Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES OF FOXES (*VULPES VULPES*) AFTER EXPERIMENTAL PRIMARY AND SECONDARY ORAL VACCINATION USING SAG_2 AND V-RG VACCINES

M. Lambot [a], E. Blasco [b], J. Barrat [b], F. Cliquet [b], B. Brochier [c], C. Renders [c], N. Krafft [b], J. Bailly [b], M. Munier [b], M.F.A. Aubert [b], P.-P. Pastoret [a]

[a] *Department of Immunology – Vaccinology, Faculty of Veterinary Medicine, University of Liège, B43b Sart tilman, 4000 Liège, Belgium*
[b] *Agence Française de Sécurité Sanitaire des Aliments, AFSSA Nancy, Domaine de Pixérécourt, B.P. 9, 54220 Malzéville, France*
[c] *Department of Rabies, Pasteur Institute of Brussels, Ministry of Public Health and Environment, 642, rue Engeland, 1180 Brussels, Belgium*

KEYWORDS: Humoral response; Cell-mediated response; *Vulpes vulpes*; Oral vaccination

ABSTRACT

Humoral and cell-mediated immune responses of 36 captive foxes to two oral vaccines against rabies currently used for foxes in Europe were studied. The Street Alabama Dufferin (SAD) mutant Gif (SAG_2) vaccine has been selected by double mutation from the SAD virus. The vaccinia recombinant virus (V-RG) expresses the rabies glycoprotein. Both vaccines induce similar humoral and cell-mediated responses after primary and secondary oral administration. We observed a typical anamnestic response, although of a limited duration, after the booster vaccination. Therefore, our results suggested that two successive oral vaccination campaigns should not significantly improve the immunisation of foxes. Lymphocyte in vitro proliferative response to the SAD antigen highlighted the presence in blood of a T-cell specific memory 6 months after vaccination. The synthesis of several vulpine cytokines was detected in peripheral blood mononuclear cells (PBMC) stimulated by SAD antigen via reverse transcription polymerase chain amplification. The data showed a concomitant expression of interleukin (IL)-4 and interferon-y in PBMC of vaccinated foxes. No change was detected in the level of IL-2, IL-10 and IL-12 synthesis, whereas the pro-inflammatory cytokine tumour necrosis factor-ex seemed involved in the activation of naive T lymphocytes.

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

Introduction

Large-scale annual campaigns of fox oral vaccination, carried out in continental Europe since the early 1980s, have led to a drastic reduction of sylvatic rabies incidence [1]. Among the vaccines used in the field, those obtained through biotechnology occupy an important position [2]. Two of them fulfil all the World Health Organisation (WHO) requirements on innocuity of anti-rabies oral vaccines. They experimentally demonstrate an increased safety for numerous target and non-target wild animal species [3,4]. This concerns the Street Alabama Dufferin (SAD) mutant Gif (SAG_2) vaccine, a double mutant of the SAD Bern rabies strain obtained by selective growth in the presence of specific monoclonal antibodies [5], and the vaccinia-rabies glycoprotein recombinant (V-RG) vaccine that expresses the rabies glycoprotein of the ERA SAD derived strain [6–8].

However, the elimination of fox rabies may be hampered for two reasons: the persistence of residual foci in some areas, and the risk of re-infection of zones already freed, which is parallel to an increase in fox population [9]. Therefore, different measures to improve the efficacy of fox oral vaccination have been tested in the field (i.e. increase in the number of vaccine baits distributed in one campaign [10], vaccination of areas with two passages separated by 15 or 30 days [11], vaccination of fox cubs by distributing vaccine baits at the entrance of fox dens [12], vaccination during summer period [13]) and need to be optimised in experimental trials on captive foxes. Furthermore, protection does not always correlate with a sound level of rabies-neutralising antibodies [14–16], suggesting the involvement of protection mechanisms other than antibodies. One of the most consistent effects of any vaccine on the immune response is related to T cells; therefore, the resistance or the susceptibility to rabies infection might be the result of a differential development of rabies-specific T-cell responses. Furthermore, alterations in cytokine profiles delivered by T cells to antigen-stimulated B cells may also contribute to differences in the production of antibodies following vaccination. Parameters regulating the post-vaccination immune response have been studied in mice and humans [17,18], but only very few data are available on the relationship between cell-mediated and humoral responses during anti-rabies vaccination in fox [19]. To evaluate their contribution in the protection against rabies, we investigated the lymphocyte proliferative and neutralising antibodies responses of 36 caged adult silver foxes primo- and secundo-vaccinated with the SAG2 or the V-RG vaccine. The production of six cytokines known to play a role in the cellular immune response in other mammalian species (i.e. interleukin (IL)-2, interferon (IFN)-g, IL-4, IL-10, IL-12 and tumour necrosis factor (TNF)-a) was also studied.

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

Materials and methods

FOXES

Thirty-six silver foxes (*Vulpes vulpes*), 1–6 years old, were purchased from the Norwegian Fur Breeder's Association (Oslo, Norway). A 1-month adaptation period was applied from their arrival until the beginning of the experiment. The animals were kept in individual cages and observed daily. They were fed with a commercial dry food for adult dogs. Water was provided ad libitum.

VIRUSES

SAD ANTIGEN PREPARATION

The SAD parental strain was used as stimulating antigen for in vitro cell stimulation tests. The virus was kindly provided by Dr Anne Flamand (Laboratoire de Génétique des Virus, CNRS, Gif-sur-Yvette, France). The virus was produced on BHK cells and the supernatant was concentrated and purified by gradient [20]. The purified SAD virus was then inactivated with b-propiolactone (Sigma, St Louis, MO, USA).

