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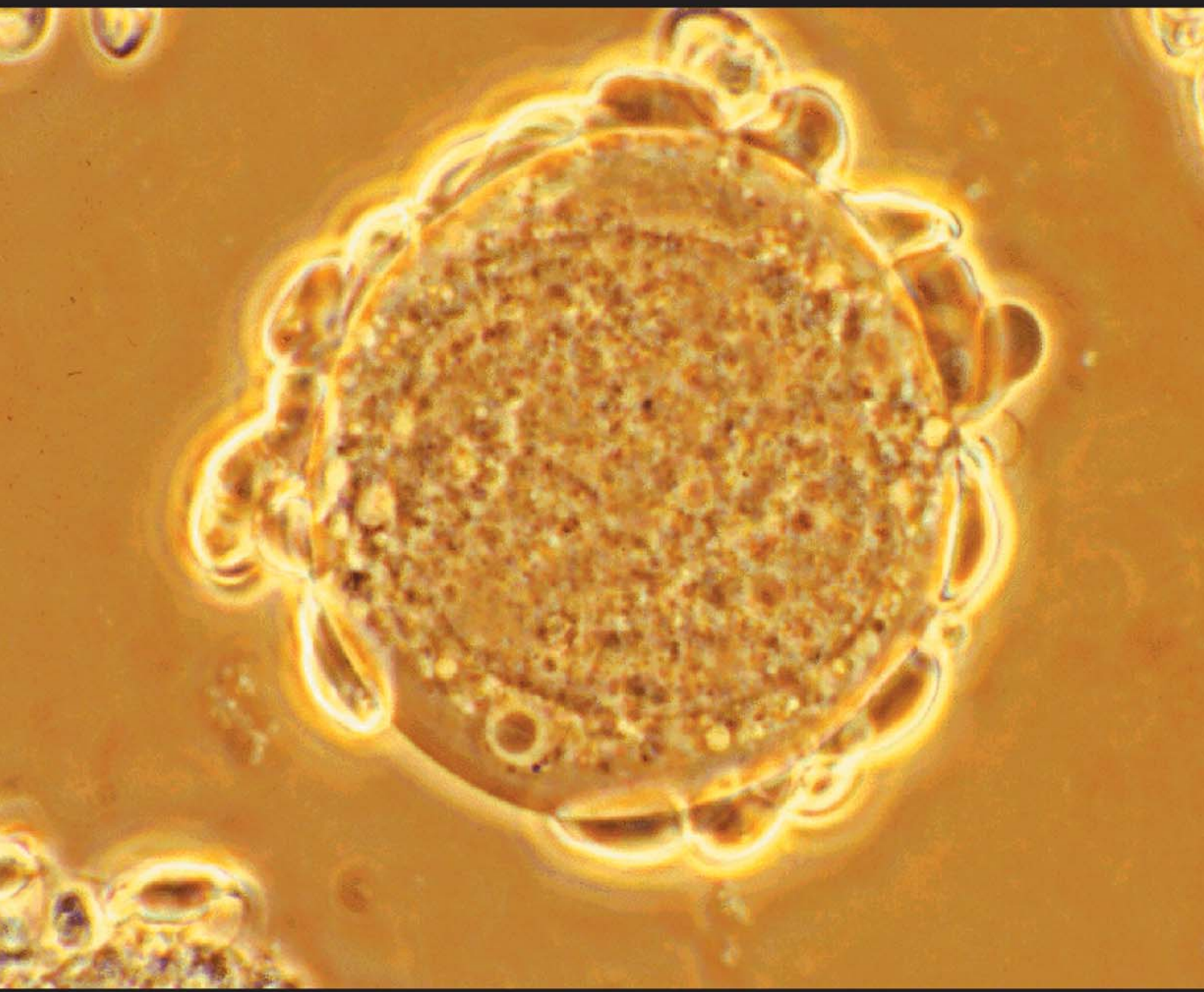

