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Replacing the Animal Component in Serum

Evaluating Raw Materials for Inclusion in Optimized Hybridoma Media

Steven C. Peppers, Daniel W. Allison, Terrell K. Johnson, Damon L. Talley, and Matthew V. Caple

With the scarcity of CGMP facilities spurring a production crisis, manufacturers need to optimize their processes and formulations for individual hybridoma cell lines and production systems. Using a factorial matrix-based process to find an optimal set of such complex variables can reduce R&D costs, shorten time to market, and secure scarce production resources early.

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The next decade is expected to see a large increase in the use of antibodies as in vivo therapeutic agents. Currently, at least 200 antibodies or antibody fragments are undergoing clinical trials (1). Development of these biopharmaceuticals is increasing the focus on the need to use cell culture media that contain no serum or any other components of animal origin (2).

Reasons for removing serum and other animal-derived raw materials from media are varied and usually include a combination of such factors as component variabilities inherent in biological sources, possible contaminants, the high cost of serum, and downstream processing issues. Because of the biological nature of serum, both the quality and performance of the final products are subject to substantial variation. Serum and animal-derived materials also have the potential for introducing contaminants and toxic elements such as viral particles and endotoxins (3). As a raw material, serum adds substantial cost to biopharmaceutical manufacturing. In addition, serum often interferes with final product purification, which increases the costs of downstream processing. Furthermore, regulatory issues associated with the use of biological materials in manufacturing processes are problematic; meeting those requirements also raises the final production costs.

We 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 for gross toxicity limits in static culture using 24- and 96-well plates. In the second stage, we optimized a complex mix of components for cell growth and antibody productivity using spinner flasks. We tested simple paired comparisons, concentration profiles, and factorial matrices, then analyzed the data using nonsubjective numerical methods and graphical techniques. In the third stage, we verified or further optimized the media while comparing them with competitor products and using various other cell lines and systems.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (www.sigma-aldrich.com) unless otherwise stated.

Cell lines. HFN 7.1 hybridoma cells, from a fusion of mouse B cells and P3×63Ag8 mouse myeloma cells, were obtained from the American Type Culture Collection (www.atcc.org, ATCC #CRL-1606). These cells secrete an IgG_1antibody directed against human fibronectin. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) until frozen at 2×10^6–1×10^7 cells/vial in DMSO-containing freezing medium (Sigma, #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) in spinner flasks for growing cell stocks. Cells were not allowed to exceed 25 passages, and cell densities were consistently maintained below 1×10^6 viable cells/mL. The stock cell density at the start of an assay was not allowed to exceed 8×10^5 viable cells/mL. Three hybridoma cell lines were obtained from Abbott Laboratories (www.abbott.com). Designated as H (clone #4-481sc184), M

