CONTINUOUS PRODUCTION OF ANTI-ERYTHROPOIETIN ANTIBODY BY IMMOBILIZED HYBRIDOMA CELLS

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A hybridoma cell line epo2C which produces anti-erythropoietin monoclonal antibody was immobilized with alginate and urethane polymer under several conditions. Concentration of the alginate affected neither the cell growth nor the antibody production when the alginate was hardened by calcium. However, the concentration was very important when strontium was used for the hardening. By coating alginate gel with urethane polymer, a long term cultivation of the immobilized cells and stable antibody production by a fluidized bed reactor were possible without destruction of the gel particles. During the cultures, the cells leaked from the alginate gel but were trapped inside of urethane coat with any types of gel. Therefore, high cell density of more than 10^7 cells/cm^3-gel was attained and volumetric productivity of the antibody was 0.8 mg/(cm^3 · d). This method will provide a promising way for effective and simple antibody production.

Introduction

Monoclonal antibodies are one of the most important bioproducts originated from mammalian cells. They are expected to be applied for therapy, diagnosis of disease and purification etc. For effective production of monoclonal antibodies, several approaches have been tried. Many researchers have tried to cultivate hybridoma cells at a high cell density around 10^7—10^8 cells/cm^3 to acheive a high level of an antibody production. For this, aeration[5] and separation of the growth inhibitors[4,8] were improved by developing special devices. Another approach is a use of recombinant DNA technology. Recently, genes encoding for antibody proteins were isolated and production of an antibody in yeast *Saccharomyces cerevisiae* was tried.[9] However, production of the antibody in the microorganism was at very low level.

Immobilized enzymes and microorganisms have been widely used for production of biomaterials, since they give stable and long term operation. Oxygen and the substrate uptakes of mammalian cells are lower than those of microorganisms,[3] therefore, we applied gel entrapment method. Easy separation of cells from products, and application of bubble aeration may be expected for immobilized animal cells. From this view, several researchers tried to immobilize animal cells.[6,7] We have reported the gel entrapment of a hydridoma cell with calcium alginate and an urethane polymer for the continuous production of a monoclonal antibody.[1] In the present paper, we applied this method for another hybridoma cell line which produces anti-human erythropoietin monoclonal antibody. Erythropoietin is a hormone related to erthropoiesis and is used for theraphy of anemia patient. Its monoclonal antibody may be useful as an affinity ligand for the purification of this hormone. We studied effects of alginate concentrations and continuous long term production of the antibody.

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1. Experimental

1.1 Cells and medium

A mouse-mouse hybridoma cell line epo2C which produces anti-human erythropoietin antibody was obtained from Snow Brand Milk Products Co. The hybridoma cells were cultured in DF medium[2] which was supplemented with 5% fetal calf serum (M.A. Bioproduct Co.). For the regular passages, the cells were cultured in 100 mm Petridishes under 95% air and 5% CO_2 at 37°C.

1.2 Immobilization of cells

Epo2C cells cultured in Petridishes were suspended in DF medium containing 5% fetal calf serum at a concentration of 4×10^6 cells/cm^3. Equal amount of filter-sterilized sodium alginate (Kimitsu Chemical Co.) solution was added to the cell suspension and the mixture was dropped into cold 0.1 M calcium chloride or strontium chloride solutions by using a pipette. The alginate gel particles were left for 5 min for hardening. The final concentrations of the alginate were 0.5, 0.6 or 1%. After washing with the medium solution, gel particles were covered with an urethane

prepolymer (PU-6, Toyo Tire and Rubber Co.) and left in a fluorocarbon (FC43, Green Cross Co.) for the polymerization as reported previously.[1] After leaving 15 min, the immobilized cells were cultured in Petridishes with DF medium containing 5% fetal calf serum for almost one week for the activation. During this period, the medium was changed every two days.

