Development of a Serum-Free, Chemically Defined Cell Culture Medium for the Production of HIV from HUT-78 Cells

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ABSTRACT

One approach to the development of an HIV vaccine is to utilize whole, inactivated HIV formulated with one or more adjuvants. This approach is currently being tested as a therapeutic vaccine in clinical trials. The virus is currently produced in stirred tank fermenters by chronically infected HUT-78 cells, a human T-cell lymphoma line, utilizing a culture medium consisting of RPMI-1640 medium supplemented with fetal bovine serum.

The goal of this project was to develop a chemically defined, protein-free medium for the production process that would eliminate the presence of all animal-derived components, improve virus yield, provide for reliable growth and scale-up of the cells, and maintain equivalence of the virus in terms of its performance as an immunological stimulant against HIV.

The first step in the project was to screen over 20 commercially available serum-free media designed for a variety of cells. Results showed that about one-third of the media formulas provided for at least equivalent cell density and virus yield (measured by p24 ELISA) compared to the serum-containing control medium. However, most of these formulas were not able to maintain reliable growth when cells were passaged repeatedly. One formula, IS MAB-CD, was able to provide for completely satisfactory long-term growth. This formula provided for high cell densities, but virus yields were significantly lower than in many of the other media. Due to the importance of reliable cell passaging, this formula was chosen as the basis for further optimization work.

A total of 18 panels of test media were prepared using the ISMAB-CD base medium in order to evaluate the effect of varying the concentrations of key ingredients, individually or in combination. Several panels resulted in "dose-response" curves indicating that virus yield could be improved by altering the concentration of certain ingredients. These panels included potassium and phosphate concentration, osmolality, lipids, metabolic intermediates, and most significantly the partial replacement of glucose with fructose. The reduction of glucose to 1-2 g/L and the addition of fructose to the medium resulted in higher cell densities, greatly increased longevity of the culture, and a three-fold increase in virus yield compared to medium with high glucose alone (6 g/L). This effect may be due to the maintenance of the culture pH above 7.0 in the presence of fructose. The concentrations of amino acids in spent media were also analyzed, and it was found that serine, methionine and tryptophan were significantly depleted. A 1.5-2X increase in the initial concentrations of these amino acids improved cell growth significantly, but did not change the amount of viral particles in the supernatant.

The final stage of the project utilized the ISMAB-CD medium with fructose as the basal medium, and the other promising changes were evaluated individually and in combination. However, only one additional change was found to be of value in increasing the virus yield; this was an increase in the concentration of a mix of metabolic intermediates.

The overall conclusions from the project were; 1) reliable cell growth was obtained with a chemically defined medium without serum or any protein constituents in the medium, 2) virus yield was improved two-fold over that obtained with serum and three-fold over the serum-free medium originally chosen, and 3) the most significant factor in the improvement of virus yield was the inclusion of fructose as a partial alternative to glucose. These results, initially obtained utilizing stationary T-flasks, were confirmed in roller bottles and WAVE cultures.

INTRODUCTION

One approach to the development of an HIV vaccine is to utilize whole, inactivated HIV formulated with one or more adjuvants. The Immune Response Corporation is currently evaluating this approach as a therapeutic vaccine in clinical trials, utilizing Freund's incomplete adjuvant and an immunomodulatory oligonucleotide (HYB2055) (ref. 1). The intent of this vaccine is to stimulate both cell and antibody mediated responses to a broad array of HIV antigens.

The HIV-1 virus used in this vaccine is the HZ321 strain. It is produced by HUT-78 cells (a human T cell lymphoma cell line) which are chronically infected with the virus. The current production process is carried out in 250L stirred tank fermenters, utilizing RPMI-1640 cell culture medium supplemented with 7% fetal bovine serum. The clarified cell culture supernatant is treated with beta-propiolactone to chemically inactivate the virus, and then purified by several steps including ultrafiltration, ion exchange chromatography and ultracentrifugation, prior to a second inactivation step with gamma irradiation.

The goal of this project was to develop a chemically defined, protein-free cell culture medium for the production process that would eliminate all animal-derived components, improve viral yield, provide reliable and consistent cell growth from the seed stocks through to full-scale production, and maintain equivalence of the virus in terms of its performance as an immunological stimulant against HIV.

RESULTS

I. Screening of Serum-Free Media Formulations

The first step in the project was to determine if currently available serum-free media formulas could provide for growth and virus production from the cell line. It was determined that FBS could not be completely removed from RPMI-1640, but a serum-free D-MEM formula with insulin, transferrin and albumin permitted passaging of the cells without FBS. Cells adapted to this medium were utilized in the screening of over twenty serum-free formulas, including those listed in Table 1. These formulas were chosen to represent a wide range of formulas in terms of the cells for which they were developed, the supplements used (proteins, lipids and hydrolysates), and the companies which supplied them. A summary of the key growth and virus yield data is shown in Figure 1. The cell densities and the kinetics of cell growth varied significantly, as did the yield of virus, and the amount of virus produced per cell. There was no consistent pattern in terms of which cell type the media had been developed for, or which supplements seemed to be important.