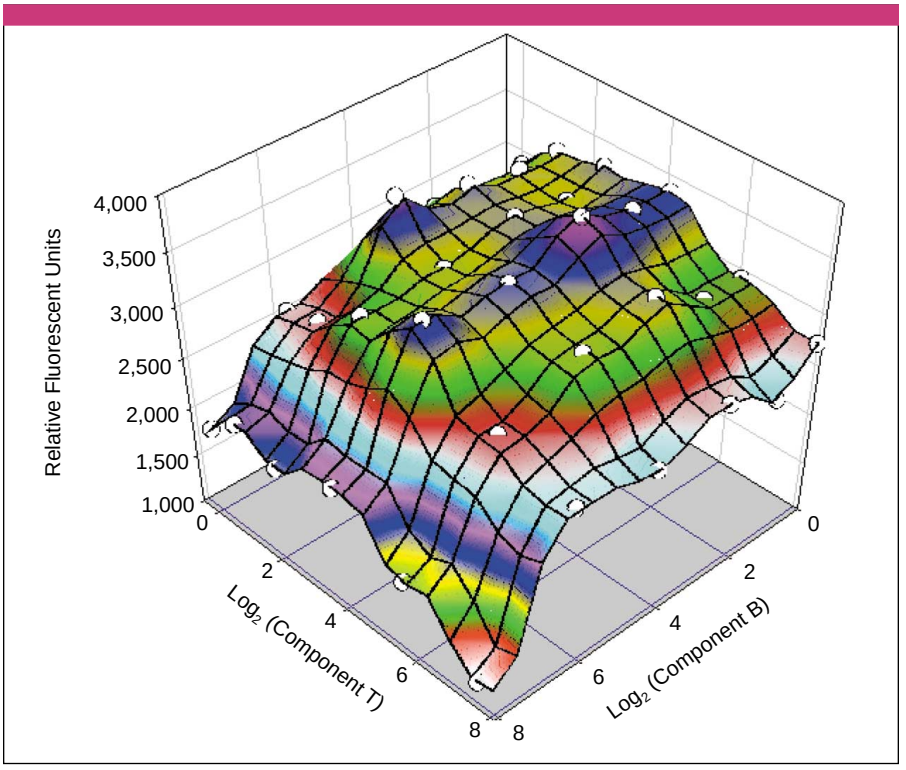

Figure 1. Two-component matrix showing the effect on cell growth as measured by the fluorescent indicator resazurin

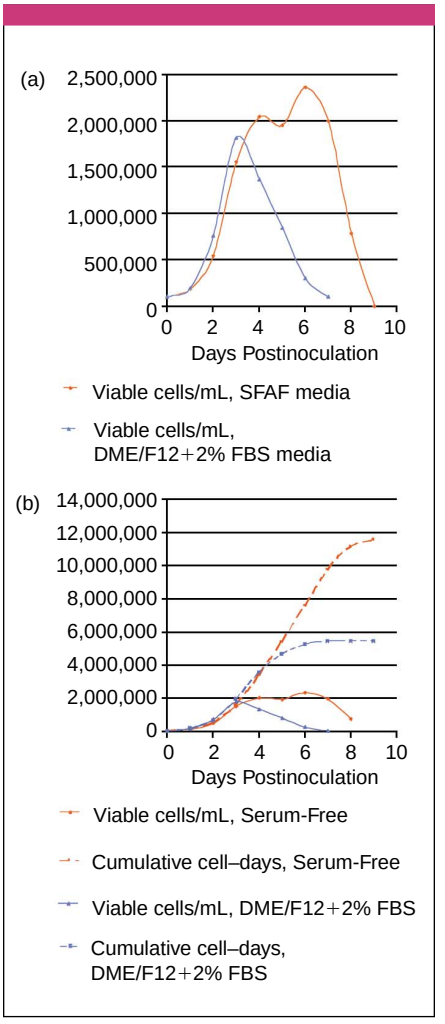

Figure 2. (a) Viable cells per day; (b) the relationship between viable cells per day and cumulative cell-days

(clone #24-168-353), and P (clone #H50C95C187), these cells were maintained similarly to the HFN cells.

Weaning into other media. For this set of assays, frozen HFN cells were thawed into DME/F12 with 10% FBS. After three days, the cells were subcultured in T-flasks containing DME/F12 with 2% FBS. Over the next 10 days, the cells were weaned from the serum into the various media by subculturing into each media using incremental passages. The first weaning step was into 50% DME/F12 with 50% other media and 1% FBS. The next passage was into 25% DME/F12 with 75% other media and 0.5% FBS. The final passage was into 100% other media (no FBS). Usually at the last weaning passage, the HFN cells were also seeded into spinner flasks to begin growing stocks for the assays.

Plate assays. Sterile polycarbonate 96-well flat-bottom culture plates (Sigma, #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/200 µL/well); the remaining perimeter wells contained 200 µL of media. The plates were placed in a humidity-controlled incubator at 37 °C and 5% CO_2. After the specified number of days, one-twentieth volume of resazurin dye (Sigma, #TOX-8) was added to each well, and the plates were incubated as above for an additional 30 minutes. Fluorescence was measured using an HTS 7000 Plus bioassay reader (PerkinElmer Instruments, www.perkinelmer.com) set at 550 nm excitation and 595 nm emission. Readings from blank wells (containing resazurin but no cells) were averaged and subtracted from each test well to give net relative fluorescence units (RFU).

Spinner assays. Spinner flasks with capacities of 125 mL or 250 mL from Bellco Glass (www.bellcoglass.com/index.htm) and Techne Inc. (www.techneuk.com) were used. Only one manufacturer, size, and style of spinner was used in each experiment. Cells were seeded into the spinner vessels at either 5×10^4 or 1×10^5 cells/mL on day zero. Total cells/mL in each spinner was determined daily using a CASY-1 cell counter (Schärfe Systems, Reutlingen, Germany). Percent viability was determined using trypan blue and a hemacytomete. The concentration of viable cells/mL was then calculated as percent viability times total cells/mL. *Cell-days*, the integral area under a cell-growth curve, was approximated using trapezoidal point-to-point summation. Unless otherwise stated, all data points are averages of two spinners. The average percent difference between each spinner in a replicate pair was approximately 12% for cell-growth data and 9% for productivity.

