

Experimental Infection of Horses with an Attenuated Venezuelan Equine Encephalomyelitis Vaccine (Strain TC-83)

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Ten horses (*Equus caballus*) were vaccinated with strain TC-83 Venezuelan equine encephalomyelitis (VEE) virus vaccine. Febrile responses and leukopenia due to a reduction of lymphocytes and neutrophils were observed in all animals. Viremias were demonstrable in eight horses, with a maximum of $10^{3.5}$ median tissue culture infectious dose units per ml of serum in two horses. Clinical illness with depression and anorexia were observed in five horses. Neutralizing (N), hemagglutination-inhibiting, and complement-fixing antibodies to the vaccine virus were demonstrable by 5, 6.5, and 7 days, respectively, after vaccination. Differential titrations of serum to six VEE strains revealed high titers of N antibody to vaccine virus, moderate titers to the epizootic Trinidad donkey no. 1 strain (VEE antigenic subtype I, variant A) from which TC-83 was derived, and low titers to two other epizootic strains (subtype I, variants B and C) in all horses at 1 month after vaccination; some animals responded with low levels of N antibody to the enzootic viruses (subtype I, variants D and E). Fourteen months after vaccination, six animals with detectable N antibody were challenged with MF-8 (subtype I, variant B), an epidemic-epizootic strain isolated in 1969 from a man in Honduras. All horses resisted challenge with the equine pathogenic strain of VEE. Marked increases of N antibody in most horses were demonstrable to some VEE strains when tested 1 month after challenge.

During the past 3 years, major epizootics of Venezuelan equine encephalomyelitis (VEE) have occurred among horses in Central America (7, 15, 17; D. H. Martin et al., *Amer. J. Epidemiol.*, *in press*). These epizootics were significantly altered from the expected pattern by the use of an experimental vaccine developed by the U.S. Army Medical Research and Development Command at Fort Detrick, Md. (10, 17; D. H. Martin et al., *Amer. J. Epidemiol.*, *in press*; R. W. McKinney, *personal communication*). The vaccine virus, strain TC-83, was produced for use in laboratory personnel engaged in studies with this highly infectious agent (1). Although initial evaluation of the vaccine was done in burros (9) prior to its use in Central America, no information was available regarding clinical effects in horses, the occurrence of viremia, the quality and quantity of antibodies produced by the vaccinated horses, and most critically, the duration of immunity against equine virulent strains of VEE virus.

The present study was undertaken to answer these and other questions.

MATERIALS AND METHODS

Horses. Ten crossbred Panamanian geldings (*Equus caballus*) from 3 to 10 years of age were vaccinated. Criteria for selection were lack of neutralizing (N) antibody to VEE virus and good health. All were housed in an insect-proof isolation unit in Gualaca, Chiriqui Province, Panama, for the duration of the acute-phase vaccination and challenge studies.

Vaccine. Strain TC-83 attenuated VEE vaccine was used in all the studies. The vaccine virus was derived from Trinidad donkey no. 1 strain of VEE and was passed serially 83 times in fetal guinea pig heart cell cultures (1, 14). The 100-dose vials of vaccine were each reconstituted with 50 ml of Hanks balanced salt solution containing 0.5% human serum albumin.

On 15 April 1970, eight horses were inoculated intramuscularly with the recommended dosage of 0.5 ml of vaccine from lot 4, produced on 17 October 1966. A ninth horse was vaccinated subcutaneously on 9 January 1971 with 0.5 ml of vaccine from another vial (same lot number), whereas a tenth horse was vac-

inated subcutaneously on 24 March 1971 with 0.5 ml of virus from lot 5, run 4, produced from the same virus pool but lyophilized in 1970. Each vial of vaccine was titrated immediately after reconstitution in NLW stock suckling mice (National Laboratory Animal Co., Creve Couer, Mo.). Serial 10-fold dilutions of 0.02 ml were inoculated intracerebrally into each 1- to 3-day-old animal.

Clinical examinations and specimens. Samples were taken from all horses before vaccination for base-line data. Rectal temperatures, pulse and respiration rates, and serum samples were obtained twice daily from the initial eight animals. Oxalated blood samples also were taken daily for 9 days for hemoglobin, hematocrit, and leukocyte determinations. Urine was collected daily from most animals; specific gravity determinations were made with a hydrometer, and other tests with Hema-Combistix (Ames Co., Elkhart, Ind.). Swab specimens were taken twice daily from the eyes, nose, mouth, and feces of each horse and suspended in veal infusion broth containing 500 units of penicillin and 500 μ g of dihydrostreptomycin per ml. The swab specimens and samples of urine and serum were all preserved as described previously (18). Urine and swab specimens were not taken from horses 9 and 10.

