The Production of Monoclonal Antibody in Growth-Arrested Hybridomas Cultivated in Suspension and Immobilized Modes

Douglas B. Seifert† and J anice A. Phillips*

Department of Chemical Engineering, Lehigh University, Bethlehem, Pennsylvania 18025

The effects of the microenvironment and the nature of the limiting nutrient on culture viability and overall MAb productivity were explored using a hybridoma cell line which characteristically produces MAb in the stationary phase. A direct comparison was made of the changes in the metabolic profiles of suspension and PEG-alginate immobilized (0.8 mm beads) batch cultures upon entry into the stationary phase. The shifts in glucose, glutamine, and amino acid metabolism upon entry into the stationary phase were similar for both microenvironments. While the utilization of most nutrients in the stationary phase decreased to below 20% of that in the growth phase, antibody production was not dramatically affected. The immobilized culture did exhibit a 1.5-fold increase in the specific antibody rate over the suspension culture in both the growth and stationary phases. The role of limiting nutrient on MAb production and cell viability was assessed by artificially depleting a specific nutrient to 1% of its control concentration. An exponentially growing population of HB121 cells exposed to these various depletions responded with dramatically different viability profiles and MAb production kinetics. All depletions resulted in growth-arrested cultures and nongrowth-associated MAb production. Depletions in energy sources (glucose, glutamine) or essential amino acids (isoleucine) resulted in either poor viability or low antibody productivity. A phosphate or serum depletion maintained antibody production over at least a six day period with each resulting in a 3-fold higher antibody production rate than in growing batch cultures. These results were translated to a high-density perfusion culture of immobilized cells in the growth-arrested state with continued MAb expression for 20 days at a specific rate equal to that observed in the phosphate- and serum-depleted batch cultures.

Introduction

A primary objective for the production of monoclonal antibodies (MAb) on the industrial scale is sustained productivity at high cell densities. There are many examples of fed-batch strategies to maintain the cell population for as long as possible (Duval, et al., 1992; Bibila and Robinson, 1995) and of modes to extend productivity in perfusion bioreactors (Tolbert, et al., 1985; Trampler, et al., 1994; Banik and Heath, 1995). For example, reducing serum to low levels for protein production in perfusion mode has been practiced for decades (Kruse, et al., 1969). MAb production is often sustained and, in many cases, enhanced in slow-growing or growth-arrested cultures. However, these findings, while prevalent, are not universal as reports of productivity in the stationary phase (i.e., nongrowth-associated MAb production) seem to vary with cell lines and culture conditions (Merten, 1988; Suzuki and Ollis, 1990; Miller et al., 1988; Al-Rubeai, 1992). De la Broise et al. (1992) describe a "grow or die" behavior of hybridomas in perfusion cultures which would suggest that higher productivity could not be achieved in a growth-arrested state for such cultures. If we could predict which cell lines or clones would be most productive in the nongrowth state and identify the appropriate environment for maintenance of viability, MAb productivity could be extended with concomitant economic benefits.

Cell immobilization has been suggested as a vehicle to protect cells for sustained viability and enhanced productivity (Lee, et al. 1993, 1994; Shen et al. 1994; Yamaguchi, et al., 1997). It is not clear if the enhanced productivity in immobilized cultures is due to decreased growth rates or to a metabolic shift attributed to the immobilized culture's microenvironment. For example, local levels of autocrine factors may be higher in the immobilized culture resulting in a different metabolic response. Possibly, the microenvironment can also play a role in sustaining productivity in the stationary phase.

Increased MAb synthesis in the G1 phase of the cell cycle has been proposed to explain the enhanced productivity of slow or growth-arrested cell populations based on cell cycle control (Suzuki and Ollis, 1990; Hayer et al., 1992; Al-Rubeai et al., 1992). Normal cells can be growth-arrested in the G1 phase by either mitogenic depletion or metabolic nutrient depletion, and these arrested states can be biochemically delineated by their metabolic consequences (Moses, et al., 1980; Basega, 1985). Unlike normal cells, transformed cell lines do not require mitogenic stimulation and their degree of cell cycle control varies with the method of transformation (Dubrow, et al., 1979). Cells transformed by DNA viruses, such as SV40, adenovirus, or HPV, will not G1-arrest due...
to viral inactivation of p53 and retinoblastoma (Rb) host proteins; these cells will continue progression through the cell cycle until nutrient depletion causes failure of cellular functions and cell death, a process often referred to as necrosis (Almansan, et al., 1995). Cells transformed by RNA viruses, chemicals, or a spontaneous event will G1-arrest due to nutrient depletion but not mitogenic depletion. In practice, protein-free media has been successfully used to grow hybridomas (Fike, et al., 1991), indicating no mitogen dependence for cell cycle progression, and hybridomas have been shown to G1-arrest (Miyatake, et al., 1995).

