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Development of Animal Recombinant DNA Vaccine and Its Efficacy in Foxes

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Rabies is prevalent in most parts of the world. An extensive reservoir of the disease is present in the population of wild animals. The fox in particular is a major vector of the disease in North America and Europe. Although attempts to control rabies by vaccination of wild carnivores with attenuated rabies virus have met with some success, this approach remains controversial. The potential of a recombinant vaccinia virus expressing the rabies glycoprotein for the protection of foxes against rabies was examined. Both the parental (wild-type) and recombinant viruses were found to be innocuous to foxes. Inoculation of live recombinant virus via the subcutaneous, intradermal, or oral routes uniformly elicited high titers of neutralizing antibodies, and animals that received 10^8 plaque-forming units of the recombinant virus in bait resisted severe challenge with live rabies virus.

Rabies is a viral disease that affects all warm-blooded animals and is widespread in most countries of the world [1]. The consequences of this disease for public health are substantial in South America, Africa, and Asia, where veterinary and sanitary structures are often lacking. However, rabies is still a subject of great concern, even in Europe and North America, because of its propagation among the wild animals that constitute a considerable reservoir of the virus: foxes in Europe, and foxes, skunks, and raccoons in North America [2]. Dogs represent the major vector of rabies in Africa and Asia, whereas in Central and South America, both dogs and bats have been implicated, with the latter being responsible for large economic losses in livestock.

The disease is transmitted through the bite of an infected animal, whose saliva contains large quantities of virus. The rabid animal undergoes behavioral changes during the final stages of the disease, and aggressive behavior facilitates transmission. As rabies is nearly always fatal in these animals, immune populations do not exist.

Prophylactic measures (other than the vaccination of domestic animals) attempt to eliminate or reduce the population of the principal reservoir through, for example, poisoning or gassing [3]. These measures have proved to be moderately successful for stray dogs, though they are less effective for wild animals. In Europe the application of these measures to foxes has reduced the number of rabies outbreaks but has not significantly contained the disease. The present upsurge of European rabies, which first appeared in Poland in 1935 in foxes and badgers [4], has since spread throughout western Europe at some 20–50 miles/year, presenting enzootic waves as in North America [5]. Vaccination of wild animals (particularly foxes) is now thought to present an alternative, and perhaps more effective, countermeasure.

Oral administration of vaccine is the only appropriate route for the vaccination of large numbers of wild carnivores and was first attempted by Baer et al. in North America [6] and by Mayr et al. in Europe [7]. In small-scale trials, live attenuated rabies virus introduced into various baits has been successfully used to vaccinate foxes [8]. Both in Switzerland [9] and in West Germany [10], field trials have been successful in eradicating rabies cases from localized areas. However, virus stability is poor and attenuated viruses remain pathogenic to some rodents and revert to virulence at a significant frequency. It is of note that inactivated rabies virus is ineffective when administered orally. For these reasons we have sought to develop a safe and effective recombinant vaccine as an alternative to attenuated rabies virus.

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Materials and Methods

Purification of vaccinia virus. Cultivated BHK-

21 (baby hamster kidney) cells were infected with vaccinia virus at 0.1 pfu/cell. After 72 hours, supernatant and infected cells were pooled and the virus was purified as described [11].

Inoculation with vaccinia virus. Suspensions of vaccinia virus (0.1 mL) were inoculated intradermally with a needle into the depilated skin of the back. For the sc route, the inoculum was delivered in a volume of 1 mL. Direct oral administration was performed by application of 1 mL into the mouth by syringe. The mucosae of four animals were scarified before administration of the virus. The bait for oral administration consisted of 1.8 mL of virus preparation sealed into "Plastipak capsules" (a gift from Dr. Wandeler), inserted into a chicken head, and distributed to test animals.

Rabies virus. The foxes were challenged with wild-type rabies virus. This preparation (batch GS6) consists of 20% of homogenized salivary glands of rabid fox as described elsewhere [12]. The animals received 17,000 fox LD_{50} units (im) at day 28 after immunization.