IgG concentration. The concentration of immunoglobulin G (IgG) secreted into the medium was determined by HPLC using a protein A binding column for capture and 280 nm absorbance for quantitation.

Contour plots. Mesh plots of data from full arrays (96-well plates) were generated using SigmaPlot version 5.0 (SPSS Science, www.spssscience.com). Response surface plots of data from central composite design experiments were generated using Statistica release 5.1, experimental design module (StatSoft Inc., www.statsoft.com).

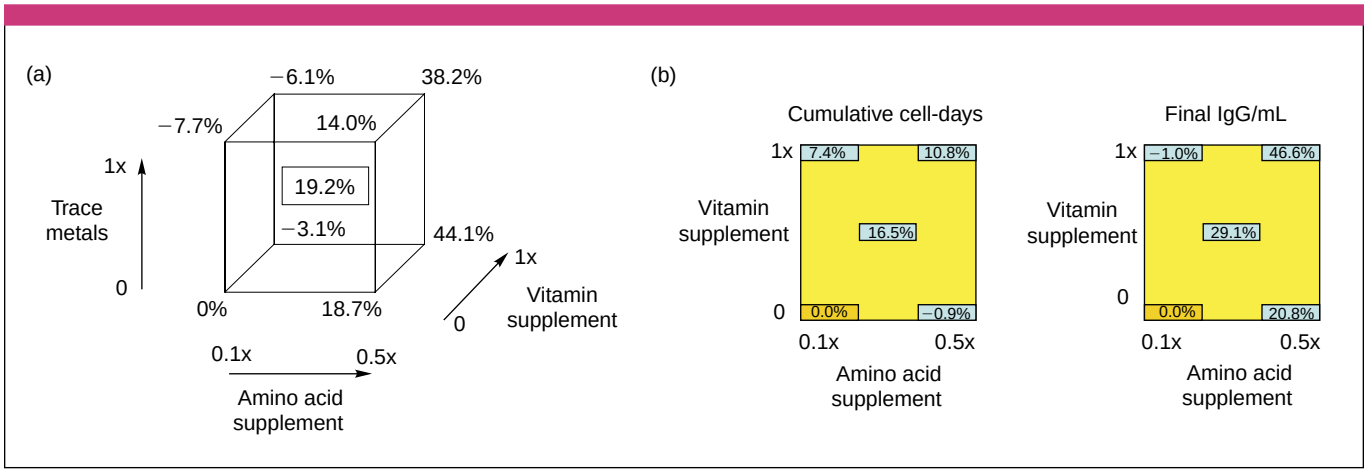

Figure 3. (a) Three-component matrix in spinners showing final IgG concentration (percent change from original); (b) the interaction of amino acid and vitamin supplements with averaged data after removing the effect of metal

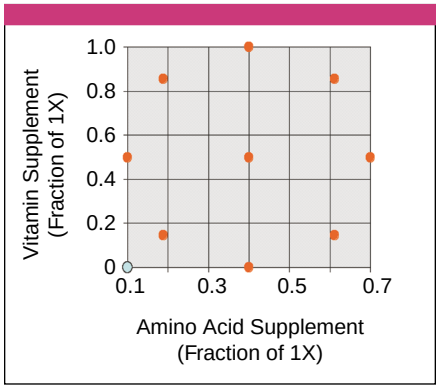

Figure 4. Two-component central composite design generating contour plots

Results and Discussion

Our media optimization process comprised three stages examining toxicity limits, best growth, and product comparisons.

Stage one: toxicity limits. In the first stage of our process to optimize serum-free or animal component-free media, we used resazurin, a fluorescent indicator, in 24- and 96-well culture plates to screen multiple agents for growth and toxicity limits. Increases in fluorescence and cell growth were tightly correlated for HFN cells (data not shown). As an example of such an experiment, Figure 1 shows the results obtained from titrating two vitamins (components B and T) in an orthogonal array (two-component matrix). This method was used to screen certain agents or mixtures for interactions in toxicity or growth. In this example, components B and T each appeared to be somewhat toxic at their highest concentrations (approximately 250 times that in DMEM/F12 medium); individually, B and T inhibited cell growth by approximately 45% and 20%, respectively. Combined, they showed an additive toxicity of approximately 65%. In addition, a slight rise in RFU was seen as T increased from the base level, but no such effect was seen for B. Subsequent trials focused on concentrations of B and T that fell within the relatively broad plateau of high activity (data not shown).

