Venezuelan equine encephalomyelitis: The goat as a sentinel for virus activity and as a serum donor for fluorescent antibody conjugates

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine

Major: Veterinary Microbiology

Iowa State University
Ames, Iowa

1973

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The insidious spread of Venezuelan equine encephalomyelitis virus (VEE) from South America through Central America to the United States has aroused great concern within the horse industry and among agricultural officials in the United States. Indeed the disease in the epizoodemic form appears to have been eliminated from the continental borders of this country but the mechanisms by which this virus can apparently reside within a given region are unknown. The term epizoodemic as employed in the text above refers to the concurrent presence of an epidemic and an epizootic due to a single disease agent (56,57). There is at present a great confusion of terminology in the literature due to the remarkable ability of the VEE virus complex to give rise to several forms of disease among man, domestic animals, and fauna of the countryside. Therefore, in an attempt to reduce this confusion, the term epizoodemic shall be employed in this study.

Due to the explosiveness of epizoodemic VEE it has become imperative to have rapid diagnostic techniques available to the virologist for confirmation of the disease agent in domestic livestock and in wildlife populations. Such a technique is readily available in the form of the fluorescent antibody test which can be used to obtain positive or negative results within a 48 hour period as compared to suckling mouse inoculation with complement fixation (CF) test confirmation which requires from 72 hours to one week for completion of diagnosis.

The invasion of the United States by VEE with subsequent eradication in 1971 has emphasized the need for a natural sentinel to detect the virus should it reenter the country in the future. Several animals have been investigated as possible sentinels by other workers. These include the canine (9), bovine, porcine (33), equine (25), laboratory (39,43,105, 107,108) and wild ungulate (67) species. Despite such intensive work, the suitability of the goat, (Capra hircus), as a sentinel species has not been investigated.

In hypothesizing the means by which epizoodemic strains of VEE run rampant through the countryside, reference has occasionally been made to the possibility of the goat acting as a silent amplifier of the virus (57,104,110). It was the purpose of this study, firstly, to investigate the suitability of the goat as a sentinel for virus activity, and secondly, to determine the sensitivity of the fluorescent antibody test on cell cultures as compared to suckling mouse inoculation with confirmatory CF test for identification of VEE virus from tissue specimens.

REVIEW OF LITERATURE

History

An encephalitic disease of equines which appeared in 1935 in the river valleys of Huila, Tolima, Valle, and Bolivar in the Andes mountains of western Colombia was tentatively diagnosed as Borna's disease or European equine encephalitis. From there the disease spread to Magdalena, Colombia in 1936 and later that year appeared in the Guajira peninsula of Colombia and Venezuela (125). In 1938 Kubes and Rios of the Venezuelan Ministry of Agriculture and Animal Husbandry isolated a filterable virus from the brain of a horse that had died with encephalitic signs (77).

Subsequent characterization of the agent was done by Kubes (76), Kubes and Rios (77) and by Beck and Wyckoff (6) in 1939 and 1944. The virus was found to be of greater virulence for equidae than any of the eastern or western equine encephalomyelitis viruses previously identified in South America. It also differed immunologically from these viruses. Due to its origin the encephalitic agent was designated "Venezuelan equine encephalomyelitis virus." A uniform system of nomenclature was not available for the complex of endemic (enzootic) and epizoodemic strains of Venezuelan equine encephalomyelitis (VEE) virus that later arose until Young and Johnson (134) in 1969 published their system embracing the antigenic variants of VEE (Table 1).

Since 1939, either epizoodemics or epidemics of VEE have been described in Peru, Ecuador, Columbia, Venezuela, Trinidad, Costa Rica, Nicaragua, Honduras, El Salvador, Guatemala, Mexico and the United States.

Table 1. Antigenic subtypes of Venezuelan equine encephalomyelitis virus (69,134)

Antigenic subtype	Isolate	Origin	Epizoodemic
IA	Trinidad donkey	Trinidad, 1943	Yes
IB	Ica	Peru, 1946	Yes
	Pergamino	Argentina, 1958	Yes
IC	P676	Venezuela, 1963	Yes
	V198	Colombia, 1962	Yes
ID	3880	Eastern Panama, 1961	No
IE	Mena II	Western Panama, 1962	No
	63A216	Mexico, 1963	No
II	Fe3-7c	Florida, 1963	No
III	Mucambo	Brazil, 1954	No
	Paramaribo	Surinam, 1963	No
	52049	Trinidad, 1959	No
IV	Pixuna	Brazil, 1961	No

Endemic foci have also been found to be widely distributed in the western hemisphere including Brazil, Trinidad, Surinam, Colombia, Panama, Nicaragua, Honduras, Guatemala, British Honduras, Mexico and Florida (57).

Current virus activity

In 1969 a severe epizoodemic of VEE arose in Guatemala and eventually reached Texas in late June, 1971 (22,24,37,39,42,56,75,114,124,130). The source of VEE virus for this and other epizoodemics remains an enigma. Three possible alternatives for the introduction of the virulent IB subtype of VEE virus into Guatemala have recently been proposed by Franck and Johnson (42). They are (1) mutation of an endemic virus subtype, (2) sudden emergence of a previously silent unrecognized virus, and (3) introduction of the virus from another region. Introduction of the IB subtype of VEE virus into Guatemala from Ecuador has been suggested by several authors (37,42,86,114, 129).

Equine deaths were first reported along the Pacific coast of Guatemala near El Salvador and shortly thereafter in northeastern El Salvador. VEE then spread north and south from each focus. The southward spread of the disease included Honduras (1969), Nicaragua (1969), and Costa Rica (1970). Susceptible equines were vaccinated with the attenuated TC-83 strain of VEE virus in Guatemala in an attempt to halt the northward spread of epizoodemic VEE. However, in August, 1969, epizoodemic VEE virus was isolated in the upper Grijalva River Valley of Chiapas, Mexico near the Guatemalan border. Equine deaths continued through the month of February and increased with the start of the rainy

season in June. From there the disease spread westward through the mountain canyons to the state of Oaxaca on the Pacific coast and was confined to that region by vaccination until September when the disease appeared just outside the vaccination zone in the state of Veracruz. Vaccination was resumed only to have epizoodemic VEE occur again just outside the barrier to the north.

Extension of the disease followed a similar pattern on the Pacific coast as it progressively appeared beyond each new vaccination barrier. Epizoodemic VEE had spread northward along the Pacific coast through the state of Guerrero to the state of Michoacah by November, 1970.

Virus activity continued unreported through the end of 1970 and into April of 1971. Equine deaths were then confirmed as VEE in Veracruz and a new vaccination barrier was established to the north. Despite all vaccination barriers epizoodemic VEE continued its northward spread across salt water lagoons of Mexico's gulf coast some 400 kilometers to Texas (125).

First case of epizoodemic VEE in the United States

The first confirmed case of epizoodemic VEE in the United States was reported on June 23, 1971, near Three Rivers, Five Oak County, Texas, approximately 320 kilometers northwest of Brownsville. The Texas epizoodemic peaked in the equine population by mid-July (30 virus isolations per day) and by the end of July had decreased to fewer than 5 viral isolates of epizoodemic VEE virus per day. A combination of ultra-low-volume aerial insecticide application and vaccination (TC-83

virus) of all susceptible equines in Texas effectively brought the invasion of Texas' equine and human populations to an end by November 7, 1971 (75). A total of 1,620 equine deaths and an additional 2,000 clinical cases of epizoodemic VEE were recorded in Texas in 1971 (86).