Neutralizing antibodies. Titration was performed in mice according to the technique recommended by the World Health Organization (WHO) [13]. Antibody titers are given as the antilog of the final neutralizing dilution (FND). Conversion to international units (IU) can be performed with use of the formula IU = 59/antilog (3.5 − log FND).

Results

Vaccinia virus (VV), a large (180 kilobases), double-stranded DNA orthopox virus, has been used extensively to control and eradicate smallpox in humans [14]. The relative innocuity of VV has stimulated its development as a live vector for viral antigens, and derivatives expressing surface proteins from influenza, hepatitis B, herpes simplex, and other organisms have been used to confer protection against the respective diseases [15–17].

Rabies virus (RV) is a rhabdovirus, an enveloped, negative-sense single-stranded RNA virus related to vesicular stomatitis virus. The glycoprotein is presented at the exterior surface of the virion, where it aggregates to form surface projections or spikes. Glycoprotein is the only protein capable of inducing or reacting with virus-neutralizing antibody (VNA) and appears to be the only viral protein capable of eliciting protection [18, 19].

We recently developed a recombinant VV (VVTGgRAB) bearing the rabies glycoprotein coding sequence and expressing the rabies surface antigen [20]. Protection of mice and rabbits against rabies after inoculation with the live recombinant virus has already been described [20–22]. VV is itself an enveloped virus, and the recombinant VVTGgRAB presenting rabies glycoprotein at its surface elicited protection against rabies even after chemical inactivation [21]. Encouraged by these results, we extended our investigations to foxes [22]. First, VV (Copenhagen and Wyeth strains) was tested for innocuity to foxes. European foxes (*Vulpes vulpes*), captured and raised in captivity as previously described [23], were inoculated with live VV by various routes. No generalized reaction to the virus was observed in any of the foxes, but mild cutaneous inflammation, which regressed spontaneously within 8 days, was observed at the site of inoculation. Identical results were obtained with the recombinant virus VVTGgRAB [24]. No impairment of digestive or alimentary function was observed when the live recombinant virus was applied orally.

We then examined the capacity of the recombinant VVTGgRAB to elicit neutralizing antibodies against

Table 1. The elicitation of virus-neutralizing antibodies (VNA) and survival of challenge with rabies virus of animals given wild-type vaccinia virus (Copenhagen strain), conventional inactivated rabies vaccine, or recombinant vaccinia virus (VVTGgRAB) by various routes.

Vaccine, route of inoculation	Dose (pfu)	Mean titer of VNA at day 28	No. animals surviving challenge/total
VVTGgRAB			
Intradermal	10^8	2.82	2/2
Subcutaneous	10^8	NA	2/2
Oral (with scarification)	10^8	2.4	4/4
Oral	10^8	2.57	8/8
Oral	10^7	ND	4/4
Oral	10^6	0.4	6/8
Oral	10^4	0.34	1/4
Oral (presentation in baits)	10^8	1.8	4/5*
Wild-type vaccinia virus, intradermal	10^8	0	0/2
Inactivated rabies vaccine, subcutaneous	. . .	1.49	2/2

NOTE. NA = not applicable (one animal had a titer of 3.03, and the other presented no detectable VNA); ND = not determined.

* Two animals were observed to have ingested only part of the vaccine.

rabies virus. Animals were inoculated by various routes (intradermal, sc, or oral), and blood was taken at days 0, 8, 14, and 28. Sera were analyzed for the presence of VNA. All animals (with one exception, an animal inoculated by the sc route) presented high titers of antibodies when vaccinated with 10^8 pfu of virus (table 1). Scarification of the oral mucosa did not significantly improve the titer of neutralizing antibody of animals that received the vaccine orally.

Direct protection testing was then performed on these animals. Animals were challenged by injection of rabies virus 28 days after vaccination. All 20 animals that had received 10^8 pfu of VVTGgRAB either orally or parenterally resisted challenge, including the one animal exhibiting undetectable levels of rabies-neutralizing antibodies. Control animals injected sc with a commercial inactivated and adjuvanted vaccine similarly resisted challenge, although virus-neutralizing antibodies were present at a reduced level (table 1). With such a vaccine, oral administration has previously been shown to be ineffective [25].

