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Review

Recombinant vaccines against the mononegaviruses—What we have learned from animal disease controls

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ABSTRACT

The mononegaviruses include a number of highly contagious and severe disease-causing viruses of both animals and humans. For the control of these viral diseases, development of vaccines, either with classical methods or with recombinant DNA virus vectors, has been attempted over the years. Recently reverse genetics of mononegaviruses has been developed and used to generate infectious viruses possessing genomes derived from cloned cDNA in order to study the consequent effects of viral gene manipulations on phenotype. This technology allows us to develop novel candidate vaccines. In particular, a variety of different attenuation strategies to produce a range of attenuated mononegavirus vaccines have been studied. In addition, because of their ideal nature as live vaccines, recombinant mononegaviruses expressing foreign proteins have also been produced with the aim of developing multivalent vaccines against more than one pathogen. These recombinant mononegaviruses are currently under evaluation as new viral vectors for vaccination. Reverse genetics could have great potential for the preparation of vaccines against many mononegaviruses.

Contents

1. Introduction

The World Organisation for Animal Health (OIE) proclaimed on May 25, 2011 that all 198 countries and territories with rinderpest-susceptible animals were free of the disease, followed by the Food and Agriculture Organization (FAO) of the United Nations declaring on June 28, 2011 that the disease had been eradicated. Rinderpest was the second virus disease, after smallpox, to be eradicated through human efforts.

Rinderpest was a highly contagious viral disease affecting several species of wild and domestic cloven-hoofed animals, notably cattle and buffalo. Many species, including sheep and goats, can show milder clinical signs of the disease when infected, but the

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mortality rate could reach up to 100% in highly susceptible cattle or buffalo herds. FAO information indicates rinderpest epizootics were associated with the fall of the Roman Empire, the conquest of Christian Europe by Charlemagne, the French Revolution, the impoverishment of Russia, and extensive famines in Africa. Efforts to fight rinderpest were connected with the 1761 founding of the world's first veterinary school in Lyon, France. Furthermore, an outbreak of rinderpest in imported animals in Belgium in 1920 was the impetus for international cooperation in controlling animal diseases, and a key factor leading to the establishment of the OIE in 1924. Outbreaks killed millions of animals in the 1980s alone in Africa, southern Asia, and the Middle East, and a 1994 outbreak in Pakistan killed tens of thousands of cattle, buffalo, and yak. Since 1994, FAO has spearheaded the Global Rinderpest Eradication Programme (GREP) with the OIE, the International Atomic Energy Agency (IAEA) and other institutional partners, governments, regional organizations such as the Interafrican Bureau for Animal Resources, and communities worldwide. The last confirmed outbreak was in 2001, when buffalo were found to be infected in Kenya, and the last vaccination took place in 2006.

In the process of this historical feat, development of live attenuated rinderpest vaccine played a most important role, along with epidemiological surveillance and the culling of infected animals. The live attenuated vaccine was developed by Plowright and Ferris (1962) by the serial passage of the virulent bovine rinderpest strain Kabete 'O' in primary bovine calf kidney cells. This effective vaccine was used in many countries and contributed to significant reduction of the incidence of rinderpest.

Only rinderpest and smallpox virus diseases have been eradicated through human efforts to date, but the elimination of certain animal diseases has been accomplished by vaccination in certain countries and regions, such as rabies in certain European countries. The obstacles to the elimination of animal disease by vaccination are the lack of a good vaccine, the difficulty of vaccinating animals, and the expense of large-scale vaccination. Thus, the development of a new, good vaccine is still desired for the control of several infectious diseases.

During the course of the eradication of rinderpest, we learned much about the strategies to develop a good vaccine. Rinderpest virus (RPV) possesses a nonsegmented negative-strand RNA genome, and is classified in the order *Mononegavirales*. The *Mononegavirales* contain many other pathogenic viruses, and thus development of a good vaccine using new strategies is required. Despite their severe pathogenic characteristics, mononegaviruses are in a way thought to be attractive candidates as viral vectors because of their desirable properties for a vector. First, much is known about attenuating mononegaviruses. Second, most mononegaviruses can infect efficiently via the intranasal route and efficiently induce local IgA, systemic IgG, and cell-mediated protective immune responses. Third, most mononegaviruses replicate in the cytoplasm and do not integrate into the host genome, obviating concerns about cellular transformation. Fourth, recombination involving mononegaviruses is extremely rare. Fifth, mononegaviruses have relatively simple genomes that encode only 5–11 proteins and the genes of mononegaviruses are nonoverlapping, making them easy to manipulate. All these reasons drive us to use mononegaviruses as viral vectors.