We obtained serum samples at 1, 2, 4, 6, 12, and 14 months after vaccination from the first group of horses, at 1 and 5 months from horse 9, and at 1 and 2 months from horse 10.

Viral isolations. In our experience, comparative titrations of the vaccine virus in suckling mice and Vero cell cultures have yielded no significant differences in sensitivity of the two systems. In the field, titrations were accomplished more easily in suckling mice than in cell cultures which were used in the laboratory. Four culture tubes of a continuous line of African

green monkey kidney (Vero) cells were inoculated with 0.1 ml of each specimen. Swab and urine samples were first centrifuged for 15 min at $770 \times g$ to remove debris.

Positive samples were titrated for virus in Vero cell cultures by using a previously unfrozen vial of serum and were prepared in serial 10-fold dilutions. End-points were determined by the method of Spearman and Karber (6). Isolates were identified as VEE using the hemagglutination-inhibition (HI) test (3) modified for Microtiter plates (13).

Serological tests. The presence of N antibody was determined by the Microtiter method of Earley et al. (4), using Vero cell monolayer cultures. Sera were heat inactivated at 56 C for 30 min and titrated in serial fourfold dilutions with an initial dilution of 1:8. The VEE virus subtypes (19) used in the tests were: strain TC-83 vaccine virus; three epizootic strains, Trinidad donkey no. 1 (variant A of antigenic subtype I), MF-8 (subtype I, variant B) from Honduras, and P-676 (subtype I, variant C) from Venezuela; three enzootic strains, 3880 (subtype I, variant D) from eastern Panama, Mena II (subtype I, variant E) isolated from western Panama, and Fe-3-7c (subtype II) isolated in Florida. End points were recorded as the highest serum dilutions giving 80% reduction of 50 to 150 plaques. Sera for HI tests were treated with cold acetone and goose erythrocytes to remove nonspecific inhibitors and were tested in serial twofold dilutions from 1:10 to 1:320. The sucrose-acetone-extracted suckling mouse brain antigen used was VEE strain TC-83. Complement fixation (CF) tests were performed in Microtiter plates (13) with twofold serial dilutions from 1:4 to 1:128. Betapropriolactone (BPL)-inactivated suckling mouse brain antigen of TC-83 was used.

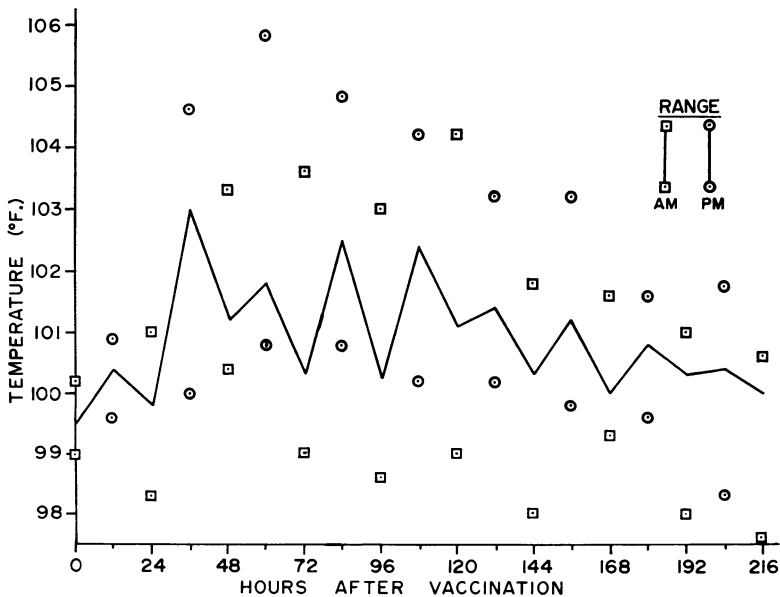


FIG. 1. Temperature medians and ranges for 10 horses vaccinated with strain TC-83 VEE vaccine.

