Filoviridae

A minireview and the latest developments in vaccines

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1. Abstract

Since their discovery, the Ebola and Marburg virus cause regular outbreaks with lethality in humans ranging from 23-90%, depending on the virus species and strain. They can infect humans through contact with infected body fluids or aerosols. Together they form the Filovirus group, in which Marburg only has one subtype and Ebola has four: Zaïre, Sudan, Reston and Ivory Coast. Filoviruses are non-segmented, enveloped, negative strand RNA viruses within the order of the *Mononegavirales*. They have a 19 kb genome and are pleomorphic particles which vary greatly in length and shape. It is thought that filoviruses are able to interfere with the host immune response, in particular the interferon response.
The viral entry and replication cycle is not fully understood yet. Many receptors are suspected to be involved, but evidence is thin.

Marburg and Ebola differ from each other, Ebola produces besides a membrane anchored glycoprotein, also a soluble version and the 3'non coding region of Marburg structurally and functionally differs from Ebola's.

Because of the high virulence and the threat of using these viruses as a bio warfare agent, a vaccine is needed. The goal of this paper is to describe the most recent findings on vaccine development for EBOV and MARV. In particular, what type of vaccines are currently available and what is the mechanism and the effectivity of these vaccines?

The latest developments are promising, there are three vaccines that have proven to be effective in non human primates: the use of Ebola or Marburg virus like particles as a vaccine, a panfilovirus vaccine based on a complex adenovirus and a vesicular stomatitis virus-based vaccine against aerosol infections.

2. Introduction

Ebola virus (EBOV) and Marburg virus (MARV) infections cause a severe form of viral hemorrhagic fever (VHF) with lethality in humans ranging from 23-90% depending on the virus species and strain. Given the high virulence in humans, these viruses are classified as biosafety level 4 and category A list pathogens (Bente et al., 2009).
These pathogens are highly infectious through contact with infected body fluids and can be aerosolized easily. Together EBOV and MARV form the Filovirus group, but although these viruses are both considered members of the family *Filoviridae*, they are distinguishable from each other by differences in structure, protein sequences, protein antigenicity and lack of serological cross-reactivity. In 2002 it was even suggested, based primarily on Van Regenmortel et al. (2000), that MARV and EBOV are two different genera. There is one type species in the MARV genus with six strains within the species. The genus *Ebolavirus* comprises four type species: Cote d'Ivoire ebolavirus, Reston ebolavirus, Sudan ebolavirus, and Zaire ebolavirus. There are 13 strains within these four species (Hart, 2003).

MARV was discovered first in 1967, when outbreaks of hemorrhagic fever occurred at the same time in Germany (Marburg and Frankfurt), and Yugoslavia (Belgrade) among laboratory workers having contact with tissues and blood from African green monkeys (*Cercopithecus aethiops*) imported from Uganda. A total of 31 cases in humans with seven fatalities occurred. A virus was isolated from blood and tissues of the patients by inoculation of guinea pigs and cell cultures and the virus was named Marburg virus after the city in which it was first characterized. The virus also appeared to be highly pathogenic for monkeys, killing all African green monkeys experimentally infected with the virus. After this dramatic episode the virus disappeared from sight until 1975, when three cases of Marburg hemorrhagic fever were reported in Johannesburg, South Africa. The index case patient died 12 days after onset of the disease, while two patients, secondarily infected, survived. The next Marburg virus outbreak occurred in 1980 when one index patient became ill and finally died in Kenya and an attending physician became infected but survived. In 1987 a single fatal Marburg case was reported in western Kenya. Two cases of laboratory infections with MARV occurred in Russia in 1988 and 1990. The first took place as the result of an accident with a contaminated needle, and the researcher died within several days. The second person infected by the serum of a laboratory animal survived after intensive therapy.

EBOV was first discovered in 1976, when more than 550 cases of severe hemorrhagic fever with more than 430 fatalities occurred simultaneously in Zaire and Sudan. Subsequently Ebola virus (named after a small river in northwestern Zaire, today the Democratic Republic of Congo) was isolated from patients in both countries and was shown to be morphologically similar to but serologically distinct from MARV. In 1979 Ebola hemorrhagic fever occurred again in the Sudan with 34 cases and 22 fatalities. In 1989 filoviruses were isolated from cynomolgus monkeys *(Macaca fascicularis*) imported into the United States from the Philippines. During quarantine in a primate facility in Virginia, numerous macaques died, some with symptoms consistent with simian hemorrhagic fever. Investigations led to a single source in the Philippines that was thought to have furnished all identified infected shipments, including monkeys sent to facilities in Texas and Pennsylvania. This filovirus isolated from the monkeys in Reston, Virginia, was clearly a strain of Ebola virus and was undoubtedly responsible for the deaths, being found in tissues of naturally infected monkeys in high concentrations and because monkeys inoculated with this virus alone died with typical filovirus disease. The use of monoclonal antibodies and genetic sequence analyses suggested differences between the Reston isolate and the Ebola viruses isolated in 1976 from Zaire or

Sudan. In addition, Reston virus appeared to be less pathogenic for nonhuman primates and humans. In 1995 a re-emergence of Ebola, subtype Zaire, in Kikwit, Zaire, and in Gabon occurred. Ecological investigations have been conducted after most filovirus outbreaks beginning with the initial Marburg episode in 1967, but the source of filoviruses in nature still remains a mystery, although fruit bats are suspected (Beer et al.,1999;Leroy et al., 2005).

2.1 Classification

Filoviruses are enveloped, non-segmented, negative stranded RNA viruses with filamentous shape that constitute the family *Filoviridae* within the order *Mononegavirales* (Dolnik et al., 2007). As mentioned before, MARV is a unique agent without known subtypes. The EBOV has four subtypes (Zaïre, Sudan, Reston, and Ivory Coast). The Zaïre subtype has the highest case fatality rates in humans, up to 90% in some epidemics, with an average case fatality rate of approximately 83% over 27 years. Sudan EBOV was the second species of Ebola emerging simultaneous with the Zaïre virus. The average human fatality rates for Sudan EBOV were 54% in 1976, 68% in 1979, and 53% in 2000/2001 (CDC, 2006). Reston EBOV is not pathogenic to humans, but hazardous to monkeys. Ivory Coast EBOV has one none infection case in humans, but fully recovered after treatment. It is lethal to monkeys. The discrepancies in pathogenicity among the Ebola virus subtypes remain to be explained, as does the extreme virulence of Ebola Zaire. There have been reports of a newly emerged fifth EBOV species in Uganda, but this species was not researched nor named yet (Beer et al.,1999;Takada et al., 2001;Swenson et al., 2008).