1.3 Continuous production of the monoclonal antibody by a fluidized bed reactor

The immobilized cells were cultured in a fluidized bed reactor. The configuration of the reactor was reported previously.[1] Packed volume of about 100 pieces of the immobilized cell particles was 24 cm^3. From the bottom of the reactor, humidified air was supplied at a rate of 25 cm^3/min. In order to suppress the foaming, 0.005% (v/v) of an antifoam (Silicone KM-72, Shinetsu Polymer Co.) was included in the medium. In addition to this, a part of the inner surface of the column was covered by a silicon grease (HIVAC-G, Shinetsu Polymer Co.). DF medium containing 5% fetal calf serum was continuously supplied at a flow rate of 48 cm^3/d and the monoclonal antibody, glucose and lactate concentrations in the overflow medium were analyzed. At suitable intervals, some gel particles were taken from the reactor and the cell density was analyzed.

1.4 Analyses

Concentration of the anti-erythropoietin antibody was measured by the enzyme-linked immunosorbent assay by using the purified antibody (obtained from Snow Brand Milk Products Co.) as a standard. Glucose was assayed by using a glucose analyzer (Model 27, Yellow Spring Instrument Co). Lactate concentration was assayed by a lactate analyzer made of an immobilized lactate oxidase membrane and an oxygen electrode (Able Co.). The cell density in the alginate was measured by the trypan blue dye exclusion method using haemocytometer after the gel was solubilized in 1% sodium citrate. Before the solubilization, PU-6 coat was removed by using a forceps. We observed that the cells leaked from the alginate gel, and accumulated in a space between PU-6 coat and alginate gel. In order to measure the cell number in this space, PU-6 coat was removed and both the alginate gel and the coat were rinsed extensively in the medium. Cell number in the medium was then counted as that in the space between the alginate and the PU-6 coat.

2. Results

Growth of the hybridoma cells in Petridishes was studied especially in terms of the serum requirement. We tried to adapt the cells in DF·ITES serum free medium,[2] but the stable growth could not be observed. Therefore, the cells were cultured in the presence of various concentrations of the serum. In the presence of low concentration of the serum, the cell growth was good. However, the antibody production was storngly affected by the serum. In the presence of 5% fetal celf serum, a fairly good antibody production was obtained. **Figure 1** shows the growth and the antibody production of epo2C cells in different media. In the presence of 5% serum, the specific production rate of the antibody was 120 pg/(cell·d). Since the serum was essential for the antibody production, defoaming method became very important for culture of the immobilized hybridoma cells in a fluidized bed reactor with direct air bubbling. As shown in **Table 1**, Silicone KM-72, a typical antifoam for microbial cultures was found to be effective and affect neither the cell growth nor the antibody production at a low concentration. The silicone grease was also inert for the cell growth and the production (Table 1). Therefore, we used both Silicone KM72 and the silicone grease as antifoam with a fluidized bed reactor.

Growth of the hybridoma cells in the alginate gel seemed to be affected by physical properties of the gel.

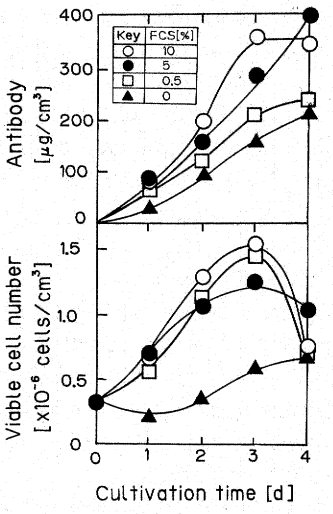

Fig. 1. Cultures of epo2C cells in the presence of various concentrations of fetal calf serum. Basal media supplemented FCS were RPMI1640 for 10% FCS and DF for 5 and 0.5% FCS. DF·ITES serum-free medium was used for 0% FCS.

Table 1. Effects of antifoams on hybridoma growth and antibody production. Hybridoma cells were inoculated at a density of 5×10^5 cells/cm^3 with a suitable concentration of Silicone KM-72 or a block of silicon grease in Petridishes

	Control	Silicon grease	Silicone KM-72 (0.1%)	Silicone KM-72 (0.01%)
Viable cells on day 2 [$\times 10^5$ cells/cm^3]	6.8	7.8	4.3	7.0
Antibody production on day 5 [%]*	100	97	65	110

* Relative values.

