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ANTIBODY RESPONSE OF SANDHILL AND WHOOPING CRANES TO AN EASTERN EQUINE ENCEPHALITIS VIRUS VACCINE

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ABSTRACT: As a possible strategy to protect whooping cranes (*Grus americana*) from fatal eastern equine encephalitis (EEE) viral infection, studies were conducted to determine the immune response of this species and sandhill cranes (*Grus canadensis*) to a formalin-inactivated EEE viral vaccine. Viral-specific neutralizing antibody was elicited in both species after intramuscular (IM) vaccination. Subcutaneous and intravenous routes of vaccination failed to elicit detectable antibody in sandhill cranes. Among the IM vaccinated cranes, the immune response was characterized by nondetectable or low antibody titers that waned rapidly following primary exposure to the vaccine. However, one or more booster doses consistently elicited detectable antibody and/or increased antibody titers in the whooping cranes. In contrast, cranes with pre-existing EEE viral antibody, apparently induced by natural infection, exhibited a rapid increase and sustained high-antibody titers. Even though EEE virus vaccine induced neutralizing antibody and produced no adverse side effects, further studies will be required to determine the protective efficacy of the antibody.

Key words: Sandhill crane, *Grus canadensis*, whooping crane, *Grus americana*, eastern equine encephalitis virus vaccine, neutralizing antibody, immunology.

INTRODUCTION

An unprecedented outbreak of fatal eastern equine encephalitis (EEE) viral infection occurred among captive whooping cranes (*Grus americana*) during the late summer and fall of 1984 at the Patuxent Wildlife Research Center (PWRC, Laurel, Maryland 20708, USA) (Dein et al., 1986). Of the resident population of 39 cranes, viral assays of tissues from five of the seven fatalities yielded EEE virus. Epizootiological observations following the outbreak revealed that 14 (44%) of the 32 surviving whooping cranes and 13 (34%) of 38 coresident sandhill cranes (*G. canadensis*) had EEE virus-neutralizing (N) antibody. Morbidity or mortality was not observed in the latter species. *Culiseta melanura*, the principal enzootic mosquito vector of EEE virus (Williams et al., 1974), was found in an area adjacent to PWRC, and EEE virus N antibody was detected in wild birds captured at PWRC immediately after the EEE epizootic in the whooping cranes. These observations indicated that EEE virus was enzootic, and may pose a serious risk to the continued successful propagation of the endangered whooping crane at PWRC. As a possible strategy for the prevention of future EEE epizootics, studies were conducted to determine the immunogenicity of a formalin-inactivated human EEE virus vaccine in sandhill and whooping cranes.

MATERIALS AND METHODS

Vaccine

The EEE virus vaccine, originally developed for human use, was provided by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID, Fort Detrick, Frederick, Maryland 21701, USA). The vaccine was prepared from the PE-6 WRAIR strain of EEE virus that was propagated in primary chick embryo cell cultures and inactivated with formalin (Maire et al., 1970). Prior to inoculation,

the vaccine was rehydrated with sterile distilled water.

Trial 1

On 7 November 1984, nine EEE virus-seronegative sandhill cranes 1 yr of age were inoculated with the EEE virus vaccine. Three cranes were inoculated with 0.5 ml intramuscularly (i.m.) in the pectoral muscle, three cranes received 0.25 ml intravenously (i.v.) in the jugular vein, and three were inoculated with 0.5 ml subcutaneously (s.c.) over the femoral musculature. These cranes were bled immediately prior to vaccination and on days 20 and 75 postvaccination.

Ten mo later, four cranes from Trial 1 were evaluated for adverse reaction to repeated i.m. administration of 1.0 ml of EEE virus vaccine. Cranes were bled and inoculated with vaccine on 1 August 1985 and every 7 days thereafter for 5 wk. Serum samples obtained before inoculation and at 3, 5, and 14 wk postinoculation were assayed for EEE viral N antibody.

Trial 2

On 21 December 1984, 16 seronegative, nonvaccinated sandhill cranes >1 yr old, and one 2-yr-old whooping crane were placed in four treatment groups. Vaccine was administered i.m. to three groups (Table 3), and one group (the control cranes) was inoculated i.m. with distilled water.