Stage two: best growth. The second stage of optimization involved testing media using 125-mL or 250-mL spinner vessels to identify the best combination of components for cell growth and antibody 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 illustrates how the DME/F12 medium containing 2% FBS appears to outperform the serum-free hybridoma medium in the initial days of testing when measuring only viable cells/mL. Because the serum-free medium is clearly superior in total growth support, comparison of cell densities alone is an inadequate single parameter for gauging cell growth. Instead, we estimated the integral area (under the final growth curve) to combine cell density and longevity into a single measurement we call 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.

Replacing the serum. Because serum is such a complex mix of undefined components, replacing it involves introducing or changing concentrations of many different interacting components. The traditional method of medium development has been to optimize each component in a linear sequence and then to repeat that cycle until the medium is declared optimized. That method inefficiently addresses the highly interactive nature of many components in a complex medium. Although simultaneously looking at all possible combinations of all components would certainly reveal their interactions, it would be far too complicated and would require a prohibitive number of experimental conditions. We employed a statistical matrix to screen for interactions and the main effects of several components. Full factorial designs were then used to examined those interactions in more detail (4).

Testing the supplements. The effects of three supplements (two concentrations each) were tested, as shown in Figure 3a, by a "2^3 plus center-point" design with a center-point at midconcentration for each of the supplements. The numbers shown are final IgG concentrations normalized to the basal level (lower left corner with 0.1× amino acid supplement, without vitamin or trace metal 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 were not beneficial. We therefore chose to ignore the effect of the trace metal supplement to gain greater statistical power in our data.

***FOR** optimizing media performance and reliability, the goal is to identify the region on the contour plots that show both high productivity and growth (the maximal region) and low variability (the most level region).*

The vertically linked pairs of data from the cube were then averaged and renormalized to the pooled basal level. These values were plotted as a square with a center point to examine the amino acid and vitamin supplements only. The squares in Figure 3b show changes in cell-days and IgG concentrations at each of these five points. Raising only the level of vitamin supplement resulted in an increase in cell-days without affecting productivity. However, raising only the level of amino acid supplement resulted in an increase in productivity without affecting cell-days. Simultaneously increasing both supplements gave substantial boosts in both growth and productivity. The center point values also suggested a nonlinear response to changes in these supplements.

Relationship between supplements and growth. To explore in more detail the relationship between the two supplements and cell growth or antibody productivity, we used a rotatable central composite design (Figure 4). This design was chosen for the generation of second order, three-dimensional, contour plots because of its inherent minimization of statistical bias (5). A test condition at the basal level was included for reference. We included HFN cells with four replicate spinners at the center point condition, with two replicates for each of the other test conditions. The entire experiment with HFN cells was repeated, except that an additional four spinners were included at the center point. Contour plots were generated from the averaged data of the combined HFN experiments using Statistica software. For optimizing media performance and reliability, our goal was to identify the region on the contour plots that showed high productivity and growth (maximal region) with low variability (most level region).

Figure 5a demonstrates that cumulative, final cell-days for HFN cells increased sharply as both the vitamin and amino acid supplements were simultaneously raised, reaching a relatively broad maximal response near the center point. Figure 5b shows that the response surface for IgG production appears slightly saddle shaped for HFN cells. Similar to cell-days, IgG concentrations also rose in response to increases in both the vitamin and amino acid supplements and became somewhat level toward the center point. The highest concentrations of vitamin supplement showed even greater IgG production, perhaps because of a general stress on the cells similar to the reported effect of high osmotic concentration (6).

