New Protein-Free Medium for Gene Therapy Supports Increased Adenovirus Production

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Abstract

The biotechnology industry needs animal component-free protein-free media for the production of cell culture derived human therapeutic agents. This need exists due to the potential presence of adventitious agents in animal-derived components commonly used in cell culture medium. This concern is particularly critical in the production of viral vectors for gene therapy applications. We report here a medium free of proteins and of animal-derived components intended for use with the Per.C6® cell line for the production of adenoviral vectors. Through modification of the medium composition, we are able to facilitate a metabolic shift of the cells, which increases the longevity of cultures without adverse effects on the rate of cell growth and cell density. In Gene Therapy Medium-3 (GTM; Sigma G 9916), cells can be adapted to grow in spinner cultures and can be sub-cultured for a minimum of 20 population doublings without changes in population doubling times. No weaning is necessary; as cultures can be initiated from frozen, serum-supplemented stocks by direct transfer of cells into this medium. Cultures reach maximum cell density (5-6 x 10^6 cells/ml) between days 5 and 6 when inoculated at 2.5 x 10^5 cells/ml. Cultures grown in this medium and infected with adenovirus vector showed an increase in virus production when compared to our prior Gene Therapy Medium-1 (GTM; Sigma G 0916) formulation and other competitor formulations available in the market.

Introduction

There is a strong demand for improved technologies in the field of gene therapy. Many vectors in gene therapy are based on viruses, with adenoviruses being used as a common delivery system. However, the presence of wild type replication competent adenoviruses (RCAs) is a major problem for the application of these vectors in gene therapy. Crucell (Leiden, The Netherlands) has developed a helper cell line called Per.C6® containing defined sequences from adenovirus serotype 5 (Ad5). The definition of sequences from Ad5 incorporated into Per.C6® cells eliminates the issue of generating RCAs by homologous recombination producing safe recombinant adenovirus vectors. The increasing demands for Per.C6® cells and virus production in the industrial and pharmaceutical sectors has created the need for an animal component-free protein-free medium in which to produce therapeutic vectors. Sigma has assisted in meeting the demands by improving the performance of our serum-free suspension medium. Modification of the GTM-1 has lead to an increase in cell density while supporting increased virus production.

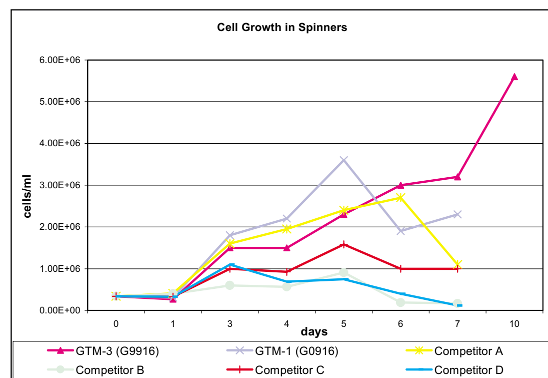

Figure 1. Comparison of cell growth of Per.C6® cells in GTM-3 and GTM-1. Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in spinner flasks with GTM-3, and GTM-1, and several other commercially available media. Samples were taken daily to monitor cell growth and viability. Each data point represents the best cell growth in the GTM-3 medium with 5.60 x 10^6 cells/ml at day nine of the culture. The Per.C6® cells growing in GTM-1 reached a maximum density of 3.60 x 10^6 cells/ml at day five of the culture.

Materials and Methods

Materials: Per.C6® cell stocks were obtained from Crucell (Leiden, The Netherlands). All materials used in this work were obtained from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise stated.

Methods: Stock cultures of Per.C6® cells were grown in GTM-3. The cells were passaged three times per week and seeded at 2.5 x 10^5 cells/ml. Cell cultures did not reach densities greater than 2 x 10^6 cells/ml. Cells were maintained in 1L spinner flasks (Techne, Inc., Princeton, New Jersey) on magnetic stir plates (Thermolyne Corp., Dubuque, Iowa) and incubated at 37 °C and 5% CO_2.

Medium Development: The medium development process began by selecting several components GTM-1 that were believed to have significant effects on cell growth. These significant effects were determined in previous assays. A growth assay was performed to identify the relative importance of the components in the medium and their effects on cell growth. Spinner flasks inoculated with 2.5 x 10^5 cells/ml in 100 milliliters medium were placed on stir plates in the incubator at 37 °C and 5% CO_2 and stirred between 50-60 rpm. Total cell counts were done using the Schärfe CASY 1 TTC unit (Reutlingen, Germany) and viability was done by the trypan blue exclusion method. The concentration of viable cells/ml was then calculated and expressed in percent.

