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A single immunization with a recombinant canine adenovirus expressing the rabies virus G protein confers protective immunity against rabies in mice

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Abstract

Rabies vaccines based on live attenuated rabies viruses or recombinant pox viruses expressing the rabies virus (RV) glycoprotein (G) hold the greatest promise of safety and efficacy, particularly for oral immunization of wildlife. However, while these vaccines induce protective immunity in foxes, they are less effective in other animals, and safety concerns have been raised for some of these vaccines. Because canine adenovirus 2 (CAV2) is licensed for use as a live vaccine for dogs and has an excellent efficacy and safety record, we used this virus as an expression vector for the RVG. The recombinant CAV2-RV G produces virus titers similar to those produced by wild-type CAV2, indicating that the RVG gene does not affect virus replication. Comparison of RVG expressed by CAV2-RV G with that of vaccinia-RV G recombinant virus (V-RG) revealed similar amounts of RV G on the cell surface. A single intramuscular or intranasal immunization of mice with CAV2-RVG induced protective immunity in a dose-dependent manner, with no clinical signs or discomfort from the virus infection regardless of the route of administration or the amount of virus.

Keywords: Rabies virus; Rabies virus glycoprotein (RVG); Rabies recombinant virus vaccines; Canine adenovirus 2 (CAV2); Canine adenovirus 2-rabies virus glycoprotein recombinant (CAV2-RV G) vaccine; Oral immunization

Introduction

Rabies is the 10th most common lethal infectious disease causing approximately 60,000 annual human deaths worldwide (Martinez, 2000). In most developing countries, stray dogs represent the major reservoir of rabies virus, whereas wildlife is important in many developed countries (Meslin et al., 1994). Parenteral immunization of dogs with killed rabies virus (RV) vaccines, which is the most effective method to control dog rabies, is very difficult to perform with stray dogs and unrealistic for wildlife. An alternative to parenteral immunization with killed RV vaccine is oral immunization with live recombinant viruses expressing the RV G or modified-live rabies viruses (CDC, 2000; Winkler and Bogel, 1992). Indeed, vaccinations using vaccinia-RV G recombinant virus (V-RG) or modified-live rabies viruses, such as the Ellen–Rockitniki–Abelseth strain (ERA), the Street–Alabama–Dufferin strain (SAD B19), and the SAD Avirulent Gif strains (SAG-1 and SAG-2), have resulted in almost complete elimination of vulpine rabies in Western Europe (Aubert et al., 1994). However, while these vaccines induce protective immunity in foxes (Aubert et al., 1994) and raccoons (Rupprecht et al., 1986), neither V-RG nor first-generation SAD- and ERA-based modified live rabies vaccines work well in dogs or skunks (Rupprecht et al., 1990; Tolson et al., 1987, 1988). Moreover, the V-RG vaccine may be associated with environmental or public health limitations (Rupprecht et al., 2001). Clearly, there is a need for additional safe and potent rabies vaccines for wild animals and stray dogs.

Because adenoviruses replicate on mucosal surfaces, they represent appropriate vectors that can be administered both orally and intranasally (Fischer et al., 2002). Replication-competent and replication-deficient human recombinant adenoviruses expressing RVG have been developed (Prevec et al., 1990) and tested in different animal models. Skunks and foxes vaccinated per os with a replication-competent recombinant

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human adenovirus type 5 were protected against challenge infection (Charlton et al., 1992), indicating the potential of recombinant adenoviruses as oral vaccines for wildlife. Live modified canine adenovirus type 2 (CAV2), which is already used worldwide for the routine vaccination of dogs against both CAV1 and CAV2 and has an excellent safety record (Fischer et al., 2002; Appel et al., 1975), represents an almost ideal vaccine vector for immunization of carnivores against rabies. Although this vaccine is usually administered subcutaneously, it is also effective when given orally (Baer et al., 1989). Whereas CAV2 did not spread after parenteral inoculation (Appel et al., 1975), oral–nasal administration resulted in replication of this virus in surface epithelium, which seems to be essential for oral immunization (Appel et al., 1975). Skunks developed only mild respiratory signs and recovered completely after 1 week following oral administration of CAV2 (Sumner et al., 1988).

