

Effect of maternal immunity on the immune response to oral vaccination against rabies in young foxes

Thomas F Müller, DVM; Peter Schuster, DVM; Ad C. Vos, PhD; Thomas Selhorst, PhD; Ulf D. Wenzel, DVM; Andreas M. Neubert, DVM

Objective—To determine effect of maternal antibodies on immune response to oral vaccination against rabies in young foxes.

Animals—250 cubs from 48 vixens.

Procedure—Sera were obtained from cubs of 36 vaccinated (maternally vaccinated [MV⁺]) and 12 nonvaccinated (MV⁻) vixens between 23 and 71 days of age and tested for neutralizing antibodies. Seventy-one MV⁺ cubs and 33 MV⁻ cubs were vaccinated orally with modified-live virus vaccine SAD B19. Geometric mean titer (GMT) was determined in these cubs approximately 21, 39, and 57 days after vaccination. In a subsequent experiment, 10 vaccinated MV⁺ cubs, 6 vaccinated MV⁻ cubs, and 6 control cubs were challenge inoculated with virulent rabies virus approximately 100 days after vaccination.

Results—Serum GMT of nonvaccinated MV⁻ cubs (0.23 U/ml) was significantly greater than that of nonvaccinated MV⁻ cubs (0.15 U/ml). The GMT of vaccinated MV⁺ cubs 21, 39, and 57 days after vaccination were 2.85, 2.11, and 0.79 U/ml, respectively, and were significantly less than those of vaccinated MV⁻ cubs (12.19, 6.76, and 4.02 U/ml, respectively). All challenge-inoculated cubs with GMT < 0.5 U/ml succumbed to rabies.

Conclusion and Clinical Relevance—Partially impaired immune response in cubs < 8 weeks old from vaccinated vixens causes insufficient protection against rabies. Inhibition of the immune response persists longer than the period during which maternal antibodies are detectable. Thus, oral vaccination campaigns for young foxes in areas where vaccination has been performed need to be reconsidered. (*Am J Vet Res* 2001;62:1154–1158)

Oral vaccination programs against rabies in foxes have been successfully used to decrease the incidence of rabies substantially in participating countries in Europe.^{1,2} However, in some areas, eradication of rabies has proven more complicated and protracted

than originally estimated.² Several factors have been identified as possible causes for these setbacks. For example, the low vaccination coverage of fox cubs was considered to be the main factor contributing to the maintenance of rabies in the Swiss Jura.³ Hence, in Switzerland and several other countries, an alternative vaccination strategy was implemented by placing baits at fox dens in early summer to improve the vaccination coverage of these animals.⁴⁻⁶ Substantially increased bait uptake by the target subpopulation, fox cubs, was observed after distribution of vaccine baits at den entrances. However, the expected benefit was not supported by serologic findings, and it was assumed that maternally transferred immunity may have interfered with the active immunization of the fox cubs.⁵

In Europe, vaccination campaigns are generally performed twice a year, in fall and spring.^{1,7} Although it is assumed that, in areas where vaccination has been attempted, most of the cubs are born to immunized vixens, no experimental data on maternally acquired immunity and its influence on the induction of a specific immune response after vaccination against rabies in foxes is available to support this assumption. From a few experimental and epidemiologic studies with dogs and mice, it is known that vaccination of dams against rabies results in transfer of maternal antibodies (maAb) to the offspring.^{8,9}

The immune response in fox cubs is the result of the ontogenic development of the immune system and possible maternally transferred immunity. The presence of maAb against rabies in the juvenile fox population is of practical relevance because maAb may influence the induction of a specific immune response after vaccination. Also, European authorities require that experiments to determine this response be performed for every vaccine intended for registration.¹⁰ Because there is a paucity of information regarding this subject, the purposes of the study reported here were to experimentally determine the influence of maternally transferred immunity on the immune response of young foxes after oral vaccination against rabies and determine whether these foxes were sufficiently protected against a relevant challenge.

