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Oral vaccination of wildlife against rabies: Differences among host species in vaccine uptake efficiency

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ABSTRACT

Oral vaccination using attenuated and recombinant rabies vaccines has been proven a powerful tool to combat rabies in wildlife. However, clear differences have been observed in vaccine titers needed to induce a protective immune response against rabies after oral vaccination in different reservoir species. The mechanisms contributing to the observed resistance against oral rabies vaccination in some species are not completely understood. Hence, the immunogenicity of the vaccine virus strain, SPBN GASGAS, was investigated in a species considered to be susceptible to oral rabies vaccination (red fox) and a species refractory to this route of administration (striped skunk). Additionally, the dissemination of the vaccine virus in the oral cavity was analyzed for these two species. It was shown that the palatine tonsils play a critical role in vaccine virus uptake. Main differences could be observed in palatine tonsil infection between both species, revealing a locally restricted dissemination of infected cells in foxes. The absence of virus infected cells in palatine tonsils of skunks suggests a less efficient uptake of or infection by vaccine virus which may lead to a reduced response to oral vaccination. Understanding the mechanisms of oral resistance to rabies virus vaccine absorption and primary replication may lead to the development of novel strategies to enhance vaccine efficacy in problematic species like the striped skunk.

1. Introduction

Oral vaccination against rabies using modified live rabies virus vaccines has been highly successful in different reservoir species. The first animal targeted was the red fox (*Vulpes vulpes*) followed by the raccoon dog (*Nyctereutes procyonoides*) [1,2]. Subsequently, the concept of oral rabies baiting was investigated for other animal species, like raccoons (*Procyon lotor*) [3,4], coyotes (*Canis latrans*) [5–7], gray foxes (*Urocyon cineroargenteus*), striped skunks (*Mephitis mephitis*) [8], small Indian mongooses (*Herpestes auropunctatus*) [9,10], and domestic dogs (*Canis lupus domesticus*) [11–13].

It became evident that not all animal species were equally susceptible for vaccination by the oral route; some species like the striped skunk seem to be extremely refractory to oral rabies vaccination, irrespective of the construct used even when high virus titres were administered [4,14–20]. Oral virus vaccines for veterinary use, e.g. rabies [21–28] and classical swine fever (CFS) virus [29–32] or recombinant poxvirus against lethal plaque [33–35] have been developed and used under field conditions in Europe and North America. However, it remains largely unknown how and where the vaccine viruses are transported across the epithelium in the oral cavity.

Several studies have revealed that the vaccine viruses are predominantly present in the tonsils and less pronounced in the oral mucosal epithelium after oral administration as shown for both attenuated and recombinant oral rabies virus vaccines [36–41] as well as for attenuated CFS vaccine virus constructs [42–44]. Lymphoreticular tissues of the pharynx assumed to be involved in efficient oral immunization, also called Waldeyer's tonsillar ring, variably comprise *Tonsilla* (*T.*) *lingualis, T. palatina, T. veli palatini, T. paraepiglottica, T. pharyngea,* and *T. tubaria* in a species-dependent pattern. Furthermore, tonsils can be subdivided based

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on their histoarchitecture into those containing epithelial crypts or those covered by a smooth epithelium as well as those bulging above the mucosal surface versus those covered within a mucosal fossa [45–48]. Because of this complexity, the exact locations of entry of the attenuated rabies virus vaccines within these tissues and the pharmacokinetics have not been investigated in detail. Hence, even after 35 years of oral vaccination of wildlife against rabies there is limited knowledge on how oral vaccination in target species actually works. Considering the difficulties in inducing a protective immune response against rabies in reservoir species other than foxes and raccoon dogs, prompted us to elucidate the mechanisms behind. Therefore, the objectives of this study were to (i) examine the immunogenicity of an oral rabies virus vaccine construct in two species that show extreme differences in susceptibility to oral vaccination; the red fox (*Vulpes vulpes*) and striped skunk (*Mephitis mephitis*), after direct oral administration, and (ii) investigate the dissemination of the vaccine virus construct in the oral cavity of these two species by establishing the hypothesis that differences in virus presence and replication in lymphoreticular tissues, in particular the palatine tonsils between the two species could contribute to the vaccine uptake efficiency.

2. Material and methods

2.1. Vaccine virus

The SPBN GASGAS vaccine virus was constructed as previously described [49]. The parental vaccine of SPBN GASGAS is the SAD B19 oral rabies vaccine virus. The SPBN construct lacks the pseudogene (ψ) and the G-gene is flanked by a Sma/XmaI and PacI-restriction enzyme cleavage site. Furthermore, the SPBN-virus contains a linker to express a (foreign) gene with two restriction enzyme sites (BsiWhI/NheI) for subsequent introduction of additional genetic information. The construct contains two glycoprotein genes with the following two modifications (site-directed mutagenesis); a change from asparagine to serine and one from arginine to glutamic acid at position 194 and 333 of the glycoprotein, respectively [49,50]. The antigen, SPBN GASGAS was produced according to the protocol given by Vos et al. [51]. Antigen with titers $>10^{8.0}$ focus forming units (FFU)/ml was concentrated via tangential flow filtration using ultrafiltration flat sheet cassettes with a Molecular Weight Cut Off (MWCO) of 300 kDa.

