

Immunogenicity of an E1-deleted recombinant human adenovirus against rabies by different routes of administration

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The immunogenic properties of an E1-deleted, human adenovirus type 5 (Ad5) vaccine virus with activity against rabies were examined in mice, foxes and dogs using different routes of administration. NMRI mice received $10^{5.8}$, $10^{5.3}$, $10^{4.3}$, $10^{3.3}$ and $10^{2.3}$ TCID₅₀ by peroral or intramuscular (i.m.) administration. Furthermore, six mice received $10^{5.8}$ TCID₅₀ intracerebrally (i.c.). The construct elicited marked seroconversion in mice after oral administration. Immunoreactivity in mice was even more pronounced i.m. and i.c. After direct oral administration ($10^{8.0}$ TCID₅₀) in foxes, six of eight animals developed rabies virus-neutralizing antibodies (VNA). All foxes immunized by direct injection ($10^{7.7}$ TCID₅₀) in the membrane of the jejunum were shown to seroconvert. Pre-existing immunity against canine adenovirus did not hinder the development of rabies VNA after oral application of the construct ($10^{8.0}$ TCID₅₀). Fox cubs (24–29 days old) born from rabies-immune vixens were shown to develop very high levels of rabies VNA after i.m. administration ($10^{8.0}$ TCID₅₀), indicating that the immunogenicity of the construct could surpass maternally transferred immunity. In dogs, the construct ($10^{8.0}$ TCID₅₀) induced a very strong immune response after i.m. administration. However, no immune response was detectable in dogs after direct oral administration ($10^{8.3}$ TCID₅₀) or after endoscopic deposition in the smaller intestine ($10^{8.0}$ TCID₅₀). Hence, it must be concluded that the construct is not suitable for oral vaccination of dogs against rabies.

Introduction

Oral vaccination of foxes (*Vulpes vulpes*) against rabies has been developed as a preferred method to control, and eventually to eradicate, vulpine rabies (Stöhr & Meslin, 1996; Müller & Schlüter, 1998). This approach has also been suggested for vaccination of dogs (*Canis familiaris*), particularly those that cannot be vaccinated by the parenteral route. However, due to the close relationship between dogs and humans, more stringent safety conditions are required for oral vaccination of dogs against rabies (Wandeler, 2000). Presently, no commercially available oral rabies vaccine is completely without risk. In order to address this issue, new vaccine

candidates have been under investigation. One such family of candidates are recombinant human adenovirus type 5 (Ad5) vectors that express the highly immunogenic rabies glycoprotein G (RG) (Yarosh *et al.*, 1996; Xiang *et al.*, 1996; Wang *et al.*, 1997). One of these prototype vaccines has already been shown to induce protective immunity in animals upon challenge with rabies virus (Prevec *et al.*, 1990; Charlton *et al.*, 1992). However, the potential for *in vivo* replication of this E3-inserted recombinant adenovirus is an undesirable attribute from a safety perspective. In response to this shortcoming, a replication-deficient, E1-deleted, human Ad5 vector has been developed that expresses RG. This particular candidate vaccine, designated human Ad5 HCMV-intron-ERA, was evaluated for its ability to induce an immune response by different routes of administration in mice (*Mus musculus*), foxes and dogs. It was also determined whether pre-existing canine adenovirus

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(CAV)-specific immunity inhibited the development of rabies antibody generation, as expressed by the adenovirus construct. Finally, the ability of the construct to surpass maternally transferred immunity against rabies in young animals was examined. The variability of construct immunogenicity in terms of target species and route of administration was determined.

Methods

■ **Vaccine virus.** The vaccine virus was produced by a homologous recombination approach using commercially available reagents (Microbix Biosystems Inc., Toronto, Canada). Briefly, cDNA for the ERA (Evelyn-Rochitniki-Abelseth) strain RG was inserted into the *Xba*I site of plasmid pCA13 just downstream of the forward-orientated HCMV immediate-early promoter and ahead of the SV40 polyadenylation signal. This generated plasmid pCA13-RG.

