Serum-Free Systems for MDBK and MDCK Epithelial Cells

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Introduction

Vaccines developed from viruses grown in mammalian cell cultures are of major importance in both human and animal health. The economic and sociological losses due to viral infections in human populations and the animal-production industry are significant, and necessitate the use of large-scale vaccination programs. Therefore, efforts are continually underway to improve cell culture systems that enhance our ability to increase the effectiveness of vaccine production, to make the process more economical, and improve the quality of life.

The kidney epithelial cell lines, Madin-Darby Bovine Kidney (MDBK) and Madin-Darby Canine Kidney (MDCK), are important and well-established culture systems used to grow attenuated viruses for vaccine production.[1, 2] The use of serum in a manufacturing process lessens the validity of data interpretation, increases downstream-processing complexity, is less cost-effective, and raises regulatory and safety concerns primarily related to adventitious agents. To address these issues, we have developed four new media formulations. These new formulations allow the growth of these cell lines to higher cell densities than those cultured under traditional serum-supplemented conditions.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Cell Lines

MDBK (NBL-1, CCL-22) and MDCK (NBL-2, CCL-34) cell stocks were obtained from ATCC (American Type Culture Collection, Manassas, VA). Stock cultures of cells were grown in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DME/F-12; 1:1) with 10% (v/v) fetal bovine serum (FBS). Cells were subcultured twice per week following standard trypsinization protocols.

Serum-free culture system

Cultures using the serum-free medium system required slightly modified protocols. The following protocol was developed for use in maintaining cells in 25 cm^2 T-flasks. Cultures were briefly washed with 0.2 ml/cm^2 Dulbecco's Phosphate Buffered Saline (DPBS) containing calcium and magnesium (Product Code: D 8662). Cold 0.5% trypsin/5 mM EDTA (Product Code: T 3924) at a volume of 0.2 ml/cm^2 was then added to the flask and allowed to cover the culture surface for 30 to 60 seconds at room temperature. After removal of the trypsin-EDTA, cultures were allowed to remain at room temperature until cells became rounded and easily dislodged from the surface by gently rapping the flask. Care was exercised to avoid excessive force, as the cells were sensitive to mechanical damage. Cells were resuspended in a 10 µg/ml solution of soybean trypsin inhibitor (Product Code: T 6414), at a volume of 0.2 ml/cm^2 of original surface area. Cells were pelleted at 500 rpm for 5 minutes, the trypsin inhibitor solution removed, and the cells were resuspended in at least 0.1 ml of DPBS/cm^2 of original surface area. Cells were then diluted into pre-warmed medium and plated in 25-cm^2 flasks at a minimum of 1-2 x 10^4 cells/cm^2 of surface area.

Medium development

Medium development was conducted using a modified Plackett-Burman method for testing various supplements.[3] Experiments were conducted by plating cells at 1 x 10^4 cells/cm^2 in 24-well multiwell plates (Corning Costar, Cambridge, MA). Growth was monitored initially by visual inspection of stained cells and later by either dye absorption or direct measurement.

Since MDBK and MDCK cell lines are frequently cultured under dynamic culture systems, such as roller bottles and microcarrier beads in suspension, it was critical to develop protocols for the use of these media under these conditions. We employed 25 cm^2 tissue culture flasks on a rocker apparatus to mimic the shear forces associated with fluid motion in dynamic culture systems. This model system was used to examine the effect of medium components and optimize culture inoculation and handling. Roller bottle cultures were inoculated at a density of 25,000 cells/cm^2 in a volume of 0.2 ml/cm^2 pre-warmed (37° C) medium to ensure optimal distribution of cells. To facilitate cell attachment, initial rotational speed did not exceed 0.2-0.3 revolutions per minute. After 12 to 24 hours, cells were firmly attached to the surface and speed was increased to 0.5 revolutions per minute. Medium changes were performed every 3-4 days to maintain optimal cell growth. To evaluate the performance of the medium system using microcarrier beads in suspension, we used 100-ml spinner flasks containing 75 ml of medium and 4 mg/ml beads. Beads were inoculated with 25,000 cells/mg of beads to ensure that a sufficient number of cells populated all of the beads. Use of a sufficient cell inoculum to populate all the beads was vital since cells were not observed to migrate from one bead to another. MDBK cell attachment and growth was evaluated on dextran-based beads (Cytodex™-1 and -2, Amersham Pharmacia Biotech, Piscataway, NJ) and polystyrene-based beads (Plastic-Plus and Pronectin®-F treated, SoloHill Engineering Inc., Ann Arbor, MI).

