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Recombinant Vaccinia Virus/Venezuelan Equine Encephalitis (VEE) Virus Protects Mice from Peripheral VEE Virus Challenge

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Mice immunized with recombinant vaccinia virus (VACC) expressing Venezuelan equine encephalitis (VEE) virus capsid protein and glycoproteins E1 and E2 or with attenuated VEE TC-83 virus vaccine developed VEE-specific neutralizing antibody and survived intraperitoneal challenge with virulent VEE virus strains including Trinidad donkey (subtype 1AB), P676 (subtype 1C), 3880 (subtype 1D), and Everglades (subtype 2). However, unlike immunization with TC-83 virus, immunization with the recombinant VACC/VEE virus did not protect mice from intranasal challenge with VEE Trinidad donkey virus. These results suggest that recombinant VACC/VEE virus is a vaccine candidate for equines and humans at risk of mosquito-transmitted VEE disease but not for laboratory workers at risk of accidental exposure to aerosol infection with VEE virus.

Venezuelan equine encephalitis (VEE) virus is a mosquito-borne alphavirus that produces encephalitis in equines, with fatality rates of 38 to 83% in various epidemics, and occasional encephalitis in humans (22). Numerous VEE virus outbreaks have been recorded in South America between 1935 and 1970 (22). In 1971, a VEE virus epidemic that originated in South America reached the United States, causing over 1,400 equine deaths in Texas (34).

Laboratory accidents have caused at least 150 human infections with VEE virus (2). Vaccines containing formaldehyde-treated VEE virus have caused clinical disease in 4% of vaccinees, despite extensive animal testing for residual infectious virus (3, 51). The live, attenuated VEE TC-83 virus vaccine developed during the 1960s by serial tissue culture passage of the virulent Trinidad donkey (TRD) strain of VEE virus is avirulent for weanling mice inoculated by the intraperitoneal (i.p.) or intracerebral (i.c.) route (3). Mice immunized with TC-83 virus resist i.p. or i.c. challenge with virulent VEE virus (3). The attenuated TC-83 virus induces long-lasting neutralization (Nt) and hemagglutination inhibition (HI) antibodies in humans (5) and equines (11, 16, 50, 54, 56). There are, however, several biological problems with the attenuated TC-83 virus that make it unsuitable for administration to humans at risk for VEE virus infection. Reactogenicity to the TC-83 vaccine includes fever and leukopenia in equines (21, 26, 50) and 40% of vaccinated humans (1, 37). Infection of fetal rhesus monkeys with TC-83 virus has caused congenital cerebral and ocular malformations, suggesting potential teratogenic properties (33).

Reversion of TC-83 virus to the virulent wild type is a major concern. Serial passage of TC-83 virus in horses resulted in higher titered viremias and increased frequency and severity of clinical manifestations (35). There was, however, no evidence of serious central nervous system infection (39) in these horses or of reversion of the vaccine virus to virulence for small laboratory animals (35). Berge et al. (3) demonstrated that attenuated VEE virus (strain TC-50) reverted to mouse virulence after three serial i.c. mouse passages. Isolation of TC-83 vaccine virus from mosquitoes in Louisiana (41) during the 1971 VEE virus epidemic, when the vaccine was administered to over two million horses in the southern United States (47), supports the possibility of introducing TC-83 virus or revertant TC-83 virus into a natural mosquito-vertebrate host cycle.

To develop an improved VEE virus vaccine, we have explored the use of recombinant vaccinia (VACC) viruses containing cDNA copies of the 26S mRNA, which encodes the structural proteins of VEE TC-83 or TRD virus. We have shown that VEE virus capsid protein and envelope glycoproteins E1 and E2 are expressed by recombinant VACC/TC-83 and VACC/TRD viruses (R. M. Kinney, J. J. Esposito, B. J. B. Johnson, J. T. Roehrig, J. H. Mathews, A. D. T. Barrett, and D. W. Trent, J. Gen. Virol., in press). We report here the protective efficacy of the recombinant VACC/TC-5A virus in mice.

MATERIALS AND METHODS

Viruses and mice. The VEE viruses used in this study included strain TRD, the live-attenuated strain TC-83, and wild-type strains P676, 3880, Mena II, Everglades Fe3-7C (EVE), and Pixuna BeAr 35645 (PIX) (18, 28, 58). The MacMillan strain of western equine encephalitis (WEE) virus was also used. Cloned cDNA encoding the structural proteins of VEE TC-83 or TRD virus was inserted into the thymidine kinase gene of the New York Board of Health (NYBH) strain of VACC virus (Kinney et al., in press). Colony-bred male Swiss NIH white mice, inbred male C3H/HeSnJ mice (Charles River Laboratories), and inbred female A/J mice (Jackson Laboratory) were used.

