Production of Recombinant Human Monoclonal Antibody Using ras-Amplified BHK-21 Cells in a Protein-free Medium

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Received November 6, 1995

A ras oncogene-amplified recombinant BHK-21 cell line (ras-rBHK-IgG) has been established, and was shown to hyperproduce the recombinant IgG chimeric human monoclonal antibody (hMAB) AE6F4, which recognizes lung cancer cells. We found that the ras-rBHK-IgG cells could be easily cultured in a protein-free ERDF medium supplemented with iron(III) nitrate, hydroxyethyliminodiacetic acid, and non-peptide synthetic attachment factor as well as in a serum-free ERDF medium supplemented with insulin, transferrin, ethanalamine, and sodium selenite. The productivity of recombinant hMAB from the cells cultured in dishes at high cell densities was higher in protein-free medium than in serum-containing medium. True high density culture of the ras-rBHK-IgG cells was done in protein-free medium using the 1cmausome, which is a novel hollow fiber bioreactor system. After culture for 30 days in protein-free culture, a total amount of about 14mg of the recombinant hMAB AE6F4 was obtained, and was shown to be reactive against lung cancer cells in tissues.

Key words: BHK-21 cells; high density culture; recombinant human monoclonal antibody; protein-free culture; ras oncogene

Lung cancer is one of the most lethal cancers in the industrialized world, and early detection is an important key for the success in the lung cancer therapy.1–3 The human monoclonal antibody (hMAB) AE6F4 produced from human hybridomas is useful for immunocytochemical detection of lung cancer cells in tissue and sputa.4,5 A computer-aided mass-screening method is now being developed for the rapid and simple detection of lung cancer in sputa. However, the low productivity of hMAB from human hybridomas is a major obstacle in mass-producing the hMAB for medical uses.6,7 Furthermore, the hMAB AE6F4 belongs to the IgM isotype,4 which is not as good as the IgG isotype for practical use because of its inferior stability and the difficulty in preparing an enzyme conjugate.

We have developed an oncogene-activated production (OAP) system in which the amplified ras oncogene activates the cytomegalovirus immediate early promoter (CMV) promoter, which controls expression of recombinant protein in BHK-21 cells, resulting in rapid establishment of hyperproducion stable cell lines.7–10 In a previous paper, we established a ras-amplified BHK-21 cell line (ras-rBHK-
IgG), which hyperproduces a recombinant IgG chimeric AE6F4 hMAB under the control of the CMV promoter.  

Protein-free culture of recombinant cells is desired for mass production because protein-free medium is usually not only more economical than serum-containing or serum-free medium, but also facilitates the purification of recombinant proteins. BHK-21 cells can be cultivated in serum-free medium supplemented with growth factors such as fibroblast growth factor, fibronectin, insulin, transferrin, high density lipoproteins, or oleic acid. However, few researchers have reported successfully culturing BHK-21 cells in protein-free medium, with the notable exception of reports describing a high density culture where cell growth was stimulated by an autocrine mechanism. In this report, we demonstrate that ras-rBHK-IgG cells can be easily cultivated in protein-free ERDF medium supplemented with iron(III) nitrate, hydroxyethyliminoacetic acid (HIDA), and non-protein synthetic attachment factor as well as in serum-free ERDF medium supplemented with insulin, transferrin, ethanalamine, and selenium. Furthermore, the long-term protein-free high density culture of ras-rBHK-IgG cells was possible using the Tecnomouse, which is a new hollow fiber bioreactor system.