Nucleotides and metabolic nutrient availability are known to be linked to the G1-arrest point, which suggests interference with protein synthesis (cyclin accumulation) and/or the surveillance activity of p53. Interestingly, Almansan, et al. (1995), found that, for fibroblasts cells, DNA damage triggered p53-derived apoptosis while nutrient limitations produced a reversible G1-arrest. When a cell is G1-arrested, the environment must be able to sustain maintenance functions or the culture would quickly degrade to cell death by either necrosis or apoptosis. For hybridomas, both necrosis and apoptosis have been implicated in the decline in viability and lack of MAb productivity in some growth-arrested cultures (Singh et al., 1994; Mercille and Massie, 1994). A known inhibitor of apoptosis, the bcl-2 protein, is expressed at various levels in cell lines (Hockenbery et al., 1993), and overexpression of bcl-2 in hybridomas has been shown to increase MAb productivity through inhibition of apoptosis (Simpson, et al., 1997). Since hybridomas, like other transformed cells, vary in their loss of p53 regulation (cell cycle control) and bcl-2 expression levels, each cell line's response to a growth-arrested state would be expected to vary. Therefore, the ability to sustain viability (maintenance functions) and MAb production would also be expected to vary from one cell line to another, as amply illustrated in the literature cited above. Screening cell lines for sustained viability and MAb production under conditions which produce a slow-growing or growth-arrested culture may be a rapid way to determine if a particular cell line's productivity can be extended into the stationary phase.

This paper focuses on the transition to a growth-arrested state to contribute to our understanding of varied performance of hybridoma cells lines upon entry into the stationary phase. The hybridoma used in these studies, HB121, produces MAb in the stationary phase. Metabolism and MAb production for this cell line was investigated with two experimental approaches. First, a direct comparison of suspension and immobilized batch cultures was made with emphasis on the nutrient and metabolic byproduct profile changes upon entry into a growth-arrested state. The objective of this comparison was to discern if the cellular response as measured by metabolic parameters and MAb productivity would be similar for two distinct microenvironments. Second, specific nutrients were artificially depleted to a maintenance level in batch cultures to determine their effect on viability and MAb expression. This methodology provides a simple, rapid screening tool to determine which nutrient limitations result in sustained viability and MAb production and if the cell line is highly susceptible to the apoptotic pathway with limited ability to remain productive in a growth-arrested state. As an extension of this approach, a high-density perfusion culture of immobilized cells was growth-arrested with continued MAb expression for an additional 20 days. While this study focused on HB121, the results may find applicability with other hybridomas.

Materials and Methods

Cell Line. The ATCC murine hybridoma cell line HB121 used in these studies was derived from a P3 × 63Ag8.653 myeloma parent line hybridized with splenocytes of BALB/c mice. HB121 was banked over liquid nitrogen and routinely subcultured in Dulbecco's modified Eagles medium (DMEM) with 3% fetal bovine serum (FBS) and 9% horse serum (HS) without the use of antibiotics. DMEM from JRH BioSciences (Lenexa, KS) was formulated from powder, and growth kinetics were monitored with the use of cell counts. HB121 contains the anti-IgE monoclonal antibody produced by hybridoma HB121 was detected by an enzyme-linked immunosorbant assay composed of an antihuman IgG (Fab specific) goat antibody coated on the surface of NUNC Immulon 1 96 well plates and an antimouse IgG (Fc specific) goat antibody-horseradish peroxidase conjugate. All immunochemicals were purchased from Sigma Chemical Co.

Metabolic Assays. Glucose was measured using a YSI Glucose Analyzer, model 23 (Yellow Springs Instrument Co., Yellow Springs, Ohio). Lactate was quantified enzymatically using a Sigma Diagnostics Kit Model 826UV. Ammonia was measured with an Orion membrane electrode probe, Model 95-12 (Fisher Scientific), after adjustment of the sample pH to above 11 to produce gaseous ammonia. Ammonia was pre-derivitized with dansyl chloride and quantified on a Microprobe C18 reverse-phase column (Rainin Instrument Co., Woburn, MA) using 12 mM pH 6.5 Na2HPO4 with 4 v/v% formamide and acetoni tre with 4 v/v% formamide as the mobile phase in a programmed gradient. Norvaline, a nonphysiological amino acid, was used as an internal reference standard. Cysteine and aspartic acid could not be resolved with this method.

Cell Entrapment in PEG-Alginate Beads for Batch and Perfusion Studies. Cells were immobilized in a modified alginate system which used polyethylene glycol to create a network of macro pores within the bead for high-density growth and minimal cell leakage. The development of this system, procedures for immobilization in batch and perfusion modes, and the method to dissolve the beads with sodium citrate to quantitatively release viable cells are described elsewhere (Seifert and Phillips, 1997). For both the batch and perfusion cultures, the cells were entrapped in uniform 0.8 mm diameter beads created by destabilization of a viscous liquid jet with a controlled vibrational disturbance. For the perfusion run, the beads were produced in a 2 L multiple nozzle bead former (Seifert, 1989) and transferred to 2 L Celligen™ bioreactor (New Brunswick Scientific, New Brunswick, NJ) for perfusion culture. The initial concentration was 2 × 10^6 cells/g of bead (after 35% shrinkage in volume by bead curing) which, at a bead loading of 19%, corresponds to 3.4 × 10^6 cells/mL bioreactor working volume. The temperature was gradually increased to 37 °C and the pH controlled at 7.3. Perfusion was initiated at a rate of 10 mL/h and increased throughout the cultivation to
maintain a rate of 10 mL/10^9 cells/h. At 230 h the perfusion medium was switched to the nongrowth medium containing 0.1 v/v% serum. The perfusion rate was maintained at 2.4 bioreactor volumes per day for an additional day to wash out the growth media before being reduced to 1.2 bioreactor volumes per day; this perfusion rate was maintained for the subsequent 20 days of culture. Samples for metabolic monitoring, antibody titer, and cell concentration were taken daily.

