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Enhanced Growth and Productivity of a Hybridoma with Recombinant Human Albumin and Lactoferrin

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The biopharmaceutical industry has seen a major shift away from the use of serum and other animal-derived components in the manufacture of biopharmaceuticals. Guidance from the EMEA and FDA for the manufacture of biopharmaceuticals and medical devices encourages the use of “animal-free” components to lessen concerns over contamination from adventitious agents such as prions, a cause of spongiform encephalopathy.^[1]

The drive away from animal-derived components has led to the rise in popularity of serum-free and animal component-free cell culture media. However, problems do arise in biomanufacturing because serum-free or animal component-free formulations typically do not offer the same performance as media supplemented with serum. Often cells do not grow as well or produce as much product in media without beneficial serum components.

InVitria has introduced animal-free recombinant human serum albumin (rAlbumin [rHSA], Cellastim™) and recombinant human lactofer-

rin (rLactoferrin [rhLF], Lacromin™) for use in cell culture applications. Cellastim has been optimized to support the health, proliferation, and productivity of cells in serum-free media (SFM). Albumin, derived from plasma, is a classic media supplement, and numerous independent studies have shown that albumin supplementation increases cell growth and productivity in SFM.^[2-7]

Albumin (66 kDa) is the most abundant serum protein and has multiple functions that support cell growth and productivity. Albumin transports nutrients such as lipids and metals.^[8,9] Each albumin molecule can carry several fatty acid molecules.^[10] Albumin also contains two binding sites for beneficial metals such as Cu, Ni, and Zn.^[11] Studies also show that albumin is an antioxidant, stabilizes cell membranes, reduces shear in bioreactors, and sequesters waste and toxins.^[12-15]

Lactoferrin (LF) is an 80 kDa protein found in milk and colostrums. LF has a single polypeptide chain and two iron-binding domains.^[16] Lactoferrin is present in human milk at 1–2 g/L and up to 6 g/L in colostrum.^[16] It is also found in other parts of the body as it is secreted by the salivary and pancreatic glands and is found in granules of neutrophils.^[16,17] LF is a multifunctional protein with many biological activities including

antimicrobial behaviors against human pathogens.^[17] LF also regulates iron absorption, immune system modulation, and anti-inflammation.^[17]

Lacromin is also multifunctional in cell culture applications. It has been shown to transport iron into cells without concerns about oxidation of other biomolecules, exhibit growth factor properties to stimulate cell growth, reduce apoptosis, and increase cell viability.^[18-20]

In this study, we examined the ability of Cellastim and Lacromin to improve hybridoma growth and productivity in a variety of serum-free media formulations. These data shows that Cellastim and Lacromin stimulate hybridoma growth and increase the production yield. These studies also show that Cellastim eases adaptation of cells away from serum dependence and increases cell survival in chemically-defined media.

Experimental Materials

Cell Lines

Sp2/0 hybridoma line AE-1 (HB-72) and NS1 hybridoma line L243 (HB-55) were obtained from ATCC (American Type Culture Collection). AE-1 and L243, respectively, secrete an IgG1 and IgG2a monoclonal product.

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Media and Supplements

Commercial hybridoma media were obtained from three manufacturers (Invitrogen, HyClone, and SAFC) and used as recommended. Media was supplemented with 2 mM glutamine (GlutaMAX, Invitrogen) when glutamine was not present in the formulation.

All classical media were further supplemented with an insulin, transferrin, selenium and ethanolamine mixture (#51500, Invitrogen) in order to support serum-free growth. Cell culture-grade plasma-derived HSA (pHSA) was obtained from Seracare. Cellastim rHSA (#777HSA017) and Lacromin rhLF (#777LAC015) were both from InVitria. Fetal bovine serum (FBS) was obtained from HyClone. Each medium was supplemented with 0.1% Pluronic F68 (BASF) shear protectant if a protectant was not included in the formulation.

Methods

The AE1 hybridoma cells were adapted to and maintained in the respective medium supplemented with 0.5% FBS in 125 ml shake-flasks. Cells were allowed to adapt to each medium for at least 30 days before initiating tests. Cells were washed twice to remove residual FBS prior to seeding at 0.8×10^5 viable cells/ml. A minimum of two shake-batch cultures were performed for each condition. Media supplemented with pHSA or 10% FBS were used as comparative controls to Cellastim.

