Philadelphia College of Osteopathic Medicine

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The Philadelphia College of Osteopathic Medicine received $19,760 in formula funds for the grant award period January 1, 2008 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Entry Mechanisms of Mouse Hepatitis Virus, a Model for Multiple Sclerosis - In order for viruses to infect cells, they must first bind to and enter potential host cells. There are several distinct pathways that viruses can use to enter cells, and understanding viral entry mechanisms provides important information on how viruses cause infections. This project investigates entry mechanisms of mouse hepatitis virus (MHV); the MHV strain used, MHV-A59, is studied as a model for multiple sclerosis (MS). Understanding more about MHV entry will provide information on how viruses infect the central nervous system, and may provide insight into how viral agents can contribute to changes observed in human demyelinating diseases such as MS.

Anticipated Duration of the Project

1/1/2008 – 12/31/2011

Project Overview

Mouse hepatitis virus strain A59 (MHV-A59) can cause an acute meningoencephalitis, followed by a chronic demyelinating disease in animals surviving the acute infection. Understanding pathogenic properties of this neurovirulent virus might shed insight on how viruses could induce cytopathology and immunopathology associated with demyelinating disease. The ability of viruses to bind to and enter specific cell types is an important determinant of what cells get infected in vivo. Furthermore, the mechanism by which viruses enter cells will influence their ability to spread to uninfected cells, as well as influence virus-induced damage to the host cell and viral interactions with the host immune response.

Viruses can enter cells either at the level of the plasma membrane, or by receptor-mediated mechanisms. Receptor-mediated pathways may involve endocytosis utilizing structures called clathrin coated pits and endosomes, or membrane invaginations associated with protein complexes called caveolae. In addition, viral entry may occur at specific regions of the plasma membrane that are high in cholesterol and sphingolipid, called lipid rafts. This project will

examine the abilities of inhibitors of clathrin-dependent entry and caveolae-dependent entry, as well as cholesterol depletion, to interfere with viral replication. It will involve assays measuring viral titers, percent infected cells, and production of viral mRNA. In addition, both fluorescent and electron microscopy will be utilized to visualize aspects of viral entry and the affect of entry inhibitors. Of particular interest will be the investigation of entry mechanisms of MHV-A59 in primary neuronal cells, including mixed glial cells and cells enriched for astrocytes and microglial cells.

This project will also investigate the relationship between a virus's ability to induce cell-to-cell fusion, or syncytia formation, and use of entry pathways. Syncytia formation could allow viral entry through the plasma membrane, and could potentially allow the virus to infect adjacent cells that do not express virus-specific receptors, which could affect the in vivo pathogenesis of MHV.

Principal Investigator

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Other Participating Researchers and Employers

None

Expected Research Outcomes and Benefits

Multiple sclerosis (MS) is a human demyelinating disease where the cause is unknown. MS is considered an autoimmune disease in which patients develop an immune response to myelin, considered an insulator for the electrical impulses of nerve cells. It has been suggested that infectious agents might trigger the immune response observed in MS. The virus examined in this project, mouse hepatitis virus strain A59 (MHV-A59), is studied as a model for multiple sclerosis (MS). Thus, understanding how MHV-A59 infects cells might help explain how viruses can trigger events that lead to the development of MS.

The focus of this project is to better understand how MHV causes disease by examining the pathways that the virus uses to enter and infect different types of cells. We know that in some cell types, MHV-A59 infection produces fusion between membranes of adjacent cells, which, during an infection in the animal, would allow the virus to pass from cell to cell without exposure to the external environment and possible removal by the immune response. However, prior results have indicated that MHV-A59 does not produce cell-to-cell fusion in cells isolated directly from the brains of mice, suggesting that in the brain MHV-A59 might enter cells by a different pathway than that used to enter continuously cultured cell lines. This project proposes to compare entry mechanisms of MHV-A59 into cells where infection produces cell to cell fusion with entry into cells where cell fusion is not observed, which may more closely resemble infections in the animal.

Summary of Research Completed

During the 2008-2009 fiscal year a real-time polymerase chain reaction (RT-PCR) assay was developed to quantify levels of viral RNA in cells at early times post infection as a measure of entry of two viruses, $RA59_{SA59}$ and $RA59_{S2}$. $RA59_{SA59}$ and $RA59_{S2}$ are recombinant viruses that express the MHV-A59 or MHV-2 S proteins in an MHV-A59 background. These two viruses differ in their abilities to induce cell-to-cell fusion when they infect different cell lines, thus might be expected to differ in the mechanism(s) used to enter cells.