Three formulas (IS MAB-CD, Gibco CD 293, and Ex-Cell Sp2/0) were chosen for further evaluation based on their ability to either support high density cell growth (IS MAB-CD) or a high specific productivity with adequate growth (CD 293 and Ex-Cell 293). Cells were

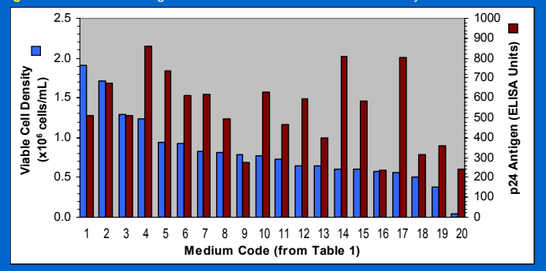
I. Screening of Serum-Free Media Formulations (cont.)

passaged in each of these media for three weeks and then re-evaluated for virus production in T flasks and roller bottles. The IS MAB-CD provided for significantly better results in passaging and yielded a comparable level of virus production. Based on its growth promoting attributes, IS MAB-CD was chosen as the basal medium for further optimization work, as described in the next section.

Table 1. Serum-Free Media Screened for Growth and Virus Production.

Code	Product	Company	Comment	Cell Type
1	IS NS0	Irvine Scientific	with lipids	NS0
2	IS MAB-CD	Irvine Scientific	CD	Hybridoma
3	293 SFMII	Gibco	protein	293
4	CD 293	Gibco	CD	293
5	CD Hybridoma	Gibco	CD	Hybridoma
6	Ex-Cell 325	JRH	soy hydrolysate	CHO
7	Hybridoma SF	Sigma	protein	Hybridoma
8	Hybridoma SFM	Gibco	protein	Hybridoma
9	Ex-Cell 293	JRH	protein	293
10	Ex-Cell Sp2/0	JRH	CD	Sp2/0
11	Ex-Cell 302	JRH	soy hydrolysate	CHO
12	RPMI-1640 (+5% FBS)	HyClone	FBS	B-cells
13	Advanced D-MEM	Gibco	protein	
14	IR-02	D. Wyatt		custom
15	HyQ-CCMI	HyClone		
16	MegaCell RPMI	Sigma		B-cells
17	IR-05	D. Wyatt		custom
18	SF/PF Hybridoma	Sigma		Hybridoma
19	IMAT	IRC	protein and lipid	HUT-78
20	Cell MAB	BD		Hybridoma

Figure 1. Media Screening – Cell Growth and Viral Production on Day 7 of Culture.



II. Optimization of the Basal Medium

The main approach to the optimization of the IS MAB-CD basal medium was to create test "panels" of media in which one or a related group of ingredients was varied. Table 2 shows the panels (prepared by Irvine Scientific) that were tested, and the over-all conclusions reached from each panel.

The first panel, which evaluated the partial substitution of fructose for glucose, was by far the most significant in the improvement of virus yield. There have been reports that virus yields after infection can be raised by the maintenance of a higher pH, and one way of increasing culture pH without feedback control is to substitute fructose (or certain other carbohydrates) for glucose (ref. 2). As Figure 2 illustrates, the reduction of glucose and the addition of fructose resulted in at least a 50% increase in virus yield and cell growth. Subsequent experiments showed that low glucose alone (2g/L) did not result in improved growth or yield.

The results from the other panels are summarized in Table 2. Several variables were found to have different optimum levels than those present in the IS MAB-CD medium, with improvements in viral yield varying from 10-50%.

An analysis of amino acids in "spent media" (with fructose) was performed as well. Six amino acids did not decrease in concentration, including cystine. The remaining 14 amino acids were depleted to varying extents as shown in Figure 3. The concentrations of three

II. Optimization of the Basal Medium (cont.)

amino acids (serine, methionine and tryptophan) were found to be significantly depleted. When the initial concentration of these 3 amino acids was raised by 1.5-2X, the growth and viability of cells after day 7 was improved significantly.

Table 2. Summary of Medium Optimization Panels and Results.

Panel	Name	Conclusion (Optimal Range)	Approximate Improvement
1	Glucose/Fructose Ratio	2:6 glucose/fructose ratio	50%
2	Osmolality	350mOsm/Kg	10-30%
3	Potassium	15mM	25%
4	Glutamine	2-15mM	--
5	Glutamine Feed	no benefit	--
6	Pluronic F-68	10x lower	30%
7	Cation DoE	1.5x K & Ca	10-30%
8	Nucleosides	0.5–6mg/L	50%
9	Reducing Agents	1-2x	--
11	Phosphate	0.6x	0-50%
12	Cation DoE Verification	1x	--
13	Trace Metals	0.5-2x	--
14	Iron	0.5-5x	--
16	Fructose	≥4g/L	50%
17	Vitamins	0.5-1x	--
18	Groups DoE	1.75x Metabolic Intermediates	20%
SM	Spent Media Analysis	3 amino acids increased 1.5-2x	

Figure 2. Effect of Varying Fructose/Glucose Concentrations on Cell Growth and Productivity (Panel 1; Day 10 Results).

