

The Development of a Screening Tool for a Reovirus T1L Mutant

**Casey Andrason Reed
North Carolina State University
Biological, Earth and Physical Sciences**

**Faculty Mentor
Barbara Sherry
North Carolina State University**

ABSTRACT

Interferons (IFNs) are cytokines that provide innate protection for essentially all cells in response to viral infections¹. Viruses can induce IFN- β , which is then secreted and binds to other cells to induce antiviral gene expression and protection in those neighboring cells. Induction of IFN- β is particularly important in protection of cardiac myocytes against reovirus-induced myocarditis (inflammation of the heart) in mice, which provides a good model for viral myocarditis in humans. In general, nonmyocarditic reoviruses, such as T3D, induce IFN- β well while myocarditic reoviruses, such as T1L, induce IFN- β poorly². T3D is highly sensitive to the antiviral effects of IFN- β , in contrast to myocarditic reovirus strains which are less sensitive to the effects of IFN- β . We hypothesized that a novel selection procedure can be developed to select T1L mutants that induce IFN. These T1L mutants could be used in the future to identify phenotypes that vary concomitantly with increased induction of IFN.

In order to select T1L mutants that induce IFN, we employed vesicular stomatitis virus (VSV), which is highly sensitive to the antiviral effects of IFN- β ³. The overall strategy is to use T3D, a virus known to induce IFN, in varying dilutions to see whether limited reovirus infection is sufficient to protect against a subsequent challenge with VSV. After finding a cell culture well that was not killed by VSV, and thus protected by reovirus, 0.1% NP-40 would be used to lyse the cells and inactivate the enveloped VSV while leaving the nonenveloped reovirus intact for isolation and further amplification. After optimizing the assay using reovirus T3D, the assay would be used to select T1L mutants for further study of parameters of viral infection that induce IFN.

INTRODUCTION

Interferons (IFNs) are cytokines made by essentially all cells in response to viral infections in the body. In general, IFNs inhibit viral replication within neighboring cells. IFN- β is controlled by multiple regulatory factors in the cell. Viruses can induce IFN- β , which is then secreted and binds to other cells to induce antiviral gene expression. This process protects adjacent cells by stopping the spread of virus.

Viruses in the *Reoviridae* (Respiratory Enteric Orphan virus) family are not enveloped and have a genome that consists of 10 segments. The two reovirus strains of interest are Type 1 Lang (T1L) and Type 3 Dearing (T3D), which differ in all 10 genomic segments. It is known that nonmyocarditic reoviruses, such as T3D, induce IFN- β well while myocarditic reoviruses, like T1L, induce IFN- β poorly. T3D is highly sensitive to the antiviral effects of IFN- β , which is unusual when compared to myocarditic reovirus strains which are less sensitive to the effects of

IFN- β . It is also known that IFN- β induction is much greater in cardiac myocytes than in cardiac fibroblasts and a mouse fibroblast cell line (L cells). The M1, L2, and S2 genes were found to be involved in IFN- β production. It is known that the M1 gene encodes the mu2 protein that is both a component of the virion structure and of viral inclusion bodies of infected cells.

VSV, vesicular stomatitis virus, is in the family *Rhabdoviridae*. VSV is enveloped and causes lytic cell death. It is highly sensitive to, and its spread can be prevented by, IFN- β , which makes VSV an ideal tool for detecting cells that produce IFN.

The overall strategy is to use T3D, a virus known to induce IFN, in varying dilutions to see whether 1 plaque forming unit (PFU)/well of the reovirus is sufficient to protect against VSV. A T1L mutant that induces IFN would be rare, so using T3D first to ensure that protection against VSV is possible is necessary before moving forward to screen for the mutant. Finding a T1L mutant that induces IFN would then be used to determine if the viral inclusion bodies induced by the mutants are altered such that they look more similar to T3D than T1L inclusion bodies. After finding a well that was not killed by VSV, and thus protected by T1L, 0.1% NP-40 would be used to lyse the cells and inactivate the enveloped VSV while leaving the non-enveloped reovirus T1L intact to be harvested. Once harvested, the mutant would be used to infect fresh cells to amplify it and thus isolate the T1L mutant that induces the most IFN.