In animals receiving $<10^8$ pfu of VVTGgRAB a clear dose-related response was observed, with one of four, six of eight, and four of four animals surviving challenge after oral administration of 10^4, 10^6, and 10^7 pfu of VVTGgRAB, respectively (figure 1).

The oral route is the only appropriate route for the vaccination of wild animals. Accordingly, the vaccine must be presented in a form appropriate for ingestion. We thus prepared capsules containing 10^8 pfu of VVTGgRAB. These were inserted into chicken heads (one capsule per head, into the beak) and distributed to the test animals (one per fox). These animals also produced high titers of rabies-neutralizing antibodies and resisted challenge with rabies virus (table 1).

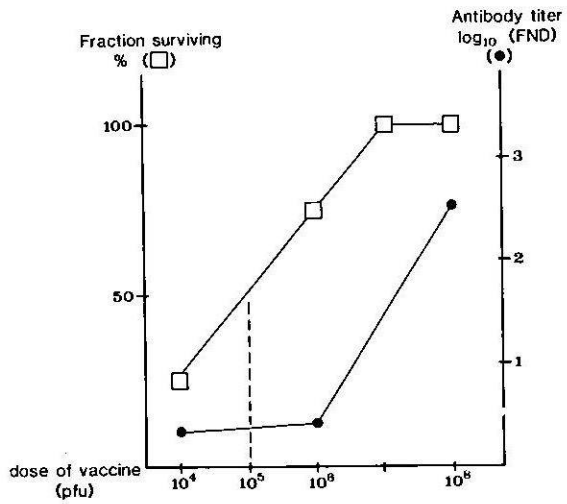

Figure 1. Relation between antibody titer expressed as the antilog of the final neutralizing dilution [FND]), resistance to challenge, and dose of orally administered vaccine.

Horizontal transmission of the recombinant virus could have an important impact on the wild population. To address this question we examined whether vaccinated animals could transmit the virus to nontreated control animals. Four animals were vaccinated by direct application of 10^8 pfu of VVTGgRAB into the mouth. Each was subsequently housed in the same cage as an untreated animal of the opposite sex (two animals per cage). Sera were analyzed 28 days after vaccination. All treated animals presented high titers of neutralizing antibodies. Surprisingly, one of the control animals (female) also possessed neutralizing activity in her serum (table 2). It is of note that this female shared a cage with a male that exhibited particularly aggressive behavior and was bitten by the male immediately after administration of the vaccine. This circumstance is likely to be rare in the wild.

Four animals were challenged 1 year after oral vaccination, at which time two animals had no detectable neutralizing antibodies and the two others showed a titer of 1.3 and 1.7. Nevertheless, all animals survived challenge, results that attest to the long duration of immunity conferred by the recombinant vaccinia virus.

Discussion

As VV has been extensively used to control and eradicate smallpox in humans, procedures for its production, stabilization, and distribution are fully established [26]. We have shown that VV and its recombinant VVTGgRAB bearing the rabies surface

Table 2. Levels of virus-neutralizing antibody (VNA) and survival in vaccinated (V) animals and the opposite-sex cagemates (C).

Sex (V/C)	VNA titers at day 28 (V/C)	Survival (V/C)
M/F	1.34/0.97	+/+
F/M	3.23/0	+/−
M/F	3.95/0	+/−
F/M	2.36/0	+/−

antigen are innocuous to foxes. The administration of 10^8 pfu of the recombinant virus elicits the production of titers of rabies-neutralizing antibodies that are equal or superior to those obtained with conventional vaccine; sc, intradermal, or oral administration uniformly confers complete protection to severe challenge infection with live rabies virus. Similar experiments with other animal vectors of rabies (notably the skunk and the raccoon [27]) are in progress. Importantly, presentation to foxes of the live recombinant virus, encapsulated and introduced into chicken heads, also enables them to resist a severe challenge infection, and the duration of immunity can exceed 1 year.

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