Three-Stage Approach of Hybridoma Media Optimization

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Abstract (#333)

Omitting serum from media and optimizing other components for cell growth and productivity require a carefully designed approach with quantitative and nonsubjective methods. We recently used a three-stage approach in optimizing our serum-free hybridoma media formulation. In the first stage numerous components were tested for general growth and toxic limits using 24-well and 96-well plates and measuring growth by resazurin fluorescence. In the second stage of optimization 125-ml or 250-ml spinner vessels were used to find the best combination of components for cell growth and IgG production, comparing simple pairs of test conditions, concentration profiles, or complex matrices with multiple components. In the last stage tandem 5-L bioreactor runs (with or without media exchange by perfusion) tested cell growth and productivity under pilot-scale culture conditions.

Several examples of this approach are being presented in this poster. A plot from an experiment with HFN cells in a 96-well matrix assay showed toxic limits and interactions of two vitamins. In another experiment, we used a "2^3 + center point" statistical design to test three supplements for potential interactions. Follow-up experiments examined interactions of two supplements (vitamins and amino acids) using a rotatable central composite design. The results were analyzed as 2nd order statistical three-dimensional contour plots of cell growth and IgG production. From these experiments, we chose the optimal combination of components for our serum-free hybridoma medium. This medium was further refined to eliminate all animal components, including transferrin. Spinners were used to compare these media to competitor media using several hybridoma cell lines. In addition, bioreactor trials demonstrated strong growth and productivity at high cell density. Thus, using the three-stage approach we have formulated media that supports high cell density and productivity for a variety of hybridoma cell lines.

Introduction

The next decade is expected to see a large increase in the use of antibodies as *in vivo* therapeutic agents. Currently there are at least 200 antibodies or antibody fragments undergoing clinical trials(1). As these biopharmaceuticals are being developed, there is increasing attention focused on the need to produce them using cell culture media that contain no serum or any other components of animal source(2,3).

We have used a three-stage approach in evaluating raw materials and optimizing the formulation of serum-free and animal-component free hybridoma media. In the first stage we tested numerous agents for general growth characteristics and gross toxicity limits in static culture using 24-well and 96-well plates. In the second stage we optimize the complex mix of components for cell growth and productivity using spinner flasks. In the third stage, we verify or further optimize this medium while comparing it to competitor products and using various other cell line and systems.

Materials and Methods

Cell Lines. HFN 7.1 hybridoma cells, from a fusion of a mouse B cell and a P3x63Ag8 mouse myeloma cell, were purchased from ATCC as number CRL-1606. Cells were initially grown in DMEM containing 10% FBS until frozen in DMSO-containing freezing medium (Sigma product C6164). During media development HFN cells were routinely thawed into DME/F12 medium containing 2% FBS and then passaged within 3-6 days into our most current version of serum-free medium (H4281) or animal component-free medium (H4409). M-2E6 hybridoma cells were also purchased from ATCC (HB 138) and three hybridomas were obtained from Abbott Laboratories and designated as "H," "M" and "P."

Plate Assays. Polycarbonate 96-well flat-bottom sterile culture plates (Sigma Product M9780) were used in this fluorescence assay. To minimize edge-related anomalies, only the inner 60 wells of each plate were inoculated with cells (5,000 cells per 200 ul per well); the remaining perimeter wells contained 200-ul of media only. The plates were placed in a humidified-controlled incubator at 37°C and 5% CO_2. After the specified number of days, one twentieth volume of resazurin dye (Sigma Product Number TOX-8) was added to each well and the plates were incubated as above for an additional 30 minutes. Fluorescence was measured using a Perkin Elmer HTS 7000 Plus BioAssay Reader set at 550 nm excitation and 595 nm emission. Readings from blank wells (containing resazurin but without cells) were averaged and subtracted from each test well to give net relative fluorescence units (RFU).