The order *Mononegavirales* comprises four families: *Rhabdoviridae*, represented by vesicular stomatitis virus (VSV) and rabies virus; *Paramyxoviridae*, including Sendai virus (SeV), human parainfluenza virus types 1, 2, 3, and 4 (HPIV1 to −4), measles virus (MeV), mumps viruses, Newcastle disease virus (NDV), human respiratory syncytial and metapneumoviruses, and Nipah virus (NiV); *Filoviridae*, containing Ebola and Marburg viruses; and *Bornaviridae*, containing Borna disease virus. Although there are some slight differences, their genome structure and intracellular replication steps are very similar. In the case of paramyxoviruses, the virions are enveloped with fusion proteins (F) and attachment proteins (hemagglutinin [H], hemagglutinin-neuraminidase [HN], which are glyco- [G] proteins) protruding on the virion surface. Matrix (M) proteins inside the envelope stabilize the virus structure and regulate viral replication and assembly. The ribonucleocapsid core is composed of the genomic RNA, nucleocapsid (N) proteins, phosphoproteins (P) and polymerase proteins (L). All paramyxoviruses share six common genes, each with its own transcription start and stop signals. These genes appear in the following order from the 3′end: N, P, M, F, H, and L.

2. Vaccines developed by classical methods

Almost all of the currently used vaccines were developed by classical methods. There are two categories of licensed vaccines used for mononegaviruses: inactivated and live vaccines. In an inactivated vaccine, an adjuvant, which non-specifically enhances the immune response to a given antigen, is necessary to give an adequate protective immunity. When an animal is vaccinated for the first time, a double vaccination is required to induce an adequate level of immunity. The Vaccine against rabies virus is an example of an inactivated vaccine. Inactivated vaccines usually give rise to high serum antibody titer. However, inactivated vaccines are rather ineffective in stimulating appropriate specific cell-mediated and mucosal immune responses. Thus, the systemic humoral immunity and immunological memory will probably contribute the most to the vaccinal immunity against the infectious diseases.

A live attenuated vaccine is generally composed of viruses that are rendered avirulent through attenuation and are still able to replicate in the host. The aim of attenuation is to weaken or eliminate the virulence of viruses without affecting their immunogenicity. Usually a single vaccination is sufficient to induce a high level of immunity. Vaccines against RPV, canine distemper virus (CDV), MeV, and NDV are examples for live attenuated vaccines. The immunological mechanisms underlying the protection from the viruses induced by live vaccine will probably involve a complex interplay between the induced humoral and cell-mediated immunity and immunological memory.

3. The first recombinant vaccines

In addition to the classical vaccines, there are other types of vaccines: recombinant and multivalent vaccines. Live attenuated vaccines have often been considered to be the ideal approach for immunization. However, it has not always been possible to attenuate every virus by conventional methods. Furthermore, even if they were attenuated, the site of the attenuating lesion was not always known and reversion could occur. To overcome these disadvantages, reverse genetics has been used to identify virulent genes of viruses and alter them either by mutations or by deleting them to develop new attenuated vaccines. Multivalent vaccines were developed because their use can reduce the number of vaccine applications, which simplifies the vaccine regimen, reduces costs for vaccination, and may reduce the risk of possible post-vaccination side effects. In this review, we will focus on the trials to develop new attenuated and multivalent vaccines of mononegaviruses by using recombination techniques.

3.1. Production of vaccinia-vectored vaccines using recombinant techniques

The vaccinia virus had a part to play in the only successful eradication of a human infectious disease to date. The widespread use of the vaccinia virus for smallpox vaccination, especially during

the smallpox eradication campaign, was a major contribution to the final eradication of smallpox (Fenner, 1989). Even before the end of the smallpox eradication campaign, the value of vaccinia virus in the development of recombinant virus vaccines was anticipated. Vaccinia virus is particularly favorable as vaccine carriers for a variety of reasons. Firstly, the large size of the viral DNA genome allows for the tolerance of the insertion of foreign genes up to a reported 30,000 base pairs (Perkus et al., 1985; Smith and Moss, 1983). Secondly, vaccinia virus is a potent inducer of both arms of the immune response and has been shown to adequately display foreign antigens to the immune response in various disease models (Pastoret and Vanderplasschen, 2003). Thirdly, a major advantage of the use of vaccinia virus as a vaccine vector is that the lyophilized vaccinia virus has heat-stability, which obviates the need for a cold chain. Because of these advantages, many vaccinia-vectored vaccines against the mononegaviruses, such as RPV (Yilma et al., 1988; Tsukiyama et al., 1989; Barrett et al., 1989), CDV (Wild et al., 1993; Welter et al., 1999, 2000), NiV (Wang et al., 2006), SeV (Takao et al., 1997), NDV (Meulemans et al., 1988; Boursnell et al., 1990), and rabies virus (Wiktor et al., 1984; Kieny et al., 1984), have been examined for veterinary use so far. Here, two examples of vaccinia-vectored vaccines: vaccinia-RPV glycoprotein recombinant vaccine, which was shown, for the first time, to be sufficiently attenuated in authoritative trials, and vaccinia-rabies glycoprotein recombinant vaccine, which is the first recombinant vaccinia mumps virus (MuV) used as a vaccine, will be discussed.