We have constructed a recombinant CAV2 that expresses the RVG under the control of the human cytomegalovirus (CMV) early promoter/enhancer and assessed the safety and protective activity of the CAV2-RV G vaccine in mice. Here we show that CAV2-RV G-infected MDCK cells express RV G protein on the cell surface at levels similar to those on MDCK cells infected with V-RG, which is currently one of the gold standards for oral rabies vaccines. CAV2-RV G administered intramuscularly (i.m.) or intranasally (i.n.) into mice resulted in the induction of RV-neutralizing antibody and protection against a lethal RV challenge infection in a dose-dependent manner.

Results

Construction and selection of a recombinant CAV2-RV G virus

Neither CAV2 nor CAV2-RV G induces a distinct cytopathic effect in tissue culture, obviating the selection and purification of recombinant CAV2-RV G virus by standard plaque selection. Thus, as proof-of-concept, we first constructed CAV2 containing green fluorescent protein as a reporter gene (CAV2-GFP). Foci of CAV2-GFP-infected cells were easily identified by GFP expression and picked up. After cloning the CAV2-GFP construct, we transfected MDCK cells with CAV2-GFP DNA together with the DNA containing the RVG expression cassette (Fig. 1). Recombinant CAV2-RV G virus was cloned, selected, and characterized using Southern blotting and PCR analysis. PCR analysis revealed the presence of both the GFP and the RV G genes in virus preparations recovered after the 1st and 12th cloning cycles. Only the RV G gene was detectable in the virus population that was recovered after the 26th cloning cycle (Fig. 2). Furthermore, MDCK cells infected with the virus recovered from the first cloning cycle showed massive GFP fluorescence, but only a few fluorescent foci were detected after incubation with anti-RV G-specific rabbit IgG followed by incubation with Alexa Fluor 610-labeled anti-rabbit IgG. By contrast, strong RV G immunostaining but no GFP fluorescence was seen in cells infected with virus recovered from the 26th cloning cycle (data not shown).

Growth of CAV2-RV G in vitro

A potential problem associated with any recombinant adenovirus carrying foreign genes is that the introduction of an extra gene into the viral genome affects the replication efficiency of the virus. However, comparison of the time courses of virus production in MDCK cells infected with CAV2 or CAV2-RV G revealed no differences (Fig. 3), indicating that the insertion of a RV G gene does not affect virus replication.

Expression of the RV G in CAV2-RV G-infected MDCK cells

Fluorescence microscopy was used to determine whether the RVG is correctly expressed by the CAV2 vector. Fluorescence microscopy revealed CAV2 antigen in both CAV2- and CAV2-RVG-infected MDCK cells (Figs. 4C, F) but RV G-specific immunostaining only in CAV2-RVG-infected MDCK cells (Fig. 4B) and not in CAV2-infected MDCK cells (Fig. 4E). Whereas CAV2-antigen staining was localized mainly within the cell (Figs. 4C, F), RV G was predominantly detected on the cell surface membrane (Fig. 4B), indicating that this protein is correctly processed and most likely retains its antigenic structure.

Immunoprecipitation analysis comparing the levels of RV G expressed by the CAV2 vector with the levels expressed by V-RG indicated similar expression levels in CAV2-RV G-infected MDCK cells and in V-RG-infected MDCK cells (Fig. 5). Flow cytometry to measure the relative cell surface expression of RVG on MDCK cells at 48 h p.i. also revealed comparable surface expression in CAV2-RVG-infected cells and in V-RG-infected cells (Fig. 6). Thus, based on the RVG expression levels, the CAV2-RVG vaccine is expected to have a potency comparable to that of the V-RG vaccine.

Immunogenicity and pathogenicity of CAV2-RV G in mice

To assess the immunogenicity of CAV2-RVG, mice were inoculated once i.m. or i.n. with serial dilutions of CAV2-RV G. While mice immunized i.m. with 10^8 infectious particles produced a VNA GMT of 47.5 IU, the VNA titer in mice immunized with 10^7 and 10^6 FFU decreased in a dose-dependent manner resulting in VNA titers of 6.7 and 0.3 IU, respectively (Table 1). Similarly, dose-dependent differences in VNA titers were also seen after i.n. immunization (Table 1). Note that mice immunized with10^7 FFU i.n. developed a VNA GMT of 12.8, indicating that i.n. administration of the virus is as effective as i.m. administration.