Spinner Assays. Cells were seeded into 125-ml or 250-ml capacity spinner vessels at $5x10^4$ or $1x10^5$ cells/ml on day zero. Viable cells/ml in each spinner was determined. Cell-days, the integral area under a cell growth curve, was approximated using a trapezoidal point-to-point summation method. Unless otherwise stated, all data points are averages of two spinners.

IgG Concentrations. The concentration of immunoglobulin G secreted into the medium was determined by HPLC using a Protein-A binding column and 280nm absorbance.

Three-Dimension Plots. Mesh plots of data from

full arrays (96-well plates) were generated using SigmaPlot version 5.0 (SPSS). Contour plots of data from central composite design experiments were generated using Statistica, Release 5.1, Experimental Design module (StatSoft).

Results and Discussion

During the first stage of media optimization, fluorescence from the indicator resazurin was used as a proxy for cell growth in 24-well and 96-well plates. As an example in this stage of media development, Figure 1 shows the results obtained from titration of two vitamins in an orthogonal array. This method was used to screen certain agents or mixtures for toxicity limits or interactions. In this example components B and T each appeared to be somewhat toxic at their highest concentrations (approximately 250 times that in DME/F12 medium) and together they showed additive toxicity. Future trials focused on concentrations of B and T that fell within the relatively broad plateau of high activity.

Two-Component Matrix: Effect on Cell Growth

(Measured by Fluorescent Indicator Resazurin)

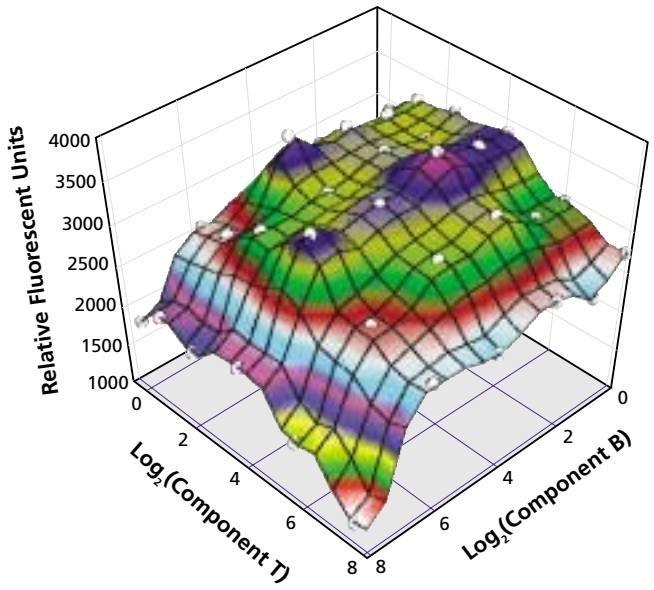

Figure 1

The second stage involved testing media in spinner vessels to identify the best combination of components for cell growth and productivity. The final concentration of antibody, usually IgG, was chosen as the best single parameter for measuring hybridoma productivity. The best single parameter for measuring cell growth is less straightforward. Figure 2a shows how a simple media containing 2% FBS might appear to outperform the serum-free medium early on the basis of viable cell/ml alone. Since the serum-free medium is clearly superior in total growth support, comparing cell densities alone is not an adequate single parameter to gauge cell growth. Instead, we have estimated the integral (area below the final growth curve) to combine cell density and longevity into a single measurement we term "Cell-Days." Figure 2b shows that the serum-free medium was more than twice as effective in supporting cell growth than the serum-containing medium.

