VACCINIA VIRUS O1L VIRULENCE GENE AND PROTEIN LOCALIZATION

by

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Abstract

Smallpox killed an estimated 500 million people in the twentieth century alone. Although this fatal disease was eradicated from the world over thirty years ago, its potential use as a bioterrorism agent remains a concern. In addition, monkeypox continues to cause human outbreaks in Africa, and in the US in 2003. Vaccinia virus, the live virus vaccine for smallpox and monkeypox, is dangerous for immunocompromised individuals, and a safer vaccine is needed. The Roper lab studies how poxviruses cause disease in mammals and which genes contribute to virulence. The vaccinia virus O1L gene is highly conserved in poxviruses, and we have shown that it is required for full virulence in mice. When the O1L gene is removed from the wild type virus, the virus becomes attenuated, and immune responses are improved. Very little is known about this protein including its molecular weight, location within the cell and its function. We raised anti O1L peptide antibodies in rabbits and are using these to investigate the localization of the O1L protein using immunofluorescence techniques. In accordance with preliminary data from western blot analysis, we hypothesized that the O1L protein is located in the nucleus of the cell. Through immunofluorescence, the O1L protein was detected in the nucleus and cytoplasm of the cell. Identifying where the protein localizes will help us to form further hypotheses as to the mechanism of O1L immunosuppression and understand how poxviruses control the immune system. This research will increase our understanding of poxvirus pathogenesis and aid in the creation of new and safer vaccines.

Introduction

Poxviruses are large, brick-shaped, viruses with a size of approximately 200 nm in diameter and 300 nm in length (Moss, 2001). These double stranded DNA viruses replicate in the cytoplasm of cells and also infect a wide range of hosts including vertebrates and invertebrates. Variola virus, a type of poxvirus, was the causative agent of smallpox. This fatal disease was responsible for over 300 million deaths in the 20th century alone ("History of Smallpox"). The World Health Organization declared smallpox eradicated from nature in 1980. Vaccinia virus (VACV) was used as the live virus vaccine during the global campaign for smallpox vaccination (Rehm, 2010). However, vaccination of the public was discontinued due to the high occurrence of adverse reactions to the virus such as progressive vaccina, eczema vaccinatum, disseminated vaccinia, encephalitis, or even death (Lederman, 2009). Virulence factors of VACV make it contraindicated in a quarter of the United States population. Those who are immunodeficient, pregnant, have a history of eczema, or have heart disease are unable to be vaccinated with the live virus vaccine (Kemper, 2002). Today, military personnel, first responders, and laboratory workers researching poxviruses are the only persons required or even allowed to receive the vaccine.

Outbreaks of other poxviruses have recently emerged since the discontinuation of smallpox public vaccination, such as Cantagalo virus in South America (Damasco, 2000), buffalopox in India (Kolhapure, 1997), Tanapox in Europe (Dhar, 2004), monkeypox in Africa and the United States (Chen, 2005), and Molluscum Contagiosum virus in the United States (Konya, 1999). Of these outbreaks, monkeypox poses the most serious threat as a zoonotic virus that causes similar symptoms as smallpox (Rimoin, 2010). Monkeypox is prevalent in the

Democratic Republic of Congo and other African countries (Chen, 2005). However, the import of African rodents to the United States in 2003 caused the disease to spread in the western part of the nation (Chen, 2005). The rate of monkey pox infections has drastically increased since the ending of the smallpox vaccination program (Hammarlund, 2005). Those who were vaccinated with VACV during the smallpox vaccination campaign were at less risk of developing monkeypox due to cross-protective antiviral immunity (Hammarlund, 2005). A pressing need exists for a safer and more effective vaccine to protect all people against monkeypox and other possible emergent poxviruses. Understanding how poxviruses cause disease in humans will aid in the development of a safer vaccine.

Poxviruses can be used as potential vaccine platforms for other diseases, because they can accept large amounts of foreign DNA into their genomes. They are also valued for their abilities to cause strong humoral and T cell mediated immune responses and to infect a wide variety of host cells (Rocha, 2004). Modified Vaccina Ankara (MVA) is an attenuated and replication-defective poxvirus strain that is being researched to use as an alternative poxvirus vaccine. MVA has already been shown as a promising vaccine platform for numerous diseases. MVA is also safer than some other attenuated strains of VACV (Wilck, 2010). Although MVA has many advantages, MVA vaccine is not able to fully protect vaccine recipients against potent poxvirus strains (Golden, 2012). The Roper lab and others have shown that by selectively eliminating specific poxviral virulence genes, it is possible to diminish virulence while preserving the immunogenicity of the VACV vaccine (Rehm, 2011; Wang, 2012).