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NS0 Batch Cell Culture Process Characterization: A Case Study

BY BRIAN KEARNS, DAVID LINDSAY, MATTHEW MANAHAN, JOHN MCDOWALL, AND DENNIS RENDEIRO

The characterization of a batch cell culture process to produce a monoclonal antibody from a GS-NS0 mouse myeloma cell line is described. Productivity and cellular metabolism were monitored during scale-up to both characterize the process and aid in assessing cell culture stability. During fermentation scale-up studies, it was found that as culture generation number increased, productivity declined. In both flask and bioreactor cultures, declining production started abruptly at approximately generation 60. In this study, we assessed whether the decline in productivity was due to genetic instability of the cell line, which resulted in the generation of a non-producer sub-population, or a shift to a less productive state of cellular metabolism.

Genetic stability of the cell line was assessed by determining the copy number of the genetic elements encoding the antibody heavy and light chains, at both early and late generations. Construct copy number instability could result in declining productivity if copy number fell as the culture aged. To address this possibility, Southern analysis techniques were developed to distinguish between single copy, tandem copy, and multiple copy integration possibilities. This analysis revealed that there was a single copy of the construct encoding the heavy and light chain antibody fragments, and copy number did not change in cultures that were passaged for 100 generations, relative to the master cell bank. Genetic instability was therefore not the cause for the drop in antibody production.

FACS analysis was employed to determine whether productivity declines could be explained by the presence of a sub-population of non-producing cells, or by a shift in the metabolic state of the culture. Fluorescently labeled anti-IgG antibodies were used to assess intracellular levels of antibody, and a mitochondrial probe was used to assess the metabolic state of the cells. This analysis revealed no evidence of a sub-population of non-producing cells. We did find, however, evidence suggesting that the metabolic state of the culture is a key factor in determining productivity.

Significant increases in both lactate production and glucose consumption occurred after cell cultures were passaged for 60 generations, suggesting that later generation cultures were utilizing oxygen less efficiently than earlier generation cultures. These late generation cultures displayed a higher specific lactate production rate and produced lower antibody titers. Those cultures with higher mitochondrial activity produced higher antibody titers, and individual cells with higher mitochondrial activity contained higher levels of intracellular antibody. As a whole, these studies demonstrate that shifts in cellular metabolism can occur as a culture ages, significantly impacting culture productivity.

Introduction

Cell culture scale-up processes involve progressively increasing culture volumes and cell numbers until a sufficient source of inoculum for a production bioreactor is obtained. Due to the slow rate of mammalian cell growth, a significant amount of time can elapse between thawing a vial of cells and inoculating a production bioreactor. During this time, cell culture conditions must be monitored and tightly controlled to maintain a healthy inoculum.[1,2,3,4] Typical parameters monitored during culture scale-up include viability, cell number, glucose and glutamine consumption, and lactate and ammonia production. Determining which of these cell culture measurements are predictive of productivity is the challenge faced by those involved in the cell culture development process. Predictive measurements may provide information such as when to inoculate a bioreactor, which of two

Corresponding author ***Brian G. Kearns****, is Associate Principle Scientist, Fermentation Scale-up and Production; Brian.Kearns2@spcorp.com.* ***David Lindsay*** *formerly with Fermentation Scale-Up and Production, is currently with MedImmune, Inc. and* ***Matthew Manahan, John McDowall,*** *and* ***Dennis Rendeiro,*** *Fermentation Scale-Up and Production, Biotechnology Development, Schering-Plough Research Institute.*