3.2. Vaccinia-RPV glycoprotein recombinant vaccines

The first vaccinia recombinant vaccines for rinderpest were developed in the late 1980s and these used the two surface glycoprotein genes of the virus, F and H protein genes, as the immunizing antigens (Yilma et al., 1988; Tsukiyama et al., 1989; Barrett et al., 1989). These were based on the WR strain of the virus but this was not considered sufficiently attenuated to be suitable for licensing in the absence of smallpox in the human population. In addition, this vaccinia strain produced severe lesions at the site of inoculation in animals (Belsham et al., 1989). An expert committee convened by OIE in 1989 to consider guidelines for the use of these vaccines recommended the use of safer strains of vaccinia which have shown to be sufficiently attenuated in authoritative trials. A rinderpest recombinant based on the LC16mO strain was tested in cattle in Britain (Yamanouchi et al., 1993) and one based on the Wyeth strain was tested in the USA (Giavedoni et al., 1991). Subsequently the duration of immunity in African (Verardi et al., 2002) and European cattle (Inui et al., 1995) was shown to be reasonably long-lasting, certainly sufficient to control an outbreak situation. Another poxvirus vector, capripox virus (the agent of sheep and goat pox) was also used to produce a recombinant rinderpest vaccine. Using the established capripox vaccine as a vector it is possible to protect cattle against two diseases, rinderpest and lumpy skin disease, the latter being caused by capripox (Ngichabe et al., 1997; Romero et al., 1993, 1994a,b). These recombinant vectors were considered to be more suitable for use in an epidemic area of rinderpest because of their heat stability. Unfortunately, controversy over the release of genetically manipulated organisms hampered the licensing of these vaccines for field use and they were not utilized for GREP.

3.3. Vaccinia-rabies glycoprotein recombinant vaccines

Rabies infection of domestic and wild animals is a serious problem throughout the world. The major disease vector is wildlife species, and rabies control has focused on vaccination and/or culling such wildlife species. Oral rabies vaccination has proved to be an obligatory strategy for rabies elimination programs in domestic and several wildlife target species. Traditional live attenuated rabies vaccines were not applicable to the oral vaccination and were heat-instable. Furthermore, all live attenuated rabies vaccines were still pathogenic for some animal species, and cases of vaccine-induced rabies were often reported. Therefore, recombinant vaccinia virus, a relatively heat-stable virus, was used for the development of safer recombinant vaccines against rabies virus. The first recombinant vaccinia virus used as a vaccine is a recombinant vaccinia virus expressing rabies virus glycoprotein that is inserted into the vaccinia virus thymidine kinase gene (Wiktor et al., 1984; Kieny et al., 1984). The vaccine is used in oral baits, which are distributed by hand or by aerial distribution from low flying aircraft over the target area. This vaccine has been widely used for the oral vaccination of raccoons, gray foxes and coyotes in North America, raccoons in Canada and for red foxes in several Western European countries (Cliquet and Aubert, 2004; Rupprecht et al., 1986, 2004; Blancou et al., 1986). The virus has also been used for the immunization of other important reservoirs of rabies, such as vampire bat (Aguilar-Setien et al., 2002). Although used in certain campaigns with great success, potential safety issues related to the use of the potent vaccinia virus hamper the expansion of its use for new target species and new areas.

4. Approaches for development of live attenuated mononegaviruses using reverse genetics

In negative strand RNA viruses including mononegaviruses, it was impossible to generate their recombinant virus from cloned cDNA of the RNA genome for a long time. In 1994, Schnell, Mebatsion, and Conzelmann developed a reverse genetic system for rabies virus that allowed the recovery of infectious virus entirely from cloned cDNA (Schnell et al., 1994). This was followed quickly by the development of reverse genetic systems for numerous other mononegaviruses. Reverse genetics is used in molecular virology to generate infectious viruses possessing genomes derived from cloned cDNA that have been modified in order to study the consequent effects on phenotype.

One of the characteristics of most mononegaviruses is that their genomes are located exclusively in the cytoplasm of infected cells and do not go through a DNA phase; thus there is no concern about unwanted integration of foreign sequences into chromosomal DNA. Therefore, this new class of “cytoplasmic RNA vector” is expected to be a safer and more efficient viral vector than existing vectors for application to human therapy in various fields including gene therapy and vaccination. Mononegaviruses require attenuation before use as potential vectors because of their high cytopathic effects. Reverse genetics is a powerful tool to introduce combined attenuating mutations or deletions in the RNA genome to fine-tune the level of attenuation and to produce vaccines sufficiently infectious and immunogenic yet attenuated and genetically stable. To this end, attenuating amino acid point mutations can be identified and introduced in desired combinations by reverse genetics. The inactivation of immune-modulating viral proteins, such as accessory proteins, by reverse genetics provides an optimized method for increasing the efficacy of vaccines and reducing their pathogenicity. The vaccine vector is improved by deleting the envelope-related genes from their genomes. For example, the deletions of all the envelope-related genes (M, F, HN) from the SeV genome are very effective for reducing cytopathic reaction (Yoshizaki et al., 2006). These modifications by reverse genetics are expected to improve the safety and broaden the range of clinical applications of this new class of cytoplasmic RNA vector. The following discussion is about the use of mononegaviruses, in particular

members of the *Rhabdoviridae* and *Paramyxoviridae*, as live vaccine vectors.