Material and Methods

Maternal antibodies—To determine the concentration of maAb, 48 captive-bred litters comprising 250 cubs were examined in 1998 and 1999. Two sets of cubs were used; 185 cubs were whelped by 36 vixens that had been vaccinated orally with modified-live virus vaccine SAD B19⁹ shortly before mating or during early pregnancy, and 65 cubs were

Received Mar 21, 2000.

Accepted Aug 3, 2000.

From the Institute for Epidemiological Diagnostics, Institute for Epidemiology, Federal Research Centre for Virus Diseases of Animals, WHO Collaborating Centre for Rabies Surveillance and Research, 16868 Wusterhausen, Germany (Müller, Selhorst); Impfstoffwerk Dessau-Tornau GmbH, PO Box 214, 06855 Rosslau, Germany (Schuster, Vos, Neubert); and Fur Animal Breeding Station, Nerzfarm Gleinermühle, 06774 Söllichau, Germany (Wenzel).

The authors thank Jeanette Burow, Astrid Schameitat, Elke Pommerening, Kathrin Teske, and Doris Balan for technical assistance.

whepled by 12 vixens that were seronegative for rabies and were not vaccinated. Henceforth, cubs born from vaccinated and unvaccinated vixens will be referred to as maternally vaccinated MV⁺ and MV⁻ cubs, respectively. Blood samples were collected from the cubs on fixed dates with mean age at sampling of 45 and 42 days for MV⁺- and MV⁻-cubs, respectively. From several cubs (n = 47 [33 MV⁺ and 14 MV⁻]) born in 1999, a second blood sample was collected 21 days after the first blood sample. Consequently, age of cubs used to determine maAb concentrations ranged from 23 to 71 days.

Immune response—To test the effect of maternally transferred immunity on the development of the immune response after vaccination against rabies, only cubs born in 1999 were used. All cubs and vixens were marked individually by use of electronic identification.^b Each litter was divided into 2 groups in order to obtain data from a wide age spectrum (23 to 71 days); the first group was vaccinated at a young age (23 to 48 days), and the second group was vaccinated approximately 3 weeks later. Seventy-one MV⁺ cubs and 33 MV⁻ cubs received 1.5 to 2.0 ml (10^{6.7} foci formatting units/ml) of SAD B19 by direct oral instillation. Immediately after vaccination, the vaccinated cubs were kept separated from their littermates for 2 hours to prevent possible horizontal transmission of the vaccine virus. Blood samples were collected immediately before vaccination and at a mean of 21, 39, and 57 days after vaccination.

Challenge inoculation—Ten and 6 MV⁺ and MV⁻ cubs, respectively, vaccinated between 23 and 54 days of age, were selected for challenge inoculation, together with 6 control cubs (nonvaccinated cubs of corresponding age and born to nonvaccinated vixens). Challenge exposure was done by IM administration of 1 ml (10^{3.3} tissue culture infective dose [TCID₅₀]) of rabies virus in the masseter muscle, 100 days (mean value) after vaccination. For vaccination, the cubs were anesthetized with 1 ml mixture of

ketamine hydrochloride (115.34 mg/ml)^c and xylazine hydrochloride (20 mg/ml).^d The challenge virus (CVS/USA/TX coyote/295/R/061893)^e was isolated from the salivary glands of a rabid coyote (*Canis latrans*) and was selected because of its high pathogenicity (100% mortality of control animals).¹¹ Furthermore, a vaccine intended for use in wildlife should be effective against all variants of rabies virus serotype 1.

Cubs that had clinical signs of rabies were euthanized by administration of 1 ml of a barbiturate solution (105 mg/ml).^f The surviving cubs were euthanized 76 days after the last cub succumbed to rabies. To prove the presence of viral antigen in all challenge-inoculated cubs, the brain was collected immediately after death for fluorescent antibody testing. A blood sample was collected at the end of the challenge test from all surviving cubs. Animal experimentation was performed according to the German Animal Welfare Act (Tierschutzgesetz) of May 25, 1998. Experimental design of the challenge test was approved by the responsible German authorities.

