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저혈청농축배지에서 세포성장 및 간염표면항원에 대한 단일클론항체 생산의 증가

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Enhancement of Hybridoma Cell Growth and Anti-Hepatitis B Surface Antigen Monoclonal Antibody Production in Enriched Media with Low Serum

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ABSTRACT

Enhancement of hybridoma cell growth and monoclonal antibody(MAb) production by the addition of a small amount of serum into both serum-free medium and enriched medium was studied. The enriched medium was constructed by mixing a basal serum-free medium and a nutrient-fortified RPMI 1640 medium. It was supplemented with human serum albumin, insulin, transferrin, and monoethanolamine. It was found that addition of low concentration of serum with other serum-free supplements was favorable for growth of a mouse hybridoma 2c3.1 cells. The concentration of serum was determined to 0.5%. The maximum cell concentration obtained in this enriched medium supplemented with 0.5% fetal bovine serum (FBS) was 3.06×10^6 cells / ml and the concentration of secreted anti-Hepatitis surface antigen (anti-HBsAg) MAb was 159.7㎍ / ml compared to 43 ㎍ / ml in RPMI 1640 medium with 10% FBS and 50㎍ / ml in previously-developed serum-free medium. The 2c3.1 cell growth and MAb production could be enhanced considerably by using the enriched medium supplemented with 0.5% FBS and serum-free supplements instead of RPMI 1640 medium or serum-free medium. The enhancement in MAb production in the enriched medium was more noticeable.

INTRODUCTION

Mammalian cells secrete a variety of proteins of biomedical interest such as monoclonal antibodies, interferons, interleukins, tissue plasminogen activators, and growth factors. For the economical production of these proteins, large scale cultivations of mammalian cells have been studied by using various cell culture techniques. In the large scale production of monoclonal antibodies to be utilized in diagnostics and clinical therapeutic applications, construction of serum-free media is critical in considering yield, cost, quality, and purification. In spite of the disadvantages of serum-free media including difficulties in optimization of serum free supplements for each cell line and slower cell growh than that in serum-supplemented medium, many attempts have been made to increase the production of monoclonal antibodies and other important products using serum free media (1-3).

In addition, in order to enhance cell growth and monoclonal antibody production in serum-free medium, various techniques were adopted. Those are further optimization of serum-free medium, feeding of serum or nutrients during the cultivation, utilization of continuous culture method, immobilization, etc(4-9).

In our institute, a serum-free medium for the general cultivation of mammalian cell lines was constructed and applied in the cultivation of mouse hybridoma U3-2-1 cells producing anti-urokinase monoclonal antibody. The medium was found to support logarithmic cell growth without serum. For the application of the serum-free medium to other hybridoma cell lines, we examined in the medium the growth of 2c3.1 hybridoma cells, which produce anti-HBsAg monoclonal antibody. The cells also propagated logarithmically without lag period. On top of that, the maximum concentration of anti-HBsAg MAb produced by 2c3.1 cells was slightly higher in the serum-free medium than that in 10% FBS medium. Although the serum-free medium was enough to substitute 10% FBS medium in respect of MAb production, it was not adequate for high density culture.

In this study, a possibility of enhancement in hybridoma cell growth and monoclonal antibody production by the addition of a small amount of serum into both serum-free medium and enriched medium was studied. Effects of serum-free supplements, inoculation density, and serum types on the cell growth and monoclonal antibody production are also presented.

MATERIALS AND METHODS

Media and chemicals

Human serum albumin(Albumin®) and fat emulsion (Intralipos®) were kindly provided by Korea Green Cross Corporation. The Intralipos® consists of 100 mg/ml of purified soybean oil, 12 mg/ml of purified ovolecithin, and 25 mg/ml of glycerin. Insulin, transferrin, monoethanolamine, glutaminase, and glutamate dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO). RPMI 1640 and Ham's F12 medium were bought from Gibco Laboratories (Grand Island, NY). Fetal bovine serum and supplemented bovine calf serum (sBCS) were supplied from Hyclone Laboratories (Logan, Utah).