The VACV O1L gene is not well characterized and very little is known about the protein. O1L is highly conserved in Orthopoxviruses, but it is fragmented or not present in poxviruses

that do not infect or do not replicate well in mammals. It is predicted to encode an approximately 78 kD protein.

The Roper lab was interested in finding out if O1L was required for virulence of the wildtype reserve (WR) strain of VACV. An experiment was conducted in which mice were infected with the WR vaccinia virus, one of the O1L deletion mutant viruses (1A1 or 3A2), or mock infected with phosphate buffered saline (PBS) solution. The average weight of mice during the days post infection with vaccinia virus was measured as a measure of morbidity (Figure 1). The WR infected mice had significant weight loss, resulting in death compared to the deletion mutant infected mice, which became sick but soon recovered to a healthy status. The mock-infected mice continued to remain healthy. This experiment showed that vaccinia virus requires O1L for full virulence.

To elucidate the function of the O1L gene and protein, the Roper lab conducted various *in vitro* experiments. Monolayers of BS-C-1 cells were infected with wildtype WR virus or O1L deletion mutants, and the formation of plaques was analyzed. The outer diameters of the plaques formed by the deletion mutants and the plaques formed by WR were of similar size. This suggested that O1L is not required for viral replication or spread. One-step growth curves and multi-step growth curves were also conducted to further investigate viral replication and spread. The WR virus and the deletion mutants produced similar numbers of virus particles at various time points. This denotes that O1L does not affect viral spread. To determine if O1L alters the ability of the WR virus to destroy the host cell, a cell viability assay was conducted. Cells infected with WR or O1L deletion mutants significantly inhibited cellular metabolism compared to uninfected cells, but O1L did not affect cell metabolism. This suggests that the presence of

O1L does not affect cell viability. The experiments previously described demonstrate that O1L does not affect viral replication, spread, or cell viability.

Here we further investigate the O1L protein. Western blot analysis was conducted to examine the size and location of O1L in the cell (Figure 2). Preliminary data indicates O1L can be detected in the nuclear fraction of cells. In this study, we further investigated the location of O1L in the cell to help determine its function. Immunofluorescence microscopy was used as an alternative method to investigate the localization of O1L.

Materials and Methods

Cells and Virus

The HeLa S3 (Dynan repository CCL-2.2) cell line was a kind gift from Dr. Isabelle Lemasson (East Carolina University Brody School of Medicine). HeLa cells were used to conduct the immunofluorescence experiments. Vaccinia virus strain Western Reserve (WR) and the O1L deletion mutant viruses were propagated using BSC-1 cells in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS).

O1L Mutant Virus

Mutant viruses without the O1L gene were created by a series of recombinant PCR reactions. The enhanced green fluorescent protein (eGFP) was amplified and attached to two flanking sequences identical to the 5' and 3' sections on either side of the O1L gene. This construct was then transfected into cells where homologous recombination occurred and eGFP replaced the O1L gene. The recombinant mutants were isolated and purified. This resulted in independently selected O1L deletion mutants: 1A1 and 3A2.

Anti-O1L Rabbit SERA

Antibodies specific to O1L were generated by synthesizing peptides from O1L and injecting them into four rabbits. The rabbits were injected with the peptides in adjuvant multiple times to develop and maintain the adaptive immune response. This allowed the rabbits to form antibodies to the O1L protein. The rabbits were bled, the blood was centrifuged, and the sera containing the antibodies specific to O1L were collected.