Preparation of virus vaccines. NYBH (Wyeth Laboratories) VACC virus and recombinant VACC/TC-83 (VACC/TC-5A) virus were grown in CV-1 monkey kidney cells. VACC and VACC/TC-5A viruses were partially purified from the cytoplasms of infected cells by centrifugation through 40% (wt/wt) sucrose (15), suspended in TE buffer (10 mM Tris hydrochloride [pH 9.0], 1 mM disodium EDTA), and stored at −70°C until being used. Plaque-purified TC-83 virus was grown in Vero or BHK-21 cell

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TABLE 1. HI and ELISA serum titers in A/J mice immunized with TC-83 or recombinant VACC/TC-5A virus

Virus	Vaccine dose[a] (PFU)	Challenge[b]	Survival[c]	Log_{10} geometric mean titer (± SE)[d]				Ratio[e]	
				Prechallenge		Postchallenge			
				HI	ELISA	HI	ELISA	HI	ELISA
TC-83	10^4	TRD	8/8	2.47 (0.09)	≥5.01	3.03 (0.14)	ND[f]	4	ND
VACC/TC-5A	10^7		8/8	1.64 (0.13)	3.17 (0.13)	1.94 (0.09)	3.24 (0.14)	2	1
VACC/TC-5A	10^7	TRD	8/8	1.56 (0.09)	3.24 (0.14)	2.77 (0.17)	4.67 (0.22)	16	29
VACC/TC-5A	10^6	TRD	7/8	1.43 (0.04)	3.03 (0.07)	2.72 (0.13)	4.75 (0.09)	19	53
VACC/TC-5A	10^5	TRD	5/8	1.18 (0.03)	2.36 (0.06)	3.10 (0.16)	5.01 (0.10)	85	445
VACC	10^8	TRD	0/8	<1.0	<1.0				
PBS		TRD	0/8	<1.0	<1.0				

[a] PFU determined in Vero cells. TC-83 and VACC viruses given by i.p. injection or tail scarification, respectively.
[b] Challenge was 100 $IPLD_{50}$ (15 PFU) of TRD virus.
[c] Number of survivors/number in group.
[d] Determined from individual titers of five (10^5 PFU of VACC/TC-5A), seven (10^6 PFU of VACC/TC-5A), or eight (all others) mice per group.
[e] Postchallenge geometric mean titer/prechallenge geometric mean titer.
[f] ND, Not done.

monolayers. Vero cell monolayers were used for virus titrations. Virus titers of freshly hydrated commercial TC-83 (National Drug Company) and VACC (Wyeth Laboratories) vaccines were 1.6×10^4 and 1.4×10^6 PFU for standard doses of 0.5 ml and 25 μl, respectively.

Immunization and challenge of mice. Five-week-old mice were immunized i.p. with 10^4 PFU of TC-83 virus in 0.2 ml of phosphate-buffered saline (PBS) or by intradermal (i.d.) tail scarification with 10^5 to 10^8 PFU of either VACC or recombinant VACC/TC-5A virus in 20 μl of PBS. Mice were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, N.J.) before scarification. Mice were challenged 3 weeks after immunization by i.p. injection of 0.2 ml of virulent VEE virus or by intranasal (i.n.) inoculation of 5 μl of virulent virus in minimally anesthetized animals. The 50% median lethal doses of challenge viruses administered i.p. ($IPLD_{50}$) or i.n. ($INLD_{50}$) were determined by titrations in 8-week-old Swiss NIH or A/J mice by the method of Reed and Muench (44).

Serology. Nt tests were performed in Vero cell monolayers by the serum dilution-plaque reduction method (24). HI tests were performed as described by Lennette and Schmidt (32). Enzyme-linked immunosorbent assay (ELISA) for anti-VEE antibodies also was used.