The RT-PCR assay was subsequently used to examine the entry of these viruses into two cell lines, L2 cells (murine fibroblast cells) and bEnd cells (murine brain endothelial cells). The ability of two inhibitors of clathrin-mediated endocytosis, bafilomycin A1 (BAF) and chlorpromazine (CZ), to interfere with viral entry was examined by quantifying viral RNA in the presence or absence of the inhibitors. The two inhibitors disrupt different steps in the endocytosis pathway; BAF inhibits vacuolar H+/ATPase, preventing acidification of endosomes, while CZ inhibits the recycling of clathrin to the plasma membrane. The RT-PCR assays begun during the 2008-2009 fiscal year were continued into the 2009-2010 fiscal year. Figure 1, which compares the relative levels of viral RNA present at 6 hours post infection (hpi) with $RA59_{S2}$ in untreated cells or in cells treated with BAF or CZ, completes these sets of experiments; similar data from $RA59_{SA59}$-infected cells and infected cells at 4 hpi was presented in the previous progress report.

It is possible the viruses studied here enter cells through either a clathrin-mediated and/or a caveolin-mediated pathway. Immunofluorescent labeling was used to visualize affects of inhibitors on the expression of clathrin and caveolin in cells. It was anticipated that labeling for clathrin might be altered by treatment of cells by CZ, since this drug can prevent recycling of clathrin to the cell surface. However, as shown in figure 2, there was no obvious difference in clathrin labeling in untreated cells compared to cells treated with CZ.

Previous experiments (2007-2008 progress report) looked at the ability of methyl-β-cyclodextran (MβCD), a drug that can remove cholesterol from membranes, to interfere with viral entry. Since caveolin is a protein that is associated with lipid rafts, which contain cholesterol, it was thought that MβCD-treatment of cells would alter caveolin expression in treated cells. As with clathrin labeling in CZ-treated cells, there was no dramatic difference in caveolin labeling in MβCD-treated and untreated cells, although there was a tendency for caveolin labeling to appear brighter along the plasma membrane of cells that were not treated with MβCD (figure 3), which may reflect partial removal of caveolin from the surface of cells treated with MβCD.

Immunofluorescent labeling and 3-D deconvolution microscopy is being investigated as a method to look for co-localization of proteins or proteins and structures within cells. Fluorescent labels will emit light regardless of whether the labeled proteins/structures are in focus or not. By knowing how light spreads out from the point of focus, it is possible to work backwards and subtract out light that is due to out of focus proteins/structures. Deconvolution involves eliminating out of focus fluorescence from an image; 3-D deconvolution is performed on a series of images taken from different planes of focus, called a Z-stack. The result is a 3-dimensional representation of fluorescently labeled cells. If two labels co-localize, then they must be at the

same location along all 3 axes (x-, y- and z-axis). The ultimate goal of the immunofluorescence/deconvolution experiments is to determine whether internalized virus co-localizes with clathrin or caveolin, thus providing additional information on the pathway used by $RA59_{SA59}$ and $RA59_{S2}$ to enter cells. Initial experiments are looking for co-localization of transferrin with clathrin and cholera toxin B with caveolin since transferrin enters cells via clathrin-mediated endocytosis and cholera toxin B enters via the caveolin pathway. The images shown in figures 4 and 5 show cells that were labeled for transferrin or cholera toxin B (red fluorescence), and caveolin or clathrin (green fluorescence). Areas of colocalization will appear yellow in the main part of image (top view; z-planes) and on the strips at the bottom (side view; x-planes) and right edge (side view; y-planes) of the image. As shown in figure 4, transferrin and clathrin show areas of co-localization along all 3 axes, while there is little co-localization of transferring and caveolin. Conversely, cholera toxin B and caveolin show more co-localization than cholera toxin B and clathrin (figure 5).