Immunofluorescence Microscopy

HeLa cells were grown on coverslips in a 24 well plate for 48 hours. The cell monolayers were infected with either WR or the O1L deletion mutants, 1A1 and 3A2, at a multiplicity of infection of 10 virus particles per cell in complete MEM, or were incubated with media alone for 3 hours. Following infection, the cells were fixed and permeabilized with 0.5 mL of a 1:1 solution of methanol and acetone for 30 seconds. The cells were washed twice for 3 minutes each in 1 mL of PBS, and were blocked in 0.5 mL of PBS-FBS (2%) solution for 30 minutes. To detect the O1 protein, rabbit anti-O1L sera were diluted (1:5000, 1:10000, and 1:50000) in PBS-FBS (2%) and incubated for 1 hour. Cells were washed twice for 3 minutes each with 1 mL PBS. Cells were stained with secondary antibody Anti-Rabbit IgG (Fc) AP Conjugate (1:500 in 0.5 mL PBS-FBS (2%) solution) for 1 hour. Cells were washed three times for 3 minutes each with 1 mL PBS and were rinsed with 1 drop distilled water for 10 seconds. The coverslips were mounted onto slides using Vectashield (Vector Laboratories, Inc.), a mounting medium for fluorescence, and the samples were viewed on a Nikon Eclipse E600 fluorescence microscope.

Results and Discussion

To gain insight into the possible functions of O1L, its localization was assessed. To determine the distribution of O1L, uninfected, wildtype-infected, and deletion mutant (ΔO1L) infected cells were fixed, permeabilized, and stained with rabbit anti-O1L sera and fluorescence-labeled secondary antibody. Figure 3 is preliminary data that demonstrates positive and negative staining, or cells infected with the wildtype virus versus cells infected with the deletion mutants. O1L was detected in the cytoplasm of the cell through punctate staining (Figure 3, top row, left). However, this is not found in the deletion-mutant infected cells (on the right). Nuclear staining was also apparent in the wildtype-infected cells (Figure 3, bottom row), but not present in the deletion mutant infected cells (right). The wildtype-infected cells also appear brighter than the ΔO1L infected cells. This staining pattern suggests that O1L possibly localizes in the cytoplasm and nucleus of the cell.

Determining the localization of O1L is important in understanding how the protein affects cells. For example, if it is located in the nucleus, O1L could affect gene expression. If O1L is located in the cytoplasm, its function could be involved with protein trafficking or intracellular signaling. Since the results collected are just preliminary data, immunofluorescence experiments must be repeated in order to confirm the initial findings. Identifying how the O1L protein functions will contribute to a better understanding of poxviruses pathogenesis and will aid in clinical applications, such as making safer vaccines.

