Journal of Biotechnology, 16 (1990) 259–278
Elsevier

BIOTEC 00551

Effect of initial cell density on hybridoma growth, metabolism, and monoclonal antibody production

Sadettin S. Ozturk and Bernhard Ø. Palsson

Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan, U.S.A.

(Received 16 August 1989; accepted 25 March 1990)

Summary

A murine hybridoma cell line (167.4G5.3) was cultivated in batch mode with varying inoculum cell densities using IMDM media of varying fetal bovine serum concentrations. It was observed that maximum cell concentrations as well as the amount of monoclonal antibody attainable in batch mode were dependent on the inoculum size. Specifically, cultures with lower inoculum size resulted in lower cell yield and lower antibody concentrations. However, in the range of 10^2 to 10^5 cells per ml, the initial cell density affected the initial growth rate by a factor of only 20%. Furthermore, specific monoclonal antibody production rates were independent of initial cell density and the serum concentration. Glutamine was the limiting nutrient for all the cultures, determining the extent of growth and the amount of antibody produced. Serum was essential for cell growth and cultures with initial cell concentrations up to 10^6 cells per ml could not grow without serum. However, when adapted, the cells could grow in a custom-made serum-free medium containing insulin, transferrin, ethanolamine, and selenium (ITES) supplements. The cells adapted to the ITES medium could grow with an initial growth rate slightly higher than in 1.25% serum and the growth rate showed an initial density dependency–inocula at 10^3 cells per ml grew 30% slower than those at 10^4 or 10^5. This difference in growth rate was decreased to 10% with the addition of conditioned ITES medium. The addition of conditioned media, however, did not improve the cell growth for serum-containing batches.

Hybridoma; Cell culture; Inoculum size; Serum effect

Correspondence to: B.Ø. Palsson, Dept. of Chemical Engineering, University of Michigan, Ann Arbor, MI 48109, U.S.A.

Introduction

Several studies have shown that mammalian cells require a minimum initial cell concentration for growth. About two decades ago Rubin and co-workers (Rubin, 1966; Rein and Rubin, 1968) showed that the growth of primary chick embryo cells depended on the initial cell density. They reported that the initial growth rate of these primary cells varied directly with the initial cell concentration and only little or no net growth occurred below some critical initial cell concentration. By the addition of 'conditioned' media, obtained from concentrated cell cultures, cell density growth was improved. These results suggested that this anchorage-dependent primary cell line was influenced by one or more growth factors produced by the cells themselves – the higher the initial cell concentration the higher the amount of these factors and the better the growth. The extent of growth in terms of maximum cell density also varied with the initial cell density. Similar observations have been reported in the literature for other primary cell lines. Stoker and Sussman (1965) have described the effect of initial cell density on mouse embryo fibroblasts and the enhancement of growth by a feeder action from BHK cells. Mered et al., (1980) reported the effect on other cell lines including Vero cells and LLC-MK2 cells growing on microcarriers.

More recently, Hu and co-workers (Hu et al., 1985; Hu and Wang, 1987) presented data on human foreskin fibroblasts, FS-4, growing on Petri dishes and on microcarriers. Their results showed no effect of initial cell density for cells cultivated in Petri dishes. However, there was found to be a critical number of inoculated cells per microcarrier bead in order for growth to occur. The data of Hu and co-workers has been interpreted mathematically by Lauffenburger and Cozens (1989) based on an autocrine growth factor-receptor model.

The influence of initial cell density and the existence of a critical inoculum size for growth may therefore be considered to be well established for normal diploid cell lines. Similar data for continuous cell lines, including hybridoma cells, is, however, scarce. Most of the data come from cloning studies, where the emphasis is directed to the cloning efficiency from a single cell level obtained in limiting dilution. The dependence of cloning efficiency of hybridoma cells on a feeder cell (or some extrageneous growth factors such as endothelial cell growth supplement, ECGS) may suggest that the cell-to-cell interactions play an important role, because the presence of feeder cells, such as spleen cells, thymocytes, or macrophages is essential in cultures containing a low number of hybridomas (Hammerling et al., 1978; Lernhardt et al., 1978; Neugartner et al., 1978). Other studies showed that the requirement of a feeder cell could be eliminated by the supply of growth factors such as human endothelial culture supernatants (HECS), endothelial cell growth supplement (ECGS) (Astaldi et al., 1980; Pintus et al., 1983), or Ewing sarcoma growth factor (ESGF) (King and Sartorelli, 1989).

The somatic cell fusion and subsequent selection using limiting dilution is the most critical step in hybridoma development. As the hybridoma cell is newly formed, it is in a stage of transition to a continuous cell line. Therefore it is quite possible that newly formed hybridoma cells need more growth factors, supple-

mented either by a feeder cell, or by external addition. However, once the clone is recovered, and passed in cell culture media, it adapts and requirements for growth become less stringent. It has been shown by several investigators that many hybridoma cell lines can grow in the absence of growth factors provided that insulin, transferrin, ethanolamine, and selenium (ITES) are added. Therefore, a study of the inoculum density effects on hybridoma growth during the early stages of the establishment of a new cell line is of limited utility for understanding behavior of the fully established cell line.

The data on the influence of inoculum density for established hybridoma cell lines is rather limited. Velez et al. (1986) reported a critical cell density at 2×10^4 cells per ml for a murine hybridoma cell line growing in spinner flasks. Glacken et al. (1988) reported that initial growth rates are enhanced with increasing initial cell density. They attributed this effect to the thiol chemistry of the medium. Takazawa et al. (1988), on the other hand, observed no effect of initial cell density on the specific growth rates for media supplemented with serum. However, there was an effect of initial density on the cell growth in a serum-free medium. It is important to note that these reports focussed on cellular growth rates.

A systematic study of the influence of initial cell density on cell metabolism and antibody production for hybridoma cells has yet to appear. Here we fill this gap in our knowledge base and report the effect of initial cell density and serum concentration on cell growth rates, metabolism and monoclonal antibody production rates.