**Alginate-Entrapped Cell-Counting Procedure.** To determine the net bead weight in a sample, we used a macroscreen-covered 15 mL centrifuge tube to gently remove the liquid contents by vacuum. The beads were then dissolved with the addition of 5 mL of 100 mM pH 7.4 sodium citrate and 5 mL of Dulbecco's phosphate-buffered saline (PBS). The cell concentration was determined by hemocytometer and viability by trypan blue exclusion. Assuming a bead density of 1.03 g/cm^3, we converted cell densities to cells per milliliter of bead, which are the typical units reported in the literature.

**Nutrient Depletion Studies.** Two growth-arrest experiments were conducted. Each involved growing a culture to mid-exponential phase, washing the cells, and resuspending the cells at the same cell density with one nutrient concentration lowered significantly below the control level to cause the cells to arrest in a nongrowth state. A custom DMEM growth media from JRH Biosciences (Lenexa, KS) without glucose, glutamine, isoleucine, or phosphate was used for the depletion studies. Rollertops were chosen for this study to provide a low-shear, relatively well-mixed environment with adequate volume for sampling for flow cytometry studies. A repeated fed batch strategy was used in which the sample volume (10% of the total volume) was replaced with the same volume of the medium used to initiate the experiment (i.e., the depleted nutrient concentration was 1% of the control). The low molecular weight nutrients contained in serum were removed by dialysis to allow accurate reductions of the selected nutrients to 1% of their control level. The 90 v/v% horse serum, 10 v/v% fetal calf serum mixture was dialyzed (1000 MWCO) against a Dulbecco's PBS solution, for which the phosphate components had been replaced with 2 mM pH 7.4 HEPES buffer. The dialysis solution was stirred at 4 °C for 36 h, filter-sterilized, and stored at 4 °C.

The inoculum for the 5 roller bottles was grown to mid-log phase in a 500 mL working volume Techne spinner flask. At harvest, the cells were washed twice in DMEM without additional nutrients and resuspended to 5 × 10^6 viable cells/mL for inoculation of the roller bottles. A 500 mL medium preparation, consisting of 10% dialyzed serum and 90% DMEM with the appropriate nutrient reduced to 1% of the control, was prepared for each depleted nutrient and the control. These solutions, stored at 4 °C, were used for inoculation of the roller bottles and all subsequent medium additions. Each roller bottle contained 90 mL of the appropriate medium formulation and 10 mL cell inoculum, resulting in an inoculation density of 5 × 10^5 viable cells/mL. The roller bottles were gassed with CO_2 to obtain an initial pH of 7.4 and incubated at 37 °C at a rotation speed of 3 rpm. During each sampling, the pH was measured, a homogeneous 0.5 mL sample was withdrawn for cell counts, and the remaining suspension was centrifuged at 150g for 5 min. For flow cytometric analysis, cells were washed in cold PBS, fixed in cold ethanol, and stored at 4 °C. The pH of the roller bottles was adjusted by CO_2 gassing to maintain the pH in the range of 7.2–7.4.

**Flow Cytometry.** The cells were prepared for flow cytometric analysis by staining with acridine orange (AO) (Darzynkiewicz, 1982). Ethanol-fixed cells were washed in cold PBS and treated with an acidic Triton X-100 solution to disassociate the RNA into single strands. The Electro Pure AO (Polysciences, Warrington, PA) solution was then added to differentially stain the double-stranded DNA and single-stranded RNA. An Epic V sorting flow cytometer (Coulter Electronics, Inc., Hialeah, FL) with an argon laser excitation wavelength of 488 nm was used to measure DNA and RNA levels. For AO staining, a 560 nm dichroic filter split the emission wavelengths; a 525 nm band-pass filter was used for the green fluorescence emission (DNA), and a 590 nm long pass filter was used for the red fluorescence emission (RNA). The instrument was routinely calibrated for AO, with a 20 μg/mL concentration being optimal for the instrument.

**Results**

1. **Suspension and Immobilized Batch Culture.** The batch culture studies focused on two objectives: quantifying cell metabolism at the transition to a growth-arrest state and comparing suspension and immobilized cultures. Since shear sensitivity has been reported in growth-arrested cultures, a low-shear bioreactor, the roller bottle, was employed in this study. The immobilized system consisted of small macroporous PEG-alginate beads, 0.8 mm in diameter, to minimize diffusional limitations. This PEG alginate system, described elsewhere (Seifert and Phillips, 1997), creates a network of radial pores within the alginate bead for high cell density growth through the interaction of PEG with the alginate during gelation. The PEG-alginate system also minimized cell leakage from the bead (<10^4 cells/mL in bulk medium), which is a prevalent problem in conventional alginate immobilizations.