MATERIALS AND METHODS

Viruses

The viruses that were used included reoviruses T3D and T1L as well as VSV. All viruses used were tested at earlier dates for their concentrations. Reovirus T3D was previously determined to be 6×10^8 PFU/ml and reovirus T1L was determined to be 5×10^8 PFU/ml. VSV stock was 3×10^8 PFU/ml. Reovirus T3D induces IFN- β while reovirus T1L does not induce IFN- β . VSV is a lytic virus and is highly sensitive to IFN- β .

Cells and Infections

The cells used were L cells, which is an immortal mouse cell line and therefore readily available. L cells were plated in 96-well clusters at 8×10^4 cells/well on the day before infection or 1.6×10^5 cells/well the day of infection, except for Figures 6 and 7 as indicated, in a 300 μ l volume of cMEM. The media, cMEM, is Modified Eagle's Medium completed with 5% fetal calf serum and 2 mM glutamine. The fetal calf serum provides growth factors for the cells as well as protein carrier for the virus while glutamine provides energy for rapidly dividing cells by feeding into the Krebs cycle. Reovirus replicates optimally at 37°C, while VSV replicates optimally at 35°C. Cells were infected with reovirus T3D or mock infected for one day at 37°C and challenged with VSV at 35°C, except in Figures 2 and 6 which omitted reovirus infection. Plates were screened for cell death two days post-VSV challenge by MTT assay, except in Figure 4 which was screened three days post-VSV challenge.

MTT Assay

The MTT assay is a colorimetric assay that measures live cells. The solution changes color (from yellow to purple) in mitochondria of live cells. The mitochondrial reductase enzyme reduces the yellow MTT into the purple form if the cells are alive. Therefore, the amount of purple is directly related to the amount of live cells in the well (Figure 1). The absorbance of

color is measured by computer software and a spectrophotometer. The wavelengths used by the software are 650 nm (background color) and 570 nm and the background 650 nm reading was subtracted from the 570 nm reading to determine the actual absorbance. The data in Figures 2 – 8 are presented as the average of the mean of three to six samples, plus or minus the standard error of the mean.

Figure 1: MTT Assay



L cells were mock-infected (columns 1, 3, 5) or infected with VSV (Multiplicity of infection (MOI) 0.1 PFU/cell; columns 7, 9, 11) and incubated for two days before MTT assay. Even-numbered wells did not contain cells.

RESULTS

0.1% NP-40 inactivates VSV but not reovirus T1L

In order to prove that VSV can be inactivated it was incubated in 0.1% NP-40 or a control media. L cells were plated in six-well clusters and incubated in a 37°C incubator overnight. One day later, 10^4 , 10^5 , or 10^6 PFU of T1L or VSV were incubated in 0.1% NP-40 or control media for 30 minutes at room temperature, and then diluted for infecting the L cell monolayers. Media was removed from the wells and they were infected with 100 µl of T1L or VSV diluted a minimum of 100-fold, to reduce the NP-40 concentration to 0.001%, which is harmless to cells. All plates were incubated for one hour at 37°C, with each being rocked 30 minutes into the incubation time. All clusters were overlaid with a mixture of 1.5 ml comp2x199 media and 1.5 ml agar. The plates infected with T1L were incubated at 37°C for seven days (fed on day three with a mixture of 1 ml comp2x199 and 1 ml agar, and stained with 1 ml neutral red/2x199/agar per well on day 6), to allow time for multiple rounds of virus infection and cell lysis. On day seven, the plates were scored for plaques. The plates infected with VSV were incubated at 35°C for two days (stained one day post infection with 1 ml neutral red/2x199/agar per well) and scored two days post infection for plaques. The results demonstrated that while 0.1% NP-40 reduced VSV infectivity to undetectable levels, T1L infectivity remained unchanged (data not shown).