Another hybridoma cell line, H (clone #4-481sc184), was examined by the same central composite design and with identical concentrations of the two supplements (Figure 6). Four replicate spinners were included at the center point and two replicates were included for each of the other conditions. For both cell growth and antibody productivity, cell line H was more responsive to increases in the vitamin

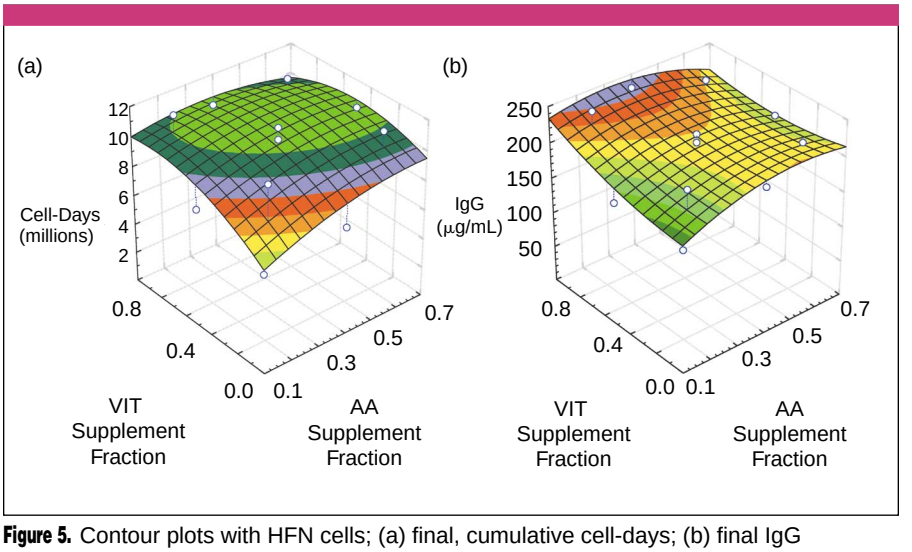

Figure 5. Contour plots with HFN cells; (a) final, cumulative cell-days; (b) final IgG concentration

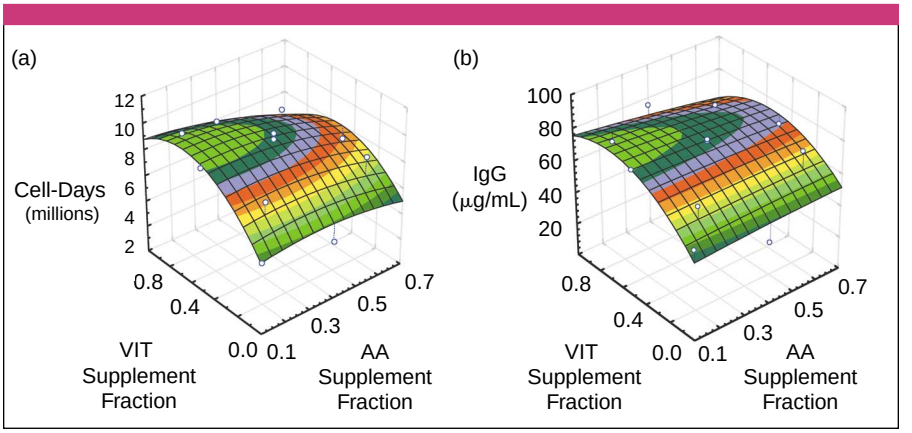

Figure 6. Contour plots with cell line H; (a) final cumulative cell-days; (b) final IgG concentration

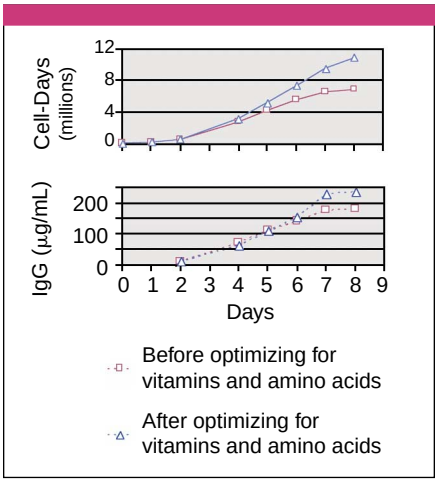

Figure 7. HFN cells showing cell-days and IgG concentrations before and after optimizing for vitamins and amino acids

supplement than to the amino acid supplement. After considering the growth and productivity contours for both cell lines (HFN and H), we chose an optimal concentration near the center point of this series for the formulation of our serum-free medium.

Figure 7 shows that the newly optimized hybridoma medium is superior to its earlier version in growth and productivity of HFN cells throughout the assay time course. During optimization of the animal component-free medium, transferrin was also replaced by an iron-chelation system that gave equivalent performance in several tested cell lines (data not shown). We finished our optimization stage with two finished media: a serum-free hybridoma medium (#H4281) containing small amounts of bovine serum albumin, bovine insulin, and human transferrin; and an animal component-free hybridoma medium (#H4409) containing no albumin, no transferrin, a small amount of recombinant human insulin, and an iron-chelation system with all components from nonanimal sources.