Epizoodemic virus activity in Mexico in 1972

Confirmed (virus isolation) epizoodemic VEE virus activity recurred in Mexico in the states of Durango and Sonora in 1972. Other nonconfirmed reports of VEE outbreaks came from the states of Nayarit, Guerrero, and Morelos. Intensification of vaccination of susceptible equines with the TC-83 strain of VEE virus in the state of Sonora in addition to that done in 1971 appears to have prevented epizoodemic VEE from advancing beyond Hermosillo, which is approximately 265 kilometers south of the Arizona-Mexico border (22,23,24,25).

Arthropod vectors and their possible role in the spread of epizoodemic VEE

Epizoodemic strains of VEE have been isolated from at least 25 species of mosquitoes consisting of 7 genera and one subgenus (22,115, 125) (Table 2). Isolation of epizoodemic VEE virus from field-trapped mosquitoes in itself is insufficient evidence to incriminate a particular species as a vector of the disease. Three additional factors must be evaluated prior to the assignment of vector status (115). They are as follows:

(1) Determination of the infection threshold and infection rate of the mosquito. Infection threshold may be defined as that level of host viremia sufficient to infect 1 to 5 percent of the mosquitoes feeding upon the virus source. Infection rate is the percent of mosquitoes that are viremic 14 to 21 days after

Table 2. Mosquito species from which epizoodemic VEE viruses have been isolated (115,117,123,125)

Mosquito species	Country
Aedes aegypti	Venezuela
Aedes angustivittatus	Colombia
Aedes scapularis	Venezuela
Aedes serratus	Venezuela
Aedes sollicitans	Texas, U.S.A.
Aedes taeniorhynchus	Colombia, Venezuela, Costa Rica; Texas, U.S.A.
Aedes thelcter	Texas, U.S.A.
Anopheles aquasalis	Venezuela
Anopheles crucians	Texas, U.S.A.
Anopheles neomaculipennis	Trinidad
Anopheles pseudopunctipennis	Texas, U.S.A.
Anopheles punctimacula	Colombia
Culex aikenii	Colombia
Culex corniger	Colombia
Culex nigripalpus	Guatemala
Culex quinquefasciatus	Colombia
Culex (Melanoconion) sp.	Guatemala; Texas, U.S.A.
Culiseta inornata	Mexico
Deinocerites pseudes	Costa Rica
Mansonia indubitans	Colombia
Mansonia titillans	Colombia, Guatemala, Trinidad
Psorophora ciliata	Texas, U.S.A.
Psorophora cilipes	Guatemala
Psorophora confinnis	Venezuela, Guatemala; Texas, U.S.A.
Psorophora discolor	Texas, U.S.A.

having fed upon a viremic host. Infection is determined by trituration of each mosquito with subsequent inoculation of one 2- to 4-day-old mouse per mosquito.

(2) Determination of transmission rate of the mosquito. This is obtained by allowing individuals one opportunity to infect a susceptible host following an incubation period (14-21 days) after the original blood meal. The transmission rate is then calculated by determining the percentage of infected individuals that transmitted the disease agent. Infection is assayed as described in (1) after allowing the arthropod to engorge.

(3) Determination of the extent of mosquito-host interaction. Field studies are employed to define this parameter of the possible vector as determined by (1) and (2). Areas of mosquito-host interaction usually considered are proximity of the host to breeding sites of the mosquito, blood meal identification, abundance of the potential vector species, attraction rate to various sentinel hosts, longevity, and flight range of the mosquito.

Some information on potential epizoodemic VEE virus vectors has been presented (Tables 3 and 4), but much remains to be done. Simulium spp. have also been implicated as biologic vectors of VEE virus. However, no laboratory data are available to substantiate this hypothesis (16,70,121).

The average host viremia needed to infect a suitable mosquito vector species with VEE virus is 5.0 $\log_{10}$ suckling mouse intracerebral lethal dose for 50% of those inoculated per ml ($SMICLD_{50}$) of viremic blood (14, 16) and an average VEE virus inoculum per mosquito bite has been stated to be 3.0 $\log_{10}$ $SMICLD_{50}$[1]. For establishment of infection and transmission capability of a mosquito two major barriers must be overcome (17). When a mosquito feeds upon a host the blood meal moves directly to the glandular midgut for digestion. To retain the blood meal within the

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[1]Chamberlain, R. W., Communicable Disease Center, Atlanta, Georgia. Personal communication. October, 1971.

Table 3. Mosquito infection and transmission rates for VEE virus IA (74)

Mosquito species	% infected	% transmitting
Culex tarsalis	100	100
Aedes triseriatus	100	90
Aedes canadensis	100	50
Psorophora confinnis	96	45
Aedes sollicitans	80	44
Aedes aegypti	79	26
Mansonia titillans	56	22
Anopheles freeborni	90	22
Mansonia indubitans	72	17
Anopheles quadrimaculatus	16	4
Culex pipiens	12	4

Table 4. Mosquito infection thresholds for VEE virus IB (115)

Mosquito species	% mosquitos infected	Viremic hosts titer[a]
Psorophora confinnis	2	4.9 - 5.2
Aedes aegypti	3	4.9 - 5.2
Aedes triseriatus	2	6.1
Aedes taeniorhynchus	4	6.7 - 7.1
Anopheles quadrimaculatus	0	>6.7 - 7.1
Culex nigripalpus	0	>6.7 - 7.1

[a]Suckling mouse lethal does 50% per ml ($SMLD_{50}$/ml) of viremic blood.

digestive tract a peritrophic membrane is usually secreted around it which then serves as a minor barrier to passage of virus from a viremic blood meal to the midgut epithelial cells. The virus may then attach to suitable receptor sites if they are available. These represent the first major barrier to infection of the mosquito. Assuming the mosquito is a suitable host species, the virus adsorbs to the midgut cells and is likely taken into the cell by the process of pinocytosis or viropexis. Upon entering the cell it is subjected to the action of a series of enzymes which uncoat the virion. Replication of the viral nucleic acid and coat materials then occurs with eventual assembly and maturation of progeny virions. In the process of maturation the assembled viral nuclear material is extruded through the cell's cytoplasmic membrane containing virus-specified envelope components. Hence, with viral maturation the progeny virions pass out of the midgut epithelial cell to enter the hemolymph of the mosquito. Having entered the primitive circulatory system the virus is then able to infect the organs of the mosquito that are bathed by the hemolymph. Viral replication ensues, but appears to continue only in the salivary glands. Within the salivary glands the virus replicates to high titers where it is considered to persist for the lifespan of the mosquito. The second major barrier is now at hand. In order for the mosquito to be infective the virus must be able to bud out from the acinar cells of the salivary glands into the salivary ducts or their precursors. If the mosquito in question is a vector species the virus buds into the salivary gland ducts and the mosquito is then infective indefinitely according to laboratory studies (16,17). The

extrinsic incubation period in the mosquito can be defined as the interval required for virus to appear in the salivary juices after feeding upon a viremic host. This period is inversely proportional to the ambient temperature of the mosquito's habitat. For species of Aedes and Mansonia the period is probably no more than 8 to 12 days or about the span of time between the first and the third blood meals. The period would probably be even less for a species such as Psorophora confinnis due to its relatively short lifespan as compared to longer-lived mosquitoes such as Aedes sp. (16).