Materials and Methods

Cell line, medium and culture maintenance

The murine hybridoma, 167.4G5.3, was kindly provided by Dr J. Latham Claflin from The University of Michigan Medical Center. The antibody produced by this cell line is an IgG_1, directed against the phosphorylcholine (Briles et al., 1984). The cells were obtained in the frozen state at an unknown passage number. They were thawed and maintained in 75 cm^2 plastic T-flasks (Bellco Glass, Inc., Vineland, NJ) using Iscove's Modified Dulbecco's Medium (IMDM, Gibco Laboratories, Grand Island, NY) containing 5% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY) supplemented with 100 U ml^{-1} potassium penicillin G, and 100 μg ml^{-1} streptomycin sulphate (Sigma Chemical, St. Louis, MO). The cells were kept at 37 °C and and 5% CO_2 atmosphere in humidified incubators (VWR Scientific, San Francisco, CA). The cultures were maintained by passing them every 2 to 3 days with a dilution factor 1 to 4 with fresh media.

Effect of inoculum size on cell growth, metabolism and MAb production

For the assessment of the effect of inoculum density, four different serum concentrations were used: 1.25, 2.5, 5, and 10% v/v. Cells were adapted to media with different serum levels for two weeks in order to minimize the influence of changes in media composition when introduced into fresh medium. For each serum concentration, cells initially maintained in 5% FBS were passed daily using the

medium with a particular serum level, and variable dilution ratios which were determined by the cell counts. Thus, cells were always kept in the exponential phase of growth with almost-fresh media. This protocol was found to be very helpful in eliminating the stresses subjected to the cells during inoculation. In this manner the lag phase can always be eliminated and accurate kinetic data on hybridoma growth and metabolism can be obtained.

Cells in the exponential growth phase were centrifuged at 1000 rpm (200 g) for 10 min. They were then washed with fresh medium and distributed into 75 cm^2 T-flasks with 120 ml of media with a cell concentration of 10^5 cell ml^{-1}. A volume of 12 ml of this suspension was then taken into 120 ml of another fresh medium to get a cell concentration of 10^4 cell ml^{-1}. A similar procedure was applied sequentially for lower inoculum densities. Finally, 100 ml of each cell suspension was distributed into two 100 cm^3 spinner flasks (Bellco Glass, Inc., Vineland, NJ) to give 50 cm^3 working volume. The flasks were then placed on magnetic stirrers (Bellco) in a humidified CO_2 incubator at 37°C with 5% CO_2. The agitation rate for the spinner flasks was 100 rpm.

Static cultures using T-flasks were used to determine cell growth at very low (100 cells per ml) or very high (more than 10^5 cells per ml) concentrations. Very low cell concentrations (100 cell ml^{-1}) cell suspensions were distributed into several 25 cm^2 T-flasks in 10 ml volumes and one flask was sacrificed every time a cell count was performed. High cell concentrations (more than 10^5 cell ml^{-1}) were obtained by concentrating the cells via centrifugation. Cells from several 75 cm^2 T-flasks were combined after centrifugation at $200 \times g$. They were then washed with IMDM containing no serum. Finally they were suspended in IMDM containing no serum and distributed into several 25 cm^2 T-flasks in 10 ml working volume.

ITES medium

The ITES media were prepared from DME-F12 media (Sigma) and IMDM mixed in half and half (Table 1). Insulin, transferrin, and selenium were added from a mixture (Sigma). Ethanolamine and BSA also were added by the concentrations given in Table 1. The cells could grow in these media after a month of adaptation with good viability at a growth rate compared to that observed in 1.25% serum.

Conditioned medium

Conditioned media were prepared from the supernatant of cultures with 1.25% serum and with ITES medium. Cells were cultivated in 30 ml of IMDM with 1.25% serum (or in 30 ml ITES medium) for 4 d in exponential phase. The culture was then centrifuged at 1000 rpm for 10 min and the supernatant was collected. Supernatant portions of 12 ml were placed inside a dialysis membrane (molecular weight cut off 12,000–14,000) and dialyzed against 500 ml of fresh media with 1.25% serum (or fresh ITES). The dialysis was carried out at 4°C for 1 d.

Analytical methods

A one milliliter sample was taken twice a day during exponential growth, but once per day during the death phase. After performing cell counts, the samples were

centrifuged and the supernatants were stored at $-80\,^{\circ}C$ for subsequent determination of metabolite and monoclonal antibody concentrations. Viable and dead cells in suspension were counted using a hemacytometer. Trypan blue exclusion method was used to differentiate dead cells from viable cells. Cell counts for samples containing low cell concentrations were obtained using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Cell counts for the cultures inoculated with 100 cells per ml were done without dilution with isotonic buffer.

Glucose and lactate concentrations were measured using a Model 2000 Glucose/L-lactate analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). Ammonia was measured with an gas sensitive electrode (Orion, Boston, MA). Samples (100 μl) were mixed by 500 μl pH adjusting buffer solution (Orion) and the response was recorded.

Glutamine was measured by ammonia electrode after enzymatic reaction by Glutamase (Sigma), using a method described by Ozturk et al. (1989). The enzyme was kept at 25 U ml^{-1} in 0.005 M acetate buffer at pH 6. For the assay, the enzyme is diluted to 5 U ml^{-1}. A mixture of acetate buffer (40 μl, 0.1 M, pH 4.9), sample (50 μl) and the glutaminase (10 μl) was incubated at $37\,^{\circ}C$ for 15 min. Then 500 μl pH adjusting buffer solution was added and the response was measured by ammonia electrode. The ammonia in the samples was measured prior to the glutaminase reaction and the additional ammonia was contributed to glutamine. Further details of this method are described elsewhere (Ozturk et al., 1989).

Antibody, IgG_1, was quantified using an enzyme-linked immunosorbent assay (ELISA). Anti-mouse IgG (Sigma) was used to coat wells of plates at a concentration of 10 $\mu g\ ml^{-1}$ of PBS overnight at $4\,^{\circ}C$. The plates were washed with detergent solution (Triton X-100 in PBS) and BSA (1% in PBS) was applied to the plates for fixation. Aliquots of culture supernatant and purified MAb as standards were then added to the wells (diluted 200 times in 1% bovine serum albumin (BSA) in PBS). After 45 min incubation at room temperature, plates were washed and alkaline phosphatase-labelled affinity purified goat antibody against mouse IgG_1 (Southern Biotechnology Associates, Inc., Birmingham, AL) was added to the plates. After 45 min, the plates were washed and alkaline phosphatase substrate was added. The optical density at 405 nm of each well was recorded by ELISA reader (Molecular Devices, Palo Alto, CA).