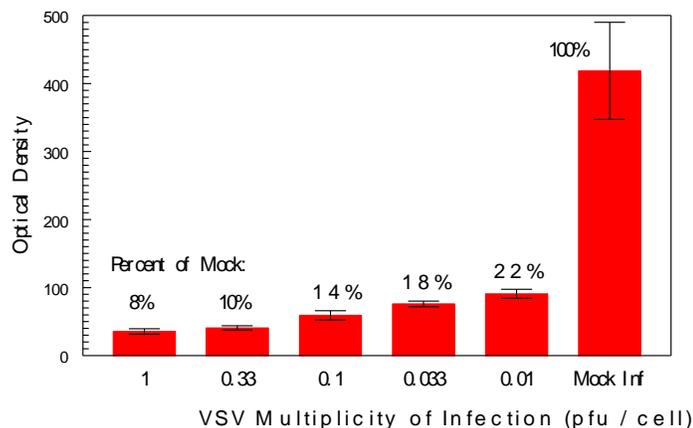
Reovirus T3D can protect against VSV

To determine if reovirus T3D could protect against VSV a series of steps were taken, as outlined below. First, we had to prove that VSV killed L cell monolayers and then assess the degree of cytopathogenicity by MTT assay. Next, we determined whether any amount of T3D could protect against VSV, and if so, what the optimum incubation time was for the assay. We also determined how many replicates were needed to get the least amount of variability in results along with the optimum cell density for experiments.

VSV kills cells in MTT assay

To prove that VSV would kill L cells, cytopathogenic effect (CPE) was monitored by MTT assay. Three 96-well clusters were plated with L cells at a density of 1.6×10^5 cells/well and incubated in a 37°C, CO₂ incubator for two hours. VSV was diluted to 1, 0.33, 0.1, 0.033, and 0.01 PFU/cell concentrations (which corresponds to 1.6×10^5 PFU/well, 5.28×10^4 PFU/well, 1.6×10^4 PFU/well, 5.28×10^3 PFU/well, and 1.6×10^3 PFU/well respectively). Six wells on each of the three plates were infected with 100 µl of VSV/well in the varying concentrations and six wells were infected with 100 µl of cMEM/well. All plates were incubated in a 35°C, CO₂ incubator. Plate #1 was read by MTT assay one day post-infection, plate #2 was read two days post-infection, and plate #3 was read three days post-infection. The MTT assay was completed by adding 25 µl of MTT to each well and incubating the plate for four hours in the 35°C, CO₂ incubator. After the incubation, the plate was centrifuged at 750xg for eight minutes. After the centrifugation, the dye and media were removed using a P200, and 100 µl of 0.04 N HCl in isopropyl alcohol was added to each well and allowed to incubate for 15 minutes on the benchtop. The plate was then tapped gently to break up the crystals and 100 µl of deionized water was added to each well. The plate was read using computer software in a spectrophotometer and the 650 nm read out was subtracted from the 570 nm read out to determine the amount of live cells within the wells. It was found that at one day post-infection, there was little CPE, while at three days post-infection, all VSV infections were highly cytopathogenic. At two days post-infection, all doses were also highly cytopathogenic (Figure 2). Because earlier harvest would preclude CPE from reovirus T3D in future experiments, we chose harvesting at two days post-infection as the optimal time for future experiments. While 0.01 PFU/cell of VSV was strongly cytopathogenic, it was not as strong as 0.1-1 PFU/cell. However, 0.01 PFU/cell is easier to protect against than the higher concentrations, so that was the best choice for future experiments.

Figure 2: Cytopathogenicity of VSV at different doses

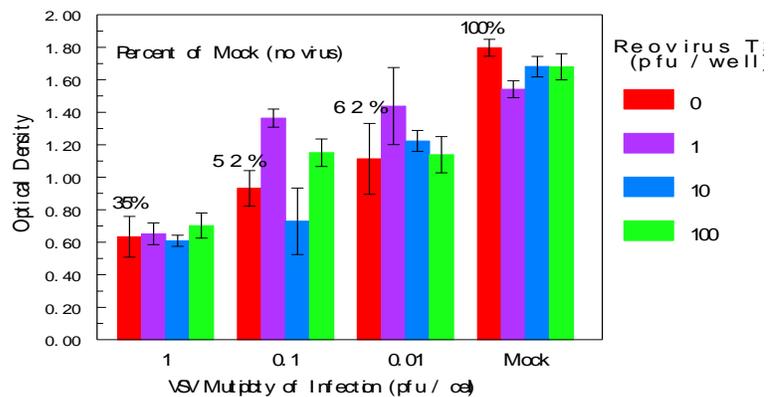


Conclusion: multiple MOIs of VSV are highly cytopathogenic; try lower end of range in future experiments because it will be easiest for detection of T3D-induced protection.