Materials and Methods

Recombinant BHK-21 cells. A ras-amplified recombinant BHK-21 cell line (ras-rBHK-IgG) was established as reported previously (Shoji et al., submitted to Human Antib. Hyridomas). Briefly, the variable region of a heavy chain gene and whole a light chain gene of the AE6F4 hMAB were cloned from human hybridoma cDNAs by PCR method. The V and CH1 region of AE6F4 µ chain gene and the CH2 and CH3 region of human IgG1 gene were fused to construct the IgG chimeric AE6F4 µ heavy chain gene. The IgG chimeric AE6F4 µ heavy chain gene and the AE6F4 light chain genes were inserted downstream from the CMV promoter to construct the pCMVP-AE6F4HL plasmid. BHK-21 C13 cells (ATCC CCL 10) were cotransfected with both pCMVP-AE6F4HL and pCMV-D-Ha-ras plasmid, which contains both the dihydrofolate reductase (dhfr) gene and the human c-Ha-ras gene, and selected with methotrexate to amplify both genes. Stable ras-rBHK-IgG clones that allow the production of the recombinant IgG chimeric AE6F4 hMAB were established and maintained in ERDF medium (Kyokuto Pharmacy Industrial Co.,) supplemented with 10% fetal bovine serum (FCS). The antibody productivity of these ras-rBHK-IgG clones was greater than 0.8 µg IgG 10^7 cells/day, which was equal to 16 times the productivity of the original human hybridoma.

Serum-free and protein-free cell culture. In serum-free culture experiments, ras-rBHK-IgG cells (1 x 10^7 cells) were plated into 35-mm plastic dishes (Falcon) and cultured in 2 ml of ERDF medium supplemented with various combinations of 5 µg/ml insulin (H) (Novo), 20 µg/ml human transferrin (T) (Sigma), 20 µM ethanolamine (E), 25 mM sodium selenite (S), or complete ITES-ERDF medium14 at 37°C in humidified 5% CO2:95% air for 5 days. In protein-free culture experiments, ras-rBHK-IgG cells (1 x 10^7 cells or 1 x 10^8 cells) were plated into 35-mm plastic dishes and cultured in ERDF medium supplemented with various iron compounds at 8 µM or with combinations of 80 µM iron(III) nitrate and 80 µM of various chelating agents at 37°C for 5 days.

Measurement of antibody production and cell number. Antibody concentrations in spent medium of the recombinant BHK-21 cells were measured by ELISA as described previously, using an anti-human IgG (γ) antibody (4100, TAGO) as the first antibody, and an anti-human IgG (γ) peroxidase conjugate antibody (2390, TAGO) as the second antibody. Cells were counted using a cell counter. All experiments were duplicated and the average value was used for analysis. Standard deviations were within 10%.

Coating with attachment factors. Non-protein synthetic attachment factors FC-44 and FC-106, which are poly(ethylene glycol) modified human serum albumin (HSA) derivatives containing the cell attachment peptide fragment (Arg-Gly-Asp-Ser), were obtained from Fuji Film Co., Ltd. Fibronectin and transferrin factors were dissolved in phosphate buffered saline (PBS) at concentrations of 0.1, 1, and 10 µg/ml and were then added to a 35-mm plastic dish and incubated at 37°C for 2 h. After being washed 3 times with PBS, the dishes were used for the protein-free culture of ras-rBHK-IgG cells.

Protein-free high density culture of recombinant cells using the Tecnomouse bioreactor system. The Tecnomouse bioreactor system (INTEGRA Bioscience Co., Germany) consists of five independent bioreactor unit (culture cassettes) for simultaneous cultivation of different cell lines or primary tissues. The culture cassette has a sandwich structure in which 400 hollow fibers are bundled among silicon membrane gas filters. The culture medium (about 2 liters) is circulated in the intracapillary (IC) space. Cells were inoculated into the extracapillary (EC) space (about 5 ml). The sandwich construction of the bioreactor guarantees not only optimal oxygenation but also a highly efficient supply of nutrient to and removal of waste from the cells. The EC space of the culture cassette was coated with a sputtered concentration of the non-protein synthetic attachment factor FC-106. Ras-rBHK-IgG cells (2 x 10^7 cells) were seeded into the culture cassette and cultured in protein-free ERDF medium supplemented with 80 µM iron(III) nitrate and 80 µM HIDA at 37°C in humidified 5% CO2:95% air. On the 16th day, the medium was changed to ERDF medium supplemented with 80 µM iron(III) nitrate and 160 µM HIDA. This medium (D) was recirculated through the culture cassette at the rate of 75 ml/hr. and the circulation medium was changed every week. The spent medium in the EC space of the culture cassette was changed every other day and the antibody concentrations in the spent medium were measured by ELISA.