**Growth and MAb Production.** The growth and monoclonal antibody profiles for hybridoma HB121 in replicate suspension and immobilized cultures are given in Figure 1. Both cultures reached a similar saturation density of 1.5 × 10^9 cells/mL, suggesting that no growth limitations occurred within the beads. The maximum growth rate of the suspension culture of 0.05 h⁻¹ was attained within the first day while the immobilized culture exhibited a 3 day lag phase before exponential growth. The transition to the nongrowth phase was chosen as the inflection point in the growth profile, corresponding to about 80 h for the suspension culture and 130 h in the immobilized culture. The subsequent analysis of metabolic rates will focus on the changes which occur at this transition point indicative of growth-arrest behavior. Antibody accumulation continued into the nongrowth phase in both cultures, reaching the same level at 270 h.

Data from replicate roller bottles were compiled to compare MAb production rates before and after the transition point using the nongrowth-associated model described by eq 1:

\[ P = \beta \int x dt \]  

The nongrowth-associated MAb production rates, \( \beta \), are listed in Table 1. Based on the 95% confidence intervals, the production rates calculated for the growth, nongrowth, and combined data for both suspension and immobilized cultures are identical, indicating no perceptible change in antibody synthesis rate per viable cell.
upon transition to the nongrowth phase. However, the specific MAb production rate in the immobilized culture was 1.5–1.8-fold higher than the suspension. Since the MAb production rate did not significantly change upon entering the stationary phase for these cultures, the responses of other metabolic markers at the transition point event were measured and compared as described below.

Glucose and Lactate Profiles. The glucose and lactate profiles, illustrated in Figure 2, show a characteristic glycolytic oxidation of glucose to lactate with apparent molar conversion rates of 0.79 and 0.77 for the suspension and immobilized cultures, respectively. At the transition point in the growth profile, the glucose utilization rate dramatically decreases in all cases; lactate production ceases and lactate is consumed in the suspension culture, as has been reported for other hybridoma cultures (Bree, et al., 1988).

Glutamine Metabolism. The profiles of glutamine and associated metabolites are illustrated in Figure 3. At the transition point, glutamine is almost completely exhausted in both the suspension and the immobilized cultures, suggesting a possible cause for the growth-arrest. A rapid shift in ammonia accumulation coincides with the exhaustion of glutamine, which would be expected given that the deamination of glutamine to glutamate is required to catabolize glutamine via the TCA cycle at α-ketoglutarate. In many cell lines, glutamate is converted to α-ketoglutarate through the transamination of either pyruvate to alanine or oxaloacetate to aspartate. Since alanine accumulation parallels glutamine depletion while aspartate accumulation was negligible, HB121 appears to use alanine aminotransferase to catabolize glutamine. Interestingly, glutamate accumulation occurred after the transition point, suggesting that glutamine may not have been limiting the culture; instead, aerobic catabolism may have been significantly curtailed as a growth-arrest response. The profiles for glutamine metabolism for both the suspension and immobilized cultures are quite similar except for the steady, albeit slow, accumulation of ammonia in the arrested phase of the immobilized culture. This is hypothesized to be due to the hindered release of the NH₄⁺ from the negatively charged alginate bead matrix.

Amino Acid Metabolism. Amino acid profiles in Figure 4 illustrate the abrupt change in utilization of certain amino acids at the transition point. While lysine, histidine, and methionine show a down regulation of their rates, glycine accumulates in both suspension and immobilized cultures during the stationary phase. Glycine accumulation in growth-arrested cultures has been reported for other cell lines (Butler and Thilly, 1982) as well as hybridomas (Reid, et al., 1987). Since glycine plays an important role in the C₁ pool for de novo purine

Table 1. MAb Specific Rates Calculated from the Nongrowth-Associated Model for Suspension and Immobilized Cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>growth phase</th>
<th>stationary phase</th>
<th>entire culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>titer/MM cells/day</td>
<td>correlation coefficient</td>
<td>titer/MM cells/day</td>
</tr>
<tr>
<td>suspension</td>
<td>96</td>
<td>0.937</td>
<td>92</td>
</tr>
<tr>
<td>immobilized</td>
<td>170</td>
<td>0.969</td>
<td>140</td>
</tr>
<tr>
<td>immobilized to suspension culture ratio</td>
<td>1.8</td>
<td>1.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*a The model was fit for the growth phase data, stationary phase data, and the entire culture data set. The correlation coefficient from linear regression is provided for each data set. The ratio of the immobilized rate to the suspension rate is also provided.
synthesis, this may indicate a reversal of this pathway in response to entry into an arrested state where nucleotides are no longer in demand.

In contrast to those illustrated in Figure 4, the amino acids profiled in Figure 5 show no clear inflection point at the transition point. The branched chained amino acids leucine and isoleucine were consumed to exhaustion in all cultures. Valine, another branched chain amino acid (data not shown), was heavily consumed in a similar manner but was not exhausted during the cultivations. Branched chained amino acids have been reported as energy sources for other cell lines (Thilly, et al., 1986), entering the TCA cycle at either acetyl CoA (leucine, isoleucine) or succinyl CoA (valine and isoleucine). The lack of an inflection point suggests that the decrease in the rates after the transition to nongrowth is probably more attributable to the low concentrations than a response to arrested growth.