Trial 3

On 25 March 1985, all (n = 15) EEE viral N antibody-seronegative whooping cranes at PWRC and four whooping cranes with pre-existing, naturally acquired N antibody received an i.m. injection of 0.5 ml of vaccine followed by a 1.0 ml booster i.m. after 30 days. After 6 mo, these whooping cranes were rebled and administered 1.0 ml of vaccine IM.

Laboratory assay

Blood specimens were obtained from cranes by jugular venipuncture, allowed to clot and then centrifuged at 800 g for 20 min. Serum was decanted and stored at −20 C until assayed for EEE N antibody. Sera were assayed for EEE viral N antibody by plaque-reduction neutralization (PRN) tests in Vero (African green monkey kidney) cells (Clark et al., 1986). An 80% or more reduction of the virus dose by a specific dilution of crane serum was considered evidence of EEE virus N antibody. All assays were performed without knowledge of the treatment of individual birds. The EEE stock virus used in the PRN test was isolated from a pool of *C. melanura* mosquitoes collected at the Pocomoke Cypress Swamp, Maryland on 3 July 1979. The virus stock had undergone two cell-culture passages.

TABLE 1. Neutralizing antibody elicited by EEE viral vaccine in sandhill cranes.

Route of inoculation (volume)	Crane number	Days postvaccination 0	20	75
Intramuscular (0.50 ml)	8444	Neg[a]	20[b]	Neg
	8455	Neg	80	Neg
	8460	Neg	10	Neg
Intravenous (0.25 ml)	8447	Neg	Neg	ND[c]
	8458	Neg	Neg	ND
	8469	Neg	Neg	ND
Subcutaneous (0.50 ml)	8439	Neg	Neg	ND
	8445	Neg	Neg	ND
	8453	Neg	Neg	ND

[a] No significant plaque reduction at 1:10 dilution.
[b] Reciprocal of highest serum dilution producing ≥80% plaque reduction.
[c] Not done.

Surveillance

To monitor the possibility of EEE virus transmission during 1985, five seronegative adult bobwhite quail (*Colinus virginianus*) were retained at each of five sites in wire mesh cages around the northern perimeter of the PWRC. In addition, 17 young (<1-yr-old) seronegative sandhill cranes located throughout the captive colony were employed as indicators of EEE virus transmission. All birds were bled weekly from 23 July through 28 October 1985. The serum component was collected following centrifugation of blood and assayed for EEE N antibody by PRN tests as described above.

RESULTS

Trial 1

As presented in Table 1, EEE viral N antibody was detected only in the sandhill cranes inoculated i.m. Although serum dilutions of 1:10 to 1:80 reduced the virus dose ≥80% on day 20 postvaccination, N antibody was not detected at day 75. Adverse reactions to the vaccine were not observed in the cranes that received the i.m. or s.c. inoculations. However, the i.v. inoculated cranes developed various degrees of temporary ataxia within 15 min.

The four sandhill cranes, originally in-

TABLE 2. Neutralizing antibody response after five weekly intramuscular inoculations with EEE viral vaccine (1.0 ml) in sandhill cranes.

Crane number	Original route of inoculation	Weeks from first booster			
		0	3	5	14
8444[a]	i.m.	Neg[b]	1,280[c]	640	320
8455	i.m.	Neg	ND	320	1,280
8460	i.m.	10	160	160	80
8439	s.c.	Neg	10	20	10

[a] Designated cranes from experiment described in Table 1 were held for approximately 10 mo before receiving additional vaccine.
[b] 1:10 dilution with <80% plaque reduction.
[c] Reciprocal of highest serum dilution producing ≥80% plaque reduction.

oculated in November 1984 and subsequently receiving five weekly i.m. inoculations of vaccine, exhibited no adverse systemic clinical signs or reactions at the injection site. Antibody titers as high as 1:1,280 were exhibited by these cranes (Table 2). Those cranes that received the vaccine i.m. originally developed higher and more persistent N antibody titers than the one vaccinated by the s.c. route.