4.1. Virus attenuation

In the case of VSV, several unique strategies for attenuation have been attempted. Truncation of the cytoplasmic tail (CT) region of G protein was used to attenuate in vitro growth and in vivo virulence of recombinant VSV vectors (Roberts et al., 1998; Schnell et al., 1998). Viruses with shortened CTs have slower growth rates, reach lower peak titers in vitro, and are less pathogenic in mice than unaltered viruses (Roberts et al., 1999). It is thought that shorter CTs reduce the rate of particle formation and peak virus titer produced in vitro, possibly due to impaired CT interaction with viral core proteins (Roberts et al., 1998; Schnell et al., 1998; Dubovi and Wagner, 1977; Jayakar et al., 2004). Next, the ability to attenuate in vitro growth and virulence of recombinant VSV by moving the N gene from the first position in the genome to downstream locations was clearly demonstrated (Flanagan et al., 2001, 2003; Wertz et al., 1998). The step-wise translocation of the N gene further away from the 3′transcription promoter leads to incremental reduction in N protein expression. Consequently, limiting N protein reduces the level of nucleocapsid available for transcription, replication, and subsequent incorporation into virus progeny. These mutants give rise to virus with a very stable attenuation phenotype because replacement of deleted sequence encoding part of a protein is very improbable. A third attenuation strategy relies on nucleotide substitutions within the M gene that ablate expression of two in-frame overlapping polypeptides initiated downstream from the M protein translation start codon (Jayakar and Whitt, 2002). Viruses that do not express these polypeptides demonstrate reduced cytopathology in a variety of cell lines and are highly attenuated in mice. Consequently, mutants that do not express these polypeptides have been called noncytopathic M mutants. More of the resulting combination mutants were growth attenuated in vitro than vectors containing either single form of mutation (Clarke et al., 2007). The results show synergistic rather than cumulative increases in attenuation and demonstrate a new approach to the attenuation of VSV and possibly other viruses.

NDV causes a serious respiratory disease in poultry. Engineered recombinant NDV expressing chloramphenicol acetyltransferase (CAT) was generated by reverse genetics (Krishnamurthy et al., 2000). The CAT gene of this recombinant NDV was maintained stably for at least eight passages without any detectable loss of the gene. However, the virus showed reduced plaque size, slower replication kinetics, an increase in mean death time for eggs and a lower intracerebral pathogenicity index in day-old chicks, implicating attenuation of the recombinant virus. Thus, introduction of an additional gene into the NDV genome represents an alternative method to achieve viral attenuation.

Recently, a unique alternative method of viral attenuation, referred as a "death by a thousand cuts" strategy, was reported (Coleman et al., 2008). As a result of redundancy of the genetic code, there is a species-specific "codon pair bias", which means that some synonymous codon pairs are used more or less frequently than statistically expected. Recombinant polioviruses with underrepresented codon pairs showed decreased rates of viral protein translation and were attenuated in mice. Furthermore, the recombinant polioviruses were used to immunize mice and provided protective immunity after challenge. This method has three key advantages in its use as a vaccine over previous methods: (i) It produces a virus encoding precisely the same amino acid sequences as the wild-type virus, and therefore inducing the same immune responses. (ii) The attenuation is not subject to reversion, simply because of the sheer number of mutations. (iii) It can be used in combination with other attenuation approaches described above. Thus, viruses attenuated by this method will be candidates for novel attenuated virus vaccines in the future.

4.2. Inactivation of accessory viral genes

The V and C proteins are produced from the P gene, and are widespread among the members of the subfamily *Paramyxovirinae* of the family *Paramyxoviridae* but are not as ubiquitous as the six major structural proteins, and therefore have been regarded as nonessential "accessory" gene products. An open reading frame (ORF) that is shifted from the ORF of the P protein gives rise to C protein. This diversity is accomplished by the use of translation initiation codons starting at different points. The frames for the C proteins therefore are accessed through "ribosomal choice". The V protein, on the other hand, is produced by a G residue insertion at an editing site within the P ORF by viral RNA polymerase, which occurs at a constant rate. The V protein therefore consists of a P/V common region and the V unique region. Viruses are subjected to various antiviral host responses upon infection. Among the responses, interferon (IFN) responses play important roles in early innate immunity and in the modulation of subsequent acquired immunity. Accessory proteins of various paramyxoviruses have been shown to have IFN-antagonist activity and play an important role in the pathogenicity in vivo. For example, C protein of Respirovirus and V proteins of most paramyxoviruses have been shown to prevent the IFN response by inhibiting IFN signaling (Gotoh et al., 2002). In addition, the V proteins of most paramyxoviruses are able to antagonize both the NF-kB and IRF-3 arms of the dsRNA signaling (Poole et al., 2002; Childs et al., 2007), and mda-5 was identified as an interacting partner for the V proteins (Childs et al., 2007; Andrejeva et al., 2004). To date, various recombinant viruses lacking accessory proteins have been established and the attenuation examined in vivo. The silencing of the accessory proteins is practical because recombinant viruses lacking these proteins frequently grow to high titers in cultured cells, a feature necessary for efficient preparation of vaccines, and of great relevance to attenuated vaccine development.