**Metabolic Rate Changes at the Transition Point.**

The change in metabolic rates of various nutrients and metabolites at the transition point was determined by the differential rate method. The specific kinetic rates over about a 48 h period before the transition point (3 data points) were compared to the rates over about 72 h after the transition point (3 data points). The rates were calculated for each culture individually by determining the slope between each time interval and averaging the results. Note that the calculations were also done at 24 h before (2 data points) and 48 h after (2 data points) the transition point with similar results, indicating that the rate change reflects an abrupt change at the inflection point and not gradual rate changes over time. The average specific rates from replicate cultures are tabulated in Table 2 for the growth phase; the stationary phase rates were calculated as a percentage of the growth phase rate. The standard deviations are below 10% in most cases, indicating the consistency between the replicates.

Most of the nutrients, which were far from being exhausted, showed a greater than 80% decrease in utilization rate upon entering the stationary phase. Interestingly, three components stand out on this list: Glutamate and glycine accumulated substantially in the growth-arrested phase and the MAb production decreased by 33% and 40% for the suspension and immobilized cultures, respectively. There was no statistically significant difference between the MAb production
rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.

As illustrated in Figure 6, the change in the specific rates upon entering the stationary phase is strikingly similar for the suspension and immobilized cultures. The rates calculated for the growth and nongrowth phases using the integral method. Comparing Tables 1 and 2 shows that the two kinetic analyses yield similar values. The MAb rates for the growth and stationary phases of the suspension culture were 96 and 92 titer/million (MM) cells/day for the integral method and 120 and 80 titer/MM cells/day for the differential method, respectively. Similarly, the MAb rates for the growth and stationary phases of the immobilized culture were 170 and 140 titer/MM cells/day for the integral method and 180 and 106 titer/MM cells/day for the differential method, respectively.
other cell lines and as a nitrogen source for the de novo biosynthesis of nucleic acids. Glucose supplies another precursor for nucleotides, the ribose moiety, through the oxidative arm of the pentose pathway in addition to its use as an energy source. Phosphate ions are utilized in signal transduction, energy metabolism, de novo nucleic acid synthesis, and membrane synthesis. Serum depletion in a hybridoma culture is not expected to result in a G1-arrest due to growth factor depletion. However, the depletion of low molecular weight components of serum, such as lipid precursors and trace elements, which are often bound to carrier proteins, would be expected to cause a G1-arrest.

Note that the conditions used for the depletion studies described here do not provide the opportunity for the selection of a genetically instable population with poor MAb production. Once the exponentially growing cells are placed in a medium sufficiently depleted in a key nutrient to arrest growth, there is negligible replication and, thus, no opportunity for outgrowth of variants under the stressed conditions.

Viability and Antibody Production in Growth-Arrested Cultures. In an initial screening study, depleting glucose to 2.1% of the control level (with 1.1% contributed by the serum) resulted in significant viability losses. Therefore, no further work on glucose depletion was pursued. Under all other depletion conditions in nondialyzed serum, significantly higher viabilities were obtained. Modest cell growth (1–2 population doublings) was seen with 1% serum (10% of the control) and a phosphate depletion to 18% of the control, illustrating that depletions of these nutrients to lower levels are required to growth-arrest the HB121 cells. In the next study, cells in the exponential phase were planted into roller bottles with full growth medium (the control) or medium where one of the four nutrients (isoleucine, glutamine, phosphate, and serum) was depleted to 1% of the control level. The serum used in all cultures was dialyzed to accurately control the concentration of the depleted component. Ten percent of the culture was sampled daily and replaced with an equal amount of the same medium used to initiate the culture. As shown by the profile of total cell counts in Figure 7, there was no appreciable increase in total cells under any of the depletion conditions examined. Since the values for total cells reported accounts for all cells removed during sampling, it can be concluded that the cultures were successfully growth-arrested. In comparison, the control culture grew to a final concentration of $2.3 \times 10^6$ total cells/mL; this is higher than the $1.5 \times 10^6$ total cells/mL achieved in the suspension roller bottle batch culture of the previous run, indicating that the procedure itself or the use of dialyzed serum did not inhibit growth and that refeeding to replace medium from sampling supported a higher density.

Culture viabilities and antibody titers for each of the nutrient-depleted conditions are given in Figure 8. Serum depletion, as shown in Figure 8a, resulted in the maintenance of over 70% viability for the first 200 h and the accumulation of more antibody than in any of the other cultures. The depletion of glutamine, depicted in Figure 8b, resulted in 75% viability after the first 150 h but with the lowest antibody production of all the cultures. The depletion of isoleucine, depicted in Figure 8c, resulted in a rapid loss in viability and antibody production, with only 25% of the population viable after 150 h. Finally, as illustrated in Figure 8d, the depletion of phosphate resulted in a drop in viability to under 50% in the first 150 h, followed by a stabilization of the population at
about 40% viability for the remaining 6 days. This behavior contrasts with that seen in a culture in an earlier study in which phosphate was depleted to 18% of that in the control: In this phosphate-depleted culture, cells grew from $2 \times 10^5$ to $8 \times 10^6$ cells/mL in 50 h and remained 84% viable after 80 h. As shown in Figure 8, the antibody accumulation under phosphate depletion was 3-fold that obtained under isoleucine depletion and approached the final titer of the serum depletion culture.

**Cell Cycle Analysis of the Growth-Arrested Cultures.** Samples taken throughout the cultivations were analyzed by flow cytometry for DNA and RNA simultaneously using acridine orange. The flow cytometry results are plotted in Figure 9 for the four depletions. The control (not shown) had a typical G1/G2 distribution during the growth phase.

