Population Balance between Producing and Nonproducing Hybridoma Clones Is Very Sensitive to Serum Level, State of Inoculum, and Medium Composition

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Received April 26, 1991/Accepted July 11, 1991

Secreting and nonsecreting hybridoma populations derived from the murine hybridoma cell line 167.4G5.3 were each grown in batch culture in low serum and serum-free media. Under serum-free conditions, a secreting population gained on a predominantly nonsecreting population and competed with the existing antibody-deficient cells effectively. It was found that this competition was sensitive to state of inoculum and medium composition. We conclude that the competition between a secreting and nonsecreting, or more generally, a producing and nonproducing, population is important; the appearance of the latter may not be a significant setback in terms of expected product titer.
Key words: hybridoma • nonproducer • instability • antibody secretion rate • flow cytometer

INTRODUCTION

Variability in the specific antibody production rate can lead to problems in predicting antibody titer. This variability is often attributed to loss of a functional immunoglobulin structural gene in at least a portion of the cell population.[5,8] This conclusion for mouse–mouse hybridoma cells has been extrapolated from experiments with either mouse or human myeloma cell lines, for the most part because of the difficulty in distinguishing the chromosomes of the hybridoma fusion partners and the large number of chromosomes in the hybrid genome. The appearance of a nonsecreting population of hybridomas is thought to be due to mutations or loss of genes associated with antibody regulation as well as antibody synthesis ability because the losses in productivity are usually irreversible.[3,11]

In support of the hypothesis that nonsecretors result from genetic mutations, it has been found that immunoglobulin genes in myelomas are more prone to mutation than other gene families after treatment with mutagenic agents.[8] Preferential loss of antibody synthesis ability is thought to occur for three reasons. First, the antibody product is not essential for cell viability, so the cell may gain a growth advantage by losing an antibody gene. Second, recombination of immunoglobulin genes is the basis of antibody diversity. Antibody genes may consequently be more susceptible than other DNA sequences to an error in gene rearrangement. Third, one allele of each immunoglobulin-related chromosome is excluded from expression regardless of whether the nonexcluded allele continues to function. Thus, the excluded allele cannot act as a backup to the initially expressed allele. It is reasonable to expect that the inherent immunoglobulin instability in myelomas be present in hybridoma cells.

In this article, we distinguish between two hybridoma cell populations derived from the same IgG_1 producing hybridoma cell line on the basis of their ability to produce IgG_1 antibody (Ab). The dynamics of this population balance in response to changes in medium composition is described. Relative producer (P) and nonproducer (NP) populations of initially stable P and NP cultures are measured with respect to time, serum level, and media composition. This aspect of adaptation is an important consideration in a process where hybridomas are first cultivated in serum containing medium and then switched to serum-free or low serum medium for production. In the following work we document a case where P cells temporarily overtake a predominantly NP population upon transfer from a low serum medium to a serum-free medium.

MATERIALS AND METHODS

Culture Maintenance

The NP cells were derived from the hybridoma cell line 167.4G5.3. This hybridoma was selected from a fusion of Balb/C spleen cells with a nonsecreting plasmacytoma fusion line P3X63–Ag8.653 and produces IgG_1 against phosphorylcholine. These cells were adapted for growth in successively lower serum levels, as described in reference 9. The NP cells arose upon adaptation in 1.25% fetal bovine serum (FBS) media and were frozen. The P cells were frozen from 5% FBS cultures. The P and NP cells were thawed and allowed to recover for 2 weeks to at least 85% viability in their respective medium (here-

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CCC 0006-3592/92/0303354-07$04.00

after known as *recovery medium*). Next, each culture was split, centrifuged, and resuspended in either 0% FBS (see Media section) or 1.25% FBS medium. (These media will hereafter be known as *experimental media.*) The seeding of P and NP cells in the experimental media is marked as day 0. The NP cells transferred to 0% FBS did not survive the fifth passage (day 10), so a second transfer was done from an older NP culture (from the third passage) at 47% viability. The cultures were maintained in 10-mL volumes in T-25-cm^2 flasks (Becton Dickinson, Lincoln Park, NJ) and sampled and passed by dilution with fresh media every 2 days with an initial seeding density of 4×10^5 viable cells per milliliter (VC/mL). If the viability was too low so that there were not enough viable cells for dilution, then the culture was not passed until sufficient viable cells were available.