According to the previously listed criteria for vector status only Psorophora confinnis and Aedes taeniorhynchus have been proven as likely vectors of the IB strains of VEE virus. Insufficient data are available at this time to unequivocally incriminate these species as vectors of epizoodemic VEE. Other probable vectors species according to habitat are as follows (115):

(1) Permanent fresh water with vegetation. Mansonia indubitans and M. titillans.

(2) Temporary fresh water pools, sunny and grassy. Psorophora confinnis, P. discolor, and Aedes thelcter.

(3) Coastal beaches, crab hole terrain. Deinocerites pseudes.

(4) Coastal areas, brackish water, inland 80 to 110 kilometers. Aedes sollicitans and A. taeniorhynchus.

(5) Woodlands, fresh water, shady pools. Aedes scapularis.

Although the role of mosquitoes as vectors of VEE virus is gradually being defined, the question of origin of epizoodemics of VEE remains unknown. Migratory birds have been considered to be a potential means

of transport of eastern and western equine encephalomyelitis viruses from one country to another, but few epizoodemic VEE virus isolations have been obtained from migratory birds to substantiate such a concept for VEE virus movement. According to Grayson (51) the only naturally-infected, viremic (epizoodemic VEE) bird reported has been a fledgling green heron (Butorides virescens), also referred to as the striated heron (B. striatus). On the basis of that isolation Grayson and co-workers (51) inoculated 9 wild-caught, serologically negative green herons with 100 suckling mouse intraperitoneal lethal dose 50% ($SMIPLD_{50}$) of the 3880, ID (endemic) strain of VEE virus. The 9 birds were all viremic by the 4th and 5th day postinoculation and were used for experimental transmission studies with Deinocerites pseudes as the vector and golden hamsters as the recipient hosts. Positive transmission was obtained indicating a possible role for the green heron in the movement of epizoodemic VEE virus strains (IA, IB, IC) from country to country along the coastal areas as occurred in the epizoodemic that spread from Guatemala to the United States. Additional supporting evidence is that green herons commonly inhabit areas where the crab-hole-breeding Deinocerites pseudes lives.

To determine if rodents (57,63) could be involved in the maintenance of epizoodemic VEE virus Zarate (135) and Walton (128) inoculated cotton rats (Sigmodon hispidus) with epizoodemic strains IC and IB respectively. With 4 to 1000 plaque-forming units of virus administered subcutaneously Zarate obtained death patterns of 1 out of 5 and 3 out of 5 rats inoculated. Walton inoculated 3 weanling S. hispidus with

1000 $SMICLD_{50}$ units of virus and had no survivors by 6 days post-inoculation. The conflicting results of these two studies and the statistically small number of experimental animals indicate further studies in this area are needed to define the possible role rodents may have in the maintenance of epizoodemic VEE virus.

Fossaert (40), Franck (41) and Johnson (68) have likewise cited the possibility of epizoodemic virus residing in natural foci much as endemic strains of VEE are known to exist. Little evidence other than that of Grayson (51) and Walton (128) exists to substantiate such a hypothesis.

Bats have also been proposed as a transport and maintenance mechanism for epizoodemic VEE by several workers (65). According to Baer, cited by Calisher (12), the Mexican freetail bat (Tadarida brasiliensis mexicana) cohabits with the vampire bat (Desmodus rotundus) and migrates hundreds of kilometers to the United States and to other regions. In August, 1970, D. rotundus was found infected with the IB strain of VEE in the state of Oxaca, Mexico during an epizoodemic (27). Epizoodemic VEE virus has also been isolated in Ecuador from D. rotundus in 1969 (51).

Vampire bats subsist on a strict blood diet and frequently feed upon equines, consuming as much as 20 to 25 ml of blood per day. While feeding the bats may either ingest viremic blood or be bitten by infectious mosquitoes (27). Sanmartin (99) has fed heparinized viremic horse blood to D. rotundus and has determined VEE virus to be present in the bat's oral cavity for 48 to 168 hours postinoculation. Transmission studies

have not yet been reported. However, after feeding vampire bats will often rest in hollow trees which may also serve as resting areas for mosquitoes. If those mosquitoes were also vector species they could then feed upon the resting, possibly viremic bats, and later become infectious to exacerbate an epizoodemic of VEE (1,29).

Another means of maintenance of epizoodemic strains of VEE virus that has been postulated is that of silent amplification. Groot (55) investigated various races of sheep from La Guijara, Colombia and found that they either developed a transient, minimal viremia or were not viremic. However, all of the sheep exhibited serologic conversion from negative to positive.

Goats have also been considered as possible silent amplifiers of VEE (57) but confirmatory data are not available at present (104). Serological surveys conducted during outbreaks of epizoodemic VEE have on the other hand shown a fairly high ratio of serologic conversion of domestic goats tested for VEE antibody by the hemagglutination-inhibition and serum-neutralization tests. Bergold (7) found a high incidence of antibody in goats in Venezuela from 1962 to 1969. By the hemagglutination-inhibition tests 102 of 332 goats were positive at a serum dilution of 1:20. Epizoodemic VEE virus was also obtained from a goat in Zulia, Venezuela over the epizoodemic period of 1968 to 1969.

In an earlier publication, Sellers, et al. (110) described hemagglutination-inhibition and serum-neutralizing antibody titers in approximately 59 percent of 39 goats sampled from November, 1962 to January, 1964 in Venezuela. During epizoodemic virus activity in

Colombia from 1967 to 1968, Mackenzie (85) obtained an overall antibody incidence of 20 percent of 31 goats tested. Serum neutralization antibodies were also found in goats in Texas during the 1971 outbreak but detailed results are not yet available (94).

The role of the dog as a silent amplifier has been evaluated by several groups. Taber, et al. (118) infected beagles with the IA subtype of VEE virus and obtained adolescent (21-23 day old) mouse viremia titers ranging from 3.1 to 4.3 $\log_{10}$ mouse $IPLD_{50}$ per ml of viremic dog blood. Bivin, et al. (9) were able to transmit IA VEE virus to beagles with A. triseriatus mosquitoes which had been infected by the hanging-drop method. Davis, et al. (30) transmitted IA VEE virus from beagles to guinea pigs with A. triseriatus at viremia titers ranging from 3.7 to 6.0 $\log_{10}$ adolescent (21-23 day old) mouse $IPLD_{50}$ per ml of blood. However, work done by Sudia (115) indicates that the threshold for infection of A. triseriatus with the IB subtype of VEE virus is considerably higher than that indicated by the work of Davis, et al. (30) with the IA subtype of VEE virus. Based on the information available it does not seem likely that the dog would serve as a silent amplifier of IB VEE virus.

Chamberlain (13) has proposed several areas which should be studied to elucidate the role of alteration of viral virulence in the sudden eruption of epizoodemic VEE in regions that previously were "free" of virus activity. They are as follows:

(1) Passage in vertebrates with either higher or lower body temperatures than the usual hosts.

(2) Incubation in vectors for either exceedingly long or very short periods.

(3) Passage by the pharyngeal route (abnormal).