Kinetic analysis of data

The results were quantified in terms of growth rates, and metabolic uptake and waste production rates. Two approaches were used for parameter estimation in the batch mode; the integral and the differential method (Levenspiel, 1972). The data was first analyzed by the differential method. The data was smoothed and the kinetic parameters, defined below, were evaluated as functions of time (transient kinetics). Then we determined the time span over which the kinetic parameters stayed constant. We took this time span to define the duration of exponential growth and we then applied the integral method to the data obtained during this period.

TABLE 1

Composition of the ITES media.

DME-F12	0.5 l
IMDM	0.5 l
Insulin	2.5 mg
Transferrin	2.5 mg
Selenium	2.5 ng
Ethanolamine	2.5 mg
BSA	5.0 g

The initial growth rates were calculated using the differential method. During the exponential phase we have that:

$$\frac{dX_T}{dt} = \mu X_v$$

where X_T and X_v are the total and viable cells, respectively, t is time, and μ is the growth rate. The growth rates calculated in this fashion remained constant over a period of time but eventually a decrease in growth rate was observed. The end of the exponential growth phase was defined as the time when the growth rate, determined by the differential method, began to decline.

The consumption and production rates were defined with respect to the viable cell counts and were determined using:

$$-\frac{dS_i}{dt} = q_i X_v \text{ (consumption)} \quad \frac{dP_i}{dt} = p_i X_v \text{ (production)}$$

where S and P are consumed substrate and product concentrations, respectively, and q and p are their specific consumption and production rates.

Ammonia and glutamine kinetics were determined by considering both the first order chemical decomposition of glutamine to ammonia and metabolic activity:

$$-\frac{d[\text{Gln}]}{dt} = k[\text{Gln}] + q_{\text{Gln}} X_v$$

and

$$\frac{d[NH_4^+]}{dt} = k[\text{Gln}] + p_{NH_4^+} X_v$$

The first order decomposition rate constants of glutamine in IMDM supplemented with different serum concentration have been reported by Ozturk and Palsson (1990). The value of k was independent of the serum concentrations, but very dependent on media pH. We used a k value of 0.0026 h^{-1} in our calculation that corresponds to a pH of 7.4, the initial pH of IMDM.

Monoclonal antibody production rates were calculated using an integral method.

$$\frac{dMAb}{dt} = q_{Ab} X_v \text{ or } MAb = q_{Ab} \int_0^t X_v \, dt \text{ if } q_{Ab} \text{ is constant}$$

where MAb is antibody concentration and q_{Ab} is the specific production rate, hence

obtained from the plot of antibody concentration (X_v) versus time integral of viable cells ($\int_0^t X_v \, dt$).

Results

The data obtained for cellular growth and metabolism of hybridoma cell line 167.4G5.3 is presented in Figs. 1, 2, and 3 for serum concentrations of 1.25%, 2.5%, and 5%, respectively. The experimental data presented was analyzed for the specific growth, consumption, and production rates and the results are presented in Table 2.

Growth kinetics

For all culture conditions examined, the viable cell concentrations increased in an exponential fashion, followed by the usual plateau and decline phases (Figs. 1A, 2A and 3A). Fig. 4A presents the maximum viable cell concentrations obtained from different initial cell densities. The initial cell density used influenced the maximum

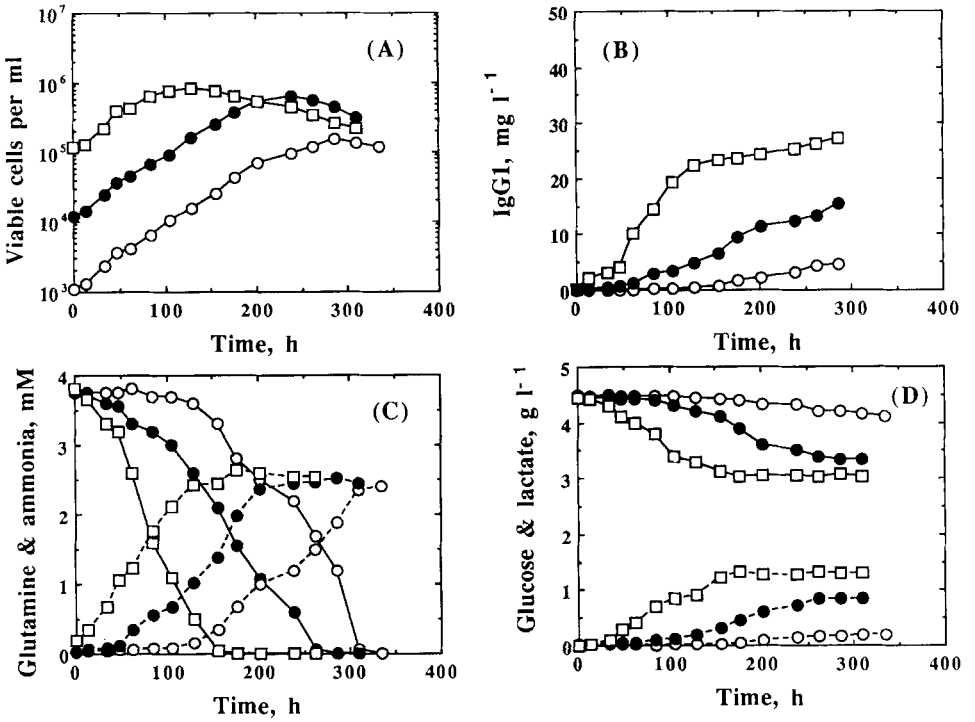

Fig. 1. Growth and cell metabolism of hybridoma cell line 167.4G5.3 in IMDM supplemented with 1.25% FBS. (A) Viable cell concentrations, (B) IgG_1 antibody concentration, (C) Glutamine (solid line) and ammonia (dashed line) concentrations, (D) Glucose (solid line) and lactate (dashed line) concentrations. Legend for initial cell concentrations: 10^3 cell ml^{-1} (open circle), 10^4 cell ml^{-1} (closed circle), and 10^5 cell ml^{-1} (open squares).

