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MICROCARRIER CELL CULTURE AND ITS APPLICATION TO THE LARGE-SCALE PRODUCTION OF HUMAN FIBROBLAST INTERFERON

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Abstract — Animal cells are important in producing several products, but anchorage-dependency of most cell strains is one of the difficulties to get over. Microcarrier was utilized in this study in order to increase the surface area for cell-anchorage and also to improve other characteristics of the cell culture system.

First the critical parameters affecting the initial attachment were determined. The best plating efficiency was found at pH=7.4 and 5% FCS concentration. Use of the intermittent stirring during the initial phase of cell culture gave better cell plating than the continuous stirring.

Next the human fibroblast interferon was successfully produced from cells cultured on microcarrier and several advantages of using microcarrier were identified. Usually 10,000 units/ml of interferon was produced from microcarrier culture as compared to 6,000 units/ml in monolayer culture. FCS concentration in cell growth stage affected the yield of interferon and gave optimum results at 5%. Antibiotics did not influence the production of interferon significantly.

The highest sensitivity in interferon assay was obtained with Hep 2 cells as the target cells and more than 3×10^5 cells/ml were needed for good result. Microcarrier culture fixed onto confluent monolayer showed results as good as suspension microcarrier culture.

INTRODUCTION

Cell culture technique has been used for many years to produce important biological materials. Many kinds of cells require surface for growth. Thus animal cells traditionally have been cultivated on the surface of petri dishes or culture flask. The development of microcarrier technology has made it possible to culture economically large numbers of anchorage-dependent cells in a single vessel. Van Wezel [1] first reported on the advantage of culturing tissue cells in suspension instead of in monolayers and of using the suspension as a substrate for virus multiplication. Microcarrier culture consists of using positively charged microspheres which are kept in suspension by gentle agitation. This technique introduces new possibilities and for the first time makes high yield culture of anchorage-dependent cells practically possible. Giard et al. [2] found that these mocrocarrier-grown cells were suitable for the production of a variety of viruses. Levine et. al [4] suggested that the use of mircrocarrier reduced the time, expense and size of culture apparatus for anchorage-dependent cells. Interferon has been produced in high yield from microcarrier cultures [5,6] and the work by Giard and Fleischaker [3] on the production of human beta-interferon using microcarrier-grown fibroblast cells is another example. A wide variety of viruses can be produced by using microcarrier [7,8,9,10].

Vaccines produced in the microcarrier system include polio, rubella, rabies, influenza, and foot-and-mouth dissease vaccines. By using microcarrier it is possible to subculture cells or scale-up cultures without using proteolytic enzyme or chelating agents [11]. Fleischaker [12] fully described about optimal conditions for the microcarrier cell culture and the production of human fibroblast interferon. A major aspect of the mass production of human fibroblast interferon from microcarrier is to obtain growth and yield of competent cells prior to interferon induction.

The objective of this study is to examine mammalian cell growth on microcarriers and the subsequent formation of an induced product, human fibroblast interferon. To increase the yield of this product and mammalian cells, major parameters such as pH, initial plating efficiency, FCS concentration and others which could affect the yields were chosen to find out their optimum values.

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MATERIALS AND METHODS

Cell Culture

Human diploid foreskin cells, FS-4, were obtained from Mr. H.W. Park (Department of Nutrition and Food Science, MIT). Human dipoid lung fibroblast cells, MRC-5, were obtained from National Institute for Biological Standards and Control, England. Africa green monkey kidney cells, Vero, and human larynx carcinoma cells, Hep2, and human amnion cells, FL, were purchased from American Type Culture Collection (Rockvile, MD, USA). Vesicular stomatitis virus, Indiana serotype, was also kindly provided by Mr. H.W. Park.

All cell types were maintained in 75-cm^2 plastic tissue culture flasks (Costar Plastics, MA, USA). All cells were grown in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% FCS. Media and FCS were obtained from Flow Laboratories. Antibiotics used were pencillin (100 units/ml) and streptomycin (100 μg/ml). Cultures were split using a trypsin solution purchased from Flow Laboratories.