Diagnostic tests—Serum samples were evaluated by use of the rapid fluorescence focus inhibition test as described by Smith et al,¹² with modifications of that method as described by Cox and Schneider.¹³ Prior to testing, sera were heat-inactivated for 30 minutes at 56 C. To calculate the titer, a 50% reduction in concentration of rabies virus in vitro was calculated by use of inverse interpolation. The rabies virus neutralizing antibody (nAb) titers were converted to international units by comparison with international standard immunoglobulin⁸ and adjusted to 0.5 U/ml, which served as a positive control.¹⁴ Detection of rabies virus antigen was done by use of a fluorescent antibody test (FAT), as described.¹⁵

Statistical analyses—For MV⁺ and MV⁻ cubs, relationships between age and nAb titers related to maternally transferred immunity were analyzed by use of linear regression.¹⁶

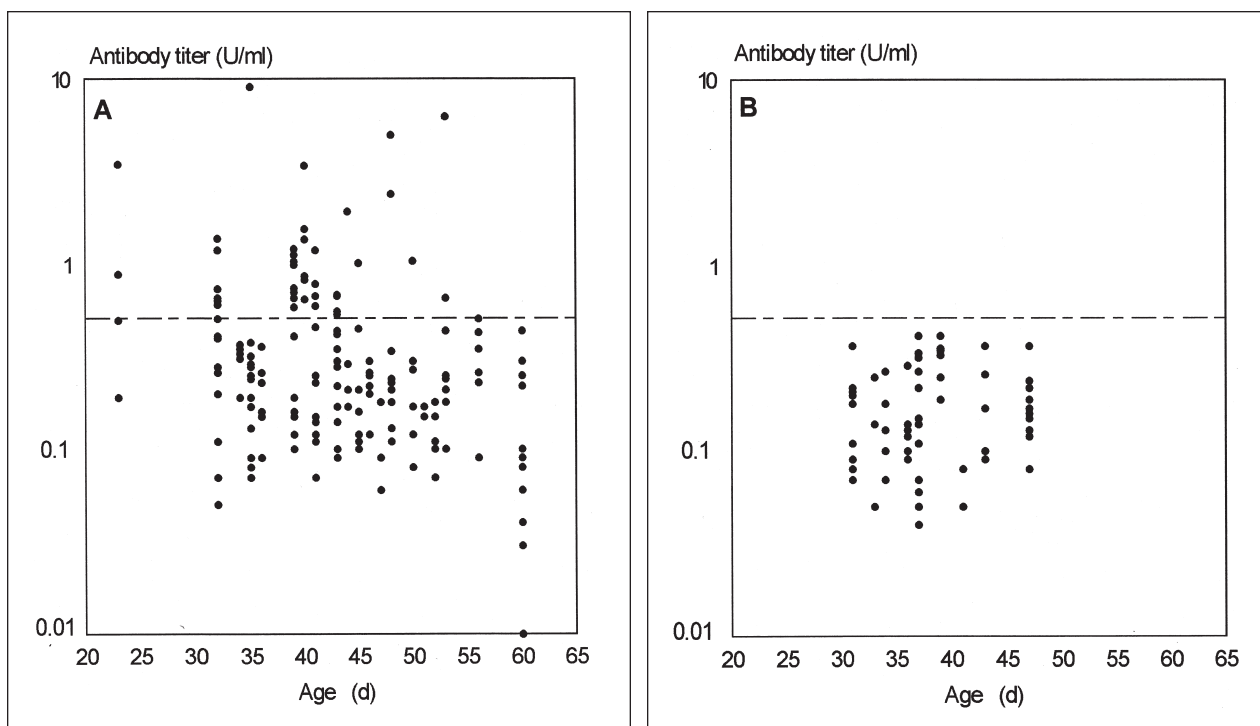


Figure 1—Rabies virus serum-neutralizing antibody titers (geometric means) determined at various ages in fox cubs of vaccinated (A) or unvaccinated (B) vixens. Dotted line at 0.5 U/ml indicates value of a standardized positive control sample.