Viable cell concentrations were determined daily by a PCA cell counter (Guava Technologies) and the concentration of produced antibody was determined on the sixth day of culture by quantitative ELISA (Bethyl Laboratories). For long-term culture studies, cells were maintained in each SFM with no additional supplementation, or supplemented with 1 g/L pHSA, 1 g/L Cellastim or 10% FBS.

Cells were grown in a humidified incubator at 37°C, 6% CO₂.

The hybridoma L243 cell line was maintained in AFM6 medium (KC Bio, LLC) which contains an iron compound. AFM6-Fe (KC Bio, LLC) was used as the iron compound-free basal medium to test the effects of various concentrations of Lacromin which ranged from 5–100 mg/L. AFM6-Fe was also supplemented with 5% FBS as a positive control. Cells were seeded at 2.0×10^5 cells/ml and cultured as a stationary suspension.

Each test point was performed in triplicate, and at day four, the cells were subcultured with a total of four passages (subculturing). At each subsequent passage, the same seeding density and culture medium was used as in the original seeding. At day four of each passage, samples were removed to determine cell viability and IgG production.

Results and Discussion

Comparing the Benefits of pHSA and Cellastim on Cell Growth and Productivity

We examined the benefits of Cellastim in a variety of classical and commercial media formulations. The rationale for this approach was to evaluate Cellastim under conditions typically employed by end-users in academic and industrial settings. Numerous published reports show that albumin supports cell growth in individualized formulations. However, there is a lack of data comparing the overall benefit of albumin across

a wider spectrum. Thus, four classical and four commercial serum-free media formulations were used in the evaluation. Furthermore, we compared the relative effects of Cellastim with pHSA.

The commercial media included a diverse group of current serum-free, protein-free, or chemically-defined formulations from different manufacturers. We evaluated the growth and productivity of hybridoma cells in each medium as recommended by the manufacturer as the basal condition. Replicate cultures

with either 1 g/L plasma-derived HSA or 1 g/L Cellastim were evaluated for comparison. Medium with 10% FBS v/v was used as an additional comparative control. Sp2/0 hybridoma, when seeded at 0.8×10^5 cells/ml typically reach late-log phase after three days of culture and peak after four days. The number of viable cells were compared after three days of incubation, in late-log phase, to compare the proliferative effect.

Figure 1 shows the concentration of viable cells after three days of culture in

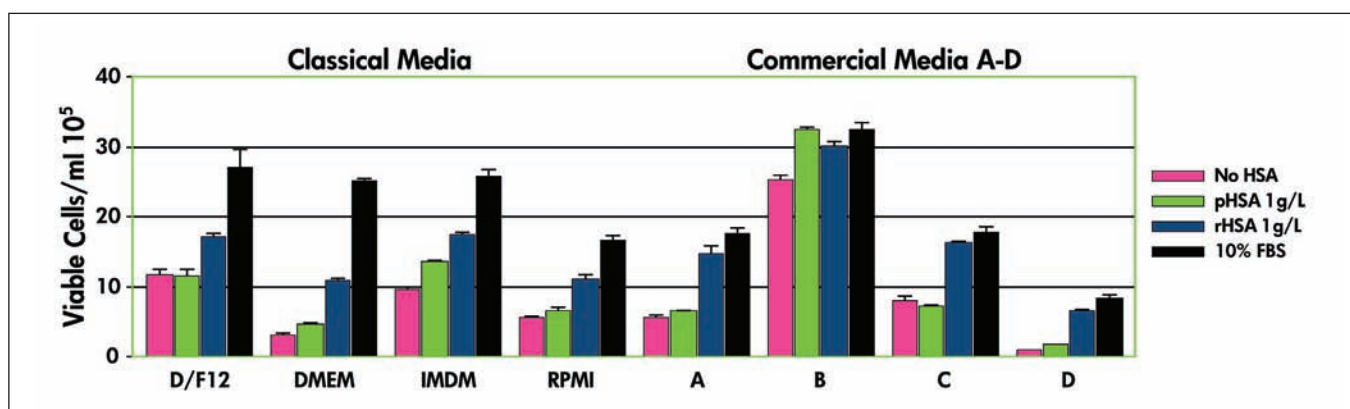


FIGURE 1. Increased proliferation of hybridoma in eight diverse classical and commercial serum-free formulations supplemented with Cellastim. Cells were seeded at 0.8×10^5 cells/ml in shake-batch culture. The number of viable cells was determined in late-log phase after three days of culture.