To test the behavior of producer and nonproducer cells in different media, five experiments were carried out with the P and NP cells, as described in Table I. In experiment 1, P and NP cells were transferred to 0% FBS experimental medium. Parallel cultures of P and NP were also transferred to 1.25% FBS and grown for more than 160 days, as shown in Figure 1B and described by experiment 2. State of inoculum was tested in experiment 3, where P and NP cells were cultivated in 1.25% FBS until day 94 before being transferred to 0% FBS experimental medium. Interaction between P and NP cells was tested in experiment 4, where producing and nonproducing cultures grown separately in 1.25% FBS were mixed and grown in 0% FBS. At two different times, the cells from the NP culture in experiment 1 were subcultured into medium of different compositions, as stated in experiment 5.

Media

IMDM basal medium with antibiotic–antimycotic solution (Sigma, St. Louis, MO) was used. The serum media contained HyClone FBS (Logan, UT) unless otherwise specified. The 0% FBS medium contained 5 g/L bovine serum albumin (BSA, cell culture grade, Sigma), 20 μM ethanolamine, and insulin–transferrin–selenium (ITS) media supplement from Sigma (5 mg/L insulin, 5 mg/L transferrin, and 5 μg/L sodium selenite). The composition of the 0% FBS was not comparable to the 1.25% FBS in the level of the five medium additions it contained: insulin and BSA levels in 0% FBS were almost 20 times that contained in the 1.25% FBS medium. The rationale behind this composition was that it provided a serum-free, low protein environment for the cells that was distinct from the 1.25% FBS. The 5% FBS and 0% FBS without BSA were also used in experiment 5.

Conditioned Medium

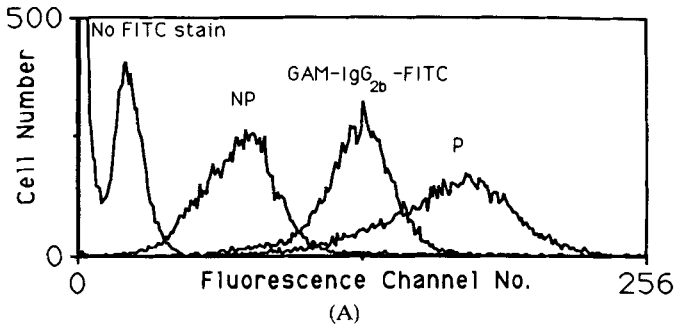
Medium from a 2-day-old culture of P cells grown in 0% FBS was passed through a 0.2-μm low protein binding filter (Gelman Sciences Inc., Ann Arbor, MI) with a syringe and used immediately for cell passage.

