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THE CAUSE OF AXL PROTEIN IN THE ENTRY OF LASSA VIRUS INTO PERMISSIVE CELLS

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Abstract— The purpose of this study was to determine the role of Axl receptor in LV entry, and to understand the effect of anti-Axl antibodies on LV infection. Polyclonal antibodies against the human Axl ectodomain were also shown to play a role in inhibiting Zaire ebolavirus entry into cells (Shimojima et al. 2006). These same antibodies (Raud D Systems) were used in this study to address LVSV binding to Axl (Figures 5-7). Within the polyclonal antibody mix, a variety of different Axl epitopes will be recognized. Therefore, it is possible that different areas of the Axl ectodomain are targeted by the antibodies. Our studies could be enhanced by using monoclonal antibodies that target known epitopes on the Axl ectodomain. Determining which monoclonal antibodies inhibit LV infection and which ones do not would help us determine the portion of Axl that LV is binding to. Antibodies that do not inhibit LV binding to Axl could also be excluded as potential therapy agents. Antibodies also have successfully been used for therapy during other viral infections, including Ebola virus infections.

Keywords: hemorrhagic fever, Lassa fever, Axl protein

1. Introduction

Lassa virus (LV) is a member of the Arenaviridae family, contains a negative- strand RNA genome (Günther and Lenz 2004), and is transmitted via rodents (Garry 2014). LV causes Lassa fever disease which is an acute viral hemorrhagic fever (VHF), and it causes the death of thousands of humans annually (Richmond and Baglole 2003). The estimated number of Lassa cases in endemic areas is 300,000 to 500,000 with 5000 to 10000 deaths annually (Ajayi et al. 2014). This virus is endemic in a large area of Africa, but it also has been reported in neighboring countries such as Mali, and Burkina Faso (Macher and Wolfe 2006). Additionally, there are more than 20 imported cases which have been reported in Japan, Europe, and North America, with the mortality rate of imported cases being more than that of nonimported cases (Günther and Lenz 2004).

Infection of LV occurs through contact with an infected animal's body fluids or excreta, and can also occur through the inhalation of aerosols generated by those infected animals. In addition, the transmission of LV to humans in endemic areas can occur through the consumption of infected rodents' meat (Yun and Walker 2012). The incubation period for this virus is from 6 to 21 days, and those infected experience gradual symptoms. Symptoms start with fever and weakness, and are followedby headache, sore throat, muscle pain, chest pain, nausea, vomiting, diarrhea, cough, and abdominal pain (Dzotsi et al. 2012).

The host for this virus is rodents, namely Mastomys natalensis (Monath et al.1974), M. erythroleucus and M. huberti (McCormick et al. 1987). The number of peopleinfected with this virus increases per year, and a vaccine against this virus has not been developed. Therefore, it is important to understand the basic biology associated with this virus.

Axl-Dependent Entry of Lassa Virus

The Lassa viral membrane has an envelope glycoprotein embedded in it, that it uses to bind to a host cell receptor to gain entry into a host cell. Because of its ability to infect rodent cells and human cells, it is assumed that Lassa virus uses multiple receptors for entry. Based on studies with other

enveloped viruses, these receptors may include Axl, Tyro3, DC-SIGN, and LSECtin (Shimojima et al. 2011). It has been shown that for other viruses, namely the filoviruses, enhancement of Axl-dependent entry into cells requires the ectodomain and cytoplasmic tail of Axl.

Axl is one of three members of the tyrosine kinases receptor family, the Tam family of receptors, and contains two domains. These domains are the conserved kinase domain and an adhesion domain (Linger et al. 2008), which Axl uses to bind to the molecule Gas6. One end of Gas6 binds to phospholipids, while the other binds to the Tam family member. The interaction between Axl protein and Gas6 cause Axl signaling, which affects the growth and the survival of different types of cells (Hafizi and Dahlbäck 2006).