Prior to analysis, data were \log_{10} -transformed to achieve normal distribution. Additionally, 95% confidence limits of the regression analysis were calculated. By use of this approach, differences between groups in regression lines are significant if the 95% confidence limits do not intersect. A Student *t*-test or a Tukey test following an ANOVA was used to compare differences between geometric mean titers (GMT) of MV⁺ and MV⁻ cubs.¹⁷

Results

Maternal antibodies—Prior to whelping, GMT of the vaccinated vixens (21 days after vaccination) and the unvaccinated vixens were 7.07 (*n* = 36) and 0.15 U/ml (12), respectively. The maAb GMT of MV⁺ and MV⁻ cubs (23 to 71 days) were 0.23 (*n* = 218) and 0.15 U/ml (78), respectively (*P* < 0.001). An exact titer was not determined for 1 MV⁻ cub born in 1998; therefore, this cub was omitted from further statistical analysis. In contrast to MV⁻ cubs, individual nAb titers of the MV⁺ cubs had considerable deviation (Fig 1). When compared with the international standard, only 44 of 218 (19.8%) blood samples from MV⁺ cubs had individual nAb titers ≥ 0.5 U/ml. During the first 7 weeks of life, MV⁺ cubs had nAb titers that were significantly higher than that of the MV⁻ cubs (Fig 2). Furthermore, a significant (*P* < 0.001) decrease of the nAb titers of MV⁺ cubs was observed from 23 to 68 days of age. Significant differences in nAb titers were not detected between MV⁺ and MV⁻ cubs from day 51 onwards.

Immune response—In blood samples obtained approximately 21, 39, and 57 days after vaccination, GMT for all vaccinated MV⁺ cubs (*n* = 71) were 2.85, 2.11, and 0.79 U/ml, respectively, and for all vaccinated MV⁻ cubs (33) were 12.19, 6.76, and 4.02 U/ml, respectively. For all 3 samples, GMT of the MV⁺ cubs was significantly (*P* < 0.001) lower than that of the MV⁻ cubs. Immune response patterns of the vaccinated MV⁺ and MV⁻ cubs, in relation to their age at the time of vaccination, were markedly different. The MV⁻ cubs that were vaccinated at 5 weeks of age developed GMT above the threshold of 0.5 U/ml; these cubs had lower GMT than MV⁻ cubs that were vaccinated at older ages, although this difference was not significant (Fig 3). The MV⁺ cubs that were vaccinated at 4 to 7 weeks of age had a significantly (*P* < 0.001) lower immune response than cubs that were vaccinated at an older age (Fig 4). The MV⁺ cubs that were vaccinated at ≥ 8 weeks of age had strong induction of nAb after vaccination, and the GMT remained high, compared with cubs that were vaccinated at a younger age. No significant difference in immune response was observed between MV⁺ and MV⁻ cubs that were vaccinated at ≥ 8 weeks of age.

Challenge inoculation—Seven of 10 MV⁺ cubs and 1 of 6 MV⁻ cubs died from rabies at a mean of 13 days after inoculation, as confirmed by use of the FAT. Also, all 6 control cubs developed typical clinical signs of rabies and died from rabies or were euthanatized 12 days after infection. Brains of cubs that survived the challenge inoculation yielded negative results for

