Loss of Antibody Productivity Is Highly Reproducible in Multiple Hybridoma Subclones

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An immunoglobulin G (IgG_{2b}) producing hybridoma cell line (S3H5/γ2bA2) was cloned and subcloned. Twenty subclones were grown in parallel while being adapted in a stepwise fashion to serum-free medium. Following adaptation to serum-free medium, it was found that 16 of the 20 subclones remained at a relatively constant proportion of nonproducing cells. Three of the remaining subclones transiently deviated from this balance but eventually returned toward this population composition. One subclone continued to lose productivity. A population balance was reached at approximately 8% of the population being nonproducers. The loss of antibody productivity was thus highly reproducible.
Key words: hybridoma • antibody productivity • kinetics • instability

INTRODUCTION

Monoclonal antibodies (MAbs) have become indispensable in many areas of medical research, diagnostics, and therapeutics. Small-scale laboratory-based production of MAbs typically utilizes serum-supplemented media to culture hybridoma cell lines, but serum use for large-scale commercial production is unsuitable for a variety of reasons. The reasons include cost of serum, downstream purification problems, batch variability, undefined composition, and foaming in culture. Serum-free culture would be advantageous, but a major problem often encountered is the loss of antibody productivity and the appearance of nonproducing hybridoma subpopulations.[2–4,6] It has been shown that the producing versus nonproducing population composition of a culture is quite sensitive to serum level, inoculum state, and medium composition.[1] A critical question thus is, given identical culture conditions, what is the variability in the loss of antibody productivity caused by underlying cellular and genetic determinants? To answer this question, 20 identical IgG_{2b} producing subclones were grown serum free under identical and parallel culture conditions to establish whether the loss of antibody productivity in a cultured population is reproducible or it must be viewed as random.

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MATERIALS AND METHODS

Culture Media

The murine hybridoma S3H5/γ2bA2 was used for study. This hybridoma secretes a γ2bA anti-idiotype antibody, directed against the 38C13. The S3H5/γ2bA2 was cloned and adapted to low-serum conditions using Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO) supplemented with 100 units/mL each of penicillin and streptomycin (Sigma) and the described percentages of fetal bovine serum (FBS, Hyclone). Dulbecco's modified Eagle's medium/Ham's nutrient mixture (DME/F12, Sigma) was used as the basal medium for serum-free growth. This medium was supplemented with 100 units/mL each of penicillin and streptomycin, insulin/transferrin/selenium (ITS: 5 mg/L insulin, 5 mg/L transferrin, 5 μg/L selenite, Sigma), and HEPES was added to bring its final concentration to 25 m*M* (equivalent to IMDM levels). No other proteins were added to this medium.

Cell Cloning and Cultivation

An IgG_{2b} producing clone was isolated from a largely producing population using limiting dilution in IMDM supplemented with 10% (v/v) FBS. This clone was then subcloned, and 20 of these subclones were then grown in parallel in T-25 flasks in a repeated fed-batch fashion. The cloning and subcloning processes were of 20- and 14-day durations, respectively. All of the subclones were subsequently adapted to serum-free medium in a stepwise fashion. This process consisted of 8 days in 5% FBS IMDM followed by 26 days in 1.25% FBS IMDM. After the subclones had been adapted to 1.25% FBS IMDM medium, they were passaged once in 1.25% FBS DME/F12 medium. Finally, the subclones were switched to serum-free DME/F12. Cells were cultured in this medium for 60 days. Growth was poor during this time, and the viability dropped to approximately 35%. Therefore, to increase viability to above 80%, a regimen of total medium replacement every third day with a seeding density of 2.5×10^5 viable cells per milliliter was begun. This time will be designated as day zero in the following section. After 60 days of the

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total medium replacement regimen, the cells were passed at an average dilution rate of 1/4.4 every 3 days to maintain equal cell densities. No change in growth or viability was noticed at this time.

