Review article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

The present and future of rabies vaccine in animals

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An effective strategy for preventing rabies consists of controlling rabies in the host reservoir with vaccination. Rabies vaccine has proven to be the most effective weapon for coping with this fatal viral zoonotic disease of warm-blooded animals, including human. Natural rabies infection of an individual is always associated with exposure to rabid animals, and the duration of clinical signs can vary from days to months. The incubation period for the disease depends on the site of the bite, severity of injury, and the amount of infecting virus at the time of exposure. The mortality of untreated cases in humans is 100%. Over the last 100 years, various rabies vaccines have been developed and used to prevent or control rabies in animals, such as modified live vaccine, inactivated rabies vaccine, and oral modified live vaccine. These have proved safe and efficacious worldwide. New-generation rabies vaccines, including recombinant rabies virus-based vaccines, vectored vaccines, DNA-based vaccines, and plant vaccines, have been explored to overcome the limitations of conventional rabies vaccines. This article discusses current and next-generation rabies vaccines in animals.

Keywords: Rabies vaccines, Bait vaccine, Vectored vaccine, Plant vaccine

Introduction

Rabies is an invariably fatal viral disease in animals and humans [1]. In Korea, rabies is designated by law as a second-class infectious disease in veterinary medicine and third-class disease in terms of public health. According to the World Health Organization (WHO), at least 500,000 people are given post-exposure vaccinations every year, and rabies infections result in approximately 55,000 human deaths annually worldwide [2]. The mass vaccination of domestic dogs has been the most effective factor in reducing human rabies. Since the first case of rabies was reported in Korea in 1907, 16,129 rabies cases have been reported nationwide as of 2011 [3]. Since 1993, the raccoon dog (*Nyctereutes procyonoides koreensis*) has played an important role in transmitting rabies virus (RABV) in Korea. In most Asian countries, the major prophylactic measures for controlling rabies in reservoirs comprise culling free-ranging dogs using various methods and vaccination. A decrease in the vector population did not prevent the spread of rabies because the decrease was insufficient [4]. Wild animals are considered maintenance hosts of RABV. It is essential to break the chain of transmission to control rabies in wild animals. An efficient method of controlling wildlife rabies is distributing bait rabies vaccine to risk regions. The purpose of rabies vaccination in

domestic animals is mainly to minimize the important economic losses, as the vaccination of pet animals protects individual animals when they are exposed to wild RABV. The distribution of bait vaccine for wildlife is intended to interrupt the transmission from rabid animals to healthy ones, and ultimately to eliminate the wild RABV from those vectors. Vaccination coverage of approximately 70% of the vector population is estimated to be sufficient to block rabies transmission [5]. This review gives an overview of rabies vaccines, focusing on inactivated, attenuated live, and oral rabies vaccines and next-generation rabies vaccines, including recombinant vectored vaccines, DNA-based vaccines, and plant vaccines.

Current Rabies Vaccines

Modified live vaccine (MLV)

The minimum requirements for live rabies vaccine are as follows: non-pathogenic in animals, ability to propagate high virus titers in cells, ability to induce protective immunity after administration, and thermal and genetic stability. To ensure the safety of candidate vaccines, most researchers modified the virus by serial passage in various cells. This technique has led to the development of attenuated live vaccines for controlling the infectious disease. The attenuated live vaccine strain Evelyn-Rokitnicki-Abelseth (ERA) was introduced from Canada in 1974. In the late 1970s, the Korean Veterinary Authority examined the ERA strain as a substitute for the Flury low egg passage vaccine, which had adverse effects caused by tissue debris present in the vaccine [6]. The ERA strain propagated in primary porcine kidney cells was cloned three times using an end-dilution method. The experimental vaccine containing the cloned ERA strain was prepared and examined using biological methods. The titers of the experimental vaccine ranged from $10^{4.5}$ to $10^{5.4}$ LD_{50}/0.03 mL in mice. Guinea pigs immunized with the experimental vaccine were effectively protected after a challenge with virulent RABV (challenge virus standard [CVS] strain). Domestic animals inoculated with the vaccine via an intramuscular route for safety, including dogs, sheep, goats, and cats, did not show any clinical signs, and the vaccine stain was not recovered from their salivary glands or brains. However, about 50% of the dogs inoculated with the vaccine via an intracranial route developed severe clinical signs such as anorexia, fever, extreme tremor, paresis, and paralysis. The animals inoculated via an intramuscular route for immunogenicity had a solid immune response and high virus neutralization antibody (VNA) titers, ranging from 6 to 132, at 14 and 30 days post-inoculation. Based on the results of a project titled "Studies on the production of rabies live vaccine," the cloned ERA strain was transferred to five Korean animal vaccine companies and commercialized in the early 1980s. The cloned ERA vaccine produced in primary porcine kidney cells has been used to immunize dogs in Korea. The vaccine containing the ERA strain can be given to dogs, cattle, horses, sheep, and goat, but is not licensed for use in cats and wild carnivores due to the safety concerns [7].