Trial 2

In further studies of i.m. vaccination, most cranes developed N antibody (Table 3). Antibody was detected among all groups of cranes except the nonvaccinated controls. Cranes in groups 1 and 3, that received a single inoculation and were positive for N antibody on days 20 and 30 postvaccination, were negative by day 60. In contrast, the four sandhill cranes and one whooping crane in group 2 that received a 1.0 ml booster on day 30 had N titers of 1:80 on day 60. Even though the antibody response was enhanced by the booster given on day 30, the titers on day 90 had waned by 1 to 3 dilutions. The whooping crane did not have detectable antibody at that time. Two of two sandhills tested after 150 days were still positive.

TABLE 3. Neutralizing antibody exhibited by cranes following intramuscular administration of EEE viral vaccine.

Group[a]	Inoculum	Days postvaccination					
		0	20	30	60	90	150
1	0.5 ml vaccine	Neg[b]	10[c]	10	Neg	ND	ND
		Neg	Neg	Neg	Neg	ND	ND
		Neg	ND[d]	20	Neg	ND	ND
		Neg	20	20	Neg	ND	ND
2	0.5 ml vaccine initially; 1.0 ml vaccine on day 30	Neg	20	10	80	40	20
		Neg	10	10	80	10	ND
		Neg	10	10	80	20	ND
		Neg	20	20	80	20	10
		Neg	20	ND	80	Neg	Neg
3	1.0 ml vaccine	Neg	10	Neg	Neg	ND	ND
		Neg	Neg	Neg	Neg	ND	ND
		Neg	Neg	Neg	Neg	ND	ND
		Neg	ND	20	Neg	ND	ND
Control							
	0.5 ml sterile water	Neg	Neg	Neg	Neg	Neg	ND
		Neg	Neg	Neg	Neg	Neg	ND
		Neg	Neg	Neg	Neg	Neg	ND
		Neg	Neg	Neg	Neg	Neg	ND

[a] All cranes were sandhill cranes except the last bird in the second group which was a whooping crane.
[b] No plaque reduction by 1:10 dilution.
[c] Reciprocal of highest serum dilution producing ≥80% plaque reduction.
[d] Not done.

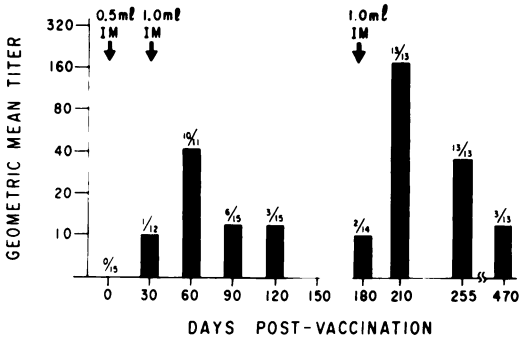

FIGURE 1. Eastern equine encaphalitis viral neutralizing antibody titers in vaccinated whooping cranes with no evidence of pre-existing antibody.

Trial 3

EEE viral N antibody titers elicited in 15 previously nonvaccinated antibody negative whooping cranes after primary and booster vaccinations are presented in Figure 1. Only one of 12 cranes tested following primary vaccination had detectable antibody. After the booster on day 30, antibody was detected in 10 (91%) of the 11 cranes bled on day 60. Only two (14%) of 14 cranes had detectable antibody on day 180 postvaccination (one of the 15 cranes died between days 120 and 180 as a result of intraspecific aggression). After a second booster with the EEE viral vaccine on day 180, all cranes (13/13) tested had antibody with a geometric mean titer of 1:160 on day 210, or a four-fold or greater increase as compared to the titers observed on days 30 or 60 after the primary inoculation and booster. On day 255 all cranes were still positive, but titers were four-fold, lower and by day 470 only three cranes exhibited detectable antibody.

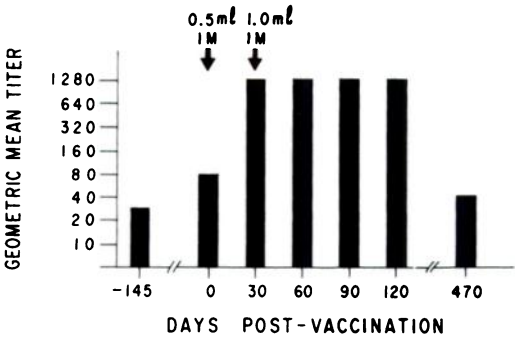

FIGURE 2. Eastern equine encephalitis viral neutralizing antibody in whooping cranes with pre-existing antibody.