Materials

Microcarriers were obtained from Pharmacia Fine Chemicals under the trade name Cytodex. Human plasma protein was obtained from Korea Green Corporation. All other chemicals were purchased from Sigma Chemical Co. (Miss., USA). Cycloheximide was prepared as a stock solution (10mg/ml), sterilized by filtration (0.22 μm filter pad), and added to unsupplemented DEME for a final concentration of 60 ug/ml. Microcarrier spinner flask was purchased from Bellco Glass, Inc. (NJ, USA). Plastic wares were purchased from Costar and Falcon.

Cell Propagation on Microcarrier

The initiation of microcarrier cultures has been described elsewhere [13]. Microcarriers were suspended in PBS at a concentration of 10mg/ml and sterilized in glass bottles by autoclaving. These microcarriers were dispersed into spinner flasks containing growth medium to give a final concentration of 3mg/ml. All vessels which were contacted with microcarrier were siliconized before use [11]. Cells were inoculated into spinner flask containing microcarriers at various inoculation densities and allowed to grow for approximately 1 week. All cultures were grown at 37°C.The pH was initially adjusted to approximately 7.4 and CO_2 was supplied to the culture vessel.

Cell Count

The nuclei counting method of Sanford et al. [14] modified by van Wezel [15] was used for counting the number of cells on microcarrier. Fo the test of cell viability, trypan blue solution was used.

Instrumentation of Culture System

Instruments and apparatus used in microcarrier cell culture are shown in Fig. 1. For optimum culture condition, 5 l fermentor (Marubishi, Japan) was used to control the variables such as pH, temperature and DO concentration. Cultures were grown in 1 l or 250ml spinner flasks and medium conditions in the spinner were held constant by recirculation through this fermentor under direct control for various process variables. The recirculation flow rate between the fermentor vessel and the spinner flask was adjusted by peristaltic pump (Tokyo Rikakikai, Japan).

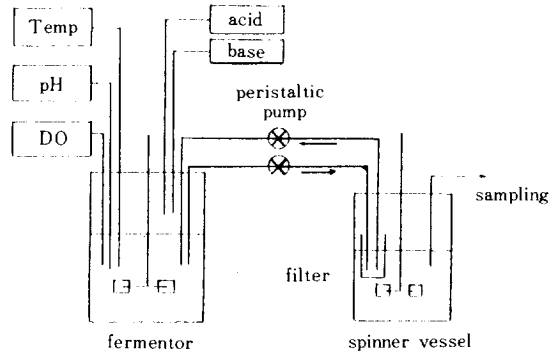

Fig. 1. Instrumentation of culture system.

Interferon Production

Modified method of superinduction reported by Havell and Vilcek [16] was used. On the seventh day, cells were washed three times with DEME without FCS and then primed at 37°C for 16 h in DEME containing 50 units/ml of human fibroblast interferon. After cells were washed twice in DEME, DEME containing 50 μg/ml of poly I:poly C and 10 μg/ml of cycloheximide were added. After 5 h of incubation at 34°C, actinomycin D was added to make 1 μg/ml. After 2h of further incubation at 34°C, the medium was removed and cells were washed two times in unsupplemented DEME. After addition of DEME containing 0.5% human plasma protein, cultures were incubated for 24 h at 37°C. Culture fluid was collected and frozen until assayed.

Interferon Assay

A modified procedure of the semimicro method by Amstrong [17] was used. The entire procedure, including dilutions, for the assay was performed in the 6mm wells of 96 well Micro Test II tissue culture plate. Each well was inoculated with $3x10^4$ Hep2 cells in 100 μl growth medium and the plate was incubated at 37°C and 5% CO_2 atmosphere. When confluent, the medium was decanted and each column was filled with 0.1ml of samples 3.2 fold serially diluted. After 24 h incubation, cells were challenged with 1,000 PFU per well of VSV. When 100% CPE was displayed in virus control, incubation was ended. After dye-fixing the cells, the optical densities of all wells were read by using Titertek