2.2 Genome organisation

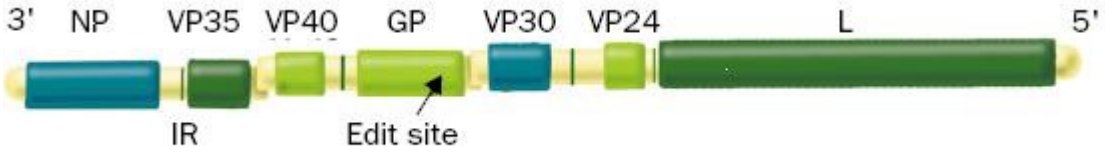

Figure 1 Filovirus genome organisation. IR=intergenic regions; GP=glycoprotein; NP=nucleoprotein; VP=viral protein; L=large protein (RNA-dependent RNA polymerase). (Mahanty et al., 2004)

Filovirus proteins are encoded by a 19 kb genome with the following gene order: 3' leader, nucleoprotein (NP), virion protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, polymerase protein (L) and 5' trailer (Fig. 1). They are the largest known genomes for negative strand RNA viruses.

In addition to common characteristics, there are others that distinguish filovirus genomes from those of rhabdoviruses and paramyxoviruses:

- transcriptional signals of filoviruses contain a common sequence 3' UAAUU (at the 5' end of start sites and at the 3' end of stop sites).
- filovirus genes possess the longest 3' and/or 5' end noncoding regions of all negative strand RNA viruses.
- the localization of overlapping genes in Ebola and Marburg virus. Gene overlaps were found between VP35 and VP40, GP and VP30, and VP24 and L genes in Ebola virus and VP30 and VP24 in Marburg virus. Overlaps are 18–20 bases in length and are limited to the conserved sequences determined for the transcriptional signals.

Seven structural proteins are encoded by the genome of which four form the helical nucleocapsid (NP-VP35-VP30-L), two are membrane-associated (VP40-VP24), and one is a transmembrane GP (reviewed by Beer et al., 1999).

NP encapsidates the viral genome and is necessary and sufficient for replication and transcription, together with L and VP35, which represents the polymerase cofactor. For

EBOV viral transcription an additional filovirus-specific transcription factor is necessary: VP30. The NPs of filoviruses are phosphorylated and appear in two forms differing in *M*r by about 2 K (94 and 92 K, respectively) (Becker et al., 1995). The NPs of filoviruses can be divided into a hydrophobic N-terminal half, which contains all the cysteine residues, and a hydrophilic C-terminal half, which contains most of the proline residues and is extremely acidic (reviewed by Beer et al., 1999).

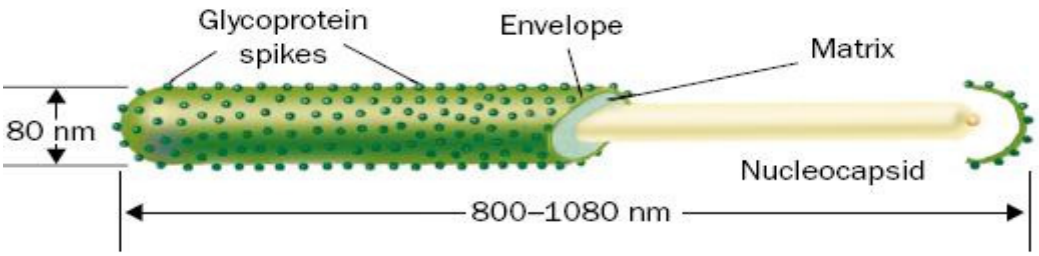

Figure 2 Structure of a filovirus Virion. (Mahanty et al., 2004)

The filovirus envelope consists of host cell-derived membrane containing the viral GP. The GP is the sole structural protein forming the virion surface spikes that mediate virus entry into susceptible host cells through receptor binding (Fig. 2)
The VP40 and VP24 are positioned underneath the viral membrane and ensure the structural integrity of the particle. The VP40 protein is believed to have a matrix protein function based on its large abundance in the virion, its hydrophobic profile, its removal from purified virion nucleocapsid by nonionic detergents under low salt conditions, and the position of its gene in the genome. The VP24 protein has a highly hydrophobic amino acid composition concentrated within five hydrophobic domains. The function of the VP24 is unclear, although it is possibly acting as a minor matrix protein or taking part in the uncoating of the virion during infection (reviewed by Beer et al., 1999).
VP24 and VP35 are also capable to antagonise the type I interferon (IFN) immune response (Basler et al., 2000).

2.3 Morphology and Structure

Marburg and Ebola viruses are pleomorphic particles which vary greatly in length, but the unit length associated with peak infectivity is 790 nm for Marburg virus and 970 nm for Ebola virus (Regnery et al., 1980). The virions appear as either long filamentous (and sometimes branched) forms or in shorter U-shaped, 6-shaped (mace-shaped), or circular (ring) configurations (Murphy et al., 1978).

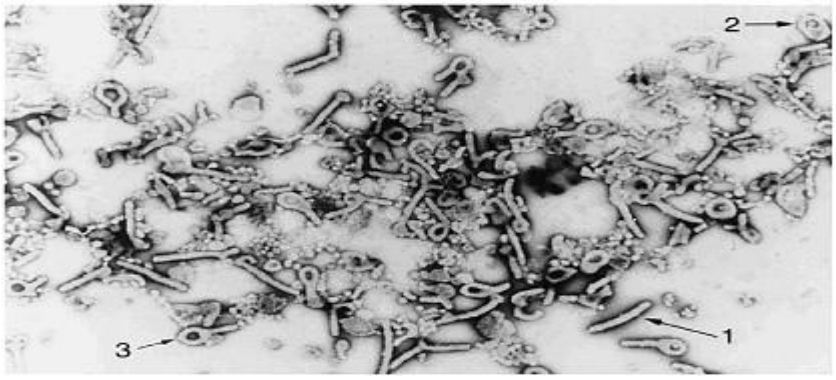

Figure 3 Marburg virus particles purified from the blood of infected guinea pigs. Stained by negative contrast medium. Different forms of the virion are shown. 1.rod shaped; 2.ring shaped; 3.mace shaped (Beer et al., 1999).

Virions have a uniform diameter of 80 nm and a density of 1.14 g/ml. They are composed of a helical nucleocapsid, a closely apposed envelope derived from the host cell plasma membrane, and a surface projection layer composed of trimers of viral glycoprotein (GP). All filoviruses contain one molecule of non-infectious, linear, negative-sense, single-stranded RNA with a *M*r of 4.2x10^6, constituting 1.1% of the virion mass (Regnery et al., 1980). Marburg and Ebola virus infectivity is stable at room temperature (20º C) (Mitchell et al., 1984).

2.4 Clinical Syndromes and Pathogenesis

Following an incubation period of 2-21 days, human EBOV and MARV infections normally show an abrupt disease onset that is characterized by flu-like symptoms (fever, chills, malaise and myalgia). The subsequent signs and symptoms indicate multi system involvement, including systemic (prostration, lethargy), gastrointestinal (anorexia, nausea, vomiting, abdominal pain, diarrhoea), respiratory (chest pain, shortness of breath, cough), vascular (conjunctival injection, postural hypotension, edema) and neurological (headache, confusion, seizure, coma) manifestations. Hemorrhagic manifestations may develop during the peak of the illness and include petechiae, ecchymoses, uncontrolled bleeding from venipuncture sites, epistaxis and other mucosal hemorrhages, and postmortem evidence of visceral hemorrhagic effusions. In addition, there is often a maculopapular rash associated with varying degrees of erythema and desquamation. In late stages of the disease, shock, convulsions, severe metabolic disturbances and diffuse coagulopathy occur. In general terms, human VHF resulting from EBOV and MARV infections is associated with fluid distribution problems, hypotension and coagulation disorders, and often leads to fulminant shock and subsequent multiorgan system failure (Bente et al., 2009).

