/milli volt meter 611 (Orion, Cambridge, MA). Standard solution of 8mM, 4mM, 2mM, 1mM, and 0.5mM of NH_4Cl in distilled water were used to generate a linear calibration curve for voltage readings (mV) vs. the log of ammonium ion concentrations.

Lactate concentration was determined by using a Sigma procedurieg 826-UV. Lactate and excess NAD^+ are converted to pyruvate and NADH by lactate dehydrogenase. The increase of absorbance at 340nm due to reduction of NAD^+ to NADH is used to measure the amount of lactate originally present in the sample. Absorbance was measured with a Bausch & Lomb Spectronic 601 spectrophotometer.

The mouse immunoglobulin, IgG_{2a} was analyzed by using RID (Radial Immuno Diffusion) methods. Anti-IgG was immobilized in the agar on the RID plates. Five μL of centrifuged sample was injected into each sample hole on RID plates and incubated at 37℃ for 36 hours. After incubation, ring was visualized by first staining with a buffalo-black stain solution and then rinsing with RID wash solution (5% v/v glacial acetic acid, 0.5% v/v glycerol).

Results and Discussions

Effect of Ammonium ion

Fig. 1 shows the effect of initial ammonium ion concentration on live cell growth kinetics. As the initial ammonium ion concentration increases, the

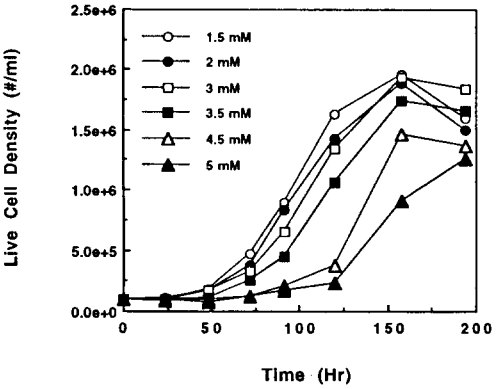

Fig. 1. Effect of ammonium ion on live cell growth kinetics of VIII H-8 hybridoma cells. Cultured in 75mL T-flasks at 37℃ and 7% CO_2 atmosphere with variation of initial ammonium ion concentration(mM).

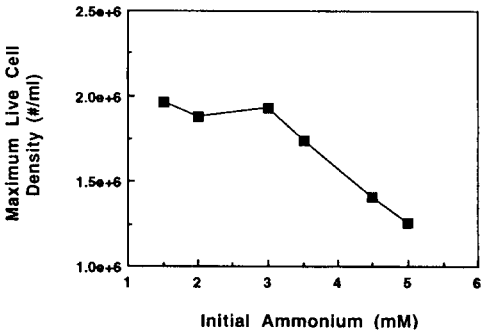

Fig. 2. Effect of initial ammonium ion concentration on maximum live cell density of VIII H-8 hybridoma cells. Same culture conditions as Fig. 1.

initial growth rate decreases. It is obvious that ammonium ion inhibits cell growth by retarding initial cell growth rate, and reducing maximum attainable cell density. Fig. 2 shows the plot of maximum cell density with respect to initial ammonium ion concentration. It shows almost same maximum cell density up to initial ammonium ion of 3mM, and cell density begins to drop more sharply beyond 3mM. Therefore, nearly 3mM seems to be the critical inhibition concentration in cell yield. Fig. 3 shows the plot of initial specific

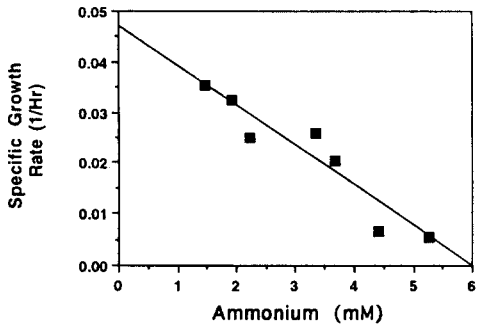

Fig. 3 Effect of ammonium ion on specific growth rate of VIII H-8 hybridoma cells. Same culture conditions as Fig. 1.

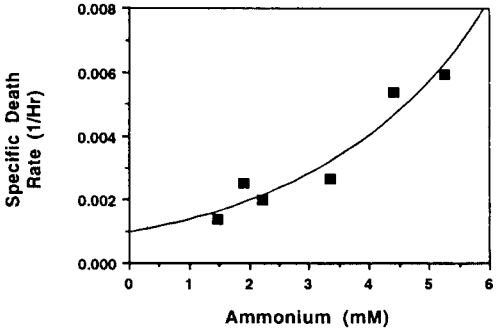

Fig. 4. Effect of ammonium ion on specific death rate of VIII H-8 cells. Same culture condition as Fig. 1.

growth rate with respect to ammonium ion concentration in the medium. As a whole, it seems to decrease continuously as initial ammonium ion concentration increases.