Can T3D protect against VSV?

To determine if reovirus T3D could protect against VSV we first infected L cells with T3D and then challenged with VSV. Two 96-well clusters were plated at an L cell density of 8×10^4 cells/well and incubated overnight in a 37°C, CO₂ incubator. One day post-plating, the cells were infected with varying concentrations of reovirus T3D. Because the goal was to provide sufficient T3D to induce protective IFN without causing CPE, T3D was added in a concentration which was much less than 1 PFU/cell and, instead is indicated here as the concentration in PFU/well. Specifically, twelve wells per plate were infected with 100 µl of either 5, 1, or 0 PFU/well of T3D. The plates were then incubated for one hour in the 37°C incubator. After incubation, 150 µl of cMEM was added to each well and the plates were left overnight in the 37°C incubator. One day after T3D infection the cells were challenged with VSV, as follows. The media was removed from each well using a P200 and each dilution of T3D (5, 1, 0 PFU/well) were challenged with 100 µl of either 0.1, 0.033, 0.01, or 0 PFU/cell of VSV. The plates were incubated for one hour in a 35°C incubator. After incubation, 150 µl of cMEM was added to each well. Both plates were left in the 35°C incubator overnight. While we expected to use data two days post-VSV infection, we read one plate by MTT assay at one-day post-infection and the other at two days post-infection, in order to gain maximum information. Results showed that at one day post-infection, there was little CPE, as expected. At two days post-VSV infection (Figure 3), 0.01 PFU/cell of VSV was almost as cytopathogenic as 0.03-0.1 PFU/cell of VSV but no concentration of VSV was as cytopathogenic as found in the previous experiment. Also, it was found that neither 1 nor 5 PFU/well of T3D protected against VSV. However, because VSV did not induce a lot of cell death (roughly only 50%), T3D protection would have been hard to see. Therefore, increasing VSV cytopathogenicity would help us to visualize T3D protection.

Figure 3: Infecting with reovirus T3D to induce IFN-β and protect against challenge with VSV.

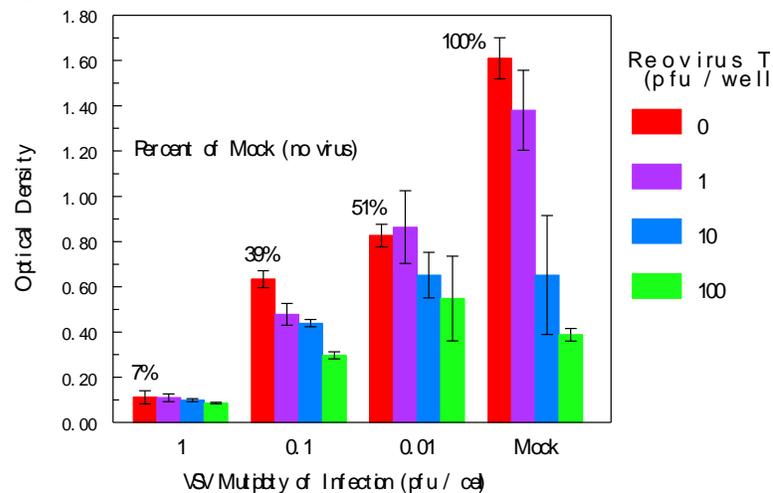


Conclusions: Even at MOI 1 and in the absence of reovirus T3D, VSV is not nearly as cytopathogenic as was previously determined (Figure 2). Something has changed, and requires further optimization so that low MOI VSV regains its previously observed cytopathogenicity. Try incubating three days instead of two days post-VSV infection.

Could longer incubation increase VSV induction of CPE?