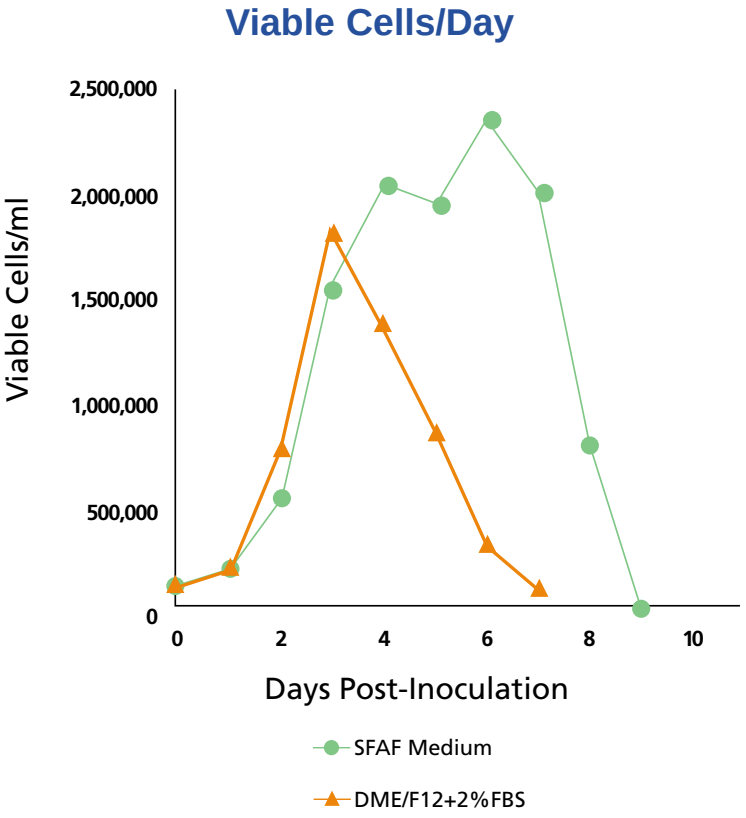

Figure 2a

Relationship Between Viable Cells/Day and Cumulative Cell-Days

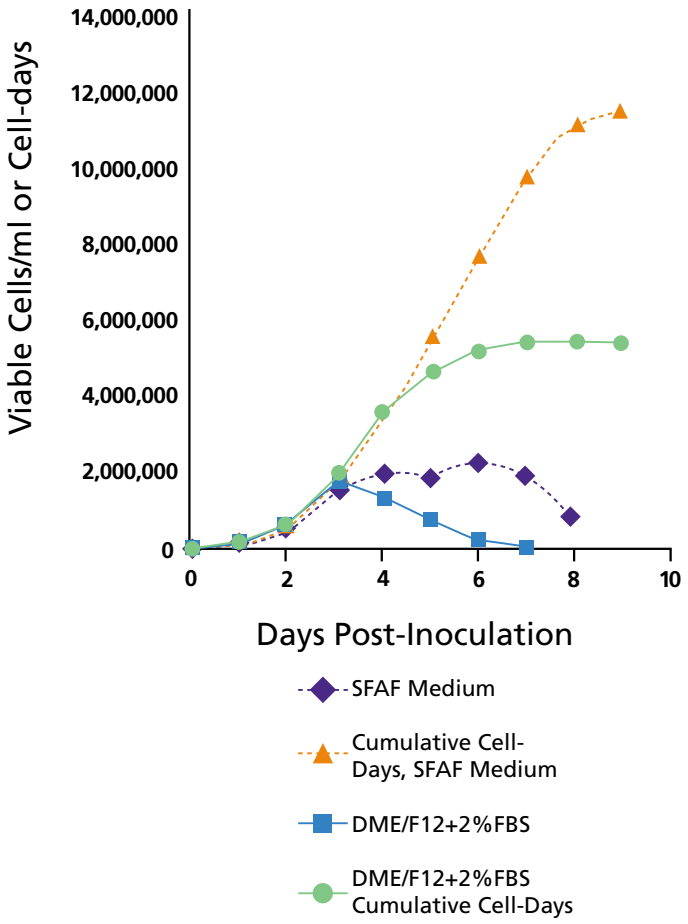

Figure 2b

The traditional method of medium development has been to optimize one component after another in a linear sequence and then to repeat this cycle until the medium is declared optimized. That method does not efficiently address the highly interactive nature of many components in a complex medium. While looking at all possible combinations of all components simultaneously would certainly reveal their interactions, it would be far too complicated and require a prohibitive number of experimental conditions. We employed a statistical matrix to screen for interactions and main effects of several components. Full-factorial designs and other methods were then used to examine these interactions in more detail(4).