The Flury strain, a chicken embryo-origin MLV vaccine, has been produced and given to animals in some Asian countries. The Street-Alabama-Dufferin (SAD) strain, which was developed using hamster kidney cells, has been used to produce MLV vaccines [8,9]. Although modified live rabies vaccine strains (ERA, Flury, and SAD) are safe and potent in dogs, the WHO stopped recommending MLV rabies vaccines for parenteral inoculation in animals in 2004. The management of MLV vs. inactivated vaccines is harder as the former is more sensitive to changes in temperature. In addition, accidents of self-inoculation with MLV rabies vaccine pose a high risk to the vaccinator. Consequently, the impact of MLV rabies vaccines is expected to decline in several countries, including Korea [2].

Inactivated rabies vaccine

Inactivated rabies vaccines require that high RABV titers be produced in tissues or cells. RABV can be grown in brain tissue, and nerve tissue vaccines (NTVs) consisting of inactivated rabies vaccine produced from RABV-infected brain tissue of sheep, goats, and mice were developed about 100 years ago and have been used in some Asian and African countries. In 2005, the WHO recommended that NTVs be replaced by cell-culture rabies vaccines because of adverse reactions in human and animals inoculated with NTVs, such as allergy. In Korea, two different inactivated rabies vaccines were produced in 1945 and 1959. The antigen for the first vaccine was obtained from RABV-infected rabbit brain and spinal cord tissue and inactivated with 0.8% phenol or 0.1% merthiolate at 37°C for 3 and 5 days. The second antigen was prepared from calf brain and spinal cord tissue infected with wild RABV circulating in Korea. Unfortunately, these inactivated vaccines had a short-acting immune response and many adverse reactions due to tissue debris present in the vaccines. Due to their inability to induce proper immunity, these NTVs are no longer produced in Korea [10,11]. Up to 2011, the Korean Veterinary Authority

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has permitted the use of seven inactivated rabies vaccines for immunizing pets and other animal species, including dogs, cats, cattle, goats, and fox, and these inactivated vaccines have been used to prevent animal rabies. Once, the inactivated rabies vaccine was considered for use as an oral immunogen in wild carnivores but, the vaccine needed large amounts of inactivated protein to induced rabies-specific VNA and revealed partial protection against lethal rabies infection [12].

Worldwide, the following RABV strains have been used for inactivated rabies vaccine: CVS 11, Pittman-Moore–NIL2, RC-HL derived from the Nishigahara strain, and Pasteur virus [13]. These inactivated rabies vaccine strains are grown in culture systems with baby hamster kidney, hamster lung, guinea pig brain, chick embryo, murine neuroblastoma, or Vero cells and are inactivated with beta propiolactone (BPL), ultraviolet light, acetylethylamine, or binary ethylenimine (BEI). The inactivating agent used most widely is BPL, but it is expensive and unstable at 37°C. Phenol and formaldehyde are no longer recommended for virus inactivation because they can alter the structure of the antigenic sites. In comparison, BEI has the advantages of good stability, low cost, and ease of preparation, and it is less hazardous to handle. After inactivating the antigen, adjuvants are added to maximize the immune response to the antigen. Commonly used adjuvants are aluminum hydroxide, aluminum phosphate, and saponin [14]. The potency and safety of the inactivated rabies vaccines via intramuscular route are quite good.

Oral MLV

Modified live rabies vaccine for oral immunization started with the SAD Berne strain, which was developed from the ERA strain in 1969 [15]. Oral rabies vaccine has been produced for free-ranging animals and wildlife species that serve as vectors. However, the SAD Berne strain had a degree of residual pathogenicity in wild animals and induced a partial immune response in young foxes [16]. The SAD strain was replaced by the Street-Alabama-Gif (SAG) 1 and SAG2 strains. The SAG2 strain was developed after two successive mutations of the arginine 333 codon using anti-glycoprotein monoclonal antibodies, and it did not produce any clinical signs in mice inoculated intracranially [17]. Raccoon dogs and dogs ingesting ten doses of SAG2 bait remained healthy, and all vaccinated animals had high rabies neutralizing antibody levels for 180 days after inoculation [18]. The SAG2 vaccine is the only oral vaccine registered with the European Medicine Agency. In Europe, other commercial MLVs for oral immunization are Lysvulpen, SAD B19, and SAD P5/88.