Due to the low CPE of VSV for the above experiment, we attempted to increase CPE by using a longer incubation period. Three 96-well clusters were plated with L cells at a density of 8×10^4 cells/well and incubated overnight in a 37°C , CO_2 incubator. One day later twelve wells on each plate were infected with $100 \mu\text{l}$ of 1, 10, 100, or 0 PFU/well of T3D. The plates were incubated for one hour and then $150 \mu\text{l}$ of cMEM was added to each well. The plates were incubated at 37°C overnight. The next day, each dilution of T3D was challenged with $100 \mu\text{l}$ of 1, 0.1, 0.01, or 0 PFU/cell of VSV. After an hour incubation at 35°C , $150 \mu\text{l}$ of cMEM was added to each well and incubated at 35°C until they were to be read by MTT assay one, two, or three days later. Plate #1 was read one day post-VSV infection, plate #2 was read two days post-VSV infection, and plate #3 was read three days post-VSV infection by MTT assay. It was found that T3D may protect 0.1 PFU/cell VSV if read by MTT assay three days post-infection (Figure 4), however a three day incubation causes T3D to become cytopathogenic to the cells.

Figure 4: Increasing incubation time to three days post-VSV infection to maximize VSV CPE



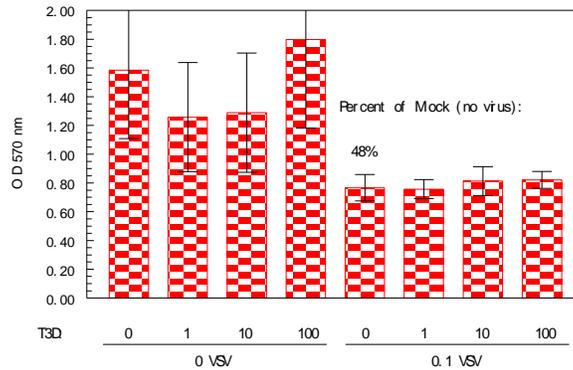
Conclusions: VSV-induced CPE was regained, but the extra day of incubation caused reovirus T3D to become cytopathogenic on its own (as seen in the mock-VSV infection).

Could greater number of replicates improve results?

Results from previous experiments were promising for T3D protection against VSV infection in L cells, so to generate more conclusive results the number of replicates was increased. Two 96-well clusters were plated with L cells at a density of 8×10^4 cells/well and incubated overnight in a 37°C , CO_2 incubator. One day later eight wells on each plate were infected with $100 \mu\text{l}$ of 1, 10, 100, or 0 PFU/well of T3D. The plates were incubated for one hour and then $150 \mu\text{l}$ of cMEM was added to each well. The plates were incubated at 37°C overnight. The next day, each dilution of T3D in plate #1 was challenged with $100 \mu\text{l}$ of 0.1 PFU/cell of VSV while each dilution of T3D in plate #2 was challenged with $100 \mu\text{l}$ of cMEM. After one hour incubation at 35°C , $150 \mu\text{l}$ of cMEM was added to each well and incubated at 35°C until they were to be read by MTT assay two days later. When plate #2, the cMEM plate, was being read by the MTT reader it was bumped, causing spilling of well contents and great variation between wells. Despite the bumping of the plate there was no indication that 100 PFU/well of T3D was cytopathogenic to the cells (Figure 5). It was found that 0.1 VSV showed

less variability with a greater number of replicates, and 0.1 VSV caused greater CPE than 0 VSV. There was no indication that T3D was protective at any dose against VSV infection because there was the same amount of cell death at every T3D concentration. Given the results, we next tested whether changing cell density could increase VSV CPE as well as increase T3D protection.