The tissue damage associated with EBOV and MARV infection can be divided into two types: those in which viral infection of host cells results in direct damage to tissues, and those in which tissue injury is brought about indirectly, through interactions between the virus and the innate and adaptive immune systems. Two factors are important for filoviruses to be able to kill a variety of cells in many different tissues. First, they can bind to widely distributed cell-surface lectins. Second, infection of these cells results in necrosis, through processes that are not fully understood yet, but they may include the toxic effects of the viral glycoproteins and other structural or matrix proteins (reviewed by Mahanty et al., 2004). Four other pathogenetic mechanisms that are based on interactions between filoviruses and the immune system also contribute to the development of lethal illness. First, systemic dissemination of virus from its point of entry is aided by suppression of innate immune responses, including type I interferon responses, in macrophages and dendritic cells. Furthermore, filovirus infection impairs the development of antigen-specific immune responses, partly by preventing dendritic cells from activating T cells. The massive "bystander" apoptosis of lymphocytes that develops over the course of infection in animals infected with Zaire ebolavirus also contributes to immunosuppression. Finally, infected macrophages produce a range of mediators that act in various ways to produce severe haemorrhagic fever. These include the cell-surface expression of tissue factor, triggering disseminated intravascular coagulation, and the release of cytokines and chemokines that induce vascular dysfunction, hypotension, and multiple organ failure. The resulting syndrome resembles septic shock resulting from infection with Gram-negative bacteria (Fig.4)(reviewed by Mahanty et al., 2004).

In contrast to the activation of monocytes/macrophages, infected dendritic cells were impaired in the secretion of pro-inflammatory cytokines, the production of co-stimulatory molecules and the stimulation of T cells. The ability of filoviruses to interfere with the host innate immune system, especially the interferon (IFN) response, has been attributed to the

virion proteins VP 35 and VP24 (reviewed by Bente et al., 2009). Limited evidence suggests that the presence of the expression of strong proinflammatory cytokine responses early in the disease course facilitates the induction of adaptive responses. In particular, the presence of IL-1β and elevated levels of IL-6 during the early symptomatic phase of the disease have been suggested as blood markers for survival, whereas the release of IL-10 and high levels of neopterin and IL-1 receptor antagonist (IL-1RA) during the early stage of disease are more indicative of fatal outcomes (Mohamadzadeh et al., 2007).

The disturbance of the blood-tissue barrier, which is controlled primarily by endothelial cells, is another important factor in pathogenesis. The endothelium seems to be affected directly by virus activation and lytic replication, as well as indirectly by an inflammatory response through mediators derived from primary target cells or viral expression products. These processes might explain the imbalance of fluid between the intravascular and extravascular tissue space that is observed in patients (Aleksandrowicz et al., 2008).

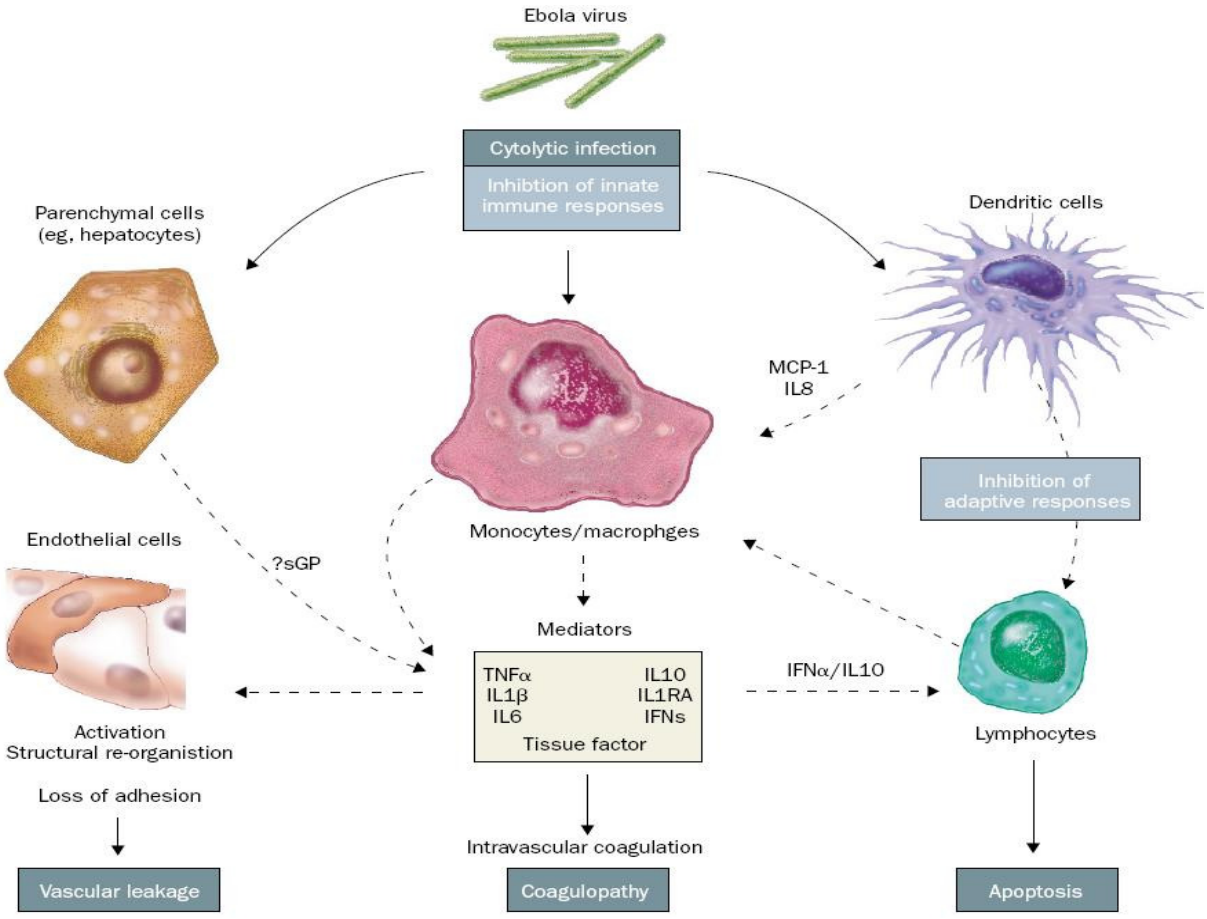

Figure 4 A model of the pathogenesis of filoviral haemorrhagic fever, based on studies of Zaire ebolavirus infection. Infection causes lysis of monocytes/macrophages, dendritic cells, and hepatocytes and suppresses innate immune responses in these cells, aiding further dissemination. Direct injury to infected cells is accompanied by indirect effects that are mediated by proinflammatory and anti-inflammatory effector molecules, including interleukin 1_ (IL1_), interleukin 6 (IL6), TNF_, interleukin 10 (IL10), and type I interferons (IFN). The severe illness results from the combined effects of widespread viral cytolysis and massive release of proinflammatory mediators. Proinflammatory cytokines and chemokines are also produced by activated endothelial cells, resulting in a feedback loop to the monocytes/macrophages. Lymphocyte apoptosis is also apparently brought about through effects of proinflammatory mediators; it may contribute to immunosuppression by weakening adaptive immune responses. The cell-surface expression of tissue factor by virus-infected monocytes/macrophages induces disseminated intravascular coagulation (DIC). MCP=monocyte chemoattractant protein; IL1RA=interleukin-1 receptor antagonist (Mahanty et al., 2004).