Flow Cytometry

One to two million cells were taken at each passaging for flow cytometric determination of intracellular antibody content. These cells were washed twice with phosphate-buffered saline (PBS) before resuspending in ice-cold 70% ethanol. The samples were then placed into a −20°C freezer until the time of flow cytometric analysis. Prior to analysis, the ethanol was removed and the cells were washed with PBS. A fluorescein isothiocyanate (FITC) conjugated heavy chain specific goat antimouse IgG_{2b} antibody (Southern Biotech.) was used to stain the cells at 4°C, and finally the cells were washed with PBS. Flow cytometric analysis was carried out using a Coulter EPICS 751. Forward-angle and 90° light scatter were used in order to gate the cell population to eliminate debris and cell clumps. From this gate, internal antibody content was plotted into a logarithmic FITC fluorescence intensity histogram. Mixed population cells with high and low internal antibody content could be easily resolved into separate peaks by this method (Fig. 1). Windows were set to obtain the percentages of each population. A correlation between the fraction of high internal antibody content subpopulation with specific monoclonal antibody production rate has previously been described with this cell line.[4] Henceforth, the low and high internal antibody content cells will be designated as nonproducers and producers, respectively.

RESULTS AND DISCUSSION

Myeloma cells have been known to exhibit many forms of loss of antibody productivity.[5] It was previously determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) that cultured nonproducers arising from the S3H5/γ2bA2 cell line do not secrete heavy or light chains.[4] A Northern blot was used to test for the presence of heavy chain mRNA (data not shown). Messenger RNA for the heavy chain could not be detected in nonproducing clones derived from this cell line. The Northern blot suggests that the loss of heavy chain production is due to either decreased or total loss of transcriptional