Figure 3 shows a "2^3 plus center point" design with HFN cells for testing the effects of three supplements (two concentrations each) with a center point at mid-concentration for each of the three supplements. The numbers in this example show final IgG concentrations normalized to the basal level (the lower left corner with 0.1X amino acid supplement and without vitamin or trace metals supplements). The data show that productivity decreased consistently when moving vertically from the lower square to the upper square of the cube, indicating that the addition of further amounts of the supplemented trace metals was not beneficial. We therefore chose to keep the media at its previous level of trace metals in future trials. Raising the level of vitamin supplement alone gave no productivity increase and raising the level of amino acid supplement alone increased productivity by 18.7%, but when these two supplements were combined productivity increased by 44.1%.

3-Component Matrix in Spinners

Final IgG Concentration (% Change from Original)

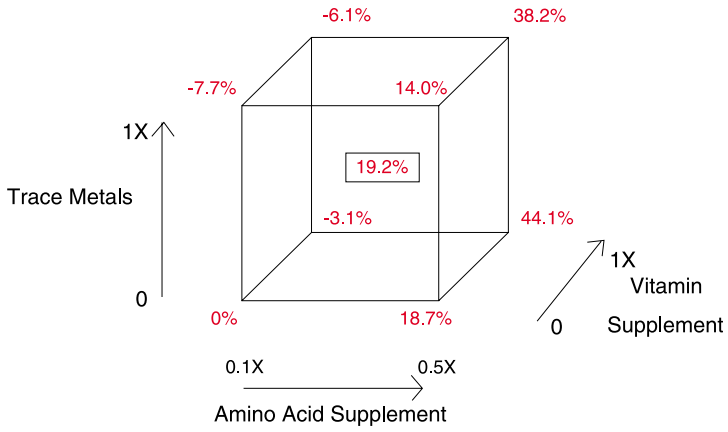

Figure 3

To explore in more detail the relationship between the two supplements and growth or productivity, we used a rotatable central composite design to generate 2nd order three-dimensional contour plots(5). HFN cells were tested in duplicate experiments with a total of 12 spinners at the center point and 4 spinners at each of the other test conditions, including four at the original condition. Contour plots were generated from the averaged data of the combined HFN experiments using Statistica software. The statistical response surface generated for cell-days (Figure 4a) was dome-shaped, showing a relatively broad maximal response near the center point. The response surface generated for IgG concentration (Figure 4b) was slightly saddle-shaped, but was also somewhat level toward the center point. The highest concentrations of vitamin supplement produced even greater IgG secretion, perhaps due to a general stress on the cells similar to high osmotic concentration(6).

HFN Cells
Cumulative Cell-Days, Final

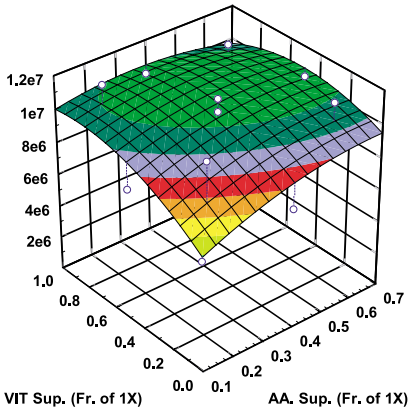

Figure 4a

HFN Cells
IgG Concentration, Final

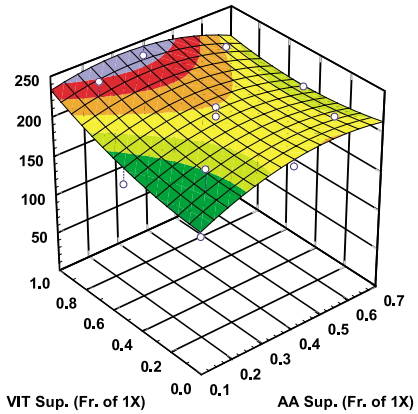

Figure 4b

Hybridoma cell line "H" (from Abbott Laboratories) was also examined in a single experiment of the same central composite design and with identical concentrations of the two supplements. In this case, four replicate spinners were included at the center point and two replicates for each of the other conditions. In both growth and productivity, cell line "H" was more responsive to vitamin supplement than to changes in the amino acid supplement (Figures 5a and 5b).