NDV edits its P gene mRNA by inserting a nontemplated G residue at a conserved editing site to produce V protein. One nucleotide substitution in the editing site resulted in reduced editing frequency and, as a result, V protein was expressed at a 20-fold-lower level (Mebatsion et al., 2001). This recombinant NDV with low-level V protein expression has been shown to be immunogenic and lack pathogenicity for chicken embryo unlike currently available NDV vaccine strains, demonstrating the potential use of this recombinant NDV as a safe embryo vaccine. In the case of SeV, C-deficient virus did not grow at all in mice and produced no lung lesions, in striking contrast to the wild-type SeV that grew vigorously and killed all the mice (Kato et al., 2007). Furthermore, it was demonstrated that the virulence of C protein in vivo is parallel to its anti IFN-α/β capacity in vitro (Kato et al., 2007). On the other hand, V-deficient virus proliferated in the lungs of normal mice as efficiently as the parental wild-type SeV until day 1 post infection. However, they were then rapidly cleared, and never lethal (Kato et al., 1997). V protein contributes to prolonged virus growth in the lung via unknown innate-immunity mechanism following IRF3 activation (Kiyotani et al., 2007). Similarly, both V-deficient HPIV2, which possesses only C protein as an accessory protein (Schaap-Nutt et al., 2010) and C-deficient HPIV1, which possesses only V protein as accessory proteins (Bartlett et al., 2008) showed significant attenuation in vivo, and undetectable level in the African green monkey respiratory tract.

However, other reports imply that accessory proteins are not indispensable for some paramyxoviruses in vivo. In the case of MeV, V- and C-deficient viruses developed attenuation of clinical symptoms and short-lived replication in rhesus monkeys, but the degree

of attenuation was mild compared to other viruses described above. Interestingly, CDV V protein is essential for rapid viral multiplication in T cells, while C-defective CDV remained fully virulent and immunosuppressive. On the other hand, C-defective HPIV3 was significantly attenuated in vivo, but V-knockout did not affect HPIV3 replication in vivo (Durbin et al., 1999).

This diversity of the activities of the accessory proteins in vivo has been confirmed from our study using RPV and NiV. The lapinized strain of the virus, RPV-L, which was derived from a vaccine virus by numerous passages in rabbits, is avirulent in cattle but highly virulent in rabbits causing clinical signs identical to those of rinderpest in cattle. In contrast, tissue culture attenuated RBOK strain is a vaccine strain and does not cause any disease in rabbits. Using these strains we constructed chimeric virus between L and RBOK in which genes were replaced with each other. As a result, recombinant viruses harboring the P gene of L strain showed clinical, virological, and histopathological signs in rabbits, indicating that the RPV P gene was considered to be a key determinant of pathogenicity (Yoneda et al., 2002, 2004). Unexpectedly, however, both V-deficient and C-deficient RPV-Ls showed severe clinical symptoms in rabbit, similar to the effects of the parental strain (unpublished data). These studies demonstrated that the accessory proteins of RPV exert little effect on the virulence in viro.

We first developed a NiV reverse genetics system (Yoneda et al., 2006). NiV was first discovered in Malaysia in 1998 in an outbreak of infection in pigs and humans and incurred a high fatality rate in humans. NiV has a broad host range, and the natural host was identified as fruit bats. The human infection induced high mortality rates (up to 70%) in recent outbreaks in Bangladesh (Kai and Yoneda, 2011). Because of the broad host range and the high mortality rates associated with the infection, NiV has been classified as a biosafety level 4 (BSL-4) agent. Thus attenuation steps are important for handling, and development of vaccines for NiV in the future is an urgent issue to prevent further outbreaks. In the case of NiV, the P gene expresses W protein, which is generated by a two G residues insertion at the editing site, in addition to P, V and C. It has been reported that all four P gene products have IFN antagonist activity in vitro. In addition, V and W inhibit IFN production. We generated recombinant NiVs lacking V, C or W protein, respectively, to analyze the functions of these proteins in infected cells and the implications in in vivo pathogenicity (Yoneda et al., 2010). Although we also confirmed the IFN antagonist activity of each accessory protein if each of the protein were expressed in cells, all the recombinants lacking an accessory protein suppressed the IFN response as well as the parental NiV (Fig. 1A), indicating that the lack of single accessory protein does not significantly affect the inhibition of IFN signaling in infected cells. The recombinant NiVs lacking each accessory protein possesses unaltered P protein, thus P protein might be enough to inhibit IFN signaling. As expected, W-deficient virus showed no apparent difference of virulence compared to that of wild-type in experimentally infected golden hamsters (Fig. 1B). However, interestingly V- or C-deficient NiV showed a significant reduction in virulence (Fig. 1B). These results suggest that V and C proteins, but not W protein, play key roles in NiV pathogenicity, while the roles are independent of their IFN-antagonist activity. This study indicated that the recombinant NiV lacking C or V could be a good candidate as an attenuated vaccine for NiV.

4.3. Replication-defective vaccines

As an attempt to generate a safer vector using a mononegavirus, a replication-defective virus has also been studied. In SeV (Li et al., 2000), the genome cDNA was constructed by replacing the F gene with an EGFP reporter gene. In addition, to provide F protein, an F-expressing packaging cell line with a Cre/loxP-inducible expression system was constructed. The recombinant SeV does not encode F protein, but instead incorporates it expressed *in trans*. Thus, the defective vector amplified specifically in an F-expressing packaging cell line but did not spread to F-non-expressing cells. Furthermore, an inserted EGFP reporter gene is vigorously expressed from this SeV vector in cells of various origins in culture. As with other viral vectors, there is induction of antibody and T-cell-mediated immune responses in mice (Yoshizaki et al., 2006). Therefore, F-deficient SeV vector is a promising vaccine tool for inducing virus-specific cellular immune responses. Currently, this system is thought to serve as a delivery vector to a wide range of dividing and non-dividing mammalian cells and tissues.