Cell line

A mouse-mouse hybridoma cell line, 2c3.1, was used. The cell line had been cloned in our institute by the fusion of CRL 1580 myeloma cells(P3 63. Ag. 8(V653)) to the spleen cells of Balb c mouse immunized against purified human HBsAg (200 μg/ml, KGCC). The hybridoma produces anti-HBsAg monoclonal antibody(IgG1). The cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum at 37℃ in humidified atmosphere with 5% CO_2.

Serum-free cell culture

Serum-free medium was a 5:1(v/v) mixture of RPMI 1640 and Ham's F12 medium supplemented with 4.5 mg/ml of human serum albumin, 10 μg/ml of insulin, 10 μg/ml of transferrin, 0.17 μg/ml of fat emulsion, 0.01 μg/ml of vitamin E, 0.02 μg/ml of vitamin E acetate, and 10 μM of monoethanolamine. The stock emulsion (IE emulsion) containing Intralipos®, vitamin E and vitamin E acetate was prepared and diluted for use in serum-free culture. The 2c3.1 cells grown in RPMI 1640 medium supplemented with 5% FBS were harvested by low speed centrifugation (1000 rpm, 5 min) when they were in the late exponential growth phase and washed once with serum-free medium. The cells were allowed to settle without stirring for initial 3 h and then inoculated at the concentration of 1×10^5 cells/ml in 250 ml of spinner flasks (Bellco) containing 100ml of medium. The flasks were placed in a CO_2 incubator and were stirred at 40 rpm.

Construction of enriched medium and cell culture

An enriched medium was a 2:1(v/v) mixture of both basal serum-free medium (a 5:1 mixture of RPMI 1640 and Ham's F12 medium, v/v) and a fortified RPMI 1640 medium. The fortified medium consisted of five times richer concentrations of glutathione, amino acids, and vitamins than those of original RPMI 1640 medium, and two and a half times richer concentration of glucose. No changes in the concentrations of salts were made in enriched medium except sodium chloride. The enriched medium was supplemented with serum-free supplements (HITE; 2 mg/ml of human serum albumin, 5 μg/ml of insulin, 5 μg/ml of transferrin, and 10 μM of monoethanolamine). The osmolality of the enriched medium was controlled to 270 mOs/kg water by lowering the sodium chloride concentration from 6 g/l to 4 g/l. Hybridoma 2c3.1 cells were cultivated with inoculation densities of 2.5×10^4 to 2×10^5 cells/ml in spinner flasks(Bellco) with 100ml of enriched medium supplemented with low concentration of serum.

Assay

The concentration of anti-HBsAg MAb in culture medium was determined by an enzyme-linked immunosorbent assay(ELISA) method of which standard protocol is as follows ; the standard anti-HBs was incubated in HBsAg-coated polystyrene strips at 37℃ for 2 h. Affinity-purified standard anti-HBs was diluted with PBS (pH 7.2) containing 1% bovine serum albumin to prepare standard solutions from 4.5 to 300 ml.U./ml. After the incubation for 2 h, the polystyrene strips were washed three times with 0.1% PBST and incubated again 37℃ for 1 h with 100 ng of HBsAg-HRP conjugate which had been diluted with normal human serum. Then they were washed five times with 0.1% PBST. The chromogenic substrate solutions were amounted to 0.1 ml / well at room temperature, followed by the addition of 0.1 ml stopping solution to each well 1 h later. Finally, color development in each well was monitored by a plate reader (EAR 30 0, SLT Lab.) at 405 nm. The standard anti-HBs used for the quantitation of produced MAb has been affinity--purified with supernatants obtained from mass culture of hybridoma 2c3.1 cells. Viable cells were counted by dye exclusion method using trypan blue.