Sampling

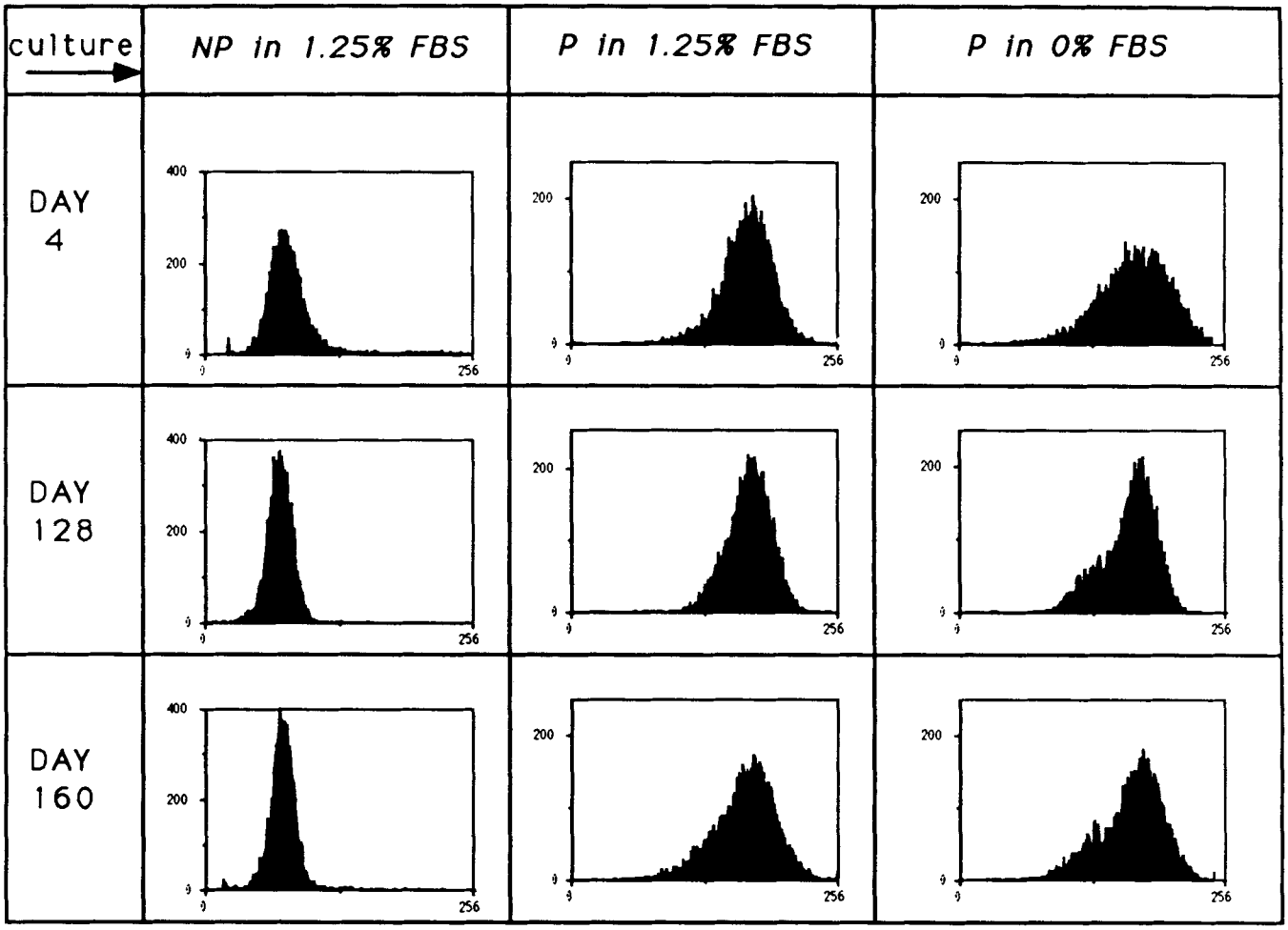
A 1-mL sample was taken once every 2 days. After counting cells with a hemacytometer and trypan blue exclusion, the samples were centrifuged and the supernatants stored at −20°C. Extracellular IgG_1 titer was measured using enzyme-linked immunosorbent assay (ELISA). The remaining cell pellets were washed once with 1 mL phosphate-buffered saline (PBS), then per-

Table I. Summary of experiments.

Experiment no.	Description
1	P recovered in 5% FBS is transferred to 0% FBS at day 0 (2 weeks after thawing) NP recovered in 1.25% FBS is transferred to 0% FBS at day 0 (2 weeks after thawing)
2	P recovered in 5% FBS is transferred to 1.25% FBS at day 0 (2 weeks after thawing) NP recovered in 1.25% FBS is transferred to 1.25% FBS at day 0 (2 weeks after thawing)
3	P grown in 1.25% FBS is transferred to 0% FBS 108 days after thawing (at day 94 of experiment 2) NP grown in 1.25% FBS is transferred to 0% FBS 108 days after thawing (at day 94 of experiment 2)
4	P in 1.25% FBS and NP in 1.25% FBS are mixed in 1:4 ratio and transferred to 0% FBS 108 days after thawing (at day 94 of experiment 2)
5	Change medium content of cells from experiment 1 (NP culture) at days 68–104 and days 138–184.