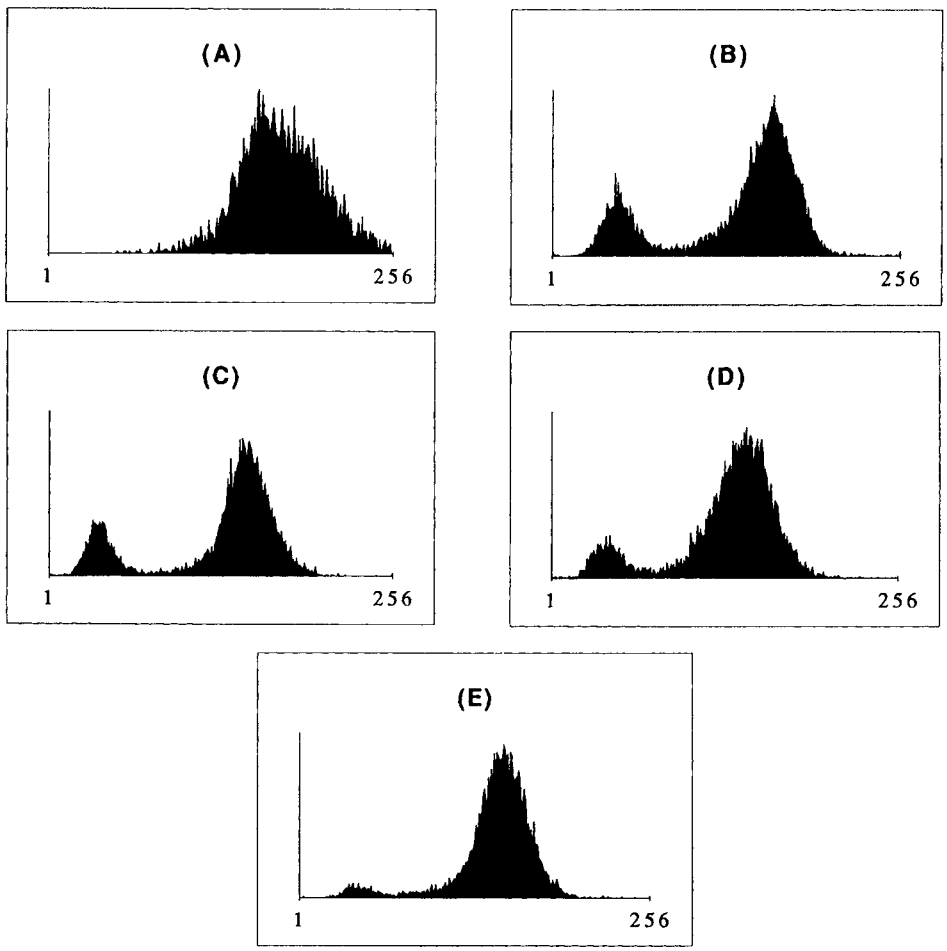

Figure 1. (a–e) Logarithmic FITC fluorescence histograms of subclone 6 on days 0, 30, 51, 72, and 137. Logarithmically sensitive channels 1–256 on the horizontal axis are plotted against number of events. Identical settings were used throughout the experiment.

capabilities of the heavy chain gene or to increased degradation of heavy chain mRNA. Mutations, gene regulation, and gene losses are three possible mechanisms that could result in decreased transcription. Downstream processes such as translation, posttranslational modifications, and secretion are therefore ruled out as the effected step in heavy chain synthesis in these nonproducers. Whatever the underlying cause may be, nonproducing clones such as these would therefore not be expected to contain any internal heavy chain and thus can be easily monitored by flow cytometry. Other types of nonproducing subpopulations which may arise might not be distinguished by using internal antibody staining.

The kinetics of loss of productivity of the 20 subclones were highly reproducible. Sixteen out of the 20 subclones showed essentially the same behavior, reaching a fairly stable population balance between producers and nonproducers within 20–30 days (Fig. 2). Two of the most stable subclones were subclone 9, with a population balance fraction of nonproducers at 0.054 (sd = 0.005, n = 7) averaged over time, and subclone 7, with a population balance fraction of nonproducers at 0.050 (sd = 0.006, n = 7) averaged over time (Fig. 2b). If the last days of sampling are examined, it is seen that the population balance is 0.077 averaged on day 137 (sd = 0.035, n = 17; Fig. 3) if the two outside samples 14 and 15 are ignored (subclone 5 data not available). The balance on day 137 is 0.107 (sd = 0.098, n = 19) if subclones 14 and 15 are included. On day 128, the balance value is 0.070 (sd = 0.035, n = 17; Fig. 3) if 14 and 15 are ignored (subclone 17 not resolved). The balance on day 128 is 0.100 (sd = 0.097, n = 19) if subclones 14 and 15 are included. The cell line S3H5/γ2bA2 has previously been observed to lose productivity under different serum-free culture conditions,[4] so this balance between producers and nonproducers is apparently quite sensitive.

Three of the four remaining subclones exhibited transient behavior of varying severity before stabilizing (Fig. 2b). These transients varied in duration and in one case exceeded 100 days. Since there was a large increase in growth rate with the change in feeding regimen, it is possible that these transients are due to fast adapting, nonproducing cells that gained temporary growth advantage while the producing cells adapted to the new culture conditions. It is also possible that the rate of switching from producers to nonproducers was not constant but transiently increased at this time. Subclone 14 exhibited anomalous behavior as compared to the other 19 subclones (Fig. 2b). The steep rise in nonproducer percentage cannot be easily dismissed as the result of a large initial change in growth rate, as in samples 6, 15, and 20. One conjecture is that this loss could be explained by a subclass switch, since the switching rate from γ2b to γ2a has been determined to be on the order of 10^{-7} per cell per generation in myelomas producing IgG_{2b}.[5] Flow cytometric analysis of