Challenge studies. Eight of the vaccinated horses were inoculated subcutaneously with approximately 10,000 suckling mouse intracerebral median lethal dose (SMICLD₅₀) units of the MF-8 strain (subtype I-B) isolated from a human during the VEE epidemic-epizootic in Honduras in 1969 (7). This virus was originally isolated in suckling mice and had been passaged once in Vero cells before inoculation. Previous use of this virus to infect horses revealed that it was an equine pathogenic VEE subtype (Walton, unpublished data). Six of the initial 8 horses were challenged at 14 months; horse 9 was challenged at 5 months and horse 10 at 2 months after vaccination. Temperatures, serum samples, and oxalated blood for hemograms were taken daily for 10 days after challenge. Serum samples were collected again 1 month later.

RESULTS

Suckling mouse titrations of TC-83 viable vaccine inocula indicated that horses 1 to 8 received 80,000; horse 9, <1,000; and horse 10, 32,000 SMICLD₅₀ units of virus.

Febrile responses [rectal temperature ≥ 101 F (ca. 38.3 C)] occurred within 1 to 4 days in all horses. Fevers persisted for periods of 12 to 96 hr and attained a maximum level of 105.8 F (Ca. 41 C) in horse 7. The maximum fevers of horses 9 and 10 were only 101.5 F (ca. 38.6 C). Median temperatures and ranges (Fig. 1) suggested that the febrile period was from 36 to 156 hr after inoculation, with significantly higher temperatures in the early evenings. With the Friedman nonparametric analysis of variance (8), a highly significant change occurred in the morning, evening, and median temperatures ($P < 0.005$). When further analyzed, by using a nonparametric post hoc comparison (12), the febrile periods were shown to occur from 1 through 4 days postvaccination for the afternoon temperatures, and from 2 through 5 days for the morning and median temperatures. The Friedman and post hoc comparisons are based upon the ranking of the data. For this reason, the assignment of statistical significance, at times, is not completely reflected in the median values presented.

Small amounts of urinary protein were detectable in five of the animals from days 2 to 6 after vaccination. Decreased urine specific gravities and pH values were transiently observed in six and five horses, respectively, during the same time period.

A leukopenia with reduction of both neutrophil and lymphocyte elements was observed from 3 to 6 days postvaccination in all 10 horses (Fig. 2). Applying the Friedman test, a significant change occurred in these values, $P < 0.005$. A post hoc comparison showed these decreased cell values from days 3 through 7 for the leukocytes and

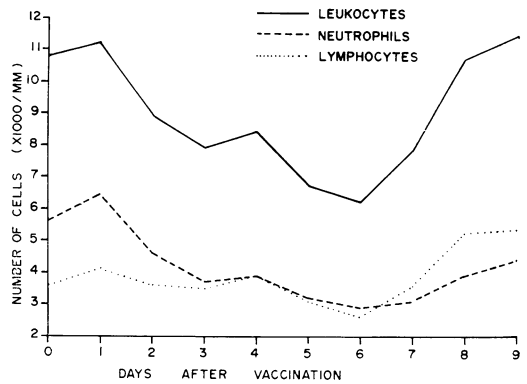


FIG. 2. Median leukocyte, neutrophil, and lymphocyte values for 10 horses vaccinated with strain TC-83 VEE vaccine.

neutrophils and on days 5 and 6 for the lymphocytes. The decrease in magnitude and duration than those shown for neutrophil and leukocyte counts, and was only of borderline significance ($P < 0.05$).

Clinical illness with anorexia, decreased water consumption, and depression was observed in five horses (no. 1, 2, 3, 5, and 8) for periods of 24 to 72 hr beginning 1 to 4 days after vaccination. Significant changes did not occur in the hematocrit and hemoglobin values.

Viremia (Fig. 3) was demonstrable in 8 of the 10 horses (exceptions were horses 1 and 9). The viremias persisted for 12 to 84 hr and reached a maximum level of $10^{3.5}$ tissue culture infectious dose (TCID₅₀) units/ml of serum in horses 3 and 7. All viremic horses had at least two positive samples; the viremia in horse 5 was detectable only in undiluted serum indicating the presence of small quantities of virus. No VEE virus was isolated from the various swab or urine specimens.