RESULTS

Titrations of VEE virus strains. The VEE complex is one of six recognized serologic complexes of alphaviruses (6) and is itself subdivided into six antigenic subtypes, with subtypes 1 and 3 exhibiting five and three antigenic variants, respectively (28). $IPLD_{50}$ determinations for five subtype 1 variants and one strain of each of the other four VEE subtypes were done in 8-week-old Swiss NIH mice. One $IPLD_{50}$ unit corresponded to 0.6, 110, and 0.1 PFU of VEE TRD (subtype 1AB), P676 (subtype 1C), and 3880 (subtype 1D) viruses, respectively. EVE virus (subtype 2) killed mice nonuniformly in three separate experiments so that a reliable $IPLD_{50}$ was not determined. Mena II (subtype 1E) virus was fatal only at high doses (1.4×10^8 PFU per $IPLD_{50}$). VEE viruses 78V-3531 (IF), Mucambo BeAn 8 (3A), PIX (subtype 4), and Cabassou CaAr 508 (subtype 5) were not lethal for 8-week-old Swiss NIH mice. The $IPLD_{50}$ for VEE TRD virus in 8-week-old A/J mice was 0.15 PFU. $INLD_{50}$ endpoints of 33 and 105 TRD PFU were obtained in 8-week-old A/J and Swiss NIH mice, respectively. LD_{50} titrations were not performed in C3H mice.

Immunologic response of A/J mice vaccinated with recombinant VACC/TC-5A virus. A/J mice were immunized i.p. with 10^4 PFU of TC-83 virus or i.d. with 10^8 PFU of VACC virus or 10^5, 10^6, or 10^7 PFU of recombinant VACC/TC-5A virus. Animals were bled 3 weeks after immunization for antibody determinations. Mice that survived virulent TRD virus challenge were bled again 2 weeks after challenge. As shown in Table 1, only mice that received TC-83 or VACC/TC-5A vaccine were protected from i.p. TRD virus challenge (100 $IPLD_{50}$). Mice that received 10^6 or 10^7 PFU of VACC/TC-5A virus developed geometric mean HI and ELISA reciprocal titers of 27 to 44 and 1,076 to 1,600, respectively. Animals given 10^5 PFU of VACC/TC-5A virus, however, developed no measurable (three mice) or much reduced (five mice) HI (1:15 mean titer) or ELISA (1:230 mean titer) antibody levels. The development of recombinant VACC-induced tail blister, antibody response, and survival after TRD virus challenge were dose related in the animals immunized with VACC/TC-5A virus. The attenuated TC-83 virus elicited higher mean HI and ELISA reciprocal titers of 293 and ≥102,400, respectively. Postchallenge antibody rises suggested a dose-related boost in VACC/TC-5A-immunized animals that was much greater than that shown in the TC-83 mouse group (Table 1). This boost in antibody titer suggested that in those mice which received the VACC/TC-5A virus, more replication of the challenge virus occurred than in the TC-83-immunized animals.

Prechallenge Nt antibody titers in individual animals receiving the various doses of recombinant vaccine are shown in Table 2. Mice immunized with VACC/TC-5A virus developed dose-related Nt antibody responses (<1:10 to 1:5,120 titers) that were generally much lower in comparison with Nt antibody levels (≥1:10,240) in TC-83-immunized mice. Three mice immunized with 10^5 PFU of VACC/TC-5A virus and one immunized with 10^6 PFU developed no Nt, HI, or ELISA reactive antibodies. These mice succumbed to TRD virus challenge. A fifth mouse (given a 10^6 dose) developed no detectable Nt or HI antibody but did have an ELISA titer of 1:200 and did survive TRD virus challenge. None of the VACC-immunized or PBS control mice developed anti-TRD Nt antibody or survived TRD virus challenge.

A/J mice immunized with 10^7 PFU of VACC/TC-5A virus were bled 18, 33, and 86 days after immunization to determine the short-term duration of immunity against VEE virus. Neutralizing antibody titers indicated that antibody

TABLE 2. VEE neutralizing antibody titers in A/J mice 3 weeks after immunization with VACC/TC-5A virus

Mouse no.	Titer[a] at vaccine dose (PFU):		
	10^7	10^6	10^5
1	2,560	160	10
2	640	<10[b]	10
3	320	80	<10[b]
4	640	<10	<10[b]
5	5,120	320	20
6	80	320	160
7	80	20	<10[b]
8	20	320	20

[a] Reciprocal of highest antiserum dilution that inhibited 70% or more of the VEE TRD virus (60 PFU) used in the test.
[b] Mouse died after challenge with 100 $IPLD_{50}$ of VEE TRD virus.

levels induced by VACC/TC-5A virus were stable for at least 3 months (Table 3).