Replication-defective virus is also useful for handling deadly diseases. Ebola viruses (EBOVs) cause hemorrhagic fevers in humans and nonhuman primates, with case fatality rates of 90% in some outbreaks, and are categorized in BSL-4. Thus, biologically contained EBOVs that resemble wild-type virus but can be handled outside BSL-4 containment are clearly needed. To this end, EBOVs that lack the essential VP30 gene were generated, and maintained by a cell line expressing VP30. The resultant viruses resemble wild-type virus in their life cycle, their morphology, and their growth properties (Halfmann et al., 2008). Next, the protective efficacy of VP30-defective EBOV in two animal models, mice and guinea pigs, was assessed. Mice immunized twice with the defective virus were protected from a lethal infection of mouse-adapted EBOV. Virus titers in the serum of vaccinated mice were significantly lower than those in nonvaccinated mice. Protection of mice immunized with the defective EBOV was associated with a high antibody response to the EBOV glycoprotein and the generation of an EBOV NP-specific CD8(+) T-cell response. Guinea pigs immunized twice with the defective virus were also protected from a lethal infection of guinea pig-adapted EBOV (Halfmann et al., 2009). These studies demonstrate the potential of the VP30-defective EBOV as a new vaccine platform.

4.4. Marker vaccines

In a global vaccine programme, it could be difficult to distinguish naturally infected animals and vaccinated animals by detection of the serological response against the intrinsic viral antigen. In fact, in the rinderpest eradication programme, the lifelong immunity induced by the vaccine confused serosurveillance from differentiating non-infected but vaccinated animals from those which had been exposed to RPV. As this was the case, part of the final strategy for eradication of rinderpest was an emergency vaccination plan to prevent spread of the virus in the event of isolated outbreaks of disease in the endemic region. To solve this problem, some types of 'marker vaccine' have been developed using reverse genetic techniques. For example, recombinant RPVs expressing HA, GFP or a membrane-anchored form of the GFP have been established (Walsh et al., 2000a,b). These vaccines were highly effective in protecting animals from virulent rinderpest challenge and enabled identification of the vaccinated animals serologically. The negative marker vaccine by replacing the N protein gene of RPV with the equivalent gene from peste des petits ruminant virus (PPRV), which is a highly contagious disease of small ruminant and classified in the *Morbillivirus* genus along with RPV, was considered to be useful even in the eradication programme, as this vaccine did not produce a strong N protein specific antibody response as found in natural infection or in traditional vaccination (Parida et al., 2007).

On the contrary, one of the recombinant PPRV vaccines was developed using the rinderpest vaccine virus genome as the backbone into which the M, F and H genes of RPV were replaced by those of PPRV (Mahapatra et al., 2006). The resulting chimeric virus proved to be a safe and effective vaccine which could protect goats against virulent challenge with PPRV. This chimeric PPR marker vaccine can be used in any endemic country without compromising