four classical media (serum-free DMEM/F12 [D/F12], DMEM, IMDM, and RPMI) and four commercial formulations (A-D). As expected, we found that each unsupplemented SFM supported proliferation to different degrees (Figure 1, pink bars). Hybridoma growth was more robust in some formulations such as D/F12, IMDM, and commercial medium B compared to other formulations such as DMEM or commercial medium D. Adding 10% FBS (Figure 1, black bars) increased the growth of cells in every formulation tested. These results show that SFM did not offer the same level of cell growth as the same medium supplemented with serum.

Figure 1 also shows the relative benefit of supplementation with 1 g/L of either pHSA or Cellastim. pHSA improved growth to some degree in the majority of media tested (Figure 1, green bars). These results indicate that pHSA provides modest benefit across diverse formulations. Note, however, that Cellastim typically produced cell growth better than pHSA (Figure 1, blue bars). 1 g/L of Cellastim resulted in a greater than 50% increase in the concentration of viable cells in seven of the eight formulations. These results indicate that Cellastim improves hybridoma growth across diverse classical and commercial formulations. Furthermore, cells grew better with Cellastim than pHSA at the same concentration.

The production of antibody product was examined after six days of growth

(Figure 2). Typically, after six days the concentration of viable cells had peaked and decreased to less than 50% of that seen at maximum cell density. Cellastim increased the production of antibody in seven of eight media (Figure 2, compare pink and blue bars). In many cases, the increase in productivity was greater than 50% (D/F12, DMEM, IMDM, and commercial media A, D). Cellastim outperformed pHSA in seven of eight formulations and improved the production of antibody in media with higher levels of productivity (D/F12, A, C) as well as in lower performing formulations (DMEM, D). We found that the total accumulation of cells in the culture, integral of the viable cell concentration (IVCC), increased in cultures supplemented with albumin (data not shown). IVCC is estimated from the area under the growth curve. Media supplemented with Cellastim produced greater IVCC than both unsupplemented and pHSA-supplemented media. This result suggests that increased productivity resulted, in part, from increased cell growth and accumulation.

Table 1 shows the average increase

in hybridoma growth and productivity in the eight media. On average, media supplemented with Cellastim produced more than double the number of cells after three days and resulted in a 92% increase in antibody production after six days. Overall, these results indicate that Cellastim is a robust supplement that improves hybridoma growth and productivity in both classical and commercial hybridoma formulations. Cells grew faster, grew to higher density, and the production of antibody increased with Cellastim in the medium (Table 1).

Cellastim Improves Cell Growth and Productivity in Long-Term Culture

The performance of a supplement over long-term culture is important. A robust media supplement should offer continued benefit over extended periods of culture. We examined the ability of Cellastim to improve cell growth and productivity over 25 days of culture. The growth of hybridoma cells was compared in serum-free DMEM/F12 medium with and without Cellastim. Throughout the 25-day period, hybridoma were maintained in

TABLE 1. Average increase in cell concentration and productivity across eight diverse SFM media supplemented with 1 g/L pHSA or Cellastim.

	No HSA	pHSA 1 g/L	Cellastim 1 g/L	10% FBS
3-Day Cell Growth	100	115	242	353
Productivity	100	108	186	266