RESULTS AND DISCUSSIONS

Effect of adding low concentrations of serum into serum-free medium

Hybridoma 2c3.1 cells inoculated with 1×10^5 cells / ml in serum free medium could not grow over 1×10^6 cells / ml. In order to examine the effects of serum addition on the cell growth rate and maximum cell concentration, sBCS was added at concentrations of 0, 0.25, 0.5, 1.0, 2.0% to the serum-free medium. As shown in Fig. 1. cultivations with addition of 0.25 to 2.0% sBCS with an inoculation concentration of 1×10^5 cells / ml caused an increase in their growth rate. The culture with 2% serum resulted in higher maximum cell concentration (2×10^6 cells / ml) than that in serum-free culture. It had been reported that the growth of human monocytic leukemia cell line, AH-01S, was increased with addition of a small amount of horse serum into serum-free medium(10).

In spite of the increase of cell growth with addition of low concentration of serum, the maximum cell concentration is not high enough and the cost of supplemented serum concentration is still high. In this context, in order to increase cell growth with less than 0.5% serum, we have constructed an enriched medium which was a mixture of both the basal serum-free medium and the nutrient-fortified RPMI 1640 medium with several serum-free supplements.

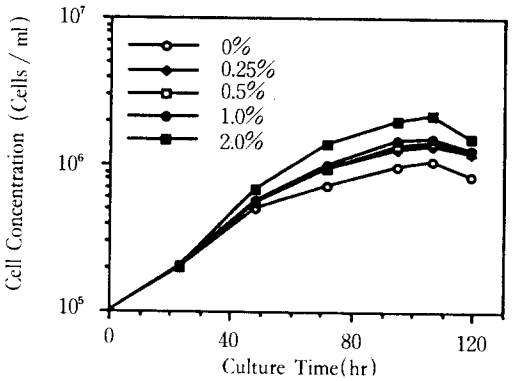


Fig. 1. Effect of sBCS on growth of 2c3.1 cells in serum-free medium.

Effect of serum-free supplements in enriched medium

In the preliminary experiments, the serum-free supplements-human serum albumin, insulin, transferrin, and monoethanolamine-were found to be essential for hybridoma 2c3.1 cell growth in serum-free medium. To investigate the effects of serum-free supplements in enriched medium, 2c3.1 cells were grown with an inoculum concentration of 2×10^5 cells / ml in enriched medium supplemented with as follows: 2 mg / ml of human serum albumin, 5 μg / ml of 'insulin and transferrin', and 10μM of monoethanolamine(HITE); human serum albumin(H); 'insulin and transferrin' (IT); monoethanolamine (E); human serum alnumin and, 'insulin and transferrin' (HIT); 'insulin and transferrin', and monoethanolamine (ITE). The results are shown in Fig 2. The concentration of fetal bovine serum was 0.5%. Control was the enriched medium without any serum-free supplements. The 2c3.1 cells in enriched medium supplemented with only one kind of serum free supplements (H, IT, E) hardly propagated. When two kinds of serum-free supplements (HIT, ITE) were added, cell growth rates became higher, but not high enough, and maximum cell concentrations were under 2×10^6 cells / ml. However, when the culture was replenished

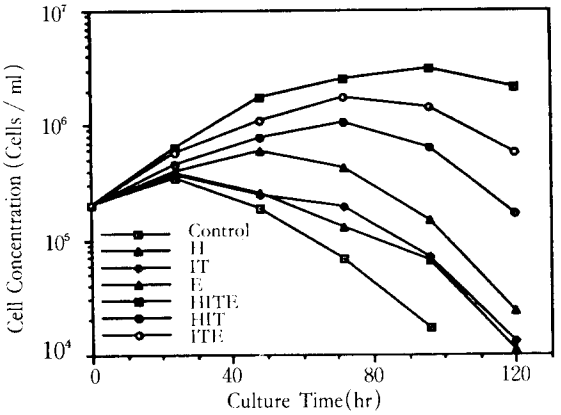


Fig. 2. Effect of serum-free supplements in enriched medium.

with all serum-free supplements (HITE), cells grew very well and 3×10^6 cells / ml of maximum cell concentration was obtained. These results indicated that serum-free supplements were unavoidably necessary for 2c3.1 cells in enriched medium with 0.5% serum to grow over 2×10^6 cells / ml which was known to be a maximun concentration obtainable in normal RPMI 1640 medium with 10% FBS.