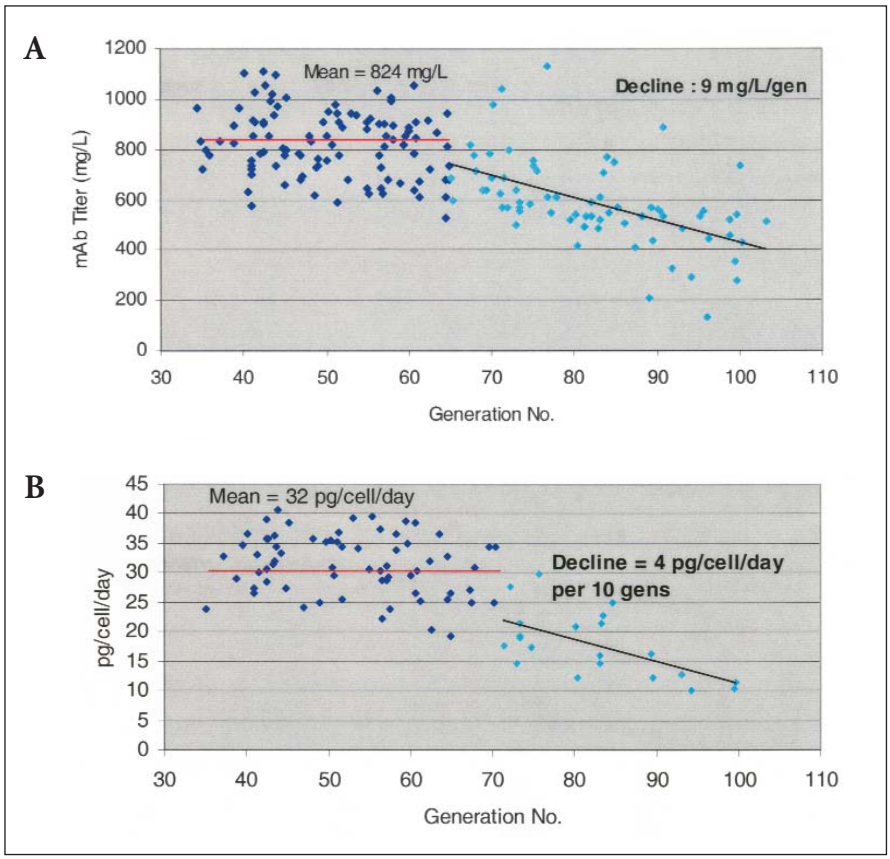

Figure 1. Antibody production is stable up to approximately 60 cell generations. After this, final batch productivity (A) and specific productivity (B) decreased significantly.

parallel cultures should be scaled-up, or whether a culture should be discarded due to its poor probability of achieving a high titer.[5]

Once a productive culture is established, it must be maintained in a highly productive state during scale-up. To maintain productivity, close attention must be paid to ensure that cellular metabolism is not altered to a less productive state during the scale-up process. One way to help control metabolism is to maintain cultures in an exponential phase of growth.[6] Maintaining this growth helps ensure that scale-up is rapid, and also prevents the culture from experiencing metabolic changes that accompany entry into stationary phase. Metabolic changes may also occur more slowly, however, as the culture is scaled-up for production. These changes are more difficult to detect and control, yet may be a significant source of decreased productivity in a production bioreactor.[7] Genetic stability of the cell line is another parameter that must be evaluated since genetic instability can result in large and sudden drops in production.

During cell culture scale-up processes, flask cultures are routinely initiated to monitor the quality of the inoculum. These cultures are instituted to more fully characterize the inoculum and determine its suitability for further scale-up, and they are cultivated well past the point at which they would normally be used for production.

In this study, we analyzed cultures involved in a scale-up process. This analysis revealed that antibody production was stable to approximately 60 cell generations, and after 60 generations a decline of 9 mg/L/generation was measured (Fig. 1A). Declines in specific productivity were also calculated in later generation cultures, and it was found that beyond 70 generations there was a decline of 4 pg/cell/day per ten generations (Fig. 1B). Specific productivity in bioreactors was similar to that measured in shake-flasks, when cultures at the same generation number were used. The result was a measured decline in productivity that was not dependent on the culture vessel type, but was a function of culture age.