2.5 Viral Entry and Replication Cycle

Infection begins with the attachment of the virion to a receptor or lectin on the cell surface. To date, the search for the filovirus receptors has been elusive and implicated a set of distinct and functionally unrelated cell surface proteins in the entry process. Possible entry factors are reflected in table 1 (Dolnik et al., 2008).

Table 1. Entry factors of filoviruses.

Cellular entry factors	Ligand specificity	Cell tropism	Filovirus	System
ASGP-R	Terminal galactose	Hepatocytes	MARV	Virus
DC/L-SIGN	High-mannose glycans	DC, macrophages / endothelial cells in liver, lymph nodes	EBOV	Virus, pseudotype*
			MARV	Pseudotype*
hMGL	N-acetylgalactosamine	DC, macrophages	EBOV	Virus, pseudotype**
			MARV	Pseudotype**
LSECtin/ CLEC4G	N-acetylglucosamine	Endothelial cells in liver, lymph nodes, DC, macrophages	EBOV	Virus, pseudotype*
			MARV	Pseudotype*
β1 integrins	Laminin, collagen, fibronectin	Wide range of cells	EBOV	Pseudotype**
FR-α	Folic acid	Wide range of cells	EBOV	Pseudotype*
			MARV	Virus, pseudotype*
Tyro3 (Axl, Dtk, Mer)	Gas6	Wide range of cells	EBOV	Virus, pseudotype*
			MARV	Pseudotype*

* lentiviral particles pseudotyped with EBOV or MARV GP
** VSV pseudotype with EBOV or MARV GP in the envelope

Recent studies have suggested that folate receptor-a, asialoglycoprotein receptor, beta1 integrins, and/or DC-SIGN/DC-SIGNR may be receptors or cofactors used by filoviruses to enter cells. Other studies indicated that cholesterol-rich lipid rafts are used by filoviruses to enter cells. Experiments using pseudotyped retroviruses packaged in glycoproteins from Marburg virus or Ebola virus indicated that the viruses do not use the same processes to interact with cells (Hart., 2003). But recently, the receptor binding domain (RBD) of the filovirus GP has been mapped to the first 200 amino acids at the highly conserved N-terminus of GP1. The GP1 subunit is analogous to the HA1 subunit of influenza virus or the SU subunit of retroviral envelopes and is responsible for receptor binding. The similarities that were found suggest a common receptor of viral entry for MARV and EBOV (Brindley et al., 2007). It is thought that C-Type lectins which belong to the pathogen pattern recognition system are hijacked by filoviruses to concentrate virus on the primary targets of infection and to facilitate interaction with another receptor(s) ultimately mediating internalization into the cells. A second group of cell surface molecules involved in adhesion, the b1 integrin adhesion receptors, was implicated in EBOV GP-mediated entry. Down-regulation of b1 integrins from the cell surface of EBOV-infected cells, which caused cell rounding and detachment, suggested a role of these proteins in EBOV entry (Takada et al., 2000). Using genetic reconstitution of entry in a nonpermissive cell line, folate receptor a (FR-a) has been described as a significant cofactor for cellular entry of filoviruses. Corresponding to the wide species and cell tropism of filoviruses, FR-a is highly conserved in many mammalian species and expressed in a variety of different cell types at variable expression levels. Importantly, not all filovirus-permissive cell types express FR-a suggesting an additional alternative factor involved in virus entry (Chan et al., 2001). Recently it was reported that three members of the Tyro3 family Axl, Dtk and Mer of receptor tyrosine kinases can also mediate entry of filoviruses (Shimojima et al., 2006).

Taken together, a model emerges in which filoviruses do not use a single common receptor to infect a broad range of cells. More likely, a combination of attachment and receptor molecules is employed to enhance and promote infection of different primary cell types. Recently it was demonstrated that infected primary cells releasing specific cytokines

seem to be able to facilitate infection of secondary target cells like endothelial cells, which play a key role in filovirus pathogenesis (Yonezawa et al., 2005).

Binding to the receptors is followed by endocytosis. Endocytosis from the plasma membrane occurs by different mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolin mediated endocytosis and clathrin- and caveolinindependend endocytosis (Conner et al., 2003). The mechanism used by filoviridae is still uncertain, but it is thought that the caveolin mediated endocytosis is used. In addition, filoviruses may infect specialized immune-defence cells as macrophages and DCs by phagocytosis and possibly also non-professional phagocytic cells via a recently described phagocytosis-like uptake (Clement et al., 2006).

Following endocytosis, the next step in the entry of enveloped viruses is the fusion of the viral membrane with endosomal membranes releasing the viral genome. It was suggested that a specific trigger such as receptor binding or exposure to acidic pH is responsible for a conformational change of filovirus GP which leads to solvent accessibility of the viral fusion peptide. The fusion peptide is then inserted into the cellular membrane which ultimately mediates fusion. Mutation analysis demonstrated that the fusion peptide at the N-terminus of GP2 is responsible for the virus-cell membrane fusion (reviewed by Dolnik et al., 2008).

A replication complex made up of VP30, nucleoprotein, VP35, and large protein then generates mRNA transcripts and copies the genome to produce full-length positive-sense RNA antigenomes, which serve as templates for genome synthesis. New genomes associate with nucleoprotein and VP30 to form nucleocapsids, which accumulate in inclusion bodies. Meanwhile, newly synthesised viral glycoprotein becomes glycosylated during its transit through the host-cell Golgi apparatus and is cleaved by a furin-like enzyme before transfer to the cell surface, producing extracellular GP1 and transmembrane GP2 segments that remain linked by a disulphide bond. The assembly of new virions takes place on the inner surface of the plasma membrane, when nucleocapsids associate with matrix proteins linked to the cytoplasmic tail of membrane-bound GP1,2. Nascent virions leave the cell through budding (Fig. 5) (Mahanty et al., 2004).