Neutralizing antibody titers were determined for both Swiss NIH and C3H mice that received 10^8 PFU of VACC/TC-5A virus i.d. (Fig. 1). The recombinant VACC/TC-5A vaccine elicited a weaker response in outbred Swiss NIH mice than in inbred C3H or A/J mice. VACC/TC-5A-induced tail lesions were also reduced in Swiss NIH mice as compared with either C3H or A/J mice.

Cross-protection studies. Five individual, high-titered anti-TC-83 or anti-VACC/TC-5A A/J mouse serum samples were pooled and tested for their ability to neutralize different antigenic subtypes of VEE virus (Table 4). The Nt cross-reactivities of anti-TC-83 and anti-VACC/TC-5A sera were very similar. Both sera neutralized epizootic VEE viruses 1AB and 1C (P676) efficiently. Subtype 1D (3880), 1E (Mena II), and 2 (EVE) viruses were neutralized at higher antibody concentrations. Anti-VACC/TC-5A serum Nt titers were lower than those of anti-TC-83 serum. Neither serum neutralized the distantly related VEE subtype 4 (PIX) virus or WEE virus.

Nt tests with anti-VACC/TC-5A serum indicated that the recombinant VACC/TC-5A vaccine should protect mice against challenge with subtype 1A-D and subtype 2 VEE viruses (Table 4). To evaluate this hypothesis, C3H and Swiss NIH mice were immunized with TC-83 or recombinant virus and challenged with VEE virus (Table 5). Both TC-83 and VACC/TC-5A vaccine viruses protected mice against at least 6×10^5 PFU (10^6 $IPLD_{50}$ in Swiss NIH mice) of TRD virus and 10^3 and 10^4 PFU of 3880 and P676 viruses, respectively. Although protection of Swiss NIH mice against EVE virus challenge was ambiguous because only three of eight PBS control animals died, C3H mice, which are most

TABLE 3. Duration of immunity in individual A/J mice immunized i.d. with 10^7 PFU of recombinant VACC/TC-5A virus

Mouse no.	Titer[a] at days postimmunization		
	18	33	86
1	320	2,560	1,280
2	640	160	320
3	2,560	640	320
4	640	2,560	2,560
5	5,120	5,120	10,240
6	20	80	40

[a] Reciprocal of highest antiserum dilution that inhibited 70% or more of VEE TRD virus (60 to 80 PFU) used in Nt test.

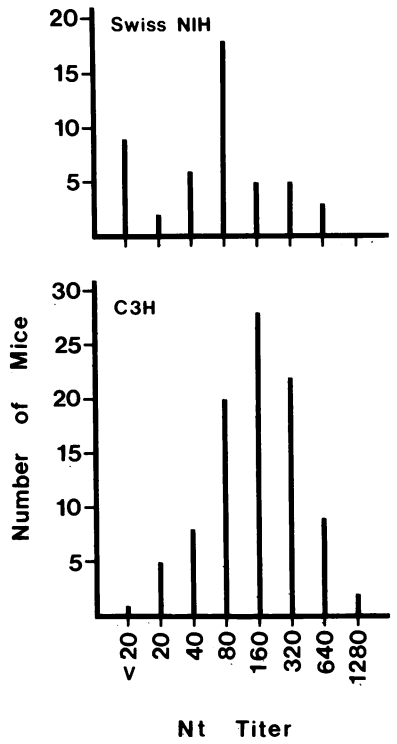

FIG. 1. Distribution of individual anti-TRD neutralizing antibody titers (70% endpoint) in outbred Swiss NIH mice (n = 48) and inbred C3H mice (n = 94).

sensitive to EVE virus, were clearly protected by both vaccines. High challenge doses of P676 and TRD viruses caused one and two fatalities, respectively, in recombinant-immunized, but none in TC-83-immunized, Swiss NIH mice (Table 5).