In this report, we present the development of a GS-NS0 batch cell culture process for production of a humanized IgG4 monoclonal antibody. Throughout these experiments, cultures were maintained in a glutamine-free medium for selection of the glutamine synthetase gene. During the course of cell culture development, we noted that the specific growth rate remained constant up to approximately 60 generations, relative to the master cell bank, and increased significantly beyond 60 generations (data not shown). Accompanying the increase in growth rate was a steady and significant decrease in antibody production, that occurred in both batch flasks and bioreactors. Production batches, that were inoculated prior to 60 generations, displayed remarkable consistency and high

final titer, helping to define an inoculation window for production. To investigate these phenomena, genetic stability of the cell line was assessed, and metabolic analyses were performed to help determine the source of the decline in antibody titer.

Materials and Methods

Culture conditions and measurements

NS0 cell cultures were maintained in shake flasks in 37°C incubators with 5% CO_2. Glucose and lactate concentrations were measured with a YSI 2700 Select analyzer, and dissolved O_2, CO_2, and pH were measured with an ABL5 radiometer. Antibody titers were measured with a Protein A, HPLC-based assay.

Southern blotting

Cells were grown in a batch process and cultured in serum-free, BSA-containing media. DNA from an early generation culture was prepared after a minimal number of subcultures, after vial thaw, and DNA from a late generation culture was prepared at generation 100. Genomic DNA was extracted and purified using a Qiagen Blood and Cell Culture Maxi kit. Cell nuclei were freshly prepared from pelleted cells, and then frozen at -20°C until used. Chromosomal DNA was purified from the frozen nuclei with Qiagen Genomic-tip 500 columns; and both Proteinase K and Qiagen protease were included in the extraction buffer to aid in the recovery of DNA. The prepared genomic DNA was quantified by taking absorbency readings at 260 nm in a spectrophotometer. Before use, DNA was diluted with TE (pH 8.0) to a final working concentration of 1 µg/µL.

All restriction enzymes were purchased from New England Biolabs. Agarose and 50x TAE buffer were purchased from Gibco Life Technologies. Amersham's AlkPhos Direct Labeling and Detection System was used to generate labeled DNA probes. The purified DNA probes were cross-linked with a heat stable alkaline phosphatase for 30 minutes, at 37°C, and then used immediately, or stored on ice for up to one hour.

Digested genomic DNA samples were resolved on a 0.9% agarose gel at 50 volts for 18 hours. Ethidium bromide (50 µg/L) was included in the gel for visualization. After the DNA was transferred to a nylon membrane, alkaline phosphatase-labeled probes were used for hybridization. Hybridization buffer was supplied with the AlkPhos Direct Labeling and Detection System, and all hybridizations were performed in roller bottles in a temperature-con-

A

Enzyme	Single copy	Tandem copies	Two or more integration sites
*Avr*II	>5981 bp, one band	>5981 bp, + 7801 bp	>5981 bp, two or more bands
*Eco*RI	6066 bp, one band	6066 bp, one band	6066 bp, one band
*Hind*III	>3888 bp, one band	>3888 bp, + 13891 bp	>3888 bp, two or more bands
*Nde*I	>5202 bp, one band	>5202 bp, + 9202 bp	>5202 bp, two or more bands