(A)



(B)

Figure 1. (A) Peak superpositions of FITC histograms showing P or NP cells with no FITC stain (off scale), NP stained with GAM-IgG_1-FITC, P or NP with GAM-IgG_{2b}-FITC (negative control), and P stained with GAM-IgG_1-FITC. (B) Peak locations of NP in 1.25% FBS and P in 1.25% FBS (experiment 2) and P in 0% FBS (experiment 1) stained with GAM-IgG_1-FITC. The x axis shows channel number of log green fluorescence, y axis shows cell number for each histogram.

meabilized with 1 mL of ice cold 70% ethanol for intracellular antibody assay and stored at −20°C for a period greater than 16 h. These cells were stained and assayed for IgG_1 content within 1 month of sampling.

ELISA

The method and reagents used are described in reference 9.

Flow Cytometry

A Coulter EPICS 751 flow cytometer was used to measure the Ab content of the cells (Coulter Electronics, Hialeah, FL). Forward angle light scatter (FALS) and 90° light scatter (90LS) data were used for the estimation of cell size and complexity. Viable cells could be differentiated from lysed, dead cells by differences in their FALS and 90LS characteristics. Nonviable cells were eliminated by gating from a 90LS-FALS histogram.

Cell Staining

Fixed cells were centrifuged for 10 min at 1000 rpm, washed with 1 mL PBS, and centrifuged again. The pellet was resuspended in 180 μL fluorescein isothiocyanate (FITC) labeled goat antimouse (GAM)–IgG_1 (at 0.02 mg/mL, stock solution from Southern Biotech) and incubated 45 min on ice. The cells were next centrifuged 10 min at 1000 rpm, the FITC solution was removed, and the cells washed with 1 mL PBS. The cells were either analyzed immediately or stored overnight at 4°C before analysis. No differences in physical (90LS and FALS) or staining (FITC) characteristics were found between these methods.

P and NP

A producing population is easily tracked by flow cytometry, in which a detectable FITC-labeled GAM–IgG_1 is used to bind to intracellular IgG_1.[1,4,9] Nonproducers are defined as those cells which do not secrete IgG_1, as measured by ELISA. The NP is represented by the lower peak of the flow cytometry FITC histogram. The mean channel number of this peak is higher than that of an unstained sample of either P or NP. The producers, secreting approximately 0.15 pg/cell h IgG_1, are represented as the higher peak of the histograms, located 60–100 channel numbers (on a log scale) above the NP mean peak depending on the activity of the FITC conjugated antibody used. Both P and NP stained with GAM–IgG_{2b}–FITC (Southern Biotech) as a control produced a single peak at a higher position than the NP peak. These results are shown in Figure 1A. Since hybridomas produce one isotype of Ab and P secretes a detectable amount of IgG_1, we attribute both the IgG_{2b} and NP IgG_1 fluorescence to a combination of autofluorescence and nonspecific binding. The P and NP peaks are not due to differences in viability since the populations lay within the same 90LS–FALS map and the viabilities (as measured by trypan blue) of the stable P and NP cultures (shown in Fig. 1B) were comparable. Although the peak locations were constant within each FITC assay for both dual- and single-peak cultures, they varied between assays depending on the activity of the FITC probe used. However, since the NP and P peaks were always distinct, we stress the importance of relative peak areas over peak locations. Percentage P was determined by the ratio of the higher peak area (P) to the total peak area (P + NP).

Figure 1A also shows there is some overlap between the P and NP peak areas, depending on the broadness of the curves. As stated above, the peak locations and thus the overlap, were dependent on the activity of the FITC-conjugated antibody used. The peaks were resolved by extrapolating from the shapes of the curves and then using MDADS II software from Coulter to allot proportions of the intersection to each peak's area. As a control for the resolving consistency of each new lot of FITC-conjugated antibody, identical samples from cultures previously showing dual-peaked histograms were stained using old and new lots of GAM–IgG_1–FITC. Although the peak locations were different, the area proportions were approximately the same for each lot. The peaks of the dual-peaked cultures were located in the same positions as the stable P and NP peaks of the single-peaked cultures, which thus acted as reference points (or internal standards) for the dual-peaked culture.

