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Pulse-Feeding of Serum Free Media for Enhancing Monoclonal Antibody Production under Perfusion Operation

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연속배양에서 단일항체 생산성 향상을 위한 무혈청 배지의 단계적 유입

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Lectin related inducer can enhance IgG_1 production rate from murine hybridoma cells by employing step-feeding of serum free media with producing about 40 mg/*l* of monoclonal antibodies. This step-feeding perfusion process also proves to be able to cutivate animal cells when serum free media can not support the growth of these cells in perfusion process, as well as to improve production rate. This process yields about 28×10^{-10} mg of MAb/cells/h compared to 11.1×10^{-10} mg/cells/h and 4.0×10^{-11} mg/cells/h for perfusion process and batch cultivation with 10% serum containing media, respectively.

It has intensively been studied to cultivate mammalian cells (normal and transformed cells, recombinants and hybridomas) *in vitro* to produce pharmaceutically important proteins from laboratory and plant scale growth systems (1-7). And many efforts have been made to grow animal cells without serum in culture media since fetal bovine serum is a major portion consisting of production costs for animal cell drived products (7-10). Even though many kinds of serum-free media have been developed for various cell lines, certain cell lines should require serum for proliferation and MAb production (11). For example, several hybridoma cells require serum to grow because it is essential to keep high cell density in secreting economic amounts of monoclonal antibodies (MAbs) (12, 13).

Therefore, it is necessary to develop a process for serum-requiring cells to grow and produce MAbs in chemically defined media. In this work a culture process is to be introduced for producing MAbs from a very fragile hybridoma which absolutely requires serum for the growth and MAb production. It will result in significantly lowering production costs for MAbs from animal cells.

Key words: Pulse-feeding, serum free media, perfusion operation
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Materials and Methods

Cells and culture conditions

Murine hybridoma 3L21 was produced by fusing P3-NS-1 myeloma cells with spleen cells (14), which produces IgG_1 that reacts with stimulatory factor, interleukin-4. Cells were grown in RPMI 1640 (Sigma, USA) enriched with 5 mM of glutamine and 10% fetal bovine serum (GIBCO, USA) in a 75 T-flask at 37°C CO_2 incubator. When cell density was reached to 1×10^6 viable cells/m*l* by changing fresh media every two or three days, cells were transferred to a 2*l* bioreactor (Celligen, NBS, USA) for batch and perfusion experiments. For serum free media, basal medium was the mixture of RPMI 1640, DME

and F12 supplemented with 5 mM HEPES (2:1:1, w/v) (Sigma, USA) and enriched with 4 μg/m*l* bovine serum albumin, 10 μg/m*l* Insulin, 9 μg/m*l* human transferrin, 1×10^{-9} M selenite, 1×10^{-5} M 2-aminoethanol, 1.8g/*l* sodium bicarbonate and 40 μg/m*l* gentamicin (Sigma, USA). 4 μg/m*l* of Leucoagglutinin PHA-L from Phaeoulus vulgaris red kindney bean was used for inducing IgG_1 production in serum free medium (15). Cell density and viability were measured every day by 0.4% trypan blue dye exclusion method (16).

Batch and perfusion cultivations

For batch operation cells from a 75 T-flask were maintained in a 2*l* bioreactor without perfusing media at 50 rpm of agitation and 0.5 *l*/min of sterilized air enriched with 5% CO_2 through the center shaft. In serum free experiment, cells grown in serum containing media were directly transferred into serum free media in a bioreactor and cell density, viability and IgG production were measured every day. Quantitative measurement of IgG_1 in spent media was analyzed by commercially available ELISA kit (Tech-4, Genzyme, USA).