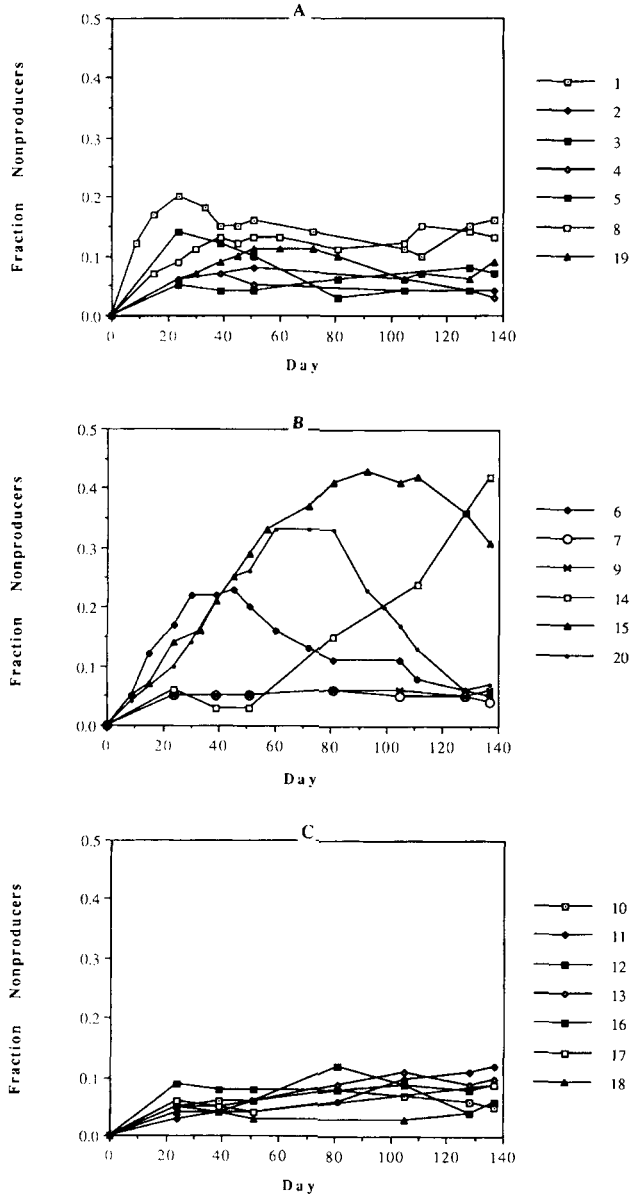

Figure 2. Fraction of nonproducers versus time in days for the 20 subclones. The numbers in the inset legend indicate the clone number.

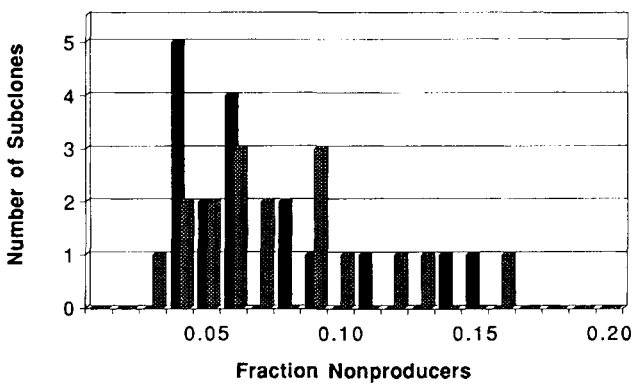

Figure 3. Population balance level histogram (■) Day 128; (■) Day 137 (subclones 14 and 15 not shown).

internal IgG_{2a} within this subclone resulted in no positives, however, so this hypothesis was dismissed. It appears that a perturbation such as this can have long-lasting effects.

In conclusion, this study supports the hypothesis that identical serum-free culture conditions result in reproducible changes in population composition. It appears that if the initial transient shifts are overlooked, the nonproducing and producing fractions are reproduced moderately well. Apparently, there will be some random fluctuations, as seen in 4 out of the 20 subclones, which cannot be predicted. These fluctuations might be minimized in a more closely controlled reactor.

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