Both the serum and glutamine cultures were predominantly a G1 phase population indicative of a G1-arrest. The RNA channel number corresponding to the G1 peak in an RNA histogram for the serum depletion was the same as that seen for the exponentially growing cells, indicating no significant decrease in ribosomal mass, which makes up about 80% of the signal. In contrast, the RNA channel number corresponding to the G1 peak for the glutamine depletion was 18% lower than the value for the serum depletion, indicating a reduction in the ribosomal machinery for antibody synthesis. Curiously, the DNA channel number for the glutamine depletion culture was also about 20% lower than the expected G1 value. This may be indicative of an apoptotic culture in which DNA was being degraded and that the viable cell population might be lower than that indicated by trypan blue exclusion. This may explain the poor antibody productivity per “viable” cell observed for this culture.

In sharp contrast to the serum and glutamine results, a significant portion of the cells in the isoleucine- and phosphate-depleted cultures, depicted in Figure 9c,d, were not in the G1 phase. Since the total cells did not increase for either culture, the DNA/RNA distributions do not represent cycling populations. Instead, the cells appear to be arrested randomly in the cycle. For isoleucine, the decreased signal with time corresponds to the viability loss seen for this culture. Cells which were cycling before the depletion apparently could not complete the cycle to enter G1, and the G1 cells also lost viability. This suggests that the concentration of this essential amino acid (1% of the control) may have been too low to sustain maintenance functions. The DNA channel number for isoleucine was about 8% lower than that seen in a healthy culture. A similar, but more dramatic, event occurred with the phosphate-depleted cultures. At 40 h a large portion of the cells were not in the G1 phase, yet by 80 h, essentially only the G1 population remained. The rapid loss of viability followed by a stabilization at 45% viable cells corresponded to the loss of cells which were not successfully G1-arrested. This result could be attributed to the phosphate concentration being too low to support DNA synthesis, resulting in the death of cells already committed to the next round of DNA synthesis. Since a phosphate depletion to 18% of the control resulted in modest growth without a large viability loss, an intermediate concentration or a more gradual decrease in concentration may eliminate this behavior. The DNA channel number for phosphate was in the range of that seen for the control. The peak RNA channel numbers for both isoleucine and phosphate were in the range of the control during the growth phase.

**Antibody Production Kinetics.** The antibody production characteristics of these cultures were correlated with the nongrowth-associated model given in eq 1. The data were normalized by dividing the antibody titer and integrated viable cells by the final integrated viable cell value for each case. The normalized titers, plotted in Figure 10, were a linear function of the normalized cumulative viable cells in all cases, indicating that a nongrowth-associated model applied. The specific antibody production rate, given as the slope of these lines, varies with the limiting nutrient. The highest specific antibody production rates (280 and 260 titer/million (MM) cells/day, respectively) were obtained under conditions of serum and phosphate depletion; the lowest rate (58 titer/MM cells/day) was under conditions of glutamine depletion. The rates in the control and isoleucine-depleted cultures were 180 and 160 titer/MM cells/day, respectively. It is noteworthy that the specific MAb production rates obtained under both serum and phosphate depletions were approximately 3 times higher than the rate obtained in batch suspension culture. The specific MAb production rate for the control was higher than in both suspension and immobilized batch cultures (89 and 140 titer/MM cells/day, respectively); this is probably due to the replenishment of nutrients through the sampling strategy. The production rate in the glutamine-depleted culture was less than half of the rate observed in batch cultures, providing additional evidence that a component besides or in addition to glutamine was probably limiting in the batch cultures. Overall, an exponentially growing population of HB121 cells exposed to various depletions responded with dramatically different viability profiles and MAb production kinetics depending on the nutrient depleted.

**Metabolism of Growth-Arrested Cultures.** The average rate of glucose utilization in the control culture during the growth phase was 8.3 $\mu$M/million(MM) cells/day which is consistent with the rates presented in Table 2 for the batch cultures. This rate dropped to 2.1 $\mu$M/MM cells/day in the stationary phase; this rate, however, was still higher than that observed during the stationary phase of the batch suspension culture (0.7 $\mu$M/MM cells/day). The average glucose utilization rates in the serum-, phosphate-, and isoleucine-depleted cultures were 6.0, 5.7, and 5.1 $\mu$M/MM cells/day, respectively, and were relatively stable ($\pm 25\%$ standard deviation) throughout the 13 day cultivation. Therefore, unlike the batch cultures that were growth-arrested by an unknown depletion(s), these growth-arrested cultures maintained a relatively high glucose metabolism. Also, the glucose utilization rates were surprisingly similar given the

![Figure 8. Percent viability (○) and antibody concentration (■) profiles for cultures with the indicated nutrient reduced to 1% of the control level.](Image 56x582 to 293x744)
disparate MAb production rates. In the case of the glutamine-depleted culture the glucose rate was 25 μM/MM cells/day, 3-fold the rate observed in the control during the growth phase; this was expected since glucose was the primary energy source for this culture.