Rates of perfusing both serum containing and serum free media were changed according to values of cell density in the reactor. Media were perfused through a decanting system in a 2 *l* bioreactor (Celligen, NBS, USA, patent #4,680,267). This perfusing filter is to recyle cells into the reactor by a membrane vertically located on the top of the vessel. Dilution rate was changed only when the system was stabilized (when changes of cell concentration were relatively stable, for example it took about nine days to stabilize the cell growth at the dilution rate of 0.02 (1/h)). Detail kinetic data are not shown in this report. Serum free media was slowly fed into the reactor where serum containing media was already perfused. Flow rates of serum free media were adjusted depending on values of cell density, that is, when the cell growth was decreased perfusion rate of serum free media was also decreased. Whenever cell density in the reactor was reached to low level (about 5×10^4 viable cells/m*l*), serum free media were switched to serum containing media and maintained with same perfusion rate for at least two days to let the system be stabilized. And then pulse-feeding was started again by gradually increasing perfusing rates of serum free media. Specific production rate of

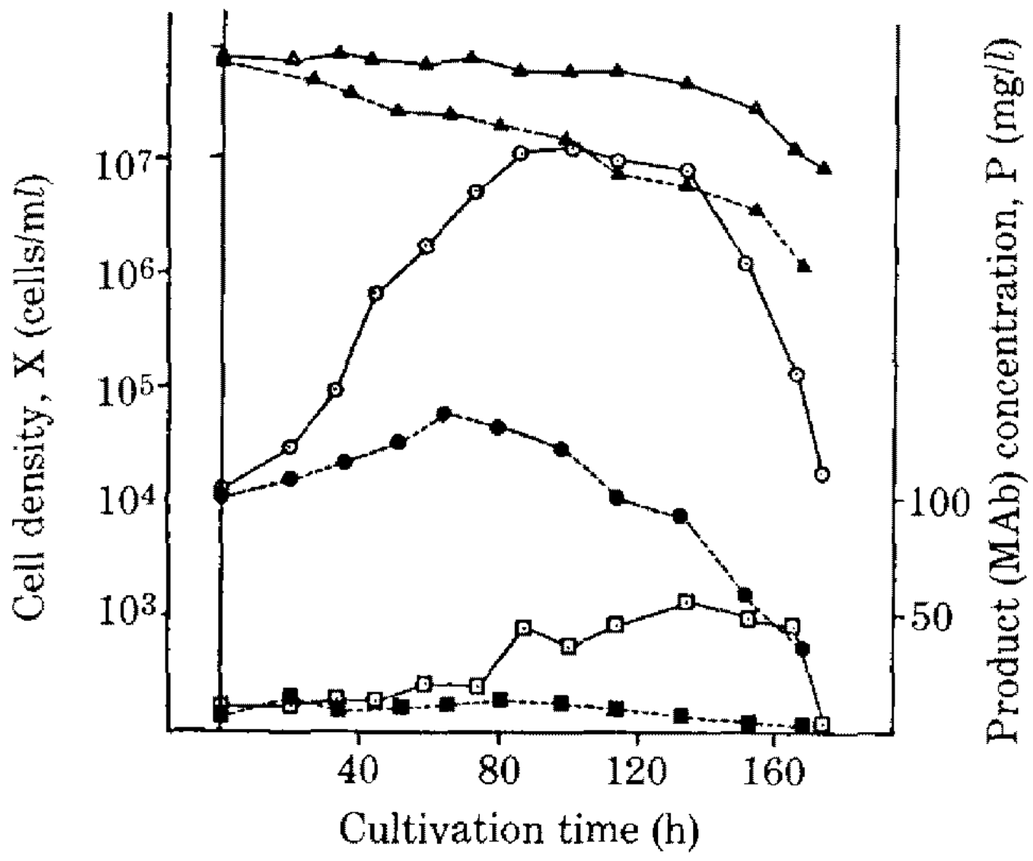

Fig. 1. Growth behavior of murine hybridoma 3L21 and product production on serum containing and serum free media under batch cultivation.
Solid line and open are results from serum-containing media and dotted line and dark from serum-free media: ⊙and ●, cell density (cells/m*l*); ⊡ and ■, product conc. (mg/*l*); ▵ and ▲, cell viability (%).