Effect of serum concentration in enriched medium

For the purpose of testing a possibility of reducing serum concentration in enriched medium, 2c3.1 cells were cultivated with an inoculum concentration of 2×10^5 cells / ml in enriched medium containing serum-free supplements (human serum albumin, 'insulin and transferrin', and monoethanolamine). The concentations of FBS were 0, 0.1, 0.3, and 0.5%. As shown in Fig. 3, both cell growth rate and maximum cell density were increased as serum concentration was increased. The serum concentration of 0.5% was found to support the best cell growth and it was apparent that decrease of serum concentration below 0.5% was not desirable for this enriched medium.

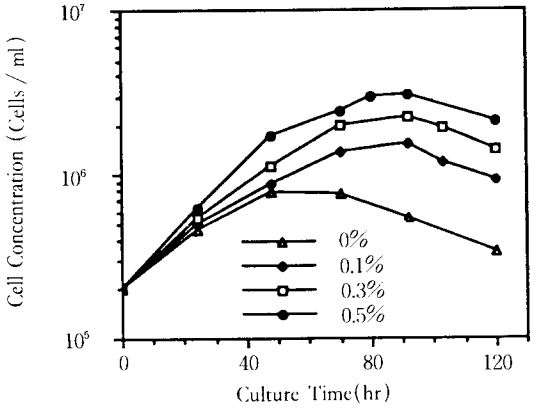


Fig. 3. Effect of serum concentration on growth of 2c3.1 cells in enriched medium.

Effect of inoculation density on growth of 2c3.1 cells

Hybridoma 2c3.1 cells were inoculated both in enriched medium with serum-free supplements(HITE) and 0.5% FBS, and in 10% FBS suppemented RPMI 1640 medium with inoculum concentrations of 2.5×10^4, 5×10^4, 1×10

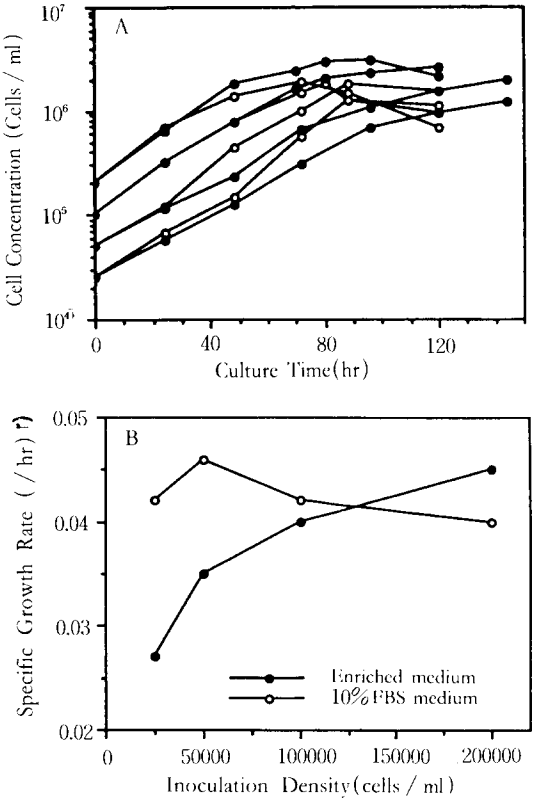
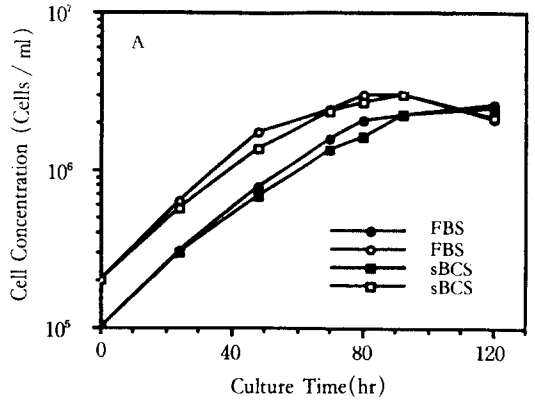


Fig. 4. Effect of inoculation density on growth of 2c3.1 cells in enriched medium (closed circle) and 10% FBS supplemented RPMI 1640 medium(open circle) :A) cell growth profiles; B) specific growth rates.