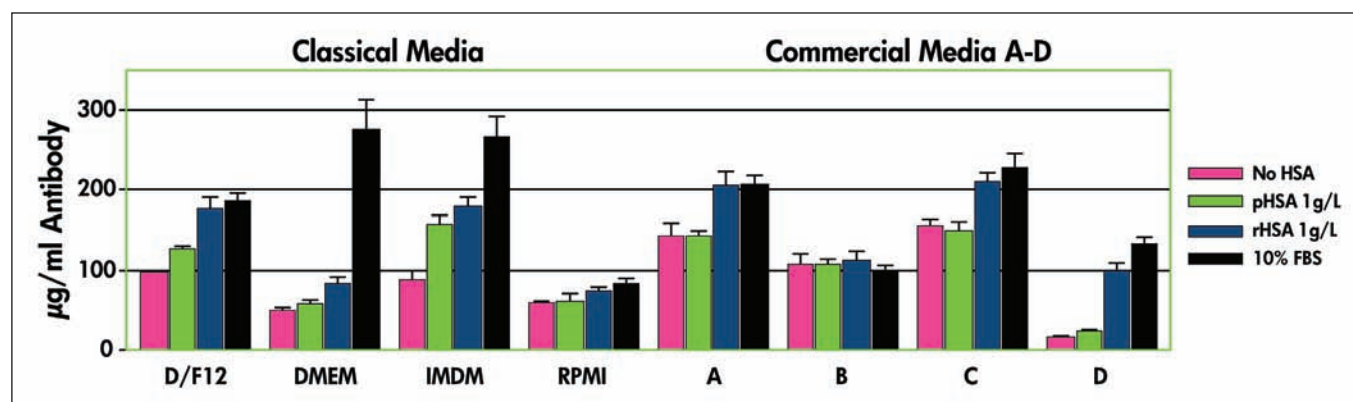


FIGURE 2. Increased production of antibody by hybridoma in eight diverse classical and commercial serum-free media formulations supplemented with Cellastim. Cells were seeded at 0.8×10^5 cells/ml in shake-batch culture. The concentration of antibody produced in the medium was determined after six days of culture.

log-phase growth via passaging three times a week. Concurrent cultures in medium supplemented with either 1 g/L pHSA or 10% FBS were passaged for comparison. At each passage, the concentration of viable cells and the cumulative number of cell-doublings was determined. Figure 3 shows the number of cell doublings achieved in media with and without supplementation through the 25 day culture period. Cells achieved more doublings (46 doublings) in media supplemented with Cellastim (blue line) compared to the 26 doublings achieved in the D/F12 media without Cellastim (pink line). The number of doublings was greater with Cellastim compared to pHSA (green line) and approached that of D/F12 plus 10% FBS (black line). Note that the lines are relatively straight, indicating that growth was stable through the 25-day period. These data shows that the improvement in cell growth with Cellastim was sustainable during long-term culture.

We examined the growth kinetics and productivity of hybridoma in shake-batch culture using cells that had been passaged in either unsupplemented or supplemented medium for 25 days, as shown in Figure 3. Cells were seeded (as described) in the same medium, and the concentration of viable cells was determined daily. The production of antibody secreted in the medium was determined on the sixth day.

Figure 4 shows the growth curve of AE-1 in shake-batch culture. Hybridoma cells grown in unsupplemented D/F12 (pink) peaked on day five at a maximum cell concentration of 16×10^5 cells/ml. Cells grown in medium with 1 g/L pHSA (green) peaked a day earlier but had no increase in maximum cell density. However, cells grown in medium with Cellastim (blue) peaked two days earlier than unsupplemented medium, on day three, and grew to higher density (23×10^5 cells/ml). The growth kinetics of hybridoma that had been passaged in medium with Cellastim was similar to that seen in medium with 10% FBS. Figure 5 shows the relative production of antibody. Cells grown in D/F12 with 1 g/L Cellastim (blue) produced 65% more antibody

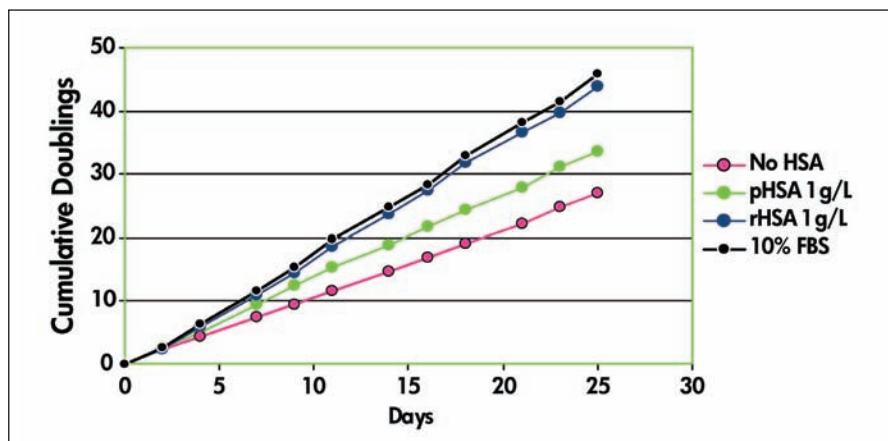


FIGURE 3. Increased cell doublings in medium supplemented with Cellastim during long-term passage of hybridoma. The number of cumulative cell doublings is shown on the left axis. Cells were passaged for 25 days in D/F12 without supplementation (pink), supplemented with 1g/L Cellastim (blue) or pHSA (green). D/F12 with 10% FBS is shown in black.