MAb was estimated as follows:

$$Q_p=\frac{P\times D}{X} \quad (1)$$

where Q_p is specific product formation rate (mg/cells/h), P is product concentration in the product stream (mg/*l*), D is media perfusion rate (1/h) and X is cell density (cells/m*l*).

Results and Discussion

Fig. 1 is the result of comparing cell growth of murine hybridoma 3L21 and MAb production from serum containing and serum free media under batch cultivation. While cell growth and viability were sharply decreased after five day cultivation for both serum containing and free media, MAb production was gradually increased in serum containing media. Maximum cell densities in serum containing and serum free media were estimated as 1×10^7 cells/m*l* and 8×10^5 cells/m*l*, respectively; however, maximum MAb production was obtained as 48 mg/*l* and 17 mg/*l* for serum-containing and serum free media, respectively after five day cultivations. It implies that the maximal production of MAb is not necessary to keep maximum cell growth because partially cells become leaky at lengthened stationary phase and/or proper environmental stresses (17). Fig. 2 is to com-

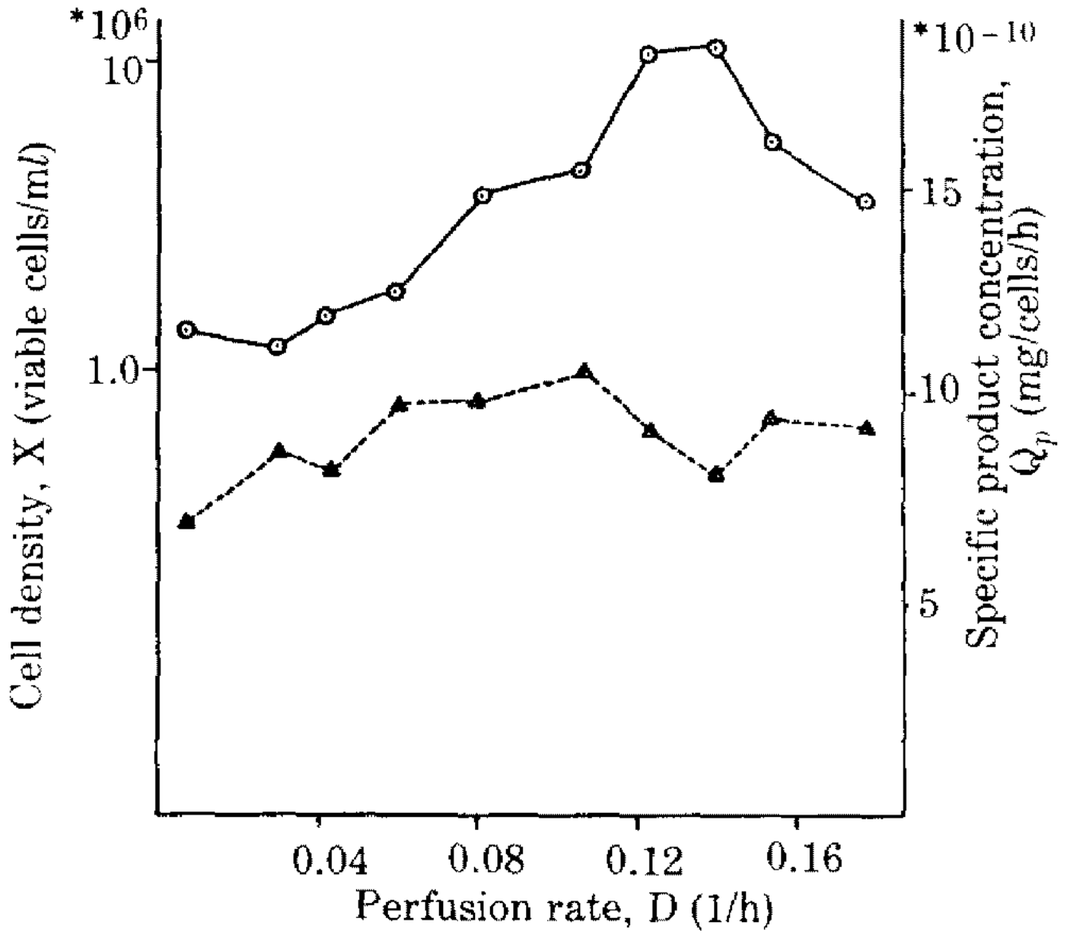

Fig. 2. Cell growth and specific product formation rate on serum containing media under perfusion chemostat operation.