A specific intron sequence, present in plasmid pMLPsp1a, as described by Berkner & Sharp (1985), is known to increase protein expression in multiple systems. It was cloned into pCA13-RG, downstream of the promoter region and before the RG gene. The intron sequence in pMLPsp1a contains the 5' splice-donor site from the Ad2 major late promoter (MLP) fused to the 3' acceptor site from an immunoglobulin light-chain gene. The primary sequence of the intron is as follows: 5' GAATTC AAG GCGTCTAACC AGTCACAGTC GCAAGGTA-GG CTGAGCACCG TGGCGGGCGG CAGCGGGTG GCGGTC-GGGGT TGTCTTGGC GGAGGTGCTG CTGATGATGTAATT-AAAGTA GGCGGTCTTG AGACGGCGGA TGGTCGAGGT GA-GGTGTGGC AGGCTTGAGA TCTGGCCATA CACTTGAGTG ACAATGACAT CCACTTTGCC TTTCTCTCCA CAGGTGTCCA CTCCAGGTC CAACCGGATC C 3'.

The intron was extracted from pMLPsp1a by PCR under standard conditions. The PCR product was restricted with *Eco*RI and ligated into similarly cut pCA13-RG to generate pCA13(int)-RG. Nucleotide orientation was confirmed by sequencing.

In order to produce recombinant human Ad5 bearing the HCMV-intron-ERA cassette, pCA13(int)-RG was co-transfected with plasmid pBHGE3, which contains the right-hand end of Ad5 (rightward of E1), into 293 cells. 293 cells are a human kidney cell line that has been transformed with left-end regions of Ad5, including E1. Homologous recombination between pBHGE3 and pCA13(int)-RG results in the production of a viral genome that will replicate in 293 cells and produce progeny virus. 293 cells provide helper E1 function. Individual virus isolates are selected by plaque purification on immobilized 293 cell monolayers.

HCMV-intron-ERA virus was propagated routinely in suspension culture using 293N3S as the host cell line. For cultivation, the suspension cells were placed in spinner flasks containing growth medium (MEM Eagle, Joklik modification, Sigma) with 10% foetal calf serum. Virus was harvested 3 days after infection of cells. HCMV-intron-ERA virus was enumerated by TCID₅₀ on 293N3S cells.

■ **Animals.** All animals were without detectable levels of rabies virus-neutralizing antibodies (VNA) prior to vaccination, with the exception of the fox cubs born from rabies-immune vixens. Foxes were marked individually with electronic identifiers and juvenile specific-pathogen-free dogs (beagles) were identified individually by a tattoo (ear). All animal tests were conducted at the Experimental Animal Facility of Impfstoffwerk Dessau-Tornau GmbH (IDT). Animal experimentation was performed according to the German Animal Welfare Act of 25 May 1998. The experimental design of the tests, as required, was approved by the appropriate German authorities.

■ Rapid fluorescence focus inhibition test (RFFIT) assays

Rabies virus. Prior to testing, sera were heat-inactivated at 56 °C for 30 min and then centrifuged at 926 *g* for 5 min. Serum samples were evaluated for rabies VNA by the RFFIT as described by Smith *et al.* (1973), with the modifications described by Cox & Schneider (1976). The rabies VNA titres were converted to international units (IU). The international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK), adjusted to 0.5 IU/ml, served as a positive control for these experiments. A level of 0.5 IU/ml is as an arbitrarily defined threshold indicative of protection against rabies infection (Anonymous, 1978) and is used here as the threshold for positivity.

CAV2. A simple inhibition test (Mayr *et al.*, 1977) was used to test sera for VNA against CAV2. Firstly, the serum samples were inactivated at 56 °C for 30 min. A virus suspension with a known titre was added to each serum dilution and the mixture was incubated at 37 °C for 60 min. This mixture was then inoculated on monolayers of receptive Madin-Darby canine kidney (MDCK) cells for virus replication. Four days later, CAV2-neutralizing antibody titres were evaluated according to Spearman-Kärber, based on the CPE. A titre of 1:4 was considered negative.