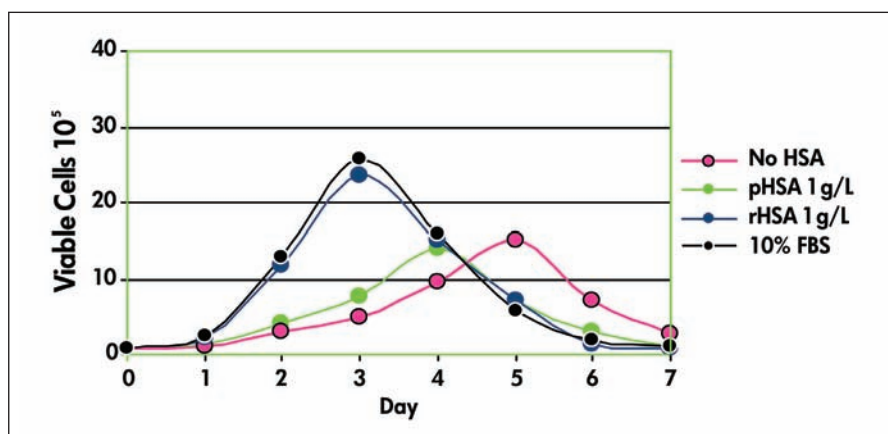


FIGURE 4. Improved growth kinetics of hybridoma in D/F12 medium supplemented with Cellastim. Hybridoma cells were previously grown in unsupplemented or supplemented serum-free D/F12 medium for 25 days (as shown in Figure 3). Cells were then seeded in a seven-day shake-batch culture.

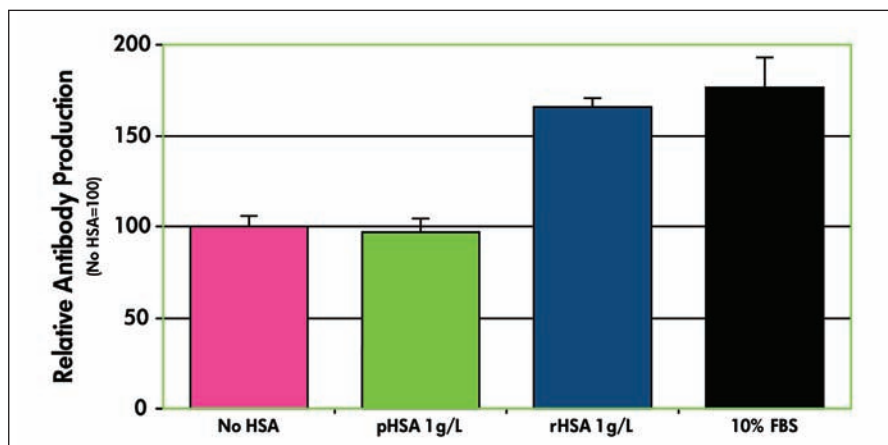


FIGURE 5. Relative increase in antibody production by hybridoma in D/F12 medium supplemented with Cellastim. Hybridoma were previously grown in supplemented or unsupplemented serum-free D/F12 medium for 25 days (as shown in Figure 3). Cells were then seeded in a seven-day shake-batch culture. The production of antibody was determined on day six. Relative values are shown with unsupplemented D/F12 (no HSA) set to a value of 100.

than medium without additives (pink). Cellastim outperformed plasma-derived HSA (green) and increased productivity similar to that of 10% FBS. The improvements in growth and productivity after 25 days for passage were similar to that seen in cells without extended passage (Figures 1 and 2). These data indicate that the substantial growth and productivity gains seen in medium supplemented with Cellastim were maintained during extended periods of culture.