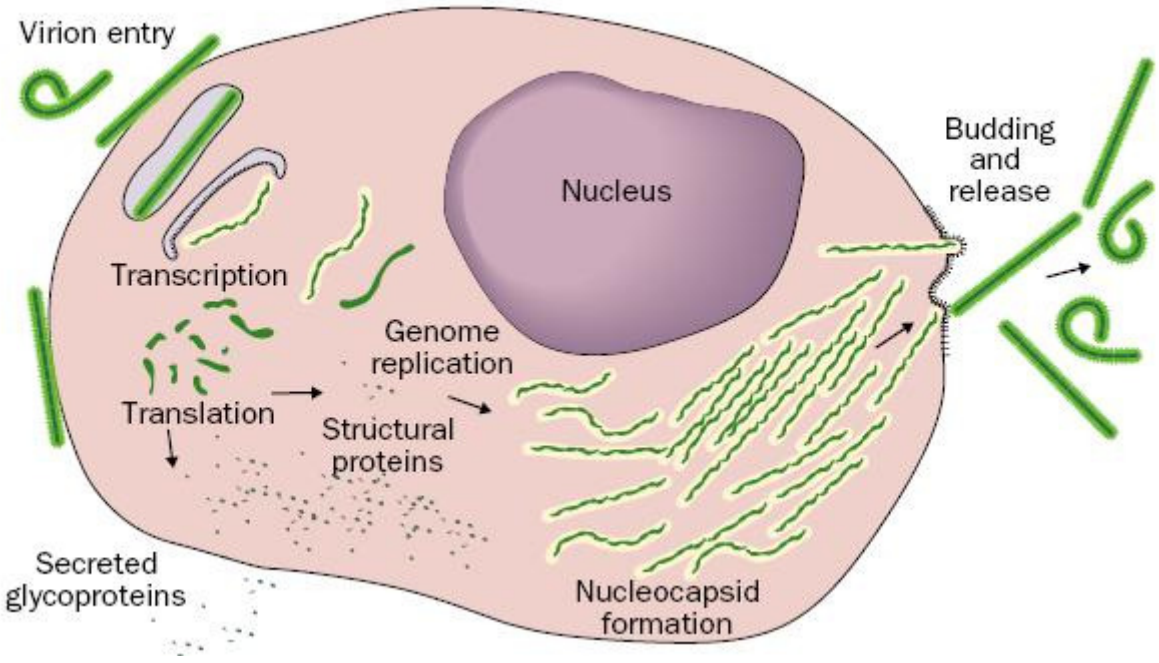

Figure 5 Filovirus replication. The steps in replication are described in the text. (Mahanty et al., 2004)

2.6. Differences between MARV and EBOV

As mentioned, MARV and EBOV differ from each other in structure, protein sequences, protein antigenicity and lack of serological cross-reactivity. The first difference between MARV and EBOV is that the MARV genome is a bit bigger (19.1 kb) than the EBOV genome (18.9 kb) and that the MARV genome has a single open reading frame encoding the GP protein. The glycoprotein gene of Ebola virus contains a translational stop codon in the middle, thus preventing synthesis of full-length glycoprotein. Besides the transmembrane form of GP, which is also present in the celmembrane of the MARV virus, this codon codes for a soluble, secretable form of GP (50–70 kDa)(sGP), synthesized in large amounts early in infection (reviewed by Beer et al., 1999). Both full-length and truncated forms of glycoprotein have been proposed to contribute to viral cytopathic effects and to suppression of host immune responses (reviewed by Beer et al., 1999). Both forms of the glycoprotein are identical for the first 295 amino acids, but then differ from each other, with sGP having a 69 amino acid carboxy region and the transmembrane form having 206 amino acids. The role of EBOV sGP in pathogenesis is unknown, but it has been suggested to function as a decoy for the immune system, perhaps by binding protective antibodies that recognise the sequences common to sGP and GP. Another possibility is that sGP binds to neutrophils and inhibits activation, thereby modulating the immune response. A third suggestion is that the cytotoxity of the glycoprotein is down-regulated by the production of sGP, which may allow an increased spread and replication by EBOV. However, this has not been evaluated in animals, and other studies evaluating Ebola virus variants failed to demonstrate significant differences in pathogenicity. Given that Marburg viruses do not make sGP and have a lower mortality rate than Ebola viruses, it will be interesting to determine the exact role of sGP in pathogenesis (reviewed by Hart, 2003).

Another difference was found by Enterlein et al.(2009), when they discovered that the MARV 3' non-coding region (NCR) structurally and functionally differs from EBOV. This region is involved in the transcription of the VP30. In EBOV, the VP30 is shown to be a transcriptional activator, necessary for efficient transcription of EBOV-specific minigenomes. Also, VP30 was shown to be essential for the rescue of recombinant EBOV and recently, involvement of EBOV VP30 in transcription re-initiation has been described (Martinez et al., 2008). The role of VP30 in the MARV replication cycle functionally differs from EBOV VP30 in not being involved in transcription initiation. VP30 in MARV is involved in secondary structure formation. Further analysis revealed that VP30-dependent transcription was regulated by an RNA secondary structure formed by the transcription start signal of the NP gene and dowstream located sequences. Computer prediction suggested an RNA secondary structure formed by the transcription start signal of the NP gene of MARV and downstream located sequences that are significantly different from the ZEBOV-specific stem-loop. These different secondary structures formed by the transcription start signal of the NP gene might account for the different transcription strategy of MARV and EBOV (Enterlein et al., 2009).

All of these differences make it difficult to develop an efficient vaccine, that works against both MARV and EBOV infections, and that is effective against all of the different strains. The goal of this paper is to describe the most recent findings on vaccine development for EBOV and MARV. In particular, what type of vaccines are currently available and what is the mechanism and the effectivity of these vaccines?

3. Vaccine development

Although Filoviruses have been studied widely over the recent years, there are no licensed Filovirus vaccines currently available to prevent the spread of an outbreak or reduce it's

severity. Besides the outbreaks caused by the unknown natural reservoir, there is another threat: the use of Filovirus as a biowarfare agent. There is anecdotal evidence that the former Sovjet Union explored the use of aerosolized MARV as a potential biowarfare agent in an offensive weapons program (Swenson et al., 2008).

A great amount of effort had been placed on developing safe and effective vaccines, but the researches encountered some difficulties. The first problem was the classifying of the viruses. The taxonomic classification is partially based on sequence and serological differences in the GP molecule. The GP is the only surface protein of these viruses and is thus the most probable target of protective immune responses and vaccine development. Vaccine development difficulties stem from the divergence between the species (the amino acid sequence of GPs from ZEBOV and SEBOV share only about 50% sequence homology). However, even for MARV, where all strains and isolates are considered a single species, there are substantial antigenic differences between some of them on the basis of evaluations with polyclonal and monoclonal antibodies. For example, the Musoke and Ravn strains, 2 MARV strains, differ by 22% in overall amino acid sequences of GPs and by over 50% in what is thought to be the antibody-binding region. These antigenic differences account for a lack of cross-protective immunity between filovirus species. In short, immunity against EBOV will not cross-protect against MARV, and vice versa. The same can even be said for immunity between ZEBOV and SEBOV species (Swenson, Wang et al., 2008). The second difficulty is that traditional vaccine platforms such as live-attenuated and killed-virus vaccines are unlikely to be used in humans due to safety risks of underattenuation or incomplete inactivation. For example, vaccination of guinea pigs with an inactivated whole-virus MARV vaccine was lethal in 20% of vaccinated animals. Therefore, much progress has been made using alternative vaccine platforms, such as recombinant viral vectors. This approach was found to be very effective in the protection of rodents and non human primates from lethal MARV infection. The same approach was effective for EBOV in rodents, but non human primates were not protected from ZEBOV infection (Swenson, Wang et al., 2008).