RABIES CHALLENGE VIRUS

The challenge virus is the supernatant of a 20% homogenate of sub-maxillary salivary glands from foxes; it is then kept frozen in liquid nitrogen until use. The virus that has been used in this experiment was first isolated from sub-maxillary salivary glands of naturally rabid foxes in 1986 [21]. That first batch (GS-7) was then passaged three successive times in a single fox [22]. The fourth passage (GS-9) is a pool of six pairs of sub-maxillary salivary glands of foxes inoculated with the third passaged suspension. The titre of this latter passage was determined by an intracerebral inoculation of mice. Immediately after inoculation, the challenge virus was back titrated intracerebrally in mice. The titre of the challenge suspension was $10^{3,3}$ median mouse intracerebral lethal doses per millilitre.

RABIES VACCINES

V-RG vaccine baits (batch 80U342) supplied by MERIAL SA (Lyon, France) were stored at +4°C until use. SAG_2 vaccine baits (batch RB2 PO91) supplied by VIRBAC Laboratories (Carros, France) were stored at −30°C until use. The titres of the vaccines were verified on receipt, before the beginning of the experiment and after each administration to foxes.

Vaccine titres were expressed in median cell culture infective dose per dose ($CCID_{50}$/dose). Briefly, titra- tions of vaccines were performed in 96-well microtitre plates. Cells (2×10^5) were distributed in each well: VERO cells (ATCC No. CCL81) in 100 µl or BSR cells in 200 µl for V-RG and SAG_2 vaccines, respectively. A one in ten serial dilution of the virus was performed; 100 µl for V-RG dilutions or 50 µl for SAG_2 dilutions were distributed in six replicates. V-RG titration plates were incubated for 120 h at 37°C with 5% CO_2. The cytopatic effect was observed on an inverted

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

microscope. SAG_2 titration plates were incubated for 72 h in the same conditions. As SAG_2 virus does not induce cell lysis, plates were stained with fluorescein-coupled rabies anti-nucleocapsid conjugate. The titre calculation was made by using the neoprobit graphic method [23]. Before the experiment, V-RG titrated $10^{8,44}$ $CCID_{50}$/dose and SAG2 titrated $10^{8,3}$ $CCID_{50}$/dose. After the primary vaccination and after the booster vaccination, titres remained unchanged.

EXPERIMENTAL DESIGN

Thirty-six foxes were divided into five groups according to age and sex: four experimental groups of eight animals (groups A, B, C and D) and a control group of four animals (group T), as detailed in Fig. 1. Vaccines were administered by direct instillation into the oral cavity with a syringe on manually restrained vigil animals. The instilled volume corresponded to a field dose of V-RG or SAG_2 vaccine (i.e. 2,7 and 1,8 ml, respectively). On day 0, groups A and B received the SAG_2 vaccine, and groups C and D received the V-RG vaccine. On day 35, groups B and D were boosted either with SAG_2 vaccine or V-RG vaccine, respectively. The four control foxes (group T) were not vaccinated. On day 195 post-vaccination, all foxes were challenged intramuscularly with 1 ml virus suspension (GS-9) into the temporal muscle. The foxes were then daily monitored for clinical signs. In order to avoid unnecessary animal suffering, it was planed to euthanise foxes that develop rabies in the terminal phase when paralyzed. The observation period had been fixed to 45 days after the death of the last control fox. Brain material and sub-maxillary salivary glands of all foxes dying of rabies or euthanised at the end of the observation period have been examined for the presence of rabies antigen by direct immunofluorescence and cell culture test.

BLOOD SAMPLING

Bleeding was carried out by jugular venipuncture on vigil animals. For the neutralising antibody test, foxes were bled on days 0 (before primary vaccination), 7, 14, 21, 28, 35, 42, 49, 53, 70, 84, 119, 161 and 182 (see Fig. 1). For both cell proliferation and cytokine production assays, blood samples were collected on day 182 only.

SEROLOGY

Rabies-neutralising antibodies were titrated in serum samples by using the fluorescent antibody virus neutralisation test [24]. The second WHO Standard for Rabies Immunoglobulin preparation, obtained from the Statens Serum Institute (Denmark) and adjusted to a concentration of 0,5 International Units (IU)/ml, was used as positive reference. Titres were expressed in IU/ml by comparison with the titre of the standard serum included in each test. The conventional level of 0,5 IU/ml has been defined in humans and is considered as indicative of protection against rabies [25]. This value is currently used in rabies epidemiological surveys of wildlife.