Cellastim Eases the Adaptation of Cells to Serum-Free, Chemically-Defined Media

Adaptation of cells from serum-containing medium to serum-free medium is a problem for many cell lines. In particular, adaptation of cells into chemically-defined medium is often difficult. Typically, significant time is required for adaptation, and the end result is often a loss of performance in cell growth and/or productivity. We examined whether supplementing media with Cellastim would improve cell growth during the transfer of a hybridoma line from a medium containing 10% FBS into serum-free, chemically-defined media.

In this approach, Sp2/0 hybridoma maintained in D/F12 supplemented with 10% FBS were immediately transferred into serum-free, chemically-defined hybridoma media without adaptation. Before transfer, cells were washed twice to remove traces of FBS and seeded in three chemically-defined formulations from different manufacturers. The effect of Cellastim was determined by replicate conditions in which the medium was supplemented with either 1, 3, or 5 g/L Cellastim. Three days after the transfer, the concentration of viable cells was determined to judge cell survival, growth, and adaptation to the chemically-defined media. Note that this approach typically induces severe stress to cells since the cells experience a sudden removal of FBS in addition to a change to a different medium formulation.

The concentration of viable cells after three days is shown in Figure 6 following the transfer of cells into the three chem-

ically-defined media. Sp2/0 hybridoma transferred into chemically-defined medium #1 did not survive without the addition of Cellastim (Figure 6). In contrast, cells survived and also proliferated 8–9 fold when the medium was supplemented with Cellastim. The concentration of viable cells was similar to that seen with 10% FBS. This indicates Cellastim improved both cell survival and proliferation. Figure 6 also shows the number of viable cells after

rapid transition into chemically defined medium #2 and #3. Note that as little as 1 g/L of Cellastim significantly increased the concentration of viable cells. Furthermore, increased concentrations of Cellastim (3–5 g/L) generally resulted in a higher concentration of cells. These results show that Cellastim significantly eased the transition to chemically-defined medium and resulted in more viable, growing cells than the unsupplemented medium.