Results of vaccination of four whooping cranes with pre-existing naturally acquired N antibody are presented in Figure 2. These cranes exhibited a high and sustained N antibody response ($\geq$1:1,280) through 120 days postvaccination. The geometric mean antibody titer of $\geq$1:1,280 on days 30 through 120 postinoculation was significantly greater ($P < 0.001$) than the peak geometric mean titer of 1:40, observed during the same time period for the vaccinated cranes (Fig. 1) with no evidence of pre-existing antibody.

Surveillance

All bobwhite quail and sandhill cranes employed as viral sentinels remained negative for EEE viral antibody during the summer and fall of 1985 at the PWRC.

DISCUSSION

Data generated by this study revealed that a formalin-inactivated human EEE viral vaccine elicited specific N neutralizing antibody in both sandhill and whooping cranes. A single dose of the vaccine given i.m. induced either no detectable antibody, or a low titered transitory antibody response. However, all seronegative cranes exhibited detectable antibody after two or more vaccinations. The immunogenicity of the vaccine for cranes was further supported by the anamnestic-like response of whooping cranes with pre-existing antibody. This response was characterized by a rapid and sustained high antibody titer and differed significantly from the low transitory antibody pattern exhibited by the seronegative cranes. This observation provided strong evidence that the immune response of seronegative cranes was induced by the EEE viral vaccine rather than possible exposure to a nat-

ural EEE viral infection. That natural EEE viral infection was not acquired was supported also by the failure to detect evidence of infection by this virus in sentinel bobwhite quail and sandhill cranes during 1985. Previous studies revealed that bobwhite were effective indicators of EEE virus transmission (Williams et al., 1972). Also, as observed during the 1984 outbreak (Dein et al., 1986), sandhill cranes retained in the same area as whooping cranes acquired natural EEE viral infection demonstrating their value as effective sentinels for EEE virus activity.

Although the EEE viral vaccine elicited antibody in whooping cranes, the protection afforded, if any, against natural infection is unknown. Attempts to employ an inactivated EEE viral vaccine to prevent mortality among ringed-necked pheasants (*Phasianus colchicus*) yielded inconclusive results (Beaudette et al., 1952; Sussman et al., 1958; Snoeyenbos et al., 1978; Eisner and Nusbaum, 1983). However, observations on pheasants were derived from single, rather than multiple vaccinations, as we administered to cranes. More recent studies involving mammalian-alphavirus models indicated that vaccine induced protective N antibody, and/or cellular immunity was demonstrable in the absence of detectable N antibody (Schmaljohn et al., 1982). While the latter observations pertained to mammals, it is conceivable that avians may respond similarly, and therefore should be considered in evaluating the efficacy of the EEE viral vaccine for whooping cranes. Currently, however, direct challenge of vaccinated cranes with live EEE virus as a measure of efficacy is considered an unacceptable risk because of the endangered status of this species; the total number at the end of 1986 was 38 captive and 138 free-ranging cranes. Alternative approaches, such as vaccinating and challenging other avian species, particularly exotic species such as pheasants that experience morbidity and mortality could be implemented. However, interspecific genetic differences may preclude any realistic extrapolation of data. Thus, the efficacy of the EEE viral vaccine-induced antibody for protecting whooping cranes cannot be readily resolved by laboratory studies. An alternative approach currently being considered is to monitor whooping cranes for protective evidence against natural EEE viral infection, in conjunction with an ongoing EEE viral surveillance program using bobwhite quail as viral sentinel animals and estimates of larval and adult *C. melanura* abundance.

The devastating impact of the EEE viral epizootic among captive whooping cranes at the PWRC must be considered as a potential risk to the successful recovery of this species. If the EEE viral vaccine is efficacious, this could provide an effective management strategy for minimizing the risk of captive cranes to EEE viral infection. The vaccine is readily available, inexpensive, and does not present any apparent adverse side effects in cranes. A booster appears necessary 30 days following the primary vaccination, and one or more shots are required annually thereafter to sustain detectable antibody. Finally, if the EEE viral vaccine fails to protect cranes from fatal EEE viral infections alternative approaches will need to be considered.

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