When Fig. 2 and 3 were compared, it is noticed that the specific growth rate decreases more sharply than maximum cell density. This demonstrates that the ammonium ion inhibits cell growth by reducing kinetic growth rate rather than stoichiometric maximum cell density. This is probably due to the fact that ammonium ion is not a nutrient. In every set of culture, same amount of nutrients were provided. Since cultures with lower NH_4Cl grow faster, soon they would be subjected to the depletion of nutrients. Therefore, live cell number of runs with 1.5 and 2mM of NH_4Cl decrease faster than other three

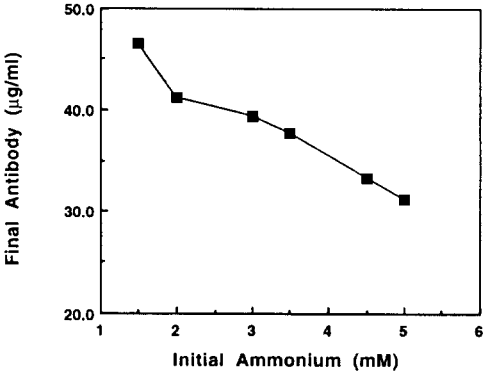

Fig. 5. Effect of initial ammonium ion concentration on final monoclonal antibody concentration in the same run as Fig. 1.

runs with 3mM, 3.5mM, and 4.5mM in the later phase, while run with 5mM still grows because nutrients are still available for its growth. Therefore in later phase, nutirent depletion seems to affect the live cell density more significantly than ammonium ion toxicity corresponding to that ammonium ion level, so cross over points occur. However, if higher concentration of NH_4Cl is added more than 4.5mM, cells cannot reach as high density as other runs with low levels of NH_4Cl can even though more glucose is available to this run (4.5mM). Therefore, ammonium ion toxicity becomes significant as ammonium ion concentration increases. Fig. 4 shows the effect of ammonium ion on specific death rate. As expected, the specific death rate of the cells increases with ammonium ion concentration in the exponential phase when nutrient depletion does not govern.

Fig. 5 shows the effect of initial ammonium ion concentration on final MAb concentration. The MAb concentration measured at 194 hr after inoculation is defined as final MAb concentration for convenience sake, eventhough it is not clear whether MAb concentration of 5 mM run is increasing further. This figure clearly demonstrates the inhibitory effect of ammonium ion on MAb production. Fig. 6 shows the effect of ammonium ion concentration on the cumulative specific

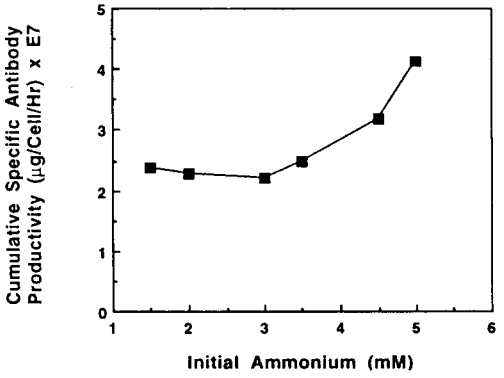

Fig. 6. Effect of initial ammonium ion concentration on the cumulative specific monoclonal antibody productivity in the same run as Fig. 1.

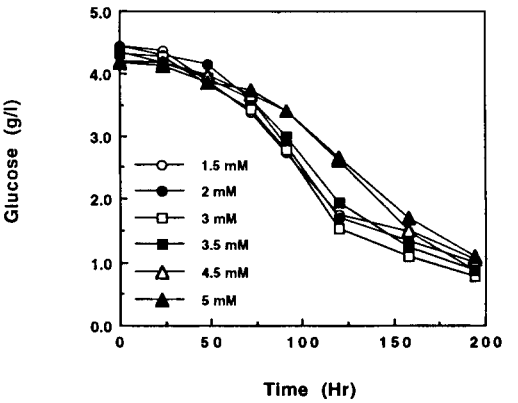

Fig. 7. Effect of ammonium ion on the glucose consumption kinetics of VIII H-8 hybridoma cells in the same run as Fig. 1.