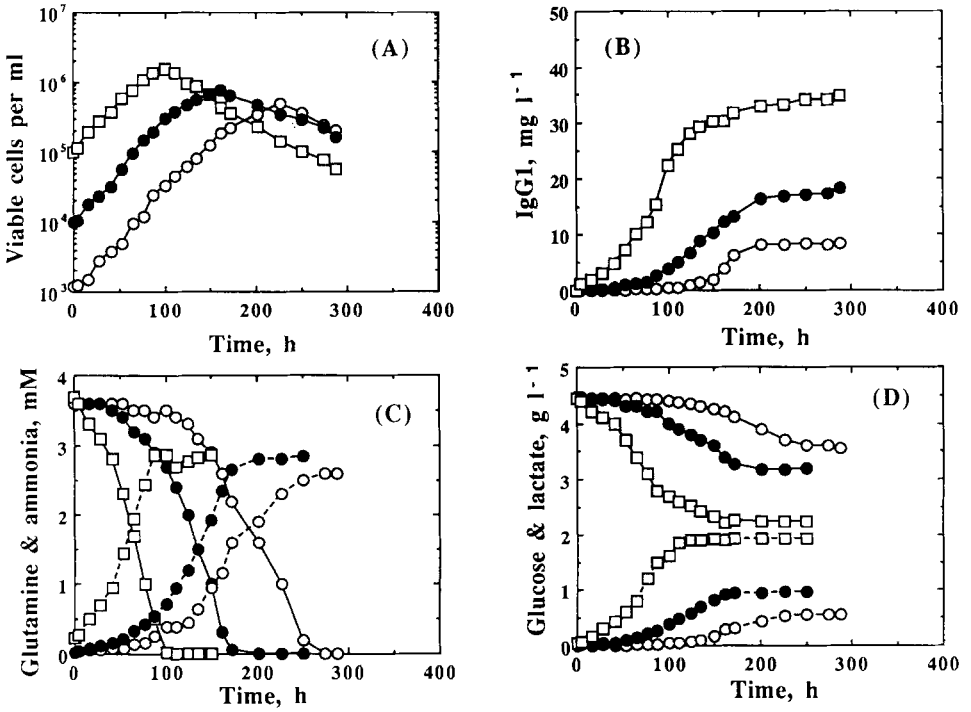

Fig. 2. Growth and cell metabolism of hybridoma cell line 167.4G5.3 in IMDM supplemented with 2.5% FBS. (A) Viable cell concentrations, (B) IgG_1 antibody concentration, (C) Glutamine (solid line) and ammonia (dashed line) concentrations, (D) Glucose (solid line) and lactate (dashed line) concentrations. Legend for initial cell concentrations: 10^3 cell ml^{-1} (open circle), 10^4 cell ml^{-1} (closed circle), and 10^5 cell ml^{-1} (open squares).

viable cell concentrations. Cultures with lower inoculum density resulted in lower maximum viable cell concentrations. Further, as seen in Fig. 4A, the serum level in the media is also important, determining the magnitude of these maxima. For each serum concentration, the maximum viable cell concentration was not directly proportional to the initial cell density. A ten-fold increase in initial cell density resulted in only about a 50% increase in viable cell concentration.

Although the cell concentrations are affected, the initial specific growth rates were not influenced significantly by the initial cell density, as seen in Table 2. For a given serum concentration, however, the cultures with the highest initial density (10^5 cells per ml) level had lower growth rates than those at 10^3 and 10^4 cells per ml. This reduction in growth rate is about 20% at serum levels of 2.5% and 5%. It is probably due to buildup of waste products, most likely ammonia, since the more dense cultures exhibit a much more rapid rise in the concentrations of waste products. Cells at 10^5 cell ml^{-1} experienced the highest ammonia concentrations during the exponential growth phase. In a separate study we have observed detrimental effects of ammonia on the growth of this hybridoma cell line and ammonia concentrations of 1 mM decrease growth rates by approximately 30%.

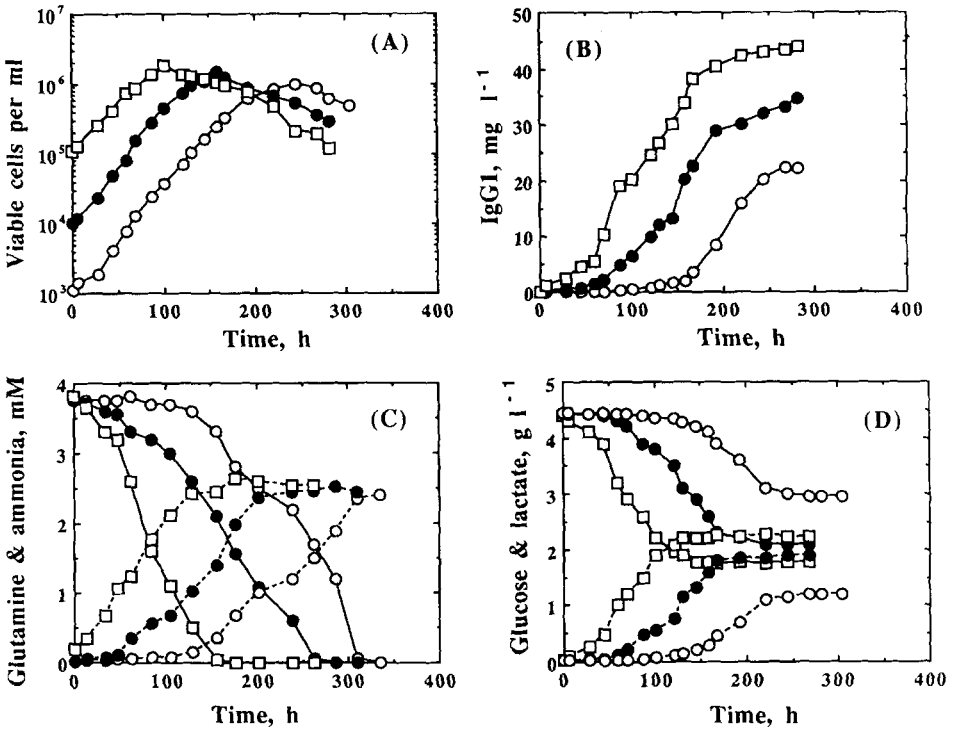

Fig. 3. Growth and cell metabolism of hybridoma cell line 167.4G5.3 in IMDM supplemented with 5% FBS. (A) Viable cell concentrations, (B) IgG_1 antibody concentration, (C) Glutamine (solid line) and ammonia (dashed line) concentrations, (D) Glucose (solid line) and lactate (dashed line) concentrations. Legend for initial cell concentrations: 10^3 cell ml^{-1} (open circle), 10^4 cell ml^{-1} (closed circle), and 10^5 cell ml^{-1} (open squares).