Direct vs. Indirect Interactions between Axl-Gas6 and Lassa Virus Glycoprotein(LV-GP)

The nature of the interaction between the Axl receptor and the Lassa virus glycoprotein (LV-GP) is important for the virus to enter the cell. There are two ways for the virus to bind with the host cell, and one of them is a direct interaction in which thevirus proteins bind directly to host cells receptors (Norkin 2010). Another way is through an indirect interaction, and occurs through bridging of the virus to target cells by Gas6, a soluble protein (Morizono et al. 2011). Morizono et al. (2011) found that Gas6 binds to phosphatidylserine (PS) on the virus and Axl receptors simultaneously, and this leads to the enhancement of uptake of enveloped viruses. The interaction between LV and cells receptor has not yet been determined.

The aim of this research was to investigate the role of Axl protein in the entry of Lassa virus into permissive cells, and determine the effect of the presence of different concentrations of anti-Axl antibody in preventing virus infection of permissive cells.

MATERIALS AND METHODS

VSV Pseudovirion Generation

Vesicular stomatitis virus (VSV) pseudovirions that were generated by replacing the G glycoprotein protein gene with green fluorescent protein gene (GFP) were used as a safe means to study LV-entry events (Takada et al.1997). Lassa virus VSV pseudovirions (LVSV) were prepared by adding VSV (helper virus) to Lassa glycoprotein expressing 293T culture cells. Helper virus will bud and thus be studded with Lassa glycoprotein (Figure 1).

Plasmid Isolation

5ml cultures of pLV-GPC were grown with 50 mg/ul Ampicillin in Nutrient Broth overnight at 37°C, 200 rpm. Bacteria was pelleted by separating 1.5ml into 10 different 1.5 ml Eppendorf tubes, and centrifuged @13,000 for five minutes. Supernatant were discarded, and tubes were labeled and preserved at -20°C. Plasmid were extracted using a High-Speed Plasmid Mini Kit protocol from IBI Scientific (Peosta, Iowa).

PEI Transfection

293T cells were removed from the flask one day before transfection and plated in 10cm dishes in fresh media (DMEM 1X, 2.5% Fetal Bovine Serum, 1% Pen Strep Glutamine 100X). In a sterile tube, plasmid DNA was diluted in 150mM NaCl (10ug DNA per plate), and in a second sterile tube, poly ethyleneimine (PEI) (1.5ug per plate) was diluted in 150mM NaCl. Both tubes were incubated for 15minutes at roomtemperature. DNA and PEI tube were mixed and vortexed for 15 second. After vortexing, tube was incubated for 7 minutes at room temperature, and then added dropwise to cells. One day after transfection, helper virus was added to each dish, and it was incubated overnight. Supernatants were then collected and stored at -80°C. Fresh media was added to cells, and cells were incubated overnight again. Supernatant was collected again and stored at -80°C. Supernatants were centrifuged at 12,000–16,000 ×g for 10 minutes, and transferred to new tubes. Supernatants were centrifuged for 24hour at 7,000 rpm, and discarded carefully. The remaining fractions with virus were resuspended with a micropipettor and aliquoted in 1.5 Eppendorf tubes.

Cell Culturing

Cells from green monkey kidneys were cultured by removing the old media from the flask, and adding 3 ml of trypsin. Trypsin releases cells by cleaving their adhesion proteins from the surface of the vessel (Rous and Johns 1916). Cells were incubated for 5 minutes at 37°C, and after they lifted, 2.5 ml from the flask was transferred to a 15ml conical vial. 14ml of fresh media (DMEM 1X, 2.5% Fetal Bovine

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Serum, 1% Pen Strep Glutamine 100X) was added to the flask and the flask was incubated for future use. In the conical vial, 5ml of fresh media was added, and 10ul of cells were counted on a hemocytometer (a chamber used to know the cells density in the suspensions) using the microscope.

Virus Titering

To determine the volume of the virus needed in each experiment, measurement of the infectivity of LV pseudovirions (LVSV) was determined. LV pseudovirions were used due to its safety. Old media in cultures was replaced by 250ul of fresh media for each well. Different volumes of the LVSV were used. The first two rows of the 48-well plate were the control, which contained cells with fresh media without the virus. Cells in the remaining wells were infected with different volumes of the virus. Infected cells were incubated for 2 days at 37°C with 5% CO_2. After the incubation period, cells were fixed by adding 300ul of 1:10 dilution of 37% formaldehyde in Hank's buffered salt solution (HBSS) for 10-15 minutes per well. After fixation, they were washed 3 times with Tris-buffered saline (TBS).