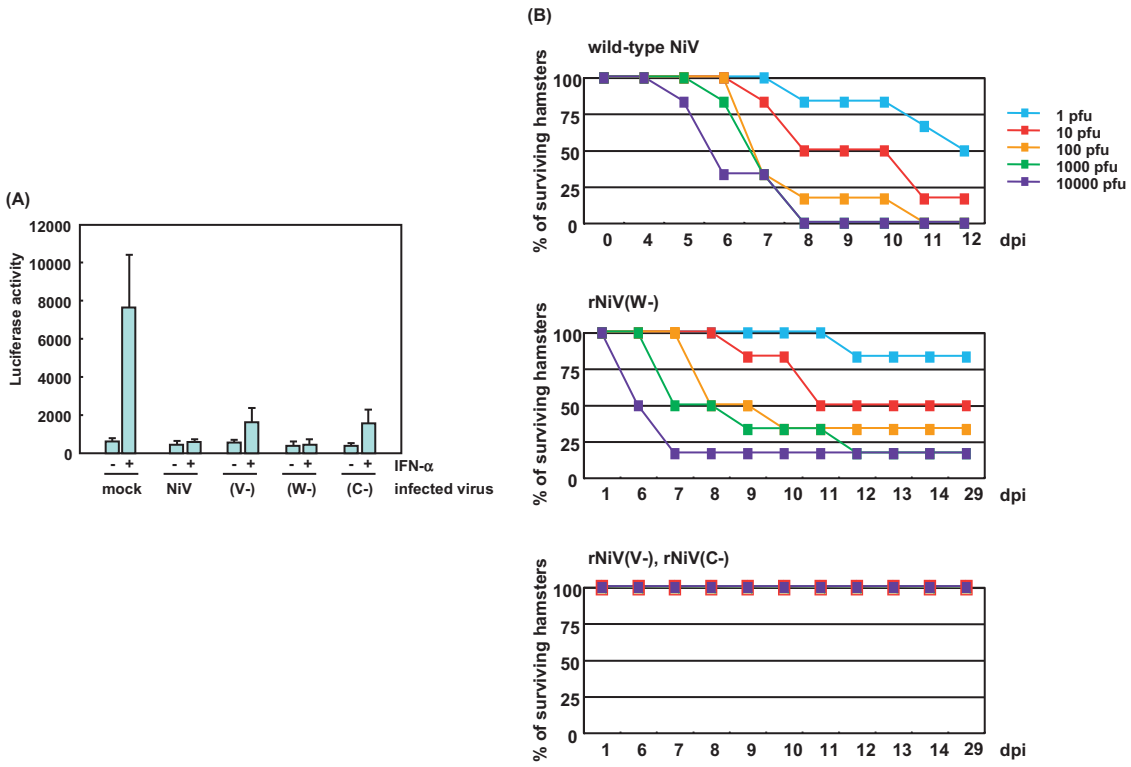

Fig. 1. (A) NiV inhibits the induction of luciferase expression from IFN responsive promoter. Vero cells were transfected with luciferase reporter plasmids under the control of the IFN-α promoter (pISRE-luc) and after 48 h, the cells were infected with NiV, NiV(V-), rNiV(W-) or rNiV(C-). At 24 h post infection, cells were treated with 1000 U of IFN-α for 24 h, lysed, and the luciferase activities were measured. (B) Survival curves of the hamsters infected with different quantities of the recombinant viruses. Hamsters (6 groups) were inoculated intraperitoneally with 10-fold serial dilutions of either NiV or rNiVs and survival rate of each group was observed for 30 days.

the global rinderpest serosurveillance effort since all antibody tests are based on either the N or H proteins which are quite distinguishable serologically between rinderpest and PPR viruses.

5. Generation of multivalent vaccine vectors

5.1. Recombinant viruses expressing foreign genes

As a logical extension of the work on obtaining improved vaccines against individual viruses, the exploration of the potential of mononegaviruses as multivalent vaccine vectors has begun. The common goal is to generate a protective immune response against other pathogens. Mononegaviruses have ideal features for live vaccines in that they do not cause homologous recombination in the infected cells, nor do they induce humoral or cellular immunity. In addition, mononegaviruses are amenable to genetic manipulation by reverse genetics systems. Transcriptional units coding for foreign genes can be inserted in between viral genes with their own start and stop signals. The foreign genes are expressed along with the viral genes during viral replication and the phenomenon of transcriptional polarity governs their levels of expression, and the corresponding protein is presented in the immunogenic context of a viral infection that may act as an adjuvant

For example, VSV has been evaluated as a vaccine vector against a number of prevalent human viruses, including HIV (Clarke et al., 2006), SARS-CoV (Kapadia et al., 2005), herpes simplex virus type 2 (Natuk et al., 2006), papillomavirus (Roberts et al., 2004), and hepatitis C virus (Buonocore et al., 2002). Furthermore, several rVSV vectors have already been generated that express various cytokines. While most of these vectors have had a weak adjuvant effect, positive results from rVSV vectors expressing IL-12 (Klas et al., 2002, 2006) or GM-CSF (Ramsburg et al., 2005) have been reported. IL-12 is a proinflammatory cytokine expressed by antigen-presenting cells as a heterodimer, and drives the development of Th1-like responses. GM-CSF recruits and activates antigen-presenting cells, including macrophages and dendritic cells.

Paramyxoviruses are currently being developed as multivalent vaccine vectors for human use. For example, recombinant MeV expressing hepatitis B virus (Singh et al., 1999; del Valle et al., 2007), HIV (Tangy and Naim, 2005; Brandler et al., 2007; Zuniga et al., 2007), West Nile and Dengue virus (Brandler et al., 2007; Despres et al., 2005; Brandler and Tangy, 2008), SARS-CoV (Liniger et al., 2008) have been established. We have also developed recombinant MeV vaccines for hepatitis C virus (Satoh et al., 2010; Kasama et al., 2011) and NiV (unpublished data). In the case of SeV, a unique attempt using SeV expressing amyloid β peptide for therapy of Alzheimer's disease has been performed (Tabira and Hara, 2006).

On the other hand, development of multivalent vaccine vectors for veterinary use is not advanced to date. In the following section, we present our recent attempts for development of canine vaccine against CDV and other pathogens.

5.2. Application of CDV as potential vaccines for other pathogens

Canine distemper is one of the lethal infectious diseases in dogs and other *Canidae* (Takayama et al., 2009; Hirama et al., 2004; Ohashi et al., 2001), and is presented with fever, pneumonia, bronchitis, leukopenia, severe diarrhea and sometimes encephalitis. CDV, the causative agent, is a member of the family *Paramyxoviridae*, in the genus *Morbillivirus* which includes MeV, RPV and PPRV. Recent dramatic epidemics of CDV in unexpected host species, such as lions (Roelke-Parker et al., 1996), other large felids (Morell, 1994), and seals (Kennedy et al., 2000; Mamaev et al., 1996; Osterhaus et al., 1988) raise additional concerns about the apparently expanding host range of CDV and the potential threat of CDV