The dependence of the initial growth rate on the serum level is pronounced at low serum concentrations but levels off at high serum concentrations. This behavior is well described by a Monod equation (Fig. 5):

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{1}$$

where S is the serum concentration, μ_{max} and K_s are the Monod parameters – the maximum growth rate and the saturation constant, respectively. The parameters μ_{max} and K_m were determined by a double reciprocal plot of $1/\mu$ versus $1/S$ and their numerical values were obtained from the slope and the y-intercept. The curve so obtained for initial density of 10^4 cells per ml is also presented in Fig. 5. The numerical values were determined to be $\mu_{max} = 0.053\ h^{-1}$ and $K_m = 1.9\%$ v/v.

Effect of serum on the growth rate was also quantified in the form of the Monod equation by Glacken et al. (1988b); Dalili and Ollis (1989), and by us (Ozturk et al., 1988). Table 3 summarizes the kinetic parameters from these studies compared to

TABLE 2

Effect of initial cell density and serum concentration on growth rates, metabolism, and monoclonal antibody productivity of hybridoma cell line 167.4G5.3.

FCS % v/v	Initial cell density cells per ml	Growth rate, μ h^{-1}	Glucose utilization rate, q_{Glu} μmol per 10^6 cells per h	Glutamine utilization rate, q_{Gln} μmol per 10^6 cells per h	Lactate yield coefficient g g^{-1}	Ammonia yield coefficient mol mol^{-1}	Antibody production rate, m pg per cell per h
1.25	10^3	0.018 (0.003)	0.24 (0.05)	0.040 (0.010)	0.63 (0.08)	0.65 (0.09)	0.26 (0.05)
	10^4	0.021 (0.003)	0.20 (0.06)	0.035 (0.003)	0.73 (0.10)	0.68 (0.12)	0.19 (0.03)
	10^5	0.020 (0.002)	0.19 (0.04)	0.038 (0.008)	0.82 (0.13)	0.69 (0.10)	0.17 (0.05)
2.5	10^3	0.031 (0.002)	0.26 (0.07)	0.032 (0.009)	0.66 (0.08)	0.71 (0.09)	0.22 (0.04)
	10^4	0.032 (0.003)	0.24 (0.05)	0.028 (0.010)	0.79 (0.06)	0.74 (0.05)	0.19 (0.03)
	10^5	0.028 (0.003)	0.19 (0.05)	0.031 (0.008)	0.89 (0.09)	0.73 (0.09)	0.21 (0.04)
5	10^3	0.039 (0.002)	0.26 (0.03)	0.036 (0.011)	0.78 (0.11)	0.67 (0.08)	0.20 (0.05)
	10^4	0.037 (0.002)	0.27 (0.06)	0.031 (0.008)	0.71 (0.02)	0.73 (0.04)	0.19 (0.02)
	10^5	0.032 (0.003)	0.25 (0.05)	0.035 (0.005)	0.85 (0.05)	0.76 (0.11)	0.21 (0.04)
10	10^4	0.044 (0.002)	0.28 (0.06)	0.040 (0.009)	0.78 (0.11)	0.76 (0.13)	0.19 (0.06)

The parameters were calculated in the exponential phase as described in Materials and Methods. The values are the average of duplicate experiments with standard deviation in the parenthesis. Note that the experiments are carried out so that there is no initial lag phase, thus the initial and exponential growth rates are the same. The glutamine utilization and ammonia yield coefficients are corrected for the chemical decomposition of glutamine as described by Ozturk and Palsson (1990).

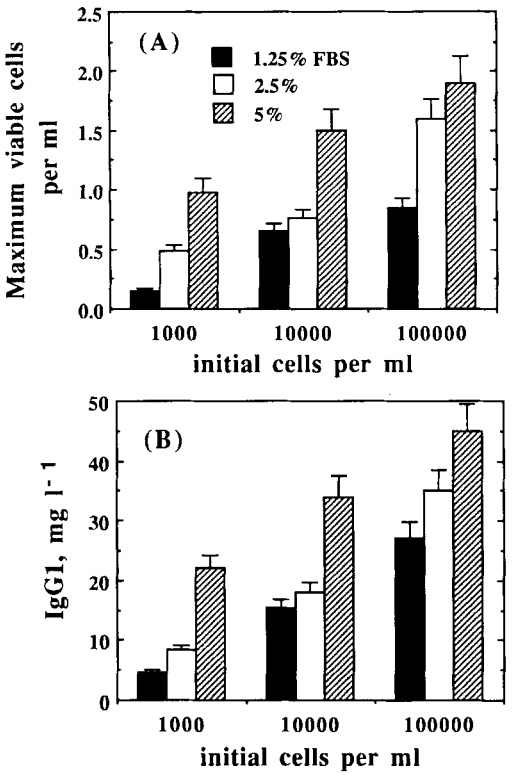

Fig. 4. (A) Maximum cell and (B) maximum IgG_1 concentrations for hybridoma cell line 167.4G5.3 in cultures with different serum levels and with different initial cell densities.

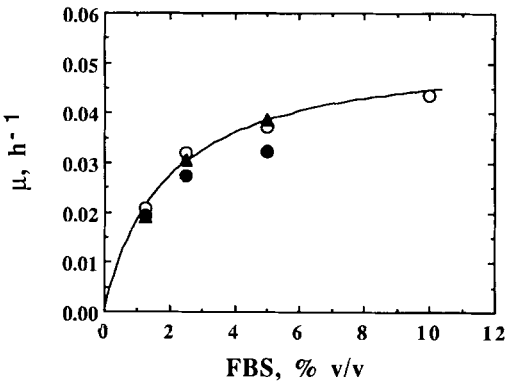

Fig. 5. Influence of serum on the specific growth rate at different initial cell densities. Legend 10^3 ml^{-1} (open circle), 10^4 cell ml^{-1} (closed circle), and 10^5 cell ml^{-1} (open squares). The solid line represents the Monod equation fit to the data with an inoculum density of 10^4 cell ml^{-1}. The values for the fitted parameters are $\mu_{max} = 0.053$ h^{-1} and $K_m = 1.9\%$ v/v.

TABLE 3

Kinetic parameters in Monod equation in describing the effect of serum on growth rate.