Thus, we checked conditions of the gel entrapment. The hybridoma cells were mixed with alginate to final concentrations of 0.5 and 1%. The suspension was dropped into 0.1 M of either calcium chloride or strontium chloride solutions, followed by the coating with PU-6. **Figure 2** shows the results of cultures of immobilized cells prepared under above four conditions in Petridishes. The medium was changed every 5 or 6 days. The hybridoma cells grew very poorly and produce a low concentration of the monoclonal antibody in 1% alginate gel hardened by strontium and the gel was obviously very hard. On the other hand, other gels seemed to be soft enough for the growth of the cells. Among them, 0.5% alginate hardened by calcium gave the best result.

From above results, we studied the continuous culture in a fluidized bed reactor with 0.6% gel hardened by strontium (0.6% Sr-gel) and 0.6 and 1% gels hardened by calcium (0.6 and 1% Ca-gels). All the gels were covered with PU-6. **Figure 3** shows the results with 1% Ca-gel. After one week activation in Petridishes, the gel particles were transferred into the bioreactor. On 17 d, the cell growth seemed to be stationary in terms of both the antibody production and the glucose consumption. The antibody concentration at the stationary phase was 390 $\mu g/cm^3$. After 18 d, the dilution rate was increased 3-times in this particular experiment since the accumulation of lactate was evident. However, increased consumption rate of glucose resulted in the increase in lactate formation and the lactate concentration went back soon to the original level. The increase of dilution rate did not affect the antibody production; the production rate did not change after increasing the dilution rate. After reaching the stationary phase, cell leak was evident and 75% of the cells were present in the space between the alginate gel and the PU-6 coat. However, the gel particles seemed to be stable after 30 days cultivation. The cell density on day 16 was 1.45×10^7 cells/cm^3-gel.

Figure 4 shows the results with 0.6% Sr-gel. The antibody concentration once became constant around 10 d, and increased again after 15 d. After 20 d, stationary antibody production could be obtained. The final antibody concentration in the medium was almost the same to that in Fig. 3. The stable production of the antibody continued for at least 30 days and the destruction of the gel particles was not observed through the culture. However, the cells leaked from the alginate gel and more than 90% of the cells were present in the space between the alginate gel and the PU-6 coat at the end of the culture. The final cell density was 1.2×10^7 cells/cm^3-gel.

Figure 5 shows the results of cultivation with 0.6% Ca-gel. The antibody concentration became constant

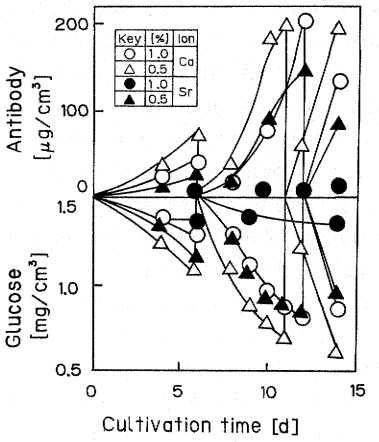

Fig. 2. Monoclonal antibody production and consumption of glucose by immobilized epo2C cells in Petridishes

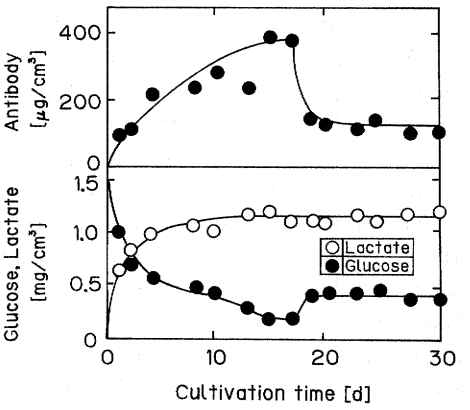

Fig. 3. Production of antibody and lactate and consumption of glucose with 1% Ca-gel

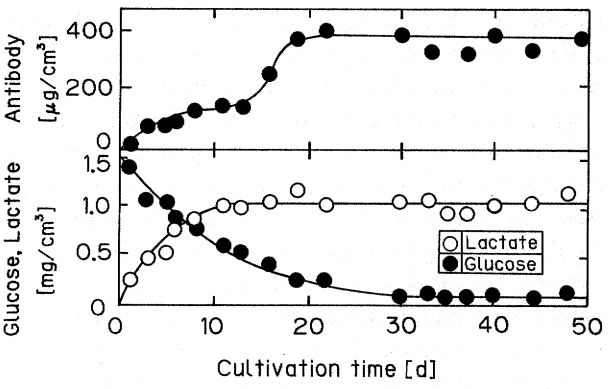

Fig. 4. Production of antibody and lactate and consumption of glucose with 0.6% Sr-gel

on 20 d. Although the gel structure was very soft, obvious gel destruction was not observed even after 50 days. The antbody concentration in the stationary phase was about 400 $\mu g/cm^3$-gel.