■ **ELISA.** The objective of these tests was to detect both general adenovirus-reactive serum antibodies and those with particular specificity for human Ad5. For detection of the former, highly purified human Ad5 hexon protein was used. This protein was isolated by DEAE chromatography and crystallization (Döhner & Hudemann, 1972). The hexon protein is the most abundant adenovirus protein (Zabner *et al.*, 1997), and antibodies directed against this protein could also be the result of earlier CAV infection. The Ad5-specific ELISA was conducted with highly purified human Ad5 fibre antigen. Fibre antigen was isolated by anion exchange and immunoaffinity chromatography.

The ELISA was performed under standard conditions using hexon or fibre antigens, at 1 µg/ml, for coating microtitre plates. Peroxidase-labelled protein A was used as the indicator reagent. Human antibodies were used as the positive control. Dog, fox and human antibodies react similarly with protein A (Yamamoto *et al.*, 1985). The absorbances of 'cut-off values' at 450/620 nm for specific antibodies were in the range of 0.5. Adenoviruses are ubiquitous; hence, it is to be expected that animals have circulating antibodies against adenoviruses. Serum samples from mice were diluted 1:10 to allow the detection of low reactivity between IgG and protein A.

■ **Statistics.** Student's paired *t*-test was used to detect possible intraspecific significance in absorbance differences between different blood samples (Zöfel, 1988).

Results

Study 1. Mice [peroral (p.o.), intramuscular (i.m.) and intracerebral (i.c.)]

Three groups of six mice were given 0.02 ml human Ad5 HCMV-intron-ERA (10^{5.8} TCID₅₀) p.o., i.m. or i.c. Animals that received the construct i.c. were anaesthetized by intraperitoneal (i.p.) injection with a 1:10 dilution of ketamine hydrochloride in saline. The animals vaccinated p.o. drank the construct voluntarily from a syringe. During a subsequent trial in mice, four groups of 10 animals each received 0.02 ml of the construct using different concentrations (10^{5.3}, 10^{4.3}, 10^{3.3} and 10^{2.3} TCID₅₀). Five animals of each group received the construct orally, the other five by the parenteral route (i.m.).

Table 1. Rabies VNA titres of mice inoculated with human Ad5 HCMV-intron-ERA by different routes of administration

Five or six animals were inoculated with the doses shown. Titres for individual animals are given in IU/ml, as determined by RFFIT. (-), No rabies VNA detectable (< 0.5 IU/ml); ND, not done.

Route of administration	Dosage (TCID ₅₀)				
	10 ^{5.8}	10 ^{5.3}	10 ^{4.3}	10 ^{3.3}	10 ^{2.3}
i.c.	269.2, 226.3, 226.3, 269.3, 320.1, 269.2	ND	ND	ND	ND
i.m.	67.3, 56.6, 56.6, 80.0, 190.3, 80.0	20.0, 20.0, 40.0, (-), 80.0	33.6, 5.0, 10.0, 8.4, Died*	(-), 56.5, 4.2, (-), 0.9	(-), (-), (-), (-), (-)
p.o.	10.0, 7.1, 10.0, (-), 67.3, 5.9	47.6, 7.1, (-), 67.3, (-)	(-), 10.0, (-), (-), (-)	(-), (-), (-), (-), (-)	(-), (-), (-), (-), (-)

* Animal died 2 days post-vaccination; cause of death unknown, but unrelated to rabies.

Table 2. Rabies VNA titres of foxes inoculated orally with 10^{8.33} TCID₅₀ human Ad5 HCMV-intron-ERA

Titres are given in IU/ml, as determined by RFFIT. (-), No rabies VNA detectable (< 0.5 IU/ml); ND, not done.

Fox	Sample				
	B0	B1	B2	B3	B4
3038	(-)	33.71	11.04	3.27	3.00
9970	(-)	3.82	3.86	0.72	0.58
1974	(-)	13.02	13.54	ND	ND
4834	(-)	(-)	(-)	(-)	(-)
5579	(-)	6.03	4.30	6.25	6.49
7443	(-)	(-)	(-)	ND	ND
1954	(-)	25.25	17.46	ND	ND
0005	(-)	1.29	6.16	ND	ND

This time, the construct was applied orally with a perfusion needle in the posterior part of the oral cavity of the animals. For this purpose, the mice were anaesthetized in an ether jar prior to administration of the construct. All mice were bled by retro-orbital puncture and subsequently euthanized 55 or 60 days post-immunization. Blood samples were examined for serum antibody titres against rabies by using RFFIT and antibodies against the human Ad5 fibre and hexon proteins (ELISA).