Results

Serum-free and protein-free culture of ras-amplified recombinant BHK cells

Ras-rBHK-IgG cells were cultured in 10% FCS-ERDF medium or ITES-ERDF medium. The ras-rBHK-IgG cells cultured in 10% FCS-ERDF medium grew faster when cultured in ITES-ERDF medium (Table 1). Even though the total amount of antibodies produced by the recombinant cells was greater in 10% FCS-ERDF medium than in ITES-ERDF medium, the hMAB productivity of the recombinant cells was about 2 times higher in ITES-ERDF medium than in 10% FCS-ERDF medium. Similarly, the ras-rBHK-IgG cells were also cultured in ITES-ERDF medium lacking insulin or transferrin. Growth in ITES-ERDF medium without insulin was slower than in complete ITES-ERDF medium, but the total amount of antibodies produced was the same in both media (Fig. 1).

Table 1. Comparison of Serum-containing and Serum-free Cultures on the Cell Growth and Recombinant Antibody Production of ras-rBHK-IgG Cells

<table>
<thead>
<tr>
<th>Media</th>
<th>Relative cell number</th>
<th>Relative antibody production</th>
<th>Relative antibody productivity</th>
</tr>
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<tbody>
<tr>
<td>10% FCS-ERDF</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ITES-ERDF</td>
<td>0.4</td>
<td>0.7</td>
<td>1.8</td>
</tr>
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* Ras-rBHK-IgG cells (1 x 10^7 cells) were cultured in a 35-mm plastic dish in 10% FCS-ERDF medium or ITES-ERDF medium for 5 days. Then, cell number and antibody production for cell growth in ITES-ERDF medium was assessed relatively to cells cultured in 10% FCS-ERDF. Relative value for cell cultured in serum-containing medium were based on cell number and antibody production values of 3.7 x 10^7 cells and 780 ng/ml respectively. 1 µg/ml of insulin, T, 20 µg/ml of transferrin; E, 20 µM ethanolamine; S, 25 mM sodium selenite.
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Recombinant cells did not grow in ITES-ERDF medium lacking transferrin (Fig. 1a). When we replaced transferrin with various iron compounds, iron(III) nitrate was found to be the best for cell growth and antibody production (Fig. 2a). The optimum concentration of iron(III) nitrate for the ras-rBHK-IgG cells was 80 μM (Fig. 2b).

**Effects of initial cell number on cell growth and antibody production**

Ras-rBHK-IgG cells were plated at two different cell numbers in 10% FCS-ERDF medium or protein-free ERDF medium supplemented with 80 μM iron(III) nitrate. At a number of 1 × 10⁵ cells, which reflects the logarithmic growth phase, both cell growth rate and antibody production were lower in ERDF medium supplemented with 80 μM iron(III) nitrate than in 10% FCS-ERDF medium. At a number of 1 × 10⁶ cells, which reflects the antibody production phase, the cell growth rate was lower in protein-free ERDF medium supplemented with 80 μM iron(III) nitrate than in 10% FCS-ERDF medium, but antibody production was 4 times higher in the protein-free medium than in 10% FCS-ERDF medium (Table II). Therefore, the antibody production of the ras-rBHK-IgG cells in protein-free medium was evaluated at the number of 1 × 10⁶ cells in the following experiments.

**Effects of chelating agents on cell growth and antibody production**

Iron(III) nitrate produces precipitates during long-term cultivation. Thus, we examined the effects of various chelating agents to supply stable iron(III) nitrate to ras-rBHK-IgG cells. As shown in Fig. 3, the combination of iron(III) nitrate with iminodiacetic acid (IDA), HIDA, or ethylenediamine-N,N'-dipropionic acid (EDDP) stimulated cell growth compared with iron(III) nitrate alone. Concerning antibody production, the combination of iron(III) nitrate with HIDA or ethylenediamine tetraacetic acid (EDTA) produced a greater amount than iron(III) nitrate alone. However, the ras-rBHK-IgG cells perished in EDTA containing protein-free medium. As a result, the combination of iron(III) nitrate with HIDA was considered to be best among the chelating agents tested. A 1:1 ratio of HIDA to iron(III) nitrate was found to be good for the cell growth and a 2 to 1 rate for antibody production (Fig. 4).