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Figures and Legends

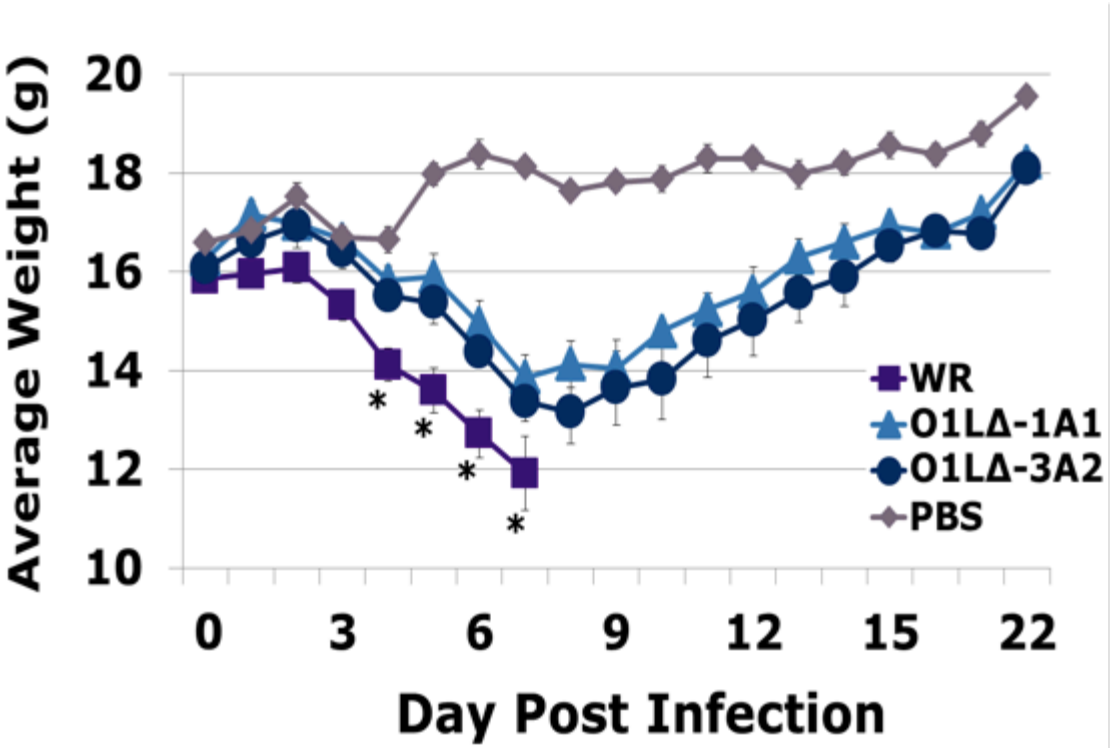

Figure 1. O1L is required for full virulence: Mice were infected intranasally with 5 x 10^4 virus particles (PFU) of WR or O1LΔ, or mock-infected with PBS. The mice were weighed various days post infection. Mouse weights are presented as mean of experimental groups ± the standard error of the mean (SEM, top). Values (*) of $p \leq 0.05$ were considered significant.

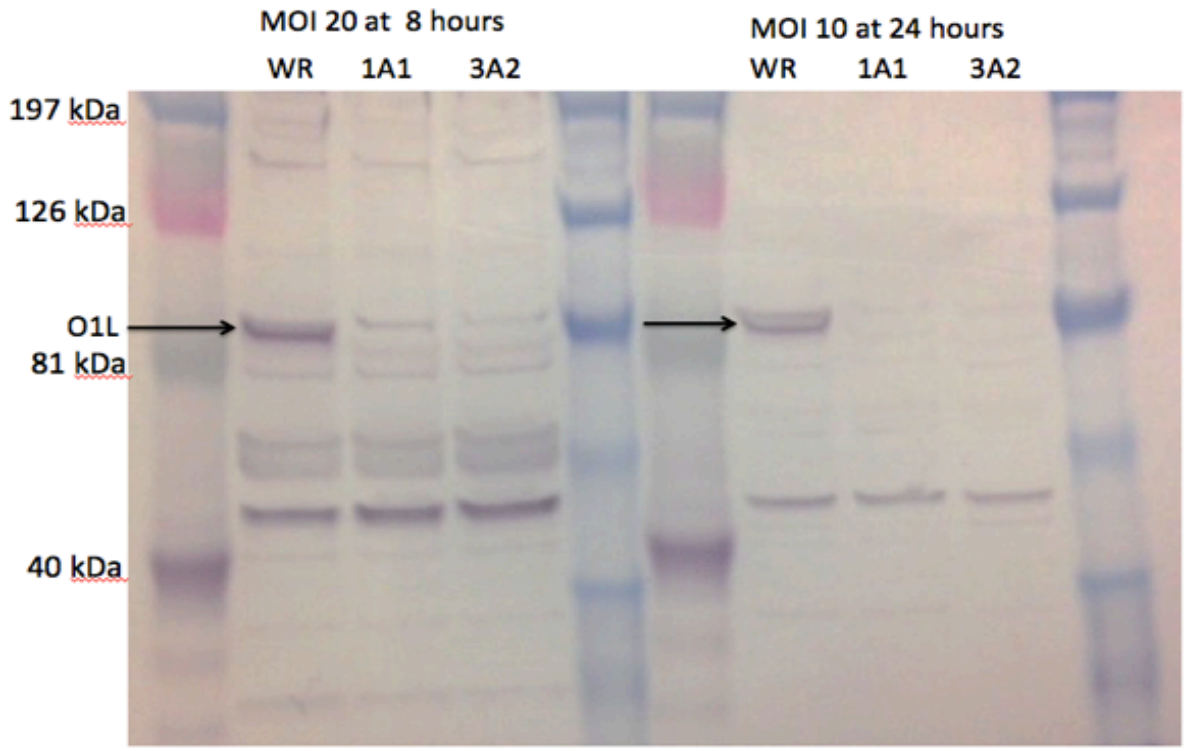

Figure 2. O1L is associated with the nucleus: HeLa cells were infected at two different MOIs and harvested at different time points. Cells were lysed and the nuclear fraction was retained and loaded into the SDS page. The data show an O1L band present in the wildtype virus infected cells and not in the deletion mutant virus infected cells.