Stage three: comparing products. In stage three of our media optimization process, we needed to compare the performance of these new media with other commercially available products. We obtained 16 other hybridoma media that were marketed as serum-free, protein-free, or animal component-free. During the weaning procedure, half of the media failed to support the HFN cells, leaving 10 cell populations weaned into eight competitor media (designated in Figure 8 by the codes A–H) and the two newly optimized media (#H4281 and #H4409). A spinner assay used the surviving HFN cell populations, and cell growth and IgG production were determined as before. Eight days later, a second spinner assay was begun using cells maintained in their respective media. For the top performing media, another identical weaning procedure was followed, leading into a third spinner assay. Figure 8 shows the average final cell-days and antibody concentrations in the three experiments, indicating the standard errors of the mean (n = 3 for #H4281, #H4409, A, B, C, and E; n = 2 for F, G, and H; and n = 1 for D). The complete weaning procedure was followed twice for each medium where n was greater than or equal to two. These weaning experiments indicate that the two newly optimized media rank higher than other media products in HFN cell growth and IgG productivity.

We also needed to test the two new media using other hybridoma cell lines. Figure 9 shows the compiled results of several experiments whereby three hybridoma cell lines from Abbott Laboratories (H, M, and P) were first grown in medium A and then transferred into the different media indicated (with the same designations as in weaning experiments). Among the serum-free media, #H4281 ranked highest of all three of the cell lines. Among animal component-free or protein-free media, #H4409 was similar to one other medium and outperformed the others in the three cell lines. Similar results were recently obtained using three additional hybridoma cell lines (data not shown). Fixed-batch, 5-L, stirred-tank bioreactors with HFN cells also gave similar results comparing #H4281 and #H4409 with the other media (data not shown).

The unique genetic make-up 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 (such as fed-batch, perfusion, and hollow-fiber bioreactors) are currently being tested, comparing #H4281 and #H4409 with others. Results to date (unpublished data) indicate that both #H4281 and #H4409 rank at the top of commercially available media products in supporting the cell growth and antibody productivity of a variety of hybridoma cell lines and growing systems.

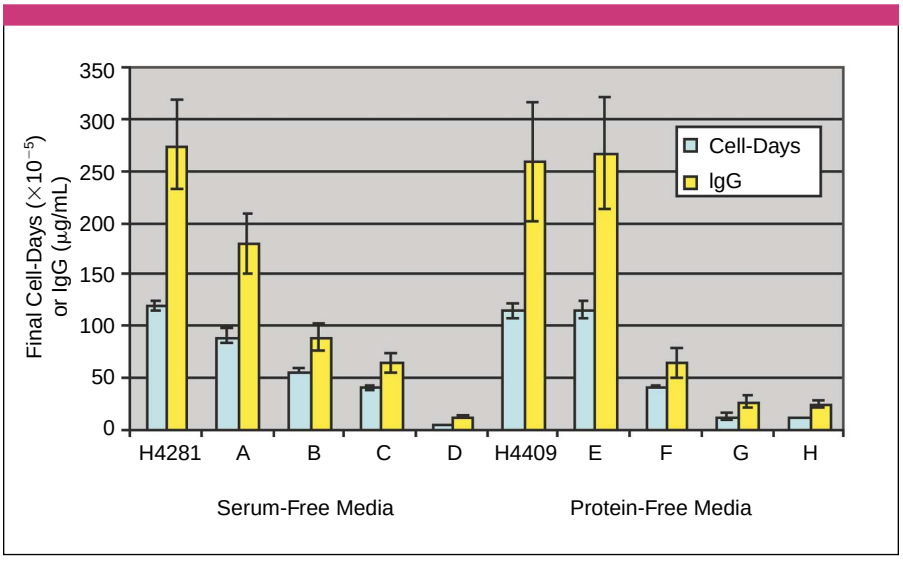

Figure 8. Serum-free or protein-free hybridoma media from other suppliers, using HFN cells and indicating standard error bars

Eliminating Animal-Derived Agents

By a systematic method involving three stages of development, we eliminated serum and other animal-derived agents from hybridoma cell culture media and replaced them with a complex mixture of components from nonanimal sources. Medium

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genetic combination of each clone suggests that no single medium will be optimal for all hybridoma cell lines.

formulations were optimized for cell growth and IgG productivity using HFN cells primarily. Two final products, a serum-free, low-protein, hybridoma medium (#H4281) and an animal component-free, hybridoma medium (#H4409) were developed. These media were tested in spinner assays with multiple hybridoma cell lines and with numerous other commercially available hybridoma media. Both #H4281 and #H4409 showed excellent cell growth and antibody production characteristics. The three-stage process was successful in finding optimal combinations of interacting components. A similar approach of factorial, matrix-based experiments has been used to optimize the formulation for a problematic hybridoma cell line (unpublished data).