Quantitation of Virus Production: The tissue culture infectious dose 50 ($TCID_{50}$), a method for infectious virus quantitation, is defined as the dilution of sample at which 50% of the replicate cell culture inoculated with the sample becomes infected. The method of Karber (1931) was used in calculating the $TCID_{50}$ value: -m= $\log_{10}$ starting dilution –[proportion of positive wells-0.5] x $\log_{10}$ dilution factor. The number of positive wells came from the data obtained by the β-gal assay.

In the histochemical assay, the virus titer is obtained from a direct count of infected cells.

$$\text{virus titer} = \frac{(\text{total number of stained cells in wells / number of wells counted})\ (\text{dilution factor for titration counted})}{\text{amount of virus inoculation per well}}$$

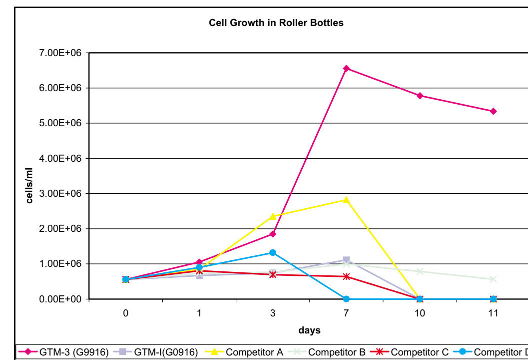

Figure 2. Comparison of cell growth of Per.C6® cells in GTM-3 and GTM-1. Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in roller bottles with GTM-3, GTM-1, and several other commercially available media. Samples were taken on a daily basis in order to monitor cell growth and viability. Per.C6® cells showed the best cell growth in the GTM-3 with 6.5 x 10^6 cells/ml at day seven of the culture.

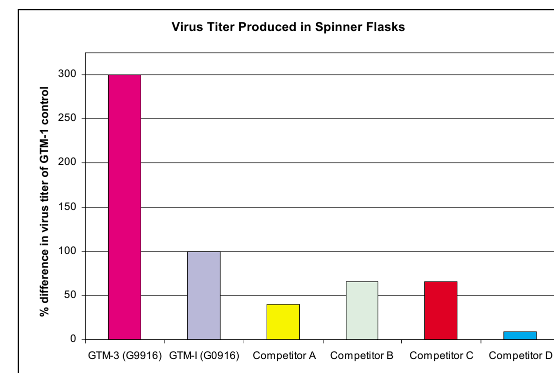

Figure 3. Comparison of virus production from Per.C6® cells in GTM-3 and GTM-1. Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in spinner flasks with GTM-3, GTM-1, and several other commercially available media. After three days of growth the cultures were infected with 3 x 10^9 virus particles (moi ≈3) of the rAd5 containing the β-galactosidase reporter gene (Crucell, Leiden, The Netherlands). Samples were taken three days post infection and assayed for virus production by using Sigma's β-gal Reporter Gene Activity Detection Kit (Sigma GAL-A) and Sigma's β-gal Reporter Gene Staining Kit (Sigma GAL-S). Evaluation of virus production was then determined by $TCID_{50}$ equation (Karber) and a virus titer calculation. Per.C6® cells grown in GTM-3 supported a three-fold increase compared to virus produced from Per.C6® cells grown in GTM-1.

Results and Discussion

Medium Development Study: Experimental Assays were performed in order to understand which components in the medium were key to cell growth and viability. The components were identified and optimal levels of each component for the medium were determined. Based on the results of the optimization assays, three modifications were made to the original medium.

Comparison Study for Cell Growth and Virus Production: Studies comparing Sigma's original medium GTM-1 to the newly formulated GTM-3 for cell growth and viral production were done. Cells were prepared and seeded in spinner flasks and roller bottles containing Sigma's original and modified medium as well as several other commercially available media as described in the material and methods. The results shown in Figure 1 indicate cells grown in our modified medium, GTM-3, reached higher cell density (5 x 10^6 viable cells/ml) than cells grown in any of the other media tested. These results also hold true as shown in Figure 2 where cells grown in roller bottles also reached higher cell density in GTM-3 than cells grown in any of the other media.