Cell Line H
Cumulative Cell-Days, Final

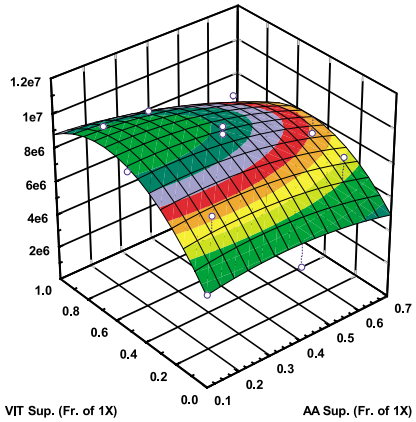

Figure 5a

Cell Line H
IgG Concentration, Final

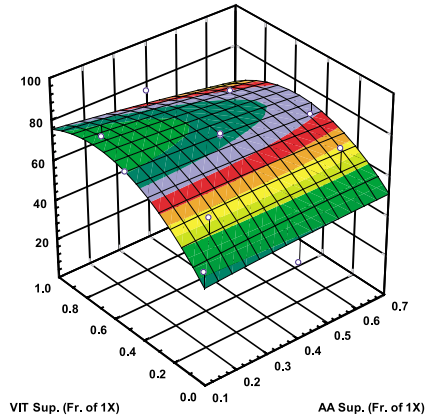

Figure 5b

Our goal in optimizing media performance and reliability was to identify the contour region in both cell lines that showed high productivity and high growth (maximal region) as well as low variability (most level region). After considering the growth and productivity contour plots for both cell lines, we chose an optimal concentration near the center point of this series for our serum-free medium.

Figure 6 shows that the newly optimized serum-free hybridoma medium (Sigma product H4281) is superior to the previous formulation in growth and productivity of HFN cells throughout the assay time course. During optimization of the animal component-free medium (Sigma product H4409), transferrin was also replaced by an iron chelation system.