The immune responses against rabies of mice inoculated with human Ad5 HCMV-intron-ERA vaccine are shown in Table 1. The highest levels of rabies VNA were observed in the mice inoculated i.c. The immune response in mice receiving the human Ad5 HCMV-intron-ERA construct orally was inferior to mice immunized i.m. with the same dose. It was also observed that the seroconversion rate was dose-dependent. With the exception of three (of six) mice vaccinated i.c., none of the animals showed high levels of antibodies against the fibre and hexon protein (data not shown).

Study 2. Foxes (p.o.)

Eight juvenile foxes received 10^{8.33} TCID₅₀ human Ad5 HCMV-intron-ERA by direct oral instillation. Blood samples were collected 0 (B0), 21 (B1), 35 (B2), 70 (B3) and 95 (B4) days post-vaccination. All blood samples were examined for the presence of rabies virus- and CAV-neutralizing antibodies by RFFIT. Detection of antibodies against the human Ad5 fibre and hexon proteins was also performed by ELISA.

Six of the eight foxes seroconverted (threshold 0.5 IU/ml). The geometric mean titres (GMT) of the sera of immunized foxes were 2.75 (B1), 2.13 (B2), 1.33 (B3) and 1.48 (B4) IU/ml (Table 2). None of the animals had detectable levels of CAV-neutralizing antibodies at any time during the study. The mean absorbances against the fibre protein of serum samples B0 and

Table 3. CAV2 VNA titres of foxes pre-immunized with $10^{7.8}$ TCID₅₀ CAV2

Titres were determined by RFFIT. (–), No CAV2 VNA detectable ($\leq 1:4$).

Fox	Sample		
	B0	B1	B2
6708	$\geq 1:64$	$\geq 1:512$	1:1722
6146	(–)	1:211	1:256
6662	1:32	$\geq 1:512$	1:2435
0804	(–)	$\geq 1:512$	1:1024

B2 were 0.296 ± 0.071 and 0.415 ± 0.112 , respectively. For the hexon protein, the following mean absorbances for B0 and B2 were obtained: 0.779 ± 0.361 and 1.439 ± 0.724 . The levels of antibodies against the hexon and fibre proteins of human Ad5 as indicated by absorbances were both significantly higher for B2 than for B0 (paired *t*-test, $T_{\text{hexon}} = -3.16$, $T_{\text{fibre}} = 2.43$, d.f. = 7, $P < 0.05$).

Study 3. Foxes (intrajejunal)

Five cubs, 8 weeks old and born from rabies-naïve vixens, received by injection 0.5 ml human Ad5 HCMV–intron–ERA ($10^{7.7}$ TCID₅₀) in the membrane of the jejunum following surgical exposure (laparotomy). For this purpose, the animals were sedated with a ketamine–xylazine (10%/2%) solution. Blood samples were collected 0 (B0), 21 (B1) and 49 (B2) days post-vaccination and examined for the presence of rabies VNA (RFFIT).

All five foxes had detectable levels of rabies VNA. Sera GMT for the vaccinated foxes were 32.2 (B1) and 11.9 (B2) IU/ml.

Study 4. Foxes (p.o.) vaccinated against CAV2

The effect of pre-existing CAV-specific immunity on the immune response against rabies, induced by the construct, was investigated by administering $10^{7.8}$ TCID₅₀ CAV2 antigen (produced at IDT) i.m. to four adult foxes after a blood sample (B0) had been taken. Twenty-nine days later, the foxes were bled (B1) and subsequently received $10^{8.0}$ TCID₅₀ human Ad5 HCMV–intron–ERA vaccine by direct oral instillation. Blood samples were taken 28 (B2) and 55 (B3) days after administration of the construct. The blood samples were examined for the presence of rabies and CAV VNA by RFFIT.