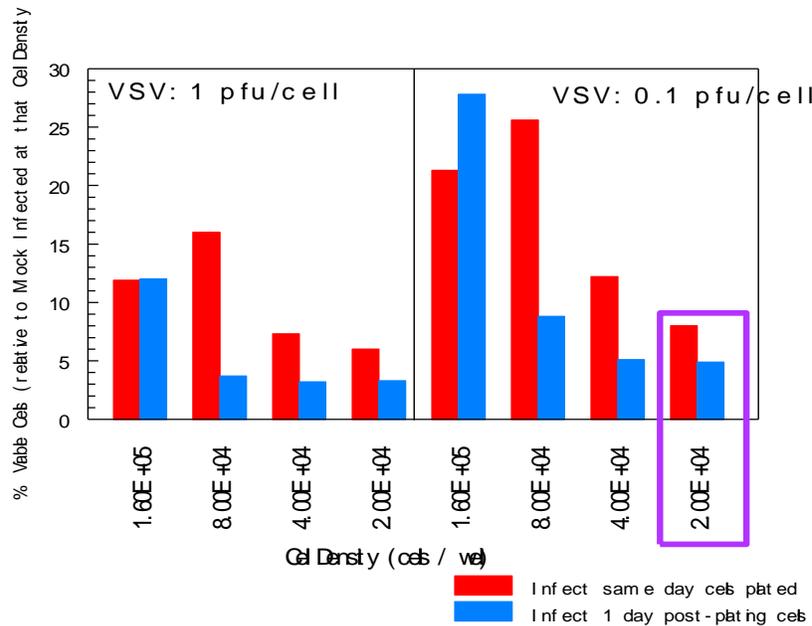
Figure 5: Increasing replicates decreases variability



Does cell density affect VSV infection?

Cells were plated at multiple densities using 300 μ l/well in two 96-well clusters. Plate #1 was plated one day before plate #2. Both plates were plated with twelve wells at each density: 1.6×10^5 , 8×10^4 , 4×10^4 , and 2×10^4 cells/well. One day post-plating for plate one and the same day as plate two was plated, four wells of each cell density were infected with 100 μ l of 1 PFU/cell of VSV, four wells of each density were infected with 100 μ l of 0.1 PFU/cell of VSV, and four wells of each density were infected with 100 μ l cMEM. Each plate was incubated in a 35°C incubator for one hour and then 150 μ l of cMEM was added to each well. Two days after infection both plates were read by MTT assay. It was found that decreasing plating density to 2×10^4 increased VSV-induced CPE (Figure 6).

Figure 6: Optimal cell plating density to maximize VSV CPE

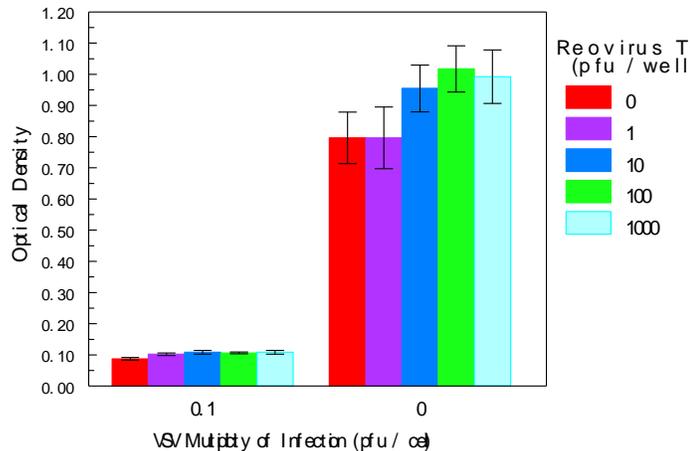


Conclusions: Plating at lower cell densities increases VSV-induced CPE dramatically. Use a low plating density (marked in purple) for future experiments.

Does T3D protect against VSV by inducing IFN- β ?

Knowing the ideal plating density, two 96-well clusters were plated at 2×10^4 cells/well. The same day, T3D was added to the plates in varying concentrations. Eight wells/cluster were infected with 100 μ l of either 0, 1, 10, 100, or 1000 PFU/well of T3D. Each plate was incubated for one hour in a 37°C incubator, after which 150 μ l of cMEM was added to each well. One day after plating and T3D infection, cells were challenged with VSV. Plate #1 received 100 μ l of 0 VSV while plate #2 received 100 μ l of 0.1 VSV. The plates were incubated at 35°C for one hour and then 150 μ l of cMEM was added to each well. Two days post-VSV infection the plates were read by MTT assay for CPE. Results showed that at a cell density of 2×10^4 , 0.1 VSV was cytopathogenic, which allowed us to monitor protection (Figure 7). However, 1000 PFU/well of T3D did not significantly protect against 0.1 PFU/cell VSV.