Before and After Optimizing for Vitamins and Amino Acids

HFN Cells: Cumulative Cell-Days and IgG Concentrations

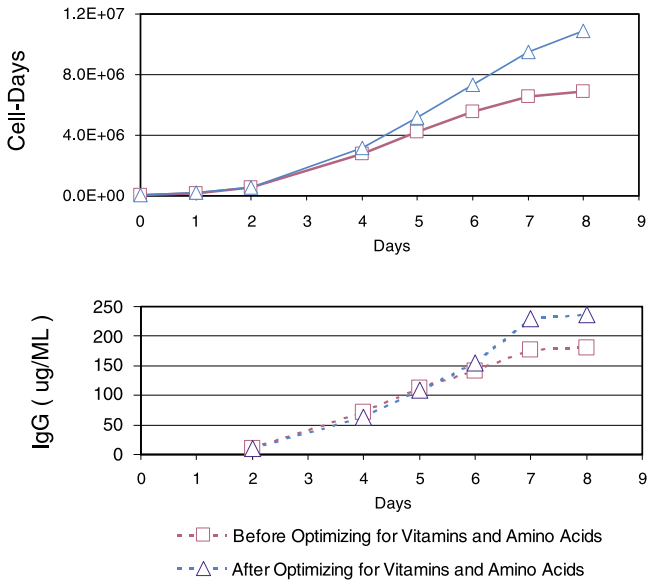

Figure 6

To compare the performance of our media with competitors, we obtained 16 other hybridoma media products that were marketed as being serum-free, protein-free, or animal component-free. For this assay, frozen HFN cells were thawed into DME/F12 with 10% FBS. They were then weaned into the different media over a ten-day period and seeded into spinner flasks for stock cultures. Eight of the media products failed to support the HFN cells during this protocol, leaving ten cell populations weaned into eight competitor media and our two media (H4281 and H4409). A spinner assay was begun using the surviving populations. Eight days later a second spinner assay was also begun using cells maintained in their respective media. An additional weaning and assay was later performed with the top few competitor media. Figure 7 shows the average growth and productivity resulting from the three experiments with standard error bars (n = 3 for H4281, H4409, A, B, C and E; n = 2 for F, G and H; and n = 1 for D). These HFN cell experiments indicate that our serum-free hybridoma medium (H4281) outperforms all other "serum-free" media tested. Our animal component-free hybridoma medium (H4409) essentially matched one competitor and outperformed all other tested "protein-free" or "animal component-free" medium products.

Serum-Free or Protein-Free Hybridoma Media Competitors Using HFN Cells (Standard Error Bars Indicated)

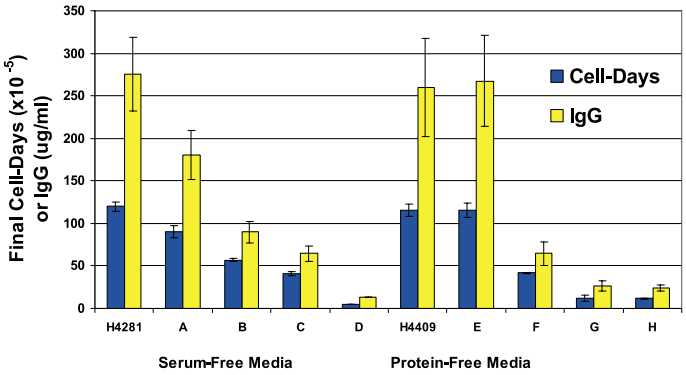

Figure 7

A different hybridoma cell line, M-2E6, was weaned into competitor media products by the weaning procedure used for HFN cells. Results from the subsequent experiment (Figure 8) indicate that both H4281 and H4409 outperform all tested competitors using M-2E6 cells.

Serum-Free or Protein-Free Hybridoma Media Competitors M-2E6 Cells (Averages of duplicate spinners)

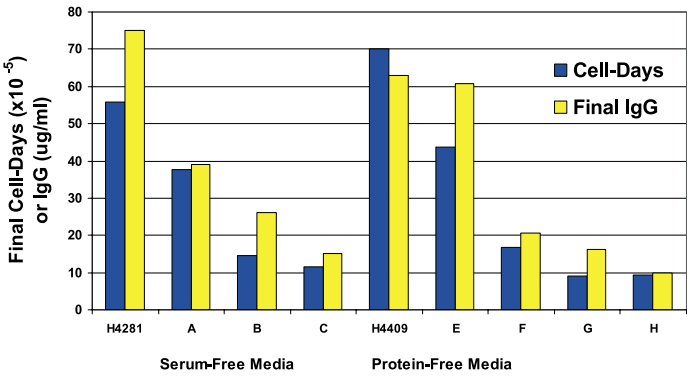

Figure 8

HFN cells tested in fed-batch operated bioreactors showed similar results. An example of a bioreactor run is shown in Figure 9.

We also compared our media with the three top competitor products using three different hybridoma cell lines from Abbott Laboratories. Figure 10 shows that our serum-free medium (H4281) outperformed the leading competitor products in all cell lines. Our animal compo-