2.2. Animals

A total of 14 and 4 foxes and 24 and 4 striped skunks were used for the vaccination and challenge and dissemination studies, respectively. All foxes and striped skunks used in this study were obtained from different commercial sources in Poland and the United States, respectively. Foxes were kept in individual cages during the entire observation period. Meanwhile, skunks were partially kept in small groups, if applicable, until challenge infection. All animals were sedated (mixture of 1.1 mg/kg Xylazin and 2 mg/kg Ketamin) during vaccine administration and challenge infection.

2.3. Ethics statement

All *in vivo* work was performed at IDT Biologika GmbH, according to European guidelines on animal welfare and care pursuant to the Federation of European Laboratory Animal Science Associations (FELASA). Study protocols were evaluated and approved by the responsible authorities (Landesverwaltungsamt Sachsen – Anhalt, Referat Verbraucherschutz, Veterinärangelegenheiten) in the federal state of Saxony-Anhalt, Germany; AZ42502-3-670 IDT (red fox – immunogenicity study), AZ 42505-3-669 IDT (striped skunk and red fox – dissemination study), AZ 42505-3-582 IDT (striped skunk – immunogenicity study).

2.4. Vaccination and challenge studies

To determine the minimum effective dose of SPBN GASGAS in striped skunks, different doses were administered by direct oral administration; $10^{7.3}$ FFU/ml (n = 3), $10^{8.0}$ FFU/ml (n = 5) and $10^{9.2}$ FFU/ml (n = 6). To mimic natural conditions, 1.5 ml (foxes) and 1 ml (skunks) of virus suspension was directly administered into the oral cavity but not targeted directly to the tonsils. Also, two skunks received the highest dose intra muscularly (i.m.). For foxes, a similar minimum effective dose as determined with the oral rabies vaccine strain SAD B19 was applied [52]. Hence, here only a single low dose of SPBN GASGAS ($10^{6.5}$ FFU/ml) by direct oral instillation was tested in 6 animals.

The vaccinated animals were inoculated with a challenge virus ($10^{5.1}$ MICLD50) between days 42 and 98 post vaccination together with control animals (N = 8). The challenge virus used was isolated from the salivary glands of a red fox (2nd passage) infected experimentally with an isolate from a naturally infected coyote (CVS/USA/TX Coyote/295/R/061893 – Centers for Disease Control and Prevention, Atlanta, USA).

2.5. Dissemination studies

For dissemination studies in each case four foxes and skunks were kept individually in groups of 2 animals each in an isolation unit within the Animal House at IDT. All animals received 1.0 ml SPBN GASGAS ($10^{7.5}$ FFU/ml) by direct oral instillation. Two animals of each species were euthanized at day 3 and 5 post vaccine administration. During necropsy different tissues (tonsils [*Tonsilla palatina, T. pharyngealis*], Supplementary Fig. 1), tongue, regional lymph nodes [*Lymphonodi (Lnn.) parotidei, Lnn. retropharyngei, Lnn. mandibulares*] and mucous membrane of the upper and lower oral cavity) were collected and examined for the presence of rabies virus vaccine construct by RT-PCR and RTCIT.

Saliva samples were collected prior to vaccine administration (S0) and 1 h (S1), 2 h (S2), 3 h (S3), 24 h (S4), 48 h (S5), 72 h (S6), if applicable, 120 h (S7) post vaccine administration. Saliva swabs were collected by swabbing of the oral cavity for 1–1.5 min. Subsequently, the cotton tips were placed in 2 ml MEM medium supplemented with antibiotics (gentamycin [50 mg/l] and amphotericin [2.5 mg/l]) and stored at −80 °C until further investigation using RT-PCR and RTCIT.

2.6. Diagnostic assays

Different regions of the brain, i.e. hippocampus, medulla oblongata and cerebellum, of animals challenged with street RABV were tested for the presence of viral antigen using the direct Immuno-Fluorescence Test (dIFT) [53]. For detection of SPBN GASGAS specific viral RNA in lymphopharyngeal tissues as well as in saliva swabs of foxes and skunks obtained during dissemination studies, RNA was extracted with TRIzol (Invitrogen)/TRIfast® (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to manufacturers' recommendations, followed by real-time RT-PCR (qRT-PCR) essentially as described [54]. The presence of viable rabies virus particles in qRT-PCR positive tissues was confirmed with the rabies tissue culture infection test (RTCIT) [55,56] using the mouse neuroblastoma cell line NA 42/13 (Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, No. 411]. Three consecutive passages in cell culture were conducted to confirm a negative result.

Blood samples were taken prior to vaccination and challenge infection from foxes and skunks from veins (*V. cephalica antebrachii*