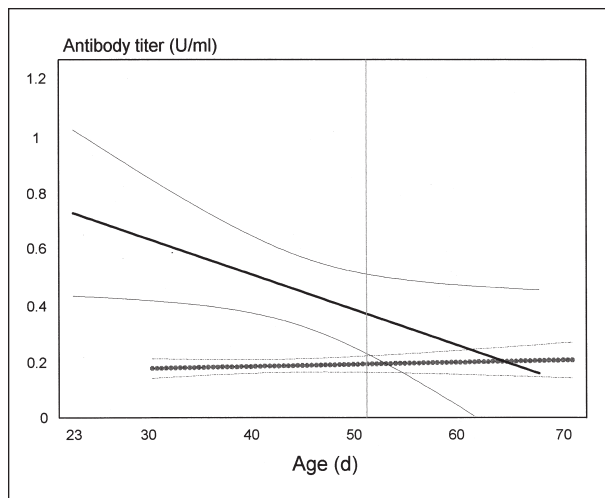


Figure 2—Results of linear regression analysis of rabies virus serum-neutralizing antibody titers (geometric means \pm 95% confidence intervals) determined at various ages in fox cubs of vaccinated (solid lines) or unvaccinated (dotted lines) vixens.

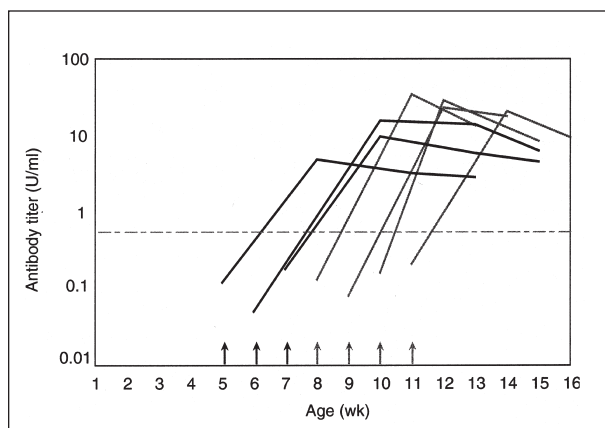


Figure 3—Immune response (geometric mean titers) in fox cubs of unvaccinated vixens. Cubs were orally vaccinated against rabies at early (dark arrows) or late (shaded arrows) ages. Dotted line at 0.5 U/ml indicates value of a standardized positive control sample.

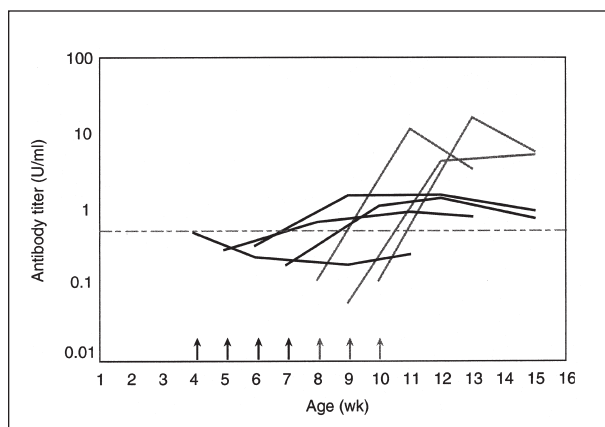


Figure 4—Immune response (geometric mean titers) in fox cubs of vaccinated vixens. Cubs were orally vaccinated against rabies at early (dark arrows) or late (shaded arrows) ages. Dotted line at 0.5 U/ml indicates value of a standardized positive control sample.