The Role of Axl in LV Entry into Permissive Cells

To address the role of Axl in LVSV entry into permissive cells, old media was replaced by 150 ul of fresh media for each well in a 48-well plate. The first two columns of the 48-well plate were the control wells, which contained cells and growth medium only. In the second two columns, 1ug of anti-Axl antibody was added to cells with growth medium. Cells were incubated at 4 °C for 1 hour. After incubation, media was replaced by 250 ul of fresh media. Cells in all wells were infected with 2.5ul of the stock virus, and incubated for four hours at 37°C with 5% CO_2. After the incubation period,oldmedia and virus were removed and replaced by 300ul of fresh media. Cells were incubated for two days at 37°C with 5% CO_2.

The Impact of Different Concentrations of Axl-Specific Antibody in LV-GPDependant Entry

To study how Axl-specific antibodies impact the entry of LVSV into cells, different concentrations of Axl antibody were tested. Cells with growth media only served as negative control wells. Cells with different concentrations of IgG were added as antibody control wells; Axl-specific antibodies ranging from 0.25ug to 1.5ug were tested. Cells were incubated at 4 °C for 1 hour, and after that media in all walls were replaced by fresh media. Cells in all wells were infected by the virus, and incubated for 4 hours at 37°C. After incubation period, virus and old media were replaced by fresh media, and cells were incubated for 2 days at37°C.

the infection rates were determined by using the ImageJ program that measured the florescence of the virus as assessed by the Evos FL imaging system (Figure 2). The intensity of the florescence in the control (non-infected cells) was normalized and set to 100% for each separate experiment. The intensity of the fluorescence for infected cells was then divided by the control and calculated as percentage of the control fluorescence.A student's *t*-test was done comparing the control, (infected cells) and the study (infected cells in the presence of anti-Axl antibody).

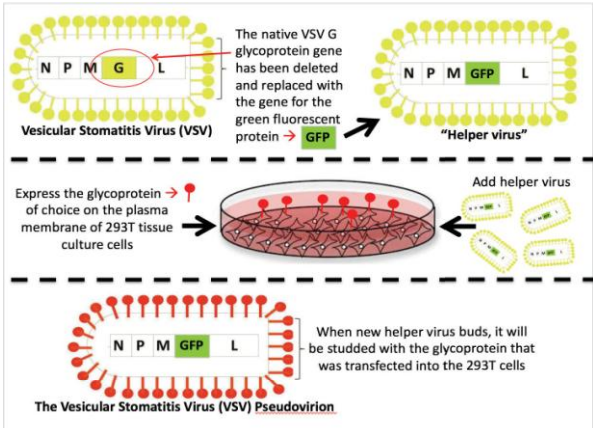

Figure 1. VSV pseudovirion generation. Helper virus contains GFP instead of VSV glycoprotein is added to expressed LV glycoprotein on 293T cells plasma membrane. Viral budding enables helper virus to stud with LV glycoprotein transfected into 293T cells.

RESULTS

Virus Titering

Viral titering was done to determine the best volume(s) of the virus to use to infect our cells. Vero cells were used due to their high expression of Axl on their surface (Brindley et al. 2011) and their susceptibility to LVSV. Cells were cultured successfully, and they were infected by different volumes of the virus. Vero cells without infection of LVSV served as a

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control in this study. Results revealed that 0.5ul of the virus showed a lower infectivity titer comparing to 2.5ul of the virus, indicating that the fluorescence intensity increased as the virus volume increased (Figure 3).