N antibody (Table 1) was initially demonstrable to strain TC-83 in two horses at 5 days, and to subtype I-B in one horse at 6.5 days after vaccination. Whereas all 10 horses had demonstrable N antibody to moderately high titers within 8 days to vaccine virus, only five seroconversions had occurred by 9 days to the epizootic virus, and antibody levels were low. One month after vaccination, N antibody was demonstrable in all 10 horses to both test viruses; titers were extremely high to the homologous strain and low to subtype I-B. After 1 year, titers had decreased, but N antibody was still present in the serum of the eight horses tested.

HI and CF antibodies (Table 1) were first demonstrable to the vaccine virus in single horses at 6.5 and 7 days, respectively, after vaccination.

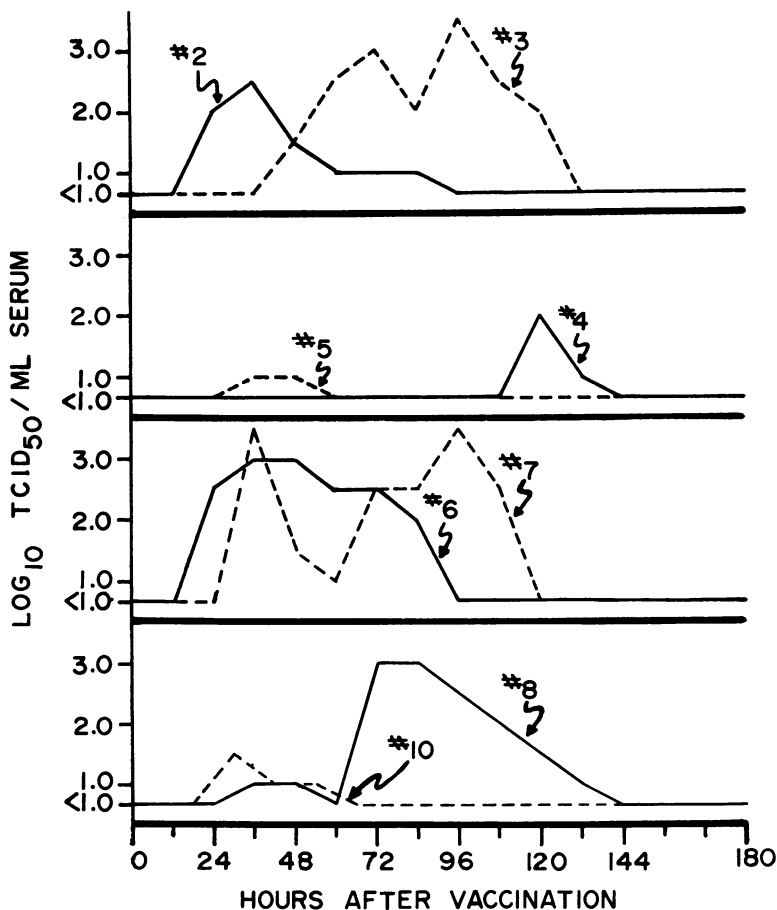


FIG. 3. Viremias of eight horses vaccinated with strain TC-83 attenuated VEE vaccine.

After 9 days, only six horses had detectable HI antibody and five animals had CF antibody in moderate concentrations. By 1 month, all 10 horses had detectable HI and CF antibody to vaccine virus. After 2 months, antibody levels and numbers of positive animals had decreased; HI and CF antibodies were barely demonstrable in six and two horses, respectively, of the eight tested 4 months after vaccination.

All of the six horses inoculated with the equine virulent VEE strain 14 months after vaccination, as well as the two animals challenged after 2 and 5 months, were protected. There was no fever, clinical illness, viremia, or change in the hemogram in any animal. Serological responses to challenge of the six horses vaccinated 14 months previously also are presented in Table 1. After 1 month, a rise in N antibody to vaccine virus was observed, but not to the levels recorded 1 month after vaccination; with the challenge virus, subtype I-B, the antibody level was threefold higher

than that attained during the initial response. Horse 9, challenged 5 months after vaccination, exhibited a twofold increase in N antibody titer to TC-83 and a fourfold increase to subtype I-B when compared to the levels 1 month after vaccination. Horse 10, challenged after 2 months, had a fourfold N antibody titer rise to epizootic virus, but no change to vaccine virus. Only small amounts of HI and CF antibodies were detectable with strain TC-83 in some animals, after challenge.