monoclonal antibody productivity. Usually the concept of cumulative specific productivity has been widely used especially for antibody production (37, 38) to compare overall performance of production when a specific productivity changes significantly with time. The cumulative specific monoclonal antibody production rate can be calculated as follows and it can be used as an index for the over- all efficiency of monoclonal antibody production.

$$\bar{q}_P=\frac{[P]_{t_c}-[P]_0}{\int_0^{t_c} X_v\,dt}$$

where $\bar{q}_P$=cumulative specific antibody production rate (μg/cell/hr)
t_C=total cell culture time (hr)
$[P]_{t_c}$=final antibody concentration (μg/mL)
$[P]_0$=initial antibody concentration (μg/mL)
X_v=viable cell concentration (cells/mL)

It shows that cumulative specific MAb productivity increases as initial ammonium ion concentration decreases. It seems that low growth rate due to inhibitory effect of ammonium ion arrset cells into G1 phase of cell cycle. Since several methods to arrest hybridoma cells include harsh condition such as serum depletion and oxygen limitation, high concentration of ammonium ion can be tried to arrest cell to G1 phase. Indeed, Suzuki included "stationary state reached after exponential growth" as an arresting methods (36). It seems that high ammonium ion concentration in the stationary phase mostly contribute to arresting cells because ammonium ion concentration is usually built up in the phase. Therefore, high concentration of ammonium ion might arrest cells into G1 phase so specific MAb productivity is increased. However, the overall effect of ammonium ion is inhibitory to MAb production as shown on Fig. 5 because ammonium ion inhibits the cell growth more than it stimulates specific MAb production rate. Therefore, it may be concluded that ammonium ion inhibits MAb production by reducing live cell numbers even though it stimulates specific MAb productivity. It is also observed that cumulative specific antibody productivity is almost constant up to initial ammonium ion concentration of 3mM and begin to increase after 3mM. This agrees with the observation that the inhibitory ammonium ion concentration for maximum cell density is approximately 3mM. Therefore, the ciritical ammonium ion concentration to arrest VIIIH-8 hybridoma cells into G1 phase is around 3mM.

Fig. 7 shows the glucose consumption kinetics.

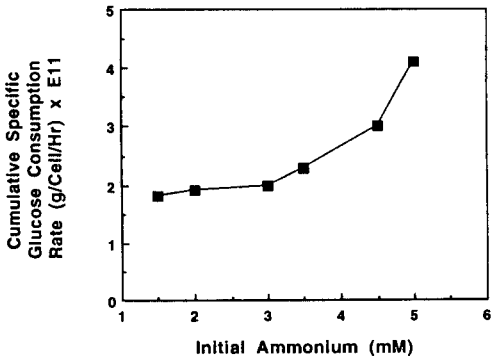

Fig. 8. Effect of initial ammonium ion concentration on the cumulative specific glucose consumption rate of VIII H-8 hybridoma cells in the same run as Fig. 1.

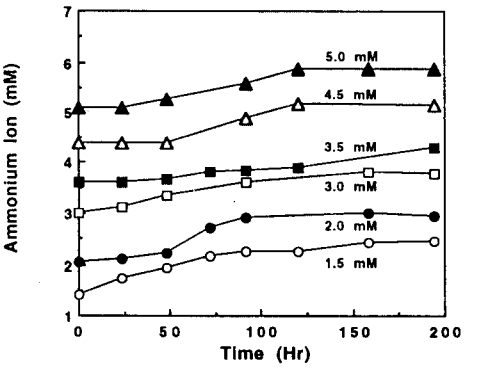

Fig. 9. Effect of ammonium ion on the ammonium ion production kinetics of VIII H-8 hybridoma cells from the same run as Fig. 1.

In case of run with high initial ammonium ion concentration, low cell growth due to ammonium ion inhibition results in low glucose consumption, and high residual glucose concentration remained in the media. However relatively little variance in glucose concentration compared with the variance in live cell number is noticeable. Thus, it can be expected that the ammonium ion toxicity leads to increase of specific consumption rate of glucose. This is proved by plotting of cummula-tive specific glucose consumption rate versus initial ammonium ion concentration as shown on Fig. 8. Miller (2, 23) also observed the dramatic increase of specific glucose consumption rate when growth rate was reduced by ammonium ion. It was also reported that observed a 20% increase of specific glucose consumption rate when ammonium ion was pulsed from 8.2mM to 18mM in the continuous culture (2, 23). It is a general phenomenon that higher nutrient consumption rate is observed when cells are exposed to inhibitory condition such as high ammonium ion concentration, low oxygen concentration, low or high pH (2, 23), or even low serum and low glutamine condition. This is possibly due to the fact that cells need more energy for maintenance in order to survive against such harsh conditions. Same phenomenon of stimulation of glycolysis by ammonium ion was observed by Stelling et al. (39) in rat adipocytes while inhibiting respiration.