**Fig. 1. Effects of Growth Factors on the Growth and Recombinant Antibody Production of ras-rBHK-IgG Cells.**

Ras-rBHK-IgG cells (1 × 10⁵ cells) were cultured in a 35-mm plastic dish in ITES-ERDF medium or ERDF medium supplemented with depleted growth factors for 5 days. Cell number and the concentration of recombinant mAb in spent medium were then measured. (a) Comparison of the cell growth (b) Comparison of the antibody production. ITES, ITES-ERDF medium. 1, ITES-ERDF medium. T, ITES-ERDF medium.

**Fig. 2. Effects of Various Iron Compounds on the Growth and Antibody Production of ras-rBHK-IgG Cells.**

Ras-rBHK-IgG cells (1 × 10⁵ cells) were cultured in a 35-mm plastic dish in ERDF medium supplemented with 8 μM various iron compounds for 5 days. ERDF medium contains 0.8 μg ferrous sulfate. (a) Effects of various iron compounds on the cell growth and antibody production. 1, 20 μg/ml of transferrin. 2, ferric citrate. 3, ferrous sulfate. 4, iron(III) nitrate. 5, iron(III) chloride. 6, none (b) Effects of iron(III) nitrate concentration on the cell growth and antibody production. T, 20 μg/ml of transferrin.

**Table II. Effects of Different Cell Number Initially Plated on the Cell Growth and Antibody Production of ras-rBHK-IgG Cells**

<table>
<thead>
<tr>
<th>Media</th>
<th>1 × 10⁴ cells</th>
<th>1 × 10⁵ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative cell number</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Relative antibody production</td>
<td>1.0</td>
<td>1.0</td>
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</table>

* Ras-rBHK-IgG cells (1 × 10⁴ or 1 × 10⁵ cells) were cultured in ERDF medium supplemented with 10% FCS or 80 μM iron(III) nitrate for 5 days. Cell number and antibody production were compared between the two media. In 10% FCS-ERDF medium, for 1 × 10⁵ and 1 × 10⁴ initially plated cells, final cell number was 4.1 × 10⁶ cells and 4.2 × 10⁵ cells, and antibody production was 720 ng/ml and 1880 ng/ml, respectively.

**Effects of non-protein synthetic attachment factors on cell growth and hMAb production of ras-rBHK-IgG cells**

Effects of non-protein synthetic attachment factors were examined for cell growth and hMAb production of
Fig. 3. Effects of Various Chelating Agents on the Growth and Antibody Production of ras-rBHK-IgG Cells.

Ras-rBHK-IgG cells were cultured in ERDF medium supplemented with iron(II) nitrate alone, chelating agents alone or the combinations of both iron(II) nitrate and chelating agents for 5 days. Left figure: effects of chelating agents on the cell growth. 1 x 10⁶ cells were initially plated. Right figure: effects of chelating agents on the antibody production. 1 x 10⁶ cells were initially plated. Solid bars, 80 μM iron(II) nitrate alone; open bars, no supplements; dotted bars, 80 μM chelating agent alone; hatched bars, combination of 80 μM iron(II) nitrate with 80 μM chelating agent. NTA, nitrotriactacetic acid; NTP, nitritopropionic acid; IDA, iminodiacetic acid; HIDA, hydroxylaminodiacetic acid; EDDP, ethylenediaminetetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

Fig. 4. Effects of the Ratio of HIDA to Iron(II) Nitrate on the Growth and Antibody Production of ras-rBHK-IgG Cells.

Ras-rBHK-IgG cells were cultured in ERDF media supplemented with various ratios of HIDA to iron(II) nitrate for 5 days. The cell growth was evaluated in cultures where 1 x 10⁶ cells were initially plated, and the antibody productions was examined in cultures where 1 x 10⁶ cells were initially plated. Solid bars, 80 μM iron(II) nitrate alone; open bars, no supplements; dotted bars, HIDA alone; hatched bars, both 80 μM iron(II) nitrate and HIDA.

ras-rBHK-IgG cells. The non-protein synthetic attachment factors, FC-44 and FC-106, are polymethacrylate derivatives containing the cell attachment peptide fragment (Arg-Gly-Asp-Ser). The structures of FC-44 and FC-106 are shown in Fig. 5. FC-106 differs from FC-44 at the linking point with the trimethylammonium group. Ras-rBHK-IgG cells were cultured in dishes coated with various attachment factors for 5 days. As shown in Fig. 6, no addition of either attachment factor resulted in poor proliferation of cells. The cells grew well in the dish coated with 5% FCS. When coated with FC-44 or FC-106, or even with the natural attachment factor fibronectin, cells cultured in ERDF medium supplemented with 80 μM iron(II) nitrate grew in a concentration-dependent manner. FC-106 at 10 μg/ml was shown to have the equivalent cell growth rate as 5% FCS. On the other hand, antibody production and productivity were not affected much by the addition of these attachment factors.