Today's shortfall in capacity worldwide for CGMP production of biopharmaceuticals is unlikely to be remedied for four to six years (7). An important strategy in dealing with this production crisis is to optimize processes and formulations for individual hybridoma cell lines and production systems. Optimization of individual "whole systems" aims for equivalent or higher product yield within shorter production times, using less medium and less production facility space. A factorial matrix-based process to find an optimal set of such complex variables is an efficient approach likely to save valuable time during product development. Whereas a linear component-titration method may ultimately result in adequately maximized production, the efficiency of the matrix-based approach offers three benefits: reduced R&D costs, reduced time to market, and early securing of scarce production resources. In all likelihood, not all developed biopharmaceutical products will actually be produced during the production capacity crisis. Advance reservation of production facilities could determine whether a product is made at all (7). Therefore, an efficient optimization approach similar to what we presented here is critical to reducing costs and in getting products to market.

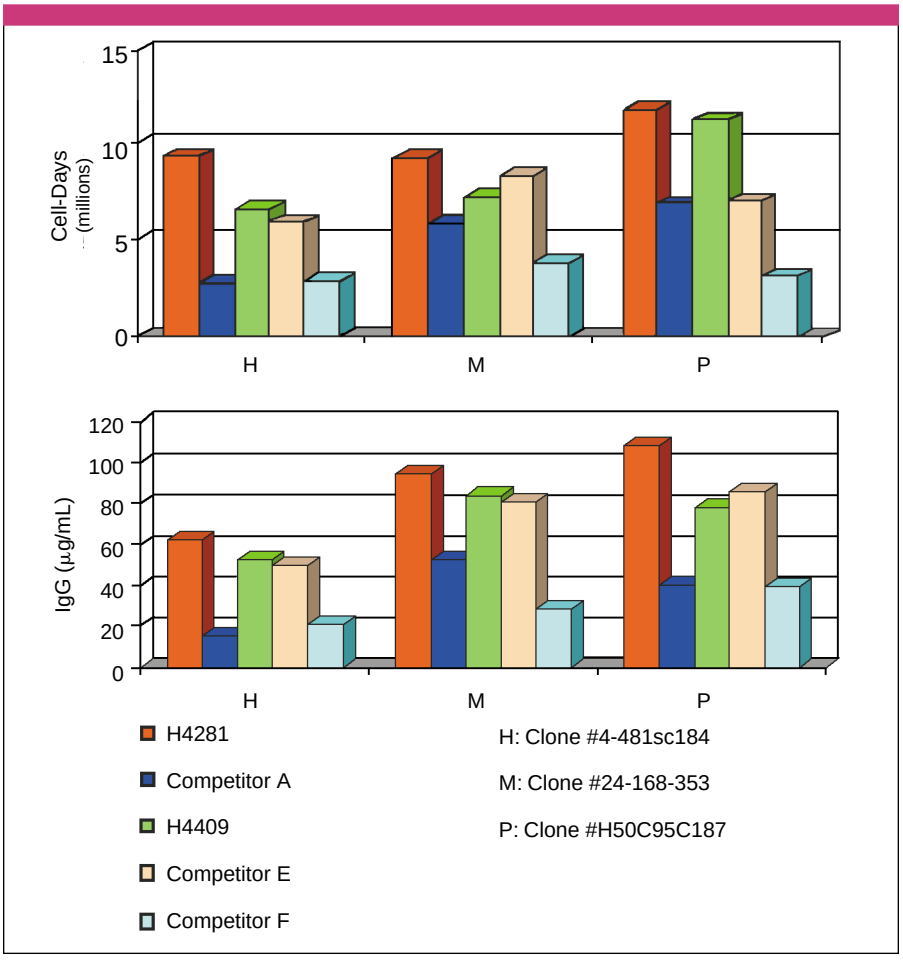

Figure 9. A comparison of commercially available media using three different hybridoma cell lines, showing both cell-days and IgG concentrations

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