Cell freezing and Recovery after cryopreservation

Successful freezing and recovery of MDBK and MDCK cells grown in these serum-free formulations required modifications to standard techniques. Actively dividing cells were trypsinized according to the protocol previously described, pelleted by centrifugation, and resuspended at a concentration of 10^6 cells/ml in serum-free cell freezing medium (Product Code: C 6295). Cells were frozen in liquid nitrogen as previously described. Cells were recovered by rapid thawing and seeded at a density of 40,000 cells/cm^2 or higher in pre-warmed MDBK "recovery" medium or MDCK culture medium without removal of the freezing medium. The "recovery" medium is a modification of MDBK-GM, which supports a slower rate of cell growth than MDBK-GM and thereby fosters improved cell recovery after thawing.

Cells were allowed to attach for 2-4 hours, then the medium was removed and replaced with fresh "recovery" or culture medium. MDBK cultures were maintained for 2 or 3 days in "recovery" medium before the addition of MDBK-GM. For

MDCK cells, an equal volume of medium was added after 48 hours and a complete medium change was performed on day 4 or 5.

Results and Discussion

Several serum-free medium formulations, including Medium K-1 for MDCK cells[4], were examined for their ability to support MDBK and MDCK cells in both static and dynamic culture systems. None of the formulas examined provided results comparable to serum-supplemented formulations. We began our development of a serum-free medium by investigating the effects of different basal medium formulations on cell growth. A variety of different basal media and various mixtures of these media were examined for their ability to support growth with low levels of serum supplementation. Based on this work, a mixture of DME and Ham's F-12 was selected as the basis of future medium development.

The most striking observations under serum-free conditions were the relatively slow cell growth rates and the rapid formation of cellular domes or other morphological changes. We sought to identify components that supported growth and normal morphology of cells upon direct transfer from serum-supplemented medium to serum-free medium. This was done using a modified Plackett-Burman system and observing cellular morphology. Based on these observations, we were able to identify several factors required for cell growth. Not surprisingly, these factors normally provided by serum, included hormones and growth factors. With the goal of making this medium regulatory friendly, animal-derived proteins were eliminated as much as possible. All hormones and growth factors used were recombinant proteins expressed in non-vertebrate systems.

Analysis of spent medium identified several amino acids and energy-producing compounds that were used in disproportionate amounts or depleted from the medium. We also observed a coordinate increase in other amino acids, such as alanine. It has been reported that limitations of essential nutrients can result in nutrient deficiency-induced apoptosis.[5] To avoid this, we then supplemented the medium with the requisite amino acids and analysis of spent medium of the new formulations showed a more consistent profile. Most amino acids were present in proportions similar to that of fresh medium. Our observations revealed the need for adaptation of traditional cell culture protocols including inoculation density, procedures to allow cell attachment, and media exchange frequency in coordination with the new medium formulations, to ensure optimal performance. The new medium formulations included a growth medium for MDBK cells (MDBK-GM) which contained a low level of animal-derived proteins (<100 µg/ml), a protein-free medium (MDCK-PFM) and a low-protein, serum-free medium for MDCK cells (MDCK-SF).

MDBK static and dynamic culture systems

Cells cultured in MDBK-GM under static conditions showed minor transient morphological differences in cell shape and vacuole formation when first transferred to serum-free medium compared to cells cultured in serum-supplemented medium (data not shown). Cells grew steadily, reaching maximal density within 5-6 days, and then appeared to lose their ability to divide as evidenced by an inability to be subcultured. This possibly was the result of the cells entering quiescence or apoptosis. We have observed that MDBK cells can undergo at least four passages in MDBK-GM medium without changes in doubling time or cell morphology when subcultured during the growth phase (data not shown).

While MDBK-GM supported the rapid growth of cells to significantly greater densities than serum-supplemented cultures, it *did not* stably maintain these densities as non-dividing cultures (dotted line in Figure 1). We reformulated MDBK-GM to minimize growth-inducing components and reduce metabolically-stimulatory components. This

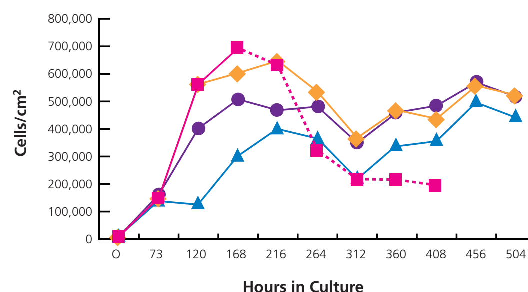

Figure 1. Growth Curve of MDBK Cells in Sigma Medium. *Serum-supplemented Control (▲); MDBK-GM (■); MDBK-GM,medium changed to MDBK-MM on day 7 (◆); MDBK-GM/MDBK-MM 50:50, medium changed to MDBK on day 7(●).*

reformulation allowed cultures to reach levels sufficient to sustain cell viability at high cell densities while minimizing cell replication. We called this new protein-free medium MDBK Maintenance Medium (MDBK-MM). MDBK-MM supported the growth of MDBK cells at a significantly reduced rate. By combining MDBK-GM and MDBK-MM into a system for culturing MDBK cells, we achieved rapid growth with no lag time and no weaning. By using MDBK-GM for 2-5 days and then switching to MDBK-MM, an inoculum of 10^4 cells/cm^2 in flasks or roller bottles expanded to 5×10^5 cells/cm^2 in five days. This could be maintained for at least two weeks in both static and dynamic cultures.