Protein-free high density culture of ras-rBHK-IgG cells using the Tecnomouse bioreactor

To mass-produce recombinant AE6F4 mAb, ras-rBHK-IgG cells were cultured in protein-free ERDF medium supplemented with iron(II) nitrate and HIDA using the Tecnomouse bioreactor system. The EC space of the culture cassette of the bioreactor was coated with the non-protein synthetic attachment factor FC-106. After inoculation of cells (2 x 10⁶ cells), spent medium in the EC space was changed every other day. As shown in Fig. 7, a high concentration of the recombinant mAb was accumulated into the EC space gradually during cultivation, reaching a plateau of 450 μg/ml 2 days on 25th day. In total, ras-rBHK-IgG cells produced about 14 mg of the recombinant mAbs in the protein-free high density culture using the Tecnomouse bioreactor. We could not count the cells during cultivation using this system. The numbers of detached cells in the spent medium was several percent of the inoculated cell number, suggesting continuing attachment of the recombinant cells in the EC space. Trypsinization of the attached cells after completing the cultivation showed that total cell number increased to 1 x 10⁶ cells.
Fig. 7. Antibody Production by ras-rBH-K-IgG Cells Cultured in the Tencosemose Bioreactor System.

**Discussion**

Host animal cells for the mass production of recombinant proteins must have the following characteristics: genetic stability, high productivity, viability in serum-free or protein-free culture, and the ability to perform normal post-transcriptional modifications. BHK cells have been widely accepted as a host cell line for the industrial production of recombinant proteins because of their chromosomal stability, accumulation of knowledge concerning virus infection and genetic mutation, easy gene amplification, and high glycosylation ability. However, productivity of recombinant proteins in BHK cells is not so high. Their requirement for growth in serum-free culture or protein-free culture is stringent. We increased the recombinant protein productivity of BHK-21 cells by activating the CMV promoter using the amplified ras oncogene. Here we demonstrate that ras-amplified recombinant BHK cells can be easily cultivated in serum-free and protein-free medium. Ras-transformed cells are known to have low growth factor requirements, because they stimulate their own growth via the autocrine mechanism.

The ITES mixture has been widely used for the serum-free culture of animal cells, especially hybridomas. Ras-rBH-K-IgG cells was shown to be viable in ITES-ERDF medium and antibody productivity was even increased in serum-free culture (Table I). It has been reported that insulin is not always necessary for serum-free culture, because ras-transformed cells secrete insulin-like growth factors. Ras-rBH-K-IgG cells in insulin negative serum-free medium grew slowly but antibody production was similar to that of insulin-containing medium (Fig. 1). Growth rate suppression of cultured cells is known to increase the productivity of recombinant proteins. These results suggested that insulin could be removed from ITES-ERDF medium without greatly affecting the antibody production of ras-rBH-K-IgG cells.

Transferrin has been considered to be an essential growth factor for various types of animal cells in serum-free cultures, and holds true for ras-rBH-K-IgG cells. It is known that large amounts of iron compounds or iron complexes with appropriate chelating agents can replace transferrin in serum-free cultures. In the protein-free culture of ras-rBH-K-IgG cells, iron(III) nitrate was shown to be preferable (Fig. 2a). In hybridomas culture, ferric citrate is widely used as a substitute for transferrin and the best concentrations of this compound are from 50 μM to 5 mM. These results suggested that iron compounds effective as transferrin substitutes would be different dependent upon cell types and protein-free culture conditions. Consequently, we could cultivate ras-rBH-K-IgG cells in simple protein-free ERDF medium supplemented with only 80 μM iron(III) nitrate.