In order to confirm the modified medium formulation also supports adenovirus production; comparison assays evaluating virus production in the various media tested were performed in parallel with cell growth assays. Following a standard system used in virus production, β-gal colorimetric and histochemical assays were performed to evaluate viral productivity qualitatively and quantitatively. At three days post infection, the GTM-3 supported a virus titer of 1.5 x 10^9 particles/ml and the GTM-1 supported a virus titer of 5 x 10^8 particles/ml. These data show a three-fold increase in virus production when cells were grown in GTM-3 compared to GTM-1 (Figure 3). The same three-fold increase in virus production was also replicated in roller bottles (Figure 4). There is a three-fold difference between GTM-3 supporting a virus titer of 2.37 x 10^8 particles/ml compared to GTM-1 supporting a virus titer of 7.50 x 10^7 particles/ml (data not shown). These data suggest that Per.C6® cells produce more virus when growing in the GTM-3 than in GTM-1 or any of the competitor media.

As noted in Table 1, the three-fold increase of adenovirus production remains constant whether cells are grown in spinner cultures or roller bottle cultures. When harvesting virus two, three, and four days post infection a three-fold difference is still apparent in cells grown in GTM-3 compared to cells grown in GTM-1.

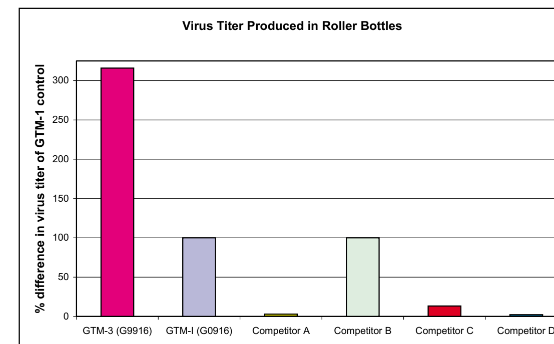

Figure 4. Comparison of virus production from Per.C6® cells in GTM-3 and GTM-1. Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in poly-HEMA coated 250cm² roller bottles rotating at 4 rpm with GTM-3, GTM-1, and several other commercially available media. After three days of growth the cultures were infected with 3 x 10^9 virus particles (moi ≈3) of the rAd5 containing the β-galactosidase reporter gene (Crucell, Leiden, The Netherlands). Samples were taken three days post infection and assayed for virus production by using Sigma's β-gal Reporter Gene Activity Detection Kit (Sigma GAL-A) and Sigma's β-gal Reporter Gene Staining Kit (Sigma GAL-S). Evaluation of virus production was then determined by $TCID_{50}$ equation (Karber) and a virus titer calculation. Per.C6® cells grown in GTM-3 supported a three-fold increase compared to virus produced from Per.C6® cells grown in GTM-1.

Conclusions

Making the necessary modifications to GTM-1 resulted in an improved animal component-free protein-free Gene Therapy Medium-3 formulated for production of adenoviral vectors in Per.C6® cells. Producing a medium free from animal-derived components supporting cell culture and adenoviral vector production will meet the needs of biotechnology and pharmaceutical manufacturers.

- When comparing the GTM-3 formulation to the GTM-1, the modification resulted in a metabolic shift in the cells in which initial growth was slower, but after six days of growth cell density was consistently >3 x 10^6 viable cells/ml.
- The cultures consistently grew to a density of 4-5 x 10^6 cells/ml versus 2-3 x 10^6 cells/ml in the original product.
- We observed consistent growth of cultures for greater than two weeks in the modified medium.
- Along with the increase in total cell density and cell longevity, we observed GTM-3 supported viral production three times greater than cells grown in GTM-1.

These experiments show the paired system of rAd5 vector and Per.C6® cells are supported by Sigma's GTM-3 cell culture medium. Not only is the system scalable to roller bottles, but we have also demonstrated that the product performs well in stirred bioreactors (data not shown).

Experiment	Medium	Fold Difference
Roller Bottle Assay	GTM-3	3.2
	GTM-1	1
Spinner Flask Assay	GTM-3	3
	GTM-1	1
Spinner Flask Assay	GTM-3	2.9
2 days post infection	GTM-1	1
3 days post infection	GTM-3	2.8
	GTM-1	1
4 days post infection	GTM-3	3
	GTM-1	1

Table 1. Comparison of virus production from Per.C6® cells in GTM-3 and GTM-1. Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in spinner flasks and roller bottles. After three days of growth the cultures were infected with 3 x 10^9 virus particles (moi ≈3) of the rAd5 containing the β-galactosidase reporter gene (Crucell, Leiden, The Netherlands). Samples were taken three days post infection and assayed for virus production by Sigma's β-gal Reporter Gene Activity Kit (GAL-A) and Staining Kit (GAL-S). In a similar spinner flask assay, samples were taken two, three, and four days post infection and assayed for virus production as previously described. In each experiment, Per.C6® cells grown in GTM-3 supported a three-fold increase compared to virus produced from Per.C6® cells grown in GTM-1.

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Per.C6® is a registered trademark of Crucell (Leiden, The Netherlands).