RESULTS

After P and NP cells were transferred from their respective recovery media to experimental media at day 0, the culture viabilities generally decreased and then increased to initial control (recovery media) levels. After 10–40 days, all culture viabilities were at least 85%. The P and NP cultures cultivated in 1.25% FBS were stable over a 200-day period: their Ab secretion rates were constant and their intracellular antibody profiles remained distinct from each other. Figure 1B shows sample FITC histograms of the 1.25% FBS cultures (experiment 2) and the P cells grown in 0% FBS, which were also stable.

The NP cells cultured in 0% FBS exhibited a changing internal Ab profile. A second peak (P) was apparent 12 days after the culture was seeded (day 12). Percentages of producing cells were determined from the internal Ab peak profiles (see ref. 9). Figure 2 (experiment 1)

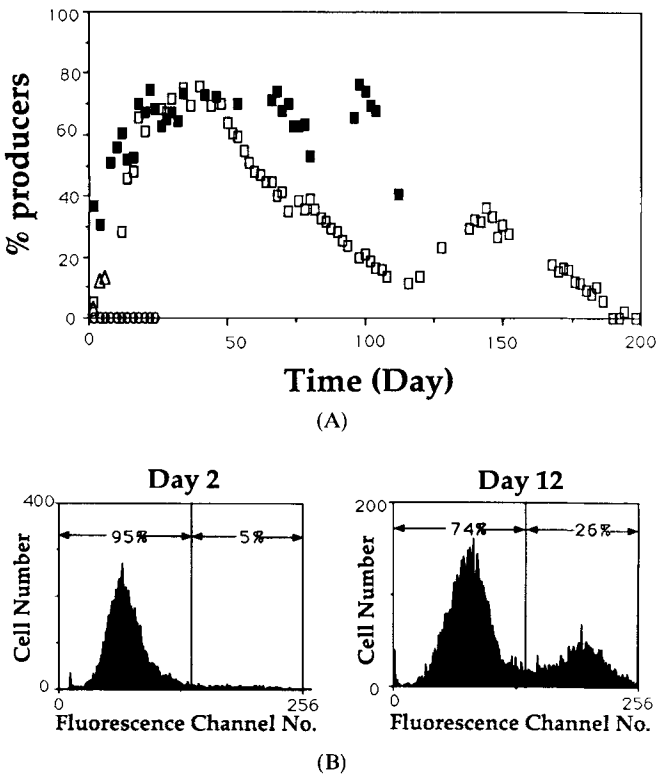


Figure 2. (A) Graph of percentage of producers in 0% FBS: (□) NP culture in experiment 1; (△) experiment 1 repeated through day 6; (○) NP culture in experiment 3; (■) experiment 4. (B) Histograms of samples from the experiment 1 NP culture at days 2 and 12.

shows how the percentage of producers in the culture changed with time. This pattern is not a reflection of the time of assay since samples taken at different times but stained simultaneously showed the same pattern as samples assayed sequentially. The transient change in the percentage of producers was confirmed by ELISA, in which culture media assayed for secreted IgG_1 showed a large peak in Ab concentration versus time followed by a smaller peak in a pattern (data not shown) very similar to that shown in Figure 2. A correlation between fraction of producers and specific Ab productivity has been established.[7,9] Another vial of NP cells from the same freezing was thawed and seeded in 0% FBS 1 week later. Less than 5% P was measured 2 days after seeding in experimental medium (day 2) and increased to 13% of the total population by day 6 (Fig. 2A), at which time this culture was terminated.

The importance of state of inoculum was tested by separately transferring P and NP cultures growing in 1.25% FBS to 0% FBS after day 94 (experiment 3). The viable cell density and percentage viability for the transferred NP and P cultures are shown in Figs. 3A and 3B, respectively. The internal Ab contents as compared by flow cytometry showed no population shifts (data not shown). The P in 0% FBS retained its specific productivity (data not shown) and viability over a 12-day period, which was expected since P seeded in 0% FBS at day 0 was also stable (experiment 1, Fig. 1B). The NP cells in experiment 3 showed a lag phase lasting 10 days, in which the viability dropped to less than 10%. After this period (note the cells were not passed when the viable cell density was less than 4×10^5 cells/mL), the cells recovered to at least 85% viability. A similar comeback was observed for NP cells transferred to 0% FBS from 1.25% FBS at day 174 (data not shown). The initial viability did not improve when conditioned medium was used for half of the passing medium volume. Average growth rates for P and NP (after the lag phase) were calculated to be 0.55 and 0.67 per day, respectively, for cultures with 80–98% viability.