None of the mice that received CAV2-RVG by the i.m. or the i.n. route developed clinical signs or showed loss of body weight within the 4-week observation period, regardless of the amount of virus administered.

The VNA data were paralleled by the results of a virus challenge experiment that revealed 90% protection of mice vaccinated i.m. with 10^8 or 10^7 FFU of CAV2-RV G and 70%

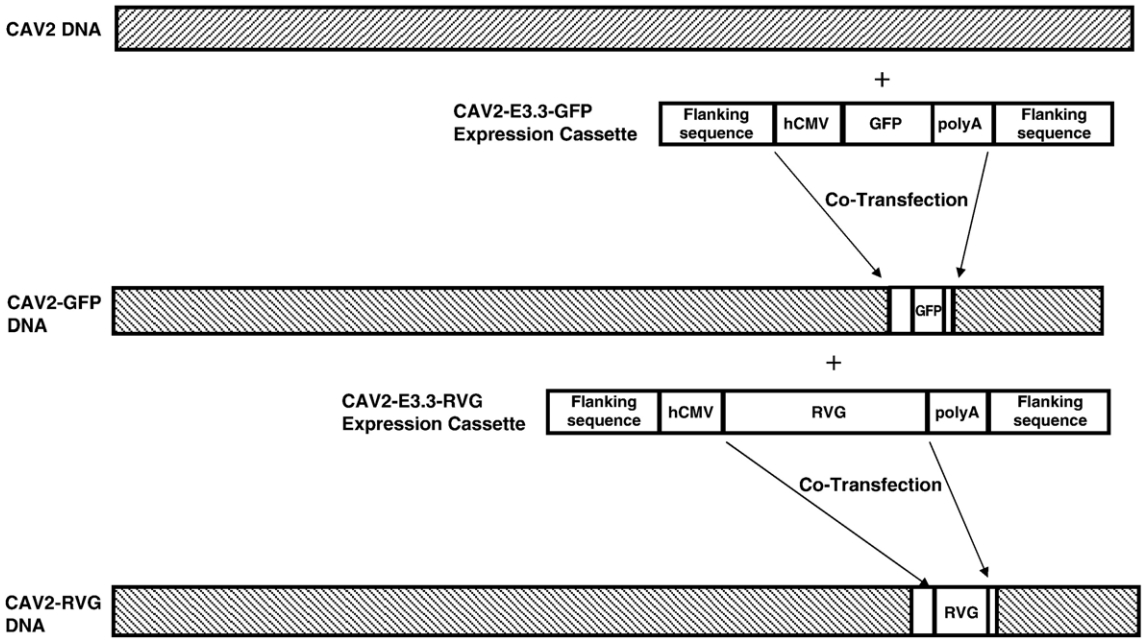

Fig. 1. Schematic representation of the construction of a full-length cDNA clone of CAV2-RV G. A GFP expression cassette was constructed consisting of 3′ and 5′ flanking sequences of the E3 region of CAV2 DNA, sequences encoding the CMV promoter and GFP, and a poly-A sequence. This cassette was transfected together with CAV2 DNA into MDCK cells, resulting in recombinant CAV2-GFP. The GFP gene of CAV2-GFP was then replaced with the RV G gene by cotransfection of MDCK cells with CAV2-GFP DNA and a RV G expression cassette, resulting in CAV2-RV G.

protection after i.n. immunization with 10^7 FFU of CAV2-RV G, respectively, and challenged i.m. with 10 LD50 of CVS-N2c (Table 1).

Discussion

RV vaccines based on recombinant viruses hold the promise of safety and efficacy (Dietzschold et al., 2003). In the past two decades, several viruses have been investigated for their ability to express RV proteins and to induce protective immunity (reviewed in Dietzschold et al., 2003). Besides attenuated RVs, the V-RG is probably the best studied and most extensively used vaccine. However, while V-RG given to a large variety of warm-blooded animals by different routes has proven to be an extremely effective RV vaccine (Brochier et al., 1991; Wiktor et al., 1984b, Tolson et al., 1987), it does not work well in skunks or dogs (Rupprecht et al., 1990; Tolson et al., 1987, 1988). Furthermore, a recent incident raised concerns regarding the safety of V-RG (Rupprecht et al., 2001). These observations underline the need for safer live RV vaccines. Because the Manhattan strain of CAV2, which is used as an effective modified live vaccine for dogs, has been safe (Fischer et al., 2002), we chose this virus as an expression vector for RV G. A suitable live recombinant RV vaccine must fulfill the following criteria: (i) ability to produce high virus titers in tissue cultures; (ii) stable expression of a correctly folded and processed RV G at