(4) Reproductive organ infection and transmission by sexual contact.

(5) Simultaneous infection of either mosquitoes or vertebrates with two strains of virus.

Due to the high magnitude of viremia produced in man by VEE virus infection he is also subject to consideration as an amplification mechanism. Viremias in excess of 5.0 $\log_{10}$ $SMICLD_{50}$ per ml of blood with a range of 3.5 to 6.0 $\log_{10}$ per ml have been reported. Man is therefore a possible vehicle of virus movement but is of secondary importance when compared to the great mobility of equines (11,36,64,101, 111,126).

The movement of viremic equines has very likely played a major role in the dissemination of epizoodemic VEE in this day of modern, rapid transportation facilities (103,109,125). McConnell (86) has stated that about 50 percent of all equines infected by the epizoodemic virus will be clinically normal. Consequently, frantic owners have been known to ship their valuable horses out of regions where virus activity is prevalent and is responsible for daily reports of equine mortality and morbidity (120). Race horses have also been incriminated as likely prospects due to their widespread travels from ranches to racetracks (93). Many factors point to equine movement as a major mode of spread of epizoodemic VEE virus but little specific evidence has been accumulated to date.

During the 1970 VEE outbreak in Central America an inactivated vaccine of poor quality was prepared in Nicaragua from IB VEE virus and distributed for use. Shortly thereafter breaks of the disease appeared to follow the path of the vaccine. When use of the preparation was suspended the equine cases of VEE immediately ceased (89).

When "new" epizoodemics of VEE appear the poor monitoring of equine deaths or complete lack of monitoring must be taken into consideration. The probability would seem to be great that epizoodemic virus activity could have been occurring undetected in isolated regions of a country with a small but significant number of equine deaths (122). The concurrent presence of endemic and epizoodemic VEE viruses in a given region would present a partially immune equine population and would likely keep equine disease to a minimum. In this type of situation the epizoodemic virus activity would probably be detected only on the periphery of the endemic virus area resulting in a very low level or complete lack of virus activity being reported.

The mechanism by which the epizoodemic virus overwinters in a given region is not known. On January 10, 1973, IB VEE virus was isolated from a pool of 50 Culiseta melanura in Xochimilco, Distrito Federal, Mexico, the first isolate of an overwintering study initiated in cooperation with the Communicable Disease Center (CDC), Atlanta, Georgia, by the Animal Health Department, Ministry of Agriculture and Livestock, Mexico (22).

Young (133) has proposed the phenomenon of recrudescence as a source of epidemics. Supportive evidence presented was the recurrence of clinical illness due to the IE subtype of VEE virus in a laboratory field worker one year after initial illness in a region of Panama where IE VEE virus had never been isolated.

Endemic (enzootic) VEE

As listed in Table 1, antigenic groups ID, IE, II, III, and IV are not epizoodemic strains. Due to their focal nature involving primarily small rodents and possibly birds they have collectively been denoted as either endemic or enzootic VEE (4,18,19,46,47,66,72,74,95,107,108,134). Endemic virus strains have been isolated from 7 species of birds, while natural antibodies against the virus have been found in at least 23 avian species. In various laboratory trials all bird species studied developed low to moderate viremias, usually of 2 or more days' duration. In mosquito-transmission experiments, some mosquitoes have become infected by feeding on birds with viremias as low as 2.6 $\log_{10}$ $SMICLD_{50}$ of endemic VEE virus per ml. However, the presence of antibodies against endemic VEE virus in field-trapped birds has been highly variable. For given regions of endemic virus activity investigators have reported a complete lack of serological evidence in field-trapped birds while other groups have found a very high incidence of endemic VEE virus antibodies in their field-trapped birds from other endemic regions. Due to the marked discrepancies at present in this area of study, the role of birds in the maintenance of endemic VEE virus activity cannot be discounted as

insignificant (32,53,54,82,83).

Endemic foci usually do not exceed one quarter of a square mile and are usually found in high rainfall areas with either a tropical or sub-tropical climate (71). The foci may be in wooded regions and have often been found to be proximal to fresh-water swamps. The endemic virus cycle is mosquito-rodent-mosquito with occasional bird hosts comingled as noted earlier. Marsupials, bats, and raccoons have recently been incriminated in the cycle with small rodents (8,52,71,80,82,100,102,106). In Florida the mammals primarily involved in the cycle with Culex (Melanoconion) spp. appear to be the cotton rat (Sigmodon hispidus), the cotton mouse (Peromyscus gossypinus), the raccoon (Procyon lotor), and the opossum (Didelphis marsupialis) (8,15,82). See Table 5 for mammals involved in the maintenance of endemic VEE virus.

A total of 38 species of mosquitoes in 14 genera and subgenera have been implicated in the transmission of endemic VEE virus (Table 6). Among them only 3 species have been reported in the literature as well-substantiated vectors. Galindo (45) indicated that 2 species of mosquitoes proven to be efficient natural vectors of endemic VEE virus are Culex (Melanoconion) aikenii and C. (M.) portesi. Mosquito groups other than Culex (M.) spp. are probably involved as secondary vectors in the natural transmission of endemic VEE virus. The third species which has been clearly implicated as a primary vector of endemic VEE is Culex (M.) cedeci (15). These vector species would seem to possess a threshold for the endemic strains of VEE virus not unlike that of Culex tarsalis and western equine encephalomyelitis. Naturally-infected rodent species have

Table 5. Mammals considered to be involved in the maintenance of endemic VEE virus activity (47,52,53,54,71,80,82,85,100,102,112,113,119, 131)

Species	Virus isolation	Antibody HI[a]	Antibody CF[b]	Antibody SN[c]
Rodent				
Cotton rat (*Sigmodon hispidus*)	X	X		X
Terrestrial rice rat (*Orzomys laticeps*)	X	X		
Rice rat (*Orzomys palustris*)		X		
Spiny rat (*Proechimys semispinosus*)	X	X		
Rice rat (*Orzomys calignosus*)		X		
Common rat (*Rattus rattus*)		X		
Thorny rat (*Hoplomys gymnurus*)		X		
Spiny rat (*Proechimys guyannensis oris*)	X	X		
Rice rat (*Orzomys capito goeldii*)	X	X		
Chisel-toothed kangaroo rat (*Dipodomys microps*)			X	
Cotton mouse (*Peromyscus gossypinus*)		X		X
Forest pocket mouse (*Heteromys anomalus*)	X	X		
Short-tailed cane mouse (*Zygodontomys brevicauda*)	X	X		
Deer mouse (*Peromyscus maniculatus*)			X	
Western harvest mouse (*Reithrodontomys megalotis*)			X	
Mouse (*Peromyscus mexicanus*)	X			

[a] Presence of hemagglutination-inhibition antibodies.

[b] Complement-fixation titers ≥ 1:16.

[c] Presence of serum-neutralizing antibodies.