Fig. 9 shows the effect of initial ammonium ion concentration on ammonium ion production and Fig. 10 shows the cumulative specific ammonium ion production rates versus initial ammonium ion concentration. Fig. 9 shows that culture of higher initial ammonium ion concentration maintains higher ammonium ion concentration than that of lower initial ammonium ion concen-tration throughout the culture period. Therefore any 'effect of initial ammonium ion concentration' can be regarded as the 'effect of ammonium ion concentration'. Fig. 10 shows that the cumulative specific ammonium ion production rate increases as ammonium ion concentration increases. The condition caused by the high inhibitory concentration of ammonium ion would require higher nutrients consumption. So higher specific glutamine and glucose consumption rates are expected. Since ammonium ion is a major product from glutamine metabolism, higher specific ammonium ion production rate is expected as ammonium ion concentration increases. The abrupt increase of cumulative specific ammonium ion productivity is observed also between 3.5 and 4.5mM of initial ammonium ion concentration. Therefore the critical inhibitory concentration of VIIIH-8 cell seems to be 3mM because abrupt change in maximum

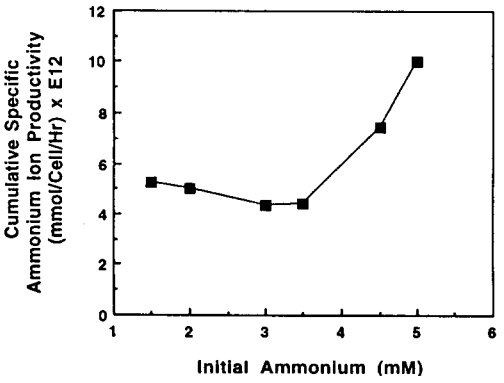

Fig. 10. Effect of initial ammonium ion concentration on the cumulative specific ammonium ion production rate of VIII H-8 hybridoma cells froms the same run as Fig. 1.

live cell density, specific MAb productivity, specific glucose consumption rate, and specific ammonium ion productivity occurred at this concentration.

In situ removal of ammonium ion

Iio et al. tried to apply synthetic zeolite ZCP-50 to the human hybridoma culture in serum free media and accomplished 45% reduction in ammonium ion concentration and 31% increase in cell number (31). However they were unable to enhance cell number in case of culture with serum -supplemented media because it seemed that serum contained a factor which suppressed the ammonium ion toxicity. Therefore, cells may be more sensitive to ammonium ion toxicity in serum free media than in serum supplemented media.

Fig. 11 shows the comparison of the effect of *in situ* removal on the live cell growth kinetics in the culture with serum free and serum supplemented media. Serum free media shows lower cell growth revealing the effectiveness of serum components. The figure demonstrates that cell growth with adsorbent beads enhances cell growth of the culture in serum free media as well as that of serum supplemented media. The significant stimu-

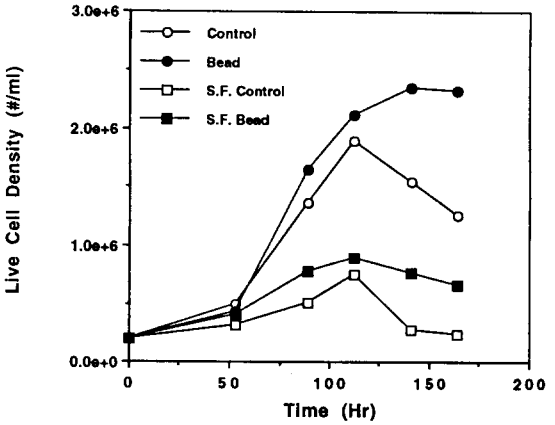

Fig. 11. Comparison of the effect of in situ ammonium ion removal on the live cell growth kinetics of VIII H-8 hybridoma cells between serum and serum free media. Cultured in 75mL T-flasks at 37℃ and 7% CO_2 atmosphere with immobilized adsorbent of calcium alginate bead type.