^{5}, 2×10^5 cells / ml in spinner flasks. As shown in Fig. 4, 2c3.1 cells in enriched medium propagated exponentially without lag period. In cultures with inoculum concentrations below 1×10^5 cells / ml, cell growth rate was generally slower in 0.5% FBS supplemented enriched medium than that in 10% FBS suppmnted RPMI 1640 medium. However, when the inoculum concentration was 2×10^5 cells / ml, the growth rate was much faster and the maximum cell concentration was 3.06×10^6 cells / ml. These results indicated that we could increase both cell growth rate and maximum cell concentration if we cultivated the cells with an inoculum concentration above 1×10^5 cells / ml in enriched medium, probably due to the enrichment of the basal components, compared to those in RPMI 1640 basal medium.

Effect of serum types on cell growth and MAb production

The effect of serum type on growth of 2c3.1 cells in enriched medium was observed. The inoculum concentrations were 1×10^5 and 2×10^5 cells / ml. FBS and supplemented bovine calf serum(sBCS) were examined with a concentration of 0.5%. Since FBS is much more expensive than sBCS, substitution of FBS by sBCS for commercial utilization is desired. The cell growth results from enriched media supplemented with 0.5% FBS and with 0.5% sBCS were similar as shown in Fig. 5A. However, as shown in Fig. 5B, anti-HBsAg monoclonal antibody production in 0.5% FBS supplemented medium was much higher than that in 0.5% sBCS medium.The maximum concentration of MAb produced in 0.5% FBS medium was 159.7 ㎍ / ml compared to 90.7 ㎍ / ml in 0.5% sBCS medium when the inoculum concentration was 2×10^5 cells / ml. In the case of cultures with an inoculum concentration of 1×10^5 cells / ml, 113.2 ㎍ / ml of MAb was produces in 0.5% FBS medium compared to 62.5 ㎍ / ml, in 0.5% sBCS medium. Therefore, substitution of FBS by sBCS was not recommendable despite of its good support in cell growth. The reason for this difference in specific MAb production is not clear.



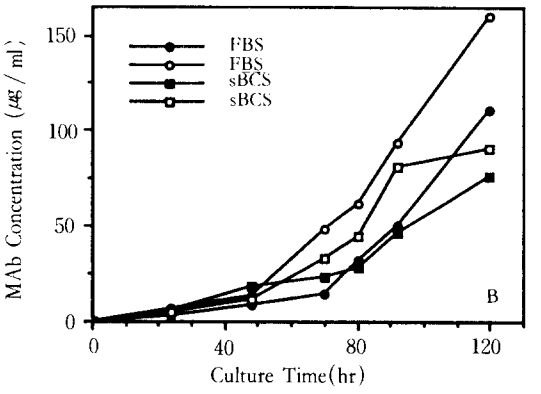


Fig. 5. Growth and monoclonal antibody production of 2c3.1 cells in enriched medium supplemented with different serum types; A) Cell growth; B) Monoclonal antibody production. Open circle and squre represent inoculation concentration of 2×10^5 cells / ml and closed circle and squre of 1×10^5 cells / ml.

Summary on growth and MAb production in various media

For the purpose of comparing and summarizing the effects of various media on the 2c3.1 cell growth and MAb production, the following media were examined together. Those were enriched medium with 0.5% FBS, enriched medium with 0.5% sBCS, RPMI 1640 medium with 10 % FBS, RPMI 1640 medium with 0.5% FBS, serum-free medium, and enriched serum-free medium. Time course profiles are illustratedin Fig. 6 and all the results were summarized in Table 1. All the experiments were done in triplicate and the results were reproducible.