Cell line	K_s, % v/v	μ_{max}, h^{-1}	Reference
S3H5/γ2bA2	1.57	0.053	Ozturk et al. (1988)
CRL-1606	1.2	0.058	Glacken et al. (1989)
MRC Ox-19	1.59	0.038	Dalili and Ollis (1989)
167.4G5.3	1.9	0.053	This study

the values of present work. The differences in the values could be attributed to the differences in the cell line or in the serum quality.

Monoclonal antibody production

Monoclonal antibody concentrations increased gradually in all the batches following the viable cell count. More antibody was produced for the cultures with higher serum and higher initial cell concentrations. Antibody production continued into the decline phase of growth. The maximum antibody concentrations are presented in Fig. 4B and they correlate with the maximum viable cell count. The antibody concentration was not proportional to the initial cell density. A ten-fold increase in inoculum size resulted in only a two-fold increase in final antibody concentration (Fig. 4B).

The specific antibody production rate was analyzed using the integral method described in Materials and Methods and Fig. 6 shows the results. The specific antibody production rate, q_{Ab}, was evaluated from the slope of the curves in Fig. 6 and the numerical values are presented in Table 2. For each culture condition, we obtain essentially the same straight line indicating that the antibody production was proceeding at a constant rate. Further, the cells are producing antibody in all phases

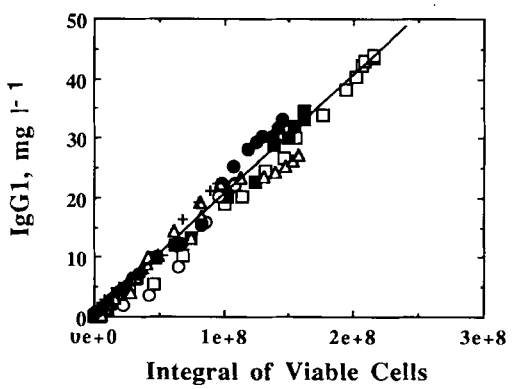

Fig. 6. The kinetics of antibody production for hybridoma cell line 167.4G5.3 in IMDM. Different symbols represent the data for different initial cell densities and serum concentrations; all of the conditions tested lead to the same (within experimental error) linear relationship between the monoclonal antibody concentration and the time integral of viable cells.

of growth, including the decline phase. The difference in specific production rates for the cultures with different initial cell density and the serum level are within the limits of experimental error.

Metabolism

When hybridoma cells are cultured, both glucose and glutamine are needed for cell growth and are utilized for energy production (Thilly, 1986; Miller et al., 1989). The metabolic utilization rates of glutamine and glucose depend on the cell line and the culture can be limited by either glutamine or glucose, or in some cases both.

The cultures were nutritionally limited by glutamine, as is evident from Figs. 1 through 3. All the glutamine was consumed by the cultures and the cessation of growth coincided with the time of glutamine depletion. The ammonia accumulation follows the consumption of glutamine. The final ammonia concentrations seem to be independent of both serum level and initial cell density, indicating that the overall ammonia yield on glutamine is constant. Glucose was also consumed in all the cultures. However, the pattern of glucose consumption was different from that of glutamine – only a portion of glucose was consumed and the extent of consumption was dependent on both serum level and initial cell density.

As seen from Table 2, the specific glucose and glutamine uptake rates were not influenced by the initial cell density. Neither were these parameters affected by the serum concentration. As the growth rate was influenced by changing the serum concentration, we can conclude that the specific uptakes of glucose and glutamine are not influenced by the growth rate. Apparently, the cells in high serum concentration use the energy generated from glucose and glutamine in a more efficient manner than the cells in low serum concentration.

It was shown that cultures with lower initial density led to lower maximum cell concentrations. When the cell yields are calculated (Table 4) we see that glucose yield is relatively constant for a given serum concentration. This is in accord with the specific glucose uptake rates. However, the glutamine yield calculated from maximum cell concentrations and the amount of glutamine consumed increases as the initial density increases. One reason for this would be more efficient glutamine utilization for cell mass. Another explanation would be the spontaneous chemical decomposition of glutamine in the cell culture media. The calculation of cell yield based on the total glutamine depletion is 'apparent' and it does not account for this decomposition. In the specific glutamine consumption rates we did consider the chemical decomposition and get a relatively constant glutamine consumption rate (Ozturk and Palsson, 1990). Hence the glutamine that chemically decomposes was excluded and actual cell yields from net glutamine were calculated (Table 4). The values for the actual yield are constant, in accordance with the specific glutamine uptake rates. The cultures with lower initial cell density consume less glutamine and most of the glutamine undergoes chemical decomposition. In fact, in the culture with 1×10^3 cells per ml in 1.25% serum, 88% of the glutamine disappears via chemical decomposition. These results emphasize the importance of glutamine decomposition and the inclusion of the decomposition rate constant in the determination of metabolic uptake rates of glutamine. The same holds for ammonia

TABLE 4

Effect of initial cell density and serum concentration on cell yields from glucose and glutamine.

FCS % v/v	Initial cell density cells per ml	Glucose yield 10^6 cells per μmol	Apparent glutamine yield 10^6 cells per μmol	Actual glutamine yield 10^6 cells per μmol	Glutamine chemically decomposed %
1.25	10^3	0.080	0.058	0.483	88
	10^4	0.105	0.125	0.597	79
	10^5	0.105	0.187	0.533	65
2.5	10^3	0.119	0.168	0.933	82
	10^4	0.133	0.275	1.146	76
	10^5	0.147	0.371	0.905	59
5	10^3	0.150	0.376	1.074	65
	10^4	0.137	0.465	1.193	61
	10^5	0.128	0.442	0.921	52
10	10^4	0.157	0.50	1.11	55

The glucose yield and the apparent glutamine yields were calculated as cells produced per substrate utilized. The actual, or true glutamine yield was calculated by considering the chemical decomposition of glutamine ($k = 0.0026\ h^{-1}$). Here only the amount that goes to the cell mass (total minus the decomposition) was used in the calculation for actual glutamine yield.

formation as the decomposition produces ammonia. In our analysis we included the decomposition and obtained the 'actual' metabolic rates.

The cell yield from glucose and glutamine increases with the serum concentration. We found that the metabolic rates are constant, although the growth rate is changing. Hence we can conclude that cells utilize these substrates at varying efficiencies.