⊙and —, cell density (viable cells/m*l*); △and- - -, specific product formation rate (mg/cells/h).

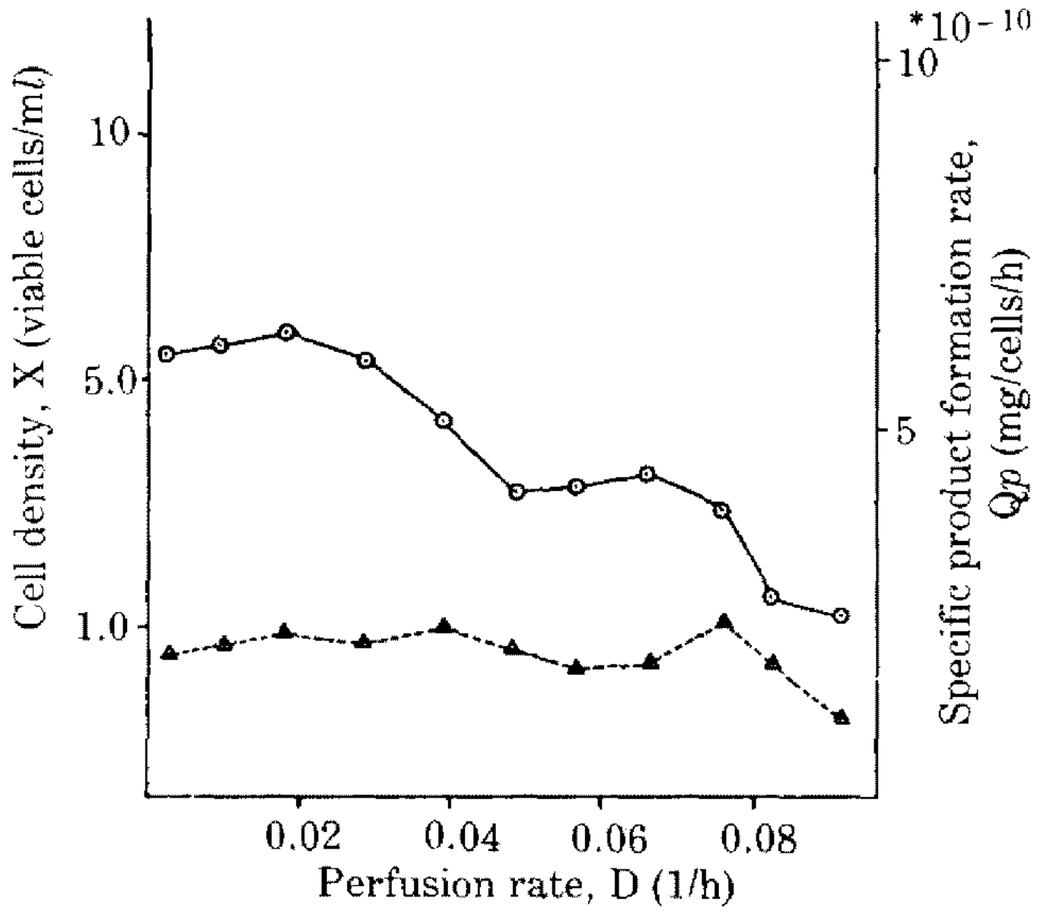

Fig. 3. Cell density and MAb production from murine hybridoma 3L21 grown on serum-free media in perfusion cultivation.