Protection of mice against i.n. TRD virus challenge. To determine the level of protection against aerosol infection afforded by immunization with recombinant VACC virus, mice were immunized with VACC/TC-5A virus and challenged i.n. with virulent TRD virus (100 $INLD_{50}$). The VACC/TC-5A vaccine protected mice against peripheral i.p. but not i.n. challenge with TRD virus, whereas the TC-83 vaccine protected mice from challenge virus given by either route (Table 6). However, 5 of 20 TC-83-immunized C3H mice succumbed to i.n. TRD virus challenge. The six VACC/TC-5A-immunized A/J mice that survived i.n. TRD virus

TABLE 4. Cross-reactivities of sera pooled from A/J mice immunized with VEE TC-83 or recombinant VACC/TC-5A virus

Virus (VEE subtype)	Endpoint titer[a] of pooled mouse serum	
	VACC/TC-5A	TC-83
VACC	80[a]	<10
VACC/TC-5A	160	<10
TRD (1AB)	1,280	≥20,480
P676 (1C)	320	≥640
3880 (1D)	10	160
Mena II (1E)	10	80
EVE (2)	40	320
PIX (4)	<10	<10
WEE	<10	<10

[a] Endpoint = 70% neutralization of 60 to 90 PFU.

TABLE 5. VACC/TC-5A and TC-83 vaccine efficacy in protecting C3H or Swiss NIH mice from i.p. challenge with four VEE virus strains

Challenge		Survival[a]			
Virus (VEE subtype)	Dose (PFU)	VACC/TC-5A		TC-83	
		C3H	Swiss	C3H	Swiss
TRD (1AB)	6×10^1	6/6	6/6	6/6	6/6
	6×10^2	6/6	6/6	6/6	6/6
	6×10^3	6/6	5/6	6/6	6/6
	6×10^5	6/6	5/6	6/6	6/6
P676 (1C)	1.1×10^4	6/6	6/6	6/6	6/6
	1.1×10^6	6/6	5/6	6/6	6/6
3880 (1D)	1×10^1	6/6	6/6	6/6	6/6
	1×10^3	6/6	6/6	6/6	6/6
EVE (2)	1×10^8	8/8	8/8	8/8	8/8

[a] Number of survivors/number of mice. All mice in 17 PBS control groups (nine virus challenge doses, two mouse strains) succumbed to virus challenge. Only three of eight PBS control Swiss NIH mice died after EVE virus challenge.

challenge all became ill after challenge and lost weight but showed no signs of paralysis. The six A/J survivors recovered very slowly and were still not normal at 3 weeks postchallenge.

In another study, six A/J mice received 30 μg of protein A-purified, anti-VEE monoclonal antibody by injection into the tail vein. This monoclonal antibody (3B4C-4) neutralizes VEE virus to high titer (46). Twenty-four hours after receiving the antibody, two mice were bled for antibody determination, and all six mice were challenged i.n. with 3,300 PFU (100 $INLD_{50}$) of TRD virus. Despite Nt antibody titers of at least 1:1,280 in the two mice tested, the TRD virus challenge was lethal for all six mice.

DISCUSSION

We have shown that immunization with recombinant VACC (VACC/TC-5A) virus encoding the structural proteins of VEE TC-83 virus protects three strains of mice against lethal i.p. challenge with virulent VEE TRD virus. Neutralizing antibody levels in these recombinant-immunized mice correlate well with Nt titers elicited by the TC-83 vaccine in equines (26) and humans (5). VACC/TC-5A-induced immunity appeared to be long lasting. Two recombinant vaccinia viruses (VACC/TRD-1A and VACC/TRD-20A) encoding the structural proteins of VEE TRD virus also protected mice from i.p. TRD virus challenge (data not shown). Recombinant VACC virus expressing the structural proteins of another alphavirus, Sindbis virus, has been reported to elicit Nt antibody in cattle (20, 45).

TABLE 6. Summary of survival of VACC/TC-5A- or TC-83-immunized mice challenged i.p. or i.n. with virulent VEE TRD virus

Mouse strain	No. of survivors/no. of mice after challenge			
	i.p.[a]		i.n.[b]	
	VACC/TC-5A	TC-83	VACC/TC-5A	TC-83
A/J	42/42	8/8	6/24	16/16
C3H	30/30	30/30	0/30	15/20
Swiss NIH	22/24	24/24	1/30	18/18

[a] Challenge dose, 15 PFU to 6×10^5 PFU of TRD virus.
[b] Challenge dose, 3,300 (A/J) or 10,500 (C3H and Swiss NIH) PFU of TRD virus.