Table 5. (Continued)

Species	Virus isolation	Antibody HI[a]	Antibody CF[b]	Antibody SN[c]
Marsupial				
Common opossum (*Didelphis marsupialis*)	X	X		
Philander opossum (*Philander* sp.)	X	X		
Woolly opossum (*Caluromys derbianus*)	X	X		X
Murine opossum (*Marmosa mitis*)	X	X		
Bats				
Artibeus lituratus		X		
Artibeus turpis	X			
Carollia subrufa	X	X		
Carollia perspicillata		X		
Glossophaga sorcina		X		
Artibeus sp.		X		
Carollia sp.		X		
Other:				
Raccoon (*Procyon lotor*)		X		
Forest rabbit (*Sylvilagus brasiliensis*)		X		
Desert cottontail (*Sylvilagus audubonii*)			X	
Nuttal cottontail (*Sylvilagus nuttallii*)			X	
Black-tailed jackrabbit (*Lepus californicus*)			X	
Paca (*Agouti paca*)		X		
Red squirrel (*Sciurus granatensis*)		X		
Variegated squirrel (*Sciurus variegatoides*)		X		
White-tailed antelope squirrel (*Citellus leucurus*)			X	
Weasel (*Mustella frenata*)		X		
Kit fox (*Vulpes macrotus*)			X	

Table 6. Mosquito species from which endemic VEE viruses have been isolated (2,3,4,15,19,46,47,53,72,74,105,113,131,135,136)

Mosquito species	Country
Aedes angustivittatus	Brazil, Colombia, Venezuela
Aedes atlanticus	Florida, U.S.A.
Aedes scapularis	Brazil, Mexico, Venezuela
Aedes serratus	Brazil, Trinidad, Venezuela
Aedes taeniorhynchus	French Guiana; Florida, U.S.A.
Anopheles aquasalis	Brazil, Venezuela
Anopheles nimbus	Brazil
Anopheles punctimacula	Colombia
Coquillettidia (Rhynchotaenia) albicosta	French Guiana
Coquillettidia (Rhynchotaenia) venezuelensis	Brazil, French Guiana, Trinidad
Culex corniger	Colombia
Culex coronator	Mexico
Culex nigripalpus	Trinidad; Florida, U.S.A.
Culex quinquefasciatus	Brazil, Colombia, Mexico, Panama
Culex thriambus	Mexico
Culex (Eubonnea) accelerans	Trinidad
Culex (Eubonnea) amazonensis	Trinidad
Culex (Melanoconion) aikenii	Brazil, Colombia, Panama
Culex (Melanoconion) albinensis	French Guiana
Culex (Melanoconion) cedeci	Florida, U.S.A.
Culex (Melanoconion) epanastasis	Trinidad
Culex (Melanoconion) iolambdis	Mexico
Culex (Melanoconion) opisthopus	Mexico
Culex (Melanoconion) portesi	Brazil, French Guiana, Trinidad
Culex (Melanoconion) spissipes	Trinidad
Culex (Melanoconion) taeniopus	Brazil, French Guiana, Panama, Trinidad
Culex (Melanoconion) thriambus	Mexico
Culex (Melanoconion) vomerifer	Brazil, Panama, Trinidad
Culex (Melanoconion) ybarmis	Trinidad
Deinocerites pseudes	Mexico, Panama
Hemagogus mesodentatus	Mexico
Hemagogus (stegoconops) spegazzinii	Brazil

Table 6. (Continued)

Mosquito species	Country
Limatus durhami	Trinidad
Limatus flavisetosus	Trinidad
Mansonia fasciolata	Mexico
Mansonia titillans	Trinidad
Psorophora ferox	Brazil, Mexico, Trinidad
Sabethini sp.	Brazil
Wyeomyia medioalbipes	Trinidad
Wyeomyia mitchelli	Mexico
Wyeomyia (Dendromyia) occulta	French Guiana

been found to develop viremias of 3.9 to 5.0 $\log_{10}$ $SMLD_{50}$ per ml of viremic blood which persist from 4 to 5 days (45,71). Within endemic foci vector species have been found to be infected throughout the year (81). The presence of susceptible host species for maintenance of the virus is greatly facilitated by the average lifespan of 6 months for ground-dwelling rodents. Continuous virus activity is further assisted by the rodents' selective habit of breeding during the rainy season when large populations of the vector species are available (71).

The fact that certain species of Culex of the subgenus Melanoconion such as C. (M.) portesi (71) will readily feed upon man presents the potential of outbreaks of disease in man due to endemic VEE. Mosquito species in the genera Aedes, Anopheles, Deinocerites, Mansonia, and Psorophora are also known to feed avidly on equines and man (115). Since species of these genera are involved in the transmission of both endemic and epizoodemic strains of VEE virus, man is indeed a very likely candidate for disease in the presence of high virus activity in a given region. The first human case of VEE in the United States was due to endemic virus activity in Florida as reported by Ehrenkranz, et al. in 1968 (35). More recently, however, the epizoodemic of VEE in Texas resulted in 88 laboratory-confirmed cases of human VEE with all but 2 of the cases occurring in July, 1971 (24).

The fluorescent antibody test

The swift spread of epizoodemic VEE and its high morbidity rate necessitates a rapid diagnostic system. The fluorescent antibody test

(FAT) as developed by Coons, et al. (26) with subsequent modifications meets nearly all the requirements for a rapid, sensitive diagnostic test when performed in a tissue culture system.

Several types of globulin separation and purification have been employed in preparation of fluorescent-antibody conjugates. The two more commonly employed techniques at present are 50 percent saturated ammonium sulfate precipitation and chromatographic separation of a gamma-globulin-rich (IgG) fraction from hyperimmune sera.

Coons, et al. orginally used fluorescein isocyanate for conjugation (26) but this compound has the disadvantages of being unstable and dangerous to prepare. The synthesis of fluorescein isothiocyanate (FITC) by Riggs, et al. in a more stable powder form has essentially replaced the isocyanate compound for use in antibody conjugation (79,97,98). The labeling of gamma globulin fractions with FITC can be done by either direct addition or by dialysis labeling. For direct conjugation FITC is ordinarily added dropwise to the globulin fraction at a ratio that has varied from 1:20 (1 mg of FITC to 20 mg of protein) up to 1:200. Current literature indicates the most suitable range for optimal tagging to occur is from 1:100 to 1:200 (44,132).

Dialysis labeling, as described by Clark and Shepard (20), is generally considered to result in more uniform tagging of the antibody molecules of the globulin preparation. Uniform labeling is also enhanced by the lack of albumin and any traces of macroglobulins (IgM). If these proteins are present their affinities for FITC are somewhat greater than that of the IgG in the globulin preparation resulting in less FITC-tagging

of IgG and a final conjugate with high background fluorescence and decreased specificity of fluorescence (88).

Dilution, tissue powder adsorption, and anion-exchange column chromatographic purification of conjugates are three methods of removal or marked reduction of antibody molecules excessively tagged with FITC after unreacted FITC has been removed by either Sephadex G-25[1] chromatography or dialysis against a buffered saline solution. Dilution is one of the most widely used techniques to reduce nonspecific staining due to excessively-tagged antibody molecules and may readily be employed with high-titered conjugates. Tissue powder adsorption and anion-exchange chromatography both reduce the antibody content of conjugates but they also result in a conjugate that is more specific in its staining characteristics. The disadvantage of the tissue powder adsorption technique is that it can lead to bacterial contamination of the finished conjugate. The advantage of anion-exchange chromatography is that only optimally-labeled antibodies are left in the conjugate (28,31,49,87).

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[1]Pharmacia Ltd., Uppsala, Sweden.