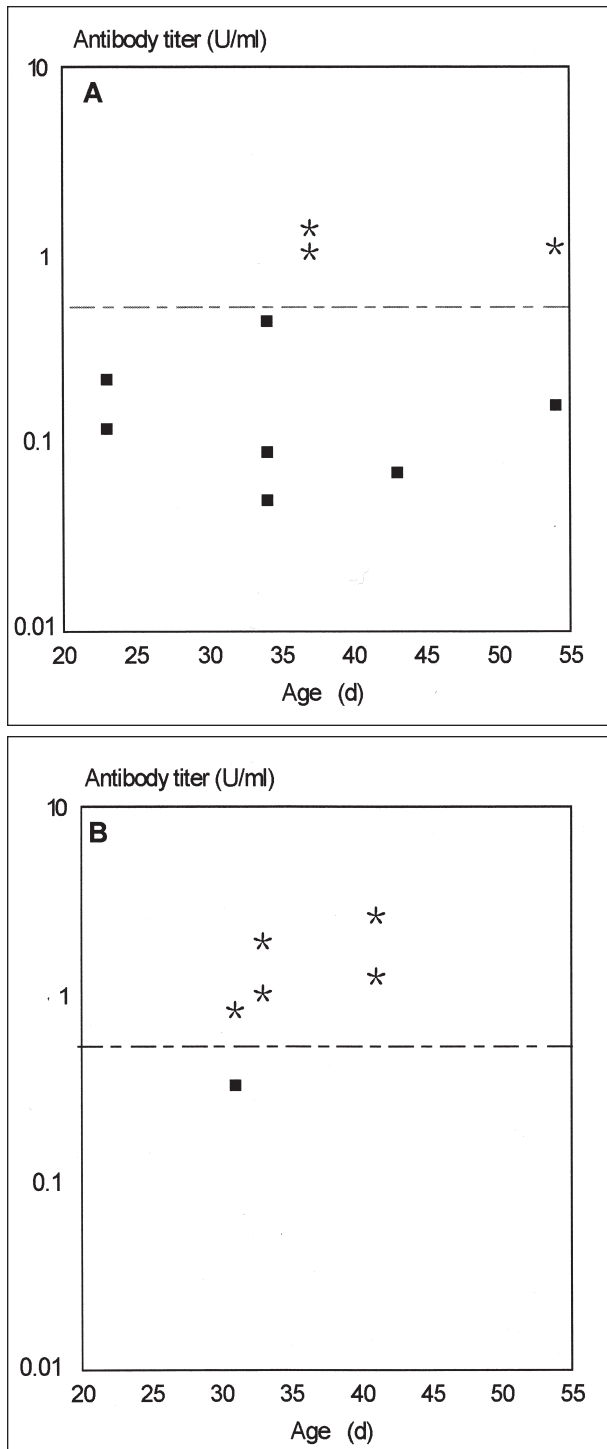


Figure 5—Serum-neutralizing antibody titers against rabies virus prior to challenge vaccination (100 days after vaccination) with virulent rabies virus in fox cubs of vaccinated (A) and nonvaccinated (B) vixens. Squares = Vaccinated cubs that survived. Stars = Vaccinated cubs that developed rabies. Dotted line at 0.5 U/ml indicates value of a standardized positive control sample.

rabies via FAT. All cubs that developed nAb titer of 0.5 U/ml or greater prior to challenge inoculation survived (Fig 5).

Discussion

In mammals, antibodies are transferred from the dam to the offspring through the placenta and via colostrum. The relative importance of each route varies among species and is related to placental cyto-architecture.¹⁸ Foxes, like dogs, have an endotheliochorial placenta, and most of the maAb transfer occurs by means of intestinal absorption of colostrum. The period during which the intestine is permeable to proteins varies among species and, to a degree, by immunoglobulin class. In general, permeability is highest immediately after birth and declines rapidly within 24 hours because of maturation of intestinal cells and establishment of intestinal flora.¹⁹

In our study, maAb at concentrations ≥ 0.5 U/ml, an arbitrarily defined threshold indicative of protection against rabies infection, were detected only in a few cubs whelped by vixens that had been vaccinated against rabies (Fig 1).¹⁴ Several MV⁻ cubs also had titers close to 0.5 U/ml; however, because nonspecific serum factors may mimic low-concentration rabies nAb titers, measurement below this threshold is difficult.

The relatively low or absent (< 0.5 U/ml) nAb titers observed in some MV⁺ cubs could be a result of the fact that maAb had already disappeared at the time of sampling or that they are not always detectable by use of seroneutralization tests. For example, in dogs, concentrations of maAb 30 days after ingestion are 1 to 3% of their initial value.¹⁹ Unfortunately, blood samples could not be collected from the fox cubs during the first 3 weeks after parturition because of the extreme susceptibility of vixens to disturbance. The youngest cubs sampled were 23 days old. At that time, the maAb titers had most probably already decreased to low concentrations, compared with the initial concentrations shortly after birth.