Selected sera were titrated differentially against several VEE virus subtypes (Table 2). All of the eight horses tested had N antibody to the vaccine virus and the three epizootic strains 1 month after vaccination. Antibody titers were highest to the vaccine strain, but were still present in all animals to all four of these virus strains 1 year later. In contrast, antibody levels and, particularly, numbers of responders were lower when tested with the enzootic subtypes I-D and I-E; horse 2.

TABLE 1. Neutralizing (N), complement-fixing (CF), and hemagglutination-inhibiting (HI) antibodies in horses after Venezuelan equine encephalomyelitis (VEE) strain TC-83 vaccination and subtype I-B challenge

No. of horses tested	Time after vaccination	No. and mean titer of sera positive to indicated virus							
		TC-83						I-B	
		N		HI		CF		N	
		Titer ^a	No. positive	Titer ^b	No. positive	Titer ^b	No. positive	Titer ^a	No. positive
10	4.5 Days	<8	0	<10	0	<4	0	<8	0
	5 Days	8	2	<10	0	<4	0	<8	0
	5.5 Days	≥20	2	<10	0	<4	0	<8	0
	6 Days	≥24	3	<10	0	<4	0	<8	0
	6.5 Days	≥26	5	10	1	<4	0	8	1
	7 Days	≥200	6	10	2	8	1	8	1
	7.5 Days	≥248	9	10	2	13	3	8	1
	8 Days	≥265	10	13	3	13	4	8	5
	8.5 Days	≥291	10	16	5	30	5	22	5
	9 Days	≥301	10	23	6	≥50	5	27	5
1 Month	≥3,580	10	55	10	≥99	10	30	10	
8	2 Months	1,232	8	10	6	6	8	14	8
	4 Months	1,040	8	10	6	4	2	14	8
	6 Months	848	8	10	3	<4	0	14	8
	12 Months	275	8	<10	0	<4	0	14	8
6	14 Months, pre-challenge	240	6	<10	0	<4	0	12	6
	8 Days, post-challenge	≥3,520	6	10	1	4	3	55	6
	1 Month, post-challenge	≥1,984	6	10	4	4	3	108	6

^a Reciprocal of geometric mean N antibody titer of positive sera based upon 80% plaque reduction of VEE virus indicated.

^b Reciprocal of geometric mean CF or HI antibody titer of positive sera.

TABLE 2. Neutralizing (N) antibody to Venezuelan equine encephalomyelitis (VEE) viruses in horse sera after strain TC-83 vaccination and subtype I-B challenge

No. horses tested	Time	VEE virus									
		TC-83	I-A	I-B	I-C	I-D		I-E		II	
8	1 Month, postvaccination	≥4,160 ^a	392	32	101	32	<u>0.88</u> ^b	15	<u>0.88</u>	NT ^c	
	12 Months, postvaccination	275	35	14	62	20	<u>0.50</u>	12	<u>0.75</u>	NT	
6	14 Months, prechallenge	240	32	12	56	20	<u>0.33</u>	20	<u>0.67</u>	9	<u>0.67</u>
	8 Days, postchallenge	≥3,520	136	55	112	56		32		56	
	1 Month, postchallenge	≥1,984	124	108	128	38	<u>0.67</u>	36		56	

^a Reciprocal of geometric mean N antibody titer of positive sera based upon 80% plaque reduction of indicated virus subtype or strain.

^b Indicates fraction of animals positive. Absence of underlined fraction indicates that all animals were positive.

^c Not tested.

did not develop N antibody to either virus. After 1 year, horses 1 and 2, and horses 1, 2, 5, and 6 lacked demonstrable N antibody to subtypes I-E and I-D, respectively.

When challenged 14 months after vaccination with subtype I-B, antibody increases were demonstrable in the six horses to most of the seven test viruses. Antibody titers to strain TC-83 and subtype I-A, from which TC-83 was derived, did not attain the levels recorded after vaccination. Threefold and twofold increases occurred with subtypes I-B and I-E, respectively, above the levels recorded for the initial response 1 month after vaccination; the responses to subtypes I-C and I-D were only slightly greater than the initial values. At 1 month post-challenge, horses 5 and 6 failed to exhibit N antibody to subtype I-D.