⊙ and —, cell density (viable cells/m*l*); △ and- - -, specific product production rate (mg/cells/h).

pare cell growth and productivity with results from batch operation, Fig. 1 for same cell line. In perfusion cultivation cell density was relatively increased as perfusion rate was increased up to 0.14 (1/h). Higher specific production rate was also obtained than in batch cultivation; 11.1×10^{-10} vs. 4.2×10^{-11}(mg/cells/h) for perfusion and batch operation, respectivley. Specific MAb production rate was calculated by values of cell density and cultivation period corresponding to maximum MAb production. 58.33 mg/*l* of maximum production of MAb was achieved at 0.107 (1/h) of dilution rate, compared with maximum value of 48 (mg/*l*) from batch cultivation in Fig. 1.

Fig. 3 is to check if murine hybridoma 3L21 can grow and produce MAbs in serum free media under perfusion chemostat condition for a long cultivation. Cell density was sharply decreased as dilution rate was increased in Fig. 3. It tells that this cell line can not be maintained in serum free media for perfusion cultivation, compared to cell growth from batch cultivation with same serum free media in Fig. 1. Specific product production rate was also low due to very low production of MAb in perfusion operation, for example, 1-7 mg/*l* of MAb production at overall ranges of dilution rates comparing to about 40 mg/*l* in Fig. 1. Both perfusion cultivations in Fig. 2 and 3 were operated for about 30 to 35 days.

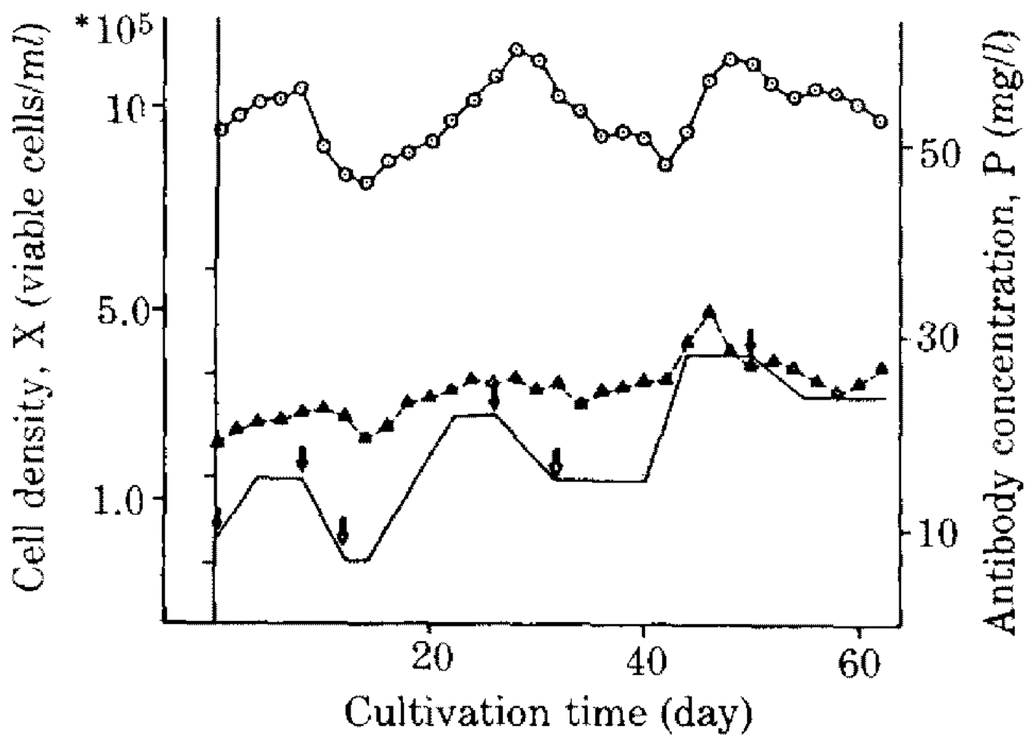

Fig. 4. The effect of stepwise feeding serum free media on cell growth and antibody production under perfusion chemostat operation.