The most common vaccine strategies used for filovirus vaccine development include protein expression from live vaccinia virus or Venezuelan equine encephalitis virus replicons, DNA vaccination and a DNA-prime with a booster of protein or recombinant adenovirus. Most of these utilise other viruses as vectors for intracellular expression of the filovirus proteins. This has the advantage of getting the immunogen produced intracellularly, where the viral proteins are likely to be processed and presented to the immune system by class I molecules. Potential disadvantages of viral vectors include cell tropisms that may not support optimal production of the immunogen, and in cases where the vector is replicated along with the immunogen (as with vaccinia), the development of anti-vector immunity that limits the future utility of similarly vectored vaccines (reviewed by Hart, 2003). In contrast to live virus vectors, Venezuelan equine encephalitis virus replicons are designed not to replicate vector structural proteins. The glycoproteins that comprise the virus spike and the nucleocapsid are deleted from the genome, leaving only the non-structural proteins involved in replication. The genes of foreign proteins are inserted in place of the Venezuelan equine encephalitis virus structural proteins. Vaccine candidates made using this platform are ‘packaged’ into particles resembling virions by the cotransfection into cells of the replicon and two helper RNAs encoding the Venezuelan equine encephalitis virus structural proteins. The use of the bipartite helper system substantially reduces the chance of recombination events leading to live virus production. Intracellular replication of all three RNAs leads to the maturation of virus-like particles carrying an RNA expressing the foreign protein (Pushko et al., 1997). The packaged replicons, which have Venezuelan equine encephalitis virus spikes, presumably bind and infect cells similar to replication-competent virus, which leads to the production of the foreign protein. In the absence of Venezuelan equine encephalitis virus

structural proteins, no additional rounds of infection are supported. An advantage to this vector is the tropism for antigenpresenting dendritic cells, but the vector may be susceptible to pre-existing immunity even if it does not induce anti-vector immunity (MacDonald et al., 2000). In contrast to virus-vectored vaccines, DNA vaccines are not subject to concerns about the induction or effects of antivector immunity. Criticisms of DNA vaccines include the number of vaccinations that may be required, the induction of suboptimal immune responses that are frequently observed, and the potential for integration into the host genome (Hart, 2003).

In the past, six proteins (glycoprotein, nucleoprotein, VP24, VP30, VP35 and VP40) have been evaluated for the induction of protection from Marburg virus and Ebola virus challenges, using at least one vaccine platform. In addition, the sGP of Ebola virus and a modified glycoprotein of Marburg virus that is secreted rather than anchored in the membrane, have also been tested. In 2003 two vaccine platforms had already demonstrated efficacy in non-human primates: the Venezuelan equine encephalitis virus replicon platform for MARV and the DNA adenovirus combination for EBOV (Hart, 2003).

3.1 Current Vaccine developments

In 2007, Warfield et al. (2007) determined the efficacy of vaccination with Ebola virus-like particles (eVLP's) in nonhuman primates. VLPs for many viruses have been developed and are based on the knowledge that expression of specific viral structural proteins results in the self-assembly of particles that morphologically resemble the authentic virus. Some of the many advantages of using VLPs as vaccines include (1) their similar morphology to the live enveloped or nonenveloped viruses from which they are derived; (2) a strong safety profile, since they are nonreplicating; (3) no concerns regarding viral vector or pre-existing antivector immunity; (4) the fact that they can be generated in large quantities by use of mammalian or insect cell lines; (5) their ability to generate innate, humoral, and cellular immunity; and (6) the fact that they have been safely and effectively administered in humans. To establish the efficacy, cynomolgus macaques were vaccinated with eVLP's containing EBOV glycoprotein, nucleoprotein and VP40 matrix protein.

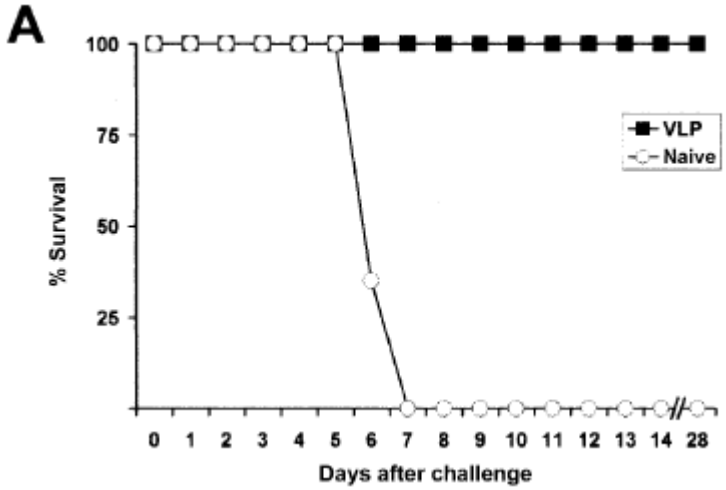

Figure 6 Protection of nonhuman primates vaccinated with Ebola virus-like particles (eVLPs) containing glycoprotein, nucleoprotein, and VP40. Cynomolgus macaques (n = 5) were vaccinated 3 times with eVLPs in RIBI adjuvant. The graphic shows survival after challenge with 1000 pfu of Zaire Ebola virus (ZEBOV), assessed 4 weeks after the last vaccination. For the purposes of representation on the Kaplan-Meier plot, results for the control monkey in the current study were combined with those for 23 historical control animals challenged with the same seed stock of ZEBOV, at the same dose and via the same route (Warfield et al., 2007)

As shown in figure 6, the results are promising: all 5 eVLP-vaccinated monkeys survived challenge without clinical or laboratory signs of EBOV infection, whereas the control animal died of infection. Serum samples from the eVLP-vaccinated nonhuman primates demonstrated EBOV-specific antibody titers (fig.7), as measured by enzyme-linked immunosorbent assay, complement-mediated lysis assay, and antibodydependent cell-mediated cytotoxicity assay.