To test the effectiveness of the MDBK medium system under dynamic culture conditions, we evaluated growth performance in roller bottles and on microcarrier beads in suspension. MDBK cultures were successfully established and maintained in roller bottles. Within five days, cells inoculated at a density of 25,000 cells/cm^2 achieved cell densities in excess of 5×10^5 cells/cm^2. These densities were maintained for over 14 days with medium changes every 3-4 days. We have also examined the growth of cells on microcarrier beads. Using an inoculum of 25,000 cells/mg of beads, we have been able to achieve uniform coverage of the beads with densities of 5×10^5 cells/mg beads. The use of sufficient cell inoculum to populate all the beads was vital since cells were not observed to migrate from one bead to another.

MDCK static and dynamic culture systems

Cells cultured in the serum-free static culture system grew steadily using either serum-free, low-protein medium or protein-free medium and reached maximal density within 4-5 days. We observed that MDCK cells could undergo at least four passages in MDCK-PFM without changes in doubling time when subcultured during the growth phase (data not shown). Cells exhibited an inability to thrive in protein-free medium at low inoculation densities and minimum inoculations of 10,000 cells/cm^2 were required.

Cell growth under dynamic culture conditions, which simulated roller-bottle cultures, was also evaluated. We observed that while the protein-free medium supported growth in static cultures, this medium did not provide

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optimal support to allow initial cell attachment under non-static conditions. During medium development, it was observed that the addition of protein(s) to the medium had a positive effect on the ability of MDCK cells to attach to the culture surface under stressful conditions. A screening of proteins revealed that several proteins could provide the

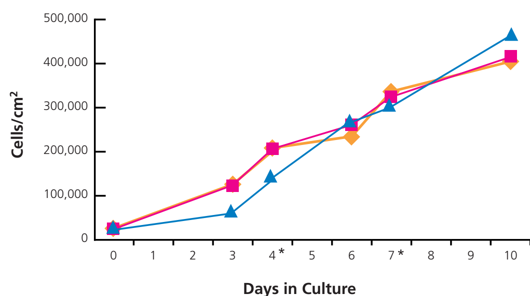


Figure 2. The Effect of a Medium Change from MDCK-LPM to MDCK-PFM. *Cells were inoculated at 20,000 cells/cm^2 from a serum-supplemented source. DMEM:F-12 with 5% FBS (▲); MDCK-LPM (■); MDCK-LPM to MDCK-PFM (◆); Media change(*).*

necessary nutritional supplementation to facilitate cell attachment at low cell densities in dynamic cultures. From this, we determined that a final protein concentration of 100 µg/ml was sufficient to allow cells to attach under dynamic culture conditions while still expressing the desired growth characteristics. We refer to this medium as MDCK low protein medium or MDCK- LPM.

MDCK-PFM or MDCK-LPM media support the growth of cells with no lag time and no weaning in static cultures. Additionally, roller bottle cultures can be initiated in MDCK-LPM to facilitate cell attachment. The medium can be replaced after 4-5 days with MDCK-PFM to minimize the amount of animal-derived protein present in the final product (Figure 2). Under these conditions, an inoculum of 2×10^4 cells/cm^2 in flasks or roller bottles expands to 4×10^5 cells/cm^2 in four days. These densities can be maintained for at least two weeks in both static and dynamic cultures. We also found that cells grown on microcarrier beads will achieve densities of 5×10^5 cells/mg beads in 4-5 days with uniform coverage of the beads in cultures at an inoculum of 25,000 cells/mg of beads.

Virus production

Both cell medium systems were evaluated for their ability to support the production of infectious viral particles. Both medium systems supported levels of viral expression similar to or superior to cells grown in medium supplemented with 10% FBS (Figures 3 and 4).