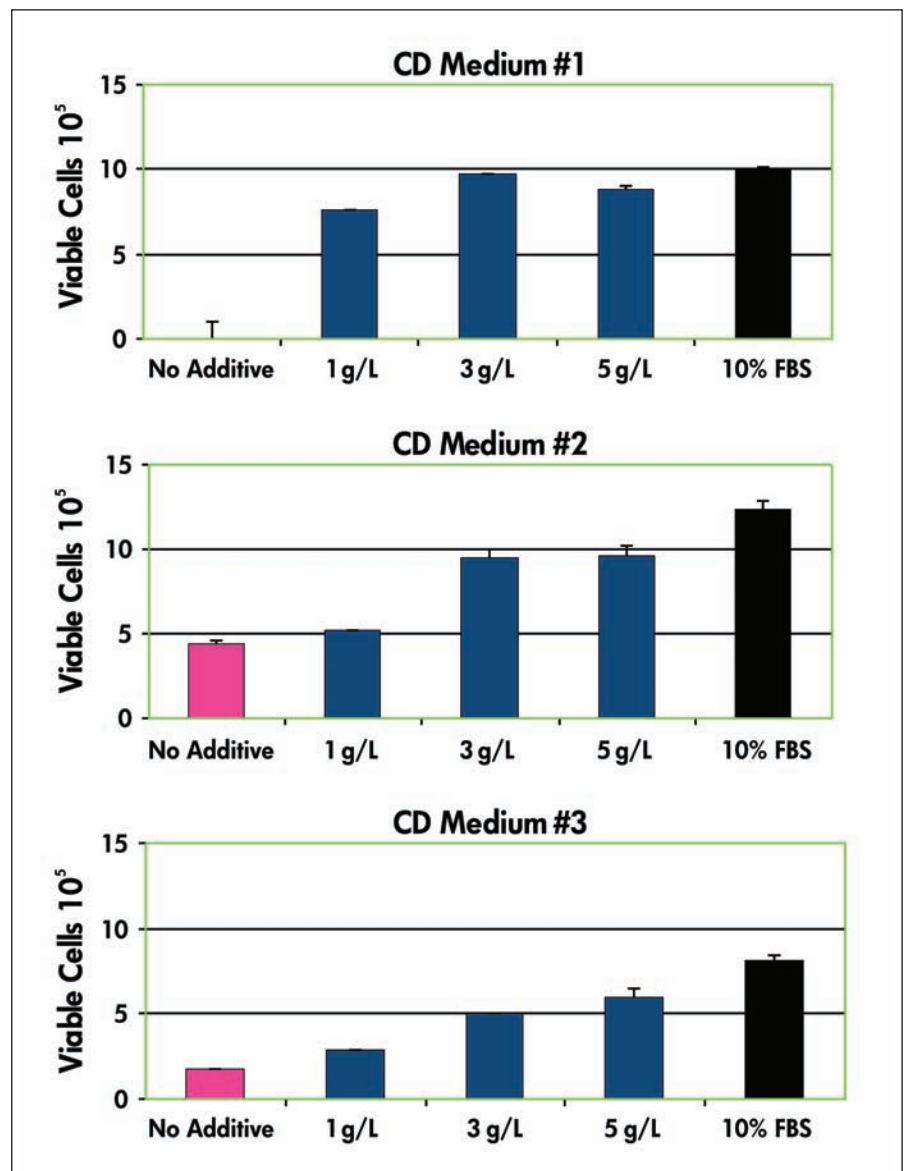


FIGURE 6. Cellastim eases serum withdrawal and the adaptation to serum-free chemically-defined medium. Sp2/0 hybridoma stock were grown in D/F12 supplemented with 10% FBS. Cells were washed and seeded in three chemically-defined, commercial hybridoma media at 1.0×10^5 cells/ml. The concentration of viable cells on the third day is shown on the left. Each medium was used as recommended by the manufacturer (pink), or supplemented with 1, 3, or 5 g/L Cellastim (blue). Medium with 10% FBS (black) is shown as a comparative control.

Lacromin Increases Hybridoma Cell Growth and MAb Production

We examined the ability of Lacromin to support cell growth and productivity in media without iron. Hybridoma L243 cells were maintained in iron containing medium, washed, and seeded at 2.0×10^5 cells/ml in the same medium without iron. Replicate cultures were supplemented with 5, 25, or 100 mg/L Lacromin. Cells were passaged four times, as described earlier.

Table 2 shows the average effect of Lacromin on cell growth over the four passages. Cells grew moderately in the medium without iron. However, the cell density was significantly increased when the medium was supplemented with Lacromin. Lacromin increased cell density in a dose-dependent manner. The greatest effect was seen at 100 mg/L where cells achieved a density roughly double that of the unsupplemented medium. Cell density at 100 mg/ml approached those seen with 5% FBS.

The effect of Lacromin on antibody production in L243 hybridoma cells was examined. The average concentration

of IgG from the four passages is shown in Table 2. Similar to cell density, there was a significant increase in antibody production in all Lacromin treatments as compared to the basal medium. The highest IgG level was seen at 5 mg/L of Lacromin where production nearly tripled that of unsupplemented medium. The level of antibody produced with each of the three Lacromin concentrations was higher than that achieved with 5% FBS. These data (shown in Table 2) show that Lacromin supports superior cell growth and productivity in SFM without iron.

Summary

Cellastim (rHSA) improved hybridoma growth and antibody production in a variety of media formulations, performing better than pHSA. The positive effect of Cellastim was stable over the long-term passage of cells. Cellastim improved the proliferation of cells experiencing serum-withdrawal and eased the transition to chemically-defined media.

Lacromin (rhLF) improved hybridoma growth and productivity. Cells grew to a higher density and produced more antibody, higher than that achieved in medium supplemented with 5% FBS.

TABLE 2. The effect of Lacromin on hybridoma growth and monoclonal antibody production.

Treatment	Lacromin mg/L	Cell Count ($\times 10^5$)		IgG level ($\mu\text{g/ml}$)	
		Mean	SD	Mean	SD
Control	0	0.39	0.18	15.78	5.63
Lacromin	5	0.63	0.10	43.58	8.24
Lacromin	25	0.81	0.20	36.10	7.54
Lacromin	100	0.78	0.26	29.73	4.28
5% FBS	0	0.98	0.12	23.88	5.50

For further information, email InVitra at info@invitria.com.

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NOTES

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