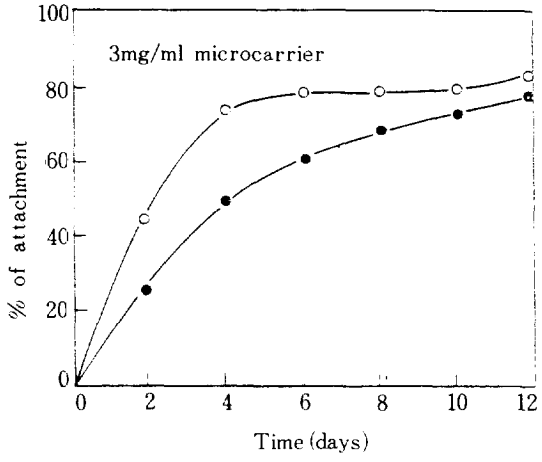

Fig. 2. Plating efficiency of Vero cells.
-●- : cultures stirred immediately after inoculation
-○- : cultures stirred intermittently (1min/every 60min)

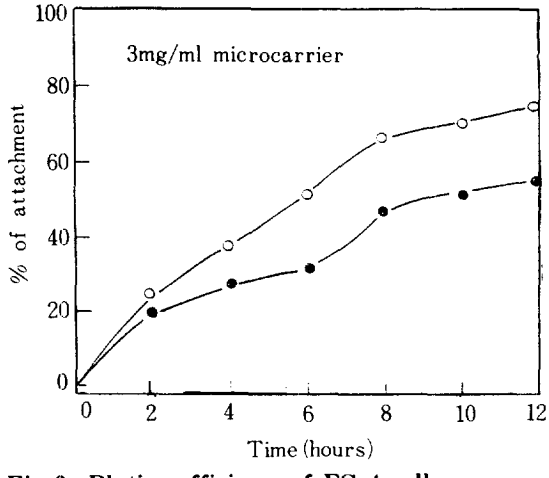

Fig. 3. Plating efficiency of FS-4 cells.
-●- : cultures stirred immediately after inoculation
-○- : cultures stirred intermittently (1min/every 60min)

multiscan (Flow Lab.) at 550nm.

RESULTS AND DISCUSSIONS

Plating efficiency

The effect of using different stirring methods was tested with the initial inoculation density of 1.2×10^5 cells/ml and the 3mg/ml microcarrier concentration. In the case of Vero cells used for VSV propagation, the result is shown in Fig. 2. Fig. 3 is for similar results with FS-4 cells. These curves indiate that twelve hours after inoculation, nearly all cells are attached to microcarriers and immediate stirring prevents the cells from initial attachment. By comparing Fig. 2 and Fig. 3, one can see that the rate of initial attachment varies with the type of cells used. Vero cells are faster than FS-4 cells in achieving attachment. It was found that for these two cell types, the plating efficiency could be improved by using a relatively small culture volume during the initial period of culture. The reduced initial volume equivalent to one third of the final volume gives cells a greater chance of coming into contact with microcarriers. Therefore plating efficiency could be improved by using a reduced initial culture volume and by stirring intermittently during the initial 12 hours period. The effect of pH on initial attachment is crucial. Plating efficiency after the first 8 hours of operation was checked at one of the selected pH values between 6.8 and 8.2 and the result is illustrated in Fig. 4.

The optimal pH for FS-4 cells was 7.4. Because pH varies widely at the beginning of culture, optimal pH should be maintained by using HEPES buffer, bicarbonate/CO_2 system or acid/base addition. Plating efficiency was also influenced by FCS concentration. As can be seen in Fig. 5, 5% FCS concentration was the best for the initial attachment of FS-4 cells. At FCS concentrations less than 5% the attachment and growth was not as good.

Cell Growth

The growth curves of the FS-4 cells in microcarrier culture and in monolayer culture are shown in Fig. 6.

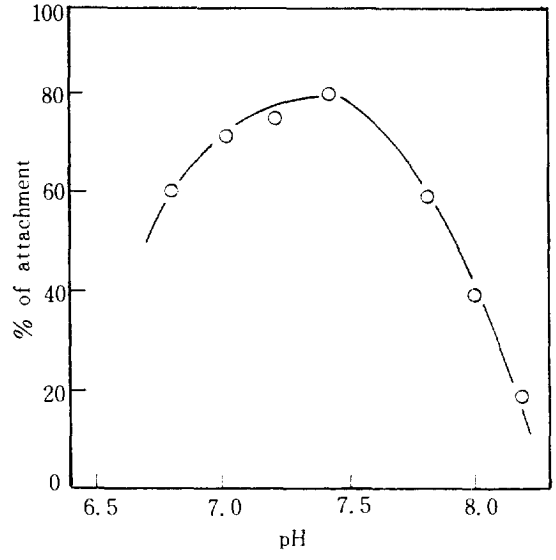

Fig. 4. Effect of pH on plating efficiency of Vero cell.