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

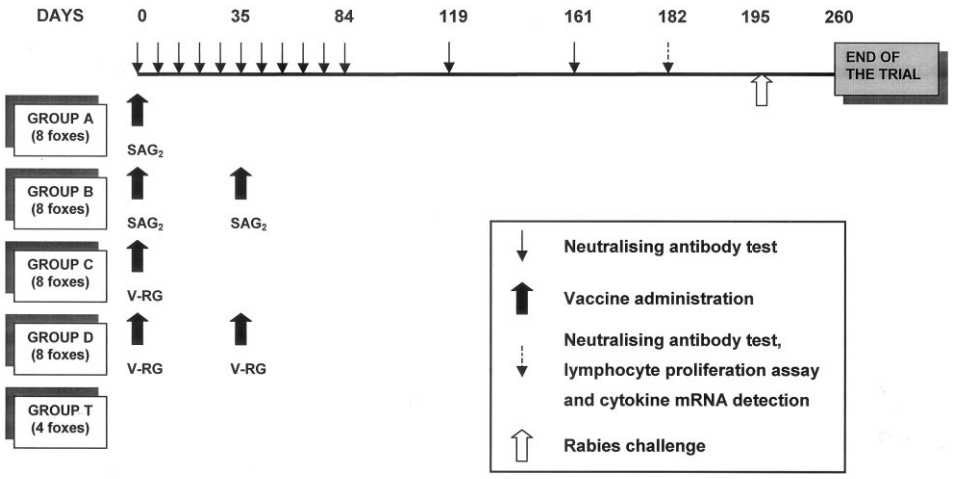

Fig. 1. Schematic representation of the experimental protocol. Animals were assigned to five groups: two groups vaccinated once or twice with the SAG_2 vaccine (group A and B, respectively), two groups vaccinated once or twice with the V-RG vaccine (group C and D, respectively) and one unvaccinated control group (group T).

CELL PREPARATION

Blood samples were collected into heparin lithium tubes (Sarstedt, Orsay, France). Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque (Pharmacia, Roosendaal, The Netherlands) density gradient as previously described [26]. PBMC were then suspended in RPMI 1640 medium (Gibco BRL, Gent, Belgium) supplemented with 1% non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 5×10^{-5} M 2-mercaptoethanol (Gibco) and 10% heat-inactivated foetal bovine serum (Gibco).

LYMPHOCYTE PROLIFERATION ASSAY

PBMC were distributed in 96-well round-bottom plates at 2×10^5 cells/well (Nunc, Paisley, Scotland). The SAD antigen preparation was then added at a final concentration of 2.5 µg/ml, previously determined as optimal. Control wells without antigen were set up in parallel. After 6 days of incubation at 37°C in a humidified atmosphere with 5% CO_2, cultures were pulsed with [3H]thymidine (0,8 µCi; specific activity, 2 Ci/mM) (Amersham, Arlington Heights, IL, USA) for the last 18 h. [^{3}H]Thymidine incorporation was then measured by liquid scintillation counting. Results were expressed as the stimulation index, i.e. the ratio between the counts per minute (cpm) mean of sextuplate cultures stimulated with antigen and the cpm mean of sextuplate cultures maintained in medium alone.

CYTOKINE MRNA DETECTION ASSAY

To measure mRNA expression of IL-2, IFN-g, IL-4, IL-10, IL-12 and TNF-a, PBMC were cultured at 1×10^6/ml in six-well plates (Nunc) for 2 days at 37°C with the SAD antigen preparation at the same concentration as already described or in medium alone. Cells were then harvested and total RNA

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

was extracted with a commercial kit (High pure RNA isolation kit; Roche). The cDNA was first obtained by using reverse transcriptase and 500 ng total RNA extracted from each sample, in a 20 µl reaction mixture according to conditions recommended by the manufacturer (Roche). The reaction mixture was then diluted 1:8 and 5 µl diluted product was used for specific amplification of vulpine cytokines. The standard polymerase chain reaction (PCR) was carried out using diluted cDNA with 1,25 U Taq polymerase (Roche), 0,2 mM each dNTP (Roche) combined with the PCR buffer provided by the manufacturer and 0,2 µM each primer pair (sense and anti- sense) for P-actin, IFN-g, IL-12, IL-2, IL-4, IL-10 and TNF-a. The primer pairs for PCR were synthesised from oligonucleotide consensus sequences already published [27]. Cycling conditions were 95°C, 3 min followed by 32–48 cycles of 95°C, 30 s/52 or 60°C, 1 min/72°C, 1 min. PCR products were assayed after a restricted number of cycles of amplification to ensure that the analysis of cytokine transcripts was performed during the exponential phase of amplification. A negative control without cDNA was included in each PCR reaction. PCR products were analysed on ethidium bromide-stained agarose gels (1.0%) and expressed as semi-quantitative values using the BIO-PROFIL gel analyser system (Vilber Lourmat, Marne La Vallée, France). Results were first standardised for the relative quantity of total mRNA used in the reverse transcriptase reaction through parallel amplification and analysis of transcripts from the housekeeping gene P-actin. The results reported for each animal were then expressed as the ratio of cytokine levels found in SAD antigen-stimulated cultures to those in the corresponding control cultures without antigen in order to consider background production of cytokine mRNAs into account. The specificity of each cytokine product was further verified by direct sequencing using the FS polymerase kit (Perkin-Elmer Cetus). Computer analysis of sequence data showed 90-96% of homology to the published canine cytokine sequences using BLAST software.

STATISTICAL ANALYSIS

Differences in the neutralising antibody results and in the lymphoproliferative responses between groups were analysed using Student's *t*-test at a 95% confidence level. Comparison of PCR results was performed using the Mann–Whitney U-test with a 95% confidence limit.