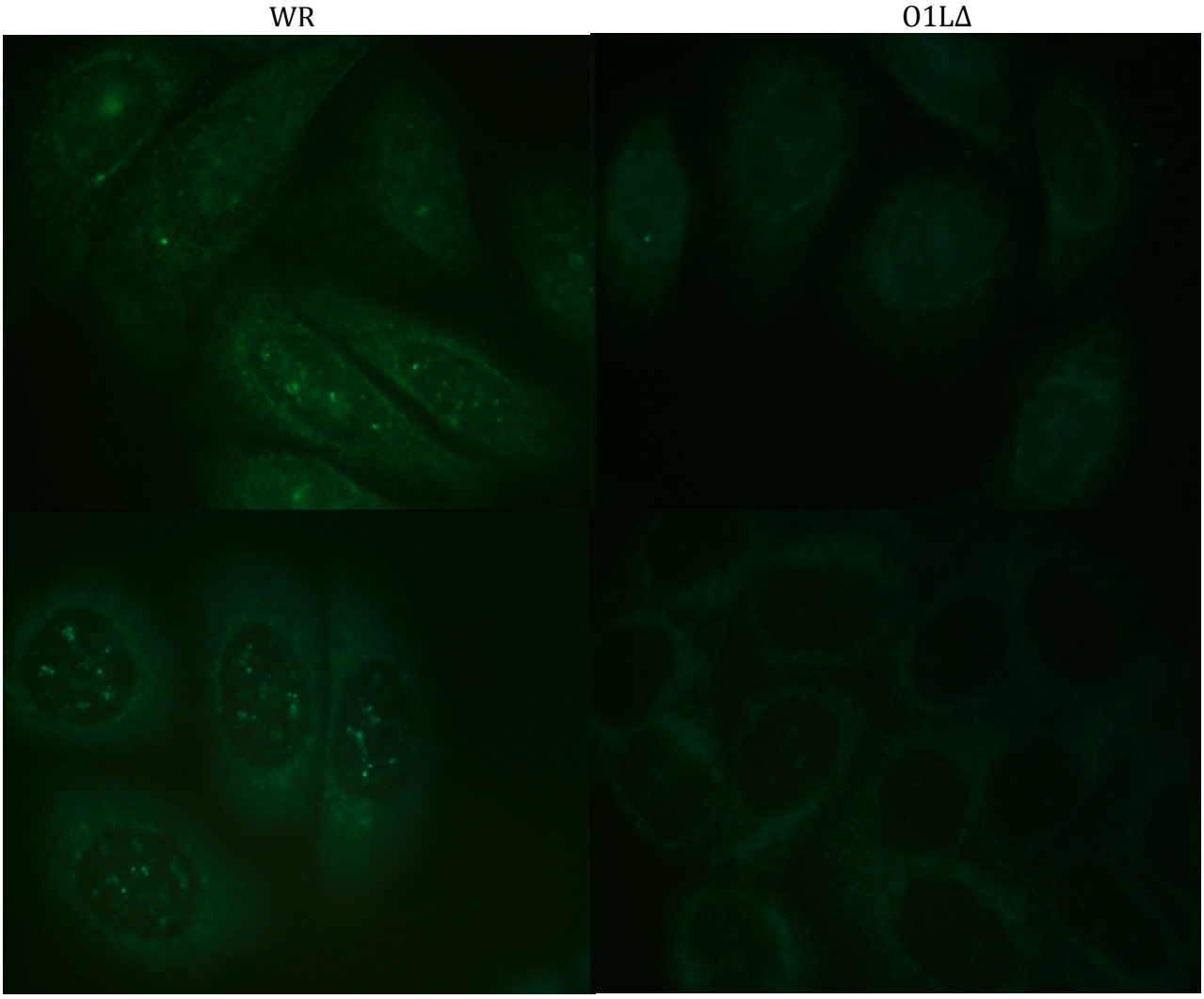

Figure 3. O1L Localization: HeLa cells were infected with wildtype WR or ΔO1L at an MOI of 10 for 3 hours and then fixed, permeabilized, labeled with rabbit anti O1L sera, and stained with secondary antibody Anti-Rabbit IgG (Fc) AP Conjugate. O1L present in the cytoplasm of the WR-infected cells (top row) and in the nucleus of the WR-infected cells (bottom row).

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