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SAG-2 oral rabies vaccine

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ABSTRACT

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The live modified rabies virus vaccine strain SAG-2 was selected from SADBerne in a two step process employing anti-rabies glycoprotein monoclonal antibodies. The first two nucleotides coding for the amino acid in position 333 of the rabies glycoprotein are mutated. Arginine at position 333, which is associated with rabies pathogenicity, was substituted first by lysine and then by glutamic acid. The two nucleotide differences at position 333 in SAG-2 to any of six possible arginine triplets translated into excellent genetic stability and apathogenicity for adult mice, foxes, cats and dogs. The vaccination of foxes and dogs by the oral route provided protection against a lethal challenge with rabies virus.

INTRODUCTION

SAG-2 is a live modified rabies vaccine for the oral immunisation of dogs against rabies. This strain represents the latest addition to the family of vaccines originating from a virus which was isolated from the salivary glands of a rabid dog in 1935. The virus was passaged in mice and adapted to chick embryos and/or various permanent cell lines. Several attenuated vaccine strains, namely SAD, ERA and Vnukovo were generated (Bunn 1991). The subclone SAD-Berne served as the parent strain for efficacious live virus vaccines like SAD B19 Tübingen, SAD Potsdam 5/88 and SAG-1. The strains have successfully contributed to fox rabies control in Europe (Blancou, Pastoret, Brochier, Thomas & Bögel 1988). With continually decreasing numbers of rabies cases in Western Europe (Aubert 1992), the public health concern is shifting to countries with a canine rabies problem. It was recognized that the traditionally close social relationships between dogs and humans not only prompt transmission of rabies to humans, but also impose exacting safety requirements for live modified and live recombinant oral candidate vaccines for dogs (Wandeler, personal communication). This paper describes the selection of the variant SAG-2 and the first experimental results of the latest and most apathogenic addition to the family of live modified virus vaccines.

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MATERIAL AND METHODS

Selection of SAG-2

Hybridomas secreting monoclonal antibodies (Mans) 50AD1 or 50AC1 with specificity for antigenic site III of the rabies glycoprotein were obtained by fusion

of myeloma SP2O cells with lymphocytes of BALB/c mice hyperimmunized with CVS virus as described by Fazekas de St Groth & Scheidegger (1980).

Following a protocol similar to the one described for the selection of CVS mutants (Seif, Coulon, Rollin & Flamand 1985), SADBerne virus was incubated with ascites fluid containing Mans 50AD1 or 50AC1. Mutants escaping neutralization were isolated by plaque purification on CER cells. Their patterns of sensitivity to Mans were determined and compared to those of the parent strain SADBerne. The mutation of the genome coding for amino acid 333 of the glycoprotein was determined by dideoxynucleotide sequencing. The pathogenicity of emerging clones was checked by intracerebral inoculation in adult mice.

Analysis of genetic stability

Six three day-old mice were inoculated intracerebrally with 10^3 pfu of SAG-2. On day 4 or 5 the animals were sacrificed and their brains dounce-homogenized in tris buffered saline. Two further intracerebral passages were performed with the homogenates. For each of the three passages six sets of three adult Swiss mice were inoculated intracerebrally with a 1/10 dilution of the brain suspensions. Clinical signs and mortalities were registered during the observation period of 30 days.

Inoculation of foxes

Fifteen foxes, approximately one year-old, which had been captured in the wild were divided into groups of five individuals. Ten were vaccinated by instillating a single dose of SAG-2 dropwise into the oral cavity of each unanesthetized animal. One group received 10^7 and the other 10^8 pfu. Serum samples obtained just before vaccination and before challenge were analyzed by rapid fluorescent focus inhibition (Zalan, Wilson & Pukitis 1979). After 29 days all foxes including five unvaccinated controls were challenged by inoculation of $10^{3,03}MICLD_{50}$ of the fox strain GS7-1-1 into a masseter muscle. The animals were observed daily throughout the experiment for clinical signs. Brain material of all foxes dying of rabies or euthanized on day 84 was examined for the presence of rabies antigen by immunofluorescence.

Inoculation of cats

Five four month-old cats were inoculated orally with 1 mℓ containing 10^9 pfu of SAG-2 as described for foxes. Blood specimens for serum were collected on day 0 and 33 of the experiment in order to determine virus neutralizing antibody titers. On day 33 all cats including three controls were challenged by inoculation of $10^{4,6}MICLD_{50}$ of the fox strain GS7-1-1 into a temporal muscle. The cats were euthanized 122 days after the challenge and their brains examined for viral antigen by fluorescence.

Inoculation of dogs

Three different concentrations (10^7, 10^8 and 10^9 pfu) of SAG-2 were administered orally to fifteen 4–5 months-old beagles by the described mentioned above. Serum specimens were drawn on days 0 and 39 and virus neutralizing antibody titers were determined. All vaccinated dogs plus five unvaccinated controls were challenged on day 39 by intra-temporal injection of $10^{3,54}MICLD_{50}$ of the dog rabies strain MA85. They were observed daily for signs of rabies. The presence of virus or viral antigen in brains and salivary glands of animals succumbing to the challenge was investigated by fluorescent antibody staining of tissue impressions and by inoculation of mouse neuroblastoma cell cultures with tissue material.