Results

HUMORAL IMMUNE RESPONSE TO RABIES VACCINATION

The kinetics of neutralising antibody response within each of the five groups is shown in Fig. 2. After primary oral administration, two different patterns of antibody response were observed depending on the vaccine used. Neutralising activity was detectable 1 week earlier in foxes of groups C and D vaccinated with the V-RG than in those of groups A and B vaccinated with SAG_2. Furthermore, the levels of antibody titres reached 21 days after primary vaccination were significantly higher in foxes that received the V-RG ($P<0.05$). Results obtained 14 days after the booster vaccination showed that neutralising activity was significantly enhanced whatever the

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

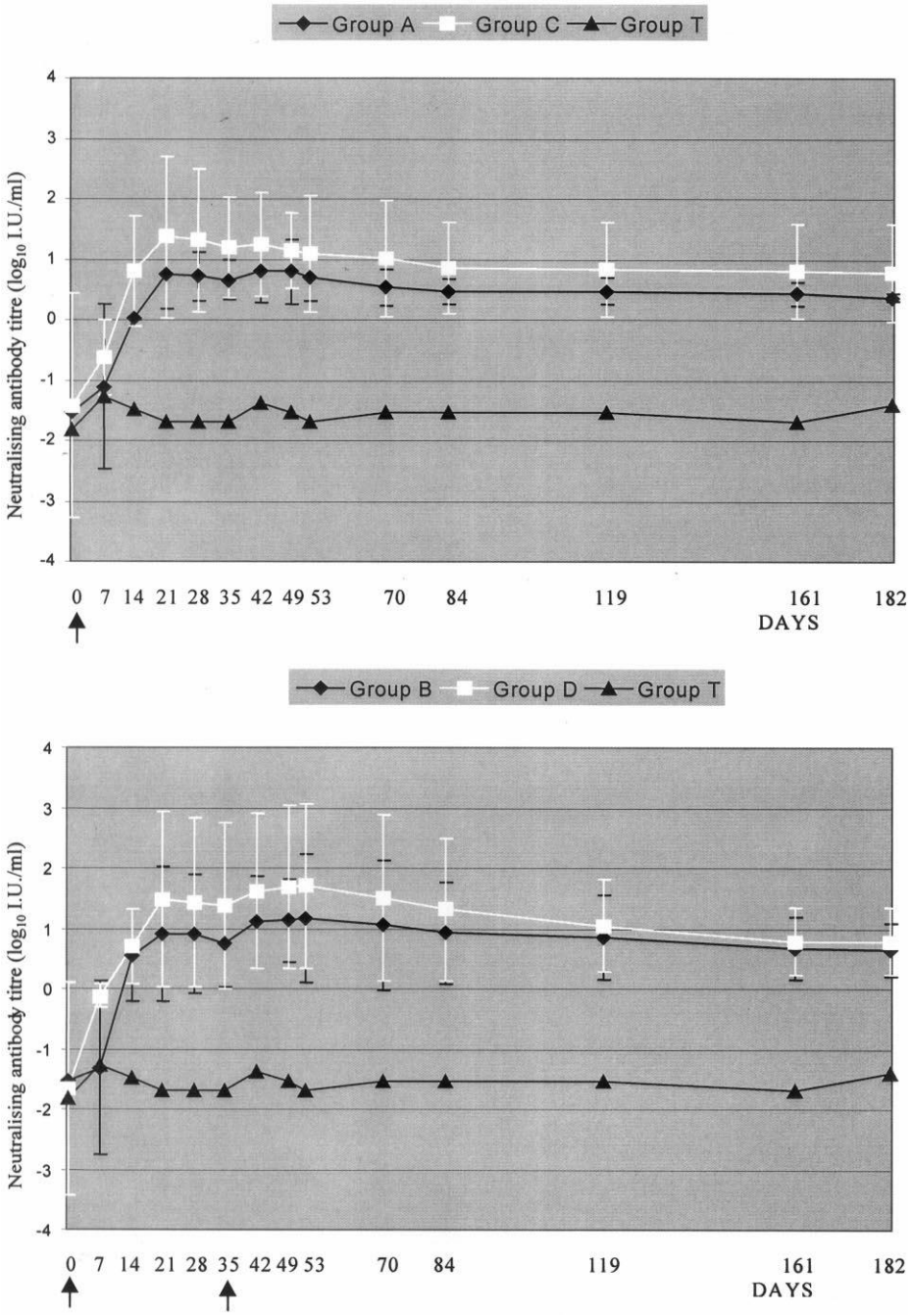

Fig. 2. (A) Kinetics of rabies virus neutralising antibody (VNA) mean titres of foxes after primary vaccination with the SAG_2 (group A) or V-RG (group C). (B) Kinetics of rabies virus neutralising antibody (VNA) mean titres of foxes after primary and secondary vaccinations with the SAG_2 (group B) or V-RG (group D). Four unvaccinated foxes served as controls (group T). The VNA mean titres are expressed in $\log_{10}$ SN_{50}. Arrows, times of vaccination.

vaccine used. This secondary response was most noticeable in animals that received the V-RG vaccine. All control foxes failed to produce any neutralising antibody. The profiles of rabies antibodies obtained with these two vaccines differed markedly in a short period of time after vaccination. At the end of a period of 6 months, there only remained a significant difference in the

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

levels of neutralising activity between the groups of vaccinated foxes and the group of control animals, whatever the vaccination protocol and the vaccine used (P<0.0001).

RESISTANCE TO RABIES CHALLENGE

All vaccinated foxes resisted to rabies challenge and all control foxes died from rabies. The mean mortality delay (time between virus inoculation and death) was 15 days. The clinical phase lasted less than 2 days. Laboratory tests (direct fluorescent antibody test and cell culture infection test) confirmed rabies in the control group but were negative on vaccinated animals at the end of the observation period.

Table 1. Rabies virus neutralising antibody (VNA) titres and lymphocyte proliferative responses of PBMC from foxes, 182 days after primary vaccination with SAG_2 (groups A and B) or V-RG (groups C and D) vaccine

Fox	VNA[a]					Lymphocyte proliferative response[b]				
Number	Group A	Group B	Group C	Group D	Group T	Group A	Group B	Group C	Group D	Group T
1	1,97	1,14	5,92	1,14	<0,3	2,30	2,38	<2,00	2,48	<2,00
2	1,14	5,92	0,66	10,26	<0,3	2,25	<2,00	5,47	2,03	<2,00
3	1,14	7,79	10,26	5,92	<0,3	<2,00	2,59	2,77	3,01	<2,00
4	3,42	7,79	10,26	10,26	<0,3	2,14	8,66	2,11	2,10	<2,00
5	1,50	4,50	1,97	4,50		<2,00	5,33	6,00	<2,00	
6	4,50	2,60	17,77	3,42		7,53	3,70	6,27	2,11	
7	2,60	0,87	0,66	10,26		3,13	<2,00	2,24	5,83	
8	2,60	5,92	0,50	3,42		2,25	<2,00	2,79	<2,00	

[a] Rabies virus (CVS strain) neutralising antibody titres are expressed in IU/ml serum. The defined level of 0.5 IU/ml is considered indicative of immune protection.

[b] Lymphocyte proliferative responses to 2.5 µg/ml inactivated SAD antigen are expressed as stimulation indexes (values>2 = significative proliferative response).

PROLIFERATIVE RESPONSE TO RABIES VIRUS

Table 1 shows the rabies-specific proliferative responses of fox PBMC 182 days post-vaccination. Among the 32 vaccinated foxes, 24 showed significant proliferative responses to the purified SAD antigen (groups A–D, *P*=0.005–0.02), in spite of an individual variability of responses. Several foxes (A3, A5, B2, B7, B8, C1, D5 and D8) did not show any proliferative response. Furthermore, the responsiveness to virus in the primo-vaccinated foxes with either the SAG_2 or the V-RG did not significantly differ in magnitude. The booster vaccination did not significantly affect the proliferative memory responses to rabies virus in any fox of groups A–B and C–D. The reactivity of the T-cell level did not correlate with the neutralising antibody titres obtained 182 days post-vaccination (Table 1): some animals with high neutralising antibody titres (foxes B2, D2 and D4) showed relatively low or no virus-specific proliferative T-cell responses and, conversely, in sera of animals with high virus-specific proliferative T-cell responses (foxes C2 and C5), low neutralising antibody titres were found.

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

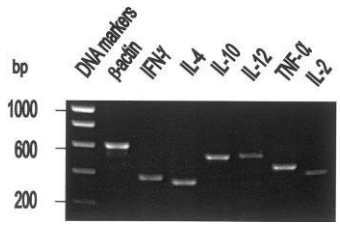

Fig. 3. Cytokine mRNAs expression in PBMC from a captive fox after 48 h of stimulation with Con A. PCR products b-actin, IFN-g, IL-4, IL-10, IL-12, TNF-a and IL-2 were analyzed on ethidium bromide-stained 1.0% agarose gel. For reference, corresponding base pair sizes of a 1 kb DNA marker are shown to the left.

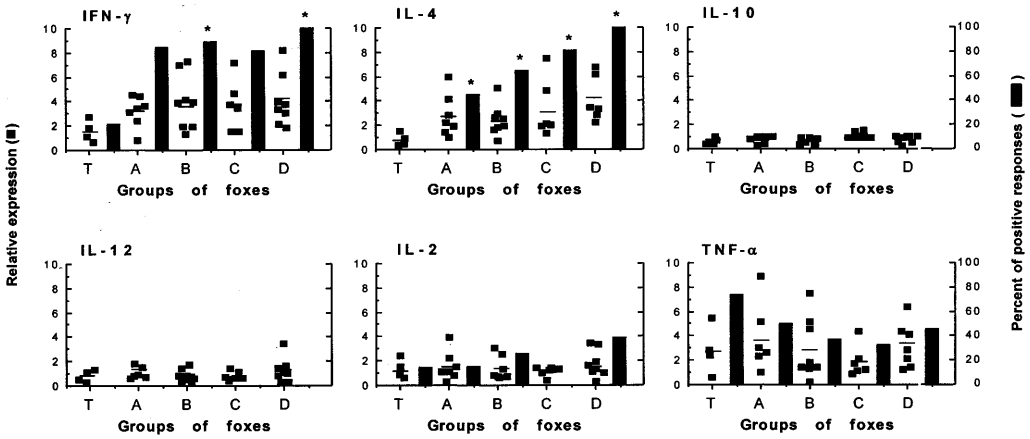

Fig. 4. Expression of IFN-g, IL-2, IL-4, IL-10, IL-12 and TNF-a in PBMC of vaccinated foxes to in vitro SAD antigen stimulation. After 48 h, cell cultures were treated for the detection of several cytokines mRNA using reverse transcriptase-polymerase chain reaction. The left ordinate represents the relative expression of the given cytokine in cells (square points) and the right ordinate represents the percentage of individual positive cytokine responses (columns). Each square point represents one fox, and the average of the expression level is indicated by a red bar. * P<0.05.

EXPRESSION OF VULPINE CYTOKINES UPON VACCINATION

The gene sequences of vulpine cytokines were not available in the literature when this study was begun. Therefore, we have assayed oligonucleotide consensus primers that have been successfully used to amplify canine, porcine, equine and primate mRNA of IFN-g, IL-12, IL-2, IL-4, IL-10 and TNF-a [27]. In an initial experiment, PBMC of an adult fox were cultured with the Concanavalin A (Con A) mitogen at a final concentration of 5 μg/ml for 48 h. Total RNA was then extracted, reverse-transcribed into cDNA and amplified by PCR using such consensus primer pairs. Reverse transcription (RT)-PCR with each cytokine mRNA were repeated in three experiments in order to check the consistency of results. As shown in Fig. 3, the RT-PCR products appear as single sharp bands and the estimated size of each respective product band corresponds to the predicted molecular weight expected from the published cytokine sequences. The absence of band with the samples in which the reverse transcriptase enzyme was omitted from the RT reaction demonstrated that only mRNA and no genomic DNA was specifically amplified (data not shown).

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

To determine the IL-2, IFN-g, IL-4, IL-10, IL-12 and TNF-a mRNA levels in blood cells following rabies vaccinations, PBMC isolated on day 182 from foxes of the five groups were first stimulated in vitro with the SAD antigen. Fig. 4 shows the data obtained from the RT-PCR experiments presented as the relative ratio of cytokine expression (left ordinate) and as the frequency of responders to SAD antigen in fox groups (right ordinate). The criteria for a positive response to stimulant was a value of cytokine level greater than the mean ± 3 S.D. of values in the mock-stimulated cultures of each group. We found that the relative expression level of IFN-y was two- to threefold higher in cell cultures of vaccinated foxes than in those of control animals. Up to 83–100% of foxes in the four rabies-immune groups produced IFN-g mRNA in response to in vitro stimulation with SAD antigen. However, a significant difference in the amount of IFN-g mRNA was only observed in foxes that had been boosted whatever the vaccine used (group B, P=0.04 and group D, P=0.01). In contrast, the average expression level of IL-4 mRNA was found to be significantly higher in all groups of animals primo- or secundo-vaccinated with either vaccine (P=0.01-0.03). The percentage of IL-4-positive responses rose to 43–100% after 2 days of culture. Inversely to the expression of IFN-g and IL-4, TNF-a mRNA was specifically induced by SAD antigen in cells of both control and vaccinated groups of foxes. The average expression levels were nearly related. Parallel RT-PCR assays using the IL-2, IL-10 and IL-12 primers revealed no specific mRNA production in PBMC of the majority of studied foxes except a slight difference, but not statistically significant, in the expression level of IL-2 in cells of vaccinated animals compared with the control ones after in vitro stimulation with the SAD antigen.

Discussion

The present study was conducted to assess the protective effect conferred to foxes by a booster vaccination 35 days after primary vaccination either with SAG_2 or V-RG oral vaccines. We have studied the kinetics of neutralising activity of experimental foxes and the induction of cellular immunity, focusing on in vitro proliferative response and cytokine production of vulpine PBMC. The present preliminary results show that, on a qualitative basis, the immune response to the SAG_2 vaccine is indistinguishable from that induced by the V-RG vaccine.

The antibody response to the first oral vaccination appeared to be induced with both vaccines, although the V-RG vaccine elicited a more rapid response than the SAG_2 vaccine. After the booster, there was evidence of priming with an increase of antibody levels typical of an anamnestic response. These results contrast with previous reports stating a long-term inhibition of the efficacy of revaccination in vaccinia-primed mice after intravenous injection of vaccinia viruses expressing a transgene product such as the glycoprotein of vesicular stomatitis virus or the nucleoprotein of lymphocytic choriomeningitis virus [28]. Since, in the present study, we performed vaccination by direct instillation into the oral cavity, the route of administration chosen for vaccination may be at the origin of this discrepancy. Further analysis of antibody responses 4 months later revealed, however, that antibody titres measured in boosted animals were comparable with those detected in foxes vaccinated once. A single dose of vaccine seemed to be sufficient not only to initiate the

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

production of memory B cells by the immune system of foxes, but also to maintain long-term memory cells and to provide protection against virus challenge.

The serological results obtained with captive foxes confirm those already observed in the field [11]: two successive oral vaccination campaigns using SAG_2 baits did not significantly improve the immunisation of foxes. In addition to neutralising antibodies, the induction of cell-mediated immunity might be an important parameter in rabies-specific protective response of foxes following vaccination with SAG_2 or V-RG vaccines. As the release of cytokines after rabies vaccination has been already studied in mouse [29] and human [30], the identification of such cytokines profiles from vulpine T lymphocytes was investigated. Foxes had significantly higher lymphoproliferative response to rabies virus post-vaccination whatever the vaccine used, as compared with unvaccinated controls. Most of them had sufficient memory cells 6 months later to be able to respond in vitro to the SAD parental strain. These results are, however, to be considered as preliminary data as the cell-mediated immune response has been studied with samples collected on one day only (day 182). Our observations suggest that both vaccines can stimulate cell-mediated immune mechanisms.

To follow-up cell-mediated response, consensus primers described by Rottman *et al*. [27] were used to measure the cytokine mRNA expression in PBMC of foxes after in vitro stimulation by SAD parental strain. It should be noted that the choice of the cytokine panel used in the study was limited because of the restricted availability of reagents for vulpine cytokines mRNA amplification. We demonstrated that PBMC of foxes immunised 6 months before with SAG_2 or V-RG vaccine significantly express IFN-g and IL-4, but little or no IL-2, IL-10 and IL-12 mRNA, when stimulated in vitro with the SAD parental antigen.

The range of responses observed after the primary vaccination was comparable with that evaluated in animals receiving a double oral administration of vaccine. The absence of detection of significant IL-2 mRNA levels in PBMC cultures of immunised foxes was surprising in view of previous studies that showed rabies-specific production of IL-2 by murine spleno cytes after in vitro stimulation with purified inactivated virus [29] or by blood lymphocytes of human vaccinated against rabies [30]. This lack of cytokine mRNA production observed in our conditions was not an artefact, as IL-2 mRNA was clearly detected in blood cells from vaccinated foxes stimulated with Con A. This discrepancy could be due to our experimental conditions, such as the route of administration or the type of vaccine (inactivated or not). The lack of IL-2 production by some human T-cell lines and clones reactive to rabies virus but that secreted IFN-g under the same conditions of antigen stimulation has been already documented [31] as a possible consequence of too limited a number of stimulated cells or too small an amount of IL-2, which is rapidly consumed by the T cells. Another possible explanation for the absence of IL-2 detection could be the weak sensitivity of our technique.

Although the biological activities of IFN-g, IL-4 and TNF-a are well studied in both mouse and human, only few data are known about their activities in foxes and even in similar species such as dog, mainly due to the lack of specific tools. Several studies have described large amounts of IFN-g induced in vivo by rabies vaccines early after the primary vaccination, pointing out the important

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

antiviral role played by this cytokine as a first-line defence mechanism of the organism (for a review, see King and Turner [32]). This cytokine was also produced in vitro by antigen-specific T cells from human recipients of rabies vaccine or by splenocytes of primed mice cultured in the presence of rabies antigenpresenting cells [31,33]. Therefore, a strong IFN-g expression in PBMC of boosted foxes with SAG_2 or V-RG vaccines may be not only an indicator of a T-cell response, but also a mean to evaluate the capability of the vaccinated foxes to control rabies infection. The significance of the concomitant expression of both IL-4 and IFN-g after in vitro stimulation with rabies anti- gens in PBMC of vaccinated foxes remain to be determined.

In summary, we have shown in this paper that humoral immune responses to oral vaccination with SAG_2 or V-RG vaccines is not significantly increased when repeating vaccination 35 days later. Our preliminary data on cell immunity yields the same conclusion. These results could suggest that only one distribution of baits is necessary in field conditions during each oral rabies fox vaccination campaign.

To our knowledge, although preliminary, this is the first study that simultaneously evaluate rabies vaccine-induced antibody response, PBMC proliferation and cytokine expression in foxes. Further studies will be needed to confirm our results by collecting more biological samples and determining the relative importance of the T-dependent cytokines identified in regulating the fox immune response to rabies virus.

Acknowledgements

The authors thank Dr Anne Flamand (CNRS 91198 Gif sur Yvette Cedex, France), who generously provided the SAD strain used in this work, and Dr Pascale Hubert for supportive interactions and critical review of the manuscript. The authors also thank the staff of the laboratory of the AFFSA Nancy for their excellent technical assistance. They are grateful to Dr Noël Tordo and Dr Pierre Perrin (Institut Pasteur, Paris, France) for helpful suggestions. Financial support for this work was provided by the European Community Commission (contract FAIR CT97-3515).

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

References

[1] Wandeler AI. Oral immunization of wildlife. In: Baer GM, editor. The Natural History of Rabies, 2nd ed. Boca Raton, FL: CRC Press, 1991:485–503.

[2] Artois M, Guittré C, Thomas I, Leblois H, Brochier B, Barrat J. Potential pathogenicity for rodents of vaccines intended for oral vaccination against rabies: a comparison. Vaccine 1992; 10:524–8.

[3] Masson E, Cliquet F, Aubert M, Barrat J, Aubert A, Artois M, Schumacher CL. Safety study of the SAG_2 rabies virus mutant in several non-target species with a view to its future use for the immunisation of foxes in Europe. Vaccine 1996; 14:1506–10.

[4] Pastoret PP, Brochier B, Blancou J, Artois M, Aubert MFA, Kiény MP, Lecocq JP, Languet B, Chappuis G, Desmettre P. Development and deliberate release of a vaccinia-rabies recombinant virus for the oral vaccination of foxes against rabies. In: Smith GL, Binns MM, editors. Recombinant Poxviruses. Boca Raton, FL: CRC Press, 1992:163–206.

[5] Flamand A, Coulon P, Lafay F, Tuffereau C. Avirulent mutants of rabies virus and their use as live vaccine. Trends Microbiol 1993; 18:317–20.

[6] Kieny MP, Lathe R, Drillen R, Spehner S, Skory D, Schmitt T, Wiktor T, Koprowski H, Lecocq JP. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 1984; 312:163–6.

[7] Schumacher CL, Coulon P, Lafay F, Benejean J, Aubert MFA, Barrat J, Aubert A, Flamand A. SAG-2 oral rabies vaccine. Onderstepoort J Vet Res 1993; 60:459–62.

[8] Blancou J, Kiény MP, Lathe R, Lecocq JP, Pastoret PP, Soulebot JP, Desmettre P. Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. Nature 1986; 322:373–5.

[9] Müller WW. Oral vaccination and high density fox population. Rabies Bull Eur 1995; 19:14–5.

[10] Bruyère V, Lemoine S, Aubert M, Melik N. La vaccination orale des renards contre la rage en 1997 nous concentrons nos efforts sur les zones plus exposées. Bull Epidémiol Mens Rage France 1996; 26:1–2.

[11] Bruyère V, Vuillaume P, Cliquet F, Aubert MFA. Oral rabies vaccination of foxes with one or two delayed distributions of SAG_2 baits during the spring. Vet Res 2000; 31:339–45.

[12] Vuillaume P, Bruyère V, Aubert M. Comparison of the effectiveness of two protocols of antirabies bait distribution for foxes (*Vulpes vulpes*). Vet Res 1998; 29:537–46.

[13] Masson E, Bruyère-Masson V, Vuillaume P, Lemoyne S, Aubert M. Rabies oral vaccination of foxes during the summer with the V-RG vaccine bait. Vet Res 1999; 30:595–605.

[14] Dietzschold B, Wang H, Rupprecht CE, Celis E, Tollis M, Ertl H, Heber-Katz E, Koprowski H. Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc Natl Acad Sci USA 1987; 84:9165–9.

[15] Sharpee RL, Nelson LD, Beckenhauer WH. Inactivated tissue culture rabies vaccine with three years immunogenicity in dogs and cats. In: Kuwert E, Merieux C, Koprowski H, Bögel K, editors. Rabies in the Tropics. Berlin: Springer-Verlag, 1985:262–73.

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

[16] Zanetti CR, de Franco MT, Vassào RC, Pereira CA, Pereira OAC. Failure of protection induced by a Brazilian vaccine against Brazilian wild rabies viruses. Arch Virol 1998; 143:1745–56.

[17] Xiang ZQ, Knowles BB, McCarrick JW, Ertl HCJ. Immune effector mechanisms required for protection to rabies virus. Virology 1995; 214:398–404.

[18] Celis E, Dietzschold B, Hoffman K, Koprowski H. Specificity and genetic of human T lymphocytes reactive with rabies virus. In: Thraenhart O, *et al*., editors. Progress in Rabies Control. Kent: Wells Medical Limited, 1989:231–73.

[19] Janot C, Blancou J, Aubert MFA. Immunité à médiation cellulaire du renard vacciné contre la rage. Comp Immun Infect Dis 1982; 5:1–3.

[20] Préhaud C, Coulon P, Lafay F, Thiers C, Flamand A. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J Virol 1988; 62:1–7.

[21] Blancou J, Artois M, Brochier B, Thomas I, Pastoret PP, Desmettre P, Languet B, Kiény MP. Innocuité et efficacité d'un vaccin antirabique recombinant des virus de la vaccine et de la rage administré par voie orale au renard, au chien et au chat. Ann Rech Vét 1989; 20:195–204.

[22] Aubert MFA, Blancou J, Barrat J, Artois M, Barrat MJ. Transmission et pathogénie chez le renard roux de deux isolats à dix ans d'intervalle du virus de la rage vulpine. Ann Rech Vét 1991; 22:77–93.

[23] Aubert MFA. Methods for the calculation of titres. In: Meslin FX, Kaplan M, Koprowski H, editors. Laboratory Techniques in Rabies, 4th ed. Geneva: World Health Organization, 1996: 445–59.

[24] Cliquet F, Aubert M, Sagné L. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods 1998; 212:79–87.

[25] World Health Organization. Expert committee on rabies. 8th Report, Technical Report Series 824. Geneva: World Health Organization, 1992.

[26] Letesson JJ, Lostrie N, Depelchin A. Production d'anticorps monoclonaux spécifiques d'isotypes d'immunoglobulines bovines. Ann Med Vet 1985; 129:131–41.

[27] Rottman JB, Freeman EB, Tonkonogy S, Tompkins MB. A reverse transcription-polymerase chain reaction technique to detect feline cytokine genes. Vet Immunol Immunopathol 1995; 45:1–18.

[28] Kündig TM, Kalberer CP, Hengartner H, Zinkernagel RM. Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination. Vaccine 1993; 11:1154–8.

[29] Joffret ML, Zanetti C, Morgeaux S, Leclerc C, Sureau P, Perrin P. Appraisal of rabies vaccine potency by determination of in vitro, specific interleukin-2 production. Biologicals 1991; 19:113–23.

[30] Perrin P, Joffret ML, Zanetti C, Bourhy H, Gontier C, Fritzell C, Leclerc C, Sureau P. Rabies-specific production of interleukin-2 by peripheral blood lymphocytes from human rabies vaccinees. Vaccine 1991;9:549–58.

[31] Celis E, Miller RW, Wiktor TJ, Dietzschold B, Koprowski H. Isolation and characterization of human T cell lines and clones reactive to rabies virus: antigen specificity and production of interferon-? J Immunol 1986;136:692–7.

[32] King AA, Turner GS. Rabies: a review. J Comp Pathol 1993; 108:1–39.

Published in : Vaccine (2001), vol. 19, n°13-14, pp. 1827–1835
DOI: 10.1016/s0264-410x(00)00321-2
Status : Postprint (Author's version)

[33] Perrin P, De Franco T, Jallet C, Fouque F, Morgeaux S, Tordo N, Colle JH. The antigen-specific cell-mediated immune response in mice is suppressed by infection with pathogenic lyssaviruses. Res Virol 1996;147:289–99.