MATERIALS AND METHODS

Cell cultures

To determine the optimal in vitro cell culture system for the isolation and identification of the IB subtype of VEE virus a comparative propagation study was undertaken. Eight types of cell cultures were analyzed for their ability to support growth of the virus with the production of visible cytopathic effects under a standard nutrient agar overlay. The cell cultures employed in this study were Vero African green monkey kidney cell line[1], human amnion (FL) cell line[1], baby hamster kidney (BHK-21) cell line[1], L cell line[1], goat kidney secondary cell culture[2], bovine turbinate cell line[2], horse kidney secondary cell culture[2], and duck embryo fibroblast (DEF) primary cell culture. All cell cultures with the exception of the BHK-21 were grown and maintained with Gibco F-15[3] medium supplemented with 10.0 ml L-glutamate and 10.0 ml sodium pyruvate per liter of medium plus serum as indicated below. The BHK-21 cell line was grown and maintained with Stoker's modification[4] of Eagle's BME[3] which is the addition of tryptose phosphate broth[5] to a concentration of 10 percent of the final medium. Antibiotics employed in the two culture media were 10,000 IU penicillin, 0.13 g streptomycin, and 2.5 mg nystatin per liter. For growth a 10 percent concentration of fetal calf serum

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[1]As obtained from the American Type Culture Collection, Rockville, Md.

[2]Developed and maintained by Diagnostic Virology, APHIS, USDA, Ames, Ia.

[3]Grand Island Biological Co., Grand Island, N.Y.

[4]Stoker, M. 1962. Virology 15:147-151.

[5]Difco Laboratories, Detroit, Mich.

was used, and cells were maintained with medium containing 5 percent fetal calf serum with the exception of the BHK-21 cell culture which was maintained with the growth medium.

For the comparative propagation study each of the 8 cell lines above was seeded into 25 cm^2 Falcon flasks[1] and was inoculated when confluent with 0.1 ml of a tenfold dilution of either cell culture or suckling mouse brain origin virus. The inoculated flasks were placed in a 37C incubator for one hour to allow virus adsorption. They were then overlaid with 5.0 ml of 1 percent Noble agar[2] containing 1x Earles BSS[3], 0.5 percent lactalbumin hydrolysate, 0.22 g sodium bicarbonate per liter, 2 percent fetal calf serum, 3.3 ml of 1:100 neutral red per 200 ml, and 400 IU penicillin, 200 μg streptomycin, and 100 μg nystatin per ml. Plaques were first counted after 48 hours incubation at 37C and again after an additional 24 hours incubation at 25C. The endpoint was calculated and expressed as the number of plaque-forming units per 0.1 ml (PFU/0.1 ml). Titers were then compared to virus titers obtained by suckling mouse inoculation to determine the relative sensitivity of each cell culture to IB VEE virus.

Viruses

The GJ9-1BJ strain of VEE virus was isolated from a pool of Psorophora confinnis mosquitoes collected near Parcelamiento Montufar, Guatemala, in

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[1]Falcon Plastics, Division of BioQuest, Los Angeles, Ca.

[2]Difco Laboratories, Detroit, Mich.

[3]Grand Island Biological Co., Grand Island, N. Y.

1969, during the course of an epizoodemic (62,116). Working stocks of this virus were prepared by inoculation of suckling mouse seed virus as supplied to Diagnostic Virology, APHIS, USDA, Ames, Ia., by CDC, Atlanta Ga., into suckling mice and two cell lines, Vero and goat esophagus[1]. A 0.5 ml quantity of a 1:100 dilution of 10 percent mouse brain suspension prepared in 0.01 M, pH 7.2, calcium-and magnesium-free phosphate-buffered saline containing 0.75 percent bovine serum albumin (BAPBS) was inoculated into each 75 cm^2 Falcon flask of confluent cells. After a one hour adsorption period at 37C, 25 ml of maintenance medium containing one percent serum was pipetted into each flask and they were returned to a 37C incubator. Fetal calf serum and normal goat serum were used in the respective cell cultures at a lower than normal concentration to allow use of the same stock viruses for tissue culture and goat inoculation studies. After a 48 hour incubation period the culture fluids were harvested as stock viruses and were maintained at -70C.

The suckling mice inoculated for stock virus preparation were observed daily for signs of encephalitis. When 5 to 15 percent of the suckling mice were either prostrate, moribund, or dead the litters were harvested. A 10 percent suspension of the pooled mouse brains was prepared in BAPBS, divided into 1.0 ml aliquots, and maintained at -70C as virus stock.

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[1]As obtained from the American Type Culture Collection, Rockville, Md.

Experimental animals

Twelve domestic goats of mixed sex from 4 to 18 months of age were used in the experiments. Prior to purchase the goats were screened by the hemagglutination-inhibition test and were found to be negative for antibodies against eastern equine encephalomylitis, western equine encephalomyelitis, and Venezuelan equine encephalomylitis viruses. They were housed in maximum biological security stalls with a filtered air intake and exhaust supply system that is more than 99.6 percent effective in the removal of viral disease agents.

Albino suckling mice, CF-1 strain, 2 to 4 days of age, obtained from Carworth Farms, Division of Becton, Dickinson and Co., New City, N. Y., were used for viremia assays and for titration of virus strains. The viruses were titrated in tenfold serial dilutions using 8 mice per dilution. Each mouse was inoculated intracerebrally with 0.02 ml of a given virus preparation and was then observed daily for 7 days. Moribund, prostrate, and dead mice were removed and stored at -70C until their brains could be processed for preparation of complement fixation (CF) test antigen to confirm the presence of VEE virus in the affected mice. The $\log_{10}$ $SMICLD_{50}$ per ml endpoint for each specimen was determined according to the method of Reed and Muench, cited by Lenette and Schmidt (79).

Serology

Hemagglutination-inhibition (HI) tests were performed by a microtiter modification of the method of Clarke and Casals (21) in twofold serum dilutions from 1:10. The test antigen was prepared from GJ9-1BJ VEE

virus infected mouse brains by sucrose acetone extraction. Ninety-six well, u-bottom plastic plates[1] with an arrangement of 8 by 12 wells were used for the test. A 0.25 percent suspension of goose red blood cells prepared in dextrose-gelatin-veronal buffer was employed for all hemagglutination-inhibition tests. The mouse brain antigen mentioned above was then titrated for 4 to 8 hemagglutination units per 0.025 ml using a 0.2% bovalbumin, pH 6.0 borate saline (BBS). Sera to be tested were likewise diluted in BBS for the HI test. Initially 0.05 ml of a 1:10 serum dilution was placed in the first well of the plate and 0.025 ml of BBS was dispensed in the succeeding wells. Twofold dilutions were then made with 0.025 ml microdiluters[1], 0.025 ml of titrated antigen was placed in each well, and the test was incubated overnight at 4C. The following morning 0.05 ml of a freshly prepared 0.25 percent suspension of goose red blood cells was added to each test well and the plate was briefly agitated for mixing of the test reagents. After one hour incubation at 37C the plate was read for the absence of hemagglutination, indicating a positive HI test.