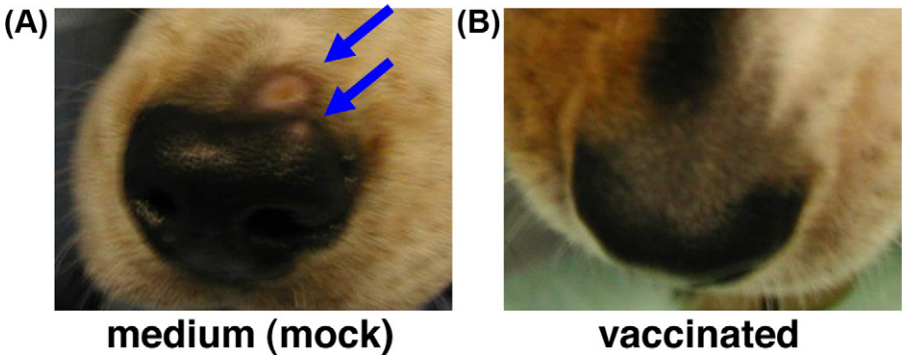

Fig. 2. Nodules on noses at 4th week post *L. major* challenge in mock-treated- (A) and vaccinated- (B) dogs. Infective-promastigotes of *L. major* were inoculated at 5×10^7 parasites per spot intradermally into noses at 56 days after the first recombinant CDV vaccination.

to wild carnivore populations in the many regions of the world where CDV occurs. In general, the introduction of live attenuated CDV vaccines in the 1950s and their extensive use have drastically reduced the incidence of canine distemper in dogs, because of its ability to elicit life-long immunity. However, canine distemper outbreaks, in which previously immunized dogs become infected, have recently been observed (Iwatsuki et al., 2000; Blixenkrone-Moller et al., 1993). This raises the question of whether the vaccines currently used efficiently protect against present-day circulating wild-types. Thus the available extremely safe, live attenuated CDV vaccine for recently prevalent strains is an ideal base for the development of multivalent vaccines.

Our previous study has demonstrated that the recently isolated CDV-Yanaka strain (Iwatsuki et al., 1997) is avirulent in dogs, and that dogs vaccinated with the Yanaka strain were completely protected against virulent CDV challenge (unpublished data). It may be expected that recombinant CDV inserted with a gene encoding neutralizing epitope against a pathogen can induce long-term immunity against both CDV and the pathogen.

Leishmaniasis is distributed in parts of 88 countries with 12 million people in tropical and subtropical regions. Leishmaniasis is caused by infection with parasite protozoa *Leishmania* and the parasites are naturally transmitted by blood-sucking sand flies among reservoir animals including rodents and dogs. Humans are accidental hosts transmitted from the animals. Epidemiologically, it was reported in Brazil that the elimination of canine leishmaniasis has been correlated with a decreased prevalence of disease in human (Dietze et al., 1997). These are, however, not effective vaccines for human or even dogs (Vanloubbeeck and Jones, 2004).

To develop the vaccine targeted to Leishmania, we constructed a recombinant CDV-Yanaka with a protective antigen of the *Leishmania major* strain of Leishmania and evaluated the efficacy of the recombinant CDV as a polyvalent vaccine against CDV and Leishmania infection. *L. major* inoculated in dogs intradermally usually proliferates at the site of inoculation and forms a nodule in the skin lesion. At the 4th week after Leishmania challenge, nodules had grown larger in the unvaccinated dogs and the tops of the nodules had been ulcerated (Fig. 2). In contrast, the sizes of the nodules were apparently much smaller in the dogs vaccinated with CDV expressing Leishmania antigen than in the controls. This result showed that the recombinant CDV vaccination induced significant protective immunity and effectively suppressed or delayed the proliferation of Leishmania at an early stage. This study showed powerful tools for the prevention of leishmaniasis epidemics by disruption of the infection root in dogs. The recombinant CDV based on the Yanaka strain is considered to be superior to conventional vaccine strains as a polyvalent vaccine vector. Using this system, we are also developing divalent vaccine against CDV and other dog viruses. Since domestic puppies are vaccinated with live attenuated CDV vaccine, the divalent vaccine against CDV and other dog viruses could be a good future candidate as an ideal vaccine for dogs.

6. Conclusions and perspective

Vaccination is the most important strategy to control virus diseases. Vaccines developed by classical methods are effective for many viruses. In fact, classical vaccines played key roles on the eradication of the two important viruses, smallpox virus and RPV. However, classical vaccines still have disadvantages and thus the development of ideal vaccines using new methods is still required. Since reverse genetics of mononegaviruses has been established, the technique has been applied for the development of vaccines against many of them. In particular, reverse genetics technologies allow us to design not only improved vaccines against the mononegaviruses themselves but also multivalent vaccines inducing humoral and cellular immune response against other pathogens. Once new vaccines are developed, the efficacy and safety of the novel vaccines are first evaluated and used for the control of animal virus infectious diseases. The studies of vaccine development for animal mononegavirus diseases give us valuable knowledge, not only for applied science but also basic issues such as molecular determinants of virus virulence, and finally contribute to our health. The development of new vaccines using reverse genetics techniques could be a promising way for ideal vaccines against the mononegaviruses.

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