B

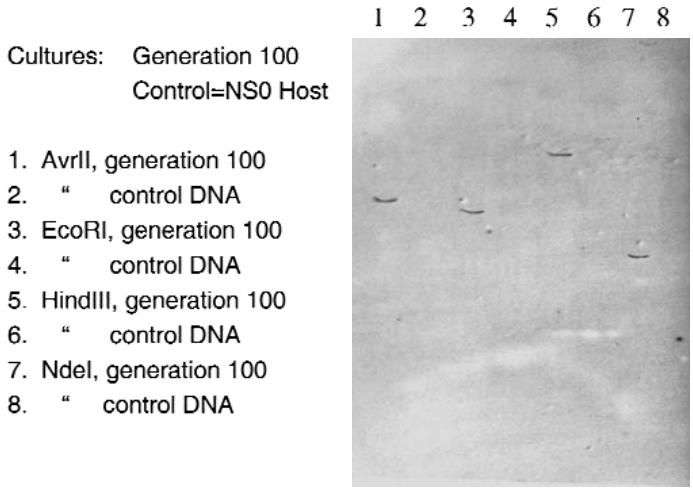

Figure 2. Predicted number of bands developed by Southern blotting after genomic DNA is digested with the indicated restriction enzymes and probed for the presence of antibody heavy chain DNA (A). The banding pattern for single copy integration, tandem copy integration, and multiple integration states can be predicted prior to experimentation. Southern blot with heavy chain probe (B). A single band is developed from the generation 100 culture and no bands are developed in the NS0 host strain control. These results are consistent with predictions for single copy integration.

trolled oven. The hybridization temperature was 70°C for the heavy chain probe and 67°C for the light chain probe. Before being directly added into the hybridization tubes, alkaline phosphatase-labeled DNA probes were brought up to 500 µL with hybridization buffer. Hybridization proceeded for 16-20 hours.

After hybridization, the membranes were washed according to the instructions in the AlkPhos Direct Labeling and Detection System, plus the addition of a third, ten minute room temperature wash with secondary wash buffer. The DNA banding pattern was detected with CDP-star detection reagent from Amersham. Bands were detected by exposing the developed blots to X-ray film, and sizes were determined by comparison to known DNA size standards.

FACS analysis

FACS analysis was performed with a Becton Dickinson FACScalibur flow cytometer. Fluorescently labeled anti-IgG antibodies and MitoTracker probes were purchased from Molecular Probes. Cells for FACS analysis were first resuspended in media containing 10% DMSO, and then frozen at -80°C. After thawing, the cells were fixed and permeabilized using a Fix and Perm kit from Caltag Laboratories. Cells were fixed for 30 minutes at room temperature, washed twice with PBS, and permeabilized for 30 minutes at room temperature. Fluorescently labeled primary antibodies were incubated with the cells during the permeabilization step.

For mitochondrial staining, the cultures were first incubated with a 500 nM solution of MitoTracker red for 30 minutes, in a 37°C incubator, with shaking to permit the cells to uptake the dye. The cells were then fixed and permeabilized. Following the antibody incubations, the cells were centrifuged, washed twice with PBS, and resuspended in sheath fluid for FACS analysis.

Results and Discussion

Southern analysis

One possible explanation for the decline in culture productivity with increasing culture age is genetic instability of the cell line. To investigate genetic stability, Southern analysis techniques were employed to determine construct copy number. Cells were harvested at a very early passage, soon after vial thaw, and at 100 generations, a generation number well beyond that required for production purposes. Genetic instability is most commonly