Initial entry assays measured viral titers at 16 hpi in the presence or absence of BAF or CZ (data shown in the 2007-2008 progress report). To better understand the effects of both these drugs on viral replication over time, viral titers were measured from 6-24 hpi in the presence or absence of BAF and CZ (figure 6). These experiments provide a more comprehensive analysis of the inhibitory effects of BAF and CZ. Results indicate that BAF inhibits replication of $RA59_{S2}$ to a greater extent than that of $RA59_{SA59}$, which is consistent with results of the RT-PCR assays that examine early times post infection. It also appears that BAF inhibits replication of both viruses in bEnd cells more than L2 cells, a trend that was observed in the RT-PCR assays on samples taken at 6 hpi. CZ, however, inhibited viral replication in L2 cells to a greater extent than in bEnd cells for both viruses; this difference was not observed in the RT-PCR assays. Furthermore, unlike BAF, CZ inhibited the replication of $RA59_{SA59}$ and $RA59_{S2}$ similarly in each cell line, also consistent with results from RT-PCR assays.

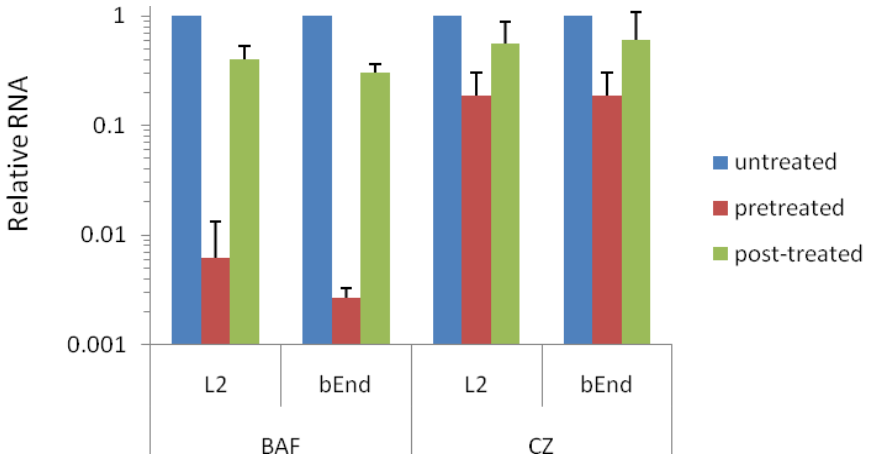

Figure 1. Inhibition of viral RNA by BAF or CZ in L2 or bEnd monolayers infected with $RA59_{S2}$ for 6 hours. Each column represents the mean + standard deviation from 3-6 separate experiments/cell line/inhibitor; for each set of samples (untreated, pretreated, post-treated) in an experiment, RNA levels are shown relative to the amount of viral RNA present in untreated monolayers.

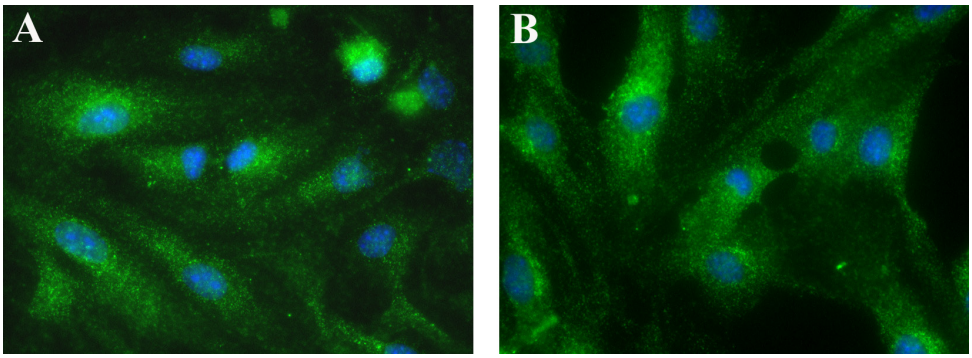

Figure 2. Clathrin (green label) in untreated cells (A) and cells treated with 20 μM CZ.