Vaccinia-recombinant glycoprotein (V-RG) consists of a Vaccinia virus (Copenhagen strain) containing the rabies glycoprotein gene from the ERA strain. The Copenhagen strain is mutated from wild Vaccinia virus and is further attenuated due to the replacement of thymidine kinase by rabies glycoprotein cDNA, conferring rabies immunity [19]. The V-RG bait vaccine comprises a plastic sachet filled with recombinant Vaccinia virus containing the rabies glycoprotein gene and paraffin wax, which serves to hold both the bait attracting wild animals and the plastic sachet. The bait contains 150 mg of tetracycline to mark the teeth of bait consumers. V-RG bait vaccines including SAG2 or SAD B19 contain a minimal titer corresponding to at least 10 times the 100% protective dose. V-RG bait vaccine containing $10^{8.0}$ $TCID_{50}$/dose is recommended for the oral vaccination of raccoons and foxes against disease caused by wild RABV. After assessing factors such as the population density of raccoon dogs, competing species, distribution methods, public awareness, and safety procedures, the V-RG vaccine is being spread in rabies-risk regions to control sylvatic rabies. A large amount of V-RG vaccine has been distributed for wild raccoon dogs in Korea since 2000, and it is thought that this has helped to reduce animal rabies. Unfortunately, the V-RG bait vaccine is not effective in skunks and dogs.

Next-Generation Rabies Vaccines

Recombinant rabies virus-vectored vaccines

Many scientists have been searching for new vaccine strains that can induce protective antibodies and effectively protect animals from rabies without raising any safety issues. Most live attenuated vaccine strains can cause rabies in wild animals, although the incidence is low. Reverse genetics can provide more stable variants of rabies vaccine strains and generate homologous virus vectors expressing a variety of foreign genes [20]. The most important modification to reduce the pathogenicity of RABV is to replace the codon for arginine at position 333 in the glycoprotein gene sequence with another amino acid codon, such as for glutamic acid, glycine, isoleucine, leucine, methionine, or cysteine. Changing the arginine codon converts the pathogenic virus into a non-pathogenic phenotype [21]. One way to enhance the immunogenicity of live rabies vaccine is to use two identical glycoprotein genes to induce high antibody levels. Indeed, the overexpressed rabies glycoproteins were identified in neuro-

blastoma cells infected with the recombinant virus, and they produced substantially higher antibody titers. When the pro-apoptotic protein cytochrome C was expressed in the recombinant rabies virus, mice inoculated with recombinant virus containing cytochrome C in the pseudogene had a lower mortality rate than animals inoculated with attenuated virus [22]. To eliminate rabies, combined vaccination and control of the stray animal population are needed in most countries. Although trap-neuter-return (TNR) programs for dogs and cats are pursued actively in several countries, the actual effect of TNR programs is unknown. Therefore, a recombinant rabies vaccine with the coding sequence of gonadotropin-releasing hormone (GnRH) inserted has been suggested, and mice immunized with such a vaccine produced GnRH-specific antibodies, which protected all immunized mice upon challenge with virulent RABV. The GnRH-carrying recombinant virus reached 1×10^9 FFU/mL in cells and has potential for rabies and animal population control [23].

Recombinant poxvirus-vectored vaccines

In the mid-1980s, a MLV using the SAD B19 strain was used in bait to immunize wild foxes in Europe. However, the vaccine strain raised serious concerns about safety in certain wild animals. A recombinant Vaccinia-rabies vaccine has been used successfully for oral bait vaccination in several species. Nonetheless, the lack of efficacy in important rabies reservoirs such as skunks and safety concerns over the use of live virus vaccine as a vector have impaired the expansion of V-RG bait to new target species and new areas [24]. Poxviruses have merit as vaccine carriers. First, poxviruses have large DNA genomes ranging from 139,000 bp for open reading frame virus to 379,000 bp for avipoxvirus, which allows the insertion of up to 30,000 bp of foreign DNA. Second, poxviruses are potent inducers of both arms of the immune response. Manipulation of the poxvirus is relatively easy in the laboratory, resulting in the development of new recombinant vaccine strains. Another advantage of using recombinant poxviruses as a vaccine is that poxviruses are thermally stable at environmental temperatures. Although recombinant poxvirus expressing rabies glycoprotein induced protective immunity in foxes, it was less effective in other animals. As a result, the canarypox virus expression vector (ALVAC) was developed as a highly host-restricted virus with interrupted replication in mammalian cells. Rabies recombinant ALVAC vector expressing a RABV glycoprotein gene has been proven safe and efficacious [25]. A rabies recombinant ALVAC vaccine including the ERA glycoprotein gene has been licensed and recommended for use in cats in the USA and Canada [26].