All four foxes developed detectable levels of CAV2 VNA after vaccination with CAV2 antigen and prior to administration of the construct (B0 and B1) (Table 3). All animals pre-immunized with CAV2 developed rabies VNA upon oral immunization with the construct. The GMT were 3.61 and

3.27 IU/ml at 28 (B2) and 55 (B3) days post-administration. These results indicate that antibodies to CAV2 did not inhibit the immune response to rabies after vaccination with the construct.

Study 5. Fox cubs (i.m.)

In order to investigate whether maternally transferred immunity to rabies virus would impair the immune response to RG upon vaccination with the human Ad5 HCMV–intron–ERA construct, 13 cubs born from four rabies-immune vixens were given the construct i.m. The vixens had been vaccinated orally with SAD (Street Alabama Dufferin) B19 just before mating. SAD B19 is a live-modified rabies virus vaccine, used extensively for oral vaccination of wildlife (Schneider & Cox, 1983; Vos *et al.*, 2000). The cubs, aged between 23 and 29 days (mean 24 ± 3 days), were bled (B0) and subsequently injected i.m. with $10^{8.0}$ TCID₅₀ human Ad5 HCMV–intron–ERA. Blood samples were collected 14 (B1), 21 (B2), 28 (B3) and 47 (B4) days post-vaccination and examined for the presence of rabies VNA (RFFIT).

The rabies VNA titres of the four vixens at the time of vaccination of the cubs were 1.81, 11.02, 11.88 and 5.12 IU/ml. All cubs born from these rabies-immune vixens developed rabies VNA upon immunization with the construct. The GMT of the sera of the cubs were 0.38 (B0), 31.08 (B1), 108.73 (B2) and 155.26 (B3) IU/ml.

Study 6. Dogs [p.o., i.m. and gastrointestinal (g.i.)]

Six dogs received $10^{8.3}$ TCID₅₀ human Ad5 HCMV–intron–ERA by direct oral instillation. Blood samples were collected 0 (B0), 21 (B1), 35 (B2), and 118 (B3) days post-vaccination. After the first oral instillation, none of the animals seroconverted. Hence, vaccination was repeated using a different lot of vaccine virus. Vaccination was repeated with $10^{8.0}$ TCID₅₀ four animals by the oral route and two by parenteral (i.m.) administration. Dogs were bled 28 (B4) and 55 (B5) days after this second vaccination attempt.

The dogs that received the secondary vaccination by the oral route did not develop detectable levels of rabies VNA. Only the i.m.-inoculated animals seroconverted, with high levels of VNA [dog 3160, 99.00 (B4) and 160.89 (B5) IU/ml; dog 3161, 18.80 (B4) and 41.67 (B5) IU/ml]. Dogs 3160 and 3161 also showed a clear immune response against the hexon and fibre antigens after vaccination by the parenteral route. The other four orally vaccinated dogs showed no detectable immune response against these proteins (Fig. 1).

The four dogs that remained seronegative after secondary oral administration received $10^{8.0}$ TCID₅₀ human Ad5 HCMV–intron–ERA by direct intestinal instillation (endoscopic deposition) in the duodenum. The animals were anaesthetized with a ketamine–xylazine (10%/2%) solution; pre-medication was with atropine–diazepam. Blood samples were taken 0 (B6), 21 (B7) and 42 (B8) days after g.i. vaccination.