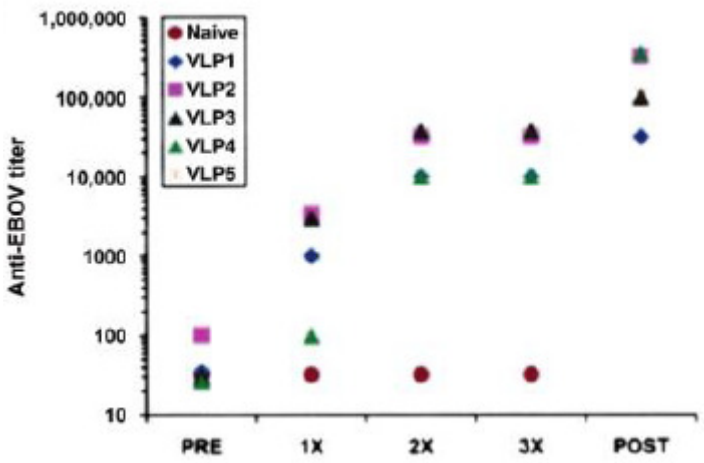

Figure 7 Antibody responses in nonhuman primates vaccinated with Ebola virus-like particles (eVLPs) containing glycoprotein (GP), nucleoprotein, and VP40. Cynomolgus macaques were vaccinated 3 times, at 6-week intervals, with 250 mg of eVLPs in RIBI adjuvant. The graphic shows total anti–Ebola virus (EBOV) antibody titers for individual animals (VLP1–5), as determined by ELISA (Warfield et al., 2007).

CD44+ T cells from eVLP-vaccinated macaques but not from a naive macaque responded with vigorous production of tumor necrosis factor–a after EBOV-peptide stimulation.

In 2008, Swenson, Warfield et al. proved that mVLP's were sufficient in the protection of guinea pigs and cynomolgus macaques against infection with multiple MARV's, and that eVLP's were able to protect guinea pigs from EBOV infection. The hybrid VLP's they created were morphologically similar to authentic filoviruses and wild-type VLPs (fig. 8).

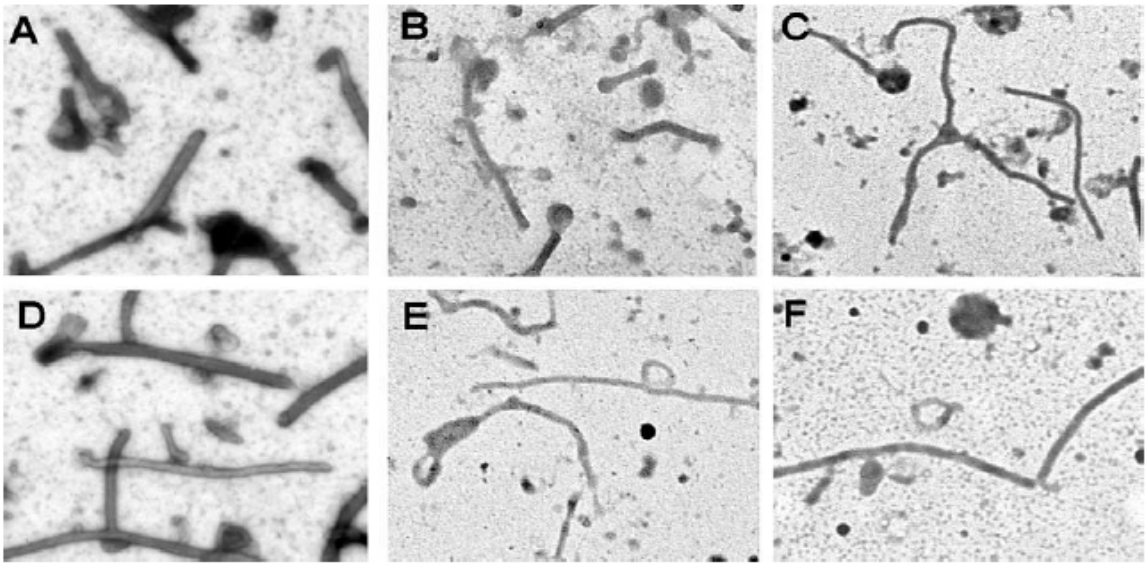

Figure 8 Hybrid VLPs were morphologically similar to authentic filoviruses and wild-type VLPs. VLPs, purified from the supernatants of 293T cells transfected with combinations of EBOV and MARV GP and VP40, were negatively stained with uranyl acetate to reveal the ultrastructure. Electron micrographs of (A) authentic EBOV, (B) Ebola virus-like particles (eVLP), (C) VLPs containing EBOV GP and MARV VP40 (e/m-VLP), (D) authentic MARV, (E) Marburg VLP's, (F) VLPS's containing MARV GP and EBOV VP40 at 40,000x.

Swenson, Warfield et al., also observed serum antibody responses to both EBOV and MARV after vaccination with eVLP's or mVLP's (fig. 9).

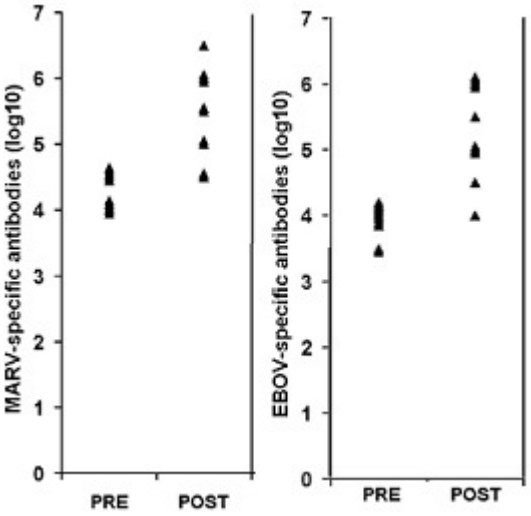

Figure 9 Serum antibody responses to EBOV and MARV after VLP vaccination. Strain 13 guinea pigs were vaccinated once with eVLPs or mVLPs. Serum samples from the guinea pigs were obtained immediately before (PRE) or 28 days post-challenge (POST). Total serum (A) anti-EBOV or (B) MARV antibodies were measured by ELISA. Antibody titers were measured in serum from individual guinea pigs and the results are graphed as the individual endpoint titers for each guinea pig in each group (n = 5–10 per group). Guinea pigs that survived lethal challenge with (A) EBOV or (B) MARV are indicated by the closed triangles.

Vaccination with mVLP's did not protect the guinea pigs against EBOV challenge, and vaccination with eVLP's did not protect guinea pigs against MARV challenge. A mixture of eVLP's and mVLP's however protected the guinea pigs against both virus infections, indicating that vaccinating with both antigens at the same time did not interfere with their ability to initiate humoral responses to the individual antigens (Svenson, Warfield et al., 2008)

Also in 2008, Swenson, Wang et al. described a panfilovirus vaccine based on a complex adenovirus (CadVax) technology that expresses multiple antigens from five different filoviruses *de novo*. Using this technique, the researchers were able to incorporate multiple genes into a single vaccine component. This incorporation enables the vaccine to protect against all relevant subtypes. In seeking the highest level of immune protection against all lethal filoviruses, a panfilovirus vaccine was developed that expresses the GP antigens of five different filoviruses covering all three significant species: ZEBOV, SEBOV, and MARV (Ci67, Ravn, and Musoke strains). The filovirus nucleoprotein (NP) is highly conserved among species, has been shown to induce effective cellular immune responses, and can enhance the efficacy of a GP-based vaccine. Therefore, since the CAdVax vaccine platform offers the advantage of multiantigen expression, the NP genes of ZEBOV and MARV Musoke were also included to maximize the breadth of immunity against the filoviruses. CAdVax vectors are able to accommodate large transgene inserts of up to 7 kilobases or six different genes, depending on the size of each respective gene. In order to ensure a balanced, high level of expression of each transgene, two filovirus genes per CAdVax vector were included. The final CAdVax-Panfilo vaccine formulation consisted of four vectors that cumulatively express five filovirus GP antigens and two filovirus NP antigens. NHP that were first challenged with MARV-Musoke strain, were back-challenged with SEBOV strain and NHP that were primarily infected with ZEBOV challenge, were back challenged with MARV-Ci67 (fig. 10).