The yield coefficients for lactate (from glucose) and ammonia (from glutamine) were also relatively constant. We have also observed that glucose and lactate concentrations correlate with each other, with regression coefficients more than 95% in all the cases. This indicates that glucose is the major supply of lactate and no or little lactate comes from glutamine. The glucose consumption and lactate production decrease in the decline phase; however, the yield coefficient of lactate from glucose was always the same in all the phases of growth. In fact, Fig. 7 shows the complete data for glucose and lactate, including that obtained during the decline phase. The figure also includes the data of all the batches. It can be seen from the figure that the yield coefficient is about 0.80 (± 0.1) g g^{-1} and independent of the phase of the growth, of initial cell density, and of serum concentrations.

Very low inoculum densities (100 cells per ml)

Experimental data was also obtained for inoculum densities of 10^2 cells per ml. The changes in substrate and waste product concentration at these low cell densities were not detectable using our assay methods and, hence, only growth rates could be evaluated. Fig. 8A shows the total cell concentrations for these cultures obtained by means of a Coulter counter. The viability could not be assessed at the early stages of growth; however, the initial viability was high (96%) at the beginning of the

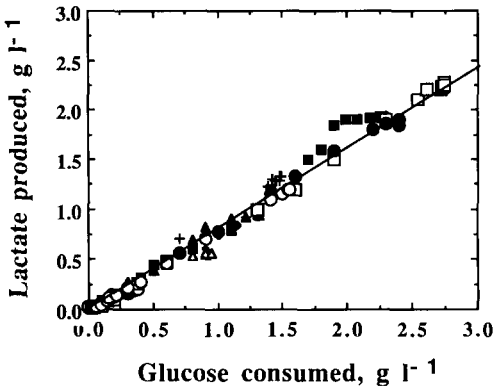

Fig. 7. The dependence of lactate production on glucose utilization. Different symbols represent the data for different initial cell densities and serum concentrations in all the phases of growth including the exponential and the death phase. The straight line corresponds to a yield coefficient of 0.80 g lactate per g glucose.

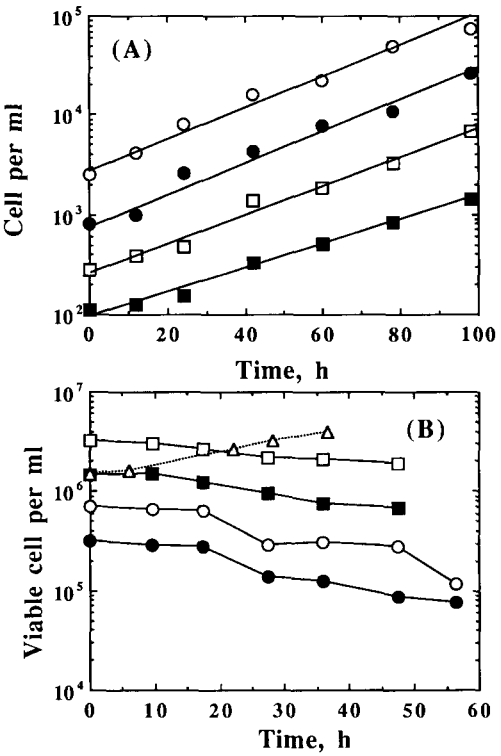

Fig. 8. Cell growth at very high and at very low cell densities. (A) Low density data with 5% serum; (B) High cell density data with zero serum. The dotted line on the high density data represents cell growth with 5% serum (control).

experiment and after 3 d of culture. Hence the growth rates from total cells could be considered to be the actual growth rates. The data show that the cells can grow at inoculum densities 10^2 cell ml^{-1} without significant changes in the growth rate. The growth rate for this inoculum size was 0.016 h^{-1}, which is only 20% lower than at the higher inoculum levels.

Very high inoculum densities (10^6 cells per ml)

It is known that some mammalian cells can produce their growth factors and it is believed that dense cultures provide a favorable environment for cell growth. At high cell densities, obtained in a perfusion mode, even the requirement for serum has been demonstrated to decrease (Heath and Belford, 1988).

To examine the hypothesis of cell growth promotion at high cell concentrations, 10^6 cell ml^{-1} – a high cell concentration in batch culture – were inoculated into media without serum. As shown in Fig. 8B, the cells could not grow without serum. The culture with 5% serum could grow up to 5×10^6 cells per ml under the same conditions. The results shown here indicate that a cell density of 10^6 cell ml^{-1} is not enough to support the growth and maintenance of the hybridoma cells used in this study in the absence of serum. Hence some of the growth requirements present in the serum are not produced by the cells at all, or if they are produced, the level is not high enough to support the growth. It should be pointed out that the cell concentration in the perfusion mode where the serum dependency is reduced, is always at least 10-fold higher than that obtained in batch mode.

Use of conditioned media

The inability to grow in media without serum, even at high inoculum densities, indicates that the cell line used may need certain factors appear to be essential for growth. The need for these factors is demonstrated by the above experiments. Besides the essential factors, these are also growth-stimulating factors that can be provided by the serum and/or by the cells. To assess how high the growth factor levels need to be for growth stimulation, we grew cells at low inoculum densities (10^4 cell ml^{-1}) in 1.25% serum with and without conditioned media. The results are shown in Fig. 9. The growth rate is not improved by the presence of conditioned media (we used spent media with 1.25% serum from cells grown into densities of 10^6 cell ml^{-1}). The growth factor content of the cultures grown in the conditioned media should be higher than for those grown in fresh media, but the growth rates are not enhanced. This indicates that the level of cell produced factors must be very low since the growth rates are sensitive to serum level at concentrations of 1.25% (Fig. 5). Hence the level of factors produced by this cell line is low and probably much less than their level in 1.25% serum, as no increase in their growth rate was observed with the conditioned media.

Use of ITES media

The above results suggest that our cell line produces a very low level of self-stimulatory growth factors. Serum contains both components that are required for growth as well as growth factors that could stimulate growth. The components in

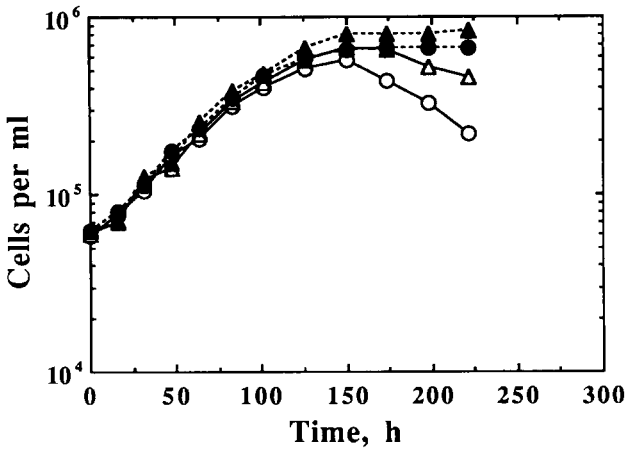

Fig. 9. Effect of conditioned media on growth when 1.25% serum level is used. Initial cell density is 2×10^4 cell ml^{-1}. Legend: fresh media (triangles) and conditioned media (circles). The total cell counts are represented by closed symbols and connected with dashed lines.

the latter category can be produced by the cells themselves and thus they can augment their own growth.