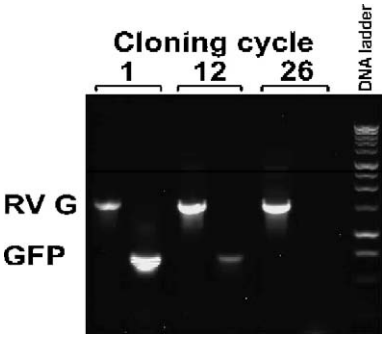

Fig. 2. PCR analysis of recombinant CAV2 DNA isolated from virus recovered after the 1st, 12th, and 26th cloning cycle. To identify the presence of the GFP gene and RV G gene, DNA was isolated from the supernatant of infected MDCK cells and subjected to PCR using RV G- or GFP-specific primers.

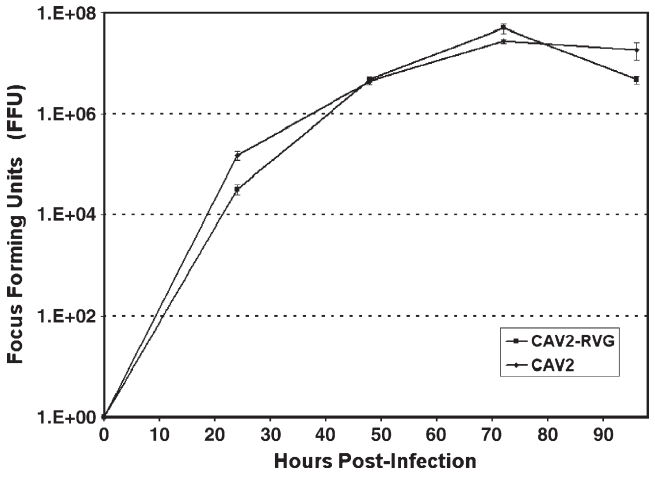

Fig. 3. Single-step virus growth curves of recombinant CAV2-RV G and wild-type CAV2 in MDCK cells. MDCK cells were infected with CAV2-RV G or CAV2 at m.o.i. of 1 and incubated at 34 °C. At the indicated times after infection, viruses were harvested and titrated. Data are the mean (±SE) of 4 virus titer determinations.