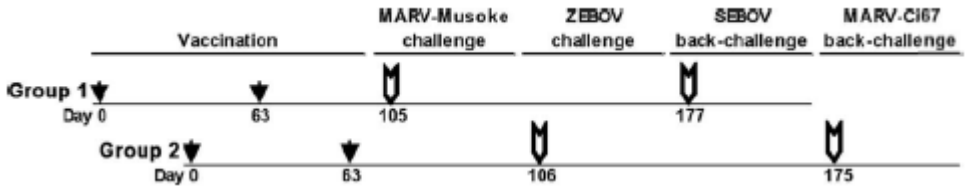

Figure 10 Experimental design for vaccination and filovirus challenge. Cynomolgus macaques were divided into two groups of five per group, and each group was vaccinated on days 0 and 63 with CAdVax-Panfilo or a control CAdVax vector. Group 1 was challenged with 1,000 PFU of MARV Musoke on day 105, and group 2 was challenged with 1,000 PFU of ZEBOV on day 106. Group 1 was subsequently back-challenged with 1,000 PFU of SEBOV on day 177, and group 2 was back-challenged with 1,000 PFU of MARV Ci67 on day 175. Filled arrows, vaccination; open arrows, virus challenge (Swenson, Wang et al., 2008).

Vaccination of Non-human primates (NHP) with CAdVax-Panfilo was 100% protective against challenge with multiple filovirus species, including ZEBOV, SEBOV, MARV Musoke, and MARV Ci67. Additionally, all vaccinated animals survived rechallenge with a completely different species of filovirus. This study provides a strong proof of concept for a single vaccine against multiple filoviruses using the CAdVax platform (Swenson, Wang et al., 2008).

In 2008, Geisbert et al. (2008a) experimented with a vesicular stomatitis virus-based (VSV) vaccine against aerosol challenge.

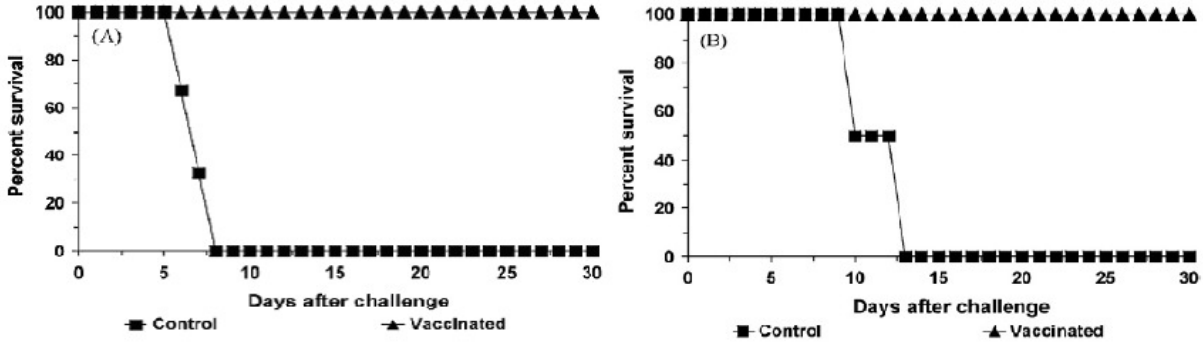

Figure 11 Kaplan–Meier survival curves for cynomolgus macaques vaccinated against ZEBOV or MARV and challenged against ZEBOV (A) or MARV (B) (Geisbert et al., 2008a).

This vaccine was proven effective against the normal challenge as well (Geisbert et al., 2008b). The recombinant VSVs expressing either the GP of ZEBOV (VSV_G/ZEBOVGP) or MARV (Musoke strain) (VSV_G/MARVGP) were generated as described recently using the infectious clone for the VSV, Indiana serotype (Garbutt et al., 2004). None of the three VSV_G/ZEBOVGP-immunized macaques became sick from the ZEBOV aerosolized challenge and all three animals were fully protected (Fig.11).

4. Conclusions

EBOV and MARV are highly infectious and outbreaks can cause a lethality from up to 90%. Since their discovery, recent outbreaks have occurred. Infection can be caused through contact with body fluids or aerosol formation. Because of the unknown identity of the natural reservoir and the threat of using EBOV and MARV as a bio warfare agent, a vaccine is needed. As described above, there are multiple vaccinetypes examined at this moment. The results are promising, all of the described vaccine trials produced positive results in NHP. And although NHPs are excellent models with which to study filovirus pathogenesis because they closely resemble the clinical disease and pathology described in humans, the next step for all the vaccine types would be clinical trials in human beings.

For the VLP's, proper glycosylation and presentation of viral proteins, as well as vaccine dose, are critical factors for successful vaccines. Another difficulty for the VLP's is that the vaccination schedule, dose and requirement for adjuvant still needs to be refined .Besides use as a vaccine, VLP's are also being used to dissect innate immune responses to filovirusses, with the goal of developing immunotherapeutics for the treatment of EBOV and MARV infections, as well as a safe surrogate model for the examination of filoviral replication, entry and assembly (Warfield et al., 2007). This is necessary, because there is still a lot unknown about these processes. A better understanding could help in the development of a vaccine, or possibly a cure.

A problem that could occur with the CadVax vaccine, is that during the production, there is a possibility of vaccine interference between gene products within a component and between different components. However, this has not been observed yet (Swenson et al., 2008).

The difficulty for the vaccine against aerosolized infection is that the pathogenesis of aerosolized infection has not been characterised fully yet. It is suspected that this exposure may be more difficult to protect against. Another problem is that because the host immune response elicited by different vaccine platforms could be different, it is not possible to say with an absolute certainty that the results for the VSV based filovirus vaccine system will hold true for all filovirus vaccine candidates and each will therefore need to be evaluated independently (Geisbert et al., 2008)

Another problem is that the groups used to investigate the vaccines, are really small. Geisbert et al. only vaccinated three macaques, and Warfield only vaccinated five. With bigger groups, the results could come out differently.

Overall, researchers are close to finding a safe vaccine against filovirus infection. The CadVax vaccine has proven to be affective against different strains of both MARV and EBOV in NHP's, a mixture of eVLP's and mVLP's has also proven effectivity against both MARV and EBOV in guinea pigs and VSV vaccine was proven to be effective against aerosol ZEBOV and MARV challenge, as well as the normal ZEBOV and MARV challenge. Although additional research still needs to be done, the current results look promising for the future vaccine development.

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