We could separate these two effects by growing the cells in ITES medium. With this medium the cells could grow as shown in Fig. 10. Under these conditions, we see that the initial cell density has a slight effect on the growth rate, Table 5. At initial densities of 10^3 cells per ml, we observe a growth rate that is approximately one half of that obtained with 1.25% serum. However, with higher initial cell densities of 10^4 and 10^5, we see a slightly accelerated growth (about 30% increase in

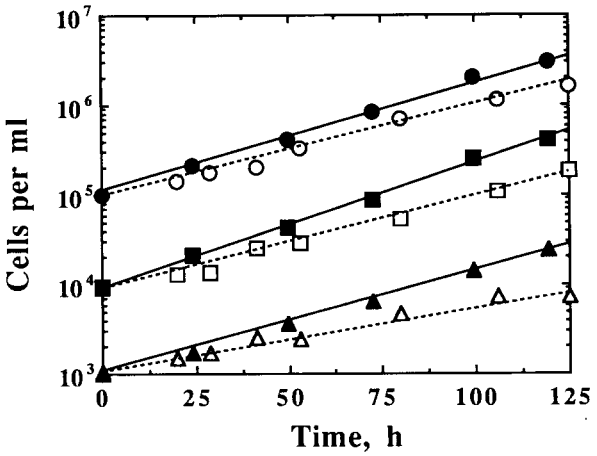

Fig. 10. The cell growth in fresh (open symbols-dashed line) and conditioned ITES media (closed symbols-solid line) at different initial cell densities.

TABLE 5

Effect of conditioned media on the growth of 167.4G5.3 hybridoma cells for ITES formulation. The growth rates tabulated correspond to the straight lines drawn in Fig. 10.

Initial cell density per ml	Growth rate, μ h^{-1} (control)	Growth rate, μ h^{-1} (conditioned media)
10^3	0.017 (0.003)	0.026 (0.002)
10^4	0.025 (0.003)	0.031 (0.002)
10^5	0.027 (0.002)	0.029 (0.003)

growth rate) which must be attributed to self-stimulatory growth factors produced by the cells themselves. This is a small, but significant, effect. Hence we can conclude that the hybridoma cell lines used in this study require very low amounts of autocrine factors. Such low sensitivity may not be observed for other hybridoma cell lines.

Figure 10 also shows growth in ITES media which have been conditioned (solid lines). For all three inoculum densities used, the conditioned media accelerate the growth slightly. Further, the 30% difference in the growth rates between the 10^3 cell ml^{-1} inocula and 10^4 cell ml^{-1} in fresh media is greatly diminished when conditioned media are used. These data further support the possibility that a low but significant amount of self-generated growth factor augments the growth rate of hybridoma cell lines.

Discussion

The assessment of the influence of initial density on hybridoma growth, metabolism, and monoclonal antibody productivity is important for several reasons. First, we would like to know the limits, if any, of inoculum size for cell growth in batch mode. It is important to know at which cell densities cells can support each other's growth and maintenance. Second, we would like to know whether the cell's metabolic, growth, and monoclonal antibody production patterns vary with cell densities. Third, the effects of initial cell density on growth and metabolic kinetic studies need to be determined. In the batch mode, the nutrient concentrations change with time and since the cells' metabolic and growth patterns are, in general, a function of medium composition, proper care should be taken. For instance, it is desirable to measure growth rates without any significant changes in medium composition. This can be achieved by employing low inoculum densities. On the other hand, changes in the medium composition are desirable for accurate measurements of metabolic activity. Hence experiments have to be carried out with different initial cell concentrations. Our results indicate that an inoculum density of 10^4 cell ml^{-1} is well suited to study both attributes.

The data presented here show that the maximum cell and antibody concentrations are determined by inoculum size. The changes in antibody concentration were due to changes in cell concentration as the specific antibody production rate was

not altered. As expected, high cell concentrations were obtained with high inoculum sizes. There was also an influence of glutamine decomposition. At low cell densities most of the glutamine disappeared via chemical decomposition. As the cultures were glutamine-limited this led to lower apparent cell yields from glutamine. The effects of inoculum size can be different under glucose-limited conditions in which all glucose would be incorporated into the cell mass. We have observed that serum or serum substitutes are essential for growth of murine hybridoma cell line 167.4G5.3. Small, but, detectable amounts of cell-derived growth factors stimulated cell growth rate in medium supplemented with insulin, transferrin, ethanolamine, and selenium (ITES). At high cell densities such as those attained in immobilized reactors, these factors can reach higher concentration levels. A detailed study is needed to characterize the cell-derived growth factors and their influence on cell growth.

It has been shown that the initial cell density has no significant effect on growth, metabolic and antibody production rates for the hybridoma cell line used over the range of conditions we investigated. Our cell growth rate data is consistent with the results of Takazawa et al. (1988). The growth rates were influenced by serum concentration and this effect was mathematically described by a Monod expression. Similar data were reported in the literature for other cell lines (Glacken et al., 1989; Dalili and Ollis, 1989). The Monod-type influence of growth by serum indicates that serum has a kinetic limitation at low concentrations. We found that the monoclonal antibody production rate is constant not only through the course of batch run but also for different serum concentrations and initial densities. The antibody production therefore appears to be non-growth associated and constant over the experimental conditions considered in this study. This result indicates that cells are acting as a catalyst for the production and the higher the cell level the higher the antibody concentration. The influence of initial cell density on metabolic and antibody production rates could not be compared as there exist no data in the literature.

Acknowledgements

This work was supported by National Science Foundation Grant No. EET-8712756. The authors thank Dr J. Latham Claflin for providing the hybridoma cell line used in this study.

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