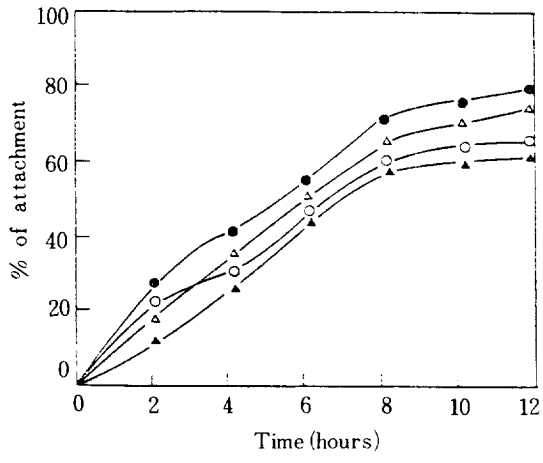

Fig. 5. Effect of serum concentration on plating efficiency of FS-4 culture.

-●-: 5% (V/V) serum concentration
-△-: 10% (V/V) serum concentration
-○-: 15% (V/V) serum concentration
-▲-: 20% (V/V) serum concentration

Results from similar experiment with Vero cell are shown in Fig. 7. In both experiments initial intermittent stirring for 12 hours in reduced culture volume was used. After the first twelve hours of operation, culture volume was adjusted to the final volume. The maximum cell concentration reached for FS-4 was $7x10^5$ cells/ml and that for Vero cells was $2.4x10^6$ cells/ml in the culture with microcarriers. In the case of monolayer culture both cells could be multiplied to about half of the

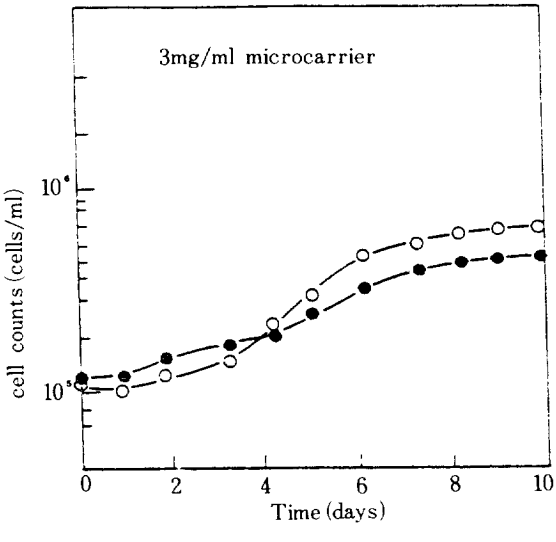

Fig. 6. Growth curve of FS-4 cells.

-○-: microcarrier culture
-●-: monolayer culture

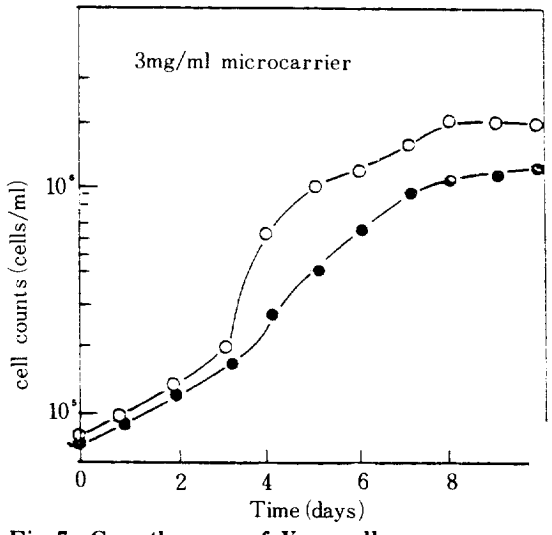

Fig. 7. Growth curve of Vero cells.

-○-: microcarrier culture
-●-: monolayer culture

final concentration attainable with the microcarri culture.