⊙ and —, cell density (cells/m*l*); △ and - - -, MAb concentration (mg/*l*).
Solid line is the perfusion rate (1/h). Open arrows indicate to start feeding serum free media and dark arrows to restart feeding serum containing media.

Therefore, it is necessary to develop a process to maintain both cell growth and product production for this cell line in perfusion cultivation by pulse-feeding of serum free media, as shown in Fig. 4. Because perfusion process is very economic system in producing animal cell culture products compared with conventional batch processes (18). This culture system was started with serum-containing media and

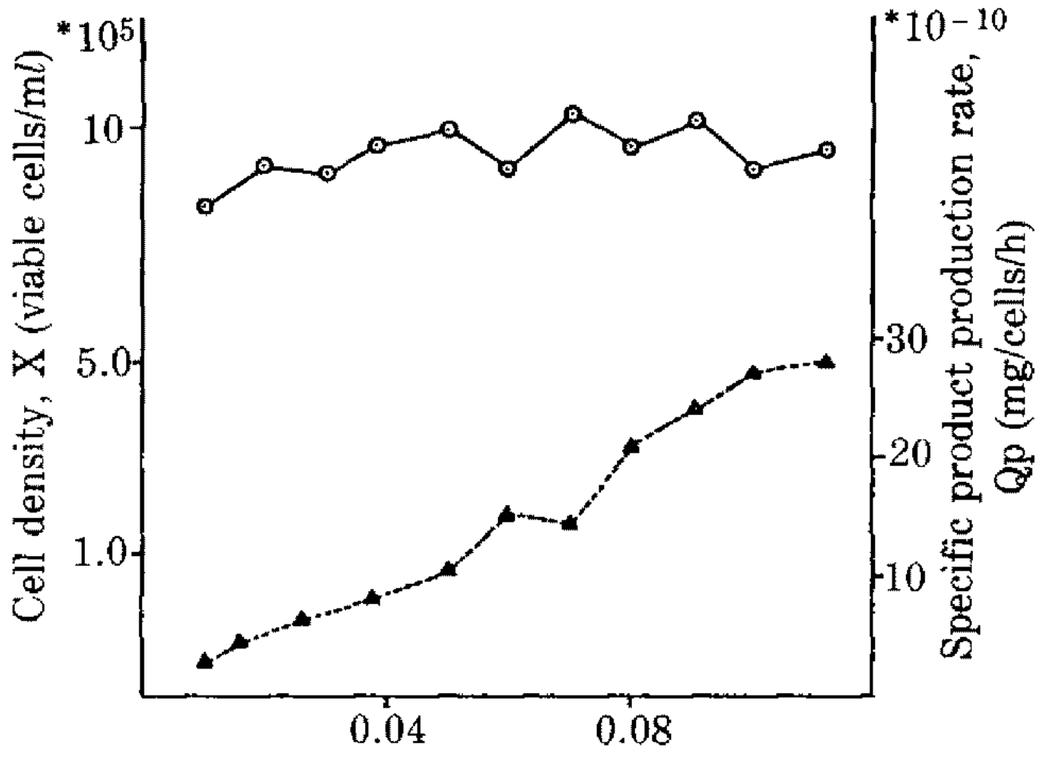

Fig. 5. Cell concentration and specific MAb production rate by step feeding of serum free medium for the growth of murine hybridoma in perfusion chemostat system from Fig. 4.

⊙ and —, cell density (cells/m*l*); △ and ···, specific product formation rate (mg/cells/h).