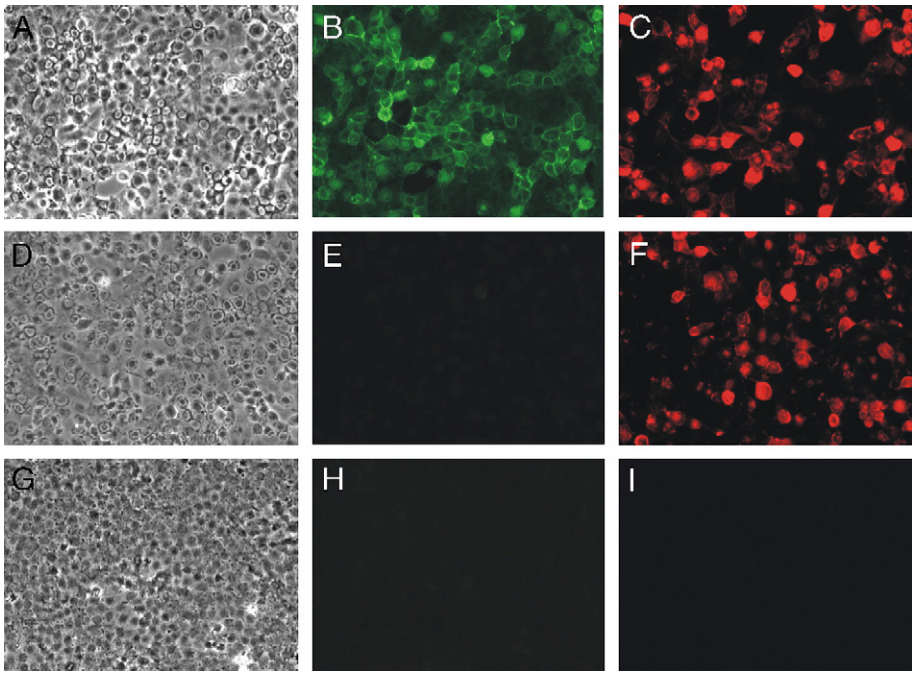

Fig. 4. Fluorescence microscopy analysis of RV G protein (green; B, E, H) and CAV2 proteins (red, C, F, I) in uninfected MDCK cells (G–I), CAV2-infected MDCK cells (D–F), and CAV2-RV G-infected (A–C) MDCK cells at 48 h p.i. Panels A, D and G show bright-field microscopy results.

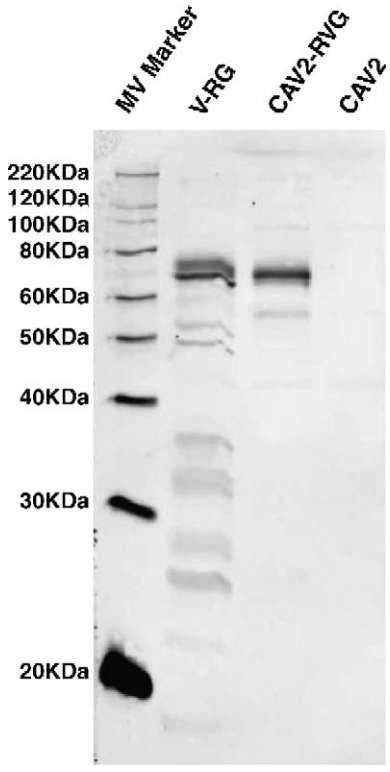

Fig. 5. Immunoprecipitation/Western blot analysis of the RV G produced in MDCK cells infected with V-RG or CAV2-RV G. Infected cells were lysed and subjected to immunoadsorption using a monoclonal antibody against the RV G. Adsorbed proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with polyclonal rabbit antibody against RVG followed by an Alexa Fluor 555-anti-rabbit IgG RV G. Bands were detected using a molecular imager.

sufficiently high levels; (iii) lack of pathogenicity; and (iv) ability to induce protective immunity after i.m. and especially after i.n. and oral immunization. With respect to virus replication, the CAV2-RVG produces virus titers in MDCK cells similar to those produced by the wild-type CAV2, indicating that the RVG gene does not affect virus replication. However, these virus titers may be too low to meet the quality requirements for an oral vaccine. Thus, efforts must be made

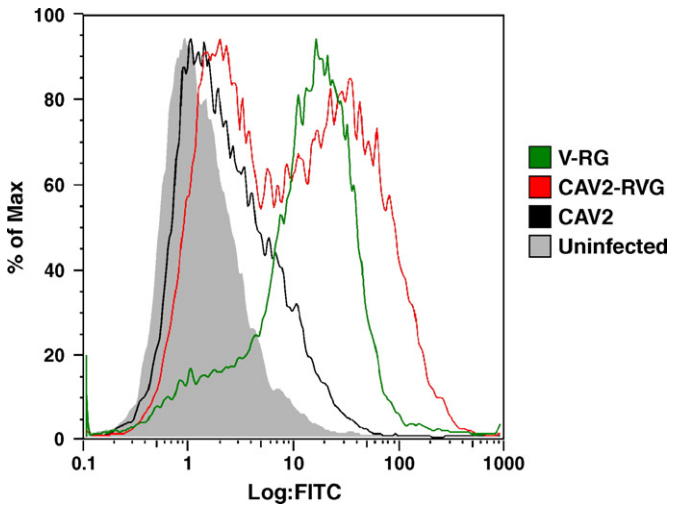

Fig. 6. Cell surface expression of RV G on MDCK cells. Cells were either mock-infected (solid histogram) or infected with CAV2 (black histogram), CAV2-RV G (red histogram), or V-RG (green histogram) at a m.o.i. of 1 for 48 h and incubated with recombinant human anti-RV G monoclonal antibody followed by FITC-conjugated anti-human IgG. Surface expression was determined by flow cytometry. Cells infected with CAV2-RV G showed RV G expression levels comparable to those seen in V-RG-infected cells.