The role of Axl on Lassa virus entry into permissive cells

Vero cells express Axl on their plasma membrane. Therefore, Axl antibody was used to study the infection rate of the virus in the presence of Axl antibody. Cultured cells were infected by the concentrated/ non-concentrated virus in the presence of Axl antibody. Infected Vero cells were used as a control, and the fluorescence of the virus was measured. The morphology of the cells did not change when they were infected by LVSV (Figure 4). For the measurement of the viral fluorescence, the same calculation method that was performed in viral titering was used in this experiment. Fluorescence intensity of cells infected in the presence of anti-Axl antibody was around 92% of the control (Figure 5). By comparing results obtained in this study with the control, I found that LVSV entry into Vero cells was significantly reduced in the presence of 1.0ug of anti-Axl antibody ($P > 0.05$) (Figure 5).

A different stock of the virus was used to investigate the role of Axl on the entry of the virus into cells. Fluorescence intensity of infected cells in the presence of anti-Axl antibody was around 93%, and in the presence of IgG antibody which is a control antibody was around 97%. By comparing control fluorescence values with those of infected cells in the presence of anti-Axl antibody, significant results were obtained when we use non-concentrated LV in the presence of 1.0ug anti-Axl (Figure 6). Infected cells in the presence of IgG antibody do not exhibit a significant reduction in the viral infectivity ($p>0.05$), and this means IgG serves as a control (Figure 6).

Effect of different concentrations of Axl antibody in inhibit LV entry into cells

Because 1.0ug of anti-Axl antibodies inhibited LVSV entry into Vero cells, the effect of the presence of different concentrations of anti-Axl antibody on LVSV infection was determined. Cells were infected by LVSV in the presence of different concentrations of Axl antibody (0ug-1.5ug). Infected Vero cells without antibody were used as a control. Results were obtained by measuring the fluorescence intensity of the virus, and it revealed that the infection of the virus was reduced as the Axl antibody increased (Figure 7).

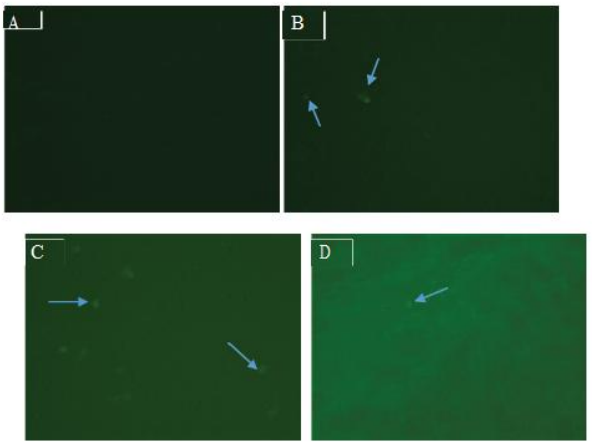

Figure 2. GFP Expression of Vero cells upon infection with different amounts of LVSV. (A) Vero cells without infection of LVSV. The infection of Vero cells with (B) 0.5ul, (C)1ul, (D)2.5ul of LVSV.

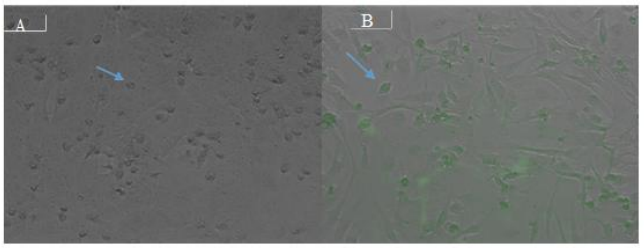

Figure 3 Morphology of LVSV infected cells. (A) Cells that have not been infected by LVSV. (B) Cells that have been infected by LVSV. These pictures indicated that the morphology of the cells does not changed when they infected by LVSV.