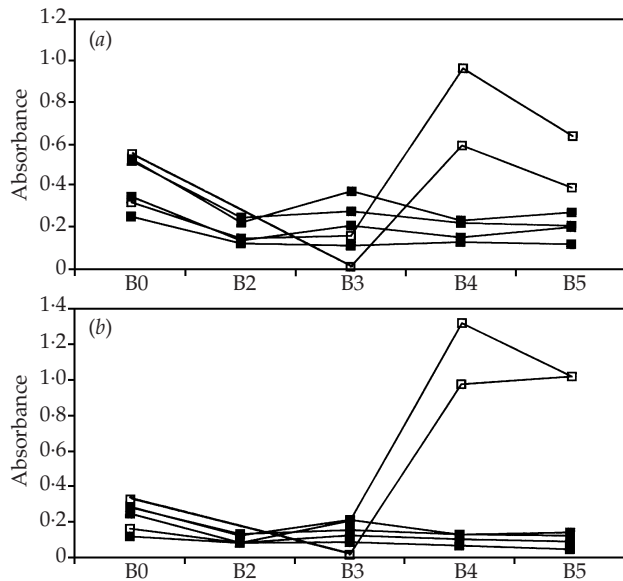


Fig. 1. Absorbances of serum samples (B0, B2–B5) from dogs inoculated with $10^{8.33}$ and $10^{8.0}$ TCID₅₀ human Ad5 HCMV–intron–ERA during primary and secondary vaccination against the human Ad5 hexon (a) and fibre (b) proteins, as determined by ELISA. Samples were included from dogs that received the construct p.o. during both primary and secondary vaccinations (■) and from dogs that received the construct respectively p.o. and i.m. during primary and secondary vaccinations (□).

Blood samples were examined by RFFIT for the presence of rabies (B0–B8) and CAV (B0–B5) VNA. Antibody levels against the human Ad5 fibre and hexon proteins were also determined by ELISA (B0–B5).

None of the four dogs developed detectable levels of rabies VNA after endoscopic deposition of the HCMV–intron–ERA construct in the duodenum.

Discussion

An effective yet safe rabies vaccine is needed for the oral immunization of a variety of wild animals, as well as the oral vaccination of (stray) dogs. Human adenoviruses have been studied intensively as virus vectors for vaccine delivery and gene therapy applications (for reviews, see Klonjowski *et al.*, 1999; Russell, 2000), and have been shown to induce protective responses after oral administration (Fooks *et al.*, 1998). The adenovirus genome has been well characterized, and recombinant versions lend themselves to the production of vaccine vectors. This application is supported further by the wide host range of human adenoviruses and the stability of the virion, which can readily be produced in large quantities *in vitro* by routine culture techniques.

The strong rabies immune response induced by replication-defective adenovirus recombinants that express RG and the absence of many of the safety risks inherent to multiplying agents led us to test the E1-deleted human Ad5 HCMV–intron–ERA as a candidate for oral vaccination of carnivores against rabies. This replication-defective human Ad5 can only

be propagated in cells that express the E1-region proteins exogenously and thereby complement the deletion in the viral genome. This virus is unable to replicate in cells that do not complement the deleted E1 region, but it is still able to infect these cells and to induce the synthesis of very high levels of foreign protein (RG). Hence, the potential spread of recombinant, replication-competent adenovirus (RCA) by horizontal transmission from vaccinated to unvaccinated animals, as mentioned by Hammond *et al.* (2000), is not a concern with human Ad5 HCMV–intron–ERA. However, the E1 deletion may not block the expression of virus genes completely following *in vivo* administration (Von Seggern & Nemerow, 1999). The absence of RCA after serial passaging in 293 and 293N3S cells attests further to the safety of this virus vector. The reason for the absence of RCA in the HCMV–intron–ERA virus preparation is not understood. All primary sequence elements required for homologous recombination have been confirmed as present by sequencing analysis. However, the absence of RCA is clearly linked to the presence of the intron moiety, as other recombinant rabies vaccines containing the HCMV promoter without the intron regularly produce RCA at frequencies in the order of 10^{-6} – 10^{-5} after five serial passages (M. Moore and K. Hughes, unpublished).

In contrast to studies with similar replication-defective human Ad5 constructs (Prevec *et al.*, 1990; Xiang & Ertl, 1999), mice developed serum antibody to rabies upon oral administration of human Ad5 HCMV–intron–ERA. However, the immune response of mice vaccinated orally was inferior to that of animals vaccinated i.m. or i.c., and was clearly dose-dependent. In rabies-naïve foxes, six of eight animals did seroconvert after direct oral administration. However, the immune response was lower than that in foxes vaccinated orally with the live-modified virus vaccines SAD B19 and SAD P5/88, showing a sub-optimal immunization process (Neubert *et al.*, 2001; Schuster *et al.*, 2001).