To determine whether the transient behavior shown in experiment 1 (Fig. 2) was caused by an interaction between producing and nonproducing cells, P and NP cells from the two 1.25% FBS cultures were mixed and seeded at a ratio of 1:4 in 0% FBS at day 94. This seeding ratio was selected and deemed appropriate considering how fast the curve from experiment 1 (Fig. 2) rose, although it is much larger than the estimated initial levels of P to NP in experiment 1 (approximately 5:95, shown in Fig. 2). This culture is described by experiment 4 (results also shown in Fig. 2). This culture behaved similar to the experiment 1 culture until the 60th day after its seeding. Whether or not this culture attained a mixed population equilibrium after this time is inconclusive.

To test the sensitivity of the population dynamics exhibited by experiment 1, short-term experiments on this transient culture were performed in either 5% FBS medium or 0% FBS medium with the BSA removed (as described in experiment 5). In both media, the nonproducing fraction of the population increased faster than in the standard 0% FBS medium (Fig. 4). The viability of both these cultures was always above 84%.

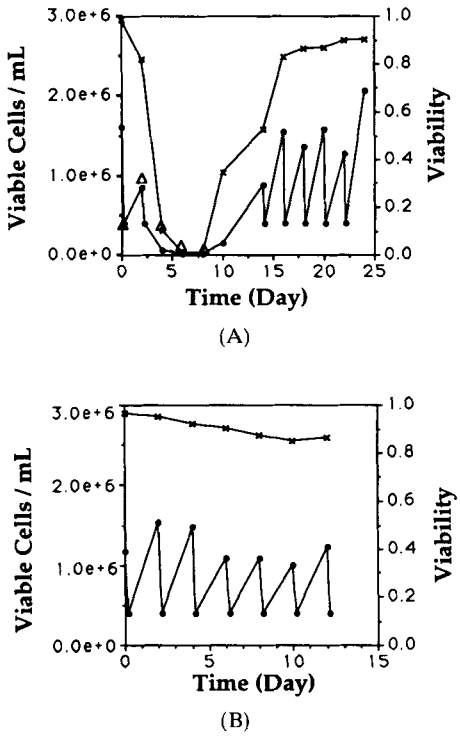


Figure 3. (A) Viable cell concentration (●) and viability (×) vs. time for NP cells in experiment 3; (△) 50% conditioned media used in passing volume. (B) Viable cell concentration (●) and viability (×) vs. time for P cells in experiment 3.

DISCUSSION

The effect of adaptation on growth rates and viabilities has been investigated in previous studies.[6,10] The difference in growth rates between populations at different stages of adaptation to media may explain why the P

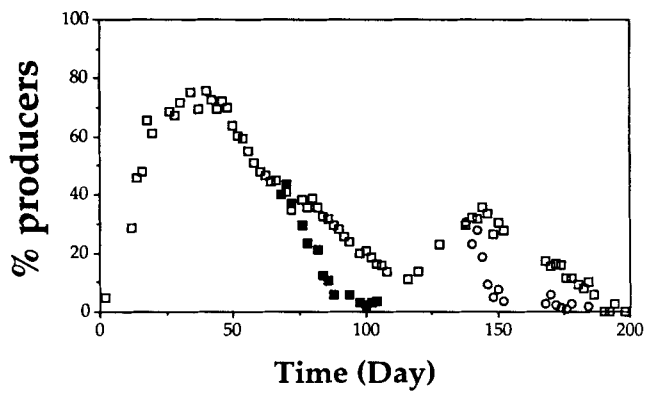


Figure 4. Graph of percentage of producers in transient culture growing in 0% FBS (□) (reproduced from Fig. 2A), 5% FBS (■), and 0% FBS without BSA (○) (experiment 5).