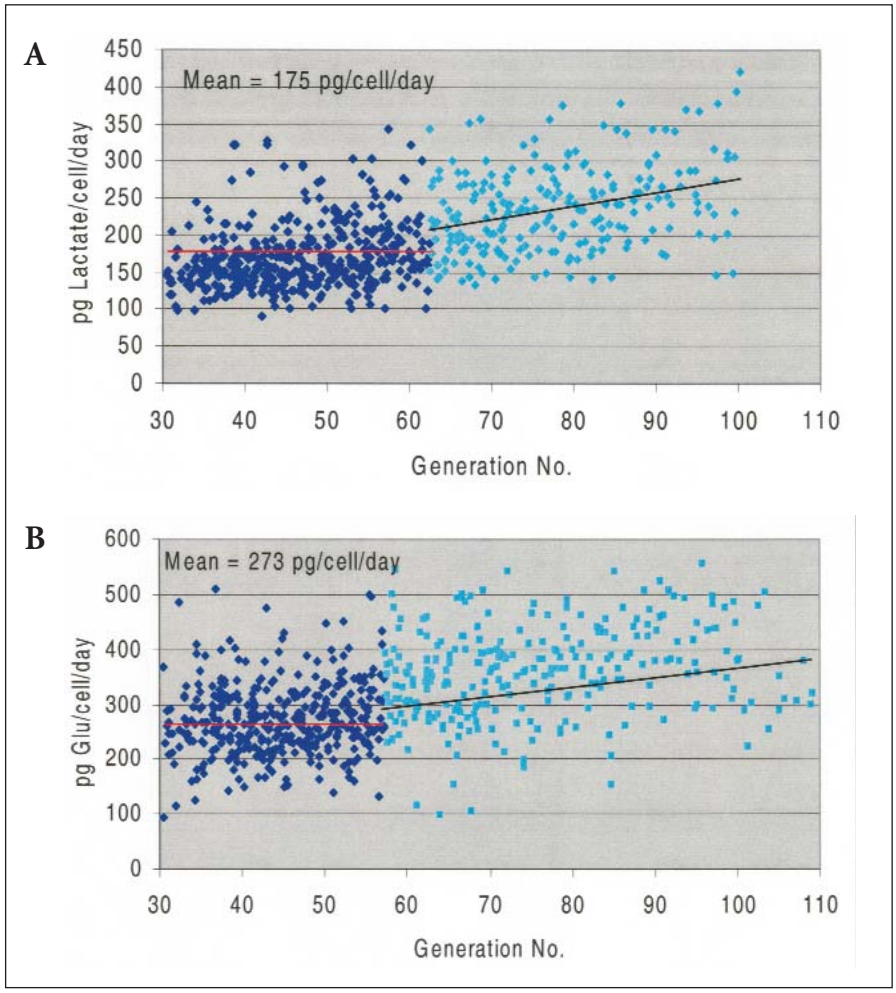

Figure 3. Significant increases in lactate production (A) and glucose utilization (B) were measured after approximately 60 cell generations.

manifested as a reduction in copy number from an initial high copy number state. Cells with reduced construct copies have a distinct selective advantage over cells that maintain a higher copy number. Their metabolic burden is reduced, enabling a faster growth rate that permits them to slowly dominate a culture. Over many generations, a small selective advantage can have a major impact. Copy number remains unchanged through many cell generations in a stable cell line.

A comparison of copy number, between late and early generation cultures, provides an indication of copy number stability. The integration state of the construct determines both the number and sizes of the bands developed by Southern blotting (Fig. 2). In this analysis, multiple developed bands are indicative of gene duplication or multiple integration events. If a single band is developed in the experimental lanes, then the copy number is one. The *Eco*R I digested DNA served as a useful control for the heavy chain-probed DNA, since only one band should be detected, regardless of the integration state of the DNA construct. Stringent conditions of hybridization were adjusted, based upon this fact. For the light chain probe, a different restriction digestion served this same purpose. The NS0 host cell line was used as a negative control.

Southern analysis revealed a single band in all digest lanes in both the early (not shown) and late generation cultures. Probes for antibody heavy chain (Fig. 2) and light chain (not shown) detected a single band, and the lengths of these bands are consistent with predictions for single copy integration. Since the copy number remained unchanged between early and late generation cultures, genetic stability of the expression system was established. One copy was integrated into the genome and was maintained to generation 100, which is a generation well beyond that used for production. The reduction in productivity observed after 60 generations cannot be attributed to genetic instability of the cell line.