Figure 6 shows the cell growth in 0.6% Ca-gel. Cell leak became evident on 10 d. During the culture, the PU-6 coat detached gradually from the alginate beads and a space was formed between them (**Fig. 7A** and **B**). Similar space was observed both with 1% Ca- and

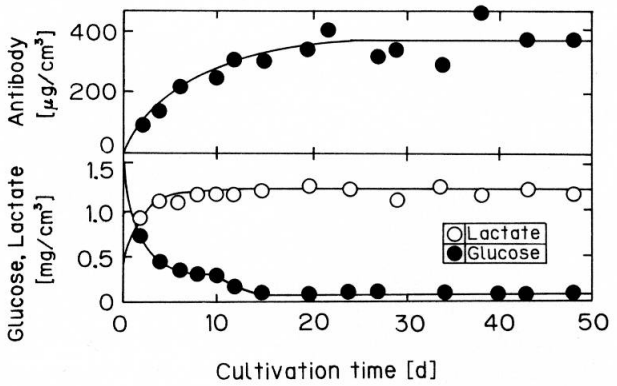

Fig. 5. Production of antibody and lactate and consumption of glucose with 0.6% Ca-gel

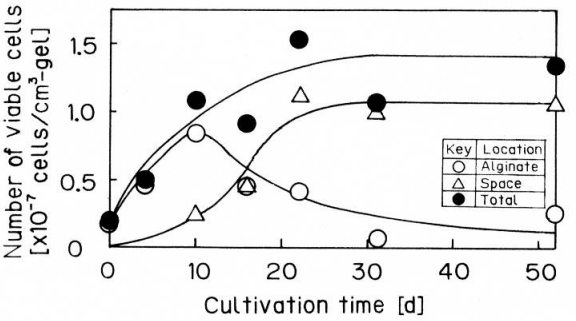

Fig. 6. Cell growth in 0.6% Ca-gel during continuous culture. Alginate: number of cells in alginate gel; Space: number of cells in the space between alginate gel and PU-6; Total: total number of cells in the particle.

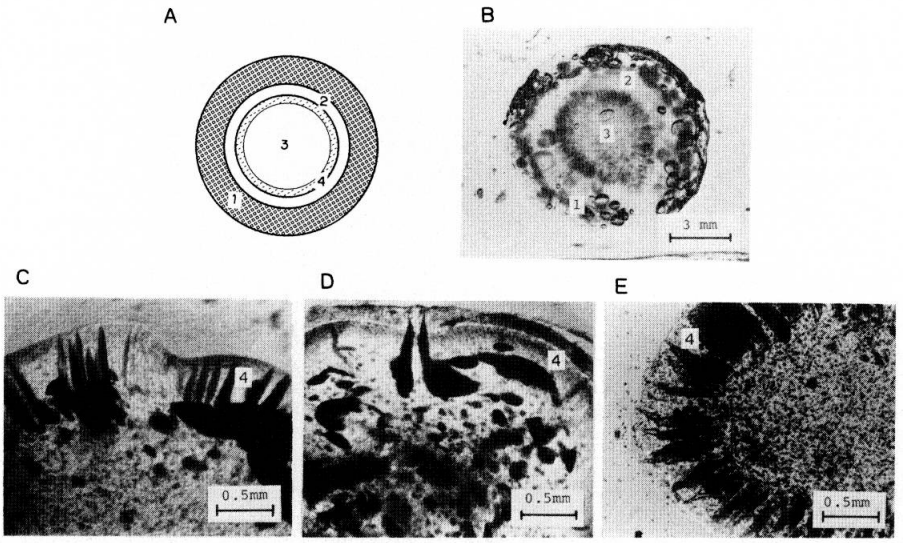

Fig. 7. Photographs of immobilized gels. A: Schematic drawing of alginate-PU6 gel; B: 0.6% Ca-gel; C: 1% Ca-gel; D: 0.6% Sr-gel; E: 0.6% Ca-gel. For photo B, a gel particle was buried in 24% polyacrylamide gel and the gel block was sliced. 1: PU-6 coat; 2: a space between alginate and PU-6; 3: alginate gel; 4: dehydrated alginate gel layer. For photos C-E, PU-6 coat was removed and alginate gel particles were then sliced.