To the authors' knowledge, this study is the first to clearly reveal partially impaired immune response in fox cubs < 8 weeks of age born to vaccinated vixens. On the basis of our results, there is evidence that inhibition of active immunization exceeded the period during which maAb were present at detectable concentrations. This is in accordance with results obtained in mice and puppies.^{20,21} Interference caused by maAb is thought to be mediated by several mechanisms, including neutralization of the vaccine by antibodies, induction of tolerance in naive B-cells by binding of complexes formed between maAb and the vaccine virus, and a putative suppressive mechanism induced in cubs by maternally transferred immune effectors.²⁰ Small amounts of antibodies, well below the detectable concentration (< 0.5 U/ml), may influence activation of the immune response by various mechanisms.⁹ Apart from maAb, other soluble factors and the cellular components of colostrum may impair the immune response.²² Hence, it seems that the response to vaccination is a more sensitive indication of the presence of maternal transferred immunity than are results of *in vitro* serologic tests.¹⁸ Also, an immune system that is incompletely developed at birth may result in vaccination failures. In our study, 5-week-old MV⁻ cubs responded to oral vaccination with nAb titers greater than the threshold of 0.5 U/ml, indicating that they

were fully immunocompetent; however, these cubs did not respond as well as older MV⁺ cubs. Concerning safety aspects linked to oral vaccination of foxes, results of this study clearly indicate that the use of the modified-live virus vaccine SAD B19 is completely safe for young foxes, as described.²³ None of the fox cubs died from vaccine virus-induced rabies, and adverse reactions were not observed.

Generally, variability of maAb concentrations between and within litters makes it quite difficult to determine the age at which young animals can be successfully immunized. However, significant differences in nAb titers between nonvaccinated MV⁺ and MV⁻ cubs did exist until 51 days of age. This is supported by the fact that a difference was not detected between groups in response to vaccination at 8 weeks of age or older.

The relevant question is: are vaccinated fox cubs effectively protected against rabies? Our results were inconclusive; most vaccinated MV⁺ cubs (70%) and 1 vaccinated MV⁻ cub (16%) succumbed to rabies during the challenge-inoculation experiment. However, all cubs that developed nAb titers > 0.5 U/ml after vaccination, irrespective of the immune status of the dam, survived challenge inoculation, whereas all cubs with titers < 0.5 U/ml succumbed to rabies. The amount of virus and pathogenicity associated with natural infection would most probably be much lower than was used in the experimental conditions reported here.

Our results suggest that maternally transferred immunity in fox cubs may interfere with the development of protective immunity induced by rabies vaccination. Fox cubs < 8 weeks of age born to vaccinated vixens have partially impaired immune responses that result in insufficient protection against rabies. This inhibition of the immune response exceeds the period during which maAb are present at detectable concentrations. In contrast, cubs of naive vixens may be successfully vaccinated by the oral route at a relatively early age (5 weeks) and are protected against relevant challenge.

In young foxes, the influence of maAb on development of the immune response after active oral vaccination is of practical relevance, because den baiting, double vaccination (2 vaccination campaigns within a 2- to 3-week period), and vaccination during early summer have been discussed as alternative strategies^{5,7} after recent set-backs in oral vaccination campaigns.^{1,2} The timing of vaccination campaigns, such as den baiting or vaccination during early summer, intended to immunize the young fox population in areas where vaccination has been undertaken previously, should be adjusted accordingly to avoid potential interference caused by maAb.

[†]Impfstoffwerk Dessau-Tornau GmbH, Roblau, Germany.

[‡]Indexel Iso transponder, Rhone Merieux GmbH, Laupheim, Germany.

[§]Ketamin 10%, Intervet GmbH, Toenisvorst, Germany.

[¶]Rometar 2%, Serumwerk BernburgAG, Bernburg, Germany.

^{**}Provided by the Centers for Disease Control, Atlanta, Ga.

^{††}Eunaron, Parke Davis, Freiburg, Germany.

^{‡‡}2nd human rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK.