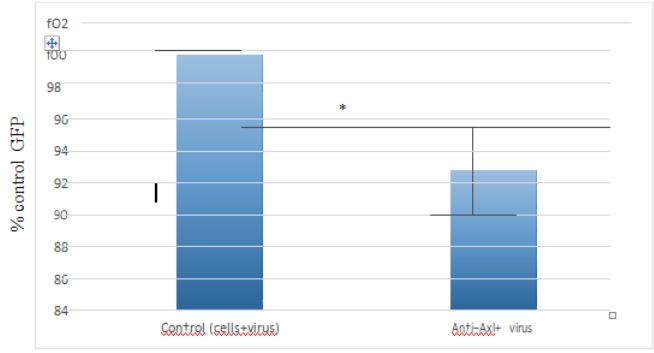

Figure 4. AXL Role in LVSV Entry into Permissive Cells. The role of Axl on LVSV entry into cells was examined

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by infecting cells in the presence of anti-Axl antibody (1.0ug). Cells infected with LVSV were used as a control. Cells were significantly reduced in the presence of anti-Axl antibody. A student's *t*-test was done to analyze the data. Data show the averages, and standard errors of five separate experiments performed in triplicate. *, P<0.05

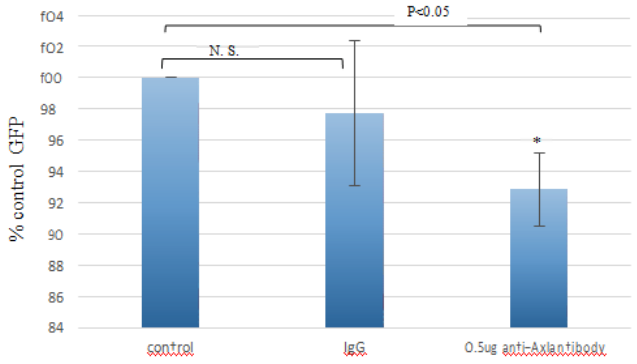
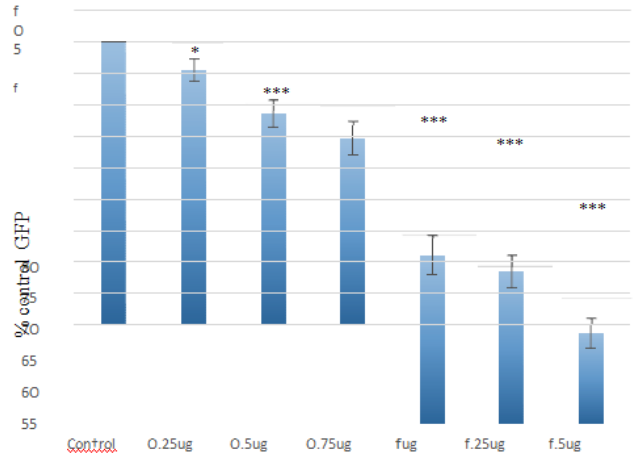


Figure-5 Axl-specific antibodies role in the entry of the non-concentrated LVSV into cells. A different stock of LVSV was used to examine the role of Axl on viral entry into cells. Cells infected with LVSV in the absence of anti-Axl antibody were used as a control. Cells were infected in the presence of IgG, a control antibody. Cells were also infected with the non-concentrated LVSV in the presence of anti-Axl antibody(0.5ug). A student's *t*-test was done to analyze the data. Data show the averages, and the standard errors of four separate experiments performed in triplicate. *, P<0.05.



Axl-Antibody

Figure 6. The role of different concentrations of Axl-specific antibody on the entry of LVSV into cells. Infected cells in the absence of anti-Axl antibody were used as a control. Data show the averages of one experiment performed in triplicate. A student's *t*- test was done to analyze the data. Data show the averages, and the standard errors of four separate experiments performed in triplicate. *, P≤ 0.05. ***, P≤ 0.001.

DISCUSSION

This research investigates the role of Axl in the entry of LV into permissive cells, and the effect of using different concentrations of anti-Axl antbody in inhibiting the entry of the virus. Vero cells are known to express Axl on their plasma membrane (Brindley etal. 2011). Axl was previously shown to play an important role in the enhancement of other virus' entry into cells, including Ebolaviruses (Brindley et al. 2011). Brindley et al. (2011) identified that the loss of Axl by RNA interference limits Ebolavirus' ability to enter cells. In a similar manner, I proposed that LV entry is enhanced by the Axl receptor, and a lack of Axl will decrease the ability of LV to enter permissive cells. By using antibodies that bind to and block access to Axl, I provided evidence that the binding of LVSV pseudovirions is inhibited by the blocking of viral access to Axl. Data in figure 5,6 and 7 support this mode of entry by LV.