Table 1
Immunogenicity and protection in mice immunized i.m. or i.n with CAV2-RV G

Vaccination [a]		VNA GMT [IU] (range)	Survivorship [b]
Route	Concentration [FFU]		
i.m.	10^8	47.5 (1.7–324)	9/10
i.m.	10^7	6.7 (0.1–108)	9/10
i.m.	10^6	0.3 (0.1–12)	4/10
i.n.	10^7	12.8 (0.1–324)	7/10
i.n.	10^6	0.6 (0.1–108)	6/10
i.n.	10^5	2.0 (0.1–36)	6/10
Control		0	1/10

[a] Mice were vaccinated with 100 μl i.m. or 10 μl i.n.
[b] Mice were challenged i.m. with 10 LD50 of CVS-N2c and observed daily for 4 weeks.

to increase virus titers either through selection of better replicating CAV2-RV G variants or by using bioreactor cell culture technology as demonstrated for the production of recombinant RVs (Dietzschold et al., 2004).

One of the most important determinants of the potency of a live rabies vaccine vector is its capacity to express the RV G. In this respect, vaccinia virus (Kieny et al., 1984) and rabies virus (Faber et al., 2002) are excellent vectors that express the RV G at very high levels. Comparison of RV G expressed by CAV2-RV G with that of V-RG revealed similar expression levels, suggesting that the potency of the CAV2-RV G vaccine is comparable to that of the V-RG vaccine.

CAV2-RV G showed no safety-related problems in mice that were immunized in this study. There were no clinical signs or discomfort regardless of the amount of virus or the route of administration. This observation promises to make CAV2-RV G a safe vaccine for use in stray dogs and wild life, with only a remote risk for humans. Nevertheless, the safety of CAV2-RV G must be assessed in target and non-target animals before it can be used in the field.

The other essential requirement for any live rabies vaccine is high efficacy, which is particularly critical when the vaccine is administered orally via vaccine-loaded baits and complete delivery of the vaccine dose cannot be guaranteed. Immunization of mice with CAV2-RV G by the i.m. or i.n. route induced rabies VNA and conferred protection against a lethal RV challenge infection in a dose-dependent manner; however, VNA titers and survivorship decreased sharply when fewer virus particles were administered. The sharp dose-dependent decrease in the immune response, which was not observed after immunization of mice with live recombinant RV (Faber et al., 2002), could rest in the relatively low susceptibility of mouse tissue to CAV2 infection, thereby limiting the virus spread and preventing the generation of sufficient antigenic mass to mount a protective immune response. This possibility is supported by the observation that neither CAV2 nor CAV2-RVG infects mouse neuroblastoma cells in vitro. The lack of sufficient susceptibility of mice to the CAV2 virus points to the need for future efficacy testing of CAV2-RV G in relevant animal target species such as canines. Furthermore, the use of other animal species for efficacy testing of orally administered CAV2-RV G vaccines is especially important because it is for unknown reason difficult to immunize mice by the oral route regardless of the vaccine used for immunization. On the other hand, most of the modified-live RV vaccines, recombinant RV vaccines, and also the modified-live CAV2 vaccine are effective in foxes and raccoons after oral administration (Baer et al., 1989; Sumner et al., 1988). The i.n. route was chosen for the immunization of mice mainly for proof of principal that protective immunity can be achieved by targeting mucosal surface membranes. Although oral immunization with CAV2-RV G did not confer protection against a lethal RV infection in mice (data not shown), a strong immune response observed after i.n. administration of CAV2-RVG is reason for optimism that this vaccine will actually work by the oral route in carnivores.

A crucial criterion for the efficacy of the CAV2-RVG is a low natural prevalence of CAV2 in the population to be vaccinated. In this regard, CAV2 is widespread among young dogs (Baker et al., 1961) and naturally occurring antibody to CAV2 has been detected in skunks, raccoons, foxes, and mongoose trapped in the field (Sumner et al., 1988). However, it has also been demonstrated that animals vaccinated orally with CAV2 had marked antibody increases, even when they already had high antibody titers to CAV2 (Sumner et al., 1988), suggesting that oral administration of CAV2-RVG can overcome the preexisting immunity to CAV2.