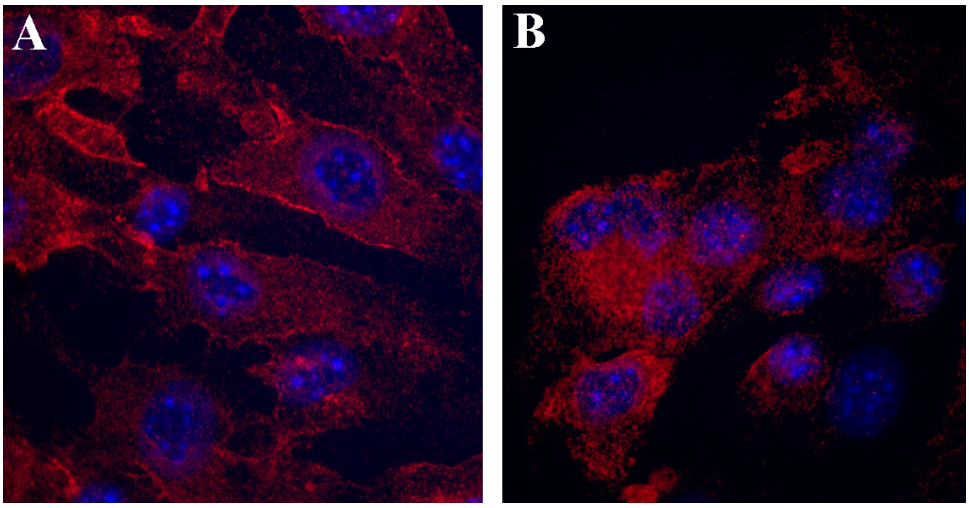

Figure 3. Caveolin (red label) in untreated cells (A) and cells treated with 5 mM MβCD.

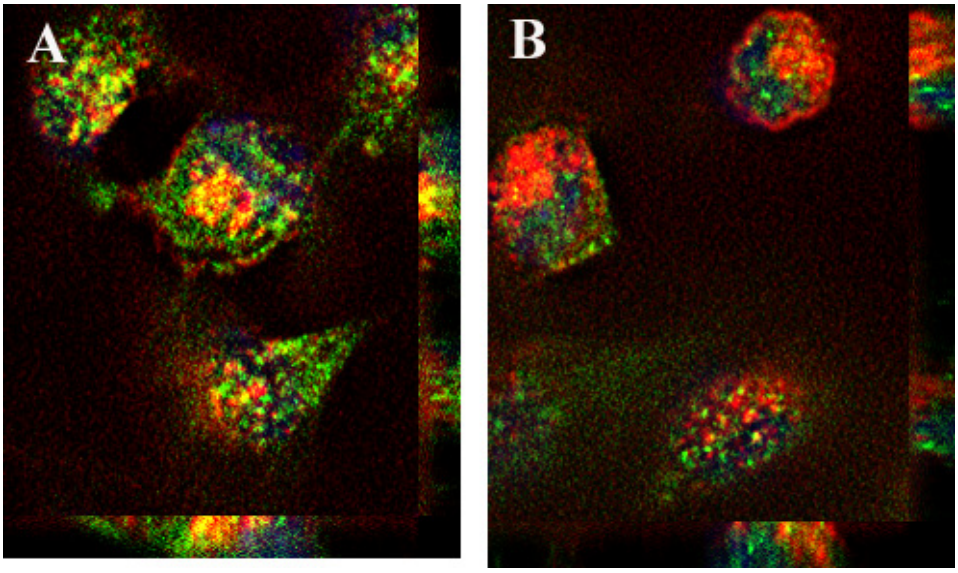

Figure 4. Cells were labeled with transferrin (red) and clathrin (green; A) or caveolin (green; B). Areas of co-localization appear yellow in all 3 planes as described in the text.

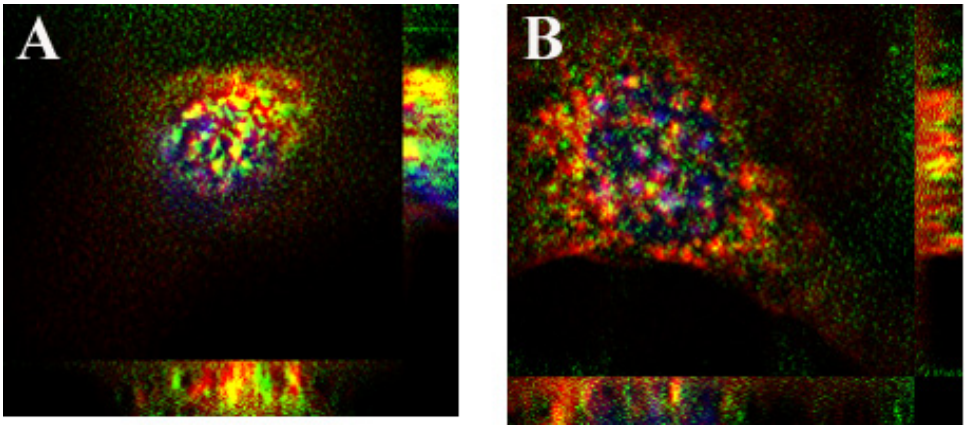

Figure 5. Cells were labeled with cholera toxin B (red) and caveolin (green; A) or clathrin (green; B). Areas of co-localization appear yellow in all 3 planes as described in the text.