The TAM family of receptors which includes Axl, were shown to play a role in the internalization of viruses indirectly via Gas6 which serves as a phosphatidylserine (PS) binding bridge (Morizono et al. 2011). Results of a previous study revealed that indirect interaction occurs between Zika virus and Axl receptors, and Axl depends on Gas6 to bridge Zika virus to cells (Meertens et al. 2017). Meertens et al. (2017) found that Gas6 binds to PS in the Zika virus envelope, and this leads to bridging of the virus to the Axl receptor. In a similar manner, I proposed that the binding of LV to Axl does not occur by an indirect interaction between LV and Axl. As Gas6 was present in all of our assays in the fetal bovine serum, (Figures 5-7), the binding of Axl to LV appears to occur directly, as the anti-Axl antibodies reduce LV binding. Furthermore, figure 7 shows that this blocking occurs in an antibody dependent manner, as increasing amounts of anti-Axlantibody decrease viral binding in a dose dependent manner. Studies without serum (and therefore Gas6) could help elucidate whether LV binds directly or indirectly to Axl.Additionally, an Enzyme-linked immunosorbent assay could help determine physical

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interactions between Axl and LV, and whether Gas6 is necessary for this interaction.

The combination of Axl and Human T-cell Immunoglobulin and Mucin-domain containing proteins (TIM proteins) were observed to play a role in enhancing viral entry by each binding PS in the viral envelope (Jemielity et al. 2013). TIM and TAM transmembrane receptors have been shown to mediate the infection of Dengue virus, and TIM proteins were shown to increase the entry of a number of other viruses such as Ebola, and West Nile viruses (Jemielity et al. 2013). Filoviruses such as Ebolavirus and Marburgvirus enter into host cells using a number of viral envelope proteins such as Axl, Dtk, Mer, and lectins (Shimojima et al. 2006). In addition to the use of Axl by LV in internalization into cells, I proposed that LV may also use several receptors for binding as we were not able to completely inhibit LV entry into cells using anti-Axl antibodies (Figure 5-7). It should be determined if other PS binding receptors also bind to LV and permit its entry in cells.

A previous study indicated that the loss of Axl reduced the ability of cells to undergo macropinocytosis (Hunt et al. 2011). Macropinocytosis is a form of endocytosis, whereby a cell takes up fluid in bulk. The loss of the Axl receptor from the cells surface rendered inhibitors of macropinocytosis ineffective at blocking the virus entry into the cells (Hunt et al. 2011). Additionally, when phospholipase C, a signaling molecule, was inhibited, it reduced Ebola virus entry into the cells (Hunt et al. 2011). These data indicate that Axl activation subsequently leads to activation of phospholipase C that in-turn induces macropinocytosis which then increases the entry of Ebola virus into cells. Our data in figures 5-7 indicate binding of Axl to LV, but we do not know if this binding leads to Axl signaling. Similar experiments may determine if Axl also facilitates macropinocytosis of LV.

The main objective of this study was to determine the role of Axl receptor in LV entry, and to understand the effect of anti-Axl antibodies on LV infection. Polyclonal antibodies against the human Axl ectodomain were also shown to play a role in inhibiting *Zaire ebolavirus* entry into cells (Shimojima et al. 2006). These same antibodies (Raud D Systems) were used in this study to address LVSV binding to Axl (Figures 5-7). Within the polyclonal antibody mix, a variety of different Axl epitopes will be recognized.Therefore, it is possible that different areas of the Axl ectodomain are targeted by the antibodies. Our studies could be enhanced by using monoclonal antibodies that target known epitopes on the Axl ectodomain. Determining which monoclonal antibodies inhibit LV infection and which ones do not would help us determine the portion of Axl that LV is binding to. Antibodies that do not inhibit LV binding to Axl could also be excluded as potential therapy agents. Antibodies also have successfully been used for therapy during other viral infections, including Ebola virus infections (Casadevall and Pirofski, 2015).

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