In enriched medium, as shown before, FBS supported better MAb production in spite of the similar cell growth. The cells in enriched medium with 0.5% FBS grew up to 3.06×10^6 cells / ml which was much higher than those obtained in serum-free medium and RPMI 1640 medium with 10% FBS. The production of anti-HBsAg MAb in enriched medium with 0.5% FBS was also the best among in the media tested(159.7 ㎍ / ml compared to 50 ㎍ / ml

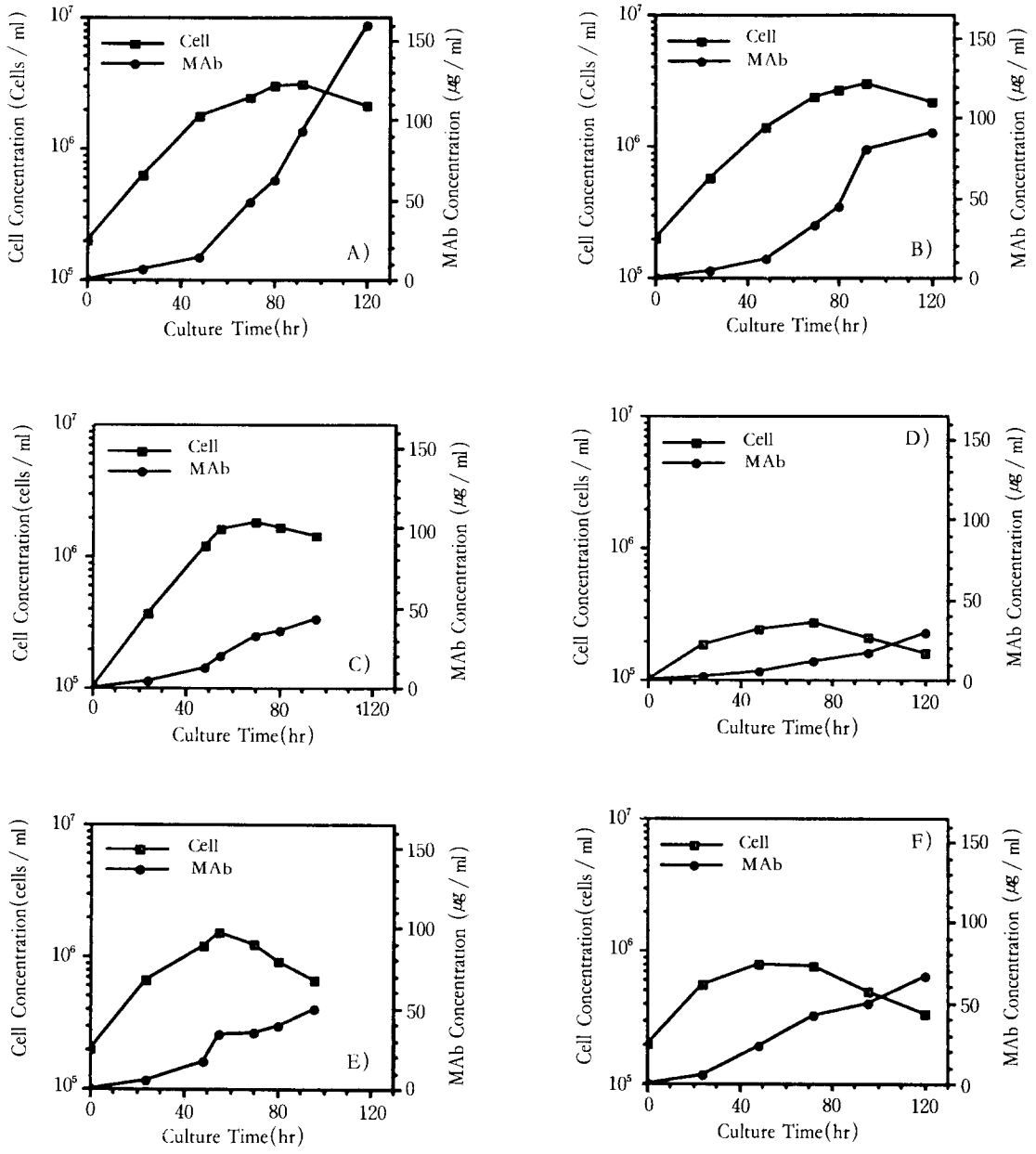


Fig. 6. Growth and MAb production of 2c3.1 cells at various media: A) enriched medium with 0.5% FBS: B) enriched medium with 0.5% sBCS: C) RPMI 1640 medium with 10% FBS: D) RPMI 1640 medium with 0.5% FBS: E) serum-free medium: F) enriched medium without serum.

in serum-free medium and 43 μg / ml in RPMI 1640 medium with 10% FBS).

Although cell growth in enriched medium without serum was almost one half of that in serum-free medium, secreted MAb concentration in enriched medium without serum (65 μg / ml) was higher than hat in serum-free medium

Table 1. Cell growth and MAb production characteristics of hybridoma 2c3.1 cells at various medium

Culture Medium	Cell Growth	Monoclonal Antibody Production	
	Specific Growth rate (1 / hr)	Maximum Cell Conc. (10^5cells / ml)	Maximum MAb Conc. (㎍ / ml)
RPMI 1640			
10% FBS	0.046	18.9	43.0
0.5% FBS	0.008	2.7	26.0
Serum-Free Enriched	0.036	15.4	50.0
0.5% FBS	0.038	30.6	159.7
0.5% sBCS	0.037	30.0	90.7
no serum	0.029	7.9	65.0