Fed-Batch Stirred Tank Bioreactor Run

HFN Cells: Cumulative Cell-Days and IgG Concentrations

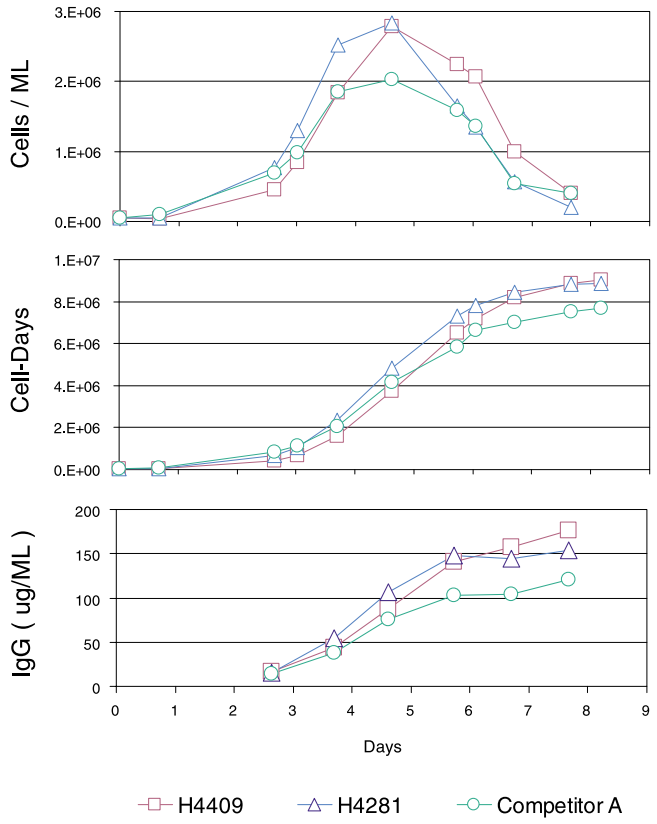

Figure 9

nent-free medium (H4409) was similar in performance to one competitor and outperformed the other leading competitors in these cell lines.

Comparison of Optimized Media with Competitors Using 3 different Hybridoma Cell Lines

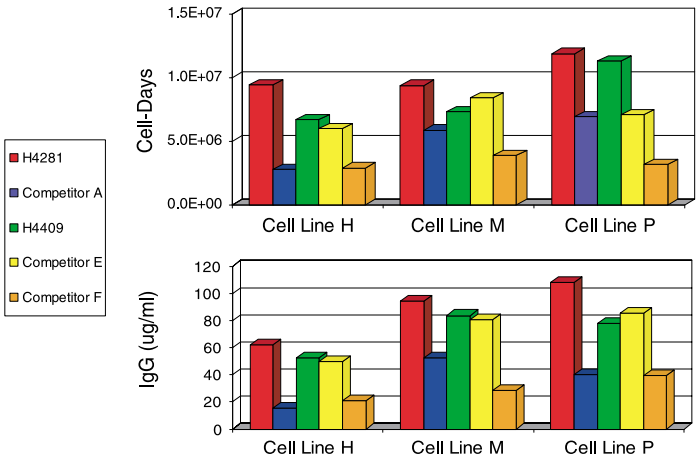

Figure 10

Altogether, our two hybridoma media products have shown excellent growth and productivity performance. The unique genetic combination of each clone suggests that no single medium will be optimal for all hybridoma cell lines. During media development we chose HFN as our model cell line because its growth characteristics were typical of many hybridoma clones used in the biopharmaceutical industry. Additional hybridoma cells lines and growing systems (e.g., perfusion stirred tank bioreactors, hollow-fiber bioreactors and roller bottles) are currently being tested. Results to date support our conclusion that both H4281 and H4409 rank at the top of all commercially available media products in supporting growth and productivity of a variety of hybridoma cell lines and growing systems.

Conclusion

By a systematic method involving three stages of development, we have eliminated serum and other animal-derived agents from hybridoma cell culture medium and have replaced it with a complex mixture of components from non-animal sources. These media formulations have been optimized for cell growth and productivity using multiple hybridoma cell lines and have been tested against many competitor products and in different culture systems. Both final products, a serum-free low-protein hybridoma medium (H4281) and an animal component-free hybridoma medium (H4409), show excellent cell growth and antibody production characteristics and rank at the top of all commercially available hybridoma media products.

Acknowledgements

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