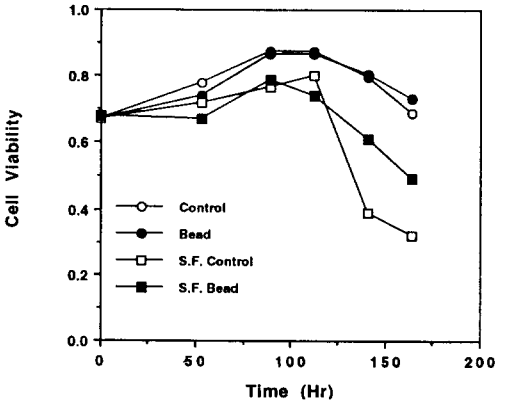

Fig. 12. Comparison of the effect of in situ ammonium ion removal on the cell viability change of VIII H-8 hybridoma cells between werum and serum free media. Same culture conditions as Fig. 11.

lation of cell growth in case of serum free media is not observed. It seems that the cells with adsorbents cannot grow further in the serum free media even though ammonium ion concentration was reduced as shown in Fig. 13 because some key nutrients such as growth factors are not sufficient in the serum free culture. Therefore signif-

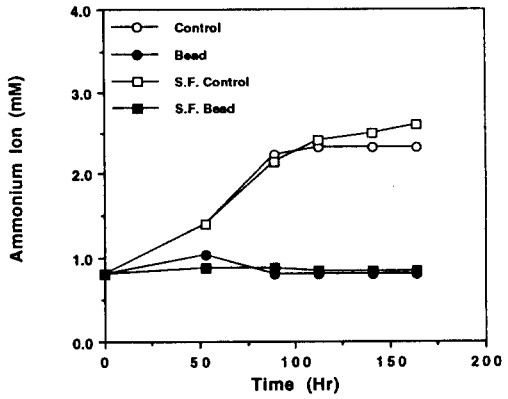

Fig. 13. Comparison of the effect of in situ ammonium ion removal on the ammonium concentration change of VIII H-8 hybridoma cell culture media between serum and serum free media. Same culture conditions as Fig. 11.

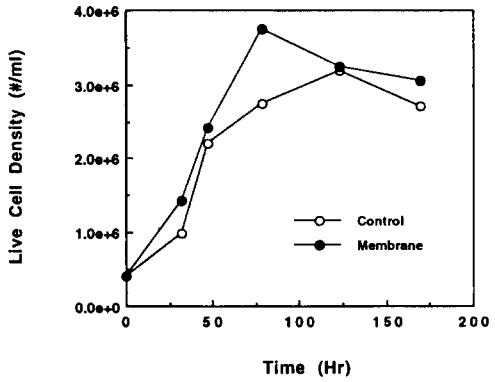

Fig. 14. The effect of in situ ammonium ion removal on the live cell growth kinetics of VIII H-8 hybridoma cells. Cultured in 150mL spinner at 37℃ and 7% CO_2 atmosphere with immobilized adsorbent of dialysis membrane type.

icant stimulation of cell growth was not observed in the culture of serum free media. Fig. 12 shows that cell viability is slightly increased by applying adsorbent beads. The enhancement of cell viability of culture with serum free media is greater than that of serum supplemented media. This implies that the cells in the serum free media is

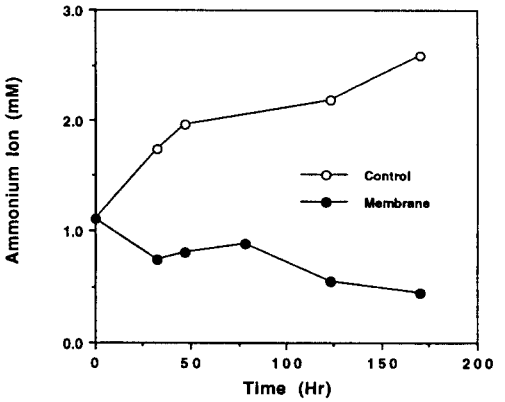

Fig. 15. The effect of in situ ammonium ion removal on the ammonium ion production kinetics of VIII H-8 hybridoma cells from the same run as Fig. 14.