The effect of inoculation desity on the growth of Ve cells in microcarrier culture is shown in Fig. 8. The thr curves illustrate cultures inoculated with 0.6x1($0.8x10^5$, and $2x10^5$ cells/ml respectively. By increasi the inoculation density, final cell density could be incre ed and the time required to reach the stationary sta could be reduced. After 7 days from inoculation, final c yield of the culture were similar for both of the two cas with the initial inoculation of $0.8x10^5$ cells/ml and 2x1 cells/ml, respectively. However, when $2x10^5$ cells/ were inoculated, stationary stage was reached fas than the culture of $0.8x10^5$ cells/ml inoculation. this reduced the culture time to approximately 4 days. Similar result was obtained with FS-4 cells. The effect of inoculation density on the growth of FS-4 cells is shown in Fig. 9. In this case three different levels of inoculation density, i.e. $1.2x10^5$, $2.0x10^5$, and $3.0x10^5$ cells/ml, were employed. As in the case of Vero cell growth, FS-4 cells with the initial inoculation density of $2.0x10^5$ cells/ml and $3.0x10^5$ cells/ml were grown to the same final cell density of about $2.0x10^6$ cells/ml. After 8 days the concentration of FS-4 cells did not increase any further and the induction for the interferon production was started. Fig. 10 shows the photograph of FS-4 cells grown for 5 days on microcarrier.

Migration of Cells from Confluent Monolayer to Microcarrier

When cultures grown in monolayer on the surface of the culture flask became confluent, 0.5mg/ml of microcarrier was added. This composite method of us-

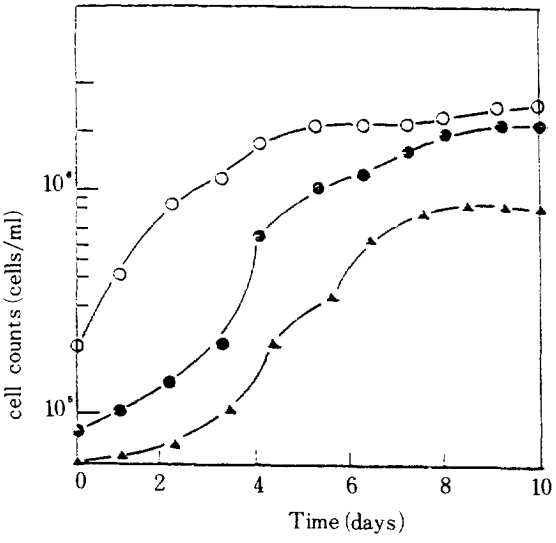

Fig. 8. Effect of inoculation density on the growth of Vero cells.

-▲- : 0.6×10^5 cells/ml inoculation
-●- : 0.8×10^5 cells/ml inoculation
-○- : 2.0×10^5 cells/ml inoculation

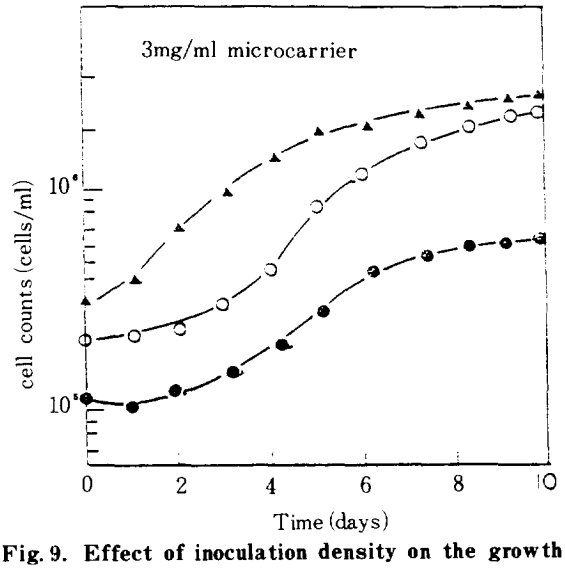

Fig. 9. Effect of inoculation density on the growth of FS-4 cells.