(50 ㎍ / ml). These results strongly suggested that 2c3.1 cell growth and MAb production in enriched medium were enhanced significantly compared to those in RPMI 1640 medium and in serum-free medium. The enhancement in MAb production was more noticeable.

In conclusion, cell growth and MAb production could be enhanced considerably by using our enriched medium supplemented with 0.5% FBS and serum-free supplements instead of RPMI 1640 medium or serum-free medium previously developed in our institute. For low serum supplementation, FBS was found to be better than sBCS in enhancing MAb production. By using this enriched medium with supplementation of low serum, it is possible to reduce the cost of medium in monoclonal antibody production.

요 약

본 논문에서는 10% fetal bovine serum(FBS)를 첨가한 RPMI 1640배지에 비하여 간염바이러스 표면항원에 대한 단일클론항체 생산에 있어서 효과적인 무혈청 배지에 낮은 농도의 혈청을 첨가하여 하이브리도마 2c3.1 세포에서 그 효과를 조사하였다. 세포성장과 단일클론항체 생산을 증가시키기위하여 기본 무혈청배지와 RPMI 1640 배지성분들의 농도를 균형있게 강화시킨 배지를 2:1 (v / v)로 혼합하여 농축배지를 조성하고, 이 배지에 2 mg / ml 인혈청 알부민, 5 ㎍ / ml insulin, 5 ㎍ / ml transferrin, 10 ㎛M monoethanolmine 등의 몇가지 무혈청 첨가물들을 첨가하였다. 이 농축 배지에 fetal bovine serum (FBS)과 supplemented bovine calf serum(sBCS)을 첨가하였을때의 세포성장과 단일클론항체 생산을 비교하여 FBS농도를 변화하여 세포성장과 단일클론항체생산의 증가를 시도하였다. 0.5% FBS를 농축배지에 첨가함으로써 세포성장과 단일클론항체생산의 증가를 보았다. 최대 세포 농도는 3.06×10^6 cells / ml이었으며, 이때 생산된 단일클론항체는 10% FBS배지의 43.0 ㎍ / ml과 무혈청배지의 50 ㎍ / ml보다 3배이상 높은 159.7 ㎍ / ml이 생산되었다.

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