Figure 7: T3D protection against VSV-induced CPE

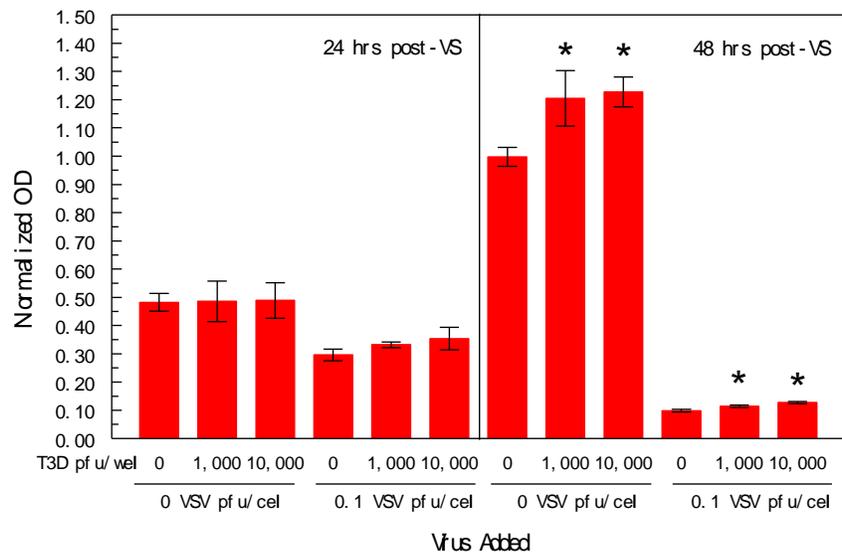


Conclusions: These conditions did ensure that VSV killed most cells, but there was no indication that reovirus T3D could protect, even at very high concentrations of 1,000 PFU/well.

What amount of T3D is needed to protect against VSV?

Two 96-well clusters were plated at 2×10^4 cells/well. The same day of plating T3D was added to the plates in varying concentrations. Sixteen wells/cluster were infected with 100 μ l of either 0, 1000, or 10,000 PFU/well of T3D. Each plate was incubated for one hour in a 37°C incubator, after which 150 μ l of cMEM was added to each well. One day after plating and T3D infection, cells on both plates were challenged with VSV. Eight wells of each dilution of T3D received 100 μ l of 0 VSV while eight wells received 100 μ l of 0.1 VSV. The plates were incubated at 35°C for one hour and then 150 μ l of cMEM was added to each well. One day post-VSV infection, plate #1 was read by MTT assay for CPE. Two days post-VSV infection, plate #2 was read by MTT assay for CPE. VSV was cytopathogenic at both one and two days post-infection and there was no statistically significant difference in VSV CPE between 0, 1000, or 10,000 PFU/well of T3D at one day post-infection (Figure 8). At two days post-infection both 1000 and 10,000 PFU/well of T3D were significantly different from 0 PFU of T3D after challenged by 0.1 PFU VSV. Therefore, T3D appears to be protective at two days post-VSV infection.

Figure 8: Protection against VSV by higher doses of T3D



Conclusions: While T3D appears to be protective at 48 hours as hoped, more experiments are needed to understand the effects of T3D when no VSV is added. Asterisks denote statistically significant differences from 0 PFU/cell.

DISCUSSION

It is known that IFN- β is induced by reovirus T3D and not by reovirus T1L. A T1L mutant that induces IFN- β would be useful in helping to determine the morphological differences in the inclusion bodies of IFN-inducing reoviruses and non-IFN-inducing reoviruses. 0.1% NP-40, a detergent, can be used to lyse the envelope of VSV, thus inactivating the virus, while leaving reoviruses intact. Cell death caused by VSV can be monitored by MTT assay and the optimum time to do the assay is two days post-VSV infection. Plating at a density of 2×10^4 cells/well is ideal when attempting to get the maximum cell death from VSV. T3D appears to be protective at two days post-VSV infection but more research is needed to understand the effects of T3D on cells when no VSV is added. Future research should continue to find the optimum reovirus T3D dilution and VSV dilution to form the screening tool to find the reovirus T1L mutant that would induce detectable amounts of IFN- β .

ACKNOWLEDGEMENTS

I would like to thank Dr. Barbara Sherry for her excellent guidance and leadership. I would also like to thank Wrennie Edwards, Jennifer Zurney, Susan Irvin, and Lianna Li for their help in getting me acquainted with the lab.

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