Material and methods

Virus culture and cells

The Manhattan strain of CAV2 (kindly provided by the Animal and Plant Health Inspection Service Center for Veterinary Biologics, Ames, IA) was grown in MDCK cells (ATCC, Manassas, VA) cultured in DMEM supplemented with 10% FBS, penicillin (50 U/ml) and streptomycin (50 U/ml). The V-RG was obtained from our virus collection at Thomas Jefferson University.

Virus titration

To determine virus yields, monolayers of MDCK cells in 96-well plates were infected with serial 10-fold dilutions of CAV2 or CAV2-RVG as described (Wiktor et al., 1977). At 48 h post-infection (p.i.), cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, incubated with a rabbit anti-RVG polyclonal antibody (1:1000), and stained with FITC-labeled goat anti-rabbit antibody (1:500, MP Biomedical, Aurora, OH). Foci were counted using a fluorescence microscope and virus titers calculated in focus-forming units (FFU). All titrations were determined in triplicate.

Virus DNA purification

CAV2 was grown in MDCK cells for 5 days as described above and concentrated by ultracentrifugation and purified by sucrose gradient centrifugation. Viral DNA was isolated using

the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation.

Cloning and sequencing of the CAV2 E3 DNA fragment

The CAV2 E3 region, which is not essential for virus replication (Fischer et al., 2002), was used to introduce RVG sequence from the SAD B19 RV strain into the CAV2 genome. After sequencing the entire E3 region (including the protein VIII precursor region and a 5′ region of fiber protein sequence), a 3.0-kbp E3 DNA fragment was PCR-amplified using high-fidelity DNA polymerase (Deep Vent, New England BioLabs, Beverly, MA), isolated CAV2 DNA as template, and primers F1 (TGTCAAGGACTCGAGTCCGG-CACAGACT) and F2 (TACTATCCGCGGACAGCTTG-CAACTGTTCGTTAATCA). The amplified fragment was cloned into the vector pBluescript II SK(+) (Stratagene, La Jolla, CA), resulting in pCAV2-E3.1. The correct sequence of the cloned DNA fragment was verified by DNA sequencing.

Generation of a CAV2 expression cassette

PCR was used to amplify a truncated fragment from pCAV2-E3.1 (1.2 kb deleted) with primers F3 (TACTAG-TACCGGTTTGGTAAGAGTCTGGAATATCA) and F4 (CATCTAGCGTACGAAGAAGACACTCCAATTTTAAT), which resemble the terminal 3′ and 5′ sequences of CAV2-E3, and using unique restriction sites AgeI and BsiWI. The amplified product was designated P1.

A second PCR was used to amplify the human CMV promoter and the BGH polyadenylation sequence from plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA) using primers F5 (TACTAGTACCGGTCGTTGACATTGATTATTGACTAG) and F6 (CATCTAGCGTACGCCATAGAGCCCACCG-CATCCCCA). The product P2 was ligated with P1, resulting in pCAV2-E3.2.

The RV G gene was introduced into pCAV2-E3.2 by the addition of an EcoRI restriction site and Kozak translation initiation sequence to the 5′ end and a PstI restriction site at the 3′ end of the RV G gene using PCR and primers F7 (TAGTCCGGAATTCAAGATGGTTCCTCAGGCTCTCCTG) and F8 (TGCACTGCAGTTATTACAGTCTGGTCT-CACCCCCA). The amplified product, P3, was ligated to pCAV2-E3.2 previously digested with EcoRI and PstI, resulting in pCAV2-E3.3.

The plasmid pCAV2-GFP was constructed by the same strategy using primers GFPf (TAGTCCGGAATTCGC-CATGGGCGTGATCAAGCCCGACATGAAGATC) and GFPr (TGCACTGCAGTTATTAGCCGGCCTGGCGGGG TAGTCCGCTGTG).

Generation of a recombinant CAV2-GFP virus by homologous recombination

A recombinant CAV2-GFP was generated by transfecting CAV2 DNA together with pCAV2-GFP into MDCK cells, picking up the green fluorescent foci detected in the transfected cell monolayers, and performing five cycles of plaque purification, resulting in a homogeneous CAV2-GFP population.