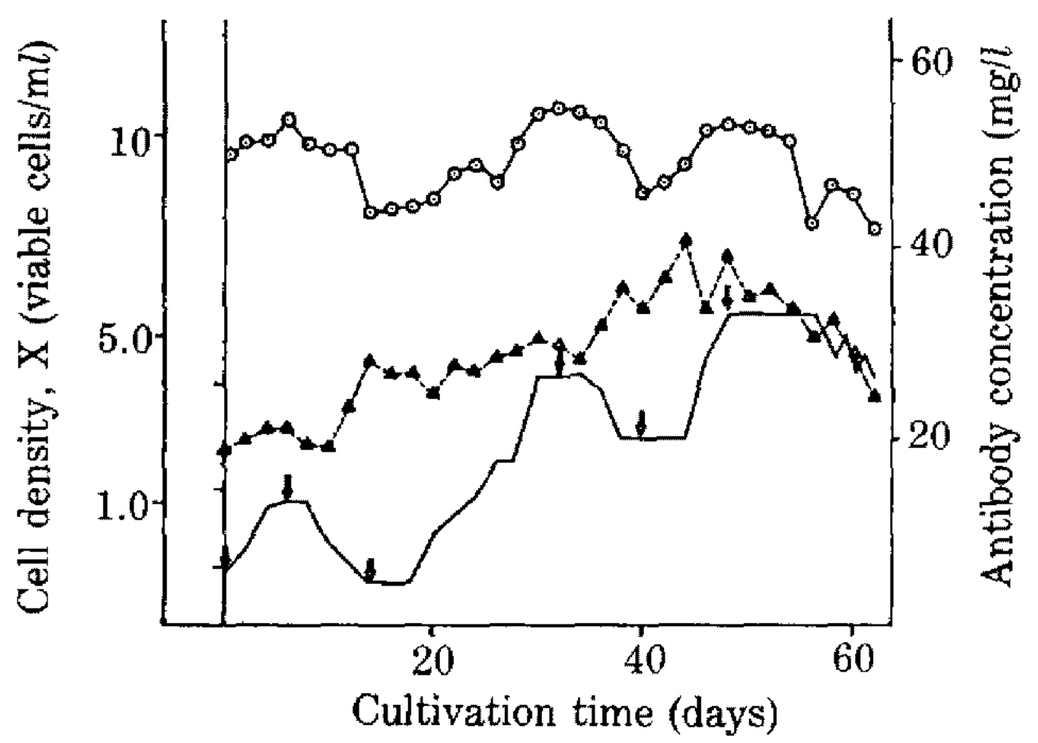

Fig. 6. Cell growth and MAb production by adding a production stimulus, PHA cultivation.

⊙ and —, cell density (cells/m*l*); △ and ····, product conc. (mg/*l*. Solid line is perfusion rate (1/h). Open arrows denote the points to start feeding serum free media containing 4 μg/m*l* of PHA and dark arrows to change serum containing media.

changed into serum free media. Serum free media were perfused when the system was stabilized at constant dilution rates to let cells be adjusted, and perfusion rate was gradually reduced according to cell density. Then, serum free media was switched to serum-containing media when the system maintained lower cell densities. While cell density was directly influenced by perfusing serum free media, MAbs were continuously produced for up to 60 days, which is absolutely longer than batch (for seven days) and only serum-free perfusion cultivations (for 30 days). Specific production rate for pulse-feeding process was increased up to 28×10^{-10} (mg/cells/h) in Fig. 5, which is better than perfusion system with serum-containing media. Because it was possible to maintain high dilution rates at low cell density even though product concentration was not so high as serum-containing system in Fig. 2. Specific production rate was gradually increased as perfusion rate was increased due to relatively constant production of MAbs in increased dilution rates.

Fig. 6 is to illustrate a possibility of increasing secretion of MAb in step-feeding process of serum free media by adding an inducer which possibly enhances release of MAbs by affecting on the cell wall (8). In adding an lectin related inducer, production of MAb was remarkably increased up to 42 (mg/*l*) from 32 (mg/*l*) even though cell density was sharply dropped compared with the results of Fig. 4, pulse-feeding of only serum free media. It also needed much time to let inducer treated cells grow up after being perfused by stimulus-containing media (about 20 days in Fig. 6) than that by only serum-free media (about 7-9 days in Fig. 4). At higher than 0.11 (1/h) of perfusion rate, cells did not grow and produce products possibly due to the closeness of wash-out point and damages of the cell wall by long exposure of an inducer, even though lectin-containing media were not perfused any more.