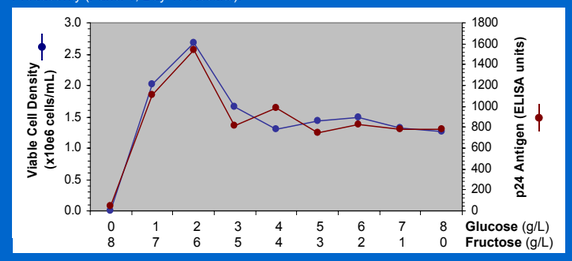
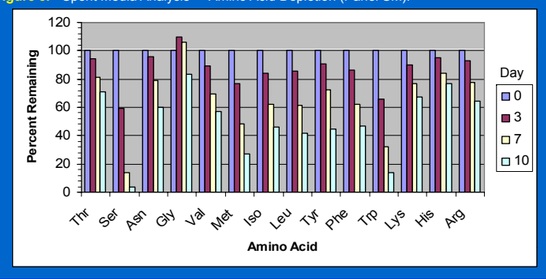


Figure 3. "Spent Media Analysis" – Amino Acid Depletion (Panel SM).



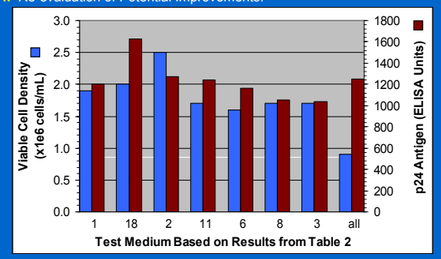
III. Final Evaluation of Changes to the Basal Medium

The potential changes to the basal medium identified in the testing of the 18 panels summarized in Table 2 were re-evaluated in a single panel, utilizing IS MAB-CD with 6g/L fructose and 2g/L glucose. The results are summarized in Figure 4. The only change that appeared to have a major impact on virus production in this experiment was the increase in the amount of the "metabolic intermediates". Additional testing confirmed that the increase in metabolic intermediates improved viral yield, and that the increase in the depleted amino

III. Final Evaluation of Changes to the Basal Medium (cont.)

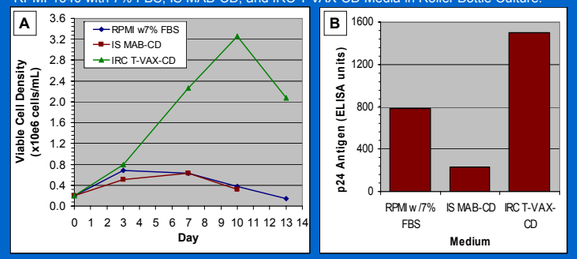
acids improved longevity of the culture. It was decided that the IS MAB-CD formula would be changed to include; a) 6g/L fructose, 2g/L glucose, b) 1.5x the concentration of the metabolic intermediates, and c) 1.5-2x increase in the three amino acids found to be significantly depleted.

Figure 4. Re-evaluation of Potential Improvements.



A 1200L GMP lot of the final formula (named IRC T-VAX-CD) was produced and compared to the original production medium (RPMI-1640 with 7% FBS) and to the IS MAB-CD from which T-VAX-CD was derived in a set of roller bottles as shown in Figure 5. The T-VAX-CD medium supported growth levels over three times as great as the other media. Virus production was double that obtained with RPMI-1640 and almost 6-fold greater than with IS MAB-CD. Analysis of the virus production in T-VAX-CD medium by several analytical techniques has shown that it is essentially equivalent to that produced in RPMI.

Figure 5. Final Comparison of Cell Growth (Panel A) and Viral Production (Panel B) in RPMI-1640 with 7% FBS, IS MAB-CD, and IRC T-VAX-CD Media in Roller Bottle Culture.



CONCLUSIONS

1. Human T cell lymphoma cell line HUT-78 (chronically infected with HIV) can be grown and reliably passaged in a chemically-defined culture medium, with cell densities exceeding 2e6 cells/mL.
2. Virus yields were improved at least two-fold over the serum-supplemented medium originally used for production through the process of screening and optimization.
3. The most significant factor in the improvement of virus yield was the utilization of a mixture of fructose and glucose as the carbon-source.
4. This optimization of our current cell culture process is being further evaluated in scale up, downstream process, and product consistency studies to enable more efficient full scale cGMP production of whole inactivated vaccines for investigational use in the treatment of HIV-1/AIDS.

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