-●- : 1.2×10^5 cells/ml inoculation
-○- : 2.0×10^5 cells/ml inoculation
-▲- : 3.0×10^5 cells/ml inoculation

ing both the culture flask surface and the microcarrier surface resulted in the increase in the final cell concentration reached. This result is shown in Fig. 11. By adding microcarrier to the confluent MRC-5 or FS-4 cell

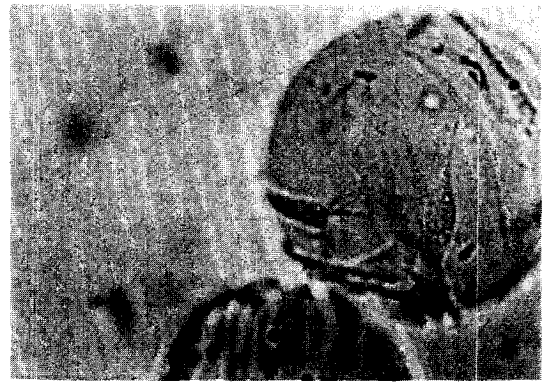

Fig. 10. Photograph of FS-4 cells grown on microcarrier.

monolayer, concentration of the diploid cells could be increased up to $1.2\text{x}10^6$ cells/ml. When the confluent cells came into contact with the surface of microcarrier, cells were slowly transferred to the microcarrier and 3-4 days after the addition of microcarrier both of the surfaces of microcarrier and culture flask became confluent. Fig. 12 is the photograph taken for such a case. In both upper and lower photographs the microcarriers added only after the formation of the confluent monolayer on the surface of the culture flask are seen to have also been covered with the confluent cells. Increased final cell concentration per unit volume could increase the yield of interferon production.

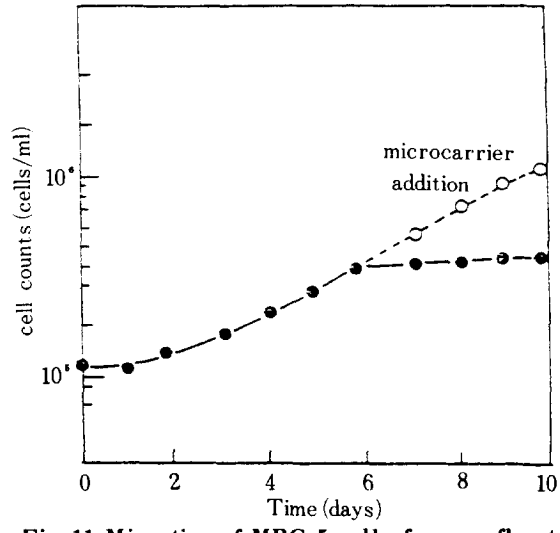

Fig. 11. Migration of MRC-5 cells from confluent monolayer to microcarrier.

-●- : monolayer culture
-○- : monolayer culture with added microcarrier

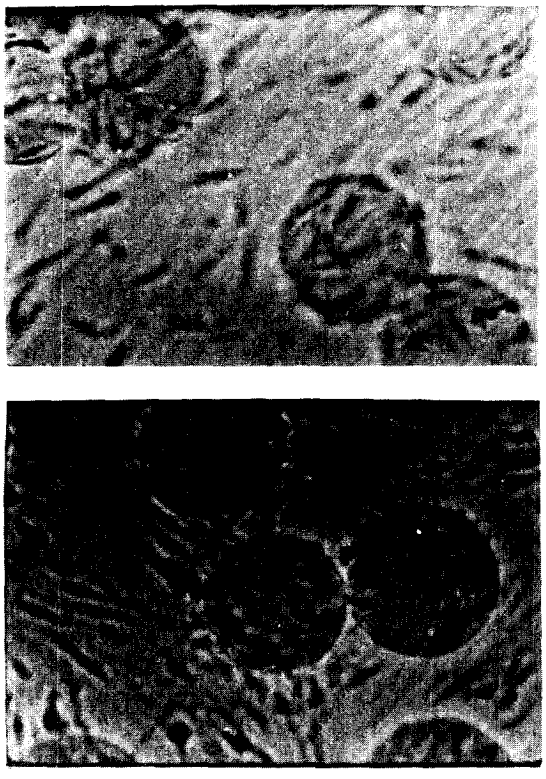

Fig. 12. Photograph showing cell migration from confluent monolayer to microcarrier.