RESULTS

Selection of SAG-2

SAG-2 was obtained in a two step selection process under neutralizing monoclonal antibody pressure. In the first step the attenuated mutant SK was selected by cocultivation of SADBerne with Mab 50AD1. Arginine in position 333 of the SADBerne-glycoprotein encoded for by the codon AGA was replaced by lysine coded for by AAA. In contrast to CVS variants bearing lysine at position 333, SK was not pathogenic for adult mice by the intramuscular route and its pathogenicity by intracerebral route was greatly reduced as compared to SADBerne.

Mutants holding a negatively charged or uncharged amino acid in position 333 were identical in their patterns of resistance to neutralization by site III-specific monoclonal antibodies. SK having the positively charged lysine in position 333 resisted neutralization by Mab 50AD1, but remained sensitive to another site III specific antibody, 50AC1. Consequently, SK was submitted to a second selection step with Mab 50AC1. Lysine in position 333 of the resulting variant SAG-2 was replaced by glutamic acid, which was encoded by GAA. Thus SAG-2 differs from SADBerne by one amino acid in position 333, but by two nucleotides from any of the six possible triplets coding for arginine. The amino acid variation between the parent strain and the variants SK and SAG-2 and the corresponding nucleotide changes in codon 333 are shown in Table 1.

Genetic stability of SAG-2

SAG-2 was passaged three times in suckling mice brains. Intracerebral injection of brain homogenate did not lead to clinical signs or mortality in any of the 54 adult individuals tested in six independent experiments. The titers of the inoculum/animal increased from 10^3 pfu in passage 1–10^5 pfu in passage 3.

TABLE 1 Characterization of SADBerne and variants S and SAG-2

Virus strain	Amino acid at glycoprotein position	Nucleotide sequence of codon 333	Neutralization by monoclonal antibody	
			50AD1	AC
SADBerne	Arginine	AGA	Yes	Yes
S	Lysine	AAA	No	Yes
SAG-2	Glutamic acid	GAA	No	No

TABLE 2 SAG-2: Innocuity of an oral dose of 10^9 pfu in cats and dogs

No. of animals	Clinical signs/ Mortality	Time of observation after vaccination
5 cats	0/5	33 days
5 dogs	0/5	39 days

TABLE 3 SAG-2: Efficacy of oral vaccination in foxes and dogs

No. of animals	Vaccine dose/ animal	Delay between challenge and vaccination	Clinical signs/ Mortality	FAT/ Brain[a] positive
5 foxes	10^8 pfu	Day 29	0/5	0/5
5 foxes	10^7 pfu	Day 29	0/5	0/5
5 foxes	Control	Day 29	5/5	5/5
5 dogs	10^9 pfu	Day 39	0/5	ND[b]
5 dogs	10^8 pfu	Day 39	0/5	ND
5 dogs	10^7 pfu	Day 39	0/5	ND
5 dogs	Control	Day 39	4/5	4/5

[a] Viral antigen detection was attempted by fluorescent antibody test (FAT) on brain smears of animals dying of rabies or euthanized at the end of the observation period

[b] ND = Not done

Sequence analysis of SAG-2 passaged 10 times in the absence of the monoclonal antibodies 50AD1 and AC in tissue culture did not reveal any changes in nucleotide sequence at codon 333.

Innocuity of SAG-2

No pathogenic effects were observed in mice inoculated with SAG-2 by the oral, intramuscular and intracerebral routes at doses as high as $10^{6,5}$ pfu/animal.

The incubation period in sucking mice was prolonged for SAG-2 and the clinical signs appeared weaker than in the closely related fox vaccine strain SAG-1. The apathogenicity of SAG-2 was further demonstrated in cats and dogs vaccinated orally with a dose exceeding by more than 10-fold the estimated field dose. No clinical signs or any negative impact on their health was detected during observation periods (Table 2). None of the foxes receiving an oral vaccine dose as high as 10^8 pfu showed any abnormal signs during the 29 days of observation prior to challenge (Table 3).

Immunizing efficacy of SAG-2

All adult Swiss mice vaccinated intracerebrally with $10^{3,5}$ $MICLD_{50}$ SAG-2 survived an intramuscular challenge with an otherwise lethal dose of CVS. All foxes vaccinated orally with 10^7 or 10^8 pfu of SAG-2/animal were protected against an intramuscular challenge performed 29 days after vaccination, while 100 % of the unvaccinated controls died. The mean virus-neutralizing antibody titer on day 29 was for 0,49 IU/mℓ (range: 0,33–0,64 IU) for the group vaccinated with the lower dose and for 0,51 IU/mℓ (range: 0,11–1,3) for the animals receiving 10^8 pfu. No rabies-specific antibodies were detectable on day 0. Rabies was confirmed in all unvaccinated controls by direct fluorescent antibody test (FAT) on brain specimens. Brains of vaccinated foxes euthanized on day 84 were free of viral antigen (Table 3).