Conclusion

A process has been developed for culturing hybridoma cells, which can not be grown in serum free media under perfusion chemostat operation, by step-feeding of chemically defined media along with serum containing media. This process can also enhance MAb production rate compared to simple perfusion process and lengthen cultivation period, up to 60 days from 40-50 days for typical perfusion system. It turns out that maximum production of MAbs was achieved as about 48 (mg/*l*) at stationary phase in batch cultivation with serum containing media. An inducer can improve MAb production by employing pulse-feeding of serum free media in relatively low cell density.

요 약

Murine hybridoma 세포배양시 lectin 계통의 생산 증진제가 무혈청 배지의 단계적 유입에 의해 약 40 mg/*l* 의 단일항체를 생산함으로서 단일항체의 생산성을 증가시킬 수 있음이 확인됐으며, 또한 이 배양공법으로 무혈청 배지에서 연속배양이 불가능한 세포의 배양이 가능했다. 이같은 무혈청 배지의 단계적 유입공법으로 약 28×10^{-10} mg/cells/h 의 속도로 단일항체가 생산된 것에 비해, 10% 혈청이 포함된 배지로는 연속배양시 11.1×10^{-10} mg/cells/h 의 속도로 생산됐으며 회분배양의 경우는 4.0×10^{-11} mg/cells/h 의 속도로 생산됐다.

References

1. Wieman, K.B., McCarthy, B. Rewick and J. Hopkins: *In vitro,* **19,** 260 (1983).
2. Tolbert, W. and J. Feder: *Ann. Rep. Fermt. Proc.,* **6,** 35 (1983).
3. Lee, H.Y. and J. Kang: *Kor. J. Chem. Eng.,* **27,** 286 (1989).
4. Jensen, M.D.: *Biotech. Bioeng.,* **23,** 2703 (1981).
5. Hu, W.S. and D.I Wang: *Biotech. Bioeng.,* **30,** 548 (1987).
6. Kang, J., H. Park and H.Y. Lee: *Kor. J. Biotech. Bioeng.,* **4,** 104 (1989)
7. Lee, H.Y.: *Kor. J. Appl. Microbiol. Bioeng.,* **16,** 266 (1988).
8. Cleveland, W.L., I. Wood and B.F. Erlanger: *J. Immunol. Methods,* **56,** 221 (1983).
9. Ham, R.G., G. Fisher and R.J. Wieser (eds.): *Hormonally Defined Media,* pp.16, Springer-Verlag, Berlin (1983).
10. Maurer, H.R.: *Cell Tissue Kinetics,* **14,** 111 (1981).
11. Ratafia, M.: *Bio/Tech.,* **7,** 574 (1989).
12. Murakami, H., M. Masui, G.M. Sato and T.K. Sueoka: *Proc. Natl. Acad. Sci. USA,* **79,** 1158 (1982).
13. Murakami, H., M. Masui and G. Sato: *Cold Spring Harber Conf. Cell. Pro.,* **9,** 711 (1982).
14. Paul, W.E.: *Nature,* **315,** 333 (1985).
15. Goldstein, I. and C. Hayes: *Adv. Carbohyd. Chem. Biochem.,* **35,** 127 (1978).
16. Kalenbach, J.P., M.H. Kalenbach and W.B. Lyons: *Exp. Cell Res.,* **15,** 112 (1958).
17. Velaz, D., S. Reuveny, L. Miller and J.C. McMillan: *J. Immunol. Meth.,* **6,** 45 (1986).
18. Tolbert, W.R. and R. Srigley: *Biopharm.,* **2,** 149 (1987).

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