Amino acid utilizations were estimated on the basis of the data obtained between 0 and 105 h. The increased glycine secretion rate in the stationary cultures was qualitatively consistent with the previous batch data. The glycine rate was 2.7-fold and 5.3-fold higher than the suspension batch growth phase rate for serum depletion, the highest MAb-producing culture, and glutamine depletion, the lowest, respectively. This suggests that glycine may be indicative of growth-arrested cultures but seems to have no, or a negative, correlation to antibody production. The utilization rates of five other amino acids (Met, Phe, Lys, His, Tyr) were significantly different between the two high MAB-producing cultures (serum- and phosphate-depleted) and the lower MAB-producing cultures (isoleucine- and glutamine-depleted). The amino acid utilization rates in higher MAb-producing cultures were, in all cases, closer to the rates seen in growing batch cultures than the lower Mab-producing cultures. This may be indicative of the latter cultures shutting down through the apoptotic pathway.

**Perfusion Culture with a Controlled Nutrient Depletion.** Immobilization results in two effects which can increase antibody productivity in perfusion bioreactors. First, the entrapment protects slow-growing or growth-arrested cells from the shear forces in a suspension culture, and second, the specific productivity is often increased probably due to the decreased growth rate. A serum depletion was selected to demonstrate sustained antibody production in a nongrowing perfusion culture. The immobilized cells were grown to a density of 1.2 × 10^7 viable cells per milliliter bioreactor volume with a growth rate of about 60% of that seen for the batch suspension cultures. The serum level was lowered to 0.1% after 230 h, and the culture was maintained for an additional 20 days. The results, illustrated in Figure 11,
indicate a cessation of exponential growth at the switch to the nongrowth medium at 230 h, with continued antibody secretion for the next 500 h. As shown in Figure 12, in perfusion culture, MAb production is nongrowth-associated and antibody is produced at a constant rate through the growth and nongrowth phases at significantly higher levels than in the batch cultures. The productivities of the perfusion reactor are compared with batch culture in Table 3; the batch culture data are from the entire culture, while the perfusion data is broken down into the growth and stationary phases. Both the absolute and specific rates of MAb production are significantly increased in perfusion culture. MAb production per liter medium used was similar between the batch and perfusion growth phases and higher for the stationary phase. In retrospect, higher viabilities may have been sustainable in the stationary culture if the perfusion rate was increased by a factor of 2; this would also have resulted in a similar MAb productivity per liter of medium as observed during the growth phase but overall higher productivity per cultivation time. Two remarkable results are seen from this run. First, a relatively constant MAb production rate could be sustained for 30 days with 20 days of the cultivation time in a nonproliferative state as indicated by the data in Figure 12. Second, the rate attained was similar to that achieved in the serum- and phosphate-depleted culture screens, suggesting that this may be the maximum MAb rate for this cell line.

Discussion

The viability and MAb production of a culture as it enters the stationary phase can vary due to inherent characteristics of the cell line, the bulk nutrient and byproduct concentrations, and the microenvironment surrounding the cell.

For MAb production, it is often unknown if the cell line will produce antibody in the stationary phase. Sensitive cell lines with poor cell cycle control may quickly become apoptotic. De la Broise et al. (1992) reported that hybridomas exhibited a “grow or die” behavior in suspension perfusion bioreactors. This result could be attributed to two underlying causes: the hybridoma could have very little cell cycle control, analogous to DNA transformed cells, and die from necrosis or the cells may be highly susceptible to the apoptotic pathway. In either case, hybridoma cell lines which behave in this way would not be expected to be high producers in slow-growing or growth-arrested cultures. This contrasts with reports, including the work here, where antibody was produced in stationary cultures: cell lines such as HB121 can be maintained in a viable, metabolically active G1-arrested state for a considerable period of time producing antibody. As shown, significant productivity can be gained if the cell line secretes MAb in a growth-arrested state. Since the degree of cell cycle control, p53 surveillance, and bcl-2 expression levels of a clone is usually not known, a simple screening experiment with selected limiting nutrients could determine if the selected clone will produce antibody in the growth-arrested state. In the

Table 3. Comparison of Batch and Perfusion Bioreactor Productivities Based on Specific MAb Rates, MAb Production per Cultivation Time, and MAb Production Per Liter of Media Used

<table>
<thead>
<tr>
<th></th>
<th>batch culture</th>
<th>perfusion growth phase</th>
<th>perfusion stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>cultivation hours</td>
<td>275</td>
<td>230</td>
<td>500</td>
</tr>
<tr>
<td>integral cells (MM)</td>
<td>270</td>
<td>800</td>
<td>2600</td>
</tr>
<tr>
<td>MAb/ml bioreactor</td>
<td>1000</td>
<td>11000</td>
<td>42000</td>
</tr>
<tr>
<td>liters used</td>
<td>2</td>
<td>21</td>
<td>34</td>
</tr>
<tr>
<td>MAb/MM cell/h</td>
<td>3.7</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>MAb/time</td>
<td>4</td>
<td>48</td>
<td>84</td>
</tr>
<tr>
<td>MAb/liter</td>
<td>500</td>
<td>520</td>
<td>1200</td>
</tr>
</tbody>
</table>

as indicated by the data in Figure 12. Second, the rate attained was similar to that achieved in the serum- and phosphate-depleted culture screens, suggesting that this may be the maximum MAb rate for this cell line.
future, screening for myelomas known to secrete antibody at high levels in the growth-arrested state and molecular intervention may be ways to decrease variation in performance.