No mortality occurred in cats that had received 10^9 pfu of SAG-2. None of the animals died of rabies following challenge 33 days later, but none of the unvaccinated controls succumbed to rabies either. The virus neutralizing antibody titer ranged between 0.01 and 0,36 IU/mℓ. The brains of vaccinated and unvaccinated animals harvested 122 days after the challenge were free of viral antigen.

Of 15 laboratory dogs vaccinated orally with 10^7, 10^8 or 10^9 pfu of SAG-2, none succumbed during 110 days of observation to a lethal challenge undertaken 39 days after vaccination. Eighty percent of the unvaccinated controls died of rabies confirmed by FAT on brain samples (Table 3). No search for viral antigen was attempted on tissue samples of vaccinated dogs since they have been conserved for hyperimmunserum production. The mean neutralizing antibody titers on day 39 had risen from zero before vaccination to 0,33 IU/mℓ (range: 0,08–0,64 IU/mℓ), 0,26 IU/mℓ (range 0,09–0,51 IU/mℓ) and 0,22 IU/mℓ

(0,14–0,31 IU/mℓ) in dogs receiving 10^7, 10^8 or 10^9 pfu respectively.

DISCUSSION

The oral fox vaccine strain SADBerne was incubated with monoclonal antibodies in order to select antigenic mutants which escape neutralization. These monoclonal antibodies possessed binding specificities for the antigenic site III (amino acids 330–338) of the viral glycoprotein. Arginine at position 333 has been linked to pathogenicity in several rabies virus strains (Tuffereau, Leblois, Bénéjean, Coulon, Lafay & Flamand 1989; Coulon 1983). Under the selection pressure of the monoclonal antibodies only site III variants of SADBerne escaped neutralization and were able to replicate in cell culture. The substitution of the arginine at position 333 of site III of the rabies glycoprotein decreased or abolished pathogenicity of several clones for adult laboratory mice. The escape variant SAG-2 was selected from SADBerne in two steps in the presence of two different anti-glycoprotein monoclonal antibodies. The first two nucleotides in the codon of the amino acid at position 333 resulting in the replacement of arginine by glutamic acid. The resulting double mutant SAG-2 lost the residual pathogenicity that characterizes in varying degrees its predecessors (ERA, SADBerne, SAD B19 Tübingen, SAD Potsdam 5/88). This effect is most clearly demonstrated in the laboratory mouse, which serves as a sensitive animal model for the detection of residual pathogenicity of live modified rabies vaccine (Leblois, Blancou, Coulon, Lafay, Préhaud, Tuffereau & Flamand 1988). Due to the exchange of the first two nucleotides in the codon of amino acid 333 of the glycoprotein, the strain cannot easily revert to the pathogenic phenotype of the parent strain SADBerne by a single mutational event as is possible for SAG-1. Following direct intracerebral passage in suckling mice and in ten subsequent passages in cell culture in the absence of site III specific antibody pressure, the integrity of the strain with regard to this mutation is preserved.

SAG-2 is apathogenic for adult Swiss mice inoculated by the oral, intramuscular and intracerebral routes. These results clearly reflect the greater attenuation of SAG-2 versus SADBerne. The latter is known to possess considerable residual pathogenicity for adult mice especially by the intracerebral route (Leblois *et al*. 1988).

The innocuity for cats and dogs at dosages that are 10 times greater than that designated for field application was shown to be complete. The short-term innocuity for the red fox of a dose greater than that intended for use in the field was confirmed. Taking these and results obtained in about 20 species with SAG-1 into consideration, it seems unlikely that SAG-2 could induce rabies. Recently SAG-2 was shown to be non-pathogenic for baboons (Bingham & Aubert, M., personal communication) which had the same origin as two that developed clinical rabies after oral inoculation with $10^{7,5}$ pfu of SADBerne in an earlier experiment (Bingham, Foggin, Gerber, Hill, Kappeler, King, Perry & Wandeler 1992).

The preliminary results on the efficacy of SAG-2 as an oral immunogen indicates that it is as immunogenic as SAG-1. As is the case with SAG-1, a single dose of 10^7 pfu of SAG-2 administered orally to both dogs and foxes conferred protection against a lethal rabies challenge carried out approximately one month after vaccination. However, the small sample size precludes definite conclusions on the efficacy of SAG-2 being made.

These preliminary results indicate that SAG-2 is a promising candidate for the development of a vaccine for the oral immunisation of dogs against rabies. Future research in compliance with WHO requirements will aim at the completion of efficacy data, especially in long term experiments. Special attention will be dedicated to the demonstration of genetic stability and innocuity for target and non-target species.

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