Serum virus neutralization (SN) testing was performed in 2 virus-tissue culture systems. The GJ9-1BJ strain of VEE virus was used with the BHK-21 cell line and the TC-83 strain of VEE virus was used with DEF cells for titration of goat sera. All sera tested were heat-inactivated at 56C for twenty minutes. The 0.2 ml allquots of tenfold dilutions of sera being tested were aseptically dispensed into sterile 12 x 75 mm stoppered tubes

[1]Cooke Engineering Co., Alexandria, Virginia.

to each of which were added 0.2 ml of virus containing 120 to 200 PFU per 0.1 ml. The resultant mixtures were then agitated briefly and placed in a 37C waterbath for a one hour period of virus neutralization. At the end of the incubation period the tubes were removed and placed in a wet ice waterbath. One tenth ml of each mixture was then pipetted into 2 25 cm^2 Falcon flasks of either BHK-21 or DEF cells according to the strain of virus being used in that test. Following a one-hour period of virus adsorption at 37C, 5 ml of the Noble agar preparation previously described was dispensed into each flask. The flasks were then placed on a level surface at 25C for 15 to 25 minutes for agar solidification. When the agar had hardened the flasks were inverted and were incubated at 37C. After 48 hours the flasks were removed and the first plaque count was made. A final count of plaques was made after 24 hours' incubation at 25C. The endpoint of a serum was that dilution producing at least a 90 percent plaque reduction.

The complement fixation test was used to confirm that suckling mice employed for virus titrations which had been either moribund, prostrate, or dead at the time of harvest were infected with VEE virus. A 10 percent suspension of mouse brain was prepared in veronal buffer diluent (VBD), and was centrifuged at 10,000 rpm for 30 minutes. The supernatant fluid was retained as the 4x CF test antigen. The microtiter CF test was performed with either VEE antiserum or mouse ascitic fluid employing dilutions of 1:8 through 1:256, a 1:20 dilution of unkown antigen, 7 hemolytic units of complement, and sensitized sheep red blood cells. The

VEE antiserum was diluted in the plates with subsequent addition of antigen and complement prior to incubation overnight at 4C for complement fixation. The following morning sensitized sheep red blood cells were added and the plate was incubated at 37C for 30 minutes, followed by centrifugation at approximately 250 g for 10 to 15 minutes to pack the remaining ghost and red blood cells. The test was read by comparing hemolysis against known color standards. From zero to 30 percent hemolysis was considered to be a positive test. Wells displaying greater than 30 percent hemolysis were considered to be negative.

Sentinel study

Six goats were inoculated intradermally with 1000 $SMICLD_{50}$ of goat esophagus cell culture origin GJ9-1BJ VEE virus (GEV). The 6 goats were divided into 2 groups with one noninfected goat per group to determine if contact transmission of GEV from goat to goat was possible. Blood samples were taken with and without heparin from the 8 goats from zero to 14 days postinoculation (DPI) for determination of viremia and for serologic assay. An additional aliquot of blood was obtained at zero DPI from each goat to provide normal goat serum for conjugation. Nasal, oral, and genitourinary swabs were also obtained 0-7 DPI to determine if VEE virus was shed by the infected goats. Serum samples were obtained from the 8 goats on a daily basis from 15-21 DPI and biweekly thereafter through 49 DPI. Rectal temperatures were recorded daily from 0-21 DPI. Goat viremia assay was done by BHK-21 plaque assay and intracerebral inoculation of suckling mice. Serologic responses were determined by HI and SN tests using viruses TC-83

and GJ9-1BJ.

Antiserum production

Two groups of goats were employed for the 2 immunization protocols. Two goats were inoculated subcutaneously (SC) with 1000 $SMICLD_{50}$ of GEV in Freund's complete adjuvant[1] at 0, 10, and 20 DPI. The 2 goats of the second group were inoculated SC with 1000 $SMICLD_{50}$ of GEV, which was followed by 4 SC injections of the virus-adjuvant mixture described above at 8, 10, 12, and 18 DPI. Serum samples were obtained at 0, 21, 30, 42, and 49 DPI for antibody assay. By 49 DPI the response of the 4 goats was not adequate (1:1000) by the SN test with virus GJ9-1BJ and the goats were subjected to challenge with 25,000 $SMICLD_{50}$ of GEV intravenously (IV) at 50 DPI. Serologic response was then monitored at 59, 63, 70, 72, and 80 DPI to evaluate the 4 goats' response following challenge. The 2 goats of the first group displayed the greatest serological response by SN testing with virus GJ9-1BJ at 63 DPI. Serum neutralization tests were done in the latter half of each week which meant that the 63 DPI results were not available until approximately 69 DPI. Serum harvest of the 2 goats by exsanguination was consequently done at 72 DPI. The goats of the second group were exsanguinated with serum harvest at the termination of this study at 90 DPI.

Seven of the 8 goats in the sentinel study were also used for the production of hyperimmune serum. One of the contact transmission goats was given 40,000 $SMICLD_{50}$ of GEV IV at 50 DPI and rectal temperature was

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[1]Difco Laboratories, Detroit, Mich.

taken daily for 7 days. The 6 previously infected goats were challenged at 50 DPI with 25,000 $SMICLD_{50}$ GEV given IV. Serologic monitoring was continued on a biweekly basis through 90 DPI. Exsanguination with serum harvest of the 7 goats was done at 70 (2 goats), 72 (1 goat), and 90 (4 goats) DPI.

VEE conjugate preparation

The serum of goat 152, which had the highest neutralizing-antibody titer to VEE using GJ9-1BJ virus, was selected for fluorescent antibody (FA) conjugate preparation. The FA conjugate was prepared by 2 different techniques to assure that one of the conjugates would be of very high quality. The first technique used was the method described by Goldman (48). Four ml of the goat 152 serum were placed in an Amicon ultrafiltration cell[1] equipped with a membrane designed to retain materials of greater than 100,000 molecular weight. Thirty-six ml of 0.1 M, pH 8 TRIS buffered saline were added to the cell and the cell was then placed on a magnetic stirrer set at a low speed to avoid denaturation of serum proteins. A pressure of 10 psi was applied to the cell for a period of approximately 45 minutes to reduce the mixture to a volume of 4 ml and to remove more than 80 percent of the serum albumin originally present. The air pressure was then removed and the filtered protein solution was applied to a 400 cc column packed with Sephadex G-200[2] equilibrated with the TRIS buffer listed above. The gamma globulin (IgG) fraction was eluted overnight by reverse

[1]Scientific Systems Division, Amicon Corporation, Lexington, Mass.
[2]Pharmacia Ltd., Uppsala, Sweden.

flow chromatography and was recorded with an ultraviolet scanning device[1]. The Lowry technique (84) was used to determine the protein concentration of the IgG fraction. The IgG fraction was then pressure dialyzed against 0.05 M carbonate-bicarbonate buffer, pH 9.0, to a 1.5 percent (15.0 mg protein per ml) concentration and was placed in 8.0 mm dialysis membrane tubing[2]. A solution of fluorescein isothiocyanate[3] (FITC) equal to 10 times the volume of the IgG fraction was prepared in a 250 ml beaker with 0.05 M, pH 9.0 carbonate-bicarbonate buffer at an FITC concentration of 0.1 mg per ml of buffer. The dialysis tubing containing the IgG and a magnetic stirring bar were placed in the FITC solution at 4C. The beaker was covered with aluminum foil to prevent evaporation and was stirred slowly overnight with a magnetic mixer.