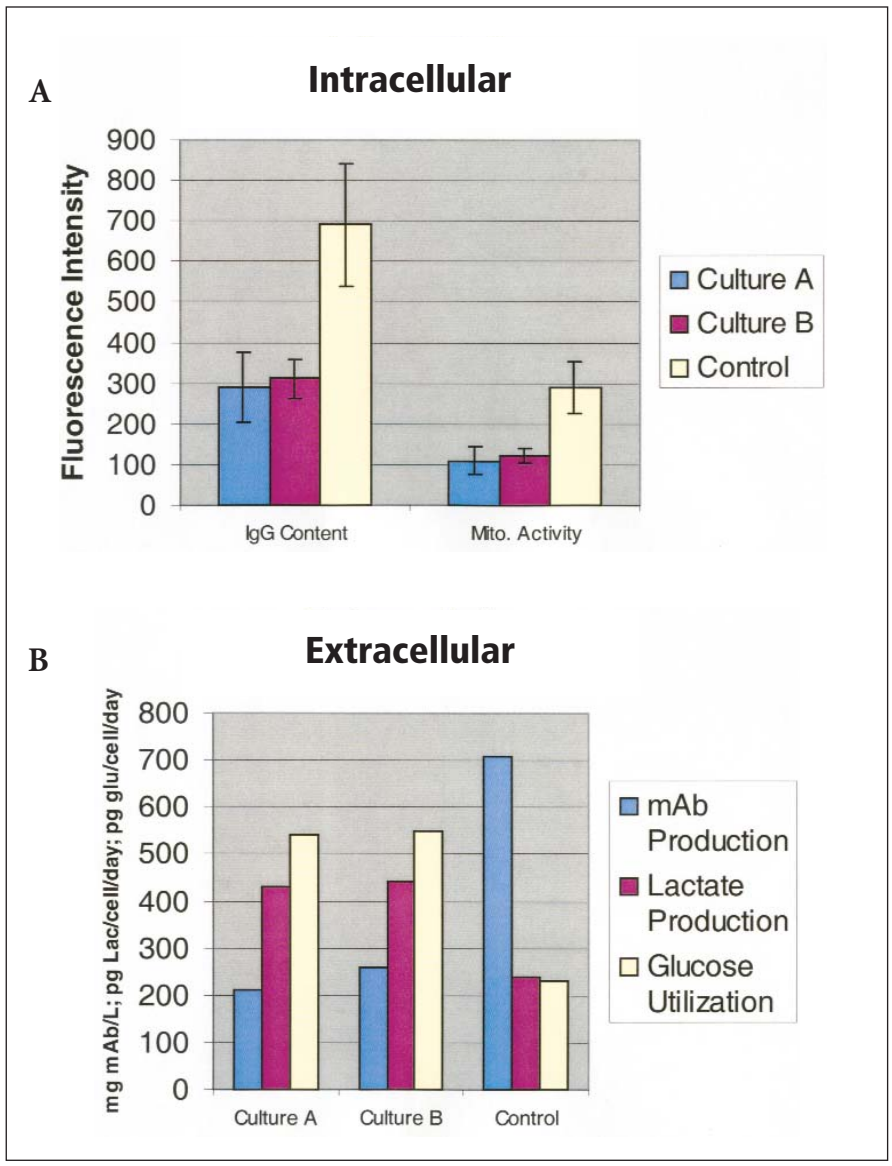

Figure 4. Intracellular (A) and extracellular (B) measurements of two low-producing cultures (cultures A and B) and a control culture. Lower producing cultures showed reduced intracellular IgG levels and mitochondrial activity, and increased glucose consumption and lactate production relative to the control, higher-producing, culture.

Metabolic and FACS analysis

Southern analysis showed that the production cell line was genetically stable. However, a sub-population of non-producers could have arisen as the culture was scaled-up. If this was the case, these subclones would be genetically identical to the producer line, and thus identical by Southern analysis, but they would be phenotypically non-productive. This population could devote more energy toward growth and reproduction, thus having a shorter generation time. At later generations this non-producing population could be large enough to represent a major percentage of the culture. The development of a non-producing population would manifest itself as decreased production over time, as the percentage of antibody-producing cells decreased. For our NS0 cell cultures, a decline of 9 mg/L/generation was measured after approximately 60 cell generations. These results are consistent with the development of a non-producer population.

