DISSERTATION

CHARACTERIZATION OF BIG BROWN BAT (*EPTESICUS FUSCUS*) RABIES VIRUS IN A MOUSE MODEL

Submitted by

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ABSTRACT

CHARACTERIZATION OF BIG BROWN BAT (*EPTESICUS FUSCUS*) RABIES VIRUS IN A MOUSE MODEL

A majority of human rabies cases in the United States are either imported from countries where dog rabies is endemic or classified as cryptic human cases, where a route of exposure is not known. Notably, essentially all rabies virus (RV) variants associated with cryptic cases of human rabies are maintained in bats. Understanding how RV is maintained in populations of bats and characterizing the diversity of bat RV is thus a high priority problem for public health. Among the knowledge gaps related to bat rabies are understanding the variation in virulence within the population of a single variant and explaining the observation that a substantial number of healthy wild bats have neutralizing antibodies to RV, but no apparent clinical illness. The work described here was designed to address both of those issues.

Nine RV isolates were isolated from big brown bats in Colorado and low-passage stocks of each were prepared. These isolates were evaluated for virulence, immunogenicity and salivary gland dissemination to investigate whether there were major differences in these characteristics within this virus population. Inoculated mice were maintained for 12 weeks after virus inoculation to assess mortality and were bled regularly to evaluate their humoral immune responses. Salivary glands from mice that developed clinical rabies were evaluated for virus replication as an indication for

potential for further transmission. The dose of RV inoculated had a greater influence on the incubation period and mortality than the individual RV isolate. There was no difference in the humoral immune response in mice between those that were protected and those that succumbed to infection. The only salivary glands that were positive for RV replication were observed from mice in the high dose inoculation groups. Collectively, the results of this experiment indicated that there was low diversity in biologic behavior within the sample of *Epitesicus fuscus* viruses tested.

The humoral immune response of mice to a big brown bat RV variant was explored to address the hypothesis that dose, route or frequency of inoculation may explain the prevalence of neutralizing rabies antibody seen in wild bat populations. Mice were inoculated via intramuscular, intradermal and intranasal routes, with two different low doses of virus and two inoculation schedules. The highest frequency of seroconversion was seen in mice inoculated intramuscularly with the higher of the two doses of RV. Mice that were inoculated intranasally experienced the highest mortality. Mice were rechallenged 3 months following the initial challenge with a high dose of virus intramuscularly to determine if the neutralizing rabies antibodies were protective and if priming of the immune system to RV had occurred in those that failed to seroconvert. The results of this experiment indicate that inoculation of low doses of virus by any of several routes can elicit a detectable humoral immune response without development of disease, which supports the hypothesis that exposure of wild bats to low doses of RV results in seroconversion without clinical disease.

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TABLE OF CONTENTS

CHAPTER ONE

LITERATURE REVIEW

History of Rabies

Rabies is one of the oldest reported diseases throughout history. The word rabies is derived from the Latin word *rabere* meaning "to rage or rave" (Wilkinson 2002) and can also be traced back to 3000 BC to the Sanskirt "*rabhas*" meaning "to do violence" (Rupprecht et al. 1996). Written records from Mesopotamia during the 23rd century BC to Chinese scholars in 500 BC all give accounts on the seriousness of rabies (Lyles and Rupprecht 2007, Wilkinson 2002). There are records from as early as the first century of various treatments that were tried to cure or protect someone from rabies (Lyles and Rupprecht 2007). In 1546, an Italian physician, Fracastoro, wrote "the Incurable Wound" which included a description of the incubation period from the time someone had been bitten by a rabid dog to the time that clinical symptoms and death occurred. The 1800s brought the first scientific proof that saliva from an individual with rabies could transmit the disease, and in 1804 Louis Pasteur carried out the first successful vaccination of a boy who had been bitten by a rabid dog (Lyles and Rupprecht 2007, Wilkinson 2002).

Rabies remains a major public health issue today. The World Health Organization (WHO) estimates that more than 55,000 people die every year from rabies virus (RV) infection, with 95% of these individuals living in Asia and Africa. RV can be found in more than 150 countries and on all continents excluding Antarctica. Every year,

roughly 15 million people worldwide receive post-exposure prophylaxis (PEP) treatment, thus preventing an estimated 327,000 rabies deaths (WHO 2010a). Transmission of RV most frequently results from a bite from an infected dog, although in the United States (US) and Canada, RV exposures are generally from terrestrial wildlife reservoirs and bats (Blanton et al. 2010, WHO 2010a).

Vaccination of domestic dogs began in the 1920s and changed the demographic of rabies in the US and Canada. There has been a marked decrease in dog and human rabies cases. In the 1960s, for the first time in history, rabies was found more often in wild animals than in domestic dogs in the US (Rupprecht et al. 1995). Aggressive oral RV vaccination programs for domestic canine and coyote RV eliminated those variants from the US in 2004 (Velasco-Willa et al. 2008). Over the last four years 92-93% of all reported US rabies cases occurred in wildlife (Blanton et al. 2007, Blanton et al. 2008, Blanton et al. 2009, Blanton et al. 2010).

Rabies Virus Variants

Wildlife rabies in the US is a disease induced by terrestrial and bat variants of RV. Different RV variants were identified, first by host species, then by monoclonal antibody panels and finally by the conserved nature of the nucleocapsid gene sequence (Constantine et al. 1968a, Nadin-Davis et al. 1999, Rupprecht et al. 1987). In the US, terrestrial rabies results in the infection of raccoons, skunks, and foxes with their individual RV variants that have their own epidemiological regions. Raccoons are the most abundant rabies-positive wild animal, followed by bats, skunks, and foxes, although bats are more frequently submitted for testing. In 2009, 27,915 bats were submitted for

rabies testing in the US (49 states and Puerto Rico) and 1,625 (~5.8%) were found positive for RV, compared to 2,327 (~11.7%) raccoons that tested positive (Blanton et al. 2010). Insectivorous bats are distributed across the US and their RV variants are found throughout 49 states; Hawaii remains rabies free (Blanton et al. 2010).

With the elimination of the canine RV variant from the US, the number of human rabies cases has drastically decreased, but a few individuals still succumb to rabies. Some of these cases are from a known exposure, but the majority of patients now have no clear history of being bitten by a rabid animal (Messenger et al. 2002, Rupprecht et al. 1996). These cases have been labeled as cryptic rabies cases and are frequently found to be caused by an insectivorous bat RV variant. In the US from 1958 to 2000 there were 35 indigenous human cases of rabies, 26 of these cases were classified as cryptic cases with 19 of them being caused by a specific insectivorous bat RV variant associated with the silver-haired bat (*Lasionycteris noctivagans*) and the tri-colored bat (*Pipistrellus subflavus*) (Messenger et al. 2002). From 2000 to 2009 there were 31 human cases in the US and the majority of these were caused by RV variants of bat origin, with some cases having no known route of exposure (Blanton et al. 2010).

Case studies indicate that people in the US who become infected with RV and die are more likely to have contracted the silver-haired bat or tri-colored bat RV variants (Smith 1996). This led to the question: Is there something unique about the silver-haired or tri-colored bat RV variant that permits easier transmission? The silver-haired bat RV (SHBRV) variant has been compared to both the coyote (COSRV) and dog (DRV) street RV in cell culture and mouse models. All three variants had similar virulence and pathogenicity in mice. However, the SHBRV can replicate to a higher concentration in

cell culture at a lower incubation temperature than the COSRV and DRV variants (Dietzschold et al. 2000, Morimoto et al. 1996). These findings led to the speculation that a lower dose of SHBRV is needed to induce clinical rabies due to its ability to replicate to a higher concentration at the site of exposure before entering the central nervous system (Messenger et al. 2002). The amount of data comparing the SHBRV to other bat RV variants is severely limited and no research has evaluated variation within this RV variant population.

Before RV variant typing was commonly done, an observation was made that there were differences in the virulence of rabies among different bat species. Leaf-nosed, Silver-haired, Hoary and *Eptesicus fuscus* bat RV was isolated from each species, were compared in inoculation experiments in carnivores and rodents (discussed later in this review) and found the Leaf-nosed bat RV variant was the most virulent with respect to causing rabies in carnivores (Constantine et al. 1968a).

Monoclonal antibodies to the RV nucleocapsid protein, glycoprotein and phosphoprotein create unique binding patterns that were first used to differentiate between terrestrial and non-terrestrial variants of RV (Rupprecht et al. 1987). Further development of monoclonal RV-specific antibodies for RV variant identification has facilitated their use as an important surveillance tool (Rohde et al. 2004). Determination of where a RV infection originated helps in developing control measures to prevent further spread of disease and to better understand how disease cycles change in an urban or sylvatic setting (De Mattos et al. 1996, Smith 1988).

Being able to identify and map where different RV variants occur has allowed scientists to look at and understand how different RV variants behave in nature. The

transmission of a virus from a reservoir host to a non-reservoir host with little or no sustained transmission in the new host is considered a spillover event (Lloyd-Smith et al. 2009). In 1999, a study focused on spillover was conducted to characterize RV variants that were found in rabid dogs and cats throughout the US, and determined the majority were infected with the local terrestrial RV variant that was dominant for that particular geographic area (McQuiston et al. 2001). Phylogenetic surveys carried out to assess if canine RV is in the US have revealed that spillover events from wildlife reservoirs into domestic dogs regularly occur (Velasco-Villa et al. 2008). Similarly, monoclonal antibody typing was used in studying RV variants in Mexico and in documenting the shift from dog to vampire bat RV variant becoming more prominent (Velasco-Villa et al. 2006).

Although RV variant typing is a useful tool, a majority of diagnostic laboratories in the US do not determine the RV variant of an animal that is infected. When suspected spillover events are observed, it is assumed that the circulating RV terrestrial variant in that area is responsible (Blanton et al. 2008). If there has been an exposure to RV, PEP should be administered regardless of the variant (Manning et al. 2008). Variant typing is only done when rabies is observed in an area that was previously RV free, in an effort to determine its origin (Leslie et al. 2006).

Another powerful tool in variant identification takes advantage of the conserved nature of the nucleocapsid gene sequence (Nadin-Davis et al. 1999). The nucleotide sequence of a segment of the N gene can be determined following RT-PCR amplification. This is more sensitive than viral variant detection with monoclonal antibodies and has largely replaced monoclonal antibody typing for variant identification (Szanto et al.

2011). Sequence of the N gene has also been used for comparing RV variants in an experimental setting (Davis et al. 2007).

Amplification and sequencing of the N gene has been used as a *post mortem* tool to diagnose RV infection and determine the causative variant in individuals who died with rabies-like clinical symptoms (Nadin-Davis et al. 2009). In addition to determining variant type, there has been a push to develop a nucleic acid-based diagnostic test that can be used before the human patient is deceased. The qRT-PCR method is a fast, sensitive test for detecting the RV genome and was developed with the hope of being able to diagnose RV infection in humans earlier (Nadin-Davis et al. 2009). This method was used in the Philippines to help diagnose rabies in two men who had been bitten by rabid dogs (Tobiume et al. 2009).

Rabies Virus and Virus Replication

Rabies virus (RV) belongs to the order Mononegavirales, family *Rhabdoviridae* and genus *Lyssavirus*. Rhabdoviruses have negative sense linear RNA genomes. The virus particles are enveloped and are generally rod-shaped 100-430 nm in length and 45-100 nm in diameter (Lyles and Rupprecht 2007). The RV genome encodes five proteins that are transcribed, translated, and assemble around a newly replicated negative sense RNA genome to make an infectious virion. Starting from the 3' end of the RV genome, the nucleocapsid (N) gene is transcribed first followed by the phosphoprotein (P), the matrix (M) protein, the glycoprotein (G) and the RNA-dependent RNA polymerase (L) (Wunner 2002). The viral genome is encapsulated by the nucleocapsid protein forming the ribonucleoprotein (RNP) core and associates with the P and L proteins. The RNP has

helical symmetry with a diameter of 30-70 nm that is surrounded by a lipid envelope made of G. The virus has a bullet-shaped appearance under electron microscopy (Banerjee 1987, Lyles and Rupprecht 2007, Mebatsion et al. 1999).

For a successful RV infection to occur several criteria must be met. An individual virus particle must bind a cellular receptor and enter the cell. Once inside the cell the virus must be able to transcribe its genome into positive strand mRNAs that are subsequently translated into viral proteins. These proteins must then assemble into the proper viral structure and bud from the infected cell to infect another susceptible cell. Our understanding of rhabodovirus replication has been based largely on studies of vesicular stomatitis virus (VSV) and it is possible that some details should not be extrapolated to RV replication (Banerjee 1987).

Once the virus enters a cell, its genome must be transcribed into viral mRNA. The (-) strand RNA genome contains a promoter sequence on its 3' end that is recognized by the viral polymerase complex for the initiation of transcription (Tordo and Kouknetzoff 1993). The polymerase complex transcribes leader RNA and five monocistronic mRNAs that encode the five viral proteins (Flint et al. 2004, Iverson and Rose 1982, Lyles and Rupprecht 2007, Trodo and Kouknetzoff 1993). Viral mRNA species maintain many of the same characteristics found in typical eukaryotic mRNA, with a 5'-terminal cap structure and a 3'-polyadenylic acid tail (Banergee 1987, Flint et al. 2004). The synthesis of viral mRNAs is nonequimolar and sequential, meaning that the mRNA of the N protein is observed first and is transcribed in the highest concentration followed by P, M, G, and L mRNAs at successively lower concentrations (Iverson and Rose 1981).

Another important event in the rhabdovirus replication cycle is the switch from transcription from mRNA to genome replication. The mechanism that causes the viral polymerase to switch from viral mRNA transcription to the (+) sense viral genome template needed for genome replication is not well understood. It has been speculated that this switch is connected to the concentration of the N protein (Tordo and Kouknetzoff 1993), which is needed to bind to the newly synthesized genome to the RNP (Banergee 1987). As the M protein concentration increases there is also a direct effect on the decrease in mRNA transcription and an increase in viral replication (Finke et al. 2003).

Viral assembly takes place at the cell membrane as the RNP and the glycoprotein form to make an infectious virion. M protein forms a bridge between the RNP, causing it to be in a condensed form that is responsible for the bullet-like morphology that is observed in RV virions, and G protein at the plasma membrane. The assembled virus then buds from the plasma membrane. The absence of the M protein severely inhibits RV budding (Mebatsion et al. 1999). Once the RV buds from the plasma membrane of an infected neuron it is then taken up by the presynaptic axon terminal of an uninfected neuron (Tsiang et al. 1991). Through double labeling of the RV, it was revealed that numerous enveloped RV particles can accumulate in an endosomal compartment and are transported along the retrograde axonal transport system to the brain (Klingen et al. 2008).

Receptors for Rabies Virus

Although RV is predominantly neuropathogenic *in vivo*, diverse cell lines support RV infection *in vitro*, including mouse and human neuroblastoma, hamster kidney, and chicken embryo fibroblast (Seganti et al. 1990). Based on the fact that there are multiple cell lines with multiple receptors, there may be more than one cellular receptor that can be utilized by the G protein of RV during infection (Dietzschold et al. 2005). Indeed, at least three cellular receptors support RV entry into the cell: the acetylcholine receptor (AChR), the neural cell adhesion molecule (NCAM), and the low-affinity nerve-growth factor receptor p75NTR. The AChR was identified as a potential RV cellular receptor when RV was found to bind to neuromuscular junctions through extensive staining of infected mouse tissue (Lentz et al. 1982, Tuffereau et al. 1998a). Although the AChR is used by RV for infection, multiple susceptible cell lines do not have AChRs (Reagan and Wunner 1985). The neural cell adhesion molecule (NCAM) has since been identified as another probable cellular receptor. Susceptible cell lines to RV have NCAM while, cell lines that are resistant to RV infection do not. However, experiments in the mouse knock-down model for NCAM, RV infection was inhibited but these mice still succumbed to rabies, indicating that although NCAM may be a receptor utilized by RV, it is likely not the only one (Thoulouze et al. 1998). A third cellular receptor that can be utilized by the RV is the low-affinity nerve-growth factor p75NTR (Tuffereau et al. 2001). The ability of the RV G protein to bind to p75NTR is dependent on the expression of a lysine and arginine in positions 330 and 333 of the RV G protein, respectively, in the antigenic site III, which has been shown to be important in the pathogenesis of rabies (Tuffereau et al. 1998b).

The viral G protein is clearly an important virulence factor. Its ability to bind cellular receptors in susceptible cell lines determines host cell range (Lyles and Rupprecht 2007). The amino acid sequence of the G protein determines protein folding and a single amino acid change at codon 333 can make a difference between a virulent and an avirulent RV strain (Tuffereau et al. 1989). Studies with recombinant viruses in which in the G protein was varied found that changes in the amino acid sequence in regions of the G protein that bind directly to the cellular receptor determine the pathology of the RV variant (Conzelmann et al. 1990, Morimoto et al. 1996, Yan et al. 2002). Although there are many contributing factors to virulence, the interaction of G protein with its cellular receptor(s) plays an important role in the pathology of rabies.

Transmission of Rabies Virus and Pathogenesis of Rabies

The CDC has defined guidelines on what constitutes an exposure to RV and when postexposure prophylaxis (PEP) should be administered for humans. Exposure routes have been broken into two categories, a bite or non-bite exposure. A bite exposure is the most dangerous and defined as "any penetration of the skin by teeth …" (CDC et al. 1999). Non-bite transmission routes are defined as "contamination of open wounds, abrasions, mucous membranes or theoretically, scratches, with saliva or other potentially infectious material (such as neural tissue) from a rabid animal…" This definition includes aerosol and oral transmission of RV (Manning et al. 2008). Due to cryptic RV cases and the fact that bat bites often go unnoticed, the CDC has additional guidelines for the administration of PEP when bats are involved. PEP should be administered if there is direct contact with a bat, or if a bat is found in the same room with a sleeping individual

or with someone who is unaware (unattended children, mentally disabled person or intoxicated person) and the bat cannot be caught to verify its rabies status, even if no bite was reported (CDC et al. 1999, Messenger et al. 2002, Blanton et al. 2010).

The incubation period for rabies in humans is typically between 1 and 3 months, and initial symptoms include fever and sometimes pain or discomfort at the wound site. Once the virus spreads from the site of inoculation into the nervous system, one of two patterns of symptoms occur. In the furious form of rabies, seen in approximately 70% of patients, individuals become anxious, hyperactive and hypersensitive to stimulation. Hydrophobia with pharyngeal spasms is another key symptom for rabies infection and diagnosis (CDC accessed 2011, WHO 2010a). In contrast, symptoms in patients manifesting the paralytic form of rabies are dominated by muscle paresis and paralysis (WHO 2010a). Unfortunately, clinical rabies of either type has a rather non-specific presentation in the beginning of disease, making early diagnosis difficult (Messenger et al. 2002). The diagnosis of clinical rabies can also be further complicated by the use of traditional medicines or herbs which can also cause abdominal, psychiatric, or neurological symptoms and influence blood analysis results (Cohen et al. 2007). Generally, once clinical symptoms of rabies start, the outcome of disease is death. Only a few cases of survival have been recorded (Blanton et al. 2010).

The clinical symptoms of rabies in animals have been categorized into three stages that overlap. First is the prodromal period where changes in behavior are observed. This is followed by the furious period in which the animal is highly excitable, behaves erratically, and becomes ferocious. The third stage is a paralytic period in which paralysis progresses from the wounded limb to the neck and head. In some cases, the

furious phase is skipped and disease progresses directly to the paralytic stage. Death is caused by respiratory failure (Lackay et al. 2008). Rabies symptoms in mice generally follow the paralytic form with paralysis of the extremities starting with the limb that was inoculated, with occasional spasticity observed (Jackson et al. 1989).

The study of natural RV infection in wild animals is difficult; therefore, experimental inoculations have helped supplement our understanding of RV transmission. Animal bite is the most common route of RV transmission throughout the world (Moran et al. 2000, WHO 2010a). In the laboratory, intramuscular inoculations have been used to mimic this route of infection (Murphy 1977). Although the intradermal route is not thought to be a major natural route of infection, it is possible and has been explored experimentally, especially for vaccine administration which will be discussed later in this review (WHO 2010b). Oral, intranasal and aerosol transmission have also been recorded in the wild (Barnard and Hassel 1981, Hubschle 1988, Constantine 1962), and laboratory experiments have again aided in expanding our knowledge of RV transmission through these routes.

Rabies Virus Transmission: Bite and Intramuscular Routes

There has been a great debate on whether RV replicates locally at the site of the bite wound before entering the nervous system or whether the virus enters the central nervous system directly. Evidence that RV first replicates in the muscle before moving into the neurons has been demonstrated through multiple laboratory techniques and in multiple species. Further, animals infected intramuscularly have shown RV replication in striated muscle cells before RV is detected in the neurons (Murphy et al. 1973, Harrison and Murphy 1978, Charlton and Casey 1979a). Denervation of the muscle allowed viral

replication at the inoculation site, but prevented spread (Charlton and Casey 1981). Although RV replication occurs first in the muscle cells, virus was taken up into the motor neurons in less than two days (Ugolini 2008).

Despite evidence supporting the replication of RV in the muscle at the inoculation site before entry into the neurons, some investigators have found that RV can directly enter the neurons without replicating in muscle beforehand. In these cases, RV could be detected in the nerves before viral replication was observed in the injected muscle (Coulon et al. 1989, Shankar et al. 1991). Nevertheless, viral entry into neurons may be dependent on the RV variant; for example, intravenous injection of SHBRV resulted in 100% fatality in mice while mice injected with a dog street variant DRV all survived. In this case SHBRV was thought to infect the brain directly (Preuss et al. 2009).

Although modern molecular techniques have advanced the understanding of RV virulence and the differences in RV variants, ground breaking studies in the 1960s had the advantage of being able to study the bite route of inoculation, and being able to inoculate a wider variety of animals in one study (Burns et al. 1958, Constantine et al. 1968a). Inoculation studies performed by Dr. Constantine and his colleges have not been repeated with more modern molecular techniques and remain the foundation for all of our understanding of rabies pathogenesis.

Differences in bat RV virulence are dependent on the species of bat, route of inoculation and the susceptibility of the species inoculated. Rabid bats have been made to bite different species of primate and carnivore and it was found that very few animals actually succumbed to rabies. These same animals were susceptible to rabies when inoculated with the same bat RV intracranially (Burns et al. 1958, Constantine et al.

1968a). Direct comparisons of RV from different species of bats through intramuscular inoculation and the bite route indicated that carnivores were more susceptible to the intramuscular route of inoculation than by being bitten (Constantine 1966a, Constantine and Woodall 1966, Constantine et al. 1968a). Similarly, a direct comparison of bat RV variants inoculated intracranially versus intramuscularly demonstrated that all the mice succumbed to RV inoculated intracranially, but that mortality of mice inoculated intramuscularly seemed to be more dependent on the RV variant than the route (Cunha et al. 2010). Interestingly, RV was not detected in any of the salivary glands taken from rabid bats or from animals that died of rabies after being bitten by rabid bats or inoculated with the bat RV intramuscularly (Constantine 1966a, Constantine and Woodall 1966). These studies demonstrate that not every bite from an infected rabid bat will result in the transmission of RV, and that there is no clear way to determine when transmission has occurred or not except to wait for the development of rabies.

Rabies Virus Transmission: Intradermal Route

Exposure to RV through a break in the skin can be the result of a shallow bite or incidental contact with a rabid animal; this is considered the intradermal route of infection. For example, there have been at least two human cases of rabies where the origin of the RV has been traced back to the butchering of a rabid animal where those who consumed the meat remained healthy. The route of transmission was assumed to be through intradermal exposure, although the aerosol and oral routes cannot be ruled out (Wertheim et al. 2009). In an experimental setting, some animals inoculated subcutaneously developed clinical rabies, but not with the 100% mortality seen following intracranial inoculation (Burns et al. 1958)

European bat lyssavirus 1 (EBLV-1) and 2 (EBLV-2) may be transmitted between bats by mechanisms similar to those used by RV. These viruses are closely related to RV, as RV belongs to lyssavirus genotype 1 and EBLV-1 and EBLV-2 belong to lyssavirus rabies-related viruses genotype 5 and 6 respectively (Nel and Markotter 2007). Insectivorous bats from both the US and Europe have been inoculated with EBLV-1 and EBLV-2 to determine how route of infection influences mortality. Not surprisingly, it was found that the intracranial route was most effective, followed by intramuscular and then subdermal routes. When the humoral immune response of surviving bats was analyzed there was some discordance between studies, with some groups reporting a robust immune response in bats inoculated subdermally (Franka et al. 2008) and others finding no response (Freuling et al. 2009). Although data on the transmission of EBLV-1 or 2 and RV in bat populations is limited, the intradermal route is thought to play a relatively small role in the transmission and maintenance of these viruses in wild bat populations (Franka et al. 2008, Johnson et al. 2008, Freuling et al. 2009).

Rabies Virus Transmission: The Oral Route

Oral transmission of RV has been observed both in nature and the laboratory and may be an important way that rabies is maintained between larger out-breaks. Theoretically, if a rabid animal is ingested by another animal, the mucous membranes of the hunter or scavenger may be exposed to infectious RV, and this pathway for transmission may have occurred in rabid lions and sled dogs (Berry 1993, Tabel et al. 1974). For scavengers, this means of transmission depends on RV remaining infectious after death of the host. The RV virion degrades more rapidly at higher temperatures and

thus the virion can survive longer in cooler climates increasing its chances of being transmitted to scavengers (Niezgoda et al. 2002, Soave 1966, Mork and Prestrund 2004).

Natural horizontal transmission of RV through saliva has been documented in multiple non-reservoir species including cattle (Dutta 1996), kudu (*Tragelaphus strepsiceros*) (Barnard and Hassel 1981, Hubschle 1988) and one human case (Fekadu et al. 1996). Infectious saliva can be transferred through shared food sources, or through licking and kissing.

Experimental oral inoculations in mice via multiple routes and dose schedules have demonstrated that clinical rabies does not result from every inoculation and seems to be more dependent on the viral dose than the RV variant that is used (Charlton and Casey 1979b). Viable RV seems to be resistant to stomach acid degradation and can be isolated from the stomach and intestine 3-6 hours following oral inoculation. The virus is taken up from the digestive tract and is fully disseminated into the whole body after five days (Correa-Giron et al. 1970, Madhusubana and Tripathi 1990). In studies involving oral inoculation, aerosol transmission was not ruled out and salivary glands were not evaluated (Charlton and Casey 1979b, Madhusuhana and Tripathi 1990).

The development of the oral rabies vaccine has been instrumental in the control of rabies in terrestrial wildlife reservoirs (Slate et al. 2009). During its development there were safety concerns for the spillover of the vaccine virus into non-target species that scavenge and ingest the vaccine bait (Winkler et al. 1976, Nicholson and Bauer 1981). Rodent experiments demonstrated susceptibility to infection with the ERA vaccine strain, with death from clinical infection, although the survivors mounted a measureable immune response (Winkler et al. 1976, Nicholson and Bauer 1981). Cannibalism

experiments designed to mimic natural scavenging among wild animals indicated that, although rodents succumbed to rabies, the chance of sustained transmission or an outbreak of rabies through this scenario was very small (Fischman and Ward 1968, Winkler et al. 1976, Nicholson and Bauer 1981).

Multiple non-reservoir species have been used to determine the effects that the age of an animal may play in susceptibility in oral inoculations (Fischman and Ward 1968, Madhusuhana and Tripathi 1990). Infection experiments with the CVS RV strain in neonatal animals compared to their weanling counterparts found that neonatal animals were more susceptible to RV infection (Fischman and Ward 1968).

Experimental oral inoculations have also been performed in natural reservoir animals for rabies. Skunks that were fed rabid mouse brains resulted in few infections, but feeding the whole carcass caused a higher infection rate (Bell and Moore 1971, Charlton and Casey 1979c, Ramsden and Johnston 1975). When evaluating the difference in RV variants and susceptibility via the oral route in cats, ferrets, skunks and foxes, only the skunk that had been fed mice infected with the silver hair bat RV variant succumbed to clinical infection. Unfortunately, this study does not indicate whether all the different RV variants were fed to each of the tested species (Bell and Moore 1971). Skunks seem to be more susceptible to RV variants that are not skunk RV variants, which may contribute to host-switching (Ramsden and Johnston 1975). This may explain the transmission that occurred in Arizona when a big brown bat RV variant was found in rabid skunks (Leslie et al. 2006). Although the oral transmission route of rabies is not the primary route of transmission for rabies, it cannot be forgotten as a way that RV may be maintained in wild reservoir populations.

Rabies Virus Transmission: Aerosol Route

Aerosol transmission have been reported for both natural and laboratory infections with RV. There have been four cases of clinical rabies possibly due to aerosol exposure in humans; two in different laboratory accidents and two in cave exposures. Both laboratory incidents involved the aerosolization of RV particles through blending brains from rabid animals and then mouth pipetting the rabid brain homogenate or the spraying of infected material (Winkler et al. 1973, Conomy et al. 1977, CDC 1977, Gibbons 2002). The other two cases of possible aerosol transmission of RV both involved exposure to RV in Frio Cave. The first case involved a scientist who worked with bat RV both in the laboratory and with wild bat populations. At the time it was thought that he contracted rabies from accidentally rubbing his neck that had a chronic skin condition with contaminated gloves (Irons et al. 1957), but the aerosol route was never ruled out. The second case was a man who worked in the guano mining industry that may have had incidental contact with a bat in Frio Cave (Kent and Finegold 1960). In both of these cases the victim had no memory of a bat bite or being exposed to RV. Although other routes of transmission could have occurred, the aerosol route cannot be ruled out.

Following these deaths, multiple experiments were set up in Frio cave to see if the transmission conditions could be reproduced. Wild carnivores were caged inside the cave in such a way that they had no access to the bats but were breathing the same air. Both coyotes and foxes succumbed to rabies through this aerosol exposure (Constantine 1962). RV was isolated from one of the coyotes and was inoculated into other mammals and was found to be transmissible, although RV was rarely found in the salivary glands

(Constantine 1966b). An additional approach to evaluating the potential for aerosol transmission of RV is to determine whether the virus is present in cave atmosphere. Air samples were collected from Frio cave with a mechanical device that traps airborne particulates, and infectious RV was isolated (Winkler 1968). This confirmed the presence of RV particles in the air of this cave, providing further support for the potential of RV transmission in such environments.

RV particles in the air may explain previous observations where some laboratory animals that were held in the same room with RV infected animals succumbed to rabies. These non-inoculated animals had no direct contact with rabid animals. Aerosol transmission was suspected due to the bat variant that was being used in aerosolization experiments nearby (Winkler et al. 1972). RV variant testing was not done to determine if the RV strain that killed the laboratory animals was the same as the bat variant used in their aerosol experiments.

Aerosolization of RV and intranasal inoculations have been used to mimic the natural aerosol route of transmission. In rodents, RV replicated in the nasal mucosa and traveled directly to the brain through the olfactory region (Hronovsky and Benda 1968). This direct entry into the brain may account for why animals inoculated intranasally experience higher mortality than those inoculated orally (Charlton and Casey 1979b, Charlton and Casey 1979c). Kudu have been reported to be particularly susceptible to rabies via intranasal inoculation when compared to cattle (Barnard et al. 1982).

Experiments comparing different RV variants via experimental intranasal or aerosol route have been performed in rodents and bats. Mice were found to be more susceptible to street RV variant than a fixed tissue culture strain when inoculated

intranasally (Selimov et al. 1969). Conversely, when bats were exposed intranasally to two different bat RV variants, none succumbed to rabies; however, anti-rabies neutralizing antibodies were detected in all inoculated bats. When these same bats were re-challenged intramuscularly, it was determine that the neutralizing antibody response was not protective since 10 out of 24 bats then succumbed to rabies (Davis et al. 2007). Although there seems to be no doubt that the bite route is the most important pathway for RV transmission, aerosol transmission may occur in special circumstances.

Immune Responses to Rabies Virus Infection

Two componensts of the immune system must function to clear a RV infection: A robust neutralizing antibody response and an inflammatory response to activate the adaptive immune response. General immunology has shown that viral infections induce the production of cytokines known as interferons (IFN). IFN released by the innate immune system initiates the Th1 response that then activates B-cells to produce neutralizing antibody that is critical in the adaptive immune response (Janeway et al. 2005). The production of IFN in a RV infection seems to be dependent on the pathogenicity of the RV strain, with non-pathogenic strains inducing a greater concentration of IFN in the brain. This higher concentration of IFN is believed to have a protective role in the clearance of virus and recovery from RV infection (Marcovistz et al. 1994).

Some strains of RV are more pathogenic than others and the concentration of the viral G protein expressed in infected cells appears to influence innate immune responses to RV infection, thereby playing an important role in the regulation of inflammation and

cellular apoptosis. The abundance of the G protein in non-pathogenic RV strains seems to be higher than in pathogenic strains and elevated expression of viral G protein seems to activate a more potent inflammatory response through the up regulation IFN-α/β (Wang et al. 2005). Similarly, the higher level of expression of viral G protein is associated with more widespread cellular apoptosis (Morimoto et al. 1999). Although additional work needs to be done to elucidate the immunological pathways behind these correlations, the level of expression of RV G protein clearly influences pathogenicity.

In a majority of human rabies cases in the US, neutralizing antibody to rabies virus cannot be demonstrated in patient serum upon initial presentation with clinical symptoms, and is found only after advanced clinical progression of disease (Noah et al. 1998). This is undoubtedly a major reason that rabies is invariably a fatal disease. Similarly, very little is known about the few individuals that have survived clinical rabies or have acquired neutralizing anti-RV antibody without developing clinical symptoms of rabies (CDC 2010, Blanton et al. 2010, Follmann et al. 1994). Abortive cases of rabies have been documented in mice, and in these cases, some mice had measurable anti-RV antibodies in their serum associated with resolution of clinical symptoms. While other mice failed to seroconvert. Interestingly, all the mice that survived the initial infection also survived a re-challenge, regardless of whether or not they produced detectable anti-RV antibody (Bell 1964). This implies that there may be more to surviving a rabies infection than just the production of neutralizing antibodies.

How neutralizing anti-RV antibody protects against infection is another question and again the mouse model has been an important tool in elucidating the pathways required for the clearance of RV from the central nervous system. There must be

collaboration between neutralizing antibody production and the inflammatory response. T-cell activity can slow down RV replication but anti-RV neutralizing antibody must somehow gain access to the CNS for complete elimination of virus (Hooper et al. 1998, Hooper et al. 2009).

Rabies Virus Vaccination and Prophylaxis

The development and history of RV vaccination has been thoroughly reviewed in other literature (Fu 1997, Johnson et al. 2010, Rupprecht et al. 2002, WHO 2010b). The effects of delivery route of the vaccine, dose, age of the animal or individual receiving the vaccine and the vaccination schedule all influence response to vaccination. For example, the route of viral inoculation influences progression to clinical disease (Franka et al. 2008, Gibbons 2002), just as the route of vaccine delivery can also influence the magnitude of the neutralizing anti-RV antibody response. Likewise, vaccine dose, and vaccination schedule are also important (Wunderli et al. 2003a, Wunderli et al. 2003b).

The National Institutes of Health has developed a specific protocol for use in certification of new batches of RV vaccines to ensure their protective properties (Wilbur and Aubert 1996). In short, the NIH protocol states that mice must be the same weight before vaccination and should be vaccinated intraperitoneally with two doses of vaccine before a lethal challenge of RV administered intracranially (Wilbur and Aubert 1996). In efforts to improve this protocol, it was found that the age of the mouse is more important than the weight of the mouse. Even a difference in two weeks of age changes the susceptibility with older mice being more resistant to rabies independent of weight (Wunderli et al. 2003a). Further evaluation has shown that reproducible results are

obtained when a single dose of RV vaccine is given intramuscularly, followed by an intramasseter muscle challenge with a lethal dose of 4 weeks post-vaccination (Wunderli et al. 2003a, Wunderli et al. 2003b).

The decision as to which humans should be vaccinated against RV is determined by the patient's potential risk for exposure to the virus. Individuals who are in high risk occupations (e.g. veterinarians, animal control officers, bat biologists or scientists who study rabies) should receive pre-exposure vaccination. Currently, such vaccination consists of a series of three doses of vaccine given on days 0, 7 and 28. Ideally, individuals should have their neutralizing anti-RV antibody titer checked following vaccination to assure there is a detectable immune response.

Individuals who have potentially been exposed to a RV should receive post-exposure prophylaxis. Several different treatments have been utilized, but most commonly, five doses of rabies vaccine are administered on days 0, 3, 7, 14 and 28 days or a four dose route of two doses on day 0 followed by single doses on days 7 and 21. For individuals that have not previously been immunized against RV, it is recommended that this vaccination schedule be supplemented by administration of human rabies immunoglobulin be as soon as possible after the exposure (Manning et al. 2008, WHO 2010).

Immune Response to Rabies Virus in Wild Bat Populations

Although wild bats do succumb to RV infection, apparently healthy bats with anti-rabies virus neutralizing antibodies have frequently been reported (Constantine et al. 1968b, O'Shea et al. 2003, Salas-Rojas et al. 2004). Approximately 1% of Mexican

free-tailed bats (*Tadarida brasiliensis Mexicana*) have been found to be rabid while an average of ~30% had anti-rabies neutralizing antibodies in their serum (Constantine et al. 1968b, Steece and Altenback 1989). Similarly, anti-rabies neutralizing antibodies have been found in multiple bat species throughout the world (Salas-Rojas et al. 2004, Jiang et al. 2010). For example, antibody to European bat lyssavirus-1 (EBLV-1), a rhabdovirus closely related to RV, has been demonstrated in *Eptesicus serotinus* bats from Spain, and some of those bats were captured repeatedly over several years and were healthy (Perez-Jorda et al. 1995). The phenomenon of bats having anti-lyssavirus antibodies over periods of time which exceed any known incubation period seems to contradict the frequently-held belief that rabies is inevitably a lethal disease in these animals and suggest that in bats, immunity to rabies can be acquired through the repeated exposure to low doses of RV and may not be the result of a productive infection (O'Shea 2003).

Several investigators have reported on experimental inoculation of big brown bats with RV to determine their susceptibility to infection and their immune responses to the virus. In some cases, bats succumbed to RV infection too quickly to develop detectable neutralizing antibody, while some of those that survived inoculation had measurable antibody titers without apparent signs of clinical infection (Jackson et al. 2008). In another study, it was found that some bats survived despite having been inoculated multiple times intramuscularly (Turmelle et al. 2010). Some of these surviving bats had neutralizing antibodies in their serum, but others never seroconverted. Repeated exposure could result in long-term immunity to RV, but other immunological factors may be at play in the clearance of RV (Turmelle et al. 2010). Factors that influence

production of neutralizing anti-RV antibody in bats remain poorly understood and merit further study.

Rationale and Hypotheses

Characterization of Rabies Viruses from Colorado Big Brown Bats (*Eptesicus fuscus*) in Mice

Bats are a major reservoir for rabies and their RV variants are responsible for the majority of human rabies cases in the United States (Messenger et al. 2002, Serres et al. 2008, Blanton et al. 2010). RV variants are most commonly distinguished by the nucleotide sequence of a portion of the nucleocapsid gene, a gene which almost certainly has, at best, only minor influences on biological behavior within hosts (Nadin-Davis et al. 1999). A single variant may consist of a population of viruses that vary considerably in regions outside the nucleocapsid gene, resulting in significant differences in traits such as virulence. Few studies have compared mortality, incubation period and the humoral immune responses following infection of animals with bat RV variants compared to terrestrial RV variants (Dietzschold et al. 2000, Morimoto et al. 1996, Preuss et al. 2009), and fewer still have compared different bat RV variants in an animal model (Constantine et al. 1968, Baer et al. 1980). There is also a near total lack of studies comparing virulence within a single bat RV variant. Understanding the spectrum of biological variation with variant populations is important to the understanding of virus maintenance in nature and transmission to terrestrial mammals, including humans. We chose to work with the big brown bat RV variant because big brown bats are distributed widely across the US and frequently live in very close contact with humans; further, bats of this species are most frequently submitted to the Colorado Department of Public Health and Environment for rabies diagnosis (Pape et al. 1999). Finally, previous work from our laboratory demonstrated that two big brown bat RV isolates with the same nucleocapsid

sequence had significant differences in virulence when evaluated in the mouse and bat animal models, confirming that significant biological variation exists within the population of a single RV variant (Davis et al. 2007). Based on this latter information, **we hypothesized that there would be a bimodal distribution in the important biological characteristics of virulence, based on mortality and incubation period within the big brown bat RV variant tested in a mouse model.** Confirming or denying this hypothesis should promote a more general understanding of the genetic basis of virulence among bat rabies viruses.

Serological Response of Mice to Multiple Low Dose Inoculations of Bat Rabies Viruses via Different Routes and Schedules

The phenomenon of healthy wild bats having anti-rabies neutralizing antibodies has been observed repeatedly throughout the world (Constantine et al. 1968, Perez-Jorda et al. 1995, Salas-Rojas et al. 2004, Steece and Altenback 1989, Jiang et al. 2010, O'Shea et al. 2003). At a given time, roughly 1% of wild bats are found to be rabid, but up to 30% have anti-rabies antibodies, which is interpreted as reflecting some type of previous exposure to RV (Constantine et al. 1968, Steece and Altenback 1989). Indeed, some seropositive bats have been identified and captured multiple years in a row, which is longer than any known incubation period for rabies (Perez-Jorda et al. 1995, O'Shea et al. 2003). The high prevalence of anti-rabies antibody in bats seems to contradict the generally-held view that rabies is an invariably fatal disease (WHO 2010). The route by which these bats acquire neutralizing antibody without development of rabies has not been elucidated, but is most likely the result of some type of exposure to RV. What is not known is whether this finding reflects some unique aspects of bat biology or results perhaps from more ecological features of their lifestyle, which likely includes repeated exposure to low doses of RV from an early age. To explore the second possibility, we evaluated a big brown bat RV variant in mice using various routes of inoculation routes,

doses of virus and schedules of virus inoculation. **Specifically, we hypothesized that frequent inoculation of mice with a low dose of RV would lead to development of a robust humoral immune response in the absence of disease, but that intranasal inoculation would likely result in a high frequency of disease.** This study was designed to address how wild bat populations may acquire natural neutralizing antibodies to rabies without progression to clinical rabies, with the assumption that bats and mice have fundamentally similar immune responses.

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CHAPTER TWO

CHARACTERIZATION OF RABIES VIRUSES FROM COLORADO BIG BROWN BATS (*EPTESICUS FUSCUS*) IN MICE

CHAPTER SUMMARY

Nine rabies viruses (RV) were isolated from big brown bats (*Eptesicus fuscus*) found in Colorado and a low-passage stock of each was prepared. These isolates were evaluated for virulence, immunogenicity and salivary gland dissemination in an outbred mouse model to investigate whether there were major differences in these characteristics within this virus population. Inoculated mice were maintained for 12 weeks to assess mortality humoral immune responses were evaluated regularly as an indication of immunogenicity. The salivary glands from mice that developed clinical rabies were evaluated for the presence of viral antigen as an indication for the potential for transmission. The dose of RV inoculated had a greater influence on mortality, incubation period and humoral immune response than the individual RV isolate itself. The prevalence of neutralizing antibody was not different in mice that survived versus those that succumbed to RV infection. The presence of viral antigen in salivary glands was observed only in mice from the high dose inoculation groups. Overall, there was low diversity in biological behavior among the isolates of big brown bat RV evaluated in these experiments.

INTRODUCTION

Bats are well known as hosts of rabies virus (RV) and populations of bats from different species maintain viruses that have relatively characteristic genomic signatures which have been exploited to define virus variants. These RV variants are commonly defined by a conserved nucleotide sequence in their nucleocapsid gene (Nadin-Davis et al. 1999). In the case of RV isolated from big brown bats, seven clades and subclades have been defined based on their phylogenetic analysis of the nucleocapsid gene sequence (Shankar et al. 2005). While this short sequence is useful for molecular typing, it clearly does not illuminate the genomic variation in other viral genes which likely have more influence on important biological characteristics of the virus, such as virulence and transmissibility. Little research effort has been devoted to comparing different bat RV variants to one another or to terrestrial RV variants in terms of virulence, induced incubation period and ability to induce an immune response. Understanding such biological variability among bat RV variants is important because, in countries where dog rabies has been eliminated, a majority of human rabies cases are caused by bat RV variants (Blanton et al. 2010, Messenger et al. 2002, Serres et al. 2008).

Understanding the extent to which different bat RV variants are alike with respect to virulence, incubation period and ability to induce immune responses is poorly developed. Comparison of viruses isolated from the leaf-nosed bat (*Macrotus waterhousii*), hoary bat (*Lasiurus cinereus*), silver-haired bat (*Lasionycteris noctivagans)* and big brown bat (*Eptesicus fuscus*) indicated that the leaf-nosed RV variant was the most virulent with regard to transmission to carnivore by the bite route from an infected bat, and that a few big brown bats were able to survive a challenge with their own RV

variant (Constantine et al. 1968). The silver-haired bat RV variant has been compared with multiple terrestrial RV variants by multiple routes of inoculation of mice. These studies revealed that the silver-haired bat RV variant may be intrinsically more virulent than terrestrial RV variants tested based on its wider range in cellular tropism, ability to replicate to higher titers at a lower temperature and the fact that it can infect the brain directly when inoculated intravenously (Dietzschold et al. 2000, Morimoto et al. 1996, Preuss et al. 2009). Evaluation of a Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) RV variant compared to a vampire bat (*Desmodus rotundus*) RV variant in a mouse model found that the Mexican free-tailed bat variant seemed to be more virulent in that it haddue to its shorter incubation period and more severe pathology in the brain (Baer et al. 1980). Each of these studies used a single representative of each bat RV variant.

Only a handful of studies have looked at the anti-rabies antibody responses to bat RV variants. In two studies it was found that inoculation of big brown bats with a big brown bat RV variant was not uniformly fatal and that some of the surviving bats seroconverted and had measurable anti-rabies antibodies (Jackson et al. 2008, Turmelle et al. 2010). Furthermore, a comparison of the virulence and immune response of both big brown bats and mice challenged by the aerosol route with three isolates of the big brown bat RV variant found that the surviving mice and bats had virus neutralizing antibodies to RV, but that this response was not protective when they were re-challenged with virus inoculated intramuscularly (Davis et al. 2007).

One issue related to bat rabies biology that has received scant attention is the degree of biological variability that exists within a population of a single RV variant.

Although the magnitude of such variability is not known, one previous study with only two big brown bat RV isolates having the same nucleocapsid gene sequence demonstrated significant differences in their virulence for mice and bats (Davis et al. 2007), leading to the hypothesis that within the population of big brown bat RV, there is a bimodal distribution in the important biological characteristics of virulence, as measured by mortality and incubation period. The work described here tested that hypothesis by characterizing the behavior of nine independent isolates of virus obtained from rabid big brown bats from Colorado.

MATERIALS AND METHODS

Experimental design

The fundamental objective of this study was to characterize a group of nine RV isolated from big brown bats in Colorado, to address the question of whether major differences in virulence, immunogenicity or salivary gland dissemination occurred within this single variant of bat RV. Outbred mice were chosen as a host to evaluate these questions. We attempted to minimize genetic changes in the virus by minimal passage in cultured cells. Virulence was determined by titrating the viruses in mice to obtain an estimate of lethal dose. Inoculated mice were maintained for 12 weeks after inoculation and bled regularly to assess humoral immune responses as an index of immunogenicity. Finally, the salivary glands of mice that developed rabies were evaluated to determine whether virus replication had occurred in this tissue, which would indicate a potential to infect a susceptible host.

Rabies Virus Isolation from Bats and Preparation of Stocks

Dead bats submitted for rabies diagnosis and tested positive by direct fluorescent antibody (DFA) testing between 2006 and 2007 were provided from the Colorado Department of Public Health and Environment. The carcasses had been kept at -80^{o}C since diagnosis and the species of each bat was confirmed by a qualified bat biologist using morphologic characteristics. Residual brain and the anterior end of the spinal cord were homogenized in 1 ml growth medium (Dulbecco's Modified Eagles medium supplemented with 5% fetal bovine serum [FBS], 100 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/ml gentamycin), using a Retsch Tissuelyser MM 300 Ball Mill homogenizer set at 20 Hz for 4 minutes. The homogenates were centrifuged and the supernatants applied to mouse neuroblastoma cells (NB) which were 50% confluent. Cells were passaged three times, with samples from each passage tested for the presence of RV by DFA staining, as described below. Clarified supernatants from passages at which 90 to 100% of the cells were infected were aliquoted into vials and stored at -80^{o}C. Viruses were named by their county of origin in Colorado and original accession number.

Detection and Titration of Rabies Virus

Cultured NB cells infected with RV were trypsinized, suspended in PBS and transferred to a 2 ml storage tube. The cells were washed twice by centrifugation and resuspension in PBS, and the final cell pellet resuspended in 200-500 μl of PBS. Drops of cell suspension were pipetted onto spot slides (Teflon Printed Slides 8-Well, 6mm diameter, Electron Microscopy Sciences) and after a few seconds, excess liquid was

aspirated, leaving a uniform film of cells on the slide. Slides were allowed to air dry, fixed in 100% acetone for at least one hour and stored at -20^{o}C until staining.

Staining for RV antigens was accomplished by applying drops of Light Diagnostics Rabies DFA Reagents (diluted 1:50) to each slide, covering with a coverslip, and incubating for 45 minutes at 37^{o}C in 5% CO_2. Coverslips were removed and slides were washed three times for five minutes each in PBS and once in water. Slides were then air dried and mounted with Dako Fluorescent Mounting Medium (Dako North America, Carpinteria, CA) and examined with an upright fluorescence microscope (Nikon Eclipse E800).

Stock viruses were titrated on NB cells using a standard quantal assay. Cells were trypsinized, counted and seeded into 96 well plates at 25,000 cells per well. The following day, serial 10-fold dilutions of virus (typically 10^{-1} to 10^{-7}) were prepared in growth medium. Medium was removed from the plates of cells by inversion and brief shaking, and 5 replicate wells were inoculated with 50 µl of each virus dilution. Plates were incubated for three to four days at 37^{o}C in 5% CO_2, then the medium was discarded, and the plates were rinsed once with PBS and fixed with 70% acetone for at least one hour. Plates were air dried and then either stained immediately or stored in the refrigerator until they were stained. Staining for RV antigens was conducted similarly to what was described above for spot slides, and the plates were evaluated using an inverted fluorescence microscope. The tissue culture infective dose 50% ($TCID_{50}$) was calculated for each sample by the Spearman-Karber method (Smith et al. 1996).

Inoculation and Monitoring of Mice

Young adult, female ICR mice 4-6 weeks of age were purchased from Charles River Laboratories, housed in groups of 5 and maintained under ABSL-3 conditions with free access to water and rodent chow throughout the experiment. Individual mice were identified by ear punch and weighed weekly to monitor their general health.

Mice were inoculated with RV in the right triceps muscle using an insulin syringe with attached 28 gauge needle at a volume of 50 µl. In the first experiment RV isolates #24046 El Paso, #21921 Mesa, #24235 Douglas, #20708 Denver and 21567 Denver were inoculated into groups of five ICR mice at three different doses (10^4, 10^3, and 10^2 $TCID_{50}$/50 µl). In the second experiment, the #25571 Boulder, #23111 El Paso, #15100 V1, #14357 V2, #24046 El Paso, #24235 Douglas, and #20708 Denver RV isolates were inoculated into mice using a single dose of $10^4 TCID_{50}$/50 µl. The isolates #24046 El Paso, #24235 Douglas and #20708 Denver were repeated from the first experiment to determine if the results seen in the first experiment were reproducible. Following inoculation, mice were monitored twice daily for clinical signs of disease. When definitive signs were observed, the mice were anesthetized with pentobarbital, bled by cardiac puncture and euthanized by cervical dislocation (Davis et al. 2007, Shankar et al. 2004). The left side of the brain and one salivary gland were fixed in 10% formalin for 48 hours and then transferred into 70% ethanol for storage. The right side of the brain and salivary gland were placed in tubes and stored at -80^{o}C for RV isolation or DFA staining, respectively. Slip smears of brainstem and minced salivary gland were prepared on standard microscope slides, fixed in acetone and stained for DFA detection of RV antigens as described above.

Isolation of Rabies Virus from the Brains of Virus-Challenged Mice

Mice that developed clinical rabies were euthanized, and their brains hemisected and frozen to -80^{o}C. The right half of the brain was subsequently thawed and homogenized in 1 ml of growth medium using a 7 ml Ten Broeck tissue grinder. Once homogenized, the mixture was centrifuged (9000 x g for 5 minutes) to remove cellular debris. One hundred microliters of the supernant was diluted into 1 ml of growth media and placed on 50-80% confluent NB cells in a 25 cm^2 (T25) flask. The cells were incubated for 30 minutes at 37^{o}C in 5% CO_2, 5 ml of growth medium was added to each flask and the cells were incubated for another 24 hours. After 24 hours the growth medium from each flask was removed, the cells were rinsed with PBS and 6 ml of fresh growth media was added back to each flask. Cells were checked daily for cellular death. After 3 days incubation, the cells were rinsed with PBS, trypsinized and split into a new T25 flask at a ratio of 1:5 and returned to the incubator for another 3 days. The remaining cells were used to make spot slides to check for RV antigens by DFA. This process was repeated up to three passages for each brain sample. If RV infection was not detected after three passages, the sample was considered negative. Samples from which RV was isolated were harvested for further analysis or use by adding 1ml FBS to the 6 ml growth media in the flask. This medium was transferred into a 15 ml tube, centrifuged for 10 minutes at 402 x g and aliquots of 0.5 ml were placed in 2 ml storage tubes and stored at -80^{o}C.

Reverse Transcriptase-Polymerase Chain (RT-PCR) Reaction and Sequencing of the Rabies Virus Nucleocapsid Gene

Total RV RNA was extracted using a commercial kit for cell culture (Qiagen Rneasy Mini Kit) following the manufactures directions. The RNA concentration was measured using a Nano Drop spectrophotometer and cDNA was synthesized using one of several kits or reagents (Thermo Scientific Verso cDNA kit, Invitrogen SuperScript II Reverse Transcriptase or Bio-Rad iScript Select cDNA Synthesis kit), following manufacturer's directions.

The primers used for amplification of the nucleocapsid gene segments were based on those described by Shankar and coworkers (2004) (Table 2-1):

Table 2-1. Primers Used to Nucleocapsid Gene Segment Amplification

Primer	Sequence	Location in RV N gene	Primer Direction
21g	5'-ATGTAACACCTCTACAATG	~base 57	Forward
304	5'-TTGACGAAGATCTTGCTCAT	~base 1514	Reverse

A 4 µl sample of cDNA from a 20 µl reaction was amplified in a total volume of 50 µl utilizing 2.5 units recombinant Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA), supplied 1X buffer, $MgCl_2$ [2.5 mM], primers [200 nM], dNTP [200 µM] and dH_2O. The dNTP and polymerase were added during a hot start of 95^oC for 5 minutes. Amplification parameters consisted of 40 cycles of 95^oC for 30 s, 37^oC for 1 minute and 72^oC for 1 minute, followed by 72^oC for 10 minutes.

PCR products were held at 6^oC or stored at 20^oC until they were run on a 1.5% agarose gel with ethidium bromide along with a 1 KB molecular weight ladder. The nucleocapsid cDNA product was extracted from the gel (Qiagen QIAquick Gel Extraction) and submitted for sequencing to Davis Sequencing (Davis, CA 95616). Sequences were manually edited and BLAST was used to compare the sequences against

known sequences in Genbank. Deduced amino acid sequences were compared using a multi-align computer program multiple sequence alignment (http://multalin.toulouse.inra.fr/multalin/multalin.html).

Rapid Fluorescent Focus Inhibition Test (RFFIT)

All serologic testing was performed using a variation of the RFFIT originally described by Smith and colleagues (1996). Blood was collected from the mice via submandibular vein puncture, allowed to clot and then centrifuged to separate the serum. Sera were inactivated at 56^{o}C for 30 minutes prior to testing for neutralizing antibody. A series of five-fold dilutions of each serum sample was prepared in a 96 well plate using growth medium as the diluent. Each assay incorporated a positive control consisting of the WHO rabies immunoglobulin standard (RIG) diluted in five-fold increments as for mouse serum, with the lowest dilution of 1 U/ml. Wells containing growth medium were included on each plate as negative controls. The CVS-11 strain of RV at a concentration of 160 $TCID_{50}$/well was used as the challenge virus. Forty microliters of challenge virus was added to 40 µl of each serum dilution and those mixtures incubated for 1 hour at 37^{o}C in 5% CO_2 to allow neutralization to occur. A 100 µl aliquot containing 40,000 baby hamster kidney cells (BHK-21) was added to each well and the plates were incubated for 20 hours at 37^{o}C with 5% CO_2. Medium was then poured off, the plates were rinsed one time with PBS and fixed in 70% acetone for at least one hour. Plates were air dried and either stained immediately or stored in the refrigerator until they were stained with anti-rabies virus DFA reagent as described above. After staining, 50 µl of PBS was added to each well and plates were stored overnight in the refrigerator. Wells were examined using an inverted fluorescence microscope and 20 fields were

classified as having or not having positive cells. Based on these numbers, the neutralization titer was calculated using the Spearman and Karber method (Manning et al. 2008, Smith et al. 1996).

Statistical Analyses

Experimental Design:

This study was conceived as a screening assay with the intent of replicating those comparisons that appeared biologically interesting. We therefore chose to use a relatively low statistical power in order to screen more isolates. Specifically, a group size of 5 was used which would allow detection of a difference in mortality of 0.95 versus 0.10 with power of 0.9 ($\alpha = 0.2$). This power calculation and all statistical analyses were performed using SAS/STAT ® software version 9.2 © 2010.

Statistical Analysis of Data:

Comparison of all nine RV isolates for ability to induce mortality and antibody response was performed using Proc GLIMMIX with RV isolate as the covariate and assuming binary responses. Due to the limits of using Proc GLIMMIX, pairwise comparisons among different RV isolates were performed using Fisher's Exact Test with Proc FREQ for analysis of mortality. A comparison of all nine RV isolates for antibody responses was performed using Proc GLIMMIX ANOVA with repeated measures over time; this comparison was conducted only for mice inoculated with the 10^4 $TCID_{50}$ dose. Following analysis of antibody responses across all nine virus isolates, a separate analysis of antibody responses was performed using Proc GLIMMIX ANOVA with repeated measures over time for those five RV isolates that were inoculated at three different doses.

Comparison of all nine RV isolates for incubation time was performed using Proc GLM with RV isolate as the covariate. Since the residuals from the analysis of variance were not independent of the mean, a $\log_{10}$ transformation of the data was used for the analysis.

RESULTS

Rabies Virus Isolation and Titration of Viral Stocks

Virus isolation was attempted from the residual brain and spinal cord of 28 big brown bats (EPFU), seven of which yielded virus within two passages in NB cells. The Colorado County of origin and titer of the resulting stocks for each of these seven viruses, plus two viruses previously described are presented in Table 2-2.

Table 2-2: County of Origin and Viral titer for Nine EPFU RV isolates from *Eptesicus fuscus*

Virus Number	County of Origin	Stock virus titer ($\log_{10}TCID_{50}$/ml)
#21921	Mesa	5.6
#24235	Douglas	5.8
#20708	Denver	5.6
#25571	Boulder	5.1
#23111	El Paso	5.0
#24046	El Paso	5.5
#21567	Denver	5.6
#15100 V1	Unknown	5.6
#14357 V2	Unknown	5.6

Rabies Virus Sequence Analysis

The nucleotide sequence of the nine RV isolates were compared to one another with a Multi-align program to determine if there were different variants of EPFU RV (Appendix 1 and 2), but a rigorous phylogenetic analysis was not performed. Based on the sequence of the nucleocapsid gene, each of the nine RV isolates were most similar to strains originally identified from *Eptesicus fuscus* bats in El Paso County, Colorado (GenBank accession numbers AY039228 and AF394888). RV AY039228 and AF394888 were submitted to GenBank directly by the CDC Rabies Laboratory in 2001 and have not been phylogenetically classified beyond the *Eptesicus fuscus* bat species they were isolated from. RV isolates #21921 Mesa, #24235 Douglas, #20708 Denver, #23111 El Paso, #24046 El Paso, #21567 Denver, #15100 V1 and #14357 V2 all had a maximum identity of 99% with Genbank accession number AY039228. The sequence of the #25571 Boulder isolate had a maximum identity of 92% with accession number AY039228, but 99% with accession number AF394888. These differences suggest that two subvariants of EPFU RV were used in this experiment as defined by the nucleocapsid gene sequence (De Mattos et al. 1996). A majority of the nucleotide differences between the two subvariants were silent, but seven amino acid differences were identified. These changes included a switch of aliphatic amino acids (isoleucine versus leucine) and a tryptophan (aromatic) versus cysteine (non-aromatic, sulfur side chain) (Mathews et al. 1996). The nucleotide and deduced amino acid sequences of the nucleocapsid gene for all nine EPFU viruses are presented in Appendixes 1, 2 and 3.

Mortality Data Separates RV Isolates Based on Virulence

We were unable to evaluate differences in mortality among the nine RV isolates at the 10^4 $TCID_{50}$ dose using SAS code Proc GLIMMIX because that procedure does not allow comparisons with no response (i.e. no mortality). Therefore, a Fisher's Exact Test was used to compare four groups that appeared *a posteriori* to have a higher percentage of mortality against those with a lower percentage of mortality. Mice inoculated with RV isolates #24046 El Paso, #20708 Denver, #21111 El Paso and #15100 V1 had mortality rates of 90, 90, 20 and 0% respectively. The mortality rates experienced by groups #24046 El Paso and #20708 Denver were different from those of both #23111 El Paso ($p = 0.017$) and #15100 V1 ($p = 0.002$). We interpreted this to indicated that the #24046 El Paso and #20708 Denver viruses were significantly more virulent than either #23111 El Paso and #15100 V1

Based on the analysis of the data from the first experiment, we repeated the inoculation of three RV isolates into mice using the 10^4 $TCID_{50}$ dose. RV isolates #24046 El Paso and #20708 Denver were chosen to see if the high mortality (4 of 5 mice) observed during the first inoculation experiment was repeatable. Likewise, #24235 Douglas was chosen due the low observed mortality rate (1 of 5) observed in the first experiment. RV isolate #24046 El Paso and #20708 Denver both induced high mortality in experiment 2, with 5 of 5 mice succumbing to rabies. However, inoculation of the #24235 Douglas virus also resulted in development of rabies in 4 of 5 mice during the second experiment, which was in direct contrast with results from the first experiment. Mortality data for both experiments is summarized in Table 2-3 and Figure 2-1.

Table 2-3: Morality Summary, Experiment #1 and #2

Virus Isolate	Number of Mice that Succumbed to RV / Total Number in the Group		
	4 $\log_{10}$ $TCID_{50}$	3 $\log_{10}$ $TCID_{50}$	2 $\log_{10}$ $TCID_{50}$
#24046 El Paso	9/10 (90%)	1/5 (20%)	0/5 (0%)
#21921 Mesa	2/5 (40%)	1/5 (20%)	1/5 (20%)
#24235 Douglas	5/10 (50%)	2/5 (40%)	1/5 (20%)
#20708 Denver	9/10 (90%)	1/5 (20%)	0/5 (0%)
#21567 Denver	3/5 (60%)	2/5 (40%)	2/5 (40%)
#25571 Boulder	4/5 (80%)	NA	NA
#23111 El Paso	1/5 (20%)	NA	NA
#15100 V1	0/5 (0%)	NA	NA
#14357 V2	2/5 (40%)	NA	NA

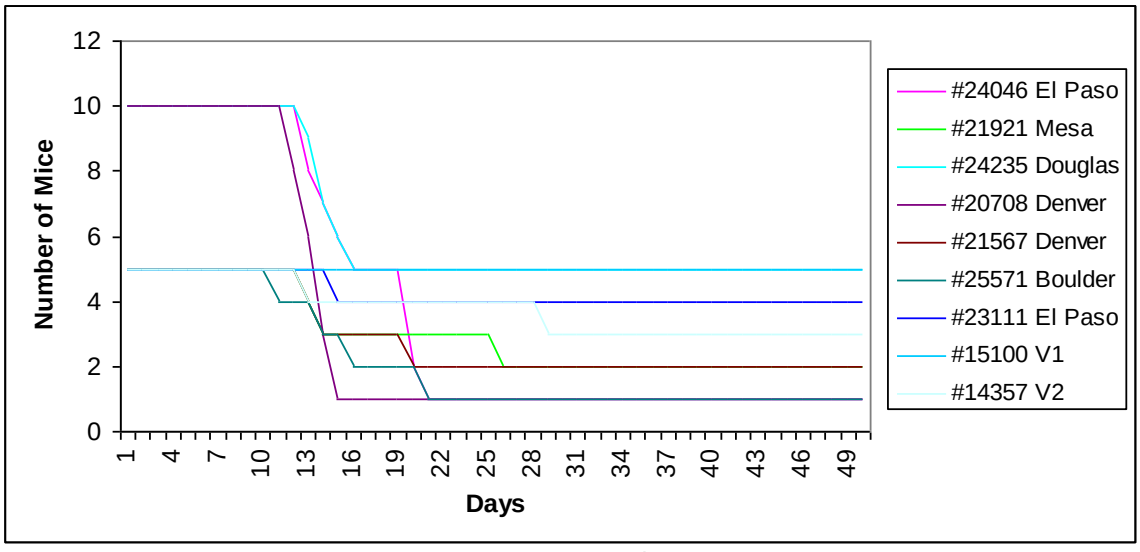

Figure 2-1: Survival of mice inoculated with 10^4 $TCID_{50}$ of RV, Experiment #1 and #2

Experiment #1: Mortality as a result of challenge with the viral dose of 10^4, 10^3, and 10^2 $TCID_{50}$ are summarized in Figures 2-4. There were no significant differences in mortality between RV isolates within the same inoculation dose. RV isolates #24046 El

Paso and #20708 Denver, at a dose of 10^4 $TCID_{50}$, induced the highest mortality with 4 of 5 (80%) mice succumbing, while the #24235 Douglas isolate induced the lowest mortality with only one mouse (20%) developing disease (Figure 2-2). Visual evaluation of mice inoculated with 10^3 $TCID_{50}$ found less overall mortality with the #24046 El Paso, #21921 Mesa and #20708 Denver isolates with the lowest mortality of only 1 of 5 mouse (20%) developing rabies; while #24235 Douglas and #21567 Denver both had two mice (40%) succumb to RV (Figure 2-3). In the viral dose of 10^2 $TCID_{50}$ groups of #24046 El Paso, #21921 Mesa and #20708 Denver, all the mice survived until the end of the study. The #24235 Douglas isolate induced rabies in 1 of 5 mice (20%) and the #21567 Denver isolate caused rabies in 2 of 5 mice (40%) (Figure 2-4).

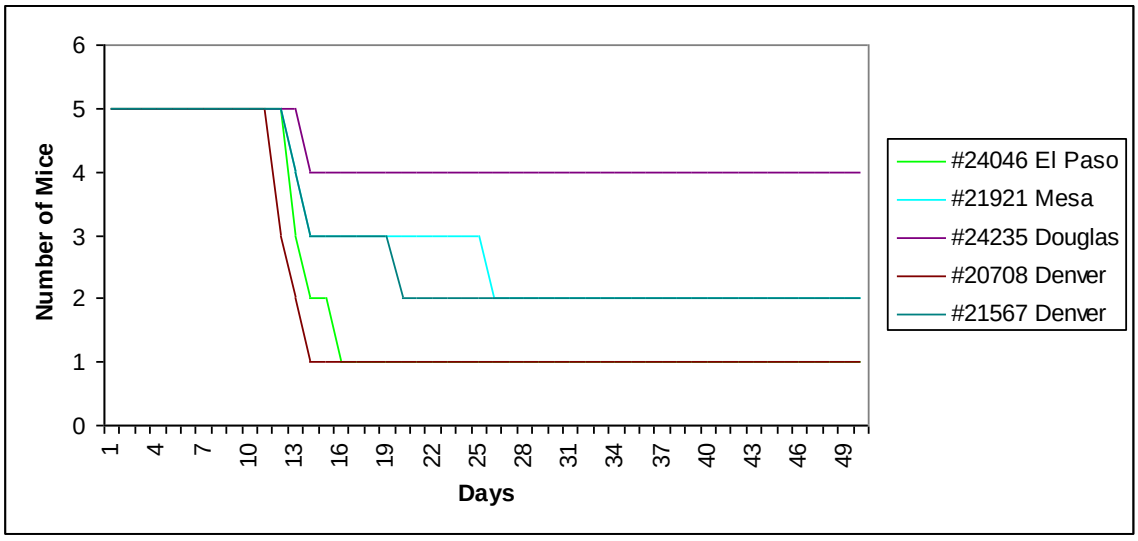

Figure 2-2: Survival of mice inoculated with 10^4 $TCID_{50}$ of RV, Experiment #1

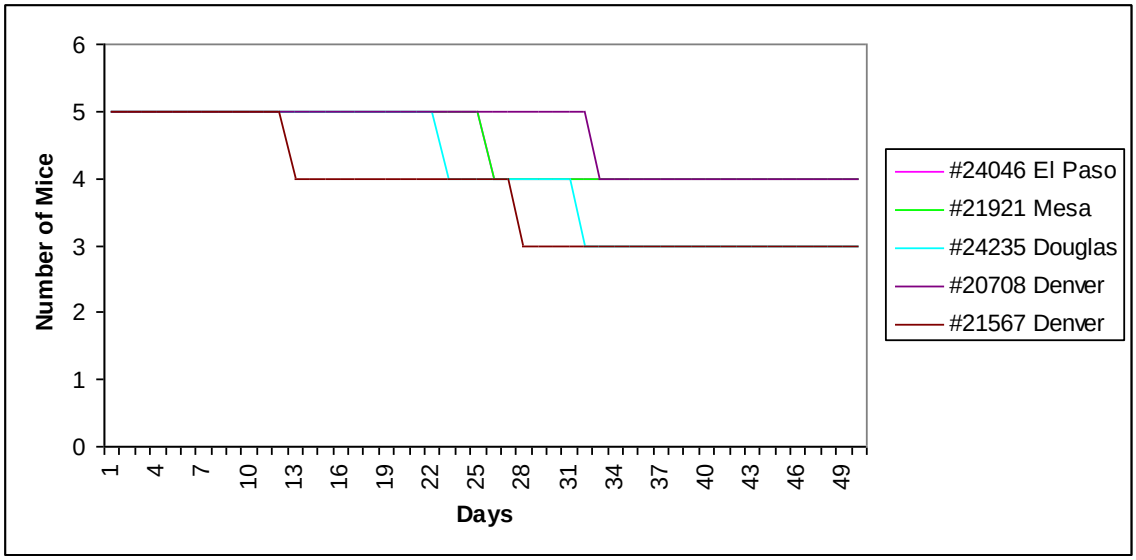

Figure 2-3: Survival of mice inoculated with 10^3 $TCID_{50}$ of RV, Experiment #1

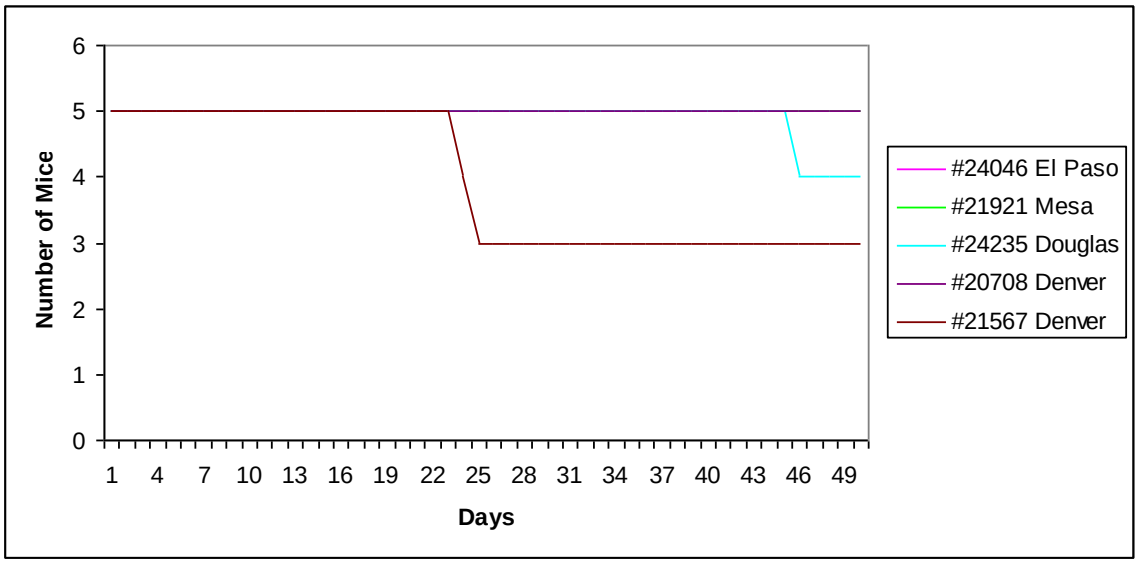

Figure 2-4: Survival of mice inoculated with 10^2 $TCID_{50}$ of RV, Experiment #1

Variation in Incubation Period

We initially attempted to use SAS Proc GLIMMIX to evaluate differences in length of the incubation period based on RV isolate at a particular dose, but such analysis was not possible because some of the mice survived until the end of the experiment,

which meant that incubation period was undefined. It is interesting to note that none of the mice inoculated with the #15100 V1 isolate succumbed to rabies within the 90 day trial (Figure 2-5).

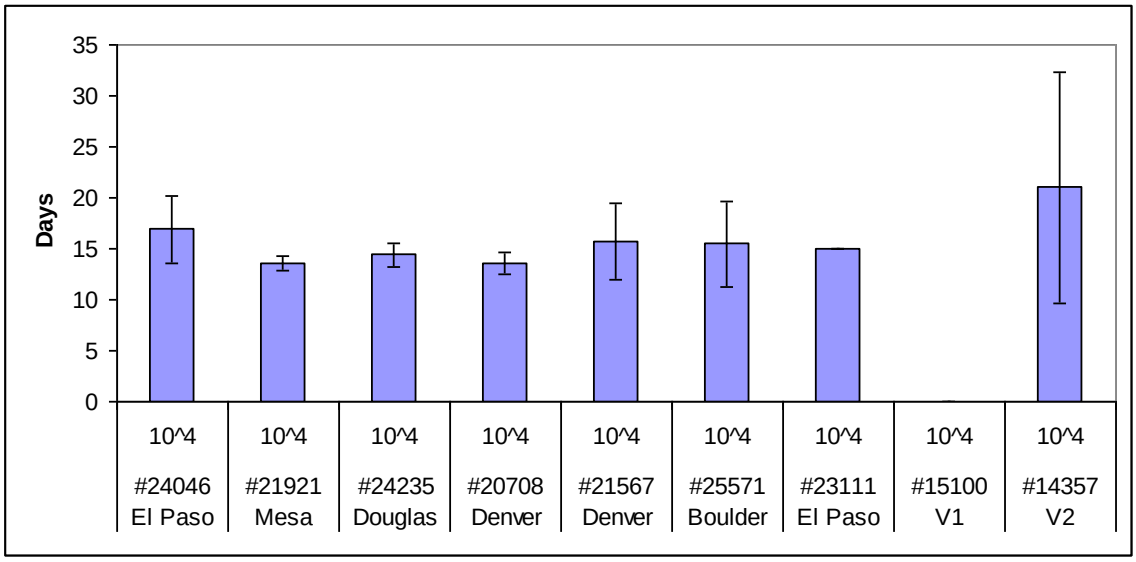

Figure 2-5: Incubation time in mice inoculated with 10^4 $TCID_{50}$ of nine RV isolates tested in Experiments 1 and 2. Bars indicate mean time from inoculation to euthanasia ± standard deviation.

Comparison of the incubation time of five RV isolates over the three different doses by Proc GLM failed to reveal significant differences among isolates. Mice inoculated at the viral dose of 10^4 $TCID_{50}$ had the shortest incubation time while a large majority of the mice inoculated with the viral dose of 10^2 $TCID_{50}$ survived until the end of the study. Comparing incubation period in mice inoculated with doses of 10^4 $TCID_{50}$ and 10^3 $TCID_{50}$ dose, and those inoculated with the higher dose appeared to have a shorter incubation time, even though that difference was not significant (Figure 2-6).

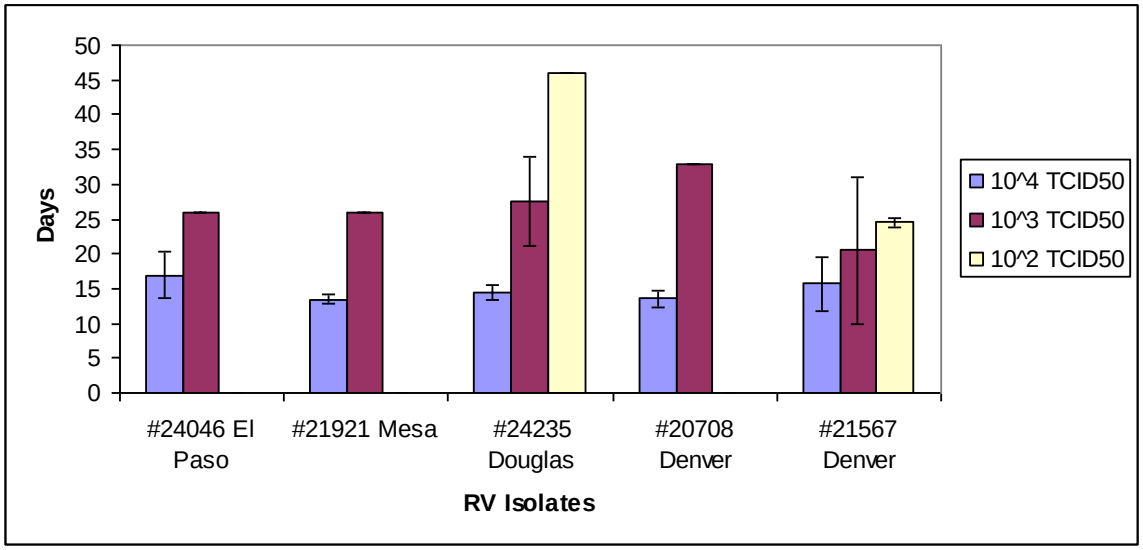

Figure 2-6: Incubation period for RV mortality in Experiment #1. Viruses that did not induce disease or mortality are not depicted. Bars indicate mean time from inoculation to euthanasia ± standard deviation.

Virus Antigen Detection in Brain Smears

Rabies diagnostic laboratories stain a smear from the brainstem of a suspected rabid animal with a polyclonal-DFA conjugate antibody to detect if there is any RV antigen present in the brain (CDC accessed 2010). Two brain smear slides were made for each mouse and immunostained for the detection of RV antigen. However, the brain smear staining results from mice that succumbed with symptoms of clinical rabies were inconclusive. Due to the inconsistency in the results, another RV virologist (Dennis Kohler, Staff Wildlife Disease Biologist with the USDA/APHIS/WS/National Wildlife Disease Program) was asked to also read the slides. He also could not distinguish between mice died from RV infection and which mice were healthy at the end of the study. Similar inconclusive results have also been documented by other research groups when using the FITC-conjugated rabies DFA reagent to detect RV in brain smears from animals that were rabid with the big brown bat RV variants (Rudd et al. 2005).

Therefore, diagnosis of RV infection was not based solely on the brain smears, but rather the ability to isolate RV from the brain tissue directly and rabies clinical symptoms. The brain smear results are combined in a table with the RV isolation in the next section (Table 2-5).

Rabies Virus Isolation

RV was isolated from the all the brain samples of all mice that succumbed to RV infection during this study (Table 2-5). RV was not isolated from any mice that died due to other causes or at the end of the study.

Virus Antigen Detection in Salivary Gland Smears was Found Only in Mice Inoculated with the 10^4 $TCID_{50}$ Dose

A total of 89% of the salivary glands from mice that died from clinical rabies were negative for RV by DFA analysis. However, 4 mice inoculated at the 10^4 $TCID_{50}$ dose with #24046 El Paso, #21921 Mesa and #20708 Denver viruses tested positive for RV antigens in their salivary glands (Table 2-5).

Table 2-5: Summary of Mortality, Virus Isolation and Antigen Detection in Brain and Salivary Glands: Experiments 1 and 2.

Virus	Virus Dose ($log_{10}TCID_{50}$)	Mortality	Presence Viral Antigen in Brain (+/number tested)	Isolation of Virus from Brain (+/number tested)	Presence of Viral Antigen in Salivary Gland (+/number tested)
#24046 El Paso	4	9/10	NC	7/7 *	2/9
	3	1/5	1/1	1/1	0/1
	2	0/5	NA	NA	ND
#21921 Mesa	4	2/5	NC	0/1 *	1/2
	3	1/5	1/1	1/1	0/1
	2	0/5	NA	NA	*
#24235 Douglas	4	5/10	NC	5/5	0/5
	3	2/5	NC	2/2	0/2
	2	1/5	NC	1/1	*
#20708 Denver	4	9/10	NC	5/5 *	1/9
	3	1/5	NC	1/1	*
	2	0/5	NA	NA	ND
#21567 Denver	4	3/5	3/3	1/1 *	0/3
	3	2/5	NC	*	0/2
	2	2/5	NC	2/2	0/2
#25571 Boulder	4	4/5	NC	4/4	0/4
#23111 El Paso	4	1/5	NC	1/1	0/1
#15100 V1	4	0/5	NA	NA	ND
#14357 V2	4	2/5	NC	1/2	0/2

NC = Not conclusive, NA = Not applicable
* Samples lost

Humoral Immune Responses

Sera were collected on day 14, and at 1, 2 and 3 months after inoculation of big brown bat RV isolates and evaluated for anti-rabies antibodies as measured by a RFFIT assay. Antiviral antibody titers varied widely within experimental groups, which was not unexpected. For example, out of five mice in the group inoculated with the RV isolate #15100 V1 at the viral dose of 10^4 $TCID_{50}$; one mouse had an antibody response above the level of detection for a RFFIT (>15625), while another mouse in the same group failed to develop a detectable antibody response (RFFIT <5) by one month post infection

(Table 2-6). A graphical representation of the neutralizing antibody response for each group over time can be found in Appendix 4.

Antibody responses of mice inoculated with 10^4 $TCID_{50}$ of the 9 RV isolates were statistically analyzed using Proc GLIMMIX Anova with repeated measures. No significant effect was found among the separate RV isolates, and within antibody responders over time. The 5 RV isolates that were inoculated at three different doses were also compared using Proc GLIMMIX Anova with repeated measures over time comparing RV isolate dose, time and all of their combinations for the humoral immune response. Significant interactions were not found, however, there was a significant different in the neutralizing antibody response related to dose ($p < 0.0001$). Since there were no interactions between RV isolate and dose over time, we analyzed the antibody data using Proc GLIMMIX to obtain the mean response for a given dose. A linear relationship was found with more mice inoculated at the 10^4 $TCID_{50}$ having measurable neutralizing antibody than mice inoculated at the 10^3 $TCID_{50}$ or 10^2 $TCID_{50}$ dose. In summary, there was a significantly larger fraction of mice that developed measurable antibodies after inoculation with 10^4 $TCID_{50}$ of virus than after inoculation with either 10^3 or 10^2 $TCID_{50}$ doses.

Mice that succumbed to RV infection also had an immune response at day 14. Due to the fact that there were no interactions between antibody response, and RV isolate over time, the analysis of survivors with a measurable antibody response and those that succumbed to rabies was measured using Fisher's Exact Test. There was no significant difference between the immune response mounted by mice who survived and mice that succumbed to RV infection ($p = 1.000$). There did not seem to be a particular pattern on

the amount of anti-rabies virus antibody produced over time. For some mice there was a decrease in the antibody response over time, while other mice had an increase in the amount of antibody produced over time. This variation in the amount of antibody seems to be a biological response and has also been seen in other studies (Manning et al. 2008, Constantine 1968, Steece and Altenbach 1989).

Table 2-6: Summary of the Anti-rabies virus antibody response						
Virus	Dose ($TCID_{50}$)	14 days	1 month	2 months	3 months	4 months
#24046 El Paso	10^4	n = 9	n = 1	n = 1	n = 1	n = 1
		213 (5 – 1108)	5931 (0)	1658 (0)	1435 (0)	6025 (0)
	10^3	n = 5	n = 5	n = 4	ND	ND
		< 5 (0)	7 (5 – 14)	7 (5 – 12)	ND	ND
	10^2	n = 5	n = 5	n = 4	ND	ND
		5 (5 – 6)	5 (0)	5 (0)	ND	ND
#21921 Mesa	**10^4**	**n = 5**	**n = 3**	**n = 3**	**n = 3**	**n = 3**
		970 (71 – 2881)	7293 (274 – 15625)	4909 (120 – 9806)	6933 (243 – 15625)	3871 (280 – 9865)
	10^3	n = 5	n = 5	n = 4	ND	ND
		7 (5 – 15)	288 (5 – 1290)	29 (5 – 55)	ND	ND
	10^2	n = 5	n = 5	n = 5	ND	ND
		< 5 (0)	< 5 (0)	< 5 (0)	ND	ND
#24235 Douglas	**10^4**	**n = 9**	**n = 5**	**n = 5**	**n = 5**	**n = 4**
		217 (5 – 542)	1679 (12 – 5137)	1057 (76 – 3857)	660 (68 – 2059)	370 (44 – 1267)
	10^3	n = 5	n = 5	n = 4	ND	ND
		61 (5 – 287)	274 (5 – 1116)	339 (5 – 1341)	ND	ND
	10^2	n = 5	n = 5	n = 5	ND	ND
		< 5 (0)	< 5 (0)	57 (5 – 267)	ND	ND
#20708 Denver	10^4	n = 10	n = 1	n = 1	n = 1	n = 1
		500 (6 – 1625)	1178 (0)	1595 (0)	758 (0)	540 (0)
	10^3	n = 5	n = 4	n = 4	ND	ND
		15 (5 – 53)	67 (5 – 242)	4553 (67 – 15625)	ND	ND
	10^2	n = 5	n = 5	n = 5	ND	ND
		< 5 (0)	38 (5 – 172)	43 (5 – 197)	ND	ND
#21567 Denver	10^4	n = 5	n = 2	n = 2	n = 2	n = 2
		65 (5 – 265)	1296 (59 – 2533)	165 (63 – 267)	131 (47 – 214)	42 (25 – 58)
	10^3	n = 5	n = 4	n = 3	ND	ND
		< 5 (0)	82 (5 – 267)	430 (5 – 1281)	ND	ND
	10^2	n = 5	n = 4	n = 3	ND	ND
		< 5 (0)	411 (5 – 1361)	< 5 (0)	ND	ND

Table 2-6: Summary of the Anti-rabies virus antibody response (continued)						
#25571 Boulder	10^4	n = 2	n = 1	n = 1	n = 1	ND
		116 (73 – 158)	2264 (0)	141 (0)	143 (0)	ND
#23111 El Paso	10^4	n = 1	n = 4	n = 4	n = 3	ND
		11 (0)	< 5 (0)	30 (5 – 106)	10 (5 – 19)	ND
#15100 V1	10^4	ND	n = 5	n = 4	n = 5	ND
		ND	3150 (5 – 15625)	3910 (5 – 15625)	1409 (5 – 7015)	ND
#14357 V2	10^4	n = 1	n = 4	n = 3	n = 3	ND
		13 (0)	417.3 (5 – 1654)	10 (5 – 19)	< 5 (0)	ND

DISCUSSION

Bat rabies is a health issue for both people and animals around the world (Calisher et al. 2006, Wong et al. 2007). A better understanding of the different *Chiropteran* RV variants that are found circulating in natural reservoirs can only help in developing programs to manage these different RV variants (Blanton et al. 2010, Whitaker et al. 2006). One major gap in our understanding of bat rabies is the phenotypic variability within a single genotypic or antigenic variant. We know such variability exists among RV variants. For example, the silver-haired bat RV variant is known to be more virulent than the coyote or the dog RV variants and is responsible for the majority of human cryptic cases and death due to rabies in the United States along with the tricolored bat RV variant (Dietzschold et al. 2000, Messenger et al. 2002, Morimoto et al. 1996, Preuss et al. 2009). We addressed this question of intravariant variability by screening nine RV isolates from Colorado big brown bats for their propensity to induce both antibody responses and mortality in mice. The nucleocapsid gene of each of these RV isolates was most closely related to a pair of previously reported isolates from big brown bats in El Paso County, Colorado.

The mortality at the high 10^4 $TCID_{50}$ dose was different among four different isolates (#24046 El Paso, #20708 Denver, #V1 15100, and #23111 El Paso). Different RV variants are known to induce different degrees in pathology in mammalian species but the difference within these different variants is virtually unknown.

During this study not all the mice that were inoculated with RV succumbed to RV infection, which was expected based on multiple other studies of experimental rabies inoculation (Morimoto et al. 1996, Preuss et al. 2009). As with all pathogens, there is a

relationship between the dose of the RV inoculated and the number of mice that survived the inoculation (Fekadu and Shaddock 1984, Hronovsky et al. 1969). The dose of virus may have been low enough and the mouse's innate immune response was strong enough to clear the infection at the inoculation site before the virus had a chance to enter into the nervous system (Faul et al. 2010, Johnson et al. 2008). Some mice showed signs compatible with the early stages of rabies, but instead of progressing towards death, these mice recovered. Although not common, this phenomenon has been observed in a human case of rabies in the United States in 2009 (CDC et al. 2010). Alternatively, the signs observed may have been induced by some other factor. Lastly, the length of the study may not have been long enough, due to varied incubation periods, to allow all the mice that were inoculated with big brown bat RV to develop clinical rabies (Madhusudana and Tripathi 1990, Mork 2004, Trimarchi et al. 1986).

The incubation period in mice that succumbed to RV infection was similar among isolates, but, as expected, mice receiving higher doses of virus appeared to manifest shorter incubation times. A varied incubation time in RV has also been observed in other natural and experimental settings (Trimarchi et al. 1986). Statistical tests comparing incubation time induced by the different RV isolates was not appropriate because in some inoculation groups all mice survived the RV challenge. Based on the graphical data, mice inoculated with the highest dose had the shortest incubation period while mice inoculated with the lowest dose of RV had the longest incubation period. Similarly, visual observation of the data indicates that mice inoculated at the highest dose had the highest mortality while mice inoculated with the lowest dose had the lowest mortality. This observation has been made in other studies and may warrant another look at these

RV isolates with a larger population in a follow-up study (Fekadu and Shaddock 1984, Hronovsky et al. 1969)

The CDC protocol for the diagnosis of RV in the brain of any mammal in the United States is done by direct fluorescent antibody testing (DFA) (CDC accessed 2010). Based on the results of this test, the decision to administer post-exposure prophylaxis (PEP) treatment to exposed individuals is made. Unfortunately, by the time someone starts showing symptoms of RV infection, treatment is often too late, so vaccinating the patient before disease develops is critical (Brass 2009, CDC accessed 2011 and WHO accessed 2010). However, the DFA protocol may be problematic when applied to big brown bat RV variants that are found in the Midwest (Rudd et al. 2005). Our results demonstrated an ambiguity for using the DFA protocol alone for the diagnosis of mice that had been inoculated with a big brown bat RV variant and died of RV-like symptoms. Instead we had to rely on clinical symptoms and virus isolation from the brains of these mice. This raises a concern since the big brown bat is the bat species most often submitted for RV testing in Colorado and other western states. Some RV positive bats may not be properly diagnosed (Pape et al. 1999). This could result in a false negative for a RV positive brain and result in PEP be not being given to an individual when in fact they should have received treatment. Diagnostic labs generally only depend on the DFA diagnosis for RV infection and do not attempt to isolate virus (CDC accessed 2010).

Guidelines for the vaccination of animals and people can be found on the CDC website (www.cdc.gov). They recommend that individuals who work with RV or have high risk jobs that could expose them to RV be vaccinated as pre-exposure protection. However, if that individual is exposed to RV they must receive two additional RV

vaccinations to insure that RV disease does not develop (Manning et al. 2008). This is because the immune response to the rabies vaccination is extremely varied from one individual to another, and the protective threshold for anti-rabies antibodies in humans is not well understood. Given the nature of RV disease, it is better to error on the side of caution (Manning et al. 2008, CDC accessed 2011, WHO accessed 2010). Likewise, the immune response observed in the ICR mice that had been inoculated with big brown bat RV isolates varied hugely, corroborating findings in humans.

There was no significant difference in the amount of anti-rabies virus antibody that was produced between mice who succumbed to RV infection and mice who survived. This raises the question of why some mice survived while other mice died when they were all inoculated with the same big brown bat RV variant by the same inoculation route. The immune response in the mice who succumbed to RV infection could have been too little of a response too late to neutralize the RV that had entered into the brain. In an epidemiological study of human rabies in the United States, they found that the majority of the serum samples from individual's hospitalized with clinical rabies had no anti-rabies antibody at the beginning of clinical symptoms. The appearance of neutralizing antibodies was generally found 7-10 days into rabies illness (Noah et al. 1998).

The amount of inflammation at the inoculation site could also be another factor in the generation of a surviving immune response (Johnson et al. 2008). All the mice were injected with 50 µl of inoculum in the right triceps muscle of the front leg. If the innate immune response was able to neutralize the RV before it entered into the neurons, the mouse will clear the RV from their system before rabies occurs (Hooper et al. 1998). We

chose to inoculate all the mice in the front right triceps muscle in the hopes of more closely replicating the natural route of infection, a bite wound (Murphy 1977). However, a sterile needle is far less traumatic and contaminated than teeth and may not cause much of an inflammatory response, and therefore, less of an innate immune response, resulting in more clinical rabies than observed naturally with this RV variant (Hooper et al. 1998, Wiktor et al. 1977).

Statistically there was no difference in the immune response between the different RV isolates at the same inoculation dose. There was however, a significant difference between the groups that received different doses of virus in the first experiment over time ($p < 0.0001$). A larger fraction of mice inoculated with the 10^4 $TCID_{50}$ dose developed neutralizing antibodies in comparison to mice inoculated with the 10^3 or 10^2 $TCID_{50}$ doses. This dose effect was found to be a linear relationship with the 10^4 $TCID_{50}$ dose being ranked the highest and the 10^2 $TCID_{50}$ group the lowest.

Overall, this study indicated that in a population of nine, independently isolated RV from individuals of a single bat species, there were not major differences in virulence or ability to induce neutralizing antibodies. At the level of confidence used in this study, we therefore rejected the hypothesis that major differences in virulence existed within the virus population tested. This is, to our knowledge, the only study in which individual isolates of RV within a single variant have been directly compared with one another. The findings of this study demonstrate that the incubation length to clinical rabies and the number of mice that succumbed to rabies is more dependent on the viral dose than the different big brown bat RV isolate. The humoral response to RV also followed this pattern, with a difference found between mice that had neutralizing antibodies in the

higher dose groups than mice in the lower dose groups. Although the RV isolates were differentiated by their nucleocapsid gene sequence, there were almost certainly other genetic differences in the viral genomes. This could be evident with the #15100 V1 isolate where all the mice survived, compared to #24046 El Paso and #20708 Denver where nine out of ten mice succumbed to rabies challenge. Even though this study showed little variation within the big brown bat RV variants, bats are a major reservoir for rabies and additional work plainly needs to be done to understand the intricacies of this virus and disease.

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CHAPTER 3

SEROLOGICAL RESPONSE TO MULTIPLE LOW DOSE INOCULATIONS VIA DIFFERENT ROUTES AND SCHEDULES OF BAT RABIES VIRUS VARIANT IN MICE

ABSTRACT

Rabies is usually considered an inevitably lethal disease, yet neutralizing antibodies to rabies virus (RV) have frequently been found in healthy, wild, non-rabid insectivorous bats in several countries, suggesting that non-lethal infections are commonplace. To study this phenomenon, we evaluated the humoral immune response of mice following exposure to a big brown bat RV variant. The experiment was designed as a factorial study to evaluate three routes of inoculation, two doses of virus and two frequencies of exposure. Mice were inoculated via intramuscular, intradermal and intranasal routes to simulate three different routes of natural infection (deep bite, shallow bite or aerosol), with one of two low doses of virus (500 versus 50 $TCID_{50}$) and using two schedules to mimic repeated versus occasional exposure to virus. We found that repeated intramuscular inoculation with the higher of the two doses of virus resulted in the highest fraction of mice developing neutralizing antibodies to RV. Mice that were inoculated intranasally experienced the highest mortality. Mice inoculated intradermally had the lowest rate of seroconversion. Mice were re-challenged at 3 months with 10^4 $TCID_{50}$ of

virus intramuscularly to determine if priming of the immune system to RV had occurred, but none of the mice, including naïve controls succumbed to the re-challenge.

INTRODUCTION

The natural phenomenon of healthy wild bats with anti-rabies antibodies seems to be in contradiction with rabies virus (RV) as a zoonotic disease that is virtually always fatal once symptoms appear (Constantine et al. 1968, Perez-Jorda, et al. 1995, Salas-Rojas et al. 2004, Steece and Altenbach 1989, Jiang et al. 2010, O'Shea et al. 2003, WHO 2010). There have been a few cases reported from the United States of human survival of RV infection, resulting in both permanent neurological damage and one case of complete recovery (Willoughby et al. 2005, CDC et al. 2010). However, there are multiple reports of wild bats with anti-rabies neutralizing antibodies (Constantine et al. 1968, Perez-Jorda, et al. 1995, Salas-Rojas et al. 2004, Steece and Altenbach 1989, Jiang et al. 2010, O'Shea et al. 2003). Some of these bats have been tagged and captured multiple years in a row, far beyond the normal incubation period for clinical disease (Perez-Jorda et al. 1995, O'Shea et al. 2003). Fluctuations in the number of bats with anti-rabies antibody occur year to year (Perez-Jorda et al. 1995, Steece and Altenbach 1989). Both natural and experimental infections have shown that bats are susceptible and can become sick and die of rabies; however, some bats survive (Blanton et al. 2010, Constantine et al. 1968, Turmelle et al. 2010, Davis et al. 2007, Jackson et al. 2008).

Although the bite route is the major mode of RV transmission, bats in particular are also exposed to RV through non-bite routes. These include aerosol transmission, as has been documented in Frio cave where bats live in very close quarters and the air does

not circulate well (Constantine et al. 1962). Bats may also be exposed to RV through a scrape of the skin or a shallow bite from a neighbor; considering the very high population densities in maternity colonies, this route of exposure may be routinely experienced.

The rationale of this experiment was to determine the neutralizing antibody response of mice to inoculation with a big brown bat RV variant, with the goal of gaining insight into possible mechanisms by which many wild adult bats acquire anti-rabies antibodies but fail to become rabid. To explore the phenomenon of seropositivity in the absence of disease, we exploited a mouse model of rabies, attempting to duplicate what may be happening in natural bat populations. We inoculated mice with a big brown bat variant of RV by three different inoculation routes, two different inoculation schedules and two different inoculation doses. We hypothesized that mice inoculated intramuscularly with repeated low doses would have a robust immune response, while mice inoculated intranasally with repeated low doses will have the highest mortality and mice inoculated intradermally with a small number of low doses will have the lowest immune response and mortality.

MATERIALS AND METHODS

Experimental design

Outbred adult (~4 months old) ICR mice were used to approximate the genetic variation observed in natural populations. Three different routes of inoculation were used. Mice were inoculated via the intramuscular route to model the bite route as the most common route of transmission, intranasally to mimic the aerosol route and intradermally to imitate shallow bites. Two different viral doses were evaluated to

simulate low dose shedding from an infected bat before its colony mates excluded it from the colony. Finally, two different inoculation schedules were used to determine if frequent exposure to low doses of RV induced a more robust neutralizing antibody response than a small number of exposures. The details of this design are depicted in Table 1 below.

Table 3-1: Experimental Groups for Low Dose Viral Inoculation

Group	Inoculation Dose ($TCID_{50}$)	Inoculation Route	Frequency of Inoculation
1	50	IM	Twice weekly for 6 weeks
2	50	IM	Days 0 and 28
3	50	ID	Twice weekly for 6 weeks
4	50	ID	Days 0 and 28
5	50	IN	Twice weekly for 6 weeks
6	50	IN	Days 0 and 28
7	500	IM	Twice weekly for 6 weeks
8	500	IM	Days 0 and 28
9	500	ID	Twice weekly for 6 weeks
10	500	ID	Days 0 and 28
11	500	IN	Twice weekly for 6 weeks
12	500	IN	Days 0 and 28

To determine whether the antibody response generated through these different inoculation criteria was protective, mice were re-challenged with the same big brown bat RV variant after 3 months with a higher dose of RV (10^4 $TCID_{50}$) intramuscularly and clinical and antibody responses monitored for an additional month.

Rabies Virus Preparation

Big brown bat RV variant #20708 Denver was propagated in mouse neuroblastoma (NB) cells as previously described (Chapter 2) with the modification of using 1% mouse serum in the growth medium instead of 5% fetal bovine serum; this modification was made to preclude the complicating factor of inoculating mice with repeated doses of fetal bovine serum proteins. RV variant was harvested for further

analysis or use of the virus by adding 1 ml mouse serum to the 6 ml growth media in the flask. This medium was transferred into a 15 ml tube, centrifuged for 10 minutes at 400 x g and aliquots of 0.5 ml were stored at -80^{o}C.

Detection and Titration of a Rabies Virus

RV was isolated from brain homogenates on NB cells and detected by direct immunofluorescence using anti-rabies antibodies. Cultured NB cells infected with stock RV were used as a positive control for immunofluorescence. These cells were trypsinized from flasks, suspended in PBS washed twice by centrifugation and resuspended in PBS. The final cell pellet was resuspended in 200-500 µl of PBS and drops of cell suspension were pipetted onto spot slides (Teflon Printed Slides 8-Well, 6mm diameter, Electron Microscopy Sciences). After a few seconds, excess liquid was aspirated, leaving a uniform film of cells on the slide. Slides were allowed to air dry, fixed in 100% acetone for at least 1 hour and stored at -20^{o}C until staining.

Immunostaining for RV antigens was accomplished by applying drops of FITC-labeled mixture of mouse monoclonal antibodies that bind to RV nucleocapsid protein (Light Diagnostics Rabies DFA Reagents; diluted 1:100) to each slide, covering with a coverslip, and incubating for 45 minutes at 37^{o}C in 5% CO_2 in air. Coverslips were removed and slides were washed three times for five minutes each in PBS and once in water. Slides were then air dried and mounted with Dako Fluorescent Mounting Medium and examined with an upright fluorescence microscope (Nikon Eclipse E800).

Stock viruses were titrated on NB cells using a standard quantal assay. Serial 10-fold dilutions of virus (typically 10^{-1} to 10^{-7}) were prepared in growth medium and 50 µl of each diluted sample was pipetted into 5 replicate wells in a 96 well plate. NB cells

were trypsinized, counted and distributed into 96 well plates at 25,000 cells per well. Plates were incubated for three to four days at 37^{o}C in 5% CO_2, then the medium was discarded, and the plates were rinsed once with PBS and fixed with 70% acetone for at least one hour. Plates were air dried and stained either immediately or stored in the refrigerator until they were stained. Staining for RV antigens was conducted as described above for spot slides, and the plates were evaluated using an inverted fluorescence microscope. The 50% tissue culture infective dose ($TCID_{50}$) was calculated for each sample by the Spearman-Karber method (Smith et al. 1996).

Inoculation and Monitoring of Mice

Young adult, female ICR mice were purchased from Charles River Laboratories, housed in groups of 5 and maintained under ABSL-3 conditions with free access to water and rodent chow throughout the experiment. Mice were 8 weeks old at the time of delivery and were housed until 4 months of age before they were inoculated with RV. Each mouse was identified by an ear punch in their right ear. Mice were visually evaluated daily for signs of disease or distress.

Twelve groups of five mice were inoculated with the big brown bat RV variant #20708 Denver by one of three routes: intramuscularly in the hind quadriceps muscle, intradermally in the hind limb just under the skin above the quadriceps muscle, or intranasally. The intramuscular and intradermal inoculations delivered a 25 µl volume using an insulin syringe with a 28 gauge needle. Mice inoculated intranasally were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (100 and 10 mg/kg respectively) and 25 µl of inoculum was delivered into the nares using a 200 µl pipette. Half of the groups were inoculated with a dose of 500 $TCID_{50}$ and half with 50

$TCID_{50}$. Again half the groups were inoculated on two different schedules either on days 0 and 28 or twice weekly for six weeks (Table 3-1). The inoculum was backtitrated on NB cells once weekly.

Following inoculation, mice were monitored twice daily for clinical signs of disease. When definitive signs of rabies were observed, the mice were anesthetized with ketamine, bled by cardiac puncture and euthanized by cervical dislocation. The brains were placed in tubes and stored at -80^{o}C for RV isolation or DFA staining. Slip smears of brainstem were prepared on standard microscope slides, fixed in acetone and stained for DFA detection of RV antigens as described above.

Isolation of Rabies Virus from Infected Mouse Brains

Mice that developed clinical rabies were euthanized, and their brains frozen to -80^{o}C. The whole of the brain was subsequently thawed, homogenized in 1 ml of growth medium using a 7 ml Ten Broeck tissue grinder and centrifuged (9000 x g for 5 min) to remove cellular debris. One hundred microliters of the supernatant was diluted into 1 ml of growth media and inoculated into a 25 cm^2 (T25) flask containing 50-80% confluent NB cells. The cells were incubated for 30 minutes at 37^{o}C in 5% CO_2, then 5 ml of growth medium was added to each flask and the cells were incubated for another 24 hours. After 24 hours the growth medium from each flask was removed, the cells were rinsed with PBS and 6 ml of fresh growth media was added back to each flask. Cells were checked daily for cellular death. After 3 days incubation, the cells were rinsed with PBS, trypsinized and split into a new T25 flask at a ratio of 1:5 and returned to the incubator for another 3 days. The remaining cells were used to make spot slides to check for RV antigens by DFA. This process was repeated up to three passages for each brain

sample. If, after three passages RV infection was not detected, the sample was considered negative.

Rapid Fluorescent Focus Inhibition Test (RFFIT)

All serologic testing was performed using a variation of the RFFIT originally described by Smith and colleagues (1996). Blood was collected from the mice via submandibular vein puncture, allowed to clot, and then centrifuged to separate the serum. Sera were inactivated at 56^{o}C for 30 minutes prior to testing for neutralizing antibody. A series of five-fold dilutions of each serum sample was prepared in a 96 well plate using growth medium as the diluent. Each assay incorporated a positive control consisting of the WHO rabies immunoglobulin standard (RIG) diluted as for mouse serum, with the lowest dilution of 1 U/ml. Wells containing growth medium were included on each plate as negative controls. The CVS-11 strain of RV at a concentration of 160 $TCID_{50}$/well was used as the challenge virus. Forty microliters of challenge virus was added to 40 µl of each serum dilution and those mixtures incubated for 1 hour at 37^{o}C in 5% CO_2 to allow neutralization to occur. A 100 µl aliquot containing 40,000 baby hamster kidney cells (BHK-21) was added to each well and the plates were incubated for 20 hours at 37^{o}C with 5% CO_2. Medium was then decanted; the plates were rinsed one time with PBS and fixed in 70% acetone for at least one hour. Plates were air dried and immunostained either immediately or stored in the refrigerator until they were stained as described above. After staining, 50 µl of PBS was added to each well and plates were stored overnight in the refrigerator. Wells were examined using an inverted fluorescence microscope and 20 fields were classified as having or not having positive cells. Based on

these numbers, the neutralization titer was calculated using the Spearman and Karber method (Manning et al. 2008, Smith et al. 1996).

Statistical Analyses

All statistical tests were calculated using SAS/STAT ® software version 9.2 © 2010. Comparison of mortality and antibody response for all 12 groups was done using the SAS procedure, Proc GLIMMIX with a binary distribution given dichotomous dependent variables. Mortality and antibody response analyses had independent variables of inoculation route, viral dose and frequency of inoculation and dependent binomial variables of death versus survival and antibody response verses no antibody response. Antibody responses were compared at different time points throughout the study. Due to the limits of using Proc GLIMMIX with zero percent mortality or no antibody response, a pairwise comparison among different RV isolates was performed. Mortality and antibody response were re-evaluated pairwise based on their percent differences using McNemars Chi square or Pearson's Chi square tests.

RESULTS

Inoculation Dose Backtitration

In previous work described in Chapter 2, RV variant #20708 Denver was found to induce clinical disease in mice at a dose of 10^4 $TCID_{50}$ but not at a dose of 10^2 $TCID_{50}$. Titration of the stock #20708 Denver RV indicated a titer of ~700,000 $TCID_{50}$/ml. Initially, a 1:35 dilution of virus in growth medium with 1% mouse serum was titrated to ensure a low enough dose was administered to the mice. This viral titration resulted in a titer of 739 $TCID_{50}$/25 µl. This value was slightly above the target of 500 $TCID_{50}$/25 µl

so 1:50 and 1:500 dilutions were used for all subsequent mouse inoculations to achieve 500 and 50 $TCID_{50}$.

Weekly titrations were performed on the 1:50 viral inoculation dilution to confirm that the inoculation dose was less than ten-fold different from the 500 $TCID_{50}$/25 µl target (Figure 3-1). The mean over the six weeks was 352 $TCID_{50}$/25 µl with a range of 158 $TCID_{50}$/25 µl to 601 $TCID_{50}$/25 µl, which was considered acceptable with respect to reproducibility of the inoculum from week to week during this experiment.

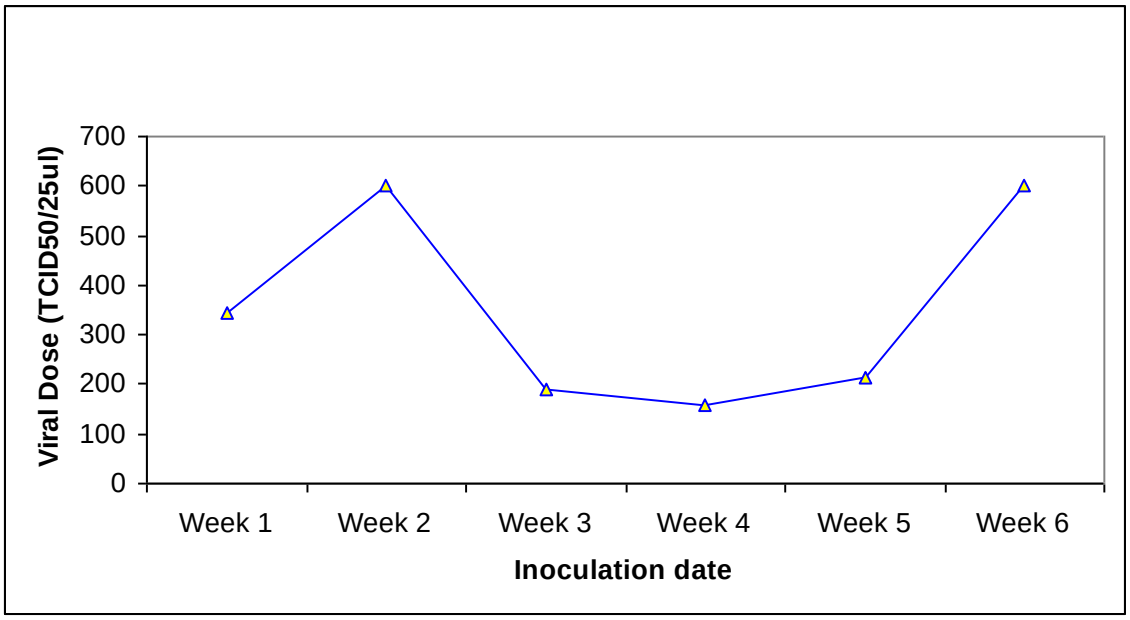

Figure 3-1: Weekly Viral Dose Inoculation Titration of Rabies Virus #20708 Denver

Mortality from Rabies Following Inoculation of Rabies Virus

Across all other factors, mice that were inoculated the intranasal route experienced higher mortality than mice inoculated intramuscularly or intradermally (Chi Square analysis, $p = 0.0013$). Those mice inoculated with the 500 $TCID_{50}$ dose were more likely to succumb to clinical rabies than mice inoculated at the 50 $TCID_{50}$ dose ($p =$

0.0025), and mice inoculated multiple times over the course of the study were more likely to experience mortality than mice inoculated only twice ($p = 0.0099$).

All mice inoculated via the intramuscular or intradermal routes survived until the end of the study. Seven mice from three different groups inoculated intranasally succumbed to clinical rabies. Four out of seven of these mice were from the five mice in group 11 that were inoculated multiple times with the 500 $TCID_{50}$ dose of virus (Table 3-2). Clinical symptoms generally were observed 24 to 48 hours before euthanasia. All mice that were euthanized due to clinical symptoms of rabies were found to have RV in their brain by DFA (Figure 3-2). Mouse 1 from group 7 was removed from the study due to non-study complications and was negative via brain smear DFA. This mouse was not showing signs of clinical rabies, so the decision was made not to attempt virus isolation.

Table 3-2: Morbidity and Mortality Summary for Each Group

Group	Inoculation Dose ($TCID_{50}$)	Inoculation Route	Frequency of Inoculation	Incubation Period (days)	Mortality Rate
1	50	IM	2X/week, 6 weeks	NA	0/5
2	50	IM	Days 0 and 28	NA	0/5
3	50	ID	2X/week, 6 weeks	NA	0/5
4	50	ID	Days 0 and 28	NA	0/5
5	50	IN	2X/week, 6 weeks	NA	0/5
6	50	IN	Days 0 and 28	27	1/5
7	500	IM	2X/week, 6 weeks	NA	0/5
8	500	IM	Days 0 and 28	NA	0/5
9	500	ID	2X/week, 6 weeks	NA	0/5
10	500	ID	Days 0 and 28	NA	0/5
11	500	IN	2X/week, 6 weeks	37 (27-45)	4/5
12	500	IN	Days 0 and 28	27	1/5

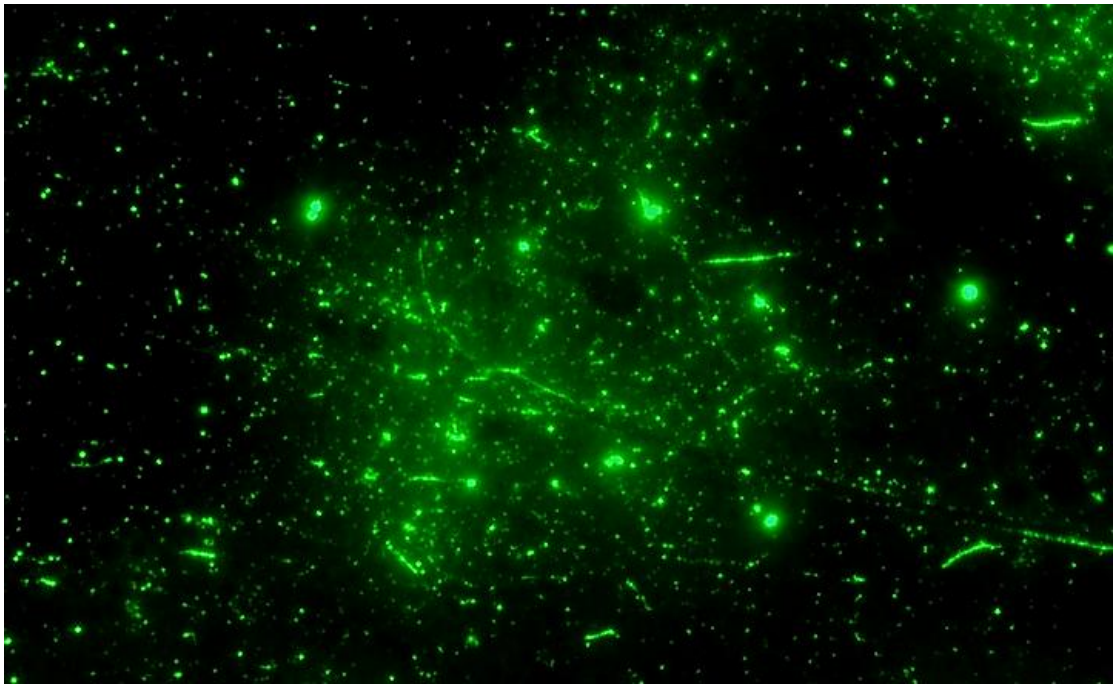

Figure 3-2: Brain smear of mouse 6-2 from group 6 that was positive for RV via DFA.

Effects of Re-challenge with a High Dose of Rabies Virus

Mice were re-challenged by intramuscular inoculation in the right triceps muscle with a dose of 10^4 $TICD_{50}$ of the #20708 Denver RV three months after the initial inoculation of virus. Seven naïve mice of the same age were also inoculated as a positive control for this viral dose. None of the mice succumbed to clinical rabies within 4 weeks of the re-challenge, including those from the naïve control group. At the time of termination, brain smears were made from the brains of every mouse to insure that they were negative for RV and did not have an asymptomatic infection. Of 61 mice tested, the results from brain smears were negative for 46 mice but were inconclusive due to high background for 15 mice. RV isolation from brain homogenates was attempted for these 15 inconclusive mice and was found to be negative for virus for all but one mouse in group 2. Surprisingly this mouse was found to have RV in the brain by RV isolation; this animal failed to have a detectable antibody response until the terminal bleed at 1 month

after the re-challenge. It seems likely that the study was terminated while this mouse was in the prodromal stage and that it would have developed clinical symptoms had she been maintained. It is possible, though unlikely, that she was in the process of clearing the infection due to previous priming of the immune system through the 2 intramuscular inoculations received 28 days apart.

Humoral Immune Response

The concentration of neutralizing antibody determined by the RFFIT assay varied substantially from mouse to mouse within groups, which is frequently seen in antibody responses to any antigen. Due to this variability in antibody titer and the fact that titer *per se* is typically not that informative, we elected to present immune responses in terms of whether a mouse did or did not have measurable antibody (i.e. titer ≥ 5). However, all antibody titer data is presented in Appendix 5.

Influence of Virus Inoculation Route on Antibody Responses

Comparison of antibody responses among the three inoculation routes, independent of inoculation schedule or dose, revealed that mice inoculated intradermally were significantly less likely to develop a detectable antibody response compared to mice inoculated by intramuscular or intranasal routes (Pearson's Chi Square, $p = 0.025$). Three of four groups inoculated intradermally failed to develop a detectable neutralizing antibody response at any point during the first three months (Table 3-3).

Table 3-3. Neutralizing Anti-Rabies Virus Antibodies in Mice Inoculated Intradermally.

Group #	Dose given ($TCID_{50}$/25ul)	Inoculation Schedule	Antibody detection*			
			14 day	1 month	2 months	3 months
3	50	Multiple	0/5	0/5	0/5	0/5
4	50	2x	0/5	0/5	0/5	0/5
9	500	Multiple	0/5	0/5	1/5	2/5
10	500	2x	0/5	0/5	0/5	0/5

* Number of mice with RFFIT titers ≥ 5 / number of mice in group

Visual evaluation of the data over all inoculation groups showed that mice inoculated intramuscularly had the highest neutralizing rabies antibody titer response and mice that were inoculated intradermally had the lowest response (Tables 3-3 and 3-4). Among mice that were inoculated by the intranasal route, neutralizing anti-rabies antibody was detected in all 7 mice that succumbed to clinical infection and in two of the 14 mice that survived to the end of the study. Neutralizing anti-rabies antibody was also detected in the terminal serum from mice that developed clinical symptoms of rabies. The phenomenon of mice first having neutralizing antibodies during clinical symptoms of rabies was also observed in our previous study (Table 3-5).

Table 3-4. Neutralizing Anti-Rabies Virus Antibodies in Mice Inoculated Intramuscularly

Group #	Dose given ($TCID_{50}$/25µl)	Inoculation Schedule	Antibody detection*			
			14 day	1 month	2 months	3 months
1	50	Multiple	0/5	0/5	1/5	1/5
2	50	2x	0/5	0/5	0/5	1/5
7	500	Multiple	0/5	4/5	4/5	4/4**
8	500	2x	0/5	0/5	1/5	0/5

* Number of mice with RFFIT titers ≥ 5 / number of mice in group
** One mouse was removed from the study due to non-study complications.

Table 3-5. Neutralizing Anti-Rabies Antibodies in Mice Inoculated Intranasally.

Group #	Dose given (TCID50/25ul)	Inoculation Schedule	Antibody detection*			
			14 day	1 month	2 months	3 months
5	50	Multiple	0/5	0/5	0/5	0/5
6	50	2x	0/5	1/5	0/4	0/4
11	500	Multiple	0/5	1/5	4/4	1/1
12	500	2x	0/5	1/5	1/4	1/4

* Number of mice with RFFIT titers ≥ 5 / number of mice in group

Influence of Virus Inoculation Frequency and Dose on Antibody Responses

When the influence of RV dose was assessed independent of inoculation route or schedule, more mice inoculated with the 500 $TCID_{50}$ dose had RV neutralizing antibody at the 1 month post-inoculation time point (Pearson's Chi Square, $p = 0.04$) compared to mice inoculated with the 50 $TCID_{50}$ dose. This pattern of significance was also observed with the two ($p = 0.001$) and three month ($p = 0.014$) serum samples, where were also analyzed by Pearson's Chi Square. Similar evaluation of the influence of inoculation schedule independent of inoculation route or dose showed that more mice on the multiple inoculation schedule had developed neutralizing RV antibody than mice inoculated twice, when assessed at two ($p = 0.011$) and three months ($p = 0.021$) following the initial inoculation.

Antibody Response After Re-challenge

Three months after the first low-dose inoculation of RV, mice were re-challenged with a high dose 10^4 $TCID_{50}$ of the same big brown bat RV variant (#20708 Denver) that was used for the original inoculations. None of the naïve or previously inoculated mice succumbed to RV infection within one month following the re-challenge. Antibody responses following re-challenge were analyzed by McNemars Chi across all treatment groups and revealed a significant increase in the number of mice with detectable RV

neutralizing antibody titer between three and three and a half months ($p = 0.03$) and a highly significant increase between three to four months ($p = 0.007$). (Table 3-7, Figures 3-2 and 3-3). This increase in the fraction of mice with antibody two weeks following re-challenge was particularly evident in group 1 and could be an indication of immune system priming to RV from the previous exposures to rabies. The increase in antibody prevalence 4 weeks after re-challenge was also observed in a majority of control mice.

Table 3-7. Neutralizing Anti-Rabies Virus Antibody Responses over the Course of the Study.

Group	Inoculation Dose ($TCID_{50}$)	Inoculation Route	Inoculation Schedule	Antibody detection**					
				0.5M	1M	2M	3M	3.5M	4M
1	50	IM	Multiple	0/5	0/5	1/5	1/5	4/5	1/5
2	50	IM	2x	0/5	0/5	0/5	1/5	1/5	2/5
3	50	ID	Multiple	0/5	0/5	0/5	0/5	1/5	1/5
4	50	ID	2x	0/5	0/5	0/5	0/5	0/4[x]	2/5
5	50	IN	Multiple	0/5	0/5	0/5	0/5	1/5	1/5
6	50	IN	2x	0/5	1/5	0/4	0/4	1/4	2/4
7	500	IM	Multiple	0/5	4/5	4/5	4/4*	4/4	3/4
8	500	IM	2x	0/5	0/5	1/5	0/5	0/5	1/5
9	500	ID	Multiple	0/5	0/5	1/5	2/5	2/5	2/5
10	500	ID	2x	0/5	0/5	0/5	0/5	1/5	3/5
11	500	IN	Multiple	0/5	1/5	4/4	1/1	1/1	1/1
12	500	IN	2x	0/5	1/5	1/4	1/4	2/4	3/4
Control	10^4	IM	1x	NA	NA	NA	0/7	1/7	4/7

* One mouse was removed from the study due to non-study complications
** Number of mice with RFFIT titers ≥ 5 / number of mice in group
[x] One mouse was not bleed due to barbering injury on the ears and scruff of the neck

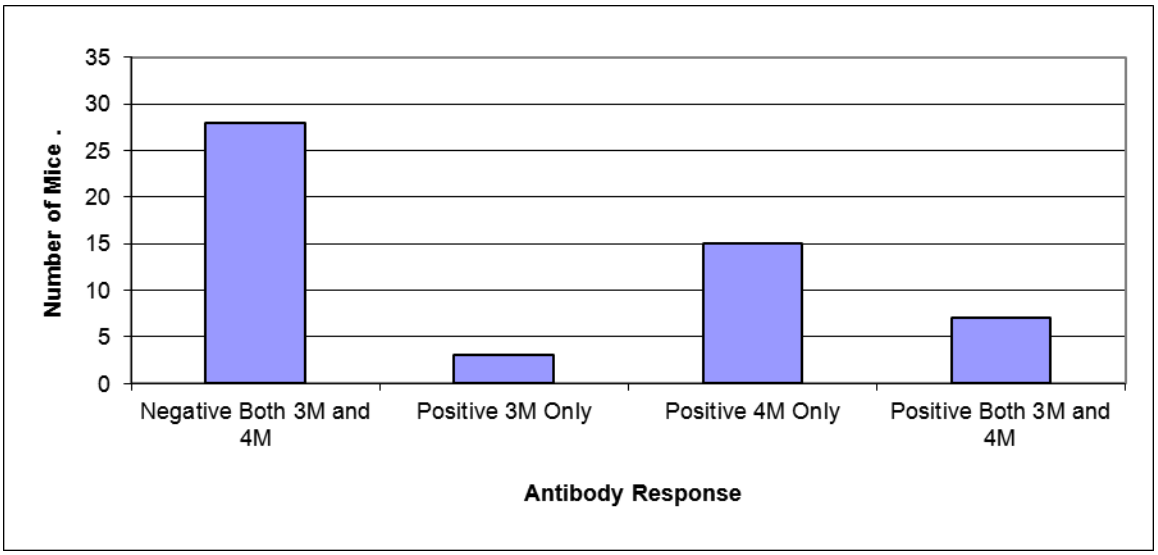

Figure 3-2. Mice with Neutralizing Antibody Response Compared Between 3 and 3.5 Months. The number of mice with neutralizing antibody significantly increased from 3 to 3.5 months.

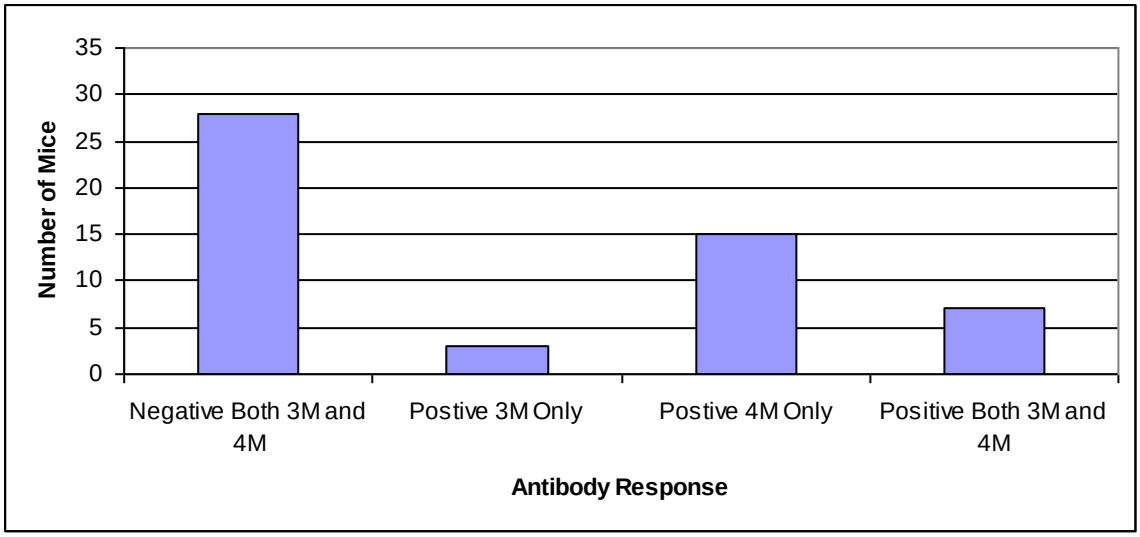

Figure 3-3. Mice with Neutralizing Antibody Response Compared Between 3 and 4 Months. The number of mice with neutralizing antibody significantly increased from 3 to 4 months.

DISCUSSION

Among the reservoir hosts for rabies viruses, bats appear to be unique in that neutralizing antibodies are frequently detected in wild, healthy bats multiple years in a row (O'Shea et al. 2003, Perez-Jorda et al. 1995), suggesting that infection frequently is

not fatal. An important question that remains to be answered, and is the focus of the study presented here, is how wild bats apparently are frequently exposed to a sufficient dose of RV to induce formation of anti-RV antibodies yet fail to progress to develop clinical disease. Multiple hypotheses have been proposed to explain this observation. First, bats may be exposed repeatedly to low and sub-infectious doses of RV such that they become immunized but not productively infected. A second hypothesis is that exposures other than through deep intramuscular bites may not lead to clinical rabies, but lead to enough virus replication to stimulate antibody production. A third possible mechanism for bats to develop RV neutralizing antibody without productive infection is by exposure to virus when they are nursing and are protected by maternal antibody. Finally, it is known that infection with other lyssaviruses (e.g. European bat lyssaviruses 1 and 2, Lagos bat virus) can induce development of antibodies that neutralize RV; however, there is no evidence to indicate that these other lyssaviruses exist in North America (Smith 2002). These alternative hypotheses are by no means mutually exclusive.

In the current study, we used a mouse model to investigate the effects of different routes of exposure, frequencies of exposures and doses of virus on seroconversion and development of clinical disease, with the objective of obtaining a better grasp on what may be occurring in wild bat populations and guiding further research on this topic.

Mice inoculated intranasally had the highest mortality. This was not unexpected, since inoculation by this route provides a direct pathway for the virus to gain access to the central nervous system. However, the high mortality made it difficult to evaluate the immunogenicity of exposure by this route. The numerically highest rate of

seroconversion was observed in mice inoculated intramuscularly (32%), and this occurred in the absence of mortality, even at the highest dose of virus. Finally, exposure to RV via intradermal inoculation was significantly less effective than either intramuscular or intranasal inoculation in eliciting development of anti-rabies virus antibodies, and failed to result in disease.

The dose of RV delivered by the bite of an infected bat with salivary gland infection is not known, but we speculate that most bites deliver a relatively low dose. In previous work with the #20708 Denver RV isolate, it was found that none of the mice succumbed following inoculation with 100 $TCID_{50}$ and only a few mice developed rabies after injection of 1000 $TCID_{50}$ (see Chapter 2). We found that a larger fraction of the mice inoculated with the higher of the low doses tested here (500 $TCID_{50}$) developed anti-RV antibody compared to those inoculated with the lower dose (50 $TCID_{50}$) with no clinical disease.

In bat colonies, the abundance of rabid bats shedding RV is about 1% of the colonies population (Constantine 1968). We speculated that healthy bats in the colony may be exposed to rabid bats and therefore RV at a moderate (monthly) or more frequent (every few days) intervals. We found that inoculation of virus multiple times during the study led to a higher fraction of mice developing anti-RV antibodies as compared to inoculation only twice. Whether bats would respond similarly remains to be tested.

Adult bats frequently become rabid and succumb to clinical rabies (Blanton 2010), however, it is unknown if the anti-rabies antibody found in wild bat populations is actually protective against RV infection. To determine whether inoculation of mice with low doses of RV lead to protection from a higher dose challenge or evidence of immune

priming, all mice that survived the initial inoculations were re-challenged with 10^4 $TCID_{50}$ of the same virus used originally. Previous studies in our laboratory demonstrated that this virus and dose was lethal in 9 of 10 mice that had no previous exposure to RV. However, none of the mice in this experiment, including naïve controls that were challenged with this dose of virus succumbed to this re-challenge, although there was a significant increase in the number of mice that seroconverted in response to re-challenge. This could be an indication that their immune systems had been primed to respond in a protective manner to an exposure to RV (Janeway et al. 2005). At the end of the study the number of mice with neutralizing antibody was increasing, however, it is unknown if this movement would have continued for a period of time, or had peaked and would have fallen off. The waxing and waning of neutralizing antibody has also been observed in wild bats over time (Steece and Altenbach 1989).

The fact that none of the mice, particularly the naïve controls, succumbed to rabies following the re-challenge was surprising. This could be due in part to the age of the mice. In our previous study, the mice were 4-6 weeks old when they were inoculated, whereas the mice in this experiment were approximately 4 months old at the beginning of this study, meaning the naïve mice were ~7 months old when they were first exposed to RV. Other studies have found that the age of the mice significantly influences susceptibility and development of clinical disease and others have observed that older mice inoculated with RV were less likely to succumb to clinical rabies than their younger counter-parts (Casal 1940). Indeed, in a previous study in which RV vaccines were evaluated, it was reported that the difference of only two weeks in age could significantly decrease the number of mice that succumbed to rabies (Wunderli et al. 2003). This has

also been observed in laboratory experiments with skunk rabies, in which it was found that older skunks were less likely to develop clinical infection than the younger pups (Ramsden and Johnston 1975).

Our study demonstrated that inoculation of mice with very low doses of RV resulted in a level of seroconversion (32%) without mortality similar to what has been observed in wild bats (~30%) living in an environment where RV is endemic (O'Shea et al. 2003). Although rates of seroconversion varied among treatment groups, some mice in each route, frequency and dose category developed a detectable humoral immune response to RV, providing support for the speculation that non-lethal RV infections can be initiated by different types of exposure. These results shed light on how natural bat populations may acquire anti-rabies antibody. The next logical step would be to repeat some of this work using bats.

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APPENDIX 1

DNA Sequences of the Nucleocapsid Gene of Nine Big Brown Bat Rabies Viruses Isolates Aligned With an *Eptesicus fuscus* Rabies Virus Sequence in GenBank.

```
                                                                                                                    1120
Virus_1435                                                                                                       AGCTGAGT
Virus_2070                                                                                                       AGCTGAGT
Virus 2156                                                                                                       AGCTGAGT
Virus_2311                                                                                                       AGCTGAGT
AY039228.1                                                                                                       AGCTGAGT
Virus_2557                                                                                                       AGCTGAAT
Virus_1510                                                                                                       AGCTGAGT
Virus_2423                                                                                                       AGCTGAGT
Virus 2192                                                                                                       AGCTGAGT
Virus 2404                                                                                                       ATGTGAGT
 Consensus                                                                                                       AgcTGAGT

            1121                                                                                                 1190
Virus 1435  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus_2070  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus 2156  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus_2311  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
AY039228.1  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus_2557  CAACTAAGAC TGAAGTGGCC TTGGCTGATG ACGGAACCGT CAATTCTGAT GACGAAGACT ACTTCTCTAG
Virus 1510  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus_2423  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus_2192  CAACAAAGAC TGATGTGGCC TTGGCAGATG ACGGAACAGT CAATTCTGAT GACGAGGACT ACTTCTCTGG
Virus_2404  CAACAANGAC TGATGTGGCC TTGGCAGATG ACGGAACAGG CAATTTTGAT GACGAGGAGT ACTTCTTTGG
 Consensus  CAACAAaGAC TGATGTGGCC TTGGCAGATG ACGGAACAGt CAATTcTGAT GACGAGGAcT ACTTCTcTGG

            1191                                                                                                 1260
Virus_1435  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus 2070  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus_2156  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus_2311  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
AY039228.1  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus 2557  TGAGACCAGG AGTCCGGAGG CAGTCTACAC TCGAATCATG ATGAATGGAG GTAGACTGAA AAGATCACAC
Virus 1510  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus_2423  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus_2192  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
Virus_2404  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC
 Consensus  TGAAACCAGG AGTCCGGAGG CAGTTTATAC TCGGATCATG ATAAATGGGG GTAGATTGAA AAGATCACAC

            1261                                                                                                 1330
Virus_1435  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus_2070  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus_2156  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus 2311  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
AY039228.1  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus 2557  ATAAGGAGGT ATGTCTCAGT CAGCTCCAAT CATCAAGCTC GCCCCAACTC ATTCGCCGAG TTTTTAAACA
Virus_1510  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus_2423  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus_2192  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
Virus_2404  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
 Consensus  ATAAGGAGAT ATGTCTCAGT AAGTTCCAAT CATCAAGCTC GCCCTAATTC ATTCGCTGAG TTTCTAAACA
```

```
            1331                                                                1400
Virus_1435  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
Virus_2070  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
Virus_2156  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
Virus_2311  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
AY039228.1  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
Virus_2557  AGACATACTC GAGTGATTCG TAAAAAGTTG AACAACAAGA TTGGAAACAC TAATAAATTG TGTACATCCT
Virus_1510  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
Virus_2423  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
Virus_2192  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATTCT
Virus_2404  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATCCT
 Consensus  AGACATACTC TAATGATTCA TAAAGAATTG ACCAACAGGA TTGTAAACAA TAATAAATTG TGTACATcCT

            1401              1421
Virus_1435  TCATGAAAAA AACTAACACC C
Virus_2070  TCATGAAAAA AACTAACACC C
Virus_2156  TCATGAAAAA AACTAACACC C
Virus_2311  TCATGAAAAA AACTAACACC C
AY039228.1  TCATGAAAAA AACT
Virus_2557  TCACGAAAAA AACTAACACC C
Virus_1510  TCATGAAAAA AACTAACACC C
Virus_2423  TCATGAAAAA AACT
Virus_2192  TCATGAAAAA AACTAACACC C
Virus_2404  TCATGAAAAA AACTAACACC C
 Consensus  TCATGAAAAA AACTaacacc c

High consensus sequence = Red
Low consensus sequence = Blue
Neutral Color = Black
```

APPENDIX 2

Nucleotide Sequence Comparison for the Nucleocapsid Gene of #25571 Boulder Rabies Virus Isolate with Two Variants in Genbank (Accession AF394888.1 and AY039228.1)

```
                                                                          558            574
AY039228.1                                                                TTGATGA CAACCCACAA
Virus_25571                                                               TTGATGA CAACCCACAA
AF394888.1                                                                TTGATGA CAACCCACAA
 Consensus                                                                TTGATGA CAACCCACAA

            575                                                                          644
AY039228.1  AATGTGCGCT AACTGGAGCA CCATACCGAA TTTCAGATTT CTAGCCGGAA CCTACGACAT GTTTTTCTCC
Virus_25571 AATGTGCGCT AACTGGAGTA CCATACCGAA CTTCAGATTT CTAGCCGGGA CCTATGACAT GTTTTTCTCC
AF394888.1  AATGTGCGCT AACTGGAGTA CCATACCGAA CTTCAGATTT CTAGCCGGGA CCTATGACAT GTTTTTCTCC
 Consensus  AATGTGCGCT AACTGGAGtA CCATACCGAA cTTCAGATTT CTAGCCGGgA CCTAtGACAT GTTTTTCTCC

            645                                                                          714
AY039228.1  CGGATCGAAC ATCTGTATTC AGCAATTAGA GTGGGCACAG TTGTCACTGC TTATGAGGAC TGCTCAGGAT
Virus_25571 CGGATTGAAC ATCTATATTC AGCGATTAGA GTGGGCACAG TTGTCACTGC TTATGAGGAC TGCTCAGGGT
AF394888.1  CGGATTGAAC ATCTATATTC AGCGATTAGA GTGGGCACAG TTGTCACTGC TTATGAGGAC TGCTCAGGGT
 Consensus  CGGATtGAAC ATCTaTATTC AGCgATTAGA GTGGGCACAG TTGTCACTGC TTATGAGGAC TGCTCAGGgT

            715                                                                          784
AY039228.1  TGGTGTCGTT CACCGGGTTT ATAAAGCAAA CAAATCTCAC CGCAAGAGAA GCAATATTAT ATTTCTTCCA
Virus_25571 TGGTGTCATT TACAGGGTTT ATAAAACAAA TAAATCTCAC TGCGAGAGAA GCACTACTAT ATTTCTTCCA
AF394888.1  TGGTGTCATT TACAGGGTTT ATAAAACAAA TAAATCTCAC TGCGAGAGAA GCACTACTAT ATTTCTTCCA
 Consensus  TGGTGTCaTT tACaGGGTTT ATAAAaCAAA tAAATCTCAC tGCgAGAGAA GCAcTAcTAT ATTTCTTCCA

            785                                                                          854
AY039228.1  TAAGAACTTT GAAGAAGAGA TAAGAAGAAT GTTTGAGCCT GGGCAGGAAA CCGCAGTTCC TCACTCCTAT
Virus_25571 CAAGAACTTT GAAGAAGAGA TAAGAAGAAT GTTTGAGCCA GGGCAAGAGA CTGCAGTCCC TCACTCCTAT
AF394888.1  CAAGAACTTT GAAGAAGAGA TAAGAAGAAT GTTTGAGCCA GGGCAAGAGA CTGCAGTCCC TCACTCCTAT
 Consensus  cAAGAACTTT GAAGAAGAGA TAAGAAGAAT GTTTGAGCCa GGGCAaGAgA CtGCAGTcCC TCACTCCTAT

            855                                                                          924
AY039228.1  TTCATCCATT TTCGTTCATT GGGCCTGAGT GGGAAATCTC CATATTCATC AAATGCAGTG GGTCACGTGT
Virus_25571 TTCATCCATT TCCGTTCGTT GGGCCTGAGC GGGAAATCTC CGTACTCATC AAATGCAGTT GGTCATGTGT
AF394888.1  TTCATCCATT TCCGTTCGTT GGGCCTGAGC GGGAAATCTC CGTACTCATC AAATGCAGTT GGTCATGTGT
 Consensus  TTCATCCATT TcCGTTCgTT GGGCCTGAGc GGGAAATCTC CgTAcTCATC AAATGCAGTt GGTCAtGTGT

            925                                                                          994
AY039228.1  TCAATCTCAT TCACTTTGTG GGATGTTATA TGGGTCAAGT AAGATCTTTA AATGCAACGG TTATTGCCAC
Virus_25571 TCAACCTCAT TCACTTTGTT GGATGTTATA TGGGTCAGGT GAGATCTCTG AATGCAACAG TGATTGCCAC
AF394888.1  TCAACCTCAT TCACTTTGTT GGATGTTATA TGGGTCAGGT GAGATCTCTG AATGCAACAG TGATTGCCAC
 Consensus  TCAAcCTCAT TCACTTTGTt GGATGTTATA TGGGTCAgGT gAGATCTcTg AATGCAACaG TgATTGCCAC

            995                                                                         1064
AY039228.1  ATGTGCCCCG CATGAGATGT CTGTTCTCGG GGGTTATCTG GGGGAGGAGT TTTTTGGAAA GGGGACTTTT
Virus_25571 ATGTGCCCCA CATGAGATGT CTGTTCTTGG GGGTTATTTG GGGGAGGAGT TTTTTGGAAA AGGGACTTTT
AF394888.1  ATGTGCCCCA CATGAGATGT CTGTTCTTGG GGGTTATTTG GGGGAGGAGT TTTTTGGAAA AGGGACTTTT
 Consensus  ATGTGCCCCa CATGAGATGT CTGTTCTtGG GGGTTATtTG GGGGAGGAGT TTTTTGGAAA aGGGACTTTT

            1065                                                                        1134
AY039228.1  GAGAGAAGAT TCTTTAGGGA CGAGAAAGAA CTGCAGGAAT ATGAGGCAGC TGAGTCAACA AAGACTGATG
Virus_25571 GAGAGGAGAT TCTTCAGGGA CGAGAAAGAA CTTCAGGAAT ATGAGGCAGC TGAATCAACT AAGACTGAAG
AF394888.1  GAGAGGAGAT TCTTCAGGGA CGAGAAAGAA CTTCAGGAAT ATGAGGCAGC TGAATCGACT AAGACTGAGG
 Consensus  GAGAGgAGAT TCTTcAGGGA CGAGAAAGAA CTtCAGGAAT ATGAGGCAGC TGAaTCaACt AAGACTGA.G
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           1135                                                                1204
AY039228.1 TGGCCTTGGC AGATGACGGA ACAGTCAATT CTGATGACGA GGACTACTTC TCTGGTGAAA CCAGGAGTCC
Virus 25571 TGGCCTTGGC TGATGACGGA ACCGTCAATT CTGATGACGA AGACTACTTC TCTAGTGAGA CCAGGAGTCC
AF394888.1 TGGCCTTGGC TGATGACGGA ACCGTCAATT CTGATGACGA GGACTACTTC TCTAGTGAGA CCAGGAGTCC
 Consensus TGGCCTTGGC tGATGACGGA ACcGTCAATT CTGATGACGA gGACTACTTC TCTaGTGAgA CCAGGAGTCC

           1205                                                                1274
AY039228.1 GGAGGCAGTT TATACTCGGA TCATGATAAA TGGGGGTAGA TTGAAAAGAT CACACATAAG GAGATATGTC
Virus 25571 GGAGGCAGTC TACACTCGAA TCATGATGAA TGGAGGTAGA CTGAAAAGAT CACACATAAG GAGGTATGTC
AF394888.1 GGAGGCAGTC TACACTCGAA TCATGATGAA TGGAGGTAGA CTGAAAAGAT CACACATAAG GAGGTATGTC
 Consensus GGAGGCAGTc TAcACTCGaA TCATGATgAA TGGaGGTAGA cTGAAAAGAT CACACATAAG GAGgTATGTC

           1275                                                                1344
AY039228.1 TCAGTAAGTT CCAATCATCA AGCTCGCCCT AATTCATTCG CTGAGTTTCT AAACAAGACA TACTCTAATG
Virus_25571 TCAGTCAGCT CCAATCATCA AGCTCGCCCC AACTCATTCG CCGAGTTTTT AAACAAGACA TACTCGAGTG
AF394888.1 TCAGTCAGCT CCAATCATCA AGCTCGCCCC AACTCATTCG CCGAGTTTTT AAACAAGACA TACTCGAGTG
 Consensus TCAGTcAGcT CCAATCATCA AGCTCGCCCc AAcTCATTCG CcGAGTTTtT AAACAAGACA TACTCgAgTG

           1345                                                                1414
AY039228.1 ATTCATAAAG AATTGACCAA CAGGATTGTA AACAATAATA AATTGTGTAC ATCCTTCATG AAAAAAACT
Virus_25571 ATTCGTAAAA AGTTGAACAA CAAGATTGGA AACACTAATA AATTGTGTAC ATCCTTCACG AAAAAAACT
AF394888.1 ATTCGTAAAA AGTTGAACAA TGAGATTGTA AACACTAATA AATTGTGTAC ATCCTTCACG AAAAAAACT
 Consensus ATTCgTAAAa AgTTGAaCAA caaGATTGtA AACAcTAATA AATTGTGTAC ATCCTTCAcG AAAAAAACT
```

High consensus sequence = Red
Low consensus sequence = Blue
Neutral Color = Black

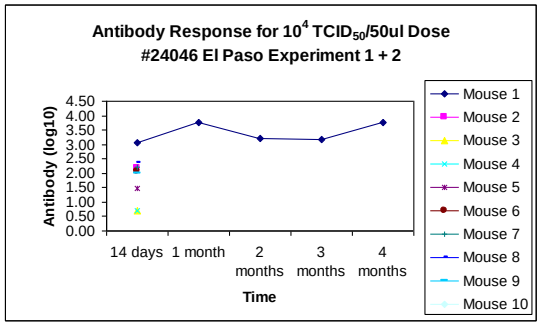
APPENDIX 3

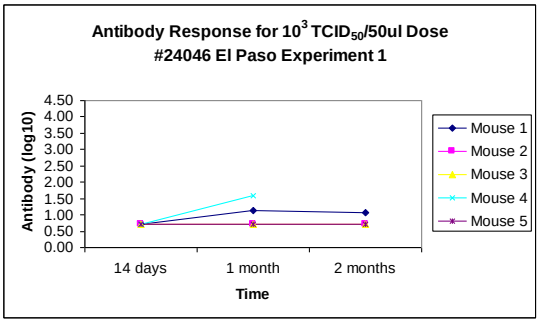
Comparison of Deduced Amino Acid Sequences of the Nucleocapsid Gene of Nine Big Brown Bat Rabies Virus Isolates

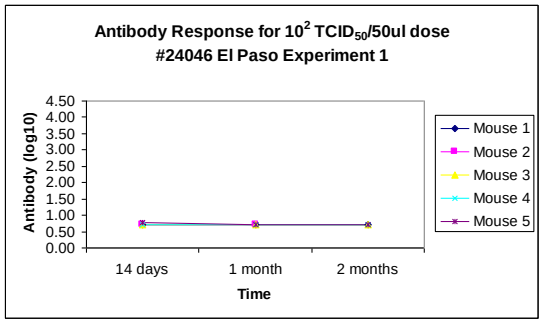
```
                                                                                                  210
14357 V2                                TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
20708 Denver                            TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
21567 Denver                            TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
23111 El Paso                           TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
AY039228.1                              TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
25571 Boulder                           TRIMMNGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
15100 V1                                TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
24235 Douglas                           TRIMINGGRL KRSHIRRYVS VSSNHQARPN SFAEFLNKTY
21921 Mesa                                                 S VSSNHQARPN SFAEFLNKTY
24046 El Paso                                                    QARPN SFAEFLNKTY
 Consensus                              triminggrl krshirryvs vssnhQARPN SFAEFLNKTY

                   211                   234
14357 V2           SNDSRIDQQD CKQIVYILHE KNHP
20708 Denver       SNDSRIDQQD CKQIVYILHE KNHP
21567 Denver       SNDSRIDQQD CKQIVYILHE KNHP
23111 El Paso      SNDSRIDQQD CKQIVYILHE KNHP
AY039228.1         SNDSRIDQQD CKQIVYILHE KN
25571 Boulder      SSDSKVEQQD WKHIVYILHE KNHP
15100 V1           SNDSRIDQQD CKQIVYILHE KNHP
24235 Douglas      SNDSRIDQQD CKQIVYILHE KN
21921 Mesa         SNDSRIDQQD CKQIVYILHE KNHP
24046 El Paso      SNDSRIDQQD CKQIVYILHE KNHP
 Consensus         SNDSRIDQQD CKQIVYILHE KNhp

High consensus sequence = Red
Low consensus sequence = Blue
Neutral Color = Black
```

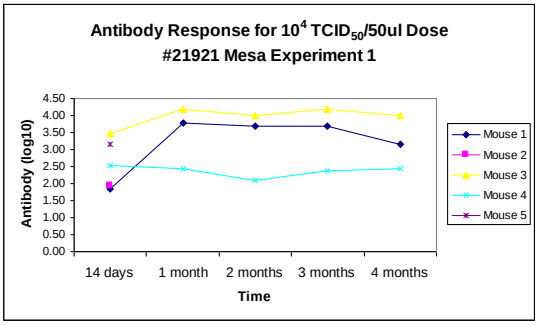
APPENDIX 4

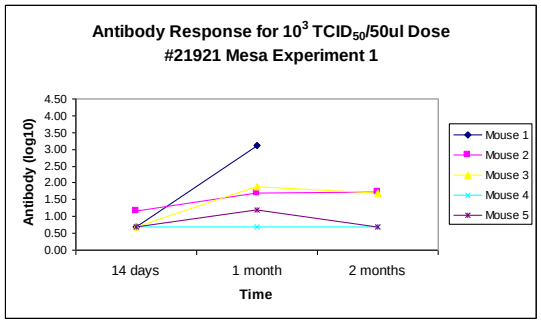
Neutralizing Antibody to Rabies Response for Individual Mice through out the Study for each Group

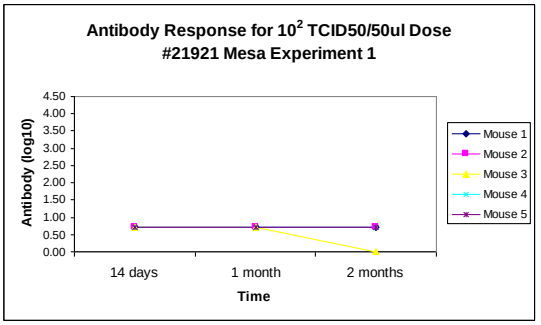


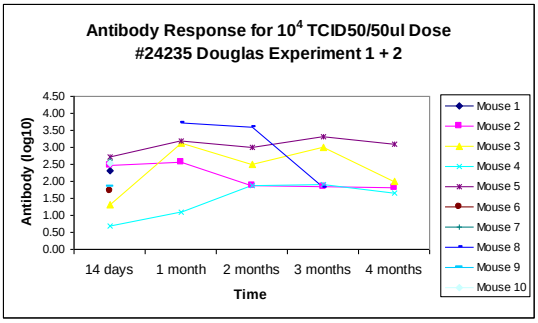


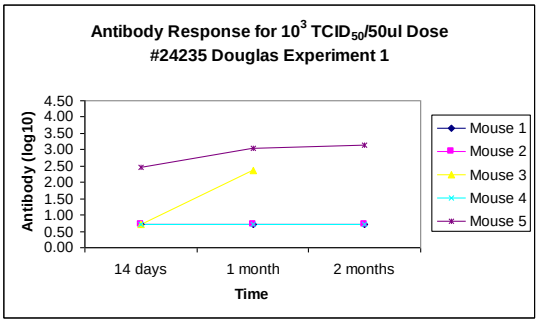


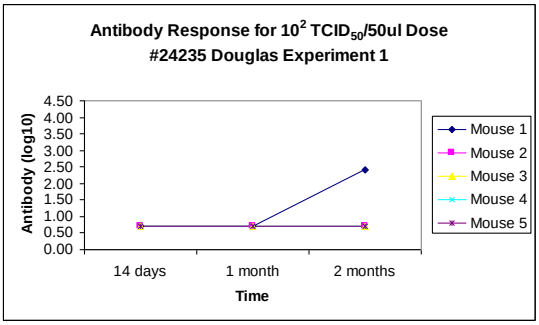


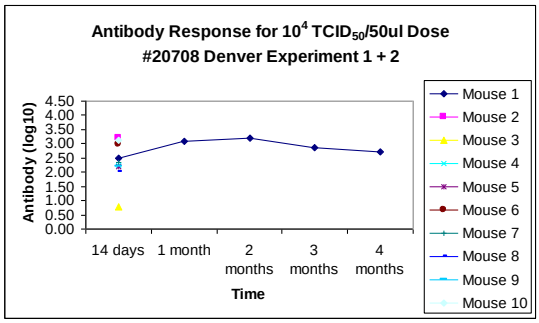


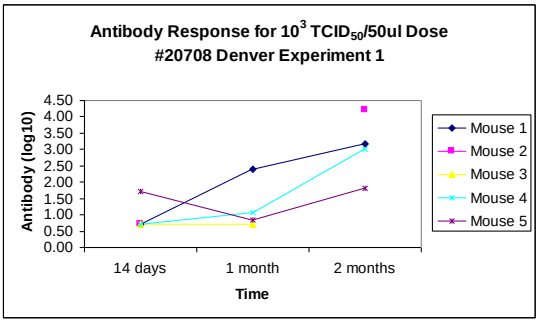


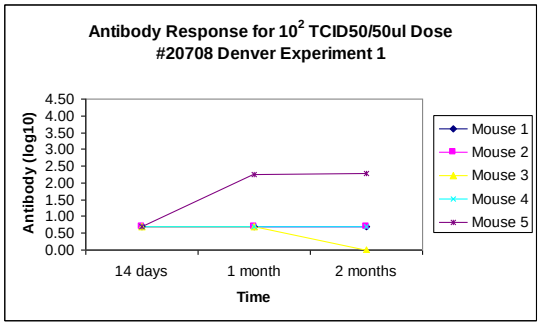

Antibody Response for 10^4 TCID50/50ul Dose
#24235 Douglas Experiment 1 + 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10

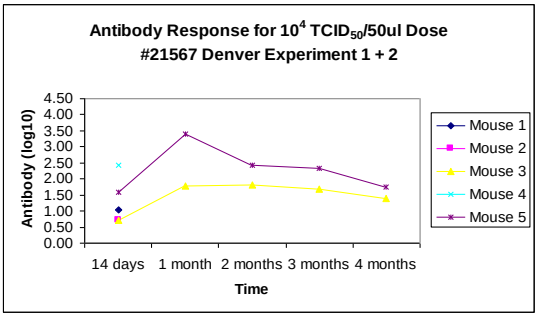

Antibody Response for 10^3 TCID50/50ul Dose
#24235 Douglas Experiment 1
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^2 TCID50/50ul Dose
#24235 Douglas Experiment 1
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^4 TCID50/50ul Dose
#20708 Denver Experiment 1 + 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10


Antibody Response for 10^3 TCID50/50ul Dose
#20708 Denver Experiment 1
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^2 TCID50/50ul Dose
#20708 Denver Experiment 1
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5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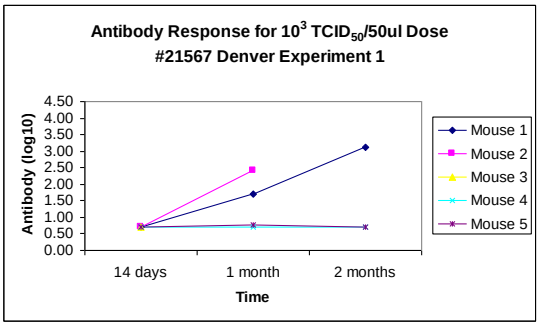
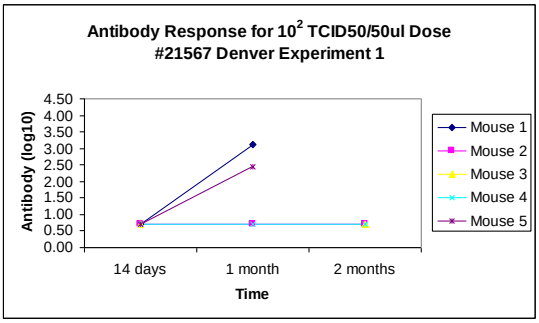
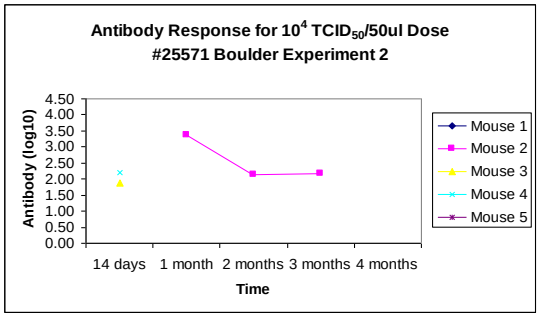
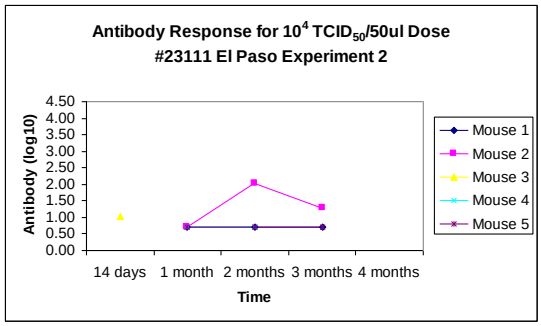
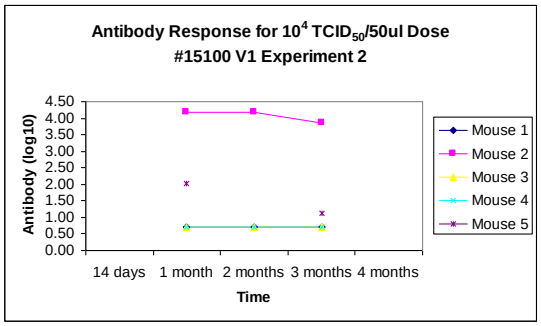
#21567 Denver Experiment 1 + 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5

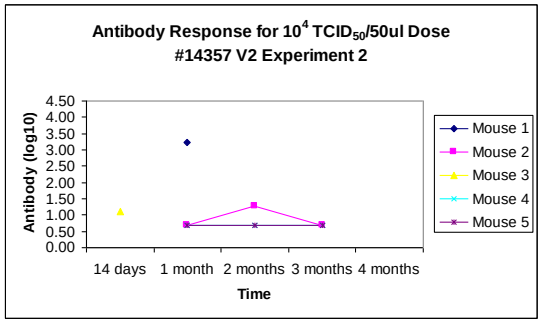

Antibody Response for 10^3 TCID50/50ul Dose
#21567 Denver Experiment 1
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^2 TCID50/50ul Dose
#21567 Denver Experiment 1
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^4 TCID50/50ul Dose
#25571 Boulder Experiment 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^4 TCID50/50ul Dose
#23111 El Paso Experiment 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^4 TCID50/50ul Dose
#15100 V1 Experiment 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5


Antibody Response for 10^4 TCID50/50ul Dose
#14357 V2 Experiment 2
Antibody (log10)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
14 days
1 month
2 months
3 months
4 months
Time
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5

APPENDIX 5

RFFIT results for neutralizing anti-RV antibody

		14 day	1 month	2 months	3 months	3.5 months	4 months
Group 1	1	<5	<5	<5	<5	7	<5
50 $TCID_{50}$	2	<5	<5	292	473	191	280
IM	3	<5	<5	<5	<5	7	<5
Multiple	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	<5	10	<5
Group 2	1	<5	<5	<5	<5	<5	206
50 $TCID_{50}$	2	<5	<5	<5	7	<5	<5
IM	3	<5	<5	<5	<5	10	12
2x	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	<5	<5	<5
Group 3	1	<5	<5	<5	<5	<5	<5
50 $TCID_{50}$	2	<5	<5	<5	<5	862	505
ID	3	<5	<5	<5	<5	<5	<5
Multiple	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	<5	<5	<5
Group 4	1	<5	<5	<5	<5	<5	<5
50 $TCID_{50}$	2	<5	<5	<5	<5	<5	<5
ID	3	<5	<5	<5	<5	NA	8
2x	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	<5	<5	163
Group 5	1	<5	<5	<5	<5	<5	<5
50 $TCID_{50}$	2	<5	<5	<5	<5	<5	<5
IN	3	<5	<5	<5	<5	10	47
Multiple	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	<5	<5	<5
Group 6	1	<5	<5	<5	<5	<5	<5
50 $TCID_{50}$	2	<5	138	NA	NA	NA	NA
IN	3	<5	<5	<5	<5	<5	<5
2x	4	<5	<5	<5	<5	<5	299
	5	<5	<5	<5	<5	11	62

		14 day	1 month	2 months	3 months	3.5 months	4 months
Group 7	1	<5	<5	<5	NA	NA	NA
500 $TCID_{50}$	2	<5	107	300	176	86	134
IM	3	<5	14	12	10	11	<5
Multiple	4	<5	231	392	229	281	101
	5	<5	62	3310	6111	3763	753
Group 8	1	<5	<5	<5	<5	<5	<5
500 $TCID_{50}$	2	<5	<5	<5	<5	<5	4914
IM	3	<5	<5	<5	<5	<5	<5
2x	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	49	<5	<5	<5
Group 9	1	<5	<5	<5	<5	<5	<5
500 $TCID_{50}$	2	<5	<5	<5	<5	12	33
ID	3	<5	<5	82	96	49	51
Multiple	4	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	7	<5	<5
Group 10	1	<5	<5	<5	<5	<5	58
500 $TCID_{50}$	2	<5	<5	<5	<5	<5	<5
ID	3	<5	<5	<5	<5	<5	83
2x	4	<5	<5	<5	<5	46	19
	5	<5	<5	<5	<5	<5	<5
Group 11	1	<5	<5	207	NA	NA	NA
500 $TCID_{50}$	2	<5	<5	75	NA	NA	NA
IN	3	<5	<5	186	243	696	395
Multiple	4	<5	<5	65	NA	NA	NA
	5	<5	108	NA	NA	NA	NA
Group 12	1	<5	<5	<5	<5	<5	1542
500 $TCID_{50}$	2	<5	<5	<5	<5	<5	<5
IN	3	<5	7	NA	NA	NA	NA
2x	4	<5	<5	<5	<5	50	280
	5	<5	<5	1064	6988	4673	1142

		3 months	3.5 months	4 months
Control Mice	A-1	<5	<5	16
10^4 $TCID_{50}$	A-2	<5	<5	25
IM	A-3	<5	<5	<5
1x	A-4	<5	<5	1190
	B-3	<5	<5	<5
	B-4	<5	<5	<5
	B-5	<5	320	214

Shaded indicates terminal blood from a mouse that was euthanized with clinical disease.

APPENDIX 6

ROLE OF POLY(rC) BINDING PROTEINS IN RABIES VIRUS REPLICATION

INTRODUCTION

Rabies virus (RV) is a member of the *Rhabdoviridae* family in the *Lyssavirus* genus and is a negative sense single strand linear RNA virus. During viral RNA transcription, mRNA is synthesized in a sequential manner by the viral polymerase starting at the 3' end of the genome with the nucleocapsid gene, followed by the phosphoprotein, matrix, glycoprotein and polymerase genes. The accepted model for mRNA synthesis is a start-stop nonequimolar transcription of mRNA, meaning that the nucleocapsid mRNA is in the highest concentration, followed sequentially by the other viral mRNAs ending with the viral polymerase mRNA at the lowest concentration (Lyles and Rupprecht 2007). However, we have experimental evidence that the steady state concentration of glycoprotein mRNA is higher than that of the matrix gene mRNA. Upon further investigation, an RNA-binding protein was found to associate specifically with the 3' untranslated region (3' UTR) of the glycoprotein mRNA. This protein was identified as poly(rC) binding protein 2 (PCBP2). This cellular protein is also known to bind to the clover leaf RNA secondary structure on the 5' end of the polio virus genome for stabilization during translation (Murray et al. 2001). PCBP2 has also been identified as a necessary protein required for poliovirus translation and may mediate the switch from viral transcription to replication (Blyn et al. 1997, Perera et al. 2007). The goal of

this study was to further characterize the interactions between PCBP2 and rabies glycoprotein mRNA and determine whether this interaction is required for normal RV replication.

MATERIALS AND METHODS

Cell Culture

Human 293T cells were utilized for all RV infection studies. They were cultured in growth media (DMEM, 10% FBS, and 100 U penicillin/ml) and incubated in 5% CO_2 at 37^{o}C. Cultures were split 1:10 upon reaching confluence.

Rabies Virus Inoculation into Cell Culture

Uninfected cells were grown to 50-80% confluence depending on the duration of the experiment. Growth media was discarded and cells were washed two times with PBS. RV CVS-11 (10^5 $TCID_{50}$/ml) variant was diluted in growth media to 10000 $TCID_{50}$/ml virus in 900µl growth media to infect a T25 flask, and 30000 $TCID_{50}$/ml virus in 700µl of growth media for a T75 flask. Cells with virus were incubated for 30 minutes at 37^{o}C in 5% CO_2. The cells were rocked every ten minutes to ensure even distribution of virus. For a T25 flask, 6ml of growth media was added at the end of incubation while 24ml of growth media was added to a T75 flask. Cells were harvested after a predetermined incubation time. Growth media was poured off and each flask was washed 2-3 times with PBS. For RNA analysis, 1ml of TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH.) for a T25 flask or 3ml for a T75 flask was added and incubated at room temperature for 5 minutes. Digested cells and TRI Reagent were pipetted into a 2 ml or 15 ml sample tube. For protein analysis, cells were harvested with

in the same volumes of RIPA buffer as used for TRI Reagent. Samples were immediately frozen at -80^{o}C until they were analyzed. All samples were transported on dry ice.

mRNA Half Life

Cells were infected in T75 flasks as described above. After a predetermined time for RV incubation, 4-Thiouridine (Sigma-Aldrich catalog number: T4509) was added to the growth media already on the cells to a final concentration of 300 μM. One flask of cells was harvested per time point per protocol with TRI Reagent. For example, 4-Thiouridine was added at 12 hours post virus inoculation and cells were harvested at 13, 14, 15, and 16 hours with TRI Reagent.

In vivo Reversible Crosslinking and Immunoprecipitation of Protein-RNA Complexes

293T cells that were 80-90% confluent were infected with RV variant CVS-11 as described in protocol above. Twelve hours later, the cells were trypsinized and suspended in 5ml of growth media. The cell suspension was placed in a 15 ml tube and centrifuged at 40 x g for 5 minutes and the cell pellet was washed twice with PBS.

To crosslink proteins to the RNA the cellular pellet was resuspended in 10 ml of PBS containing 1% formaldehyde (270μl of formaldehyde in 10 ml), and incubated on a rocker or platform moving slowly at room temperature for 10 minutes. Glycine (2M, pH 7.0) was added to the cell suspension to a final concentration of 0.25 M, and the mixture incubated at room temperature for an additional 5 minutes. Cells were harvested by centrifugation at 250 x g for 4 minutes, the cellular pellet was washed twice with ice-cold PBS and then either stored as a pellet at -80^{o}C or analyzed immediately.

The cellular pellet was resuspended in 1.1 ml cold RIPA buffer with 1x Protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN) and divided into two 1.5 ml microcentrifuge tubes for cellular lysis by sonication. All sonication of rabies infected cells was done in a biosafety hood with cotton gaze over the tube opening to minimize the amount of aerosol that was released into the air. Proper personal protection was worn at all times. Cellular suspensions were sonicated on ice to avoid heat disruption of the crosslinked protein/RNA complexes. A probe sonicator (Sonic dismembrator model 100, Fischer Scientific) was used to lyse cells through 5 rounds of 3 second pulses each at an amplitude setting of 7 (output 8-9 W). The probe was cleaned between each sample. Cell lysis was confirmed microscopically. The cell lysate was centrifuged at 16000 x g for 10 minutes at 4^{o}C. The supernatant was transferred to a new microcentrifuge tube and the cellular pellet was saved on ice. The cellular supernatant was labeled as lysate and was used in later steps.

In a separate tube, a protein A Sepharose bead slurry (Sigma Aldrich, St. Louis MO.) was prepared by putting 50 µl of protein A Sepharose bead powder in 500 µl of RIPA buffer, mixing well and centrifugating at 16000 x g for 30 seconds. The buffer was discarded and the remaining protein A Sepharose bead slurry was mixed with 540 µl of lysate (step 3) and 1 µl of tRNA 10 mg/ml (Sigma Aldrich). This mixture was incubated by rocking for 1 hour at 4^{o}C and was centrifuged at 1300 x g for 5 minutes at 4^{o}C. The precleared supernantant was transferred into a clean tube and 25 µl of this was saved in a separate tube for RNA extraction as the Input sample (positive control).

A second protein A Sepharose bead slurry was made by adding ~20 µl of protein A Sepharose beads to 500 µl RIPA buffer, mixed well and centrifuged at 16000 x g for 30

seconds. The supernatant was discarded and the protein A Sepharose bead slurry was resuspended in 160 µl RIPA buffer. In a separate microcentrifuge tube, 20 µl of protein A Sepharose bead slurry was mixed with 12 µl of antibody in 100 µl RIPA buffer. This mixture was also made using 12 µl IgG (Calbiochem, San Diego, CA) as a nonspecific antibody (negative) control. The antibody mixtures were incubated by rocking for 2 hours at 4^{o}C. After incubation, the antibody mixture was washed 2 times with 1 ml of RIPA buffer containing Protease inhibitor cocktail and centrifuged at 16000 x g for 1 minute at 4^{o}C. The majority of the supernatant was discarded leaving a little in the tube to ensure the pellet was not lost. Six µl RNAsin (Invitrogen, Carlsbad, CA.) was added to the pellet mixture which was mixed carefully and rocked for 10 minutes at 4^{o}C. At this point the precleared lysate and the protein A Sepharose with antibodies were ready to mix. A 250 µl of sample of precleared lysate was mixed with 250 µl of RIPA buffer and that mixture was added to the tube containing the specific antibody-protein A Sepharose bead mixture. This step was repeated with the mixture for the non-specific antibody. These mixtures were incubated on a rocker for 90 minutes at 4^{o}C then centrifuged at 16000 x g for 1 minute at 4^{o}C. The supernatant was placed in another tube and stored at -80^{o}C for RNA extraction, leaving ~50 µl on the pellet. The pellet was washed by adding 1 ml HIGH stringency RIPA buffer to each tube, incubating at room temperature for 10 minutes (for the first 3 washes) and centrifuging at 16000 x g for 1 minute. The supernatant was discarded, being particularly careful not to discard the pellet. After the final wash, the pellet was resuspended in 100 µl of crosslink reversal buffer that was made freshly for each assay.

Resuspended pellets were incubated at 70^{o}C for 45 minutes to reverse crosslinks. Samples were centrifuged with a quick spin to remove condensation from the lid, followed by addition of 300 µl of TRI Reagent to each sample including the 25 µl Input sample. These samples were stored at -80^{o}C until they are transported on dry ice for RNA extraction in the collaborators laboratory.

Reagents:

Antibodies Used: Anti-PCBP1 antibody was supplied from Novus Biologicals, catalog number: Lo42V1. Anti-PCBP2 antibody was supplied from Ribonomics (Medical and Biological Laboratories Ltd), catalogue number: RN025P. IgG was purchased from Calbiochem, San Diego, CA. Anti-HuR (3A2) antibody and anti-CUGBP1 (3B1) antibodies were supplied from Santa Cruz Biotechnology (Santa Cruz, CA).

RIPA Buffer: 50mM Tris (pH 7.5), 1% MP-40, 0.5% sodium deoxycholate 0.05% SDS, 1mM EDTA, 150mM NaCl

HIGH Stringency RIPA Buffer: 50mM Tris (pH 7.5), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1M NaCl, 1M urea, 0.2mM PMSF, DI water

Buffer for Crosslink Reversal: 50mM Tris (pH 7), 0.5 mM EDTA, 10mM DTT, 1% SDS, DI water

Growth Curve of Rabies Virus in Knocked Down Cell Lines

Knock down cell lines for PCBP1, PCBP2, PCBP1+2 and a pLKO-1 vector control were kindly provided by Dr. Saiprasad G. Palusa. Cells were inoculated with RV according to the protocol described above except that after a 30 minute incubation period, cells were rinsed 3 times with PBS then fresh growth media was added to each flask. A 200 µl sample of the medium was then collected and frozen at 0^{+}, 6, 12, 24, 36, 48, 60

and 72 hours later for virus titration. Cell samples were also harvested at 2, 4, 6, 12, 24, 48 and 72 for both RNA and protein analysis per the protocol found above.