and NP mixed culture (experiment 4, Fig. 2) does not show the same unsteady behavior as the transient culture (NP culture in experiment 1, Fig. 2) after the lag period. The higher growth rate of NP over P in the latter part of experiment 3 can be used to approximate the growth rates of the cells in experiment 1 after the lag phase, but the population balance observed in experiment 1 probably results from small variations from these growth rates. The P and NP grown separately in 1.25% FBS were stable over the 200-day period. Since NP in experiment 4 had been cultured for more than 3 months in 1.25% FBS before being seeded in 0% FBS, its growth rate history (or state of adaptation to 1.25% FBS) was very different from the same NP-derived population transferred to 0% FBS at day 0. This difference in growth history may also account for the difference between experiments 1 and 3, where the cells were transferred to 0% FBS 2 weeks after defrosting at 47% viability and 13 weeks after defrosting at more than 90% viability, respectively.

The instability observed in 0% FBS (experiment 1) provides a dramatic illustration of the importance of relative growth rates of P and NP populations. This consideration is applicable to low serum or serum-free media where stressful conditions caused by nutrient or growth factor limitations may change the metabolism of the populations unexpectedly. It is also possible that nonproducers can arise from mutations within the producing population,[1] but this is probably not significant here since the producers seeded in 0% FBS at day 0 were stable (Fig. 1B). The presence of producers in the experiment 1 inoculum is supported by a similar increase in percentage P when another culture from the same freezing was thawed, recovered, and grown in 0% FBS medium.

State of inoculum was tested in experiment 3, in which P and NP cells grown in 1.25% FBS for 94 days were then seeded into 0% FBS. (In experiment 1, P and NP cells were grown in recovery media for 2 weeks before being transferred to 0% FBS.) In experiment 1, a transient population balance was observed in the NP culture, and in experiment 3, no population shift occurred. The difference in the states of inoculum tested was the difference in adaptation to medium before transfer to 0% FBS, but the results suggest this difference may also include the population balance within the inoculum. The idea of population balance between two populations is supported by the initial lag phase of the NP culture upon transfer to 0% FBS and the lack of an adjustment period in the P culture. Thus, the initial increase of P in experiment 1 may be due in part to the lag phase of the NP cells. Figure 2 shows that aside from the initial lag phase, the P and NP populations shift with time, indicating that they may compete for some factors (either in the fresh medium or produced by the cells in limiting amounts) required for growth. Beyond adding conditioned medium to NP experiment 2 cells, the identities of these factors were not pursued. In this experiment, the conditioned medium did not eliminate the lag phase.

Finally, high serum levels will not necessarily prevent the appearance of a nonproducing population. Bimodal FITC histograms have been observed in high serum cultures,[1,4] and heterogeneous populations occur frequently in newly cloned cultures.[2,3] Also, increasing the serum level does not necessarily give the producing population dominance. Figure 4 shows the producing population was taken over more rapidly by the nonproducers in the presence of 5% FBS than in the 0% FBS medium, suggesting that the growth advantage of one population over the other is mediated by media composition rather than only serum level.

In summary, a population shift occurred in a 0% serum culture that temporarily favored the growth of an Ab-producing fraction of hybridomas over the nonproducing clones. This shift was dependent on state of inoculum (stage of adaptation) and serum level. The 0% FBS medium was not necessarily more stringent for cell growth compared to the 1.25% FBS medium; it merely provided an environment that allowed the small population of producers to dominate the culture for a finite period of time. Although NP is a clone derived from P, NP lacked the ability to adapt as quickly as P to the 0% FBS media. This difference in adaptation is an example of how Ab-producing and Ab-nonproducing cultures must be considered as two separate populations, with different responses to different media compositions. A nonproducing population does not necessarily have a growth advantage over a producing population. As a rule, it is important to measure the integrity of one's culture to track the purity of Ab producers. This can be done using flow cytometry. Purity of population is as important a variable as viability and growth rate in predicting Ab titer and should be monitored as such.

This work was supported by National Science Foundation Grant No. BCS-9009389. The authors thank Sadettin Ozturk for valuable discussions, Latham Claflin for providing the hybridoma cell line 167.4G5.3, and Mark Cameron for help with flow cytometry.

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