Conclusions

While the advantages of reducing or eliminating serum and other animal-derived products in medium used for manufacturing are obvious, the development of new medium formulations are complicated by various limitations. In general, cell lines have many nutritional requirements in common. However, each cell line also possesses its own requirements. Many cell types, although similar, may demonstrate different needs. The difficulty of medium development is exemplified by contrast between MDBK and MDCK cell lines. While both lines are derived from kidney epithelial cells, media developed for one line does not adequately meet the requirements of the other. Similarly, media developed for the culture of cells in static cultures may not give satisfactory results when used in dynamic culture systems, such as roller bottles and stirred cultures. Protocols employed for serum-supplemented cultures did not give positive results when employed in serum-free systems. Thus, the development of new culture media is not merely the modification of existing formulas but rather the coordinate development of cell culture systems of new or modified handling protocols that work in conjunction with modified medium formulations.

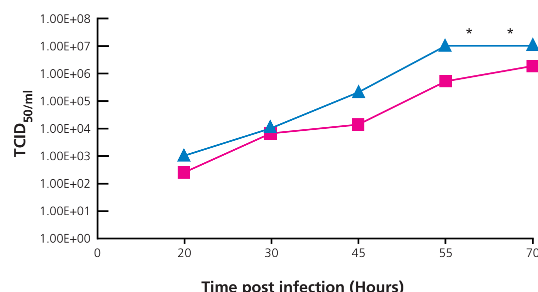
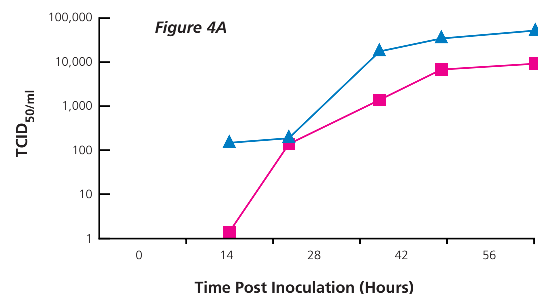


Figure 3. Viral Titers of Infectious Bovine Rhinotracheitis in MDBK cells. *Sigma MDBK System (▲); 5% Serum-Supplemented Control (■); Maximum detection level (*).*



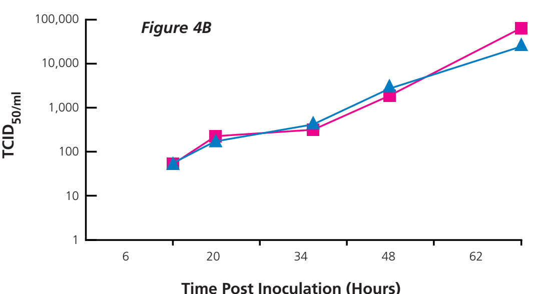


Figure 4. Viral titers of MDCK cells. A) *Canine adenovirus Type 2 and* ***B)*** *Canine herpes virus. Sigma MDCK Media (▲); 5% Serum-Supplemented Control (■).*

We have developed a serum-free medium system for MDBK cells (Product Codes: M 3553 and M 0682) that supports rapid cell growth and maintains high cell densities for over 14 days through feeding of the cultures. For MDCK cells, we have developed a low-protein, serum-free medium (Product Code: M 3803) and a protein-free medium (Product Code: M 3678). Both cell lines have achieved cell densities in excess of 5×10^5 cells/cm^2, which is comparable to medium supplemented with 10% FBS. Based on initial studies, the viral productivity of cells grown under serum-free conditions

appears to be comparable to serum-supplemented cultures. Additionally, the ability to maintain high cell densities for extended periods of time provides the opportunity to combine this medium system with a "multi-harvest" process such as that described by Noe et al.[6] which further enhances yield and reduces costs. These products offer a number of advantages over traditional methods using serum-supplemented systems, such as improved medium definition, reduced cost, improved downstream processing and fewer regulatory concerns. The performance of our new media formulations combined with the advantages previously cited offer the potential to significantly improve the efficiency of batch manufacturing processes used for growing viruses for use in vaccine production.

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About the Author

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ORDERING INFORMATION

Product Code	Product Name	Unit	1-11	12-59	60+
M 3553	MDBK-GM Serum-free Medium	1 liter	$42.60	$38.40	$34.10
M 0682	MDBK-MM Protein-free Medium	1 liter	$42.60	$38.40	$34.10
M 3803	MDCK Serum-free Medium	1 liter	$43.90	$39.60	$35.20
M 3678	MDCK Protein-free Medium	1 liter	$47.00	$42.30	$37.60
G 7513	L-Glutamine Solution (200 mM)	20 ml	$5.40	$4.90	
		100 ml	$16.80	$12.60	

RELATED PRODUCTS

Product Code	Product Name	Unit	Price
A 1345	Anti-oxidant Supplement	5 ml	$14.95
P 8483	Polyamine Supplement	5 ml	$14.95

SUPPORTING LITERATURE

MDBK/MDCK brochure (CRT)
Media for Biotechnology/Products for Formulation brochure (DOJ)
Life Science Catalog 2000-2001, pages 353-354