The FITC-tagged IgG preparation was then removed from the dialysis tubing and was placed on a 2.0 x 20.0 cm column of Sephadex G-25[4] which was equilibrated with 0.01 M, pH 6.8 phosphate buffered saline (PBS). The conjugate was then eluted with the same buffer and was collected as a single colored band from the column.

A 2.0 x 20.0 cm column of QAE-Sephadex[4] (diethylaminoethyl-Sephadex) was prepared in 0.01 M, pH 6.8 PBS for purification of the conjugate. The conjugate was adsorbed to the column and was subsequently eluted by stepwise addition of 0.1, 0.2, and 0.3 M NaCl to the PBS. Two column bed

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[1] Instrumentation Specialties Co., Lincoln, Nebraska.

[2] Union Carbide Corporation, Films-Packaging Division, Chicago, Ill.

[3] The Sylvana Company, Milburn, N. J. Lot No. 1043.

[4] Pharmacia, Ltd., Uppsala, Sweden.

volumes, approximately 130 ml, were collected for each change of buffer. The fractions were then concentrated to the volume of the original IgG fraction by pressure dialysis, evaluated for fluorescent antibody activity, and stored at -20C.

The second technique involved globulin precipitation with ammonium sulfate, direct tagging with FITC, and tissue powder adsorption of the conjugate (90). A 15.0 ml aliquot of antiserum from goat 152 was fractionated at 4C by dropwise addition of an equal volume of 100 percent saturated ammonium sulfate solution to obtain a gamma-globulin precipitate. The resultant solution was stirred overnight at 4C and was then centrifuged at 250g for twenty minutes. The supernatant fluid was discarded and the precipitate was dissolved in distilled water to a volume of 15.0 ml. The protein solution was then reprecipitated twice at 4C over a 3-hour period as described earlier and the final precipitate was redissolved in distilled water to a volume of 8.0 ml. The resultant protein solution was dialyzed against repeated changes of 0.85% NaCl solution at 4C until free of sulfate ions (SO_4) as determined by reacting a portion of the 0.85% NaCl solution with a saturated solution of barium chloride. If SO_4 ions were present a white precipitate would have been observed.

The protein concentration of the globulin solution was determined by the biuret method (50) using a Coleman spectrophotometer at 540 and 560 mμ. The protein solution was then adjusted to a concentration of 1% by the addition of 0.1 M, pH 7.2 PBS (PBS^1). The globulin solution was tagged

directly at a ratio of 1:20 (0.50 mg FITC[1] per mg globulin). A solution of FITC equal to one-tenth the volume of the globulin solution was prepared with 0.5 M, pH 9.0 carbonate-bicarbonate buffer and was added dropwise to the globulin solution with constant stirring. The FITC-globulin mixture was then stirred overnight at 4C with a magnetic mixer.

A 2.0 x 20.0 cm column of Sephadex G-25 equilibrated with PBS' was used to remove FITC as described for the first conjugation preparation technique. The conjugate was next adsorbed with acetone-extracted rabbit liver powder. One g of liver powder was dissolved in 2.5 ml of PBS' for each 20 ml volume of conjugate. The rabbit liver powder slurry and conjugate were then mixed overnight at 4C in a 250 ml beaker with the aid of a magnetic stirring bar. The following morning the mixture was centrifuged at 30,000 rpm for 1 hour and the supernatant fluid was retained for further treatment. After dialysis against PBS' for 60 hours the conjugate was dispensed into 1.0 dram screw-cap vials and maintained at -20C. Conjugate staining specificity of two conjugate preparations was evaluated by staining VEE-virus-infected BHK-21 cells.

Normal conjugate preparation

Four ml of pooled normal goat serum were fractionated by reverse flow Sephadex G-200 chromatography. The resulting IgG fraction was then conjugated according to the first technique described. To obtain a working dilution the normal conjugate was diluted to equal the intensity of

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[1]The Sylvana Company, Milburn, N. J. Lot. No. 1043.

background fluorescence of the VEE conjugate when both conjugates were used to stain normal BHK-21 cell sheets.

Specimens for examination by FAT

In addition to FA endpoint titrations of suckling mouse GJ9-1BJ and DEF TC-83 VEE viruses, 10 percent tissue suspensions were examined by FAT. The VEE (GJ9-1BJ)-infected equine tissue suspensions were prepared from spleen, tonsil, liver, salivary gland, pancreas, adrenal gland, thymus, lung, cerebellum, renal lymph nodes, splenic lymph nodes, mesenteric lymph nodes, and prescapular lymph nodes.

Preparation of slides

Baby hamster kidney (BHK-21) cells grown on coverslips in Leighton tubes were used for all fluorescent antibody tests (FAT). Four tubes were employed for each test. When the cells were 85 to 100 percent confluent the growth medium was decanted and each tube was inoculated with 0.1 ml of the material to be tested. The tubes were then returned to a 37C incubator for a one hour period of adsorption. At that time a 1.25 ml aliquot of maintenance medium was dispensed into each tube. Two tubes per test were decanted and rinsed twice with PBS[1] at 24 and 48 hours postinoculation. The cell monolayers were then fixed at 4C for a minimum of twenty-four hours by the addition of 10 ml of acetone to each tube.

For staining the fixed BHK-21 coverslips were removed from their tubes and were allowed to air dry at 25C. Approximately 0.05 ml of VEE conjugate was applied to each coverslip as a thin film. The cells were then stained for a minimum of 20 minutes in a moist chamber at 37C. After

incubation the coverslips were rinsed first in PBS and next in distilled water. After drying at 37C, the stained coverslips were mounted cell surface down on 2.5 x 7.5 cm clear glass microscope slides with a mounting medium of 50% glycerin in PBS[1].

Two conjugate specificity controls were employed for FAT. Normal conjugate was applied to VEE-virus-infected cell monolayers, and VEE conjugate was applied to normal cell monolayers. Eastern and western equine encephalomyelitis virus-infected cell monolayers were also stained with VEE conjugate to evaluate the conguate's specificity of staining.

Microscopy

Incident-light fluorescence microscopy was used for examination of fluorescent antibody stained preparations (96). A Leitz Orthoplan microscope stand with a Leitz fluorescence vertical illuminator and mercury arc lamp (OSRAM HBO 200) was equipped with the following filter system: The excitation filters were 1 2.0mm KG-1[1] to block the infrared spectrum, 2 4.0mm BG-38[1] filters to absorb the red spectrum, 2 KP-490[2] short-wave pass-interference filters to obtain an excitation wavelength of 490nm, and a 45 degree angle beam splitter TK-510[3] which reflected the 490nm light beam through the objective to the specimen and allowed only light of $\geq$ 510nm to be transmitted back to the eyepieces. The barrier filter system included the beam splitter as described, one TK-515[3] barrier

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[1]Schott and Gen., Mainz, Germany.

[2]Balzars, Vaduz, Lichtenstein.

[3]E. Leitz, Wetzler, Germany.

filter to pass light of ≥515nm, and one K-530[1] barrier passing light of ≥530nm to the eyepieces. The 2 KP-490 filters combined with the TK510 beam splitter produced a narrow-band filter system with both high transmittance (≥80%) around 490nm and very low transmittance (≤0.0001%) around 525nm (96).

Fluorescence photomicrography

The Leitz Orthomat fully automatic camera system and Kodak high speed Ektachrