

THE ROLES OF CHEMOKINES ON RABIES VIRUS PATHOGENICITY AND
IMMUNOGENICITY

by

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ABSTRACT

It has been found previously that induction of innate immunity is an important mechanism of rabies virus (RABV) attenuation. To evaluate the effect of overexpression of innate immune molecules on RABV immunogenicity, chemokines MIP-1 α , RANTES and IP-10 were initially cloned into the genome of attenuated RABV strain HEP-Flury. These recombinant RABVs were evaluated in the mouse model for virulence and immunogenicity. It was found that overexpression of MIP-1 α further attenuated RABV virulence when compared to the parental RABV. On the other hand, overexpression of RANTES and IP-10 increased RABV virulence by inducing excessive infiltration of immune cells into the CNS, especially CD3⁺ T cells and neutrophils. Furthermore, up-regulation of RANTES and IP-10 enhanced blood-brain-barrier (BBB) permeability. Immunization of mice with the recombinant HEP- MIP1 α by the intramuscular route induced significantly higher titers of virus neutralizing antibodies (VNA) and provided more protection against lethal challenge than with parental virus, HEP-MIP1 α (-) virus, as well as viruses expressing RANTES or IP-10. Our data thus demonstrate that overexpression of chemokines are not always beneficial during RABV infection. Expression of MIP-1 α can induce innate and enhance adaptive immune responses against rabies while further

attenuate RABV virulence. On the other hand, over-expression of RANTES or IP-10 increased RABV virulence. Together, these studies indicate that overexpression of MIP-1 α can further attenuate RABV virulence and enhance RABV immunogenicity and thus HEP- MIP1 α have the potential to be developed as live avirulent RABV vaccines.

INDEX WORDS: *Rabies virus (RABV), virulence, immunogenicity, BBB permeability, innate immunity, adaptive immunity, vaccine.*

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DEDICATION

I dedicate this work to my family, my advisor and all my friends for their support throughout these years.

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CHAPTER 1

INTRODUCTION

Rabies, or hydrophobia (fear of water), is a viral neuroinvasive disease that cause acute encephalitis in mammals (6). It is mainly caused by a bite of rabid animal, but it was also found in some cases with plant transportation (9). In the beginning stages of rabies, the symptoms are headache, malaise and fever. In later stages it includes acute pain, violent movements, uncontrolled excitement and the inability to swallow water, followed by lethargy and coma. Death generally caused by respiratory insufficiency. Once clinical symptoms occur, the disease is usually fatal (1).

Rabies has been known since 3000 B.C. and it is still an important viral zoonosis in the world. Although there are some rabies-free countries and islands, such as Japan, England, New Zealand, Greece, Portugal, Chile, and Barbados, rabies is prevalent in all the continents (2). Among human infections, rabies is believed to be the tenth most common cause of death (7). It is estimated that rabies kills around 60,000 people each year, mostly in Asia and Africa (4, 11). India has been reported as having the highest rate of human rabies in the world, reporting 30,000 deaths per year (5). Even by rudimentary surveillance, one person dies from rabies every 15 minutes and more than 300 others are exposed (WHO Fact Sheet N 99, Revised June 2001).

The causative agent of rabies is the rabies virus, which survives in wide-spread and varied reservoirs, in both domestic and wildlife animals. In Asia, Latin America and large parts of Africa, dogs remain the principal host, where most human rabies cases occur (7). In the United States, dogs are under control through pet vaccination program and only a few incidents occur each year. However, wildlife rabies emerges as a more challenging problem. Bat, fox, skunk, and raccoon remain the main reservoir for rabies in North America (3). In the past decade, most of the human cases in US were associated with bats, especially silver-haired bats (8, 10).

Prevention of human rabies is usually carried out by post-exposure treatment with rabies virus vaccine and anti-rabies immunoglobulins immediately after exposure. Mass vaccination of domestic pet animals and wildlife animals can also control rabies. However, the cost of post-exposure treatment including vaccine and anti-RV immunoglobulin is estimated to be more than US\$3,000 per case in US. Around 6 million people worldwide receive post-exposure treatment, which cause an economic burden exceeding \$1 billion annually (2). Therefore, extensive research on rabies pathogenesis, virus-host interaction and vaccine development are still needed for more effective therapeutic intervention in clinical rabies and understanding neuroinvasive diseases.

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CHAPTER 2

LITERATURE REVIEW

Rabies virus: Rabies virus (RABV), the agent of one of the oldest recognized infectious diseases, belongs to the genus *Lyssavirus* in the family *Rhabdoviridae* (82). RABV is an enveloped, nonsegmented, negative-stranded RNA virus. It has helical symmetry, with a length of about 180 nm and a cross-sectional diameter of about 60 nm (10). RABV has a 12kb length genome which encodes five proteins in the order of: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (L)(53). Viral RNA is encapsidated by the N, which together with P and L, forms the ribonucleoprotein (RNP) complex. By encapsidating the genomic RNA, N switches the viral polymerase from the mode of viral transcription to replication (81). RNP aggregates in the cytoplasm of virus-infected neurons and form Negri bodies (NBs), which are the characteristic histopathological lesion for RABV infection. A recent study showed that Toll-like receptor-3 (TLR3) is a major molecule involved in the formation of NBs, which indicates that RABV can exploit cellular proteins for their own benefit (47). The RNP complex surrounded by a lipid bilayer associated with G and M. RABV G spans the membrane and forms an array of trimeric spikes extending from the viral envelope (55). RABV M bridges the RNP and the cytoplasmic domain of G to form the bullet-shaped virion (46, 53).

Viral proteins: All the five proteins, N, P, M, G, and L, are structural proteins of the virion and are essential for virus replication and spread. N contains 450 amino acids and is the major component of RNP core. The amino acid sequence of N is highly conserved, reflecting its important function. N is required for encapsidation of genomic RNA and protection of the RNA template from ribonuclease activity (67). N also regulates RNA transcription and viral replication by promoting read-through of the termination signals (85, 86).

P is a noncatalytic cofactor and regulatory protein, which plays a role in viral transcription and replication. Besides the full length P protein, the P gene encodes additional shorter P products (P2, P3, P4 and P5), which are translated from downstream and in-frame AUG codons by a leaky scanning mechanism (9). It is phosphorylated by two kinases, rabies virus protein kinase and protein kinase C (26). P exhibits several phosphorylation states and binds to the ribonucleocapsid with several different affinities (65). It stabilizes L to the RNP and binds to the soluble N, keeping it in a soluble form for encapsidation of viral RNA. P also interacts with cytoplasmic dynein light chain (LC8) via a conserved (K/R) XTQT motif (54). A most recent study showed that RABV P-LC8 interaction is not directly involved in the retrograde axonal transport of RABV from the periphery to the CNS (70). Detailed analysis using different cell lines demonstrated that RABV P-LC8 interaction was important for viral transcription (70). Recently, P was found to be responsible for immune evasions by antagonizing interferon (IFN). P inhibits the phosphorylation of interferon regulatory factor-3 (IRF-3) by the upstream kinase TBK-1 and prevents the dimerization of IRF-3 (5). P prevents IFN- α/β - and IFN- γ -stimulated JAK-STAT signaling so that activated STAT1 and STAT2 are unable to accumulate in the nucleus (6). In another study, Vidy et al. found that P directly interacts with signal transducer

and activator of transcription 1 (STAT1) and promyelocytic leukemia protein (PML), inhibiting the IFN-induced antiviral response (74). P protein and the nuclear P3 isoform inhibit the binding of STAT1 or ISGF3 to the DNA promoters of IFN- γ or IFN- α -responsive genes (75).

RABV M is the smallest and most abundant protein in the rabies virions, which plays a regulatory role in viral transcription and replication (36). It associates with the core forming a coiled structure called skeleton, condenses the RNP core in the cytoplasm and binds cores to the membrane in preparation for budding (21). M mediates binding of RNP to the host membrane at the marginal region of the cytoplasm and initiates RABV budding from the cell plasma membrane (24). Several homologous sequence motifs, termed late domains, play an important role in virus budding, which includes a proline-rich region, PPxY or PY, and a P(T/S)AP sequence (36). Recently, it was found that RABV M can inhibit eukaryotic translation by interacting with the cellular translation machinery eIF3 (36).

RABV G is a trimeric, externally oriented, and membrane-spanning protein, which binds the cell surface receptors and antibody binding sites. RABV G is related to cell attachment, induction of apoptosis and immune responses (63). G is the major inducer of rabies virus-neutralizing antibodies (VNA), which are considered to be the major effectors in the immune defense against a lethal rabies virus infection. G plays an important role in viral pathogenicity. The direct involvement of RABV G in axonal transport was revealed by Mazarakis, who showed that equine infectious anemia virus (EIAV) with RABV G possessed the ability to travel from the gastrocnemius muscle to the spinal cord (42). Mutations in RABV-G play a critical role in viral pathogenesis. A single amino acid substitution at 333 from wild-type Arginine (Arg) to

glutamine (Gln) or serine (Ser) could significantly reduce the virulence of RABV by changing the rate of virus spread from cell to cell and the neuronal pathway that the virus takes to CNS (12, 64). Studies showed that high levels of G expression were found to be responsible for induction of apoptosis in infected cells (17, 49, 51, 60, 84). We also showed that RABV G determines the pattern of infection in the brain (83).

L is the largest protein and is the catalytic component of the polymerase complex. It contains four conserved motifs in the central part of L, which maintains the same linear arrangement and location in all RNA dependent RNA polymerase. One tri-amino acid core sequence GDN in motif C is extensively conserved in all non-segmented negative-strand RNA viruses (61). Study showed that not only the GDN core sequence but also specific amino acids downstream from the core sequence are crucial for the maintenance of polymerase activity (61).

Viral transcription and replication: After adsorption, the virus penetrates the host cell membrane, uncoats its envelope, and releases the viral cores into the cytoplasm. Then, the negative-strand RNA genome is transcribed into a 48-nucleotide leader RNA and 5 individual mRNAs encoding the N, P, M, G and L, by the RNA-directed RNA polymerase (L). The mRNA of the genes most proximal to the 3' leader promoter is the most abundant and the amounts of transcribed mRNA decrease with the distance from the leader sequence (19). Once viral proteins are synthesized, viral transcription is switched to replication. The balance between RNP replication and mRNA transcription is regulated by the M protein (18, 20).

Replication is characterized by production of a full-length positive RNA strand complementary to the entire parental template, followed by production of full-length negative-strand RNAs. Unlike transcription, replication requires active, ongoing translation, particularly of viral N and P proteins. After synthesis of N, P and L proteins, association of these proteins in the cytoplasm with replicated genomic RNA occurs to form RNP. RNP complex associates with M, which condenses the RNP core from outside (36). The M localizes the RNP core at the cellular membrane, where the nascent G protein is concentrated. Then M interacts with G and initiates budding through the host cell membrane (46) .

Pathogenesis: It is a puzzle to understand the mechanism by which RABV infection of neurons causes neurological diseases since rabies patients show few gross and histopathological lesions. Most clinical signs of rabies are related to abnormal neuronal functions such as altered neurotransmission. Humans become infected with RABV generally through the bite of rabid dogs and cats, or other wild carnivorous species. The precise sites of viral sequestration remain unknown: virus may replicate in muscle tissue, or directly enter the peripheral nervous system. After initial replication in the peripheral site, RABV transports to the central nervous system (CNS) in a retrograde way. Several receptors in CNS have been proposed to facilitate virus entry into neurons: the neural cell adhesion molecule, the p75 neurotrophin receptor (p75NTR) and the acetylcholine receptor (7, 73). The virus may enter the peripheral nervous system via the neuromuscular junctions and moves rapidly to CNS, particularly to the nearest sensory or motor neuron in the dorsal root ganglion or anterior horn of the spinal cord where it replicates (32). After replication, the virus travels along the corticospinal tract to the brain and infects neurons in almost all brain regions (23).

The neurological disease in rabies results from neuronal dysfunction rather than neuronal death, which could be explained by a defect in cholinergic synaptic neurotransmission as previously reported (34). Dysfunction of ion channels has been demonstrated in infected culture cells (33). Our study also showed that infection with wt RABV results in alteration of host protein expression, particularly those involved in ion homeostasis and docking and fusion of synaptic vesicles to presynaptic membrane (11). Infection of primary neurons with pathogenic RABV resulted in the destruction of neuronal processes and disappearance of microtubule-associated protein 2 and neurofilament immunoreactivity, which suggests that pathogenic RABV causes degeneration of neuronal processes possibly by interrupting cytoskeletal integrity (41).

Several mechanisms are used by RABV to evade the host immune responses. As mentioned before, P was the responsible interferons (IFNs) antagonist, which could inhibit IFNs production. Our previous study showed that wild-type (wt) but not attenuated RABV could evade the host innate immune responses mainly by restricting G expression (77). By inhibiting G expression, RABV can prevent apoptosis and thus trigger a protective immune response. Wt RABV does not induce apoptosis in the brains of experimentally infected mice (84). Highly pathogenic RABV strains also have less impact on the expression of major histocompatibility complex (MHC) class II in the infected brain (31). RABV can maintain the integrity of neurons but up-regulate FasL levels, which induce apoptosis of T-cells shortly after they cross the blood-brain barrier (BBB) (2). Recently, it was been shown that RABV infection could cause neurons to express programmed cell death ligand 1 (PD-1), which contributed to the exhaustion or eradication of CD8 T cells and thus favors the escape of virus infection from the host immune response (38).

Recent studies showed that the lethal outcomes of infection with highly pathogenic strain RABV, such as SHBRV and dog rabies virus (DRV) were associated with the inability to deliver immune effectors across the BBB. It was hypothesized that pathogenic strain RABV had enough time to spread to the CNS and activate the processes inhibiting immune cell invasion before the infiltrated cells could protect (56, 57). Our most recent study showed that laboratory-attenuated RABV induced expression of chemokines and infiltration of inflammatory cells into the CNS. Upregulation of chemokines might have triggered the change in BBB permeability, which helps infiltration of inflammatory cells into the CNS, and thus attenuation of RABV (37).

The role of innate immunity in the pathogenesis of rabies: The unquestionable general rule is that infection of RABV is fatal, but several investigators have reported animal or human cases where spontaneous resistance and survival occurred (1, 30). These atypical cases suggest that some innate resistance mechanisms exist, the understanding of which may open an avenue for the development of new therapeutic strategies (40). Innate immunity is the first line host defense mechanism that operates to protect organisms from infectious microorganisms. Innate immunity is also the oldest mechanism of defense against microbes and is present in all multicellular organisms. The mechanisms of innate immunity exist before an encounter with microbes and are rapidly activated by microbes before the development of adaptive immune responses. The innate immune system interacts with the adaptive immunity in two ways: innate immunity stimulates adaptive immunity and adaptive immunity uses the effector mechanisms of innate immunity to eliminate microbes. Janeway was among the first to point out the importance of innate immunity in 1989 (35). As proposed by Janeway, innate immunity is in fact predicated upon pattern recognition receptors (PRRs) detection of abundantly expressed pathogen-associated molecular

patterns (PAMPs), which are conserved among broad classes of microorganisms (35). These molecules are critical for pathogen replication and/or survival and are unique to microorganisms. Thus, they are absent from host cells and thereby endow the host with an efficient, nonself-reactive means to detect invading pathogens. In the late 1990s, the discovery of Toll-like receptors significantly advanced the understanding and definition of PRR-mediated innate immunity (78). The component of the innate immune system includes epithelial barriers, leukocytes (neutrophils, macrophages, and NK cells), circulating effector proteins (complement, collectins), and cytokines (e.g., IFN- α/β , chemokines, TNF, IL-1).

When facing a viral infection, most cell types in the body respond by secreting high levels of IFN- α/β . This induction occurs within a few hours of the infection, and IFN- α/β has a number of antiviral functions. First, IFN- α/β directly induces antiviral activities in the uninfected, neighboring cells. This prevents viral spread by increasing the resistance of uninfected cells toward the virus. Second, IFN- α/β can activate natural killer (NK) cell-mediated cytotoxicity toward virus-infected cells (25). Third, there is accumulating evidence that IFN- α/β contributes to driving the adaptive-immune response in the T helper cell type 1 (Th1) direction via stimulation of IFN- γ expression. IFN- α/β has been shown to potently enhance immune responses in vivo through stimulation of dendritic cells and serves as a signal linking innate and adaptive immunity (39). RABV infection triggers the expression of IFNs in vitro and vivo. Nakamichi et al. reported the regulation of IFN- α/β in RAW macrophages after stimulation with lab-adapted RABV (50). In another paper, high levels of IFN occurring in serum early during infection with rabies virus contribute to the resistance of inoculated mice (48). In our previous study, not only IFN- β but also IFN- $\alpha 2$, IFN- $\alpha 4$, and IFN- $\alpha 5$ are found to be up-regulated by infection with

attenuated RABV. IFN- α/β induced proteins implicated in the antiviral activities include PKR, 2', 5'-OAS, ADAR, Mx, and MHC I (77). These molecules are involved in mRNA translation, RNA degradation, RNA editing, and cytotoxic T-lymphocyte responses. The role of IFN- α/β in resisting RABV infection has previously been investigated. Direct administration of IFN- α/β or IFN-inducing poly (I:C) resulted in various degrees of protection against RABV infection in mice, hamsters, rabbits, or monkeys (28). Hooper et al. reported that higher virus titers were detected in IFN- α/β receptor knocked (IFNAR^{-/-}) mice than intact mice when infected with an attenuated CVS-F3. It also took a longer time for the IFNAR^{-/-} mice (21 days) than normal counterparts (8 days) to clear the virus from the CNS (29). In addition, fully immunocompetent mice developed higher levels of virus neutralization antibodies than IFNAR^{-/-} mice (29). All these data indicate that IFN- α/β plays a role in RABV resistance through both innate and adaptive immune response.

In addition to IFN- α/β , attenuated RABV also stimulates the expression of many genes encoding chemokines. Chemokines are a group of small (~8-14 kDa), mostly basic, structurally related molecules (87). There are approximately 43 chemokines identified to date, which are classified according to the configuration of cysteine residues near the N-terminus into four families: C-, CC-, CXC-, and CX3C (87). The large number of chemokines classified into 'inflammatory' and 'lymphoid' chemokines based on the site of production and the eliciting stimuli (87). Inflammatory chemokines were the first to be discovered and found to attract primarily neutrophils and other cells of the innate immune system. Subsequently they were shown to attract also different types of effector and memory lymphocytes. The inflammatory chemokines are produced by several cell types such as endothelial, epithelial, and stromal cells

as well as leukocytes. They are induced to high level of expression by inflammatory stimuli such as LPS, IL-1, and TNF- α . Classical examples include IL-8, RANTES, eotaxin, MIP1 α , MCP-1, and IP-10. Many of the inflammatory chemokines (both C-C and C-X-C families) are highly up-regulated, particularly MCP-1, -3, and -5, MIP1 α , RANTES, IP-10, and MIG in RABV-infected animals (87). Besides attraction of leukocytes to the sites of infection, chemokines also have a role in the development of a Th1 versus Th2 immune response (13). Mice lacking CCR2 display an impaired Th1 cytokine profile in the brain after MHV infection despite higher viral titers (8). In agreement with this, a study in humans showed that in patients infected with HIV, expression of CCL3, CCL4, and CCL5 correlated with a Th1 type of immune responses (72).

Activation of Toll-like receptors (TLRs) also induces inflammation. In our previous research, TLR1, TLR2 and TLR3 are found to be up-regulated in RABV-infected mice (77). TLRs are transmembrane proteins that detect invading pathogens by binding conserved, microbially derived molecules and that induce signaling cascades for proinflammatory gene expression. TLRs utilize leucine-rich-repeat motifs for ligand binding and a shared cytoplasmic domain to recruit the adaptors MyD88, TRIF, TIRAP, and/or TRAM for downstream signaling (78). It is now well accepted that TLRs, which are expressed in cells at the front line of the host-defense system (in particular epithelial cells, macrophages, dendritic cells), mediate activation of the transcription factor NF- κ B in response to a broad repertoire of microbial molecules. In particular, a subset of TLRs is involved in the sensing of viral infections, and regulating expression of type I interferons (IFNs). TLR-mediated cell activation proceeds through two distinct pathways. The first pathway depends on an adaptor molecule, myeloid differentiation factor 88 (MyD88), which transduces downstream events leading to nuclear factor κ B (NF- κ B)

activation (68). MyD88 associates with the proximal Toll-IL-1 receptor homology domain (TIR) of TLRs, initiating a signaling cascade that involves activation of interleukin-1 (IL-1) receptor-associated kinase (IRAK) family members and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This pathway is triggered by TLR2, TLR4, TLR5, TLR7, TLR8, and TLR9 (68). Toll-IL-1 receptor domain-containing adaptor-inducing IFN- β (TRIF) and the TRIF-related adaptor molecule (TRAM) mediate a MyD88-independent alternative pathway. Once TRIF binds to activated TLR3 or TLR4, it interacts with TRAF6, inducible IKK, and TANK-binding kinase 1, mediating the induction of IFN- β through the activation of NF- κ B and IRF-3. Overexpression of these adaptor molecules is proven to turn on downstream cellular signaling in the absence of TLRs or TLR ligands (69).

Rabies vaccines: Over 100 years ago, in 1885, Louis Pasteur developed a crude nerve tissue vaccine for postexposure treatment of rabies. Since then, numerous other rabies vaccines for human use have been developed and used with varying degrees of effectiveness and safety. In the 1950s, embryonated tissue vaccines came on the market, such as the duck embryo vaccine (DEV). DEV was used extensively for about 25 years in the USA until the early 1980s. This vaccine produced a poor antigenic response in a significant proportion of recipients and often caused severe adverse reactions. Now we are in the modern era of cell culture vaccines, several of which have been produced. The human diploid cell vaccine (HDCV) is developed by Dr. Hilary Koprowski and his colleagues at the Wistar Institute (80). HDCV is considered the gold standard with which others are considered. Some of the derivatives of tissue culture vaccines are similar to HDCV and they are both effective and well tolerated. They include the purified chicken embryo cell vaccine (PCEC), the purified Vero cell rabies vaccine (PVRV) and the

purified duck embryo cell vaccine (PDEV). When used appropriately, new cell culture vaccines provide nearly 100% protections with high degree of safety. But over 40,000 people world-wide die from rabies each year. The most important reason is the huge global rabies reservoirs, in both domestic and wildlife animals. Humans serve only as accidental hosts.

Particular emphasis must be put on domestic animal vaccinations to provide a buffer zone between potentially rabid wild animals and the human population. In most developing countries, dogs represent the major reservoir of rabies virus where most human rabies cases occur. A mass vaccination of canines in these regions could virtually eliminate rabies, but the high cost of vaccines hampers this undertaking. In countries where dog rabies is controlled through animal vaccination, the number of human cases has decreased considerably.

At the present, almost all the vaccines used for human and domestic animals are inactivated virus vaccines. The safety and effectiveness of an inactive rabies vaccine, especially a vaccine derived from cell culture, have been so well accepted. But it is too expensive for vaccination of people and animals in developing countries. The high cost of production of an inactivated rabies vaccine is due mainly to the complicated production process. A single rabies vaccine dose causes about \$200.00. A course of post-exposure treatment will cost about \$1,000. It costs more than \$3,000 for post-exposure treatment per person including vaccine and immunoglobulins. Thus it is prohibitive for most of the people in the developing countries. Moreover, multiple vaccinations are needed when inactivated vaccines are used. For post-exposure treatment, a total of five doses are required over a period of 90 days. Many people may not be able to follow through the course of vaccination and thus come down with the diseases. For pre-exposure

prophylaxis, three doses are required initially and booster immunization later. For animal vaccination, it is performed yearly or every three years. An attenuated live vaccine, on the other hand, is able to efficiently elicit a protective immune response with a smaller amount of the virus because the vaccine virus propagates and produces viral antigens. This vaccine can be generally produced at a lower cost than an inactivated vaccine and be delivered by needle-free methods such as oral inoculation. But the attenuated vaccine virus sometimes causes rabies in the inoculated animal by its residual virulence or pathogenic mutation during viral propagation in the body (14, 79). More efforts should be taken to improve the safety of attenuated live vaccines before they can be widely used in human and domestic animals.

Rabies in the wildlife presents a more challenging problem. Fox rabies has been endemic in Europe and North America for many years. Raccoon rabies has spread from a focal point in Florida to all the eastern states in the US by the 1990s (23). Other important reservoirs include coyotes in Asia, Africa and North America, skunks in North America, and the insectivorous bats in the Americas. Oral immunization of wildlife with attenuated live vaccines such as the modified live virus vaccines SAG-1, and SAG-2 or the recombinant vaccinia virus expressing rabies virus glycoprotein (VRG) is the most effective method of controlling and eventually eliminating rabies in terrestrial wildlife (76). Vaccination with modified live attenuated or recombinant vaccines has resulted in almost complete eradication of vulpine rabies in Western Europe. On the other hand, while these vaccines induce protective immunity in foxes, neither SAD-based modified live rabies vaccines nor recombinant vaccinia viruses work well in skunks (59, 71). Only 50% of the badgers orally administered with $10^{8.3}$ TCID₅₀ of VRG vaccines were protected against rabies (4). Administration of more than 10^9 infectious virus particles is

required for minimum effect in dogs in the laboratory, and the widespread use of this amount of material is currently beyond feasible commercial production capacities. Furthermore, current modified live rabies virus vaccines may actually cause disease. A live avirulent RABV, SAG-2, has also been used for immunization of wildlife against rabies in many parts of Europe (3). These vaccines are effective, however, they have problems. Human exposure to VRG has been associated with intensive local inflammatory reaction and generalized erythroderma (58). Low virus neutralizing antibody (VNA) response has been reported for oral immunization with live attenuated SAG-2 (27).

Thus more effort is needed to develop a cheaper, safer and more effective vaccine for prevention of rabies, especially in developing countries. Developing more affordable and efficacious attenuated live avirulent vaccine is a promising direction. But before it can be widely used in human and domestic animals, we must improve its safety.

Reverse genetics and novel RABV vaccines: Reverse genetics, as the term is used in molecular virology, describes the generation of viruses possessing a genome derived from cloned cDNAs. In 1994, Conzelmann and colleagues generated recombinant RABV from cloned cDNA (62). It was the first time to produce a negative-strand RNA virus from cloned cDNA. By using reverse genetics, RABV genome has been used to express foreign proteins or extra copy of RABV protein. Foreign proteins such as HIV Gag or Env (43), HCV envelope proteins E1 and E2 or a modified version of E2 (66), and severe acute respiratory syndrome coronavirus (SARS-CoV) envelope spike protein (S) (16) have been expressed in RABV vector and it has been shown that expression of these proteins resulted in induction of potent humoral and cellular immune

responses in immunized animals. Expression of foreign proteins which can stimulate the host immune responses has also been used for development of RABV vaccines. Cytochrome c have been cloned into RV genome and the recombinant virus can induce accelerated apoptosis, which contributes to enhanced immunogenicity and attenuated pathogenicity (52). The expression of cytokines at the time of virus infection has the potential to enhance immune responses. Overexpression of TNF α an important proinflammation cytokine, in a recombinant RABV can induce stronger CNS inflammation, thus exerting its protective activity (15). Expression of IL-2 from RABV-based vaccines vector was found to modulate the immune responses (44). A recombinant RABV that expresses an extra copy of G has also been cloned into RABV genome (17). Because RABV G is the only surface protein that is capable of inducing the production of VNA (22), thus overexpression of RABV G results in enhanced adaptive immune responses and better protection (17). Besides these, gene mutation on both the P and G (45) and deletion of the P were also proved to effective to construct novel avirulent RABV vaccines (65).

We propose to develop avirulent RABV vaccines by expressing innate immune molecules in RABV genome using reverse genetics technology since it has been found that induction of innate immunity is an important mechanism of RABV attenuation. In these studies, innate immune molecules such as INF, chemokines and TLR adaptors are cloned into RABV genome. Their pathogenicity and immunogenicity are determined in the mouse model. It was found that recombinant RABV expressing chemokine MIP1 α and TLR adaptor molecules not only further attenuated RABV pathogenicity, but also enhanced RABV immunogenicity. Together, these results indicate that RABV expressing innate immune molecules have the potential to be developed as live avirulent RABV vaccines.

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CHAPTER 3

THE ROLES OF CHEMOKINES IN RABIES VIRUS INFECTION: OVER- EXPRESSION MAY NOT ALWAYS BE BENEFICIAL

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Abstract

It has been found previously that induction of innate immunity, particularly chemokines, is an important mechanism of rabies virus (RABV) attenuation. To evaluate the effect of overexpression of chemokines on RABV infection, chemokines MIP-1 α , RANTES and IP-10 were individually cloned into the genome of attenuated RABV strain HEP-Flury. These recombinant RABVs were characterized *in vitro* for growth properties and expression of chemokines. It was found that all the recombinant viruses grow as well as the parent virus and each of the viruses expressed the intended chemokine in a dose-dependent manner. When these viruses were evaluated for pathogenicity in the mouse model, it was found that overexpression of MIP-1 α further decreased RABV pathogenicity by inducing a transient innate immune response. In contrast, overexpression of RANTES or IP-10 increased RABV pathogenicity by causing neurological diseases, due to high level expression of chemokines, excessive infiltration and accumulation of inflammatory cells in the CNS, and severe enhancement of blood-brain-barrier (BBB) permeability. These studies indicate that overexpression of chemokines, although important in controlling virus infection, may not always be beneficial to the host.

Introduction

Rabies virus (RABV) is a negative-strand RNA virus belonging to the *Rhabdoviridae* family, genus *Lyssavirus*, which causes rabies (fatal encephalomyelitis) in many species of mammals (3). More than 55,000 humans die of rabies each year worldwide (16). Once clinical signs develop, rabies is always fatal (2, 8). Despite the lethality of rabies, only mild inflammation and little neuronal destruction were observed in the central nervous system (CNS) of rabies patients (20, 21). Adaptation of wild-type (wt) RABV in laboratory animals and/or cell culture led to attenuation in phenotype and thus laboratory-adapted RABV have been used for vaccine development (1, 7). To delineate the mechanism(s) of RABV attenuation, previous studies compared the host responses to infection with either laboratory-attenuated or wt RABV (37). It was found that laboratory-attenuated RABV induced extensive inflammation, apoptosis, neuronal degeneration, as well as induction of expression of innate immune genes in the CNS; however, wt RABV caused little or no neuronal damage and avoided the activation of expression of the genes involved in innate immunity. Other investigators also reported the induction of innate immunity in mice or neuronal cells infected with laboratory-attenuated viruses (13, 22, 26). The mostly up-regulated genes in the innate immune responses after infection with attenuated RABV include genes encoding for inflammatory chemokines and type I interferon (IFN) and IFN-related proteins (13, 22, 26). Further studies have shown that the expression of chemokines (mRNA and proteins), particularly MIP-1 α , RANTES, and IP-10, correlates with the infiltration of inflammatory cells and enhancement of blood-brain-barrier (BBB) (14).

Chemokines are a group of small (~8-14 kDa), basic, structurally related molecules that can attract inflammatory cells along concentration gradients and enhance leukocyte-endothelial cell interactions (40). The tertiary structure of chemokines is highly conserved, containing at least

four cysteine residues that form two disulfide bonds (35). Chemokines have been divided into major subfamilies on the basis of the arrangement of the two N-terminal cysteine residues, CC and CXC. CC chemokines act primarily upon monocytes while CXC family members are specific for neutrophils and lymphocytes (9). Chemokines regulate cell trafficking of various types of leukocytes through interactions with G protein-coupled receptors with seven transmembrane regions (40). Most chemokine receptors are stimulated by more than one chemokine and one ligand might stimulate more than one receptor (35). This combination of redundancy and promiscuity might act as a safety factor to ensure adequate host defenses (19). Chemokines have direct anti-viral activities and/or recruit inflammatory cells to the site of infection to kill virus-infected cells (23). It has been reported that overexpression of IP-10 from the mouse hepatitis virus (MHV) genome promoted protection from coronavirus-induced neurological and liver diseases (36). In another study, IP-10 expression modulates the pathogenesis of liver disease in adenovirus-infected mice by attracting CD8⁺ T lymphocytes into the liver (39). However, due to their ability to direct migration of inflammatory cells, overexpression of chemokines might be harmful in some diseases, especially in the process of autoimmune inflammation. In an experimental autoimmune encephalomyelitis (EAE) model, IP-10, MCP-1 and MIP-1 α were strongly up-regulated (9).

In this present study, the roles of chemokines in RABV infection was further investigated by cloning and expressing MIP-1 α , RANTES and IP-10 in the genome of the RABV HEP-Flury strain. The effect of overexpression of chemokines on RABV infection was investigated in the mouse model. It was found that overexpression of MIP-1 α decreased the pathogenicity by inducing transient expression of chemokine and infiltration of inflammatory cells into the CNS.

In contrast, recombinant RV expressing MIP-1 α , RANTES and IP-10 induced persistent expression of chemokines and extensive infiltration of inflammatory cells into the CNS, causing neurological diseases and death.

Materials and methods

Cells, viruses, antibodies, and animals.

Mouse neuroblastoma (MNA) cells were maintained in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). BSR cells, a cloned cell line derived from BHK-21 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech) containing 10% FBS. Recombinant RABV strains were propagated in BSR cells. CVS-11 was propagated in MNA cells. CVS-24 was propagated in suckling mouse brains as described previously (38). Fluorescein isothiocyanate (FITC)-conjugated antibody against the RABV N protein was purchased from FujiRab (Melvin, PA). Anti-RABV nucleoprotein (N) monoclonal antibody 802-2 was obtained from Dr. Charles Rupprecht, Centers for Disease Control and Prevention. Antibodies used for flow cytometric analysis such as CD3 (17A2), Ly6G (RB6-8C5), CD45 (30-F11), and CD11b (M1/70) were purchased from BD Pharmingen (San Jose, CA). Anti-CD3 polyclonal antibody was purchased from Abcam (England). Biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). Female BALB/c mice at the age of 6-8 weeks were purchased from Harlan and housed in temperature- and light-controlled quarters in the Animal Facility, College of Veterinary Medicine, University of Georgia. All animal experiments were carried out as approved by the Institutional Animal Care and Use Committee.

Construction of recombinant RABV clones.

Mouse MIP-1 α , RANTES and IP-10 cDNA were amplified from RNA extracted from RABV-infected mouse brain using SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen-Life Technology). The primer sets used for PCR were designed by Primer3 (<http://primer3.sourceforge.net/>) (Table 3.1). The PCR products were digested with BsiWI and NheI (New England Biolabs, Beverly, MA) and then ligated into RABV vector pHEP-3.0 (ref) that had been previously digested with BsiWI and NheI. The resulting plasmids have each of the chemokine genes cloned between RABV glycoprotein (G) and the polymerase (L) genes and were designated pHEP-MIP1 α , pHEP-RANTES and pHEP-IP10, respectively (Fig. 3.1).

Rescue of recombinant RABV.

Recombinant RABVs were rescued as described previously (12). Briefly, BSR cells were transfected with 2.0 μ g of full infectious clone, 0.5 μ g of pH-N, 0.25 μ g of pH-P, 0.1 μ g of pH-L, and 0.15 μ g of pH-G using SuperFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacture's protocol. After incubation for 4 days, the culture medium was removed and fresh medium added to the cells. After incubation for another 3 days, the culture medium was transferred into NA cells and examined for the presence of rescued virus by using FITC-conjugated antibody against the RABV N protein.

Virus titration.

Viruses were titrated by direct fluorescent assay in NA cells. NA cells in 96-well plate were inoculated with serial 10-fold dilution of virus and incubated at 34°C for 2 days. The culture

supernatant was removed and the cells were fixed with 80% ice-cold acetone for 30 min. The cells were then stained with FITC-conjugated anti-RABV N antibodies. Antigen-positive foci were counted under a fluorescent microscope (Zeiss, Germany) and viral titers were calculated as fluorescent focus units (FFU) per milliliter. All titrations were carried out in quadruplicate.

Enzyme-linked immunosorbent assay (ELISA) and multiplex ELISA.

Brains were homogenized in nine-fold volume of PBS containing 0.1% NP-40 and Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN). The homogenates were centrifuged at 13,000 rpm, 4 °C for 30 min to remove the debris and the supernatants were taken out carefully and aliquoted in microtubes at 0.5ml/ tube. The supernatant was subjected to ELISA to quantify the amount of MIP-1 α , RANTES and IP-10 individually in cell culture supernatants or mouse brain suspensions by using the murine MIP-1 α , RANTES and IP-10 ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacture's protocol. Multiplex ELISA kit (Quansys Biosciences, Logan, Utah) was used to quantify a panel of 16 cytokines (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFN γ , TNF α , MIP-1 α , GM-CSF and RANTES) in brain extracts according to the manufacture's protocol.

Quantitative real-time RT-PCR.

To determine viral load, real-time RT-PCR was performed on the RNA samples using G gene-specific primers (5'-ccatctggatgcctgagaat-3' and 5'-ggcaccatttggtctcatct-3') in a Stratagene Mx3000P (Stratagene, La Jolla, CA). With 100 ng sample RNA or no-template control, PCR was performed in two steps; only one primer was used for cDNA synthesis at 50°C for 30 min, and

both primers were used in PCR amplification. Each reaction was carried out in duplicate. The reverse transcriptase and DNA polymerase were from One-Step Brilliant II SYBR Green QRT-PCR Master Mix Kit (Stratagene). For absolute quantitation, a standard curve was generated from serial diluted RABV G RNAs of known copy numbers, and the copy numbers of samples were normalized to 1µg of total RNA. The RNA standard was prepared from pH-G by Reverse Transcription System (Promega) according to the manufacture's protocol.

Histopathology and Immunohistochemistry.

For histopathology and immunohistochemistry, animals were anesthetized with ketamine-xylazine and perfused by intracardiac injection of phosphate-buffered saline (PBS) followed by 10% neutral buffered formalin as described previously (38). Brain tissues were removed and embedded with paraffin. Histopathology was performed by staining the paraffin-embedded sections with hematoxylin and eosin. For immunohistochemistry, paraffin-embedded brain sections were heated at 70°C for 10 min and then dipped in CitriSolv (Fisher Scientific) three times for 5 min and dried until chalky white. Slides were incubated with proteinase K (20 µg/ml) in 10 mM Tris-HCl (pH 7.4 to 8.0) for 15 min at 37°C and rinsed three times with PBS. The primary antibody and then secondary antibodies were used for immunological reaction as described (ref). Finally, diaminobenzidine was used as a substrate for color development.

Leukocyte isolation from CNS.

Mouse brains infected with different recombinant viruses were harvested on days 3, 6 and 9 post infection (p.i.) and digested with 2 µg/µl collagenase D (Worthington Biochemical Corporation, Lakewood, NJ), 1 µg/µl DNase I (Sigma-Aldrich) in Hanks balanced salt solution

(with Ca^{2+} , Mg^{2+}) for 1 h to dispersed the tissue into single-cell suspension. Viable cells were separated by discontinuous Percoll gradient (70/30%) centrifugation for 25 min (650g at room temperature, without brake). After being washed once with Hanks balanced salt solution (without Ca^{2+} , Mg^{2+}) (Invitrogen) and counted, cells were stained for CD3 (17A2), Ly6G (RB6-8C5), CD45 (30-F11), and CD11b (M1/70) with directly conjugated antibodies (BD Pharmingen) for 30 min at 4°C and then fixed with 1% paraformaldehyde. Data collection and analysis were performed with a BD LSR-II flow cytometer and BD FACSDiva Software (BD Pharmingen).

Rapid fluorescent focus inhibition test (RFFIT).

Blood was collected from each mouse for measurement of virus neutralizing antibodies (VNA) by using the RFFIT as described previously (33). Briefly, 50 μl of serial five-fold dilutions of serum were prepared in Lab-Tek Chamber slides (Nalge Nunc International). 50 FFD50 (50% Fluorescing Foci doses) of CVS-11 was added to each chamber and incubated at 37 °C for 90 min. 0.2 ml of MNA cells (5×10^5 cells/ml) were added into each chamber and the slides were incubated at 37 °C for 20 hr. Then the slides were fixed with 80% ice-cold acetone and stained with FITC-conjugated anti-RABV N antibodies for 1 hr. Twenty fields in each chamber were observed under a fluorescent microscope, and the 50% endpoint titers were calculated according to the Reed-Meunch formula (28). The values were compared with that of the reference serum (National Institute for Biological Standards and Control, Herts, EN6 3QH, UK) and normalized to international units (IU/ml).

Measurement of Blood-brain barrier (BBB) permeability.

BBB permeability was assessed using a modification of a previously described technique (24, 34) with the following markers: sodium-fluorescein (NaF, 100 μ l of 100mg/ml, i.v.); fluorescein-dextran (FITC-dextran) of molecular mass 10,000 Da (200 μ l of 100mg/ml, i.v.); fluorescein-dextran (FITC-dextran) of molecular mass 150,000 Da (200 μ l of 37.5mg/ml, i.v.). Mice received these markers intravenously under anesthesia. After 10 min circulation for NaF and FITC-dextran-10,000 and 4 hour circulation for FITC-dextran-150,000, peripheral blood was collected. Serum (50 μ l) was recovered and mixed with 15% trichloroacetic acid (TCA). After centrifugation for 10 min at 10,000 g, the supernatant was recovered and made up to 150 μ l by adding 30 μ l 5M NaOH and 7.5% TCA. The brain was perfused with PBS injected through the left ventricle to flush out intravascular fluorescein. Then the brain tissues were homogenized in cold 7.5% TCA and centrifuged for 10 min at 10,000 g to remove insoluble precipitates. After addition of 30 μ l 5M NaOH to 120 μ l supernatant, the fluorescence was determined using a BioTek Spectrophotometers (Bio-Tek Instruments, INC) with excitation at 485 nm and emission at 530 nm. Markers uptake into tissue is expressed as (μ g of fluorescence cerebrum/ mg brain tissue)/(μ g of fluorescence / μ l serum) to normalize uptake values of the dye for blood levels of the dye at the time of tissue collection (34).

Statistical Analyses.

All experiments were repeated at least three times. Statistical significance of the differences between different treatment groups was performed with SigmaStat software (Systat Software Inc., San Jose, California). One-way ANOVA test with Holm-Sidak method was used to analyze

clinical score, body weight, VNA titer, chemokine/cytokine concentration and immune cell infiltration into CNS.

Results

In vitro characterization of recombinant RABVs.

Our previous studies indicate that induction of chemokines, particularly MIP-1 α , RANTES and IP-10, is important for RABV attenuation (14, 37). To further investigate the effect of chemokines on RABV infection, gene encoding murine MIP-1 α , RANTES or IP-10 was amplified from virus-infected mouse brain and cloned into rHEP (11) between the G and the L genes (Fig. 3.1A). Recombinant viruses were rescued in BSR cells as described previously (11) and these viruses are designated as HEP-MIP1 α , HEP-RANTES and HEP-IP10, respectively. To characterize these viruses in vitro, the growth kinetics of these viruses were examined in NA cells. As shown in Fig. 3.1B, no significant difference in growth kinetics was observed between each of the recombinant viruses and the parental virus, indicating that viral growth was not affected by the insertion of chemokines. The ability of the recombinant RABV to produce chemokines was determined by measuring chemokine production in virus-infected cells with ELISA kits. As shown in Fig. 3.1C, production of the intended chemokine was detected in NA cells infected with each recombinant RABV in a dose-dependent manner. No chemokine was detected in NA cells infected with parent virus rHEP.

Pathogenicity of recombinant RABVs in mice.

To determine of effect of chemokine on RABV infection, BALB/c mice (10 per group) at 4-6 weeks of age were infected with 10⁵ FFU of recombinant viruses by the intracerebral (i.c.)

route. Infected mice were monitored twice daily for two weeks. Body weight was measured and mouse examined for the development of diseases and death. The animals were scored for clinical signs with: 0, normal mouse; 1, disorder movement; 2, ruffled fur; 3, trembling and shaking; 4, paralysis; and 5, dead.

As shown in Fig. 3.2A, mice infected with HEP-MIP1 α were similar to sham-infected mice. Neither obvious weight loss no clinical signs were observed in these two groups of mice. Mice infected with parent virus rHEP lost about 7% of their body weight and one mouse developed mild symptoms including rough fur and slow movement at day 6 to 9 p.i. and then recovered. Mice infected with HEP-RANTES lost about 14% of their body weight and 30% of the mice developed severe symptoms such as rough fur and emaciation, but no paralysis. One mouse in this group died at day 12 p.i. (Fig. 3.2C). Mice infected with HEP-IP10 lost about 21% of their body weight. Seventy percent of the mice in this group developed severe symptoms and 30% of the mice succumb to infection at days 10 and 11 p.i. (Fig. 3.2C). The observed symptoms in HEP-IP10-infected mice occurred significantly more frequently ($P \leq 0.01$) than in mice infected with the parental virus (Fig. 3.2B). Mice infected with HEP-IP10 lost more body weight ($P \leq 0.05$) than those infected with the parent virus (Fig. 3.2A). These results indicate that recombinant RABV expressing MIP-1 α is more attenuated while viruses expressing RANTES or IP-10 enhanced RABV pathogenicity when compared to the parental virus.

To determine if the effects of chemokines on pathogenicity are associated with virus replication, virus titers, viral antigen, and viral genomic RNA in the mouse brain was determined at days 3, 6 and 9 p.i. No virus was detected in the brains of mice infected with any of the viruses

during the period (data not shown). Viral antigen (N) was only detected sparsely in the brain at only day 3 p.i. in mice infected with each of the virus, but not in sham-infected mice (data not shown). Quantification of viral genomic RNA by real time-PCR revealed the copy number of viral genomic RNA in mouse brains was highest at day 3 p.i.. However, there is no significant difference for the quantity of the genomic RNA among mice infected with the parent or recombinant viruses (Fig. 3.2D). The data indicates that overexpression of chemokines has no apparent effect on viral replication in adult mice. Thus, the rate of replication is not a determinant for the pathogenicity of different recombinant viruses expressing different chemokines.

Expression of Chemokines and Cytokines in mouse brain after infection with recombinant RABVs.

To investigate the mechanism(s) of HEP-MIP1 α attenuation relative to rHEP, and exacerbated disease associated with HEP-RANTES and HEP-IP10 viruses, the expression of these chemokines was determined by ELISA. A multiplex ELISA was also performed to measure the expression of other inflammatory chemokines and cytokines. As shown in Fig. 3.3, all the recombinant viruses induced the expression of the intended chemokine to high levels at day 3 post infection (p.i.) except HEP-IP10 that induced high level of IP-10 expression at day 6 p.i. As expected, expression of one chemokine led to the expression of other chemokines. Virus expressing MIP-1 α induced only a transient high level of MIP-1 α at day 3 p.i. (significantly more than the parent virus) and its expression declined quickly by days 6 and 9 p.i. Interestingly, expression of MIP-1 α did not induce high level of expression of the other chemokines. In contrast, viruses expressing RANTES or IP-10 not only induced high and persistent expression

of the respective chemokines, but also induced high expression of other chemokines. HEP-RANTES induced significantly higher expression of RANTES at day 3 p.i. than any other virus ($P \leq 0.05$) and the level persisted with slight reduction by day 9 p.i. This virus also induced higher level of IP-10 expression ($P \leq 0.001$) at day 6 p.i. than the parent virus and HEP-MIP1 α . HEP-IP10 induced significant higher level of IP-10 expression (up to 250 pg per mg brain tissue) at day 6 p.i. than the parent virus and HEP-MIP1 α ($P \leq 0.05$). The IP-10 expression was slightly reduced by day 9 p.i. in HEP-IP-10-infected mice, but still significantly higher than in mice infected with other viruses. In addition, HEP-IP10 induced higher expression of other chemokines or cytokines as well. It induced highest expression of MIP-1 α at day 6 and RANTES at day 9 p.i. It also induced the highest expression of MCP-1, TNF α , and IL-6 at days 6 and 9 p.i. The parent virus rHEP induced the highest expression of RANTES at day 6 p.i. and only low expression of all these chemokines or cytokines. Overall, parent rHEP and HEP-MIP1 α induced low expression of chemokines and cytokines while HEP-RANTES, particularly HEP-IP10, induced high expression of not only the intended chemokine, but also other chemokines and cytokines. Expression of IP-10, MCP-1, and TNF- α at high levels correlates well with the development of diseases in the animals.

Induction of inflammation in mouse brain by recombinant RABV.

Chemokines and cytokines produced in large quantities may cause the huge influx of inflammatory cells into the brains. Histopathology was performed to analyze the inflammatory cells present in brain tissue. Less infiltration of inflammatory cells was observed in the brain of mice infected with HEP-MIP1 α than that in rHEP-infected mouse brain at days 3, 6, and 9 p.i. At day 6 p.i., HEP-RANTES and HEP-IP10 induced much more inflammatory cells infiltration than

rHEP. By day 9 p.i., infiltration of inflammatory cells decreased in rHEP-infected mice, while infiltration of inflammatory cells continued to persist or increase at day 9 p.i. in HEP-RANTES- and HEP-IP10-infected mice. No inflammatory cells were seen in brains of sham-infected mice (Fig. 3.4A). To quantify the infiltration of inflammatory cells, immunohistochemical analysis revealed that fewer CD3-positive cells were detected in the HEP- MIP1 α -infected mouse brains than in those infected with rHEP at days 3, 6, and 9 p.i.; while significantly more CD3-positive cells were detected in the brains of mice infected with HEP-RANTES and HEP-IP10 at 6 and 9 day p.i. ($P \leq 0.001$). By day 9 p.i., the number of CD3-positive cells decreased in mice infected with rHEP, but continued to increase in mice infected with HEP-RANTES and HEP-IP10 (Fig. 3.4B and 3.4C).

Differentiation of Inflammatory cell infiltration in mouse brain after infection with recombinant RABV.

To better understand the infiltration of inflammatory cells into the CNS after infection with different RABV, leukocytes were recovered from brains and analyzed by flow cytometry. The populations of activated microphage, neutrophils and CD3⁺ T cells was examined using cell surface markers CD11b, Ly6G, CD3 and CD45. The cells of each type was found to be less than 3×10^3 / brain at day 3 p.i. and quickly increased to more than 10^4 / brain at day 6 p.i. Compared with parental virus rHEP, HEP-MIP1 α induced less while HEP-RANTES induced more cell infiltration into the CNS although no significant difference was detected. HEP-IP10 induced significant higher infiltration of activated macrophages ($P=0.0042$), neutrophils ($P=0.0056$) and CD3⁺ T cells ($P=0.0041$). By day 9 p.i., the number of CD11b^{hi}/CD45^{hi} macrophage decreased in all groups (Fig. 3.5). In contrast, the number of neutrophils and CD3⁺ T cells remained the

same or continued to increase in HEP-RANTES- and especially in HEP-IP10-infected mouse brains. Taken together, the histopathological and flow cytometric analyses suggested that the increased neutrophils and CD3⁺ T cells trafficking to and accumulation in the mouse brain correlates with the pathogenicity of HEP-RANTES and HEP-IP10.

Enhancement of BBB permeability after infection with recombinant RABV.

To investigate if infection with each of the recombinant viruses induces different changes in BBB permeability, the leakage of the sodium fluorescein (NaF) from the circulation into the CNS tissues was measured in the cerebrum, cerebellum and spinal cord of mice. No significant change in BBB permeability was observed in cerebellum and spinal cord of mice. BBB permeability was significantly enhanced in the cerebrum of mice infected with all the viruses by 6 days p.i. when compared to sham-infected mouse brain. BBB permeability in mice infected with HEP-RANTES and HEP-IP10 was significantly higher than that of rHEP or MIP-1 α -infected mice (P=0.0259 and P=0.0235, respectively). By day 9 p.i., BBB permeability in mice infected with all the viruses returned to the level as observed at day 3 p.i. (Fig. 3.6A). These data indicate that infection with all the viruses enhanced BBB permeability at day 6 p.i. when compared to sham infection. Furthermore, HEP-RANTES and HEP-IP10 induced significantly more enhancement of BBB permeability than parent virus and HEP-MIP1 α .

To investigate if overexpression of different chemokines can induce BBB changes so that large molecules can easily enter the CNS, different size markers such as NaF (376 Da), FITC-dextran-10,000, FITC-dextran-150,000, and Evans blue (960 Da), were used to measure changes of BBB permeability at day 6 p.i. As shown in Fig. 3.6B, molecules of 150 KDa or larger did not

infiltrate into the cerebrum for any mice in all the groups. Only HEP-IP10 induced significantly higher permeability to a 10 KDa marker ($P \leq 0.001$) than any other virus. This indicates that overexpression of chemokines induced enhancement of BBB permeability to allow small molecules (NaF, 376 Da) enter into the CNS and overexpression of IP-10 can significantly enhance the permeability to allow large molecules (10 KDa) cross the BBB.

To investigate if the enhancement of BBB permeability is associated with chemokine expression in the brain or in the serum, the concentration of chemokines (MIP-1 α , RANTES and IP-10) was determined at day 6 p.i. As shown in Fig. 3.6C, the concentration of chemokines (MIP-1 α , RANTES and IP-10) in the brain is much higher than that in the serum. Overall, infection with different viruses by the IC route did not significantly affect the chemokine concentration in the serum. In the mouse brain, HEP-RANTES induced a significantly higher production of RANTES and IP-10 while HEP-IP10 induced a significantly higher production of IP-10. Only the IP-10 level correlated well with the enhancement of BBB permeability.

Discussion

RABV genome has been used to express foreign proteins including antigens from other viruses (17) or host proteins (17). Expression of host proteins such as cytochrome c, TNF α , or IL-2 has led to enhanced immunogenicity and attenuated pathogenicity (4, 18, 27). Expresses an extra copies of G has also been shown to reduce the pathogenicity of RABV (5). In the present study, chemokines MIP-1 α , RANTES, or IP-10 were cloned into the genome of RABV HEP-Flurry strain. Although expression of MIP-1 α further reduced RABV pathogenicity, expression of RANTES or IP-10 enhanced RABV pathogenicity in the mouse model.

The rationale to clone chemokines into RABV genome was to further investigate the role of chemokines in RABV infection. In our previous study, laboratory-adapted RABV B2C strain induced the expression of innate immune genes including type I IFN and chemokines while wt SHBRV did not (20). Furthermore, expression of chemokines, particularly MIP-1 α , RANTES, and IP-10, at the mRNA and the protein level were found to correlate with the infiltration of inflammatory cells into the CNS and the enhancement of BBB permeability (9). It is thus hypothesized that laboratory RABV attenuates RABV pathogenicity by inducing innate immune responses, particularly the chemokines, which in turn attracts the infiltration and inflammatory cells into the CNS and alters the BBB permeability. Inflammatory cells attracted by chemokines and other immune effectors entered the CNS through the enhanced BBB permeability help clear RABV-infected cells, thus attenuating RABV pathogenicity (9, 20). To further attenuate RABV pathogenicity, MIP-1 α , RANTES, and IP-10 were over-expressed in RABV genome. However, expression of these chemokines is not always beneficial to the host. This is not surprising since chemokines have both beneficial and detrimental effects in virus infections (10, 15).

There are many possibilities as to why expression of MIP-1 α attenuated while RANTES and IP-10 enhanced RABV pathogenicity, for example, the ability of these chemokines to inhibit or help virus replication, the ability to induce expression of the intended and other chemokines/cytokine, or the ability to recruit different subsets of inflammatory cells into the CNS. To determine if any of these abilities is responsible for the observed effects on RABV pathogenicity, virus titers, viral antigen, and viral genomic RNA were measured in the mouse brain. No virus was detected in mice infected with any of the viruses. Viral antigen was detected

only sparsely in these animals. Quantitative RT-PCR revealed no difference in the copy numbers of viral genomic RNA. These results suggest that expression of these chemokines did not change the rate of RABV replication and thus could not account for the difference in RABV pathogenicity. Then the expression of chemokine and cytokine was quantified using a multiplex ELISA. Each of the recombinant viruses expressed high levels of the intended chemokines at days 3 or 6 p.i. However the level of MIP-1 α in mice infected with HEP-MIP1 α subsided quickly. In addition, only low to moderate levels of other chemokines are induced in these mice. In contrast, HEP-RANTES, particularly HEP-IP10, not only induced high and persistent expression of the intended chemokine, but also induced high expression of other chemokines. Transient expression of chemokines may help attenuated while the high and persistent expression of these chemokines, particularly IP-10, may be responsible for the enhanced pathogenicity.

Expression of different chemokines may attract infiltration of different inflammatory cells. For examples, MIP-1 α is a monocyte chemokine and may activate resident microglia, which constitutes 20% of the total glial cell population within the brain (29). Microglia are constantly moving and analyzing the CNS and able to recognize and swallow foreign antigens, and act as antigen-presenting cells (29). In contrast, RANTES and IP10 potently attract activated T cells to the CNS. These activated T cells produced IFN- γ and TNF α and activated mononuclear cells to express inflammatory cytokines such as TNF α and IL-1 (9). In our study, it was found that HEP-MIP-1 α induced low and transient infiltration of inflammatory cells at day 6 p.i. and by day 9 p.i. infiltration of inflammatory cells returned to the level found in sham-infected animals. On the other hand, HEP-RANTES and HEP-IP10 induced high and persistent infiltration of inflammatory cells in the CNS. Differentiation of inflammatory cells into macrophages

(activated microglia), neutrophils, or T lymphocytes by flow cytometry did not reveal the preference of cells recruited into the CNS by any of the recombinant viruses. However, it confirmed that RANTES, particularly IP-10, induced more and persistent infiltration of all the inflammatory cells detected than HEP-MIP1 α or rHEP.

Recently it has been reported that enhancement of BBB is one of the important mechanism for RABV attenuation (25, 30, 31). Attenuated RABV induced enhancement of BBB permeability to allow small molecules (presumably immune effectors) to enter the CNS (6). In our study, we found that all the recombinant RABV enhanced the BBB permeability; however, HEP-RANTES and HEP-IP10 induced more enhancement of BBB permeability than HEP-MIP-1 α or rHEP. Furthermore, HEP-IP10 induced BBB permeability to the extent that allowed large molecules (10 KDa) to enter the CNS, which may have allowed more inflammatory cells or other toxic substances enter into the CNS, leading to enhancement of RABV pathogenicity.

Previous data indicated that expression of chemokines, particularly IP-10, correlates with infiltration of inflammatory cells into the CNS and enhancement of BBB permeability, thus attenuating RABV pathogenicity (9). In the present study, it is found that only low and transient expression of chemokines, infiltration of inflammatory cells, and enhancement of BBB permeability is associated with RABV attenuation. High and persistent expression of chemokines, infiltration of inflammatory cells, and enhancement of BBB permeability is responsible for enhancement of RABV pathogenicity. Some of the laboratory attenuated RABV, for example, B2C strain, can induce diseases and death when high doses are used and extensive inflammation and apoptosis were found in these animals (32, 37). The findings in the present

study thus support our previous hypothesis that laboratory-attenuated RABV induced neurological diseases by immune-mediated pathogenesis (37).

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Figure Legends

FIG 3.1. Construction and *in vitro* characterization of recombinant RABV expressing select chemokines. (A) Construction of full-length recombinant RABV. Chemokine genes, MIP-1 α , RANTES and IP-10 were individually inserted between BsiWI and NheI sites of pHEP-3.0 vector. (B) Growth curves of the recombinant and parental rabies viruses in NA cells. NA cells were infected with different recombinant RABV at 0.01 MOI (Multiplicity of Infection). At days 1, 2, 3, 4 and 5 p.i., culture supernatants were recovered and virus titers determined in NA cells. (C) Chemokines production in infected cells by recombinant viruses. NA cells were infected with different recombinant RABV at 0.001, 0.01, 0.1 and 1 MOI. After 24 hr incubation at 34°C, the culture supernatants were recovered and the concentration of the indicated chemokine was determined by ELISA.

FIG 3.2. Effects of overexpression of chemokins on virus pathogenicity. Body weight (A), clinical score (B), survivorship (C), and viral genomic RNA (D) were monitored in BALB/c mice (N=10) after i.c. infection with 10⁵ FFU of different recombinant RABV or medium (sham-infection) as described in the materials and methods. Data are obtained with 10 mice (3 mice for genomic RNA) each group and given as mean values \pm standard error. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the indicated experimental groups.

FIG 3.3. The inflammatory reaction induced by recombinant RABV by H&E staining (A) or by immunohistochemistry (B). Quantification of CD3⁺ lymphocytes in the hippocampal sections was performed with CD3-positive cells are expressed as mean values \pm SEM obtained from 3

mice at each time point (C). Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the indicated experimental groups.

FIG 3.4. Concentration of chemokines and cytokines in mouse brains. BALB/c mice were infected i.c. with 10^5 FFU different recombinant RABVs. At days 3, 6, and 9 days p.i., brains of were harvested and homogenized. After centrifugation, the suspension was used to measure the concentration of indicated chemokines and cytokines by multiplex ELISA kits. Experiments were performed with three mice for each time point and condition. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the indicated experimental groups.

FIG. 3.5. Differentiation of inflammatory cells infiltrated into the CNS by flow cytometric analysis. BALB/c mice were infected i.c. with 10^5 FFU different recombinant RABVs and brains were harvested after extensive perfusion at days 3, 6 and 9 p.i. CNS leukocytes were isolated by Percoll centrifugation and analyzed by flow cytometry with indicated antibodies. The absolute numbers of specific inflammatory cells in brains after different recombinant RABV infection were calculated (3 mice per group per day). Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the indicated experimental groups.

FIG. 3.6. Determination of changes in BBB permeability. BALB/c mice were infected i.c. with 10^5 FFU different recombinant RABV. At days 3, 6 and 9 p.i., BBB permeability was determined by uptake of NaF (A). The extent of BBB permeability to different size markers were compared (B). The concentration of chemokines in both serum and brains were assayed by

ELISA (C). Each set of data have at least triplicates. Data are given as mean values \pm standard error; asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the indicated experimental groups.

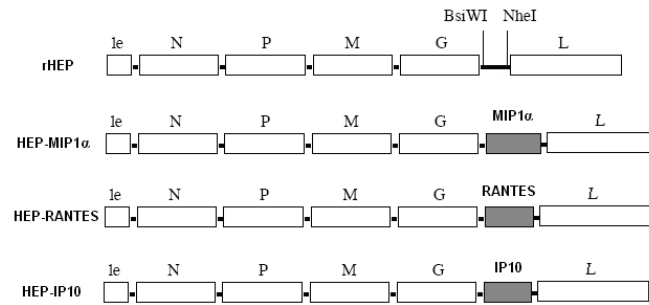
Tables

Table 3.1. Primers used for amplification of chemokines.

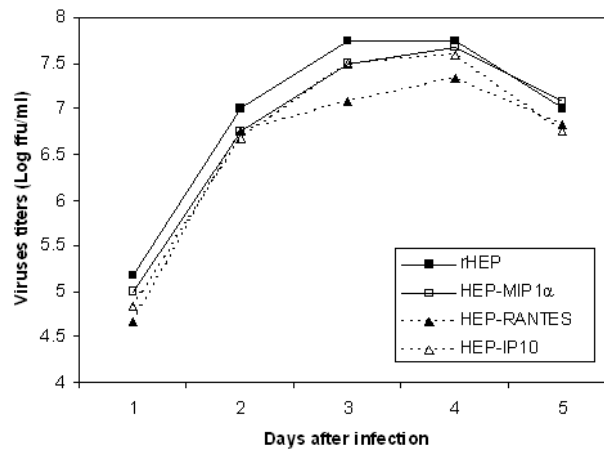
Gene	Forward primer (5'-3')	Reverse primer (3'-5')
MIP-1 α	ctgctccgtacgatgaaggtctccaccact	cctccagctagctcaggcattcagttccag
RANTES	gccgcgcgtacgatgaagatctctgcagct	aaacccgctagcctagctcatctccaata
IP-10	cccatccgtacgatgaaccaagtgtgcc	gcttcagctagcttaaggagccctttaga

Figures

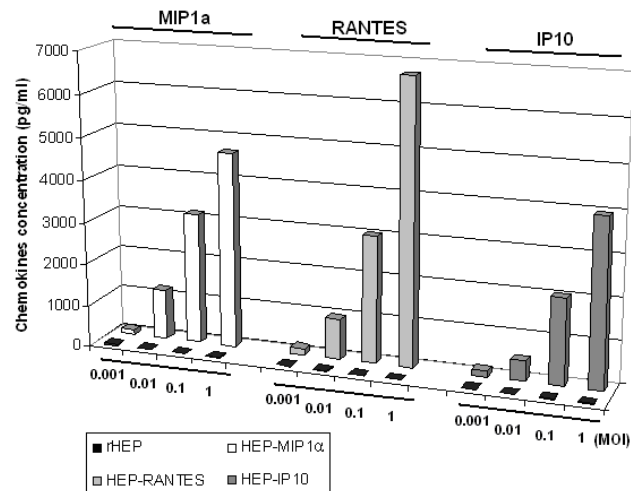
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(B)

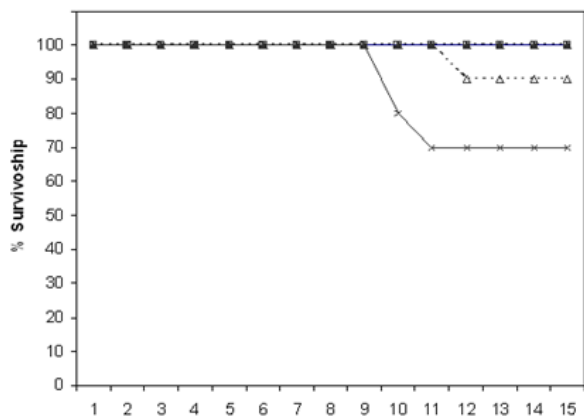


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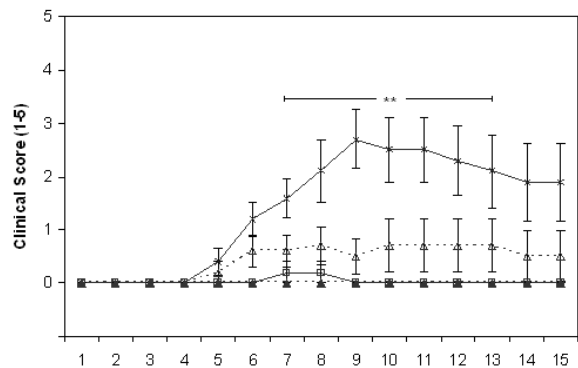


Zhao et al., Fig. 3.1.

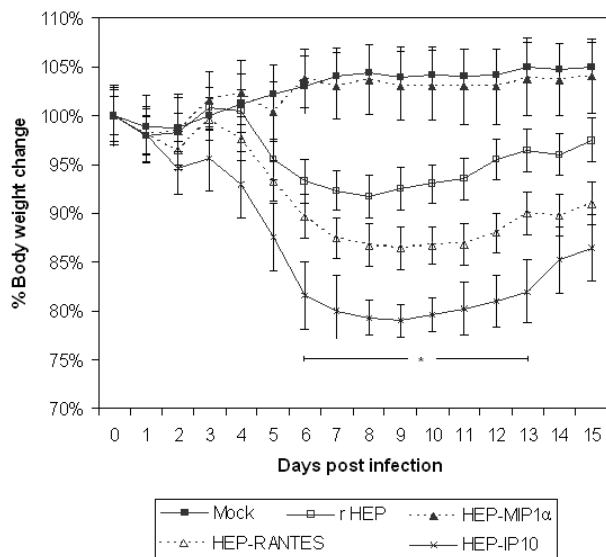
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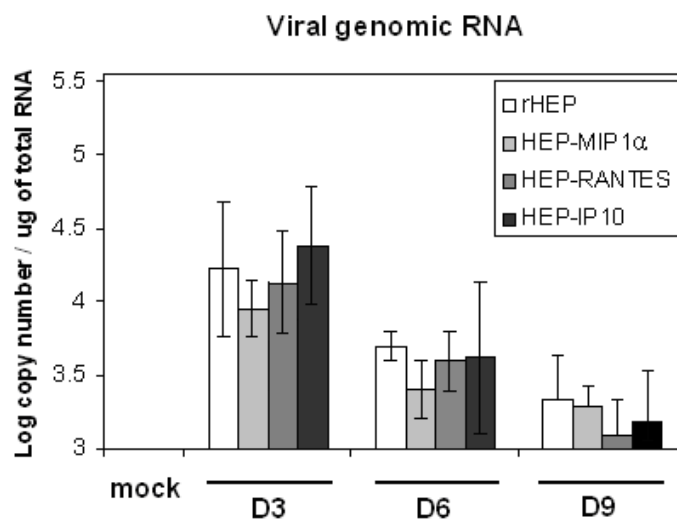
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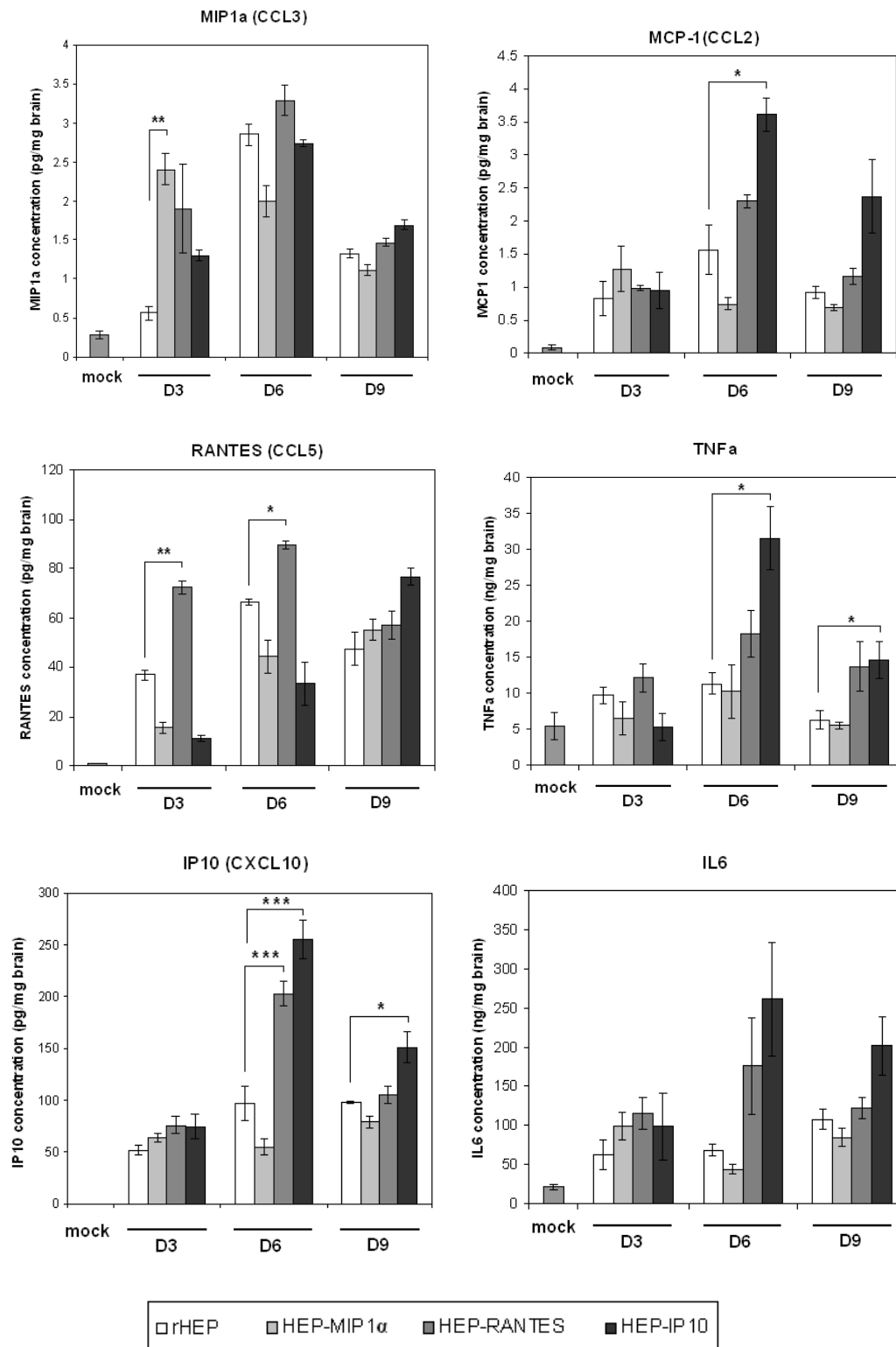
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(D)

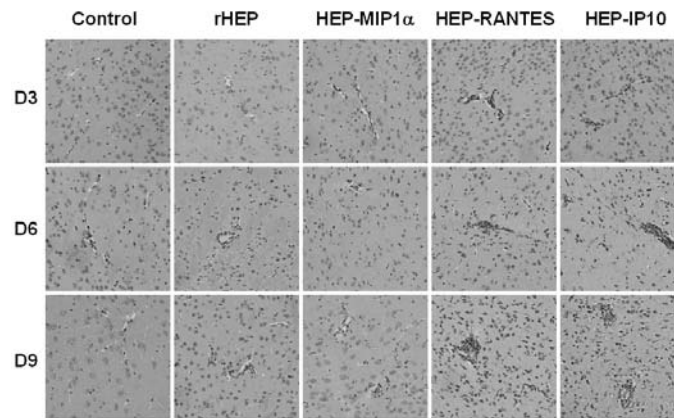


Zhao et al., Fig. 3.2.

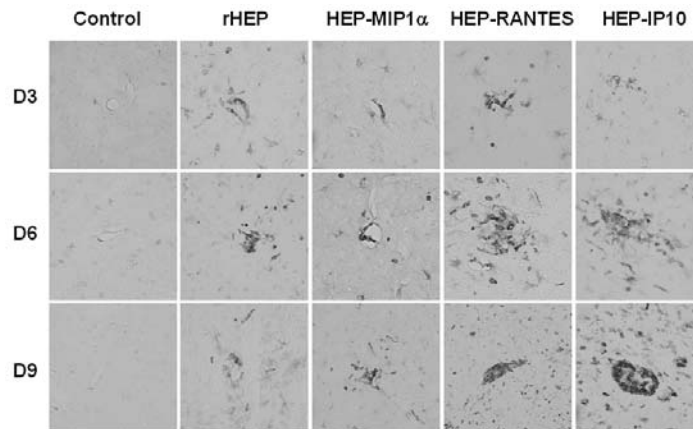


Zhao et al., Fig. 3.3.

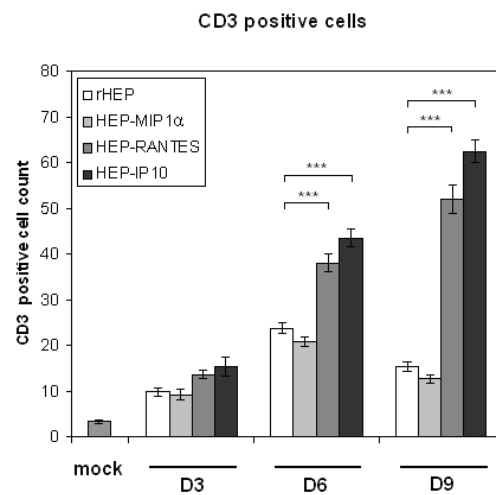
(A)



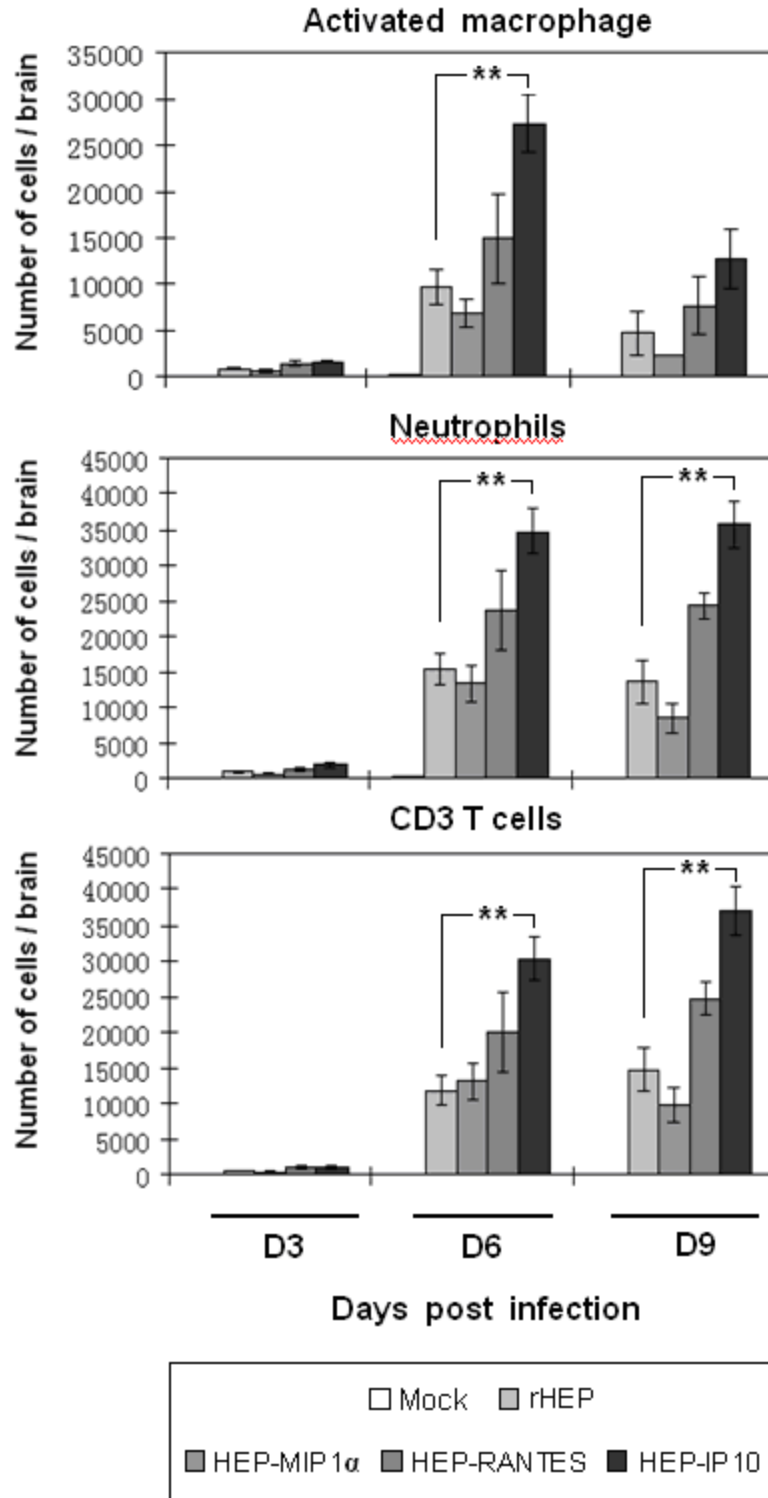
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(C)

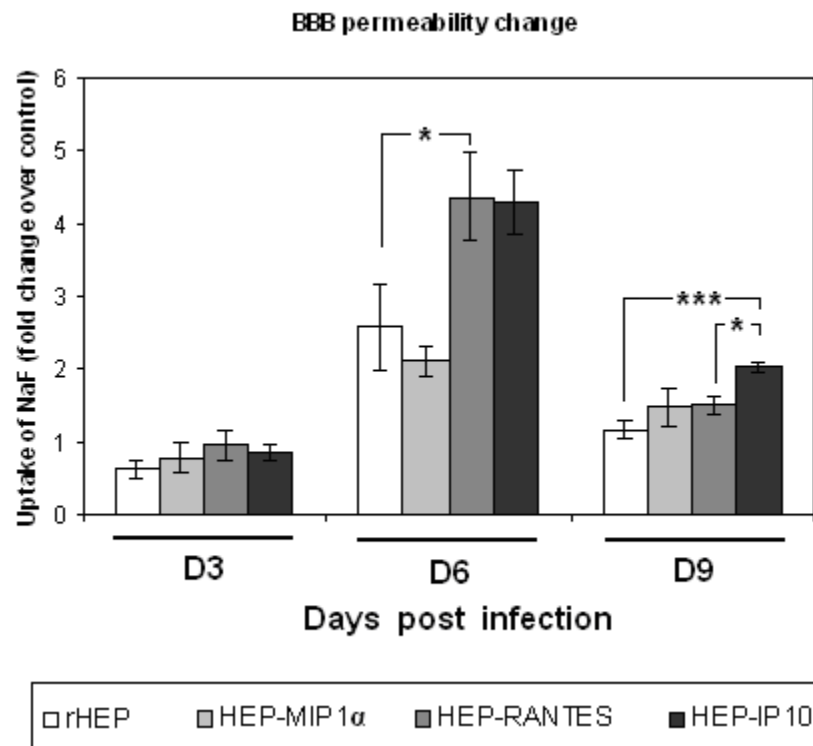


Zhao et al., Fig. 3.4.

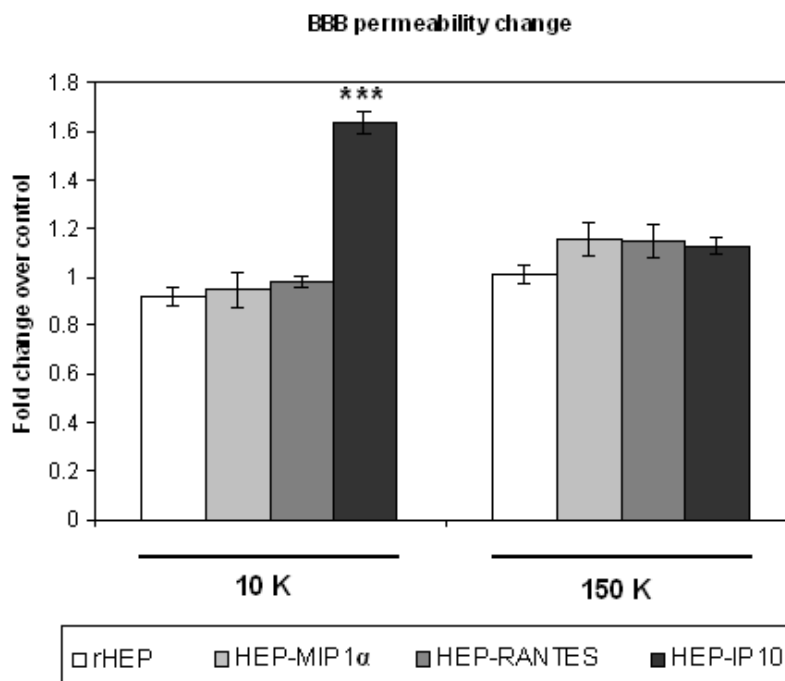


Zhao et al., Fig. 3.5.

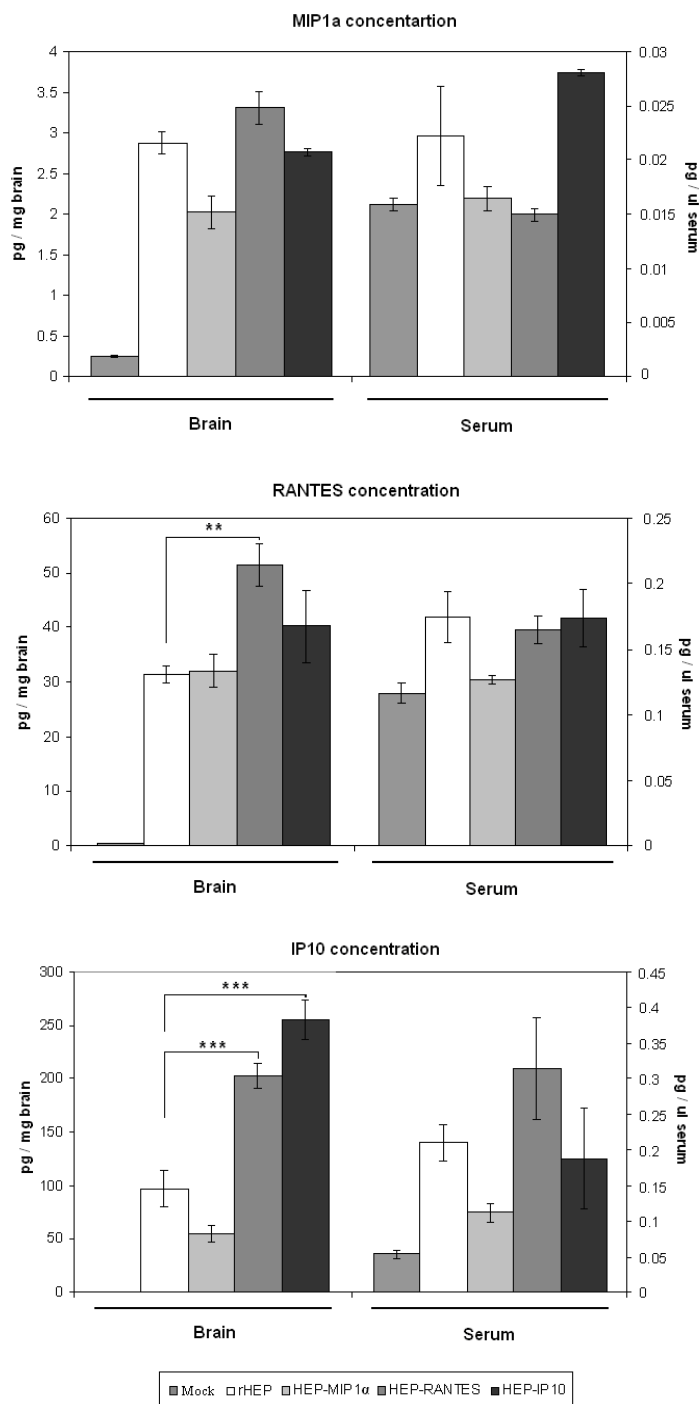
(A)



(B)



(C)



Zhao et al., Fig. 3.6.

CHAPTER 4
EXPRESSION OF MIP-1 α (CCL3) BY A RECOMBINANT RABIES VIRUS
ENHANCES THE INNATE AND THE ADAPTIVE IMMUNITY¹

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Fu: In preparing for *Vaccine* (co-first author)

Abstract

Previously we showed that the induction of mediators of innate immunity such as interferon and chemokines is one of the mechanisms for rabies virus (RABV) attenuation. To further enhance the immune responses, chemokine MIP-1 α (CCL3) was cloned into the RABV genome. It was found that the recombinant RABV HEP-MIP1 α expressed MIP-1 α in a dose-dependent manner in cell culture while MIP-1 α was not detected in cells infected with parental RABV or recombinant HEP-MIP1 α (-) that contains the MIP-1 α gene but the protein is not expressed by introduction of premature stop codons. Inoculation of the recombinant RABV into mice by the intracerebral route did not induce any disease, however, infection with this recombinant HEP-MIP1 α results in a higher level of MIP-1 α expression and increased numbers of inflammatory cells in the brain than the parental virus or HEP- MIP1 α (-) virus at 3 days after infection. The expression of MIP-1 α and infiltration of inflammatory cells were transient and subsided by day 6 after infection. Immunization of mice with the recombinant HEP- MIP1 α by the intramuscular route led to the production of significantly higher titers of virus neutralizing antibodies than with parental virus or HEP-MIP1 α (-) virus. More animals immunized with the recombinant HEP- MIP1 α were protected against lethal challenge than those with parental RABV or HEP-MIP1 α (-) virus. Our data thus demonstrate that expression of MIP-1 α enhances innate as well as adaptive immune responses against rabies and the recombinant RABV expressing MIP-1 α has the potential to be developed as a live avirulent RABV vaccine.

Keywords: Rabies virus; MIP1 α overexpression; innate immunity, adaptive immunity, neutralizing antibodies, inflammation

Introduction

Rabies virus (RABV) causes fatal encephalomyelitis in many species of mammals (25). RABV is a single-strand, negative-sense RNA virus of the family *Rhabdoviridae* and its genome encodes for five structural proteins in the order of: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) (56). Despite the fact that rabies is one of the oldest human infections, it continues to present worldwide health problems today. Each year, 50,000-60,000 human deaths from rabies are estimated and millions need post-exposure prophylaxis (29). Most of the human cases occur in the developing nations of Asia and Africa where dog rabies remains to be the main source for human exposure (18). In the developed countries, human rabies has dramatically declined during the past 50 years as a direct consequence of routine vaccination of pet animals. However, wildlife rabies has emerged as a major threat (43). Therefore, controlling rabies and protecting humans from rabies requires multi-layered control strategies, particularly vaccination of humans after exposure, routine vaccination of pet and wildlife animals.

Current human rabies vaccines are made from inactivated RABV cultured on cells. Although cell culture vaccines are safe and efficacious, multiple doses (at least 5) must be administered over an extended period of time (90 days) to stimulate optimal immune responses (1). Furthermore, the high cost of cell culture vaccines makes it difficult to effectively utilize in developing countries where they are needed most (50). Routine vaccination of pet animals (dogs and cats) is carried out by using inactivated vaccines (7). Although these vaccines provide adequate protection, they do induce local reactions (55) and multiple immunizations are required

to maintain sufficient immunity throughout the life of the animal (37). Live attenuated RABV vaccines or recombinant live vaccines have been licensed, particularly for wild animals. A recombinant vaccinia virus expressing the RABV G (VRG) has been used for large scale elimination of fox rabies in Europe (53)) as well as coyote and raccoon rabies in North America (20). A live avirulent RABV, SAG-2, has also been used for immunization of wildlife against rabies in many parts of Europe (4). These vaccines are effective, however, they have problems. Human exposure to VRG has been associated with intensive local inflammatory reaction and generalized erythroderma (42). Low virus neutralizing antibody (VNA) response has been reported for oral immunization with live attenuated SAG-2 (21).

Recently we found that activation of the innate immune responses is one of the mechanisms by which RABV is attenuated (54). Infection with laboratory-attenuated RABV (B2C) induced the expression of genes associated with innate immune responses, particularly the type 1 interferons and chemokines, while many of these genes were not activated or activated to a lesser extent in mice infected with wild type SHBRV (54). The induction of innate immunity has been confirmed by other investigators when laboratory-adapted viruses were used to infect mice or neuronal cells (27, 33, 36). Induced innate response genes include inflammatory chemokines (RANTES, MIP-1 α , IP-10, etc) and cytokines (IL-6, IL-1 β , and TNF- α), IFN and IFN-related genes (IFN- α , STAT1, Mx-1), and Toll-like receptors (TLRs1-3). These innate immune responses play important roles in resistance to virus infections. Type I IFNs, by binding to IFN- α/β receptors, activate signal transducer and activation of transcription (STAT) family of proteins, leading to induction of antiviral state (6). Cytokines and chemokines can have direct

anti-viral activities (34) and/or recruit inflammatory cells to the site of infection to kill virus-infected cells (32).

In this study, chemokine MIP-1 α was cloned into RABV genome in order to enhance its immunogenicity. It was found that recombinant RABV expressing MIP-1 α induced earlier and transient innate immunity that led to enhanced adaptive immune responses and better protection.

Materials and methods

Cells, viruses, antibodies, and animals

Mouse neuroblastoma (MNA) cells were maintained in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). BSR cells, a cloned cell line derived from BHK-21 cells, were maintained in Dulbecco's modified Eagle's medium (D-MEM; Mediatech) containing 10% FBS. Recombinant RABV strains were propagated in BSR cells. CVS-11 was propagated in MNA cells. CVS-24 was propagated in suckling mouse brains as described previously (57). Fluorescein isothiocyanate (FITC)-conjugated antibody against the RABV N protein was purchased from FujiRab (Melvin, PA). Anti-RABV nucleoprotein (N) monoclonal antibody 802-2 was obtained from Dr. Charles Rupprecht, Centers for Disease Control and Prevention. Anti-CD3 polyclonal antibody was purchased from Abcam, England. Biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). Female ICR mice at the age of 6 weeks were purchased from Harlan and housed in temperature- and light-controlled quarters in the Animal Facility, College of Veterinary Medicine, University of Georgia. All animal experiments were carried out as approved by the Institutional Animal Care and Use Committee.

Construction of recombinant RABV clones

Mouse MIP-1 α cDNA was amplified from RABV-infected mouse brain RNAs, using SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen-Life Technology) and the gene-specific primers MIP1AF (5'-ctgctcCGTACG**at**gaaggtctccaccact-3' [a BsiWI site is underlined, and the start codon is shown in bold]) and MIP1AR (5'-cctccaGCTAGCttagcatt**ca**gttccag-3' [an NheI site is underlined, and the stop codon is shown in bold]) to introduce BsiWI and NheI recognition sites before and after the MIP-1 α coding region. The PCR product was digested with BsiWI and NheI (New England Biolabs, Beverly, MA) and then ligated into rabies virus vector pHEP-3.0 that had been previously digested with BsiWI and NheI. The resulting plasmid was designated pHEP-MIP1 α (Fig. 1A). To construct a RABV vector that contains the MIP-1 α gene, but does not express MIP-1 α protein, two stop codons were introduced into the N terminus of MIP-1 α by overlapping PCR strategy. One pair primers (5'-GTCATATCTTCATGGGAG-3' and 5'-GTCAGCTC**CTA**ATGGCGCT**CA**GAAGACTTG-3'; two modified stop codons are shown in bold) were used to amplify the part of the fragment from the 3' end of G gene to the mutant region of MIP-1 α . Another pair of primers (5'-CAAGTCTTCT**AG**AGCGCCAT**TA**GGAGCTGAC-3' and 5'-ATCTTGATATGGGTCTCG-3') was used to amplify another part of the fragment from the mutant region of MIP-1 α to the 5' end of L gene. Overlapping PCR was performed and the PCR product was digested with BsiWI and NheI, and then ligated into pHEP-3.0 that had been previously digested with the same enzymes. The resultant clone is designated as pHEP- MIP1 α .

Rescue of recombinant RABV

Recombinant RABVs were rescued as described previously (24, 49). Briefly, BSR cells were transfected with 2.0 µg of full infectious clone, 0.5 µg of pH-N, 0.25 µg of pH-P, 0.1 µg of pH-L, and 0.15 µg of pH-G using SuperFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. After incubation for 4 days, the culture medium was removed and fresh medium added to the cells. After incubation for another 3 days, the culture medium was transferred into NA cells and examined for the presence of rescued virus by using FITC-conjugated antibody against the RABV N protein.

Virus titration

Viruses were titrated by direct fluorescent assay in NA cells. NA cells in 96-well plate were inoculated with serial 10-fold dilution of virus and incubated at 34°C for 2 days. The culture supernatant was removed and the cells were fixed with ice-cold 80% acetone for 30 min. Then the cells were stained with FITC-conjugated anti-RABV N antibodies. Antigen-positive foci were counted under a fluorescent microscope (Zeiss, Germany) and viral titers were calculated as fluorescent focus unit (FFU) per milliliter. All titrations were carried out in quadruplicate.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify the amount of MIP-1α in cell culture supernatants or mouse brain suspensions by using the murine MIP-1α ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Rapid fluorescent focus inhibition test (RFFIT)

Blood was collected from each mouse for measurement of VNA by using the RFFIT as described previously (48). Briefly, 50 μ l of serial five-fold dilutions of serum were prepared in Lab-Tek Chamber slides (Nalge Nunc International). 50 FFD50 (50% Fluorescing Foci dose) of CVS-11 was added to each chamber and incubated at 37 °C for 90 min. 0.2 ml of MNA cells (5×10^5 cells/ml) were added into each chamber and the slides were incubated at 37 °C for 20 hr. Then the slides were fixed with ice-cold 80% acetone and stained with FITC-conjugated anti-RABV N antibodies for 1 hr. Twenty fields in each chamber were observed under a fluorescent microscope, and the 50% endpoint titers were calculated according to the Reed-Meunch formula (39). The values were compared with that of the reference serum (National Institute for Biological Standards and Control, Herts, EN6 3QH, UK) and normalized to international units (IU/ml).

Statistical Analyses

Statistical significance of the differences between different treatment groups was determined using student t-test. One-way ANOVA test was used to analyze VNA data.

Results

Construction of recombinant RABV expressing MIP-1 α

To enhance the innate and adaptive immunogenicity of RABV vaccines, a recombinant RABV, HEP-MIP1 α , was constructed to express murine MIP-1 α (Fig. 4.1A). The parental virus,

HEP-Flury strain, is highly attenuated (28) and does not kill adult mice even after intracerebral (i.c.) inoculation (49). To ensure that any observed effect was not due to the presence of a foreign gene in the RABV genome, a recombinant virus with a MIP-1 α gene cloned, but without MIP-1 α protein expressed, was constructed by adding two stop codons in the N terminus of MIP-1 α gene. Insertion of the MIP-1 α or the mutant MIP-1 α gene was confirmed by sequencing these fragments within the infectious clones. All the recombinant RABVs were rescued using the procedures as described by Inoue et al (24). The parental virus HEP-Flury is designated as rHEP as described previously (24) and the recombinant RABV are designated as HEP-MIP1 α and HEP-MIP-1 α (-), respectively. The insertion was confirmed by RT-PCR and sequencing.

In vitro characterization of recombinant RABVs

To characterize further the recombinant RABVs in vitro, the growth kinetics of these viruses were examined in NA cells. As shown in Fig. 4.1B, no significant difference was observed between recombinant viruses and the parental virus, indicating that viral growth was not affected by the insertion of MIP-1 α gene. The ability of the recombinant HEP-MIP1 α construct to produce MIP-1 α was determined by measuring MIP-1 α in virus-infected cells with an ELISA kit. As shown in Fig. 1C, production of MIP-1 α was detected in NA cells infected with HEP-MIP1 α in a dose-dependent manner. No MIP-1 α was detected in NA cells infected either with rHEP or HEP-MIP1 α (-).

Effect of MIP-1 α overexpression on pathogenicity

To determine if overexpression of MIP-1 α has adverse effect in animals, adult mice were infected with 10⁵ FFU of recombinant viruses by the intracerebral (i.c.) route. Infected mice were

monitored twice daily for two weeks for development of diseases. As shown in Fig. 4.2, no animal was sick during the observation period in mice infected with HEP-MIP1 α . Only one mouse each from those infected with rHEP and HEP-MIP-1 α (-) developed mild symptoms including rough fur and slow moving at days 5-7 post infection (p.i.) and recovered very quickly.

Effect of MIP-1 α overexpression on the induction of adaptive immunity

To determine if the induction of earlier innate immune responses by overexpression of MIP-1 α has any effect on the activation of adaptive immunity, mice were immunized once by the intramuscular (i.m.) route with different doses of RABVs. Blood samples were collected 21 days p.i. and serum samples were used for determination of virus neutralizing antibodies (VNA) by an RFFIT assay (48). Overall the production of VNA is dose dependent for all the viruses (Fig. 4.3A). At low doses (5×10^3 ffu/mouse), the level of VNA was similar in mice infected with each of the viruses. However, significantly higher VNA titers were induced by HEP-MIP1 α than those by rHEP or HEP-MIP1 α (-) when high doses (4×10^5 and 5×10^5 ffu/mouse) were used to immunize mice. The average VNA titers reached 12.76 IU in mice immunized with 5×10^5 FFU of HEP-MIP1 α . At the same time the average VNA titers were only 7.58 IU and 8.31 IU in mice immunized with the same doses of rHEP and HEP-MIP1 α (-) respectively.

Immunized mice were then challenged with 50 LD₅₀ of CVS-24 viruses on day 21 after vaccination and observed for development of disease and death for two weeks. As depicted in Fig. 4.3B, survivorship is correlated with the dose of vaccine and the level of VNA in mice immunized with each of the viruses. More survivors were observed in mice immunized with HEP-MIP1 than those immunized with rHEP or HEP-MIP1 α (-) particularly at virus doses of

5×10^3 and 5×10^4 FFU. All the data together indicate that overexpression of MIP-1 α leads to enhanced innate as well as adaptive immune responses. The enhanced immunity resulted in more protection.

Discussion

By using reverse genetics, RABV genome has been used to express foreign proteins or an extra copy of RABV protein. Foreign proteins such as HIV Gag or Env (30), HCV envelope proteins E1 and E2 or a modified version of E2 (47), and severe acute respiratory syndrome coronavirus (SARS-CoV) envelope spike protein (S) (12) have been expressed in RABV vector and it has been shown that expression of these proteins resulted in induction of potent humoral and cellular immune responses in immunized animals. Expression of foreign proteins which can stimulate the host immune responses has also been used for development of RABV vaccines. Cytochrome c have been cloned into RABV genome and the recombinant virus can induce accelerated apoptosis, which contributes to enhanced immunogenicity and attenuated pathogenicity (38). The expression of cytokines at the time of virus infection has the potential to enhance immune responses. Overexpression of TNF α , an important proinflammation cytokine, in a recombinant RABV can induce stronger CNS inflammation, thus exerting its protective activity (11). Expression of IL-2 from RABV-based vaccines vector was found to modulate the immune responses (31). A recombinant RABV that expresses an extra copy of G has also been cloned into RABV genome (13). Because RABV G is the only surface protein that is capable of inducing the production of VNA (17), thus overexpression of RABV G results in enhanced adaptive immune responses and better protection (13). In this study, we cloned chemokine MIP-1 α into RABV genome and found that overexpression of MIP-1 α resulted in the induction of an

earlier and transient innate immune response, which led to an enhanced adaptive immunity and increased protection when used to immunize mice.

The rationale to insert MIP-1 α into RABV genome was based on our recent findings that induction of innate immunity is one of the mechanisms by which RABV is attenuated (54). In that study MIP-1 α was found to be highly up-regulated in mice infected with laboratory-adapted RABV, but not in mice infected with wild-type RABV. MIP-1 α plays an important role in protective inflammatory responses to invading pathogens. For example, a recent study demonstrated that MIP-1 α responses in the CNS of mice during *C. neoformans* infection are protective by clearing disseminated infection (23). MIP-1 α has been found to have a direct antiviral activity against HSV-1 by binding to HSV-1 envelope glycoprotein gB (34). MIP-1 α can modulate the activation state of the macrophages through autocrine mechanisms. Treatment of murine macrophages with MIP-1 α stimulates proinflammatory cytokine expression (TNF α , IL-1) (14). MIP-1 α also plays an important role in host antiviral responses by recruiting various effector (inflammatory) cells such as monocytes, T cells, neutrophils, eosinophils, basophils, and natural killer cells into infected areas (45). Recently, this role is highlighted by the observation that infection of MIP-1 α knockout mice with influenza virus results in a substantially reduced recruitment of CD8⁺ T cells into the infected lung as compared with that seen in wild-type mice (5). This failure to recruit CD8⁺ T cells to the lung leads to a significant reduction in the infection-associated pneumonitis and a pronounced delay in clearance of the viral infection. In addition, the clearance of pneumonia virus of mice, murine cytomegalovirus, and mouse hepatitis virus (MHV) is also delayed in the absence of MIP-1 α (10, 44, 51). For MHV, the absence of MIP-1 α impaired the cytokine production and cytolytic activity of CD8⁺ T cells. In

addition, macrophage accumulation within the CNS was significantly decreased in infected MIP-1 α ^{-/-} mice (51). On the other hand, overproduction of MIP-1 α has also been reported to underlie disease-related pathology in certain instances, such as infections with Coxsackie virus, respiratory syncytial virus, and herpes simplex virus 1 (5, 19, 52). In our present study, it was found that overexpression of MIP-1 α did not result in detrimental effect in the CNS of infected animals. In fact, animals infected with HEP, but not those infected with HEP-MIP1 α showed brief episode of clinical signs such as rough fur and slow movement, suggesting that HEP-MIP1 α is more attenuated than the parental HEP. This could be due to at least in part to the fact that HEP-MIP1 α induced an earlier inflammatory response (at day 3 after infection) than parental HEP (at day 6 after infection). In addition, the inflammatory response induced by infection with HEP-MIP1 α was transient and was resolved by day 6 after infection. CNS inflammation has been reported to play an important role in blocking RABV spread in the CNS and the clearance of virus from the CNS (2, 22). Among the inflammatory cells, CD8⁺ cells may directly kill the infected neurons. Microglia is a strong candidate for the clearance of apoptotic cells by phagocytosis. Virus neuroinvasion might be controlled if microglia and activated T cells eliminate the infected neurons (3). Overexpression of MIP-1 α may also have the added benefit by increasing the permeability in the blood brain barrier (BBB), which has recently been reported to allow infiltration of immune effectors into the CNS, thus attenuating RABV virulence (35, 40, 41).

Like rHEP or HEP-MIP1 α (-) virus, HEP-MIP1 α induced dose-dependent production of VNA when used to immunize mice by intramuscular route. At higher doses (5×10^4 or 5×10^5 FFU), HEP-MIP1 α induced significantly higher VNA titers than rHEP or HEP-MIP1 α (-) virus,

indicating that overexpression of MIP-1 α has the ability to enhance the adaptive immune responses. VNA is the primary immune effector for rabies virus clearance (8). Our data also indicate that high production of VNA when immunized with HEP-MIP1 α led to more protection in mice when challenged with virulent RABV. The mechanism for MIP-1 α to enhance the adaptive immunity is not precisely known, the increased innate immune responses by MIP-1 α can enhance the adaptive immunity. As has been shown that overexpression of HEP-MIP1 α in the CNS can attract infiltration of inflammatory cells, it is possible that overexpression of HEP-MIP1 α can activate macrophages, which in turn recognize foreign antigens, swallow them, and act as antigen-presenting cells (APCs). MIP-1 α is also a potent dendritic cell chemoattractant, suggesting a role in the initiation of the adaptive immune responses via the recruitment of these potent APCs (9). The epitopes presented by APCs can also be recognized by T cells, which can provide help for B cells to produce large quantities of antibodies (26).

The ability of HEP-MIP1 α to enhance the production of VNA and provide more protection may suggest that this recombinant RABV could be developed as an avirulent RABV vaccine. Attenuated RABV such as SAG-2 has been developed as an oral vaccine for wildlife animals (46). Large scale testing has demonstrated its safety and efficacy (16, 46). However, immunized animals may not develop high VNA although they can be protected (15, 21). Since the level of VNA has been used as an indicator for immunity and protection, avirulent RABV expressing chemokine such as MIP-1 α may provide an alternative to the currently available vaccines for wildlife animals since it stimulates higher production of VNA and provide better protection than the parental RABV.

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Figure legends

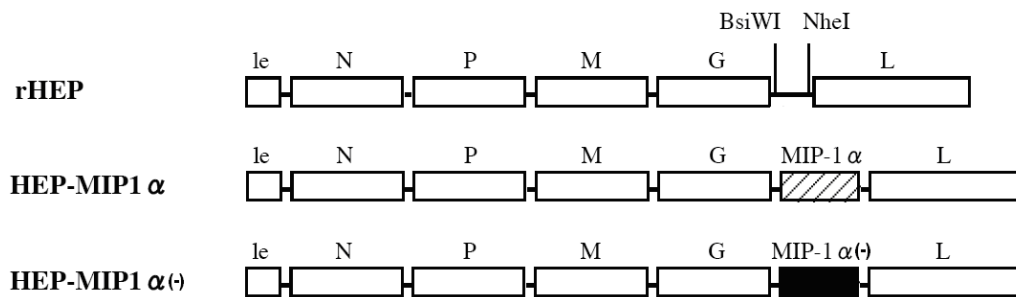
Fig. 4.1. Construction of recombinant RABVs. (A) Schematic diagram of the parental and recombinant RABV genomes. The full-length cDNA plasmid of RABV HEP-Flury strain, pHEP-3.0 (Inoue et al., 2003) was used to construct the recombinant RABV. HEP-MIP1 α was constructed by inserting the murine MIP-1 α gene between RABV G and L genes at the BsiWI and NheI sites of pHEP-3.0 vector. HEP-MIP1 α (-) was constructed by introducing stop codons in the N-terminus of the MIP-1 α gene (B) Growth curves of recombinant RABVs in NA cells. NA cells were infected with recombinant viruses at 0.01 MOI. After incubation at 37 °C for 1 hr, virus inocula were removed and cells were washed twice to remove unabsorbed virus. The cells were replenished with RPMI 1640 containing 2% FBS and incubated at 34 °C. At days 1, 2, 3, 4 and 5 after infection, the culture supernatant was harvested and viral titers were determined in NA cells with FITC-conjugated anti-rabies antibodies. (C) Expression of MIP-1 α by recombinant RABVs. NA cells were infected with recombinant viruses at 0.001, 0.01, 0.1, or 1 MOI. After incubation at 37 °C for 1 hr, virus inocula were removed and cells were washed twice, and then replenished with RPMI 1640 containing 2% FBS. After 24 hr incubation at 34 °C, the culture supernatant was harvested and the amount of MIP-1 α determined by ELISA kit. The positive control (MIP-1 α) is supplied with the ELISA kit.

Fig. 4.2. Effects of overexpression of MIP-1 α on the pathogenicity of recombinant RABVs. I.C. infection with recombinant RABV on morbidity of adult mice. ICR mice at 6 weeks of age were inoculated i.c. with 10⁵ FFU of recombinant viruses. For mock infection, mice were inoculated with DMEM medium.

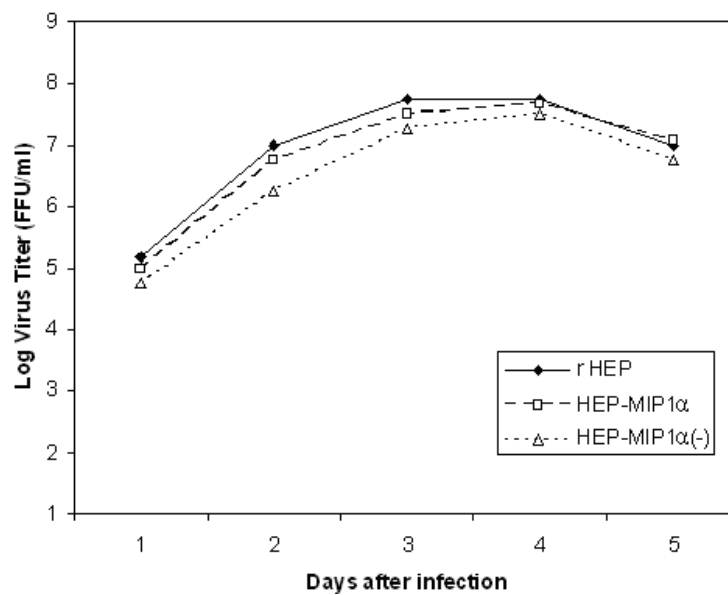
Fig. 4.3. Immunogenicity of recombinant RABVs. (A) Groups of 10 ICR mice were injected with serial 10-fold dilutions of the recombinant rabies viruses by the i.m. route. At day 20 after immunization, blood was obtained and serum used for determination of VNA using the RFFIT assay. Titers were normalized to IU using the WHO standard and given as geometric mean titers. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the indicated experimental groups, calculated by one-way ANOVA test. (B) Three weeks after immunization, the mice were challenged i.c. with 50 LD₅₀ of CVS-24 and observed daily for 2 weeks, and survivorship was recorded.

Figures

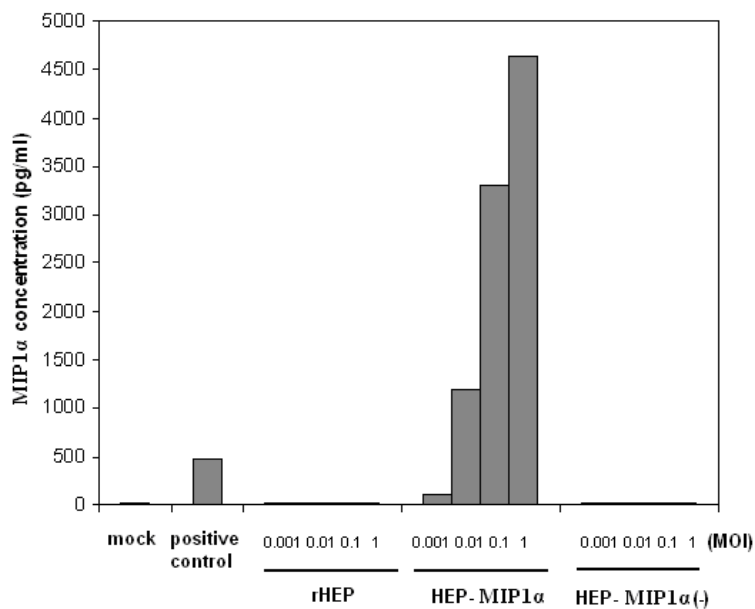
(A)



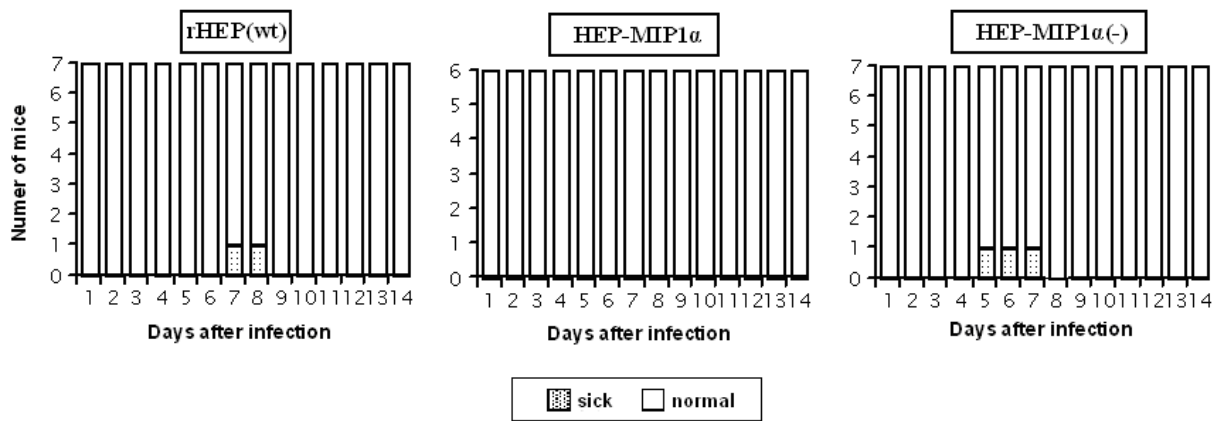
(B)



(C)

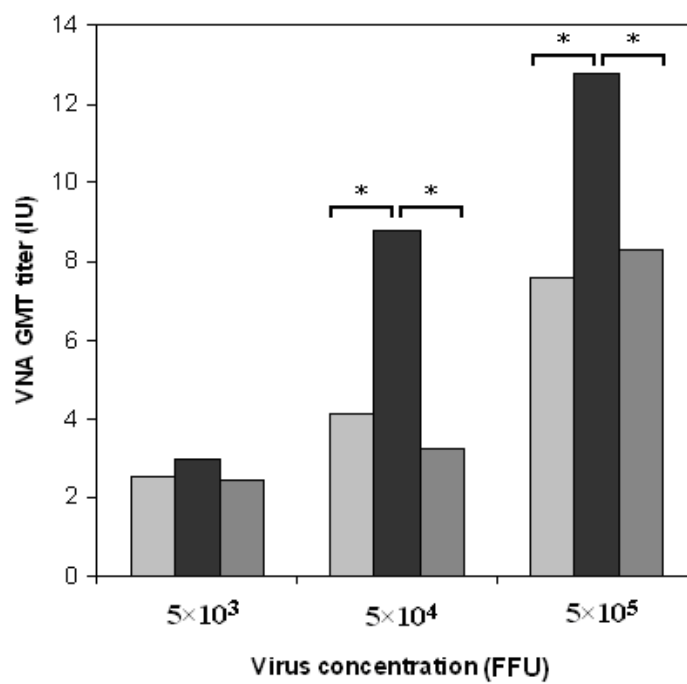


Zhao et al., Fig. 4.1.

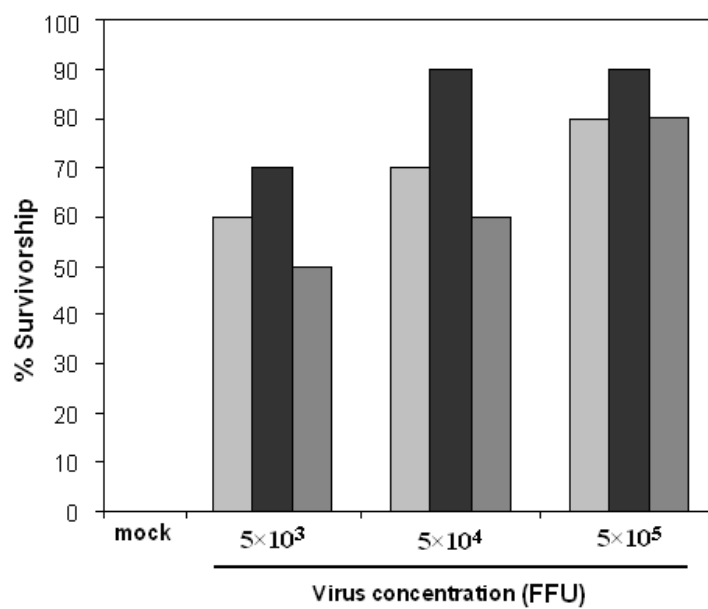


Zhao et al., Fig. 4.2.

(A)



(B)



Zhao et al., Fig. 4.3.

CHAPTER 5

DICUSSIONS AND CONCLUSIONS

In this study the roles of chemokines on RABV pathogenicity and immunogenicity were determined by cloning and expressing chemokines, MIP-1 α , RANTES or IP-10, into RABV genome. It was found that expression of MIP-1 α not only further attenuated RABV pathogenicity, but also enhanced RABV immunogenicity. Immunization with this recombinant virus induced significantly more VNA and protected more vaccinated mice against challenge infection than the parental virus. On the other hand, expression of RANTES or IP-10 increased RABV pathogenicity by inducing extensive and persistent inflammation in the CNS.

The rationale to express these chemokines was based on our previous studies that attenuated RABV induces innate and enhanced adaptive immune responses while wt RABV evades innate immune responses. The most upregulated innate immune genes include type I interferon and chemokines, particularly, MIP-1 α , RANTES or IP-10. Expression of these chemokines in RABV genome did not alter the growth characteristics in vitro, but expressed the intended chemokines in a dose-dependent manner. Inoculation of these viruses directly into the brain of mice revealed that virus expressing MIP-1 α did not induce any clinical signs or reduce body weight, similar to the sham infection. The parent virus induced significant weight loss than HEP-MIP1 α and one mouse developed mild clinical sign, but recovered. On the other hand, recombinant virus HEP-RANTES or HEP-IP10 induced significantly more weight loss than the parent virus, severe

clinical signs, and deaths in infected mice. These results indicate that expression of chemokines is important in controlling RABV infection, but not always beneficial to the host.

To delineate the mechanism(s) why expression of MIP-1 α further attenuate while RANTES or IP-10 enhance RABV pathogenicity, virus production, viral antigen expression, and viral RNA replication were determined in the infected brain. No virus was found and virus antigens were only detected sparsely. Viral genomic RNA was detected in mice infected with each of the virus, but no significant difference was found among them. Thus the reduction or enhancement of pathogenicity by over-expression of chemokines is not due to the rate of virus replication.

Next, the expression of inflammatory chemokines and cytokines, infiltration of inflammatory cells, as well as the changes in BBB permeability were investigated in mice infected with each of the viruses. It was found that HEP-MIP1 α induced a transient high expression of MIP-1 α at day 3 p.i. and low expression of other chemokines or cytokines. Only low and transient infiltration of inflammatory cells (macrophages, neutrophils, and T lymphocytes) was found in mice infected with this virus. On the other hand, HEP-RANTES and HEP-IP10 induced high and persistent expression of not only the intended chemokines, but also other chemokines and cytokines during the observation period. Infection with HEP-RANTES, particularly HEP-IP10 resulted in extensive and persistent infiltration of inflammatory cells (macrophages, neutrophils, and T lymphocytes) into the CNS. Infection with each of the viruses induced significantly more enhancement of BBB permeability than sham infection. Yet, enhancement of BBB permeability in HEP-RANTES or HEP-IP10-infected mice was significantly more than that in mice infected with rHEP or HEP-MIP1 α . Furthermore infection

with HEP-IP10 enhanced the BBB permeability to the extent that allowed large size (10 KDa) to enter the CNS while infection with other viruses only allowed small (376 Da) molecules to enter the CNS. Together these data indicate that induction of low and transient innate immunity (inflammation) is associated with RABV attenuation while extensive and persistent inflammation is detrimental to the host.

Since HEP-MIP1 α further attenuated RABV pathogenicity, its immunogenicity was determined. Mice were immunized with different doses of the recombinant virus, bled for the determination of VNA, and challenged with a lethal dose of virulent RABV. To ensure that the observed effect is due to the expression of MIP-1 α , another recombinant virus, HEP-MIP1 α (-), was constructed by introducing stop codons into the MIP-1 α coding region. In vitro and in vivo characterizations indicate that the HEP-MIP1 α (-) behaved just like the parent rHEP. HEP-MIP-1 α induced significantly more VNA and protected more mice against challenge than the rHEP or the HEP-MIP1 α (-).

In conclusion, expression of chemokines may not always be beneficial. Expression RANTES and IP-10 enhanced RABV pathogenicity. However, expression of MIP-1 α not only further attenuated RABV, but also increased its immunogenicity and protection, thus having the potential to be developed as an avirulent RABV vaccine.