Chelating agents can be used to solubilize iron compounds for maintaining a stable supply in the long-term culture and for suppressing the generation of free radicals in serum-free and low percentage serum-containing cultures. HIDA was selected as the best chelating agent in protein-free culture for ras-rBH-K-IgG cells. Although addition of EDTA, a common chelating agent, resulted in the detection of a high concentration of recombinant mAb, the high chelating ability of EDTA might cause a deficiency of essential metal ions which was followed by cell death. Therefore, it was considered that the high antibody production in EDTA containing protein-free medium might be a result of cellular lysis.

As shown in Table II, the antibody production of ras-rBH-K-IgG cells cultured in the protein-free medium was greatly increased especially when initially plated at a high density. This result suggested that at a high cell density the cells might produce cellular protein factors that can stimulate antibody production. This hypothesis is now under investigation.

In the protein-free high density culture of the ras-rBH-K-IgG cells, effects of new non-protein synthetic attachment factors were examined (Fig. 5). The synthetic non-protein attachment factors are polymethylacrylate derivatives containing the cell attachment peptide fragment. Cell attachment and cell extension activities are important indicators to assess the ability of different attachment factors. Taking attachment and cell extension activities into account, FC-106 was shown to be optimal for the culture of ras-rBH-K-IgG cells in the protein-free medium (Fig. 6). Since these synthetic attachment factors are not
only economical but also autoclavable, coating of sterilized culture vesse or a bioreactor cartridge with any of them may be beneficial for protein-free culture of animal cells.

The Tecnomouse bioreactor system is a novel hollow fiber hybrid bioreactor in which nutrient perfusion through common hollow fibers is combined with direct oxygenation via silicone membranes. The Tecnomouse bioreactor has been developed for the culturing of hybridomas and production of monoclonal antibodies replacing ascites fluid from mice. Murine hybridomas have been shown to run for 188 days in a bioreactor, in which about 850 mg MAb's was produced (Tecnomouse user report). Few papers have been published about culturing adheses cells using this high density culture system. We found that ras-rBHKG-IgG cells could be successfully cultivated under protein-free culture conditions in the Tecnomouse bioreactor system for at least one month. Finally cells could be maintained at a density of [1 x 10^6] cells/5 ml in the EC space. This cell density is considered to be high when compared with the density of hybridoma cells (1 x 10^6 cells/ml) in other hollow fiber bioreactor system. Even in a typical high density microcarrier culture system, the cell density range is 2-6 x 10^6 cells/ml for Chinese hamster ovary (CHO) cells. The spent medium contained recombinant hMAb at a maximum concentration of 450 μg/ml. The production value for this recombinant hMAb is on the same level as recombinant mouse-human chimeric antibody produced from mouse myeloma NSO cells using the glutamine synthetase gene amplification method (560 μg/ml) and of recombinant human growth hormone produced from CHO cells using the human metallothionein II A inducible promotor system (200 μg/ml).

All spent media were pooled and the recombinant hMAb was purified by protein A affinity column chromatography with a recovery rate of over 80%. Immunoblot analysis and immunohistochemical staining showed that the light and heavy chains of the obtained hMAb, which have the same molecular weight as an authentic IgG antibody, can form a functional molecule that is reactive to lung cancer cells in tissue (data not shown).

Thus we conclude that the efficient mass production of the lung cancer recognizing recombinant hMAbs AE6F4 is possible using ras-amplified recombinant BHK-21 cells, which has increased the recombinant hMAb productivity, in protein-free ERDF medium supplemented with only [111] nitrate, HIDA and synthetic attachment factor. It is expected that this study will contribute to the development of a mass-screening procedure for the detection of lung cancer cells in sputum and mass production of pharmaceutical proteins using recombinant BHK-21 cells in protein-free medium. Ras-amplified BHK-21 cells may be also suitable as a general host cell line for the mass production of other recombinant proteins. Since BHK-21 cells transfected with the c-Ha-ras oncogene is known to exhibit modified cell cycle, it is expected that glycosylation of recombinant proteins produced by these ras-amplified cells should be investigated.

Acknowledgement. We are grateful to Fuji Film Co., Ltd. for kindly providing the non-protein synthetic attachment factors FC-44 and FC-106.

References