DISCUSSION

There seems little doubt that the TC-83 vaccine for VEE is immunogenic and protective. The development of specific antiviral VEE antibodies after vaccination has been documented in field studies conducted in Panama, Costa Rica (5), and Nicaragua (17), as well as in experimental studies performed in the U.S. (10, 14). In the present study, N antibody to the vaccine virus was detectable in all animals within 8 days, reaching titers of at least 1:512 after 1 month. Antibody development to an equine virulent strain (MF-8) occurred less rapidly and to lower levels, and similar antibody patterns also were demonstrable in all animals to the other epizootic viruses. When tested against the enzootic, equine avirulent strains (Walton, *unpublished data*), very low levels of antibody were detected in some animals. Similar differences in N antibody titers to homologous and heterologous VEE viruses have been described previously in field studies (5, 17; D. H. Martin, *Amer. J. Epidemiol.*, *in press*). After 1 year, N antibody to the vaccine virus, and the epizootic viruses tested, was still readily detectable in all animals. By contrast, development of CF and HI antibodies to the vaccine virus more closely followed the production of N antibody to epizootic I-B virus. After 4 months, CF antibody was barely detectable in two horses; low levels of HI antibody were demonstrable in three animals after 6 months. As in previously described studies (10, 14), challenge of two horses shortly after vaccination (2 and 5 months) was resisted. In the face of challenge by equine-virulent virus 14 months after vaccination, horses with minimally detectable levels of antibody to the challenge virus were likewise completely immune. A marked increase in N antibody to the challenge virus subtype I-B, and to a lesser

degree to subtype I-E, occurred after challenge. However, these titers did not attain the levels seen in horses recovered from a primary infection with subtype I-B (Walton, *unpublished data*). More vaccinated animals must be studied over longer periods of time to determine whether there is, in fact, a positive correlation between presence of N antibodies to a given VEE subtype and resistance to challenge, and whether a single vaccination will protect a horse for life.

There was a significant variation in vaccine virus titer among different vials of this vaccine. The vial of vaccine used for inoculation of horse 9 was markedly acid after reconstitution of the product with diluent of a satisfactory initial pH. Suckling mouse titrations indicated <1,000 SMICLD₅₀ units/0.5 ml of vaccine; the response of the horse was mild with low fever, minimal leukocytic changes, and no viremia, but development of N antibody was stimulated. In field studies with this vaccine in Nicaragua (17) and Panama (5), considerable variation in incidence and magnitude of N antibody responses was observed. One of us (T. W.) observed significant colorimetric variation in bottles of Hanks diluent prepared and used in Nicaragua. Diluent varied in color from alkaline (red-violet) to acid (yellow-orange); the TC-83 strain has been reported to be highly acid-sensitive (R. W. McKinney, *personal communication*), and the viable virus in a 100-dose vial with acid pH might soon be inactivated. Another possible explanation for differences in the vaccine response may be related to the vaccine virus itself. The attenuated virus stocks used in these studies were all produced at the same time. Lot 4 vaccine was dispensed and lyophilized on 17 October 1966. By contrast, the lot 5 vaccine was initially frozen at -70 F (ca. -21 C) for 4 years and not lyophilized in multi-dose vials until 1970. It is reasonable to suggest that some changes could have occurred in the virus during the intervening storage period and after different methods of handling and lyophilization were used. The variations in response may also reflect different routes of inoculation although results in our first group of eight horses inoculated intramuscularly closely paralleled those reported by Henderson et al. (10) using the same vaccine lot and the subcutaneous route.

Mild, clinical illness with fever in TC-83-vaccinated horses has been reported previously (10). We were unable to correlate clinical signs, duration, and level of viremia, fever, or changes in the hemogram. Changes in the urinalyses probably reflected effects of fever and anorexia rather than specific viral effects upon the kidneys.

The leukopenia observed in all 10 horses was

due to an absolute neutropenia and lymphopenia. Kissling (11) reported similar results in horses infected with the Trinidad donkey strain. It has been found in mice, guinea pigs, and rabbits that the effects of VEE virus are upon the lymphomyelopoietic system, causing necrosis as severe as that from intense radiation (16).

Viremias observed in eight animals were of a magnitude probably below the threshold necessary to infect mosquitoes. Nonetheless, the possibility of vaccine virus transmission to mosquitoes by viremic horses and the effect of such transmission on equine virulence deserves further study. Whatever the theoretical objections to general use of TC-83 vaccine in equines, there seems little doubt that it deserves to be employed in mass campaigns in any area actually threatened by a VEE epizootic. In our opinion, the 1971 outbreak that reached Texas from Mexico demonstrated this fact beyond all reasonable doubt (2).

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