Table 1. Interferon production from three types of cultures (units/ml).

Cell Lines / Culture Types	FS - 4	MRC-5
Monolayer culture	6, 000	4, 500
Microcarrier culture	9, 000	6, 000
Microcarrier culture in confluent monolayer	10, 000	6, 000

Table 2. Effect of serum concentration on yield of interferon.

Serum concentration (%, V/V)	Interferon Production (Units/ml)
5	11, 000
10	10, 000
15	7, 000
20	6, 000

Comparision between the Production of Human Fibroblast Interferon from Monolayer and Microcarrier Culture

Human fibroblast interferon was produced from microcarrier cultures with volumes of 100ml, 250ml, and 1 1, respectively. Different scales of production can be achieved by proportionately altering the volume, the number of inoculated cells and the quantity of microcarrier. At the end of growth phase the cell concentration reached approximately $8x10^5$ cells/ml. According to the procedure shown in methods, normal confluent monolayer culture produced approximately 6,000 units/ml of interferon. When the microcarrier culture was used, the yield of interferon production could be increased. Cultures obtained by adding microcarrier to the confluent cell monolayer also resulted in the enhanced production of interferon. These differences are summarized in Table 1. Among these three methods, the mixed culture of microcarrier in flasks was the best producer. Because of the difficulties to control the microcarrier culture vessel, cells on microcarriers were not good for the production of interferon as compared to the mixed culture. The enhanced production in the case of the mixed culture may also have been caused by the increased surface area and hence the surface to volume ratio.

Effect of FCS Concentration on Interferon Yield

FS-4 cells grown in 5, 10, 15, and 20% FCS in DMEM were induced to produce interferon. As seen in Table 2, when cells were grown at low FCS concentration, higher titers of interferon were obtained.

Other Parameters Affecting the Yield of Interferon

Several chemicals such as ascorbic acid, DEAE-Dextran and L-glutamine were tested for the possibility of improving the yield of interferon, but the result was not much different from that obtained in its absence.

The effect of antibiotics was also tested. In the absence of antibiotics, the yield was slightly increased as compared to the case in the presence of antibiotics. In the absence of antibiotics, 8,000 units/ml of interferon was produced whereas 6,000 units/ml of interferon could be obtained in its presence. Lowering the temperature in the production phase could not increase the yield of interferon production.

Interferon Assay

Several parameters affecting the assay of interferon was examined. Vero, Hep2, FL, and FS-4 cells were examined to estabilish good assay system. Comparison among these cells is shown in Table 3. Hep2 and FL cells were good for assay of interferon, but FS-4 and Vero cells did not show good sensitivity in assay. For these cell strains, $3x10^5$ cells/ml or more were required for initial plating. At this concentration, clear reading

Table 3. Comparison of infecting cells used for the assay of interferon.

Cell Lines	Cell required (cells/ml)	Sensitivity
Vero	more than 3×10^5	bad
Hep 2	more than 3×10^5	very good
FL	more than 3×10^5	good
FS-4	more than 3×10^5	average

could not be done. Time taken from inoculation to assay did not influence the result. pH in virus infection medium was optimal at 7.7.

CONCLUSION

By using microcarrier, several anchorage-dependent cells could be cultured in suspension and production of human fibroblast interferon in high yield was studied.

Human diploid fibroblasts, FS-4 and MRC-5 for interferon production, and Africa green monkey cell, Vero, were grown on microcarrier under the controlled environmental conditions and the results were as in the following.

It was confirmed that effects of stirring, pH, serum concentration on the initial attachment of cells were crucial. Optimal pH for initial attachment of FS-4 cells was 7.4, and serum concentration for this case was optimum at 5%. Usually 10,000 units/ml of human fibroblast interferon was produced by microcarrier culture as compared to 6,000 units/ml for the case of monolayer culture.

Interferon assay showed high sensitivity to the slight changes of pH, cell concentration, and VSV titer, etc. Cell concentration of $5x10^5$ cells/ml was needed for good assay. As described in previous sections, microcarrier culture was an efficient method for larger-scale production of cell and its products.

Further studies on the control of the culture system and the critical parameters affecting the yield of cell and its products are necessary.

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