Epidemics of VEE disease have been associated with VEE subtype 1AB and 1C viruses, which also cause significant clinical disease in experimentally infected monkeys (38) or horses (23, 55). Mosquito-transmitted human disease has occurred with VEE subtype 2 (12) and 1D (19, 27, 58) viruses, and laboratory infection with the more distantly related VEE subtype 3 virus has been reported (9). Pooled sera from mice immunized with TC-83 or recombinant VACC/TC-5A virus neutralized VEE TRD (subtype 1AB) and P676 (subtype 1C) virus to high titer and EVE (subtype 2), 3880 (subtype 1D), and Mena II (subtype 1E) viruses somewhat less effectively. PIX (subtype 4) virus was not neutralized by serum from mice immunized with either vaccine. This pattern of cross-reactivity is typical of TC-83-immune serum in humans (5, 17), of antiserum elicited against purified VEE E2 envelope glycoprotein (18, 28), and of anti-E2 monoclonal antibody (46). TC-83- and recombinant-immunized mice were protected from lethal i.p. challenge with high doses of VEE TRD, P676, 3880, and EVE viruses (Table 5) as well as Mena II virus (3 $IPLD_{50}$), although only 50% of unimmunized control mice succumbed to Mena II challenge (data not shown). Both vaccines appeared to protect against epidemic VEE 1AB and 1C viruses. For long-term immunity to 1D, 1E, and more distantly related VEE subtype viruses, other immunogens will probably be necessary.

VACC/TC-5A-induced Nt antibody levels were lower in outbred Swiss NIH mice than in inbred A/J or C3H mice. Mice immunized with the recombinant virus developed lower anti-VEE antibody levels than did TC-83-immunized mice. This differential response to either vaccine was expected since TC-83 virus replicates in most tissues of mice (30, 31). TC-83 virus can be recovered in nasopharyngeal secretions of immunized humans (37) and monkeys (38). Replication of TC-83 virus in the nasopharynx would be expected to stimulate local secretory immunity. This stimulation may account for mouse survival after i.n. challenge with TRD virus in TC-83-immunized mice but not in mice immunized with the VACC/TC-5A recombinant vaccine. Invasion of the olfactory tract by virulent VEE virus has been demonstrated in monkeys (7, 8) and hamsters (10). Therefore, aerosol infection may lead to central nervous system involvement by a nonhematogenous route. Although passive monoclonal Nt antibody (immunoglobulin G), protects mice from peripheral VEE virus challenge (36), we showed here that it did not protect mice from i.n. challenge. Locally stimulated immunoglobulin A production is also important in resistance to respiratory infection with rubella virus (40), influenza virus (52), and rhinovirus (42).

Recombinant VACC (WR VACC strain) viruses encoding surface glycoproteins of human respiratory syncytial virus (RSV) (13, 57) and influenza A virus (48) protected rodents from i.n. challenge with RSV and influenza viruses, respectively. However, passive Nt antibody decreased replication of RSV in lungs of cotton rats but had a reduced effect on RSV replication in nasal tissues (53). Similarly, passive antibody protected A/J mice from lethal influenza pneumonitis, but not tracheitis, after influenza A virus challenge (43). Mice that were immunized i.d. with recombinant VACC virus expressing influenza A virus hemagglutinin developed serum anti-hemagglutinin antibodies that prevented lung, but not nasal, infection after i.n. challenge with

influenza virus. Unlike the i.d. route of immunization, which failed to induce nasal secretory antibody, i.n. administration of the recombinant VACC virus elicited serum and nasal secretory anti-hemagglutinin antibodies and protected the lung and upper respiratory tract against infection by the challenge virus (49). Thus, resistance to nasal infection appears to be dependent on nasal secretory antibody, whereas circulating antibody may be adequate to protect the lower respiratory tract. Perhaps the WR vaccinia virus strain provided greater stimulation of mucosal immunity than did the NYBH strain used in this study, since the WR strain is reported to be more invasive in mice (4). Recently, Esposito et al. (14) reported that mice immunized with recombinant VACC-(NYBH) virus expressing the rabies virus glycoprotein survived intracranial challenge with rabies virus. Rabies virus may have been neutralized by immune serum at the inoculation site.

Although aerosol transmission of VEE virus may occur naturally under appropriate conditions (29, 59), VEE virus infection in equines and humans is usually acquired naturally through mosquito transmission. TC-83 and VACC/TC-5A vaccine viruses both appear to protect against peripheral challenge with epidemic VEE virus strains (1AB and 1C). The recombinant VACC vaccine, however, is apparently less effective than the attenuated live TC-83 virus vaccine in protecting mice from i.n. VEE virus challenge. Recombinant virus elicited immunity that was more like that induced by formaldehyde-inactivated C-84 VEE virus vaccine (25).

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