Generation of recombinant CAV2-RV-G virus by homologous recombination

pCAV2-E3.3 was extracted using the EndoFree Plasmid kit (Qiagen), digested with AlwNI, and purified using QIAquick PCR purification kit (Qiagen). Linearized pCAV2-E3.3 fragment was transfected together with CAV2-GFP DNA using Lipofectamine 2000 CD (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the CAV2-GFP DNA/pCAV2-E3.3 fragment/liposome mix was transfected into MDCK cells, and plaques were transferred to nitrocellulose filters and then to replica filters. The original filters were hybridized with a specific RV G DNA probe to identify recombinant CAV2-RV G virus-containing foci. After retrieval from the replica filter, the virus was used to infect monolayers of MDCK cells. Three days after infection, the tissue culture supernatant was removed, the virus was transferred from the infected cells to nitrocellulose filter and then to replica filter, and CAV2-RV G containing foci were identified in situ as described above. In addition, the supernatant from each culture was subjected to PCR to determine the presence of RV G and GFP DNA. This process was repeated until only RV G DNA but no GFP DNA could be detected in the supernatant of the infected cells.

Immunofluorescence analysis

To analyze cell surface expression of RV G, MDCK cells were infected with CAV2, CAV2-RV G, or V-RG at an m.o.i. of 1, and at 48 h p.i., infected cells were fixed with 4% paraformaldehyde for 20 min at room temperature and treated with 0.1% Triton-X100. After washing 3 times with PBS, cells were incubated with RVG-specific rabbit antiserum (1:2000), followed by Alexa Fluor 488-conjugated anti-rabbit IgG (1;1000, Molecular Probes). Surface expression of G was determined using a fluorescence microscope.

Immunoprecipitation/Western blot analysis

MDCK cells were grown in T25 tissue culture flasks and infected with CAV2-RVG, CAV2, or V-RG at a m.o.i. of 5, incubated for 48 h at 37 °C, and lysed with lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate). The lysate (500 μl) was mixed with 50 μl of recombinant human anti-RV G monoclonal antibody SO57 (Prosniak et al., 2003), immobilized on Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA), and incubated overnight with agitation at 4 °C. After washing the immobilized antibody/antigen complexes with lysis buffer, protein was eluted with 50 μl 0.1 M of diethanolamine and resolved by sodium dodecyl

sulfate–10% polyacrylamide gel electrophoresis. The separated proteins were transferred onto a nitrocellulose membrane, incubated with a polyclonal rabbit antibody against RV G followed by an Alexa Fluor 555-conjugated anti-rabbit IgG (Molecular Probes). RV G bands were detected using a molecular imager (FX Pro Plus; Bio-Rad, Hercules, CA).

Flow cytometry

MDCK cells were infected with CAV2-RV G or V-RG at a m.o.i. of 1 and incubated for 48 h at 34 °C. Cells were suspended in PBS containing 50 mM EDTA, pelleted at 130×*g* for 5 min, resuspended in 50 μl of PBS, and fixed in suspension by addition of 500 μl of 4% paraformaldehyde solution. After 20 min, cells were washed twice with PBS containing 10 mM glycine and 1% bovine serum albumin and incubated with recombinant human anti-RVG monoclonal antibody SO57 (1:4000) followed by a FITC-conjugated affinity-purified goat anti-rabbit antibody (1:500; MP Biomedical, Aurora, OH). Flow cytometry was performed on an EPICS profile analyzer.

Assessment of the protective activity of recombinant viruses

Groups of 10 female Swiss Webster mice (6-week-old) were immunized i.m. with 100 μl or i.n. with 10 μl of 10-fold serial dilutions of the recombinant virus in PBS. Thirty-two days after immunization, the mice were challenged i.m. with 10 LD50 of CVS-N2c challenge virus (Morimoto et al., 1998), and observed daily for 4 weeks for clinical signs of rabies.

Virus neutralization assay

At 25 days after immunization of mice, blood was collected from the retroorbital sinus, sera were heat inactivated at 65 °C for 30 min, and the RV-neutralizing activity was determined as described (Wiktor et al., 1984a). The virus-neutralizing antibody (VNA) titers were transformed into international units (IU) using the WHO anti-rabies virus antibody standard, and the VNA geometric mean titer (GMT) was calculated for each experimental group.

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