0.6% Sr- gels. Almost all cells were present at this space between the alginate gel and the PU-6 coat layer after 30 d (Fig. 6). As checked by a microscope, there were quite a lot of holes on the PU-6 coat, but it was still effective for preventing cell leak. The final cell density was about 1.4×10^7 cells/cm^3-gel.

Figure 7 shows photographs of the immobilized cells at the end of the cultures. In all photographs, the cells did not grow at the dehydrated gel layer on the surface of the alginate gel as reported previosuly.[1] In the cases of Ca-gels (Fig. 7C and 7E), many cracks were observed at this layer. The cracks were occupied by colonies of the cells but a few colonies were observed within the alginate gel. On the other hand, such cracks could not be observed with the Sr-gel (Fig. 7D) and the cells formed colonies within the alginate gels.

3. Discussion

In the previous paper,[1] we reported a novel gel entrapment method of mammalian cells with alginate and an urethane polymer. By coating the alginate gel with PU-6, the outside surface of the alginate gel was dehydrated. This portion of the gel seems to be relatively hard since no cell growth was observed in this shell. This shell seemed to prevent cell leak and led to the high cell density and higher rate of antibody production for short term culture as reported previously.[1] In order to elucidate the possibility of practical application, we used this gel entrapment method to epo2C hybridoma cells and tried long term cultivation around 50 days. **Table 2** summarizes results of the cultures. With any types of the gels, a long term culture and the continuous antibody production were possible. The specific antibody production rates of the immobilized cells were similar to that of free

Table 2. Summary of immobilized cell cultures using a fluidized bed reactor

	Calcium alginate 1%	Calcium alginate 0.6%	Strontium alginate 0.6%	Free (not immobilized)
Location and shape of colony in the gel	surface, radial	surface, radial	inside, round	—
Culture period [d]	31	55	49	—
Specific growth rate* [d^{-1}]	0.2	0.2	0.2	0.8
Specific antibody production rate** [pg/(cell·d)]	110	110	110	120

* Calculated from viable cell count in the initial part of cultures.

** Calculated from the antibody production rate in the stationary phase of cultures.

cells. However, the specific growth rates of the immobilized cells in the initial part of the cultures were smaller than that of free cells. For the gel-entrapment of animal cells, hardness of gel structure may be very important; harder gel gave poor growth of mammalian cell (Shirai *et al.* personal communication). In our experiments, the structure of 1% Sr-gel seemed too hard for the cell growth. However, with other gels, concentration of the alginate and the difference between calcium and strontium did not affect the final antibody concentration, although the distribution of colonies was different between Ca and Sr-gels (Table 2 and Fig. 7). In our previous experiments,[1] autoclaved highly viscous alginate (Wako Chemical Co.) was used as a gel material, and cracks and radial distribution of the cell colonies were scarcely observed with this Caalginate gel.

By this immobilization method, high cell density more than 10^7 cells/cm^3-gel could be obtained because the cells were entrapped in the space between the dehydrated gel layer and the PU-6 coat layer, although the cells leaked from the alginate gel. Therefore, at the final stage of the cultures, structure of the gel particle was like microencapsulated cells (Fig. 7A). This will explain the fact that both Sr- and Ca-gels gave the similar antibody concentrations and production rates. It is not suspicious that a part of leaked cell went out through the PU-6 coat. However, the PU-6 still could keep high concentration of the cells over 30 days. This, in turn, means that diffusion rates of the substrates and the antibody were fairly high.

Calculated from the data shown in Figs. 1, 3—5, the volumetric productivity of the antibody in the fluidized bed reactor was 0.8 mg/(cm^3·d) and this was more than 80-fold of that obtained with Petridishes. If a reactor volume of 1 m^3 is used, 0.8 kg of the antibody will be produced everyday. Furthermore, the high level production could be maintained more than 30 days. Therefore, this immobilization method is one of the promising way for effective monoclonal antibody production.

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