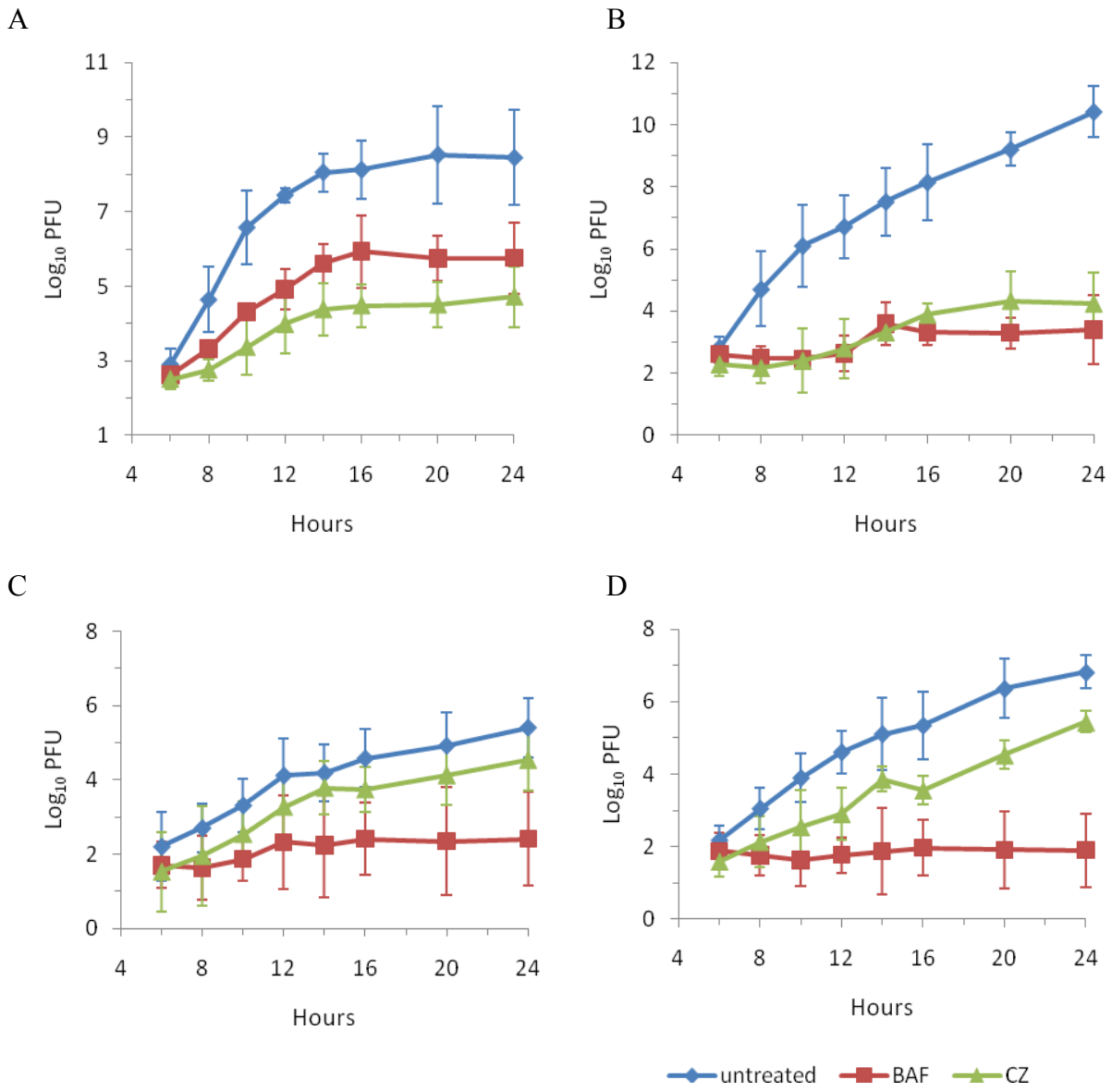

Figure 6. Inhibition of $RA59_{SA59}$ (A, C) and $RA59_{S2}$ (B, D) replication by BAF or CZ in L2 cells (A, B) and bEnd cells (C, D). Viral titers represent the mean ± SD from 5 separate experiments.