Recombinant adenovirus-vectored vaccines

Both the E1 locus of adenovirus required to initiate viral replication and the E3 gene locus that downregulates the expression of major histocompatibility complex antigens were deleted to develop human adenovirus serotype 2 and 5 (Ad-Hu2 and 5) vectors. Recombinant adenovirus expressing the rabies glycoprotein induced an immune response to RABV, even in the presence of maternally transferred immunity to RABV [27], and induced high titers of anti-RABV VNA in dogs previously immunized with conventional rabies vaccine [28]. Recombinant chimpanzee adenovirus serotype 68 based vaccine (AdC68) expressing the rabies glycoprotein administered via intranasal or oral routes induced rabies-specific VNA in newborn mice [29]. These results indicate that a recombinant adenovirus-based vaccine can be efficacious in young, pre-exposed individuals when delivered orally. The advantages of recombinant adenovirus are as follows: they elicit superb B cell and $CD8^+$ T cell responses, achieve good responses with a single moderate subcutaneous or intramuscular dose in an experimental mouse model, and provide full, long-lasting protection against virulent rabies challenge [29]. Additionally, E1-deleted and replication-competent recombinant canine type 2 adenovirus expressing the rabies glycoprotein was constructed, and it prevented both canine adenovirus type 2 infection and rabies in dogs [30]. Recently, a live adenovirus recombinant oral bait vaccine consisting of AdHu5 vector containing the ERA glycoprotein gene, plastic blister, wax, and fat-based matrix containing tetracycline hydrochloride was developed and distributed in Canada under an experimental permit for controlling rabies in skunks and raccoons. The recombinant oral bait vaccine was effective in these wild animals [31].

DNA-based rabies vaccines

One approach for developing new-generation rabies vaccines is to use a DNA-based or plasmid vaccine encoding the rabies glycoprotein gene. Advanced recombinant DNA technology has made it possible to generate a variety of DNA vaccines against infectious agents. DNA-based vaccines developed to induce a broad-spectrum immune response when delivered to the host have several advantages, such as action in the presence of maternal antibodies, strong stability, mass production, and cost effectiveness. DNA-based vaccine should

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provide efficient ways to induce a cell-mediated cytolytic $CD8^+$ T cell response, $CD4^+$ T cells, and VNA [32]. Numerous studies have demonstrated the relative effectiveness of DNA-based rabies vaccines at inducing RABV-specific VNA based on various parameters, including the plasmid dosage and inoculation route. However, DNA-based rabies vaccines were not successful at protecting non-human primates following pre- and post-exposure vaccination [33]. More studies of DNA-based rabies vaccines are needed to obtain a protective vaccine for animals. For example, alternative delivery systems with greater transfection efficiency and the use of new kinds of cytokines need to be examined.

Oral rabies vaccines derived from plants

Plants have provided new systems for the large-scale production of recombinant proteins at low cost, simplifying the production process. A variety of genetically engineered vaccines using tobacco mosaic virus and tomato bushy stunt virus have been developed for expressing foreign antigens in plants [34]. Rabies antigen expressed in plant tissue was immunogenic and protective in mice immunized intramuscularly and orally. One of the more advanced approaches for expressing foreign antigens in plants is to construct transgenic plants. To produce a plant-derived rabies antigen, the native signal peptide within the rabies glycoprotein gene was replaced with that of the pathogenesis-related protein of *Nicotiana tabacum*. Codon optimization of the rabies glycoprotein gene is necessary for providing plant-preferred codons. Plant-derived antigens induced strong mucosal and humoral immune responses after administration via either an oral or an intramuscular route in mice [35]. These antigens have several advantages, including post-translational modifications, stability for storage, and ease of delivery. The rabies glycoprotein has been expressed in several plants, including tobacco, tomato, spinach, carrot, and maize. These antigens obtained from transgenic plants conferred protection to mice against challenge [36,37]. Although plant-derived antigens have many advantages, some problems should be solved before oral rabies vaccines originating from plants are given to domestic animals. The most important are the need to improve the expression in raw transgenic plants and to reduce the substantial variability in the level of protein expression among different lines and subsequent generations. Additionally, it is necessary to shorten the time needed to obtain antigens from different plants [35].

Conclusion

The main reservoir of rabies is the dog, which is responsible for almost 99% of fatal rabies cases in humans. Although the rabies vaccines available for animals are safe, immunogenic, and efficacious when administered as recommended, vaccines that do not contain proper immunogenic antigens should be identified and discarded. The immunization of animals has saved millions of humans from this fatal disease, and the World Organization for Animal Health (OIE) introduced the concept of regional vaccine banks for dog vaccination in 2011. The major reason that alternative rabies vaccines are being studied is that inexpensive vaccines are urgently needed in developing countries to replace the rabies vaccines made from nerve tissues, which have undesirable side effects and raise animal welfare issues. Therefore, new rabies vaccines, that are inexpensive and safe and, if possible, that require one or two administrations by an oral route must be developed. Additionally, it is necessary to develop novel adjuvants such as immune stimulating complex targeting the immune system after both parenteral and mucosal administration.

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