Viral aliquots from each time point were titrated on mouse neuroblastoma (NB) cells using a standard quantal assay. Serial 10-fold dilutions of each sample (typically 10^{-1} to 10^{-7}) were prepared in growth medium and 50 µl from each dilution was placed in 5 replicate wells of a 96-well plate. NB cells were trypsinized, counted and seeded into the wells at 25,000 cells per well. Each plate contained two time points and a control dilution series of stock CVS-11 virus. Plates were incubated for three to four days at 37^{o}C in 5% CO_2, then the medium was discarded, the plates were rinsed once with PBS and fixed with 70% acetone for at least one hour. Plates were air dried and either stained immediately or stored in the refrigerator until they were stained. Staining for RV antigens was accomplished by applying drops of Light Diagnostics Rabies DFA Reagents (diluted 1:100) to each well, and incubated for 45 minutes to 1 hour at 37^{o}C in 5% CO_2. The plates were washed three times in PBS and were evaluated using an inverted fluorescence microscope. The tissue culture infective dose 50% ($TCID_{50}$) was calculated for each sample by the Spearman-Karber method (Smith et al. 1996).

RESULTS

In vivo Crosslink/Immunoprecipitation Demonstrates an Interaction between the PCBP2 Protein and the Rabies Virus Glycoprotein mRNA

In vitro assays performed in the Wilusz laboratory with RV transcripts to the different rabies viral 3' UTRs determined that PCBP2 binds to the 3' UTR region of the Glycoprotein (G) mRNA in uninfected cells. To determine if the PCBP2 also bound to G

mRNA produced during an active viral infection, an in vivo crosslink/immunoprecipitation assay was performed twice. The first experiment was performed only with a non-specific antibody (IgG) as a negative control and PCBP2 as our target protein. The second experiment was performed with IgG, PCBP2, PCBP1 and HuR antibodies. PCBP1 antibody was included due to the high homology of PCBP1 with the PCBP2 proteins. HuR antibody was included as a protein that is known to bind specifically to cellular and viral mRNAs and act as a stabilizing factor (Sokoloski et al. 2010). Messenger RNA levels were determined by both semi-quantitative and quantitative RT-PCR by Dr. Saiprasad G. Palusa in the Wilusz laboratory.

Results from the first assay demonstrated by semi-quantitative RT-PCR that PCBP2 binds only to the G mRNA and not the other viral mRNAs (Figure A6-1). The second assay confirmed the results from the first assay in that the PCBP2 protein clearly binds to the G mRNA. This assay also established that the PCBP1 and HuR proteins do not detectably associate with RV mRNA (data not shown). Thus PCBP2 seems to be unique in its interactions with the G mRNA.

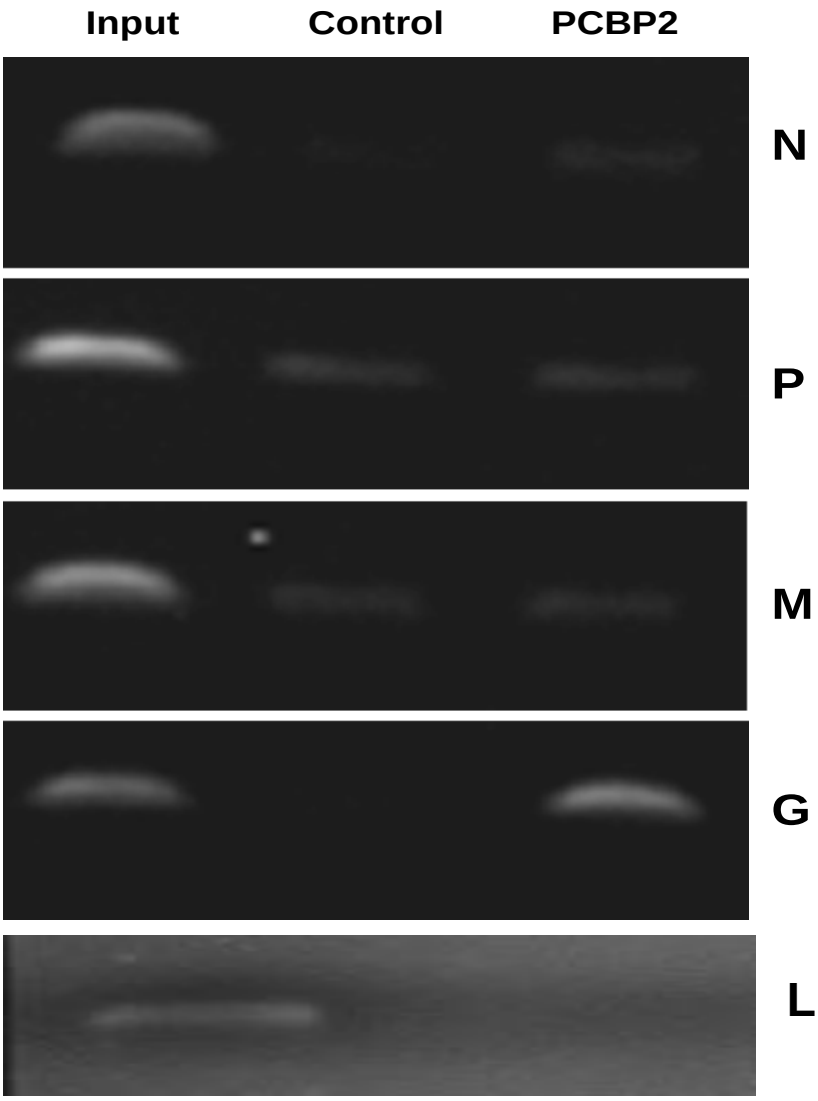


Figure A6-1: Semi-quantitative RT-PCR results for viral mRNA crosslinked to IgG or PCBP2 protein. (RV mRNA, N = Nucleocapsid, P = Phosphoprotein, M = Matrix, G = Glycoprotein, L = Viral Polymerase). (This figure kindly was provided by Dr. Saiprasad G. Palusa)

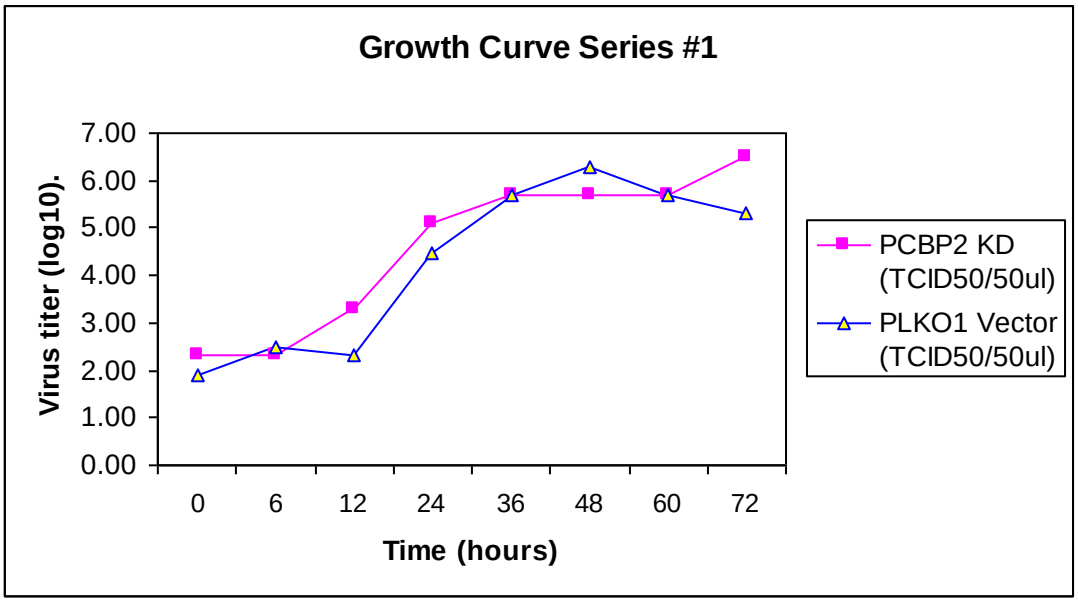
Rabies virus replication is not influcenced by the depletion of PCBP2 and/or PCBP1.

Previous data from the Wilusz laboratory and the in vivo immunoprecipitation crosslink assay indicated that the PCBP2 protein binds specifically to the 3' UTR of the glycoprotein (G) mRNA. To determine if the binding of PCBP2 affects RV replication, the viral titer was determined over multiple time points (a viral "growth curve") during

cellular infection of knockdown (KD) cell lines with RV. Three different KD cell lines were established using lentivirus shRNA technology (Sigma) to analyze the effects of PCBP1 and PCBP2 had on rabies viral replication. PCBP1 and PCBP2 have 83% identical nucleic acid sequence and have ~90% identical amino acid sequence, but viral replication studies with poliovirus have established that only PCBP2 and not PCBP1 is required for viral translation to occur (Walter et al. 2002). The KD cell lines for PCBP1 alone, PCBP2 alone, and both PCBP1 and PCBP2 were compared against a control cell line with just the pLKO-1 vector integrated into 293T cells. Western blot analysis was performed by Dr. Saiprasad G. Palusa on these different KD cell lines to ensure that the appropriate PCBP protein remained knocked down for the duration of the viral growth curve experiment. Four different growth curve series were performed throughout this section. The data for each individual series is presented below.

RV replication titers over 72 hours in PCBP2 knocked cell line compared to control lines (Series #1)

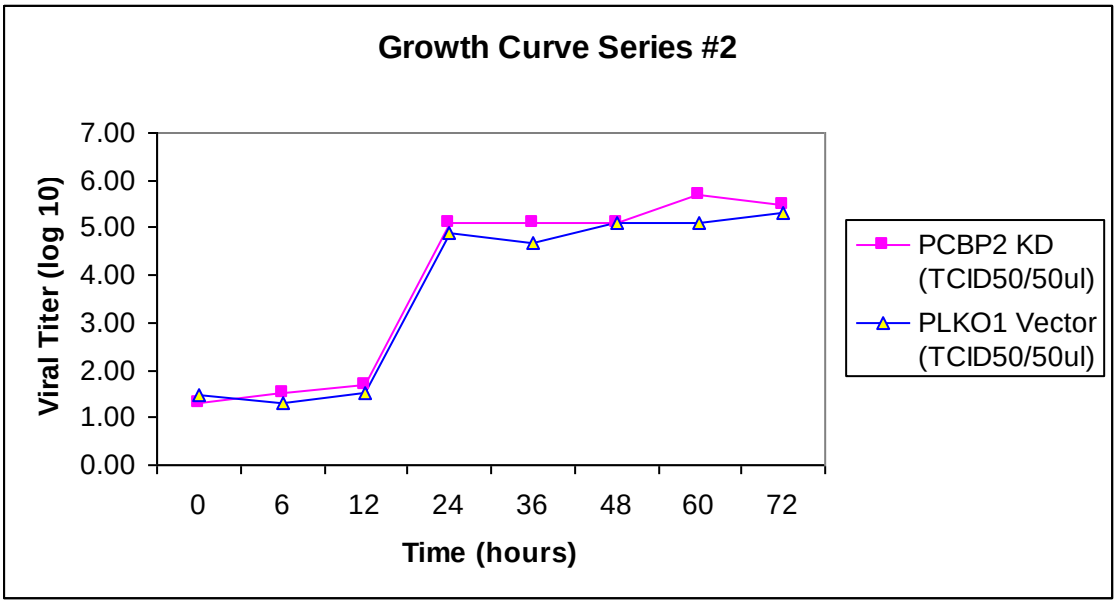
Time (hours)	PCBP2 KD (Log_{10} $TCID_{50}$/50µl)	pLKO-1 Vector (Log_{10} $TCID_{50}$/50µl)
0	2.30	1.90
6	2.30	2.50
12	3.30	2.30
24	5.10	4.47
36	5.70	5.70
48	5.70	6.30
60	5.70	5.70
72	6.50	5.30



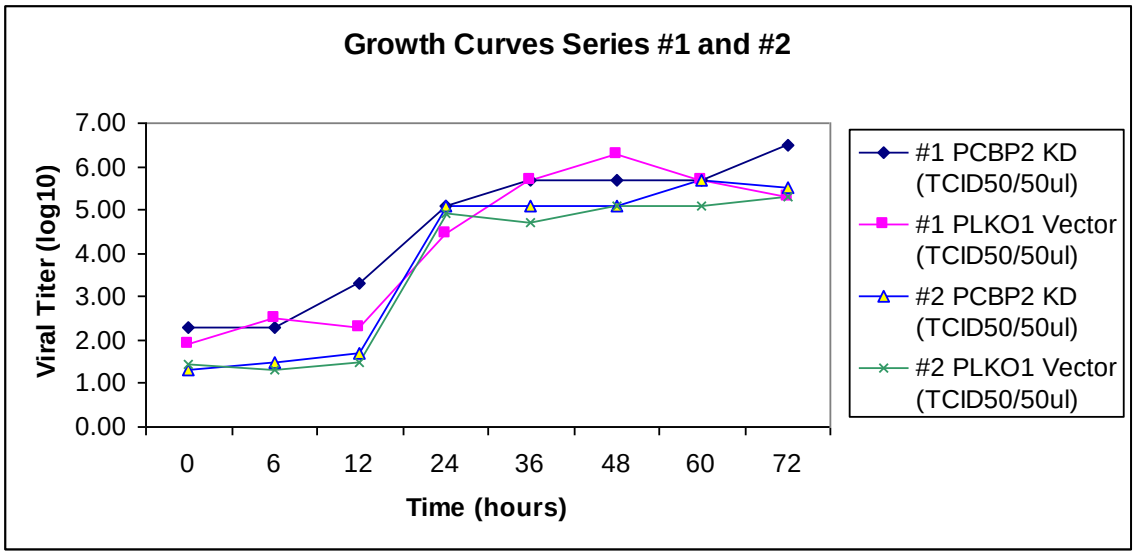
The PCBP2 mRNA expression level was reduced by 78% as assessed by qRT-PCR at the beginning of the RV infection. Based on the RV titers measured over time, there does not appear to be a difference between the pLKO-1 vector control and the PCBP2 KD cell line.

RV replication titers over 72 hours in PCBP2 knocked cell line compared to control lines (Series #2)

Time (hours)	PCBP2 KD (Log_{10} $TCID_{50}$/50µl)	pLKO-1 Vector (Log_{10} $TCID_{50}$/50µl)
0	1.31	1.46
6	1.50	1.32
12	1.70	1.50
24	5.10	4.90
36	5.10	4.70
48	5.10	5.10
60	5.70	5.10
72	5.50	5.30



The RV growth curve for the pLKO-1 vector control cell line and PCBP2 KD cell line was repeated to determine if the results were reproducible with the same KD cells from Series #1. The growth curve from Series #1 and Series #2 did not reveal a difference in the production of RV over time. The comparison of the two different growth curves demonstrated that these results were reproducible.



Upon further analysis, after both series #1 and #2 viral growth curves had been performed, the PCBP2 mRNA concentration was found to be only 60% reduced with an

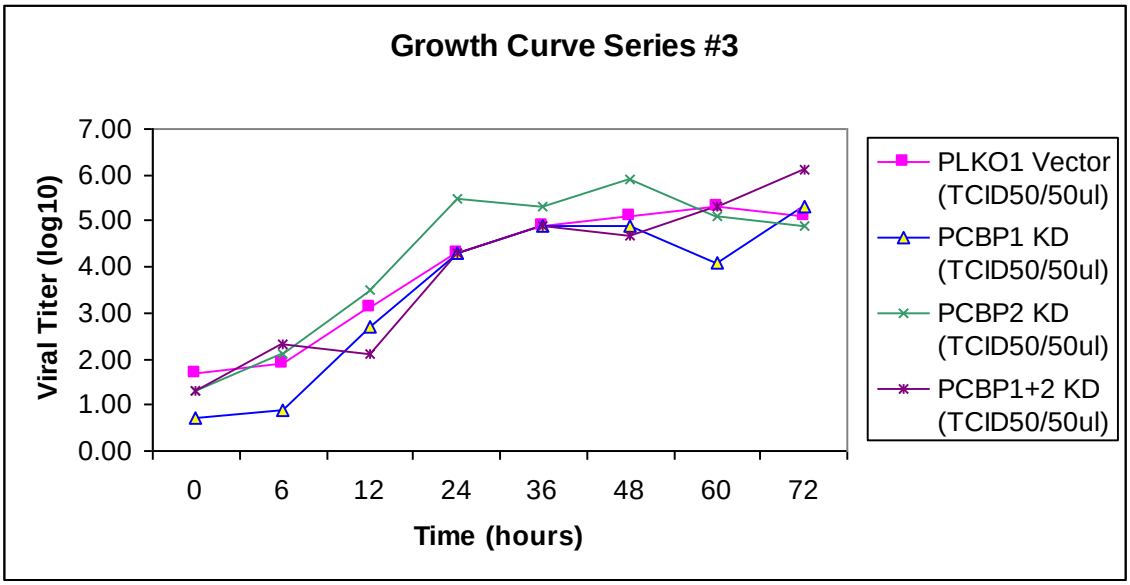
mRNA expression level of 40% by qRT-PCR. We therefore inferred that the PCBP2 KD cell line was not sufficiently knocked down to determine if RV titer was affected in a PCBP2-depleted environment.

RV replication titers over 72 hours in PCBP1, PCBP2 and PCBP1+2 knocked cell lines compared to control lines (Series #3)

New PCBP1, PCBP2 and double PCBP1+2 KD cell lines were made by Dr. Saiprasad G. Palusa. RV growth curves were performed and the data is shown below.

Upon further analysis it was found that the PCBP1+2 KD cell line had reverted back to normal production of the PCBP1 protein. Protein concentrations were measured by western blot and found to be 90% KD for PCBP1 alone cell-line, 80% for PCBP2 alone cell-line and ~75% KD for PCBP2 and 38% KD for PCBP1 in the PCBP1+2 double KD cell-line. Based on the growth curve there does not appear to be a difference in rabies viral titer between the pLKO-1 vector control cell lines and the KD cell lines over time.

Time (hours)	pLKO-1 Vector (Log_{10} $TCID_{50}$/50µl)	PCBP1 KD (Log_{10} $TCID_{50}$/50µl)	PCBP2 KD (Log_{10} $TCID_{50}$/50µl)	PCBP1+2 KD (Log_{10} $TCID_{50}$/50µl)
0	1.70	0.70	1.30	1.30
6	1.90	0.90	2.10	2.30
12	3.10	2.70	3.50	2.10
24	4.30	4.30	5.50	4.30
36	4.90	4.90	5.30	4.90
48	5.10	4.90	5.90	4.70
60	5.30	4.10	5.10	5.30
72	5.10	5.30	4.90	6.10

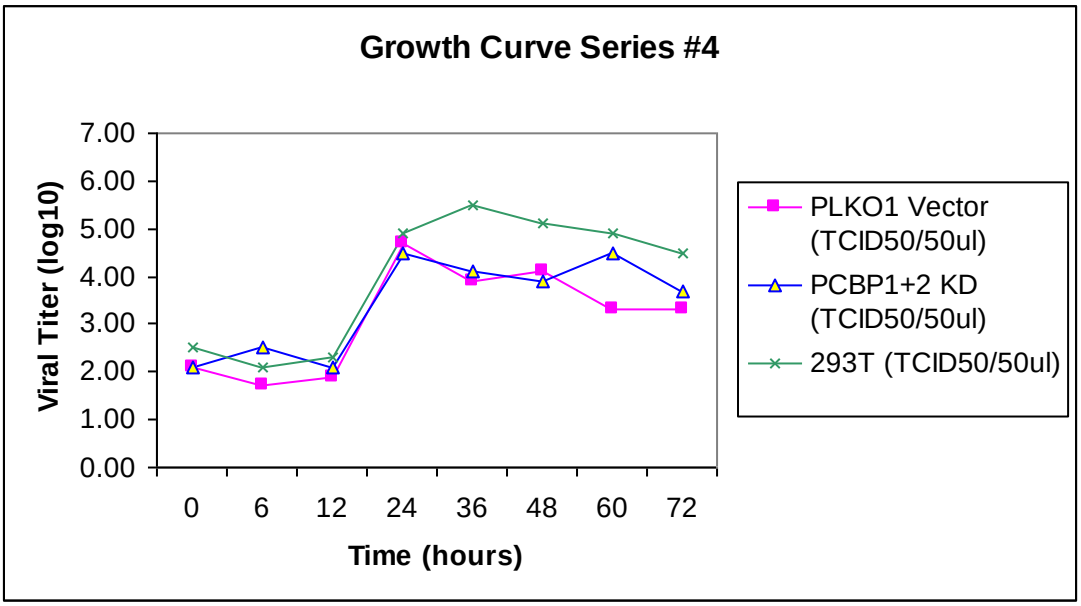


RV replication titers over 72 hours in PCBP1+2 knocked cell line compared to control lines (Series #4)

Another PCBP1+2 KD cell line was made by Dr. Saiprasad G. Palusa with the PCBP2 being knocked down 90% and the PCBP1 knocked down 76% measured by western blot. Another growth curve was performed with this cell line, the pLKO-1 vector

control and an unaltered 293T cell line that was the backbone cell line for the pLKO-1 vector control and knock down cell lines.

Time (hours)	pLKO-1 Vector (Log_{10} $TCID_{50}$/50µl)	PCBP1+2 KD (Log_{10} $TCID_{50}$/50µl)	293T (Log_{10} $TCID_{50}$/50µl)
0	2.10	2.10	2.50
6	1.70	2.50	2.10
12	1.90	2.10	2.30
24	4.70	4.50	4.90
36	3.90	4.10	5.50
48	4.10	3.90	5.10
60	3.30	4.50	4.90
72	3.30	3.70	4.50



There does not appear to be a difference in the growth curves of the pLKO-1 vector control cell line and the PCBP1+2 KD cell lines. There is a slight difference between the 293T cells and the pLKO-1 and PCBP1+2 KD at the 36 hour time point but

this could be an anomaly based on previous results with the pLKO-1 vector control growth curves in series 1-3.

DISCUSSION

Collaborative work with the Wilusz laboratory revealed that the RV G mRNA is found at a higher concentration during infection than what would be predicted from the start-stop model for transcription. The accepted model for mRNA synthesis based on vesticular stomatitis virus replication has the nucleocapsid mRNA with the highest concentration followed in decreasing concentration the other viral genes, ending with the viral-polymerase mRNA with the lowest concentration (Iverson and Rose 1981). We found that the PCBP2 protein binds to in vitro transcribed RNA consisting of the 3' UTR region of G mRNA in extracts from uninfected cells. Moreover, during RV infection PCBP2 is specifically associated with the G mRNA but not other RV mRNAs. We hypothesized that due to the higher concentration of G mRNA that the PCBP2 may be stabilizing the mRNA as has been seen with poliovirus (Murray et al. 2001). We hypothesized that PCBP2 may be important for RV replication as it could influence the abundance and/or translation of the G mRNA. If PCBP2 stabilizes G mRNA than we might expect a decrease in RV titers in PCBP2-depleted cells infected with RV. However, the results of our experiments do not show a difference in RV titer between cells having <20% PCBP2 relative to control cells. Based on these data, the PCBP2 protein does not appear to have an important influence on replication of CVS-11 RV.

One possible explanation for the lack of effect in viral replication of RV in the PCBP knock down cell lines could be the RV variant that was used. Preliminary testing

done in the Wilusz laboratory was done with sequence from the Pasteur virus strain that is a popular vaccine strain that is considered non-pathogenic (Wilkinson 2002). Infection experiments carried out for the growth curves were done with the Challenge Virus Strain-11 (CVS-11); CVS RV strains are used by the NIH for vaccine quantification (Wilbur and Aubert 1996) and are considered pathogenic in mice. There is a difference in the G mRNA 3' UTR region of these two viruses of ~350 nucleotides. The Pasteur strain has a 3' UTR region of ~72 nucleotides while the CVS-11 strain has a 3'UTR region of ~421 nucleotides (Personal communication from Dr. Saiprasad G. Palusa). Although CVS11 G mRNA clearly associates with PCBP2 during an infection, the role of PCBP2 may be less important in the presence of additional regulatory elements found in the longer 3'UTR in this strain. This difference in the region that PCBP2 recognizes could explain the lack of effect on the growth curve. To test this theory the growth curve assay should be repeated with the Pasteur or the ERA vaccine strain that have identical intergenic regions, to determine if the PCBP2 protein affects their viral replication.

Another possibility is that other cellular proteins are compensating for PCBP2. The human genome encodes five poly(C) proteins – hnRNPs K/J and PCBP1-4 (Makeyex and Liebhaber 2002). We have shown that PCBP1 does not significantly associate with G mRNA during an infection with normal 293T cells, but we did not investigate other PCBPs. We have only knocked down PCBP1 and PCBP2. It would be extremely challenging to obtain good knockdowns of all five factors but it is possible that we could investigate the importance of the PCBP binding site using morpholinos to block the binding of any proteins to the PCBP recognition site, or by deleting that region in a recombinant RV clone.

In conclusion, we have found a novel protein, PCBP2 that binds to the 3' UTR region of the G mRNA that may explain why the G mRNA is found in higher concentration than expected. When comparing the knocked down cell lines for PCBP1 and 2 to the control cell lines we did not see any difference in RV titers measured over a 72 hour period. Therefore, in this experimental setting the PCBP1 or 2 proteins are not essential for the efficient replication of the CVS-11 RV.

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