To investigate this possibility, we performed intracellular FACS analysis on early and late generation cultures, and on cultures with high and low anti-

body titers. For this study we employed a fluorescently labeled anti-IgG antibody to probe intracellular antibody levels. This type of investigation can establish if a culture is phenotypically uniform, and can detect the presence of a non-producing sub-population. The NS0 host cell line served as a negative control.

The results of this study revealed no evidence for the presence of a non-producing population. Uniform populations in both high and low-producing cultures were observed by FACS analysis. High-producing cultures revealed higher degrees of staining, and lower degrees of staining were found in low-producing cultures. Moreover, intracellular antibody staining correlated well with extracellular antibody measurements. No evidence of a non-producer sub-population was found, and so this explanation does not account for the loss in culture productivity.

Since an increase in growth rate, glucose consumption, and lactate production were measured at the point of declining productivity, we explored whether a change in cellular metabolism was related to the decline in antibody titer. High rates of glucose consumption and lactate production are hallmarks of anaerobic metabolism, since energy is derived much less efficiently from glucose in the absence of oxidative metabolism. Larger quantities of glucose must be consumed for the same energy return. We measured significant increases in specific lactate production and glucose consumption in cultures that were passaged for over 60 generations (Fig. 3A, 3B). Moreover, higher specific lactate production and glucose consumption during exponential growth were found to be characteristics of lower-titer batch cultures. These measurements suggested that as cultures aged, their metabolism shifted toward a more anaerobic and glycolytic mode.

To investigate metabolism at the level of the cell, rather than at the level of the culture, growing cultures were labeled with a cell-permeant MitoTracker probe. The reduced probe does not fluoresce until it enters an actively respiring cell, where it is then oxidized to the corresponding fluorescent mitochondrion-selective probe and sequestered in the mitochondria. The level of fluorescence correlates with mitochondrial oxidative activity and can be quantified in a flow cytometer.

This analysis showed a correlation, on a per-cell basis, between mitochondrial activity and intracellular antibody content (Fig. 4A). Cells with the highest mitochondrial staining displayed the highest levels of intracellular antibody, and lower mitochondrial activity correlated with reduced levels of intracellular antibody staining. Thus, a link was established between oxidative metabolism and antibody productivity. Cells in a more oxidative mode of metabolism, as evidenced by lower glucose consumption, lower lactate production, and higher mitochondrial staining; produced higher antibody titers (Fig. 4B). Greater glucose consumption, lactate production, and lower mitochondrial staining were hallmarks of poor antibody-producing cultures. For our GS-NS0 cell line, the transition to a less oxidative mode of metabolism occurred at approximately 60 generations, leading to less productive cultures at these later generations.

The cause of this switch in metabolism is unknown, but the faster growth rate of these less productive cells suggests that they may have a selective advantage over the more productive cells. Since glucose is readily available, efficient glucose utilization may provide no selective benefit. On the contrary, less efficient glucose utilization, coupled with higher growth rates and poor production, may permit these cells to quickly dominate a culture, once the switch in metabolism occurs. This result could affect significant decreases in culture productivity at later generations. The use of early generation cultures may not always guarantee high productivity, if a switch in metabolism to a less productive state has already occurred. An understanding of culture metabolism must be attained in order to avoid scaling-up unproductive inoculum.

Conclusions

In this report we showed that a recombinant GS-NS0 cell line performed best when it was growing in a more oxidative mode. These studies have stressed that cultures should be closely monitored for evidence of shifts in cell metabolism. Methods to help control metabolism such as pH, temperature, and dissolved oxygen control, plus control of glucose and glutamine levels should be examined carefully to ensure highest culture productivity and stability. These studies have also shown that a genetically stable cell line can be phenotypically unstable. Whereas little can be done to improve genetic stability, cell culture conditions are likely to play a pivotal role in phenotype stability. As such, cell culture conditions should be optimized with both productivity and stability in mind.

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