As exposure to CAV is relatively common in wild and domestic canids (Garcelon *et al.*, 1992; Laurenson *et al.*, 1997; Gese *et al.*, 1997; Truyen *et al.*, 1998; Cypher *et al.*, 1998; Spencer *et al.*, 1999), it is to be expected that many animals have developed VNA against CAV. However, pre-immunity to CAV did not impair the ability of the human Ad5 HCMV–intron–ERA construct to elicit an immune response to rabies. We have shown previously that cubs whelped by rabies-immune vixens developed an impaired immune response upon oral immunization with the highly efficacious vaccine virus SAD B19. This inhibition of the immune response outlasted the presence of detectable levels of maternal antibodies (Müller *et al.*, 2001). The rabies-specific VNA response to the human Ad5 HCMV–intron–ERA construct, after i.m. administration in cubs whelped by rabies-immune vixens, was significantly higher than the response elicited to the oral rabies virus vaccine SAD B19 in cubs born from naïve vixens (Müller *et al.*, 2001). This demonstrates that maternal immunity to rabies does not effect the immune response to RG,

as presented by the human Ad5 recombinant vaccine. Therefore, the construct is highly suitable for neonatal immunization of dogs. Many human rabies cases are caused by bites from rabid puppies that are too young to be eligible for vaccination.

Dogs that received the human Ad5 HCMV–intron–ERA construct by direct oral administration did not develop detectable levels of VNA against rabies. Furthermore, the construct did not elicit an immune response after g.i. immunization by endoscopic deposition. This supports the results of Papp *et al.* (1997), who could not observe an immune response after g.i. immunization of cotton rats (*Sigmodon hispidus*) with another replication-defective human Ad5 expressing glycoprotein D of bovine herpesvirus-1. The relative inefficiency of mucosal delivery for adenovirus infection is striking, since adenoviruses naturally infect mucosal tissues and human epidemiological data suggest frequent adenovirus infection. Indeed, human Ad5 is a common pathogen that infects most humans at an early age (Xiang *et al.*, 1996; Wang *et al.*, 1997; Walters *et al.*, 1999). A likely explanation is that wild-type Ad5 infection requires a large initial inoculum (Walters *et al.*, 1999). This explains the relative inefficiency of replication-defective adenovirus as virus vectors for vaccine delivery when compared with similar recombinant RCA.

Entry of human adenoviruses into cells is a stepwise process. After binding to the fibre receptor (CAR; coxsackievirus/adenovirus receptor), interaction of penton base with integrins facilitates internalization via receptor-mediated endocytosis (Dmitriev *et al.*, 2000). It has been suggested that the adenovirus fibre receptors are polarized to the basolateral plasma membrane of the epithelium, and the lack of fibre binding at the apical surface appears to be the rate-limiting step that explains insufficient adenovirus-mediated gene transfer (Walters *et al.*, 1999). According to Zabner *et al.* (1997), intentional injury of the epithelium increased the efficiency of gene transfer. However, in our study, the lumen epithelia in the intestines of three dogs were damaged (gastritis) and the animals still did not respond to the vaccine delivery. This suggests that the cells of the intestinal smooth muscle lack the appropriate cell-surface receptors for adenovirus attachment (Dmitriev *et al.*, 2000; Schmidt *et al.*, 2000). Surprisingly, direct injection of the construct in the membrane of the jejunum in foxes elicited a strong immune response. It seems that the inefficiency of recombinant replication-defective adenovirus vaccines in eliciting an immune response after oral delivery is partially species-dependent (Monteil *et al.*, 2000). Although human Ad5 HCMV–intron–ERA was able to induce an immune response in mice after oral delivery, dogs could not be vaccinated against rabies by this route and foxes developed only relatively low VNA titres. It must, therefore, be concluded that, despite high levels of RG expression *in vitro* and significant VNA production by i.m. administration, the human Ad5 HCMV–intron–ERA construct is not a suitable candidate for oral vaccination of the tested carnivores against rabies.

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