In the stationary phase, MAb production competes with maintenance functions and failure to sustain maintenance functions will result in necrosis or apoptosis. What is limiting growth in the culture is often unknown so that the antibody production can be affected by different degrees depending upon what nutrient(s) are limiting the culture. In addition, rapidly metabolized nutrients can be easily exhausted, so the cell transitions from a growth metabolism to a state where there is insufficient nutrients for maintenance. As shown, an exponentially growing population of HB121 cells exposed to various depletions of nutrients to 1% of their control level for a 13 day period responded with dramatically different viability profiles and MAb production kinetics. The ability of the cell to remain viable depends on the cell's ability to sustain maintenance functions with a nutrient at a fraction of the control concentration (1% in this work with 10% of the culture medium exchanged daily). The antibody production depends obviously on cell viability and any competition with maintenance requirements for the limiting nutrient.

Key nutrients involved in energy metabolism such as glucose, glutamine, and most likely amino acids are central to maintenance functions yet are rapidly metabolized. Severe limitations of these nutrients have been shown to trigger apoptosis (Singh et al., 1994; Mercille and Massie, 1994); controlled limitations of glucose, glutamine, and isoleucine in this work resulted in low productivity, possibly because the available nutrient concentration was rapidly exhausted by energy metabolism. The “grow and die” phenomenon may be characteristic of the exhaustion of these nutrients. In contrast, depletion of phosphate and serum (limiting nutrient unknown in the latter case) appeared to sustain a growth-arrested state with continued high production of antibody in this work. This is not surprising if one considers that phosphate demand is growth-related and that reduced serum has been used to establish a “production phase” for decades (Kruse et al., 1969). The similarity in the rates for the phosphate and serum limitations and the perfusion culture suggests that, in these cases, the antibody synthesis was not hampered by competition with maintenance functions. The concentration of the limiting nutrient is important, not only for the maintenance of viability and antibody synthesis but also to successfully arrest the cells. In the case of the phosphate depletion study, the phosphate concentration was probably lowered too rapidly for cells committed to DNA synthesis to complete the process. Due to the sustained viability of the remaining G2-arrested cells for 5 days in the phosphate depletion case, phosphate may be an interesting candidate for the control of cell cycling in bioreactors. One approach to eliminating the viability loss may be to lower the concentration gradually in a perfusion bioreactor as was done with the serum-depleted run in this work.

In practice, limiting nutrients are often depleted rapidly to exhaustion, so the cell has no opportunity to establish a stable nongrowth state. Rapid cell death due to apoptosis has been reported in these cases. In addition, nutrient replenishment strategies drive to higher and higher cell densities until an unknown nutrient(s) limits the culture; nutrient utilization is largely devoted to increasing biomass. The selection of the nutrient to be limited to maintenance levels, such as phosphate- or serum-free additives, could provide growth control to maintain a slow-growing or arrested culture with high MAb productivity. In addition, this operating mode is envisioned as a way to maintain the culture at a density optimal for robust bioreactor control and high viability.

Since the turnover rate of membranes and proteins is significantly slower in nongrowing cells, they are more susceptible to (or less able to recover from) shear damage. High-density suspension systems often have a slow turnover where dead cells are replaced by new cells emerging at a low growth rate. Therefore, a low-shear microenvironment, such as provided by immobilization, may be advantageous to sustain antibody production in a nongrowing culture. Suspension and immobilized cultures of the HB121 cell line were shown to have similar metabolic transitions into growth-arrested states characterized by significant decreased utilization of many nutrients and increased production of glutamate and glycine. For this cell line, antibody synthesis continued for 9 days for both cultures in a growth-arrested state. While the growth and metabolic rates were slower for the immobilized culture, monovalent antibody synthesis and glycine synthesis were 1.5 and 2.8-fold higher, respectively. The striking similarity in the metabolic profiles during the shift to stationary phase for the immobilized and suspension cultures suggests that the microenvironment does result in slower growth but does not significantly perturb the cell’s metabolic balance. While the suspension and immobilized batch cultures produced antibody at different rates during the growth phase (due to different growth rates), one may expect that in the stationary phase, the antibody production would be similar if they both were arrested in G2. This was not the case for these cultures: the immobilized cell culture productivity was higher. One explanation for this is that the maintenance requirements are higher for a suspension culture since cells are exposed to shear and do not benefit from a close proximity to neighboring cells. Therefore, the immobilized culture devoted more resources to protein synthesis.

The results we obtained with HB121 may or may not be representative of hybridoma behavior in general. The nutrient depletion studies illustrated here suggest that screening of a cell line for viability and antibody production under various limiting nutrients may lead to approaches for extended productivity into the stationary phase. One may envision controlling the growth of the culture with a known limiting nutrient to maintain the culture in a viable, productive, slow-growing or non-growth state at a cell concentration which can be robustly controlled in a bioreactor.

Acknowledgment

The authors acknowledge the support for this research from the National Science Foundation Grant EET-8451109 and matching funds from Monsanto, Schering-Plough, and New Brunswick Scientific Co.

References and Notes


Dobrow, R.; Riddle, V.; Pardee, A. Different Responses to Drugs and Serum of Cells Transformed by Various Means. Cancer Res. 1979, 39, 2718–2726.


Accepted May 1, 1999.

BP990068M