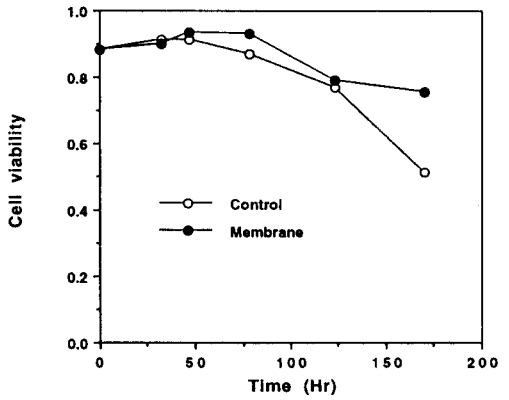

Fig. 16. The effect of in situ ammonium ion removal on the cell viability of VIII H-8 hybridoma cells from the same run as Fig. 14.

more sensitive to ammonium ion than serum supplemented media as reported by Ii (31). Therefore, significant improvement in the cell growth, cell viability and MAb production is expected if the system of *in situ* removal of ammonium ion is applied to the serum free culture of high ammonium ion accumulation. Figurel 13 shows the ammonium ion concentration change in the same run. It shows that adsorbent bead can effectively reduce ammonium ion concentration in the media

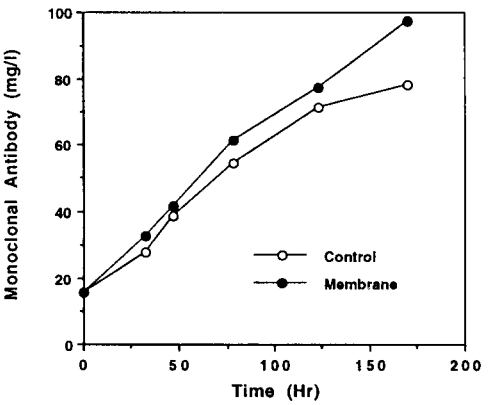

Fig. 17. The effect of in situ ammonium ion removal on the monoclonal antibody production kinetics of VIII H-8 hybridoma cells from the same run as Fig. 14.

regardless of medium type. It is also interesting to notice that ammonium ion production of cells in serum free media is almost same as that in serum supplemented media. This implies that the specific ammonium ion production rate of cells in serum free medium is higher than that of serum supplemented medium because cell growth in serum free media is lower than that of serum supplemented medium. As discussed earlier, cells are exposed to inhibitory condition such as low serum or serum free condition, so higher nutrients consumption rates are expected in serum free culture. Therefore, high specific ammonium ion production rate is expected due to high glutamine consumption rate in case of serum free culture.

Fig. 14 shows the result of live cell growth kinetics of hybridoma cells in the modified spinner reactor equipped with a tube form immobilized adsorbent on impeller. This is another form of immobilized adsorbent which can be easily prepared. Zeolite powder can be just put inside of the dialysis membrane and used as a immobilized adsorbent. The live cell growth kinetics shows the stimulation of cell growth in the culture with adsorbent. This is due to maintaining of low ammonium ion level as shown in Fig. 15 with the aid of immobilized adsorbent. Fig. 16 shows higher cell viability in the culture with tube type immobilized adsorbent than that of culture without adsorbent as expected. The enhanced cell growth and viability due to low ammonium ion concentration stimulate also the production of monoclonal antibody as shown on Fig. 17. Therefore, the developed immobilized adsorbent can be used efficiently for *in situ* removal of ammonium ion in a hybridoma cell culture system in order to increase cell growth and monoclonal antibody productivity.

요 약

하이브리도마 세포의 성장속도, 모노클론 항체의 생산성 및 세포 대사에 미치는 암모늄 이온의 영향에 대해서 조사하였다. 항체 IgG_{2a}를 생산하는 mouse-mouse hybridoma VIII H-8 세포가 모델로 이용되었다. 암모늄 이온은 세포 성장 및 항체의 생산을 저해하는 것으로 나타났다. 이러한 암모늄 이온의 저해효과를 완화시키기 위해 새로운 고정화 흡착제가 개발되었다. 세포 배양액으로부터 암모늄이온의 동시제거를 위하여 암모늄 이온 선택성 zeolite인 phillipsite-Gismondine을 calcium alginate bead 또는 투석막 안에 포획하여 하이브리도마 세포배양에 적용하였다. Calcium alginate bead 형태의 고정화 흡착제를 혈청첨가 배양 및 무혈청 배양에 모두 적용하여 봄으로써 암모늄 이온의 동시제거가 세포의 성장에 미치는 영향을 조사하였고 그 결과 고정화 흡착제의 첨가로 인하여 세포 성장이 향상됨을 알 수 있었다. 또한 투석막 형태의 고정화 흡착제를 혈청첨가 배양에 적용하여본 결과 세포성장, 세포생존율 및 모노클론 항체의 생산이 향상됨을 알 수 있었다.

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