References

1. Stöhr K, Meslin FX. Oral vaccination of wildlife in Europe. In: Dodet B, Meslin FX, eds. *Rabies control in Asia*. Paris: Elsevier, 1996;2–34.
2. Stöhr K, Meslin FX. Progress and setbacks in the oral immunisation of foxes against rabies in Europe. *Vet Rec* 1996;139:32–35.
3. Breitenmoser U, Kaphegyi T, Kappeler A, et al. Significance of young foxes for the persistence of rabies in northwestern Switzerland, in *Proceedings*. 3rd Congr Eur Soc Vet Virol 1995; 391–396.
4. Breitenmoser U, Zanoni R. An adapted concept for the elimination of sylvatic rabies in Switzerland. *Rabies Bull Eur* 1995; 19(4):13–16.
5. Vuillame P, Bruyere V, Aubert M. Comparison of the effectiveness of two protocols of antirabies bait distribution for foxes (*Vulpes vulpes*). *Vet Res* 1998;29:537–546.
6. von Schloss A. Darstellung der Tollwutsituation in Nordrhein-Westfalen-1995; Auftreten und Bekämpfungsstrategie. *Tierärztl Umsch* 1997;52:540–564.
7. Müller T, Schlüter H. Oral immunization of red foxes (*Vulpes vulpes*) in Europe—a review. *J Etlik Vet Microbiol* 1998;9:35–59.
8. Winters WD. Time dependent decreases of maternal canine virus antibodies in newborn pups. *Vet Rec* 1981;108:295–299.
9. Xiang ZQ, Ertl HCJ. Transfer of maternal antibodies results in inhibition of specific immune responses in the offspring. *Virus Res* 1992;24:297–314.
10. EU-directive 92/18/EEC on analytical, toxicologic-pharmaceutical and veterinary or clinical regulations and proofs for experiments with animal medicaments, European Community, Brussels, Belgium, March 20, 1992.
11. Office Internationale des Epizooties. *Manual of standards for diagnostic tests and vaccines*. 3rd ed. Paris: World Organisation for Animal Health 1996;207–217.
12. Smith JS, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. *Bull World Health Organ* 1973;48:535–541.
13. Cox JH, Schneider LG. Prophylactic immunization of humans against rabies by intradermal inoculation of human diploid cell culture vaccine. *J Clin Microbiol* 1976;3:96–101.
14. World Health Organization. WHO/IABS Developments in biological standards. Symposium on the standardization of rabies vaccines for human use produced in tissue culture (rabies III) 1978;40:268–270.
15. Dean DJ, Abelseth MK, Athanasiu P. The fluorescence antibody test. In: Meslin FX, Kaplan MM, Koprowski H, eds. *Laboratory techniques in rabies*. 4th ed. Geneva: World Health Organization, 1996;88–93.
16. Sokal FJ, Rohlf FJ. *Biometry*. 3rd ed. New York: WH Freeman & Co, 1995;887.
17. Zöfel E. *Statistik in der Praxis*, 2. Stuttgart, Germany: Auflage Gustav Fisher Verlag, 1988;426.
18. Pollock RVH, Carmichael LE. Maternally derived immunity to canine parvovirus infection: transfer, decline, and interference with vaccination. *J Am Vet Med Assoc* 1982;180:37–42.
19. Chappuis G. Neonatal immunity and immunisation in early age. *Vaccine* 1998;16:1468–1472.
20. Aghomo HO, Oduye OO, Rupprecht CE. The serological response of young dogs to the Flury LEP strain of rabies virus vaccine. *Vet Res Commun* 1990;14:415–425.
21. Wang Y, Xiang Z, Pasquini S, Ertl HCJ. Immune response to neonatal genetic immunization. *Virology* 1997;228:278–284.
22. Bianchi A. Silent memory induction in maternal immune young animals. *Vet Q* 1998;20:89–92.
23. Vos A, Neubert A, Aylan O, et al. An update on safety studies of SAD B19 rabies virus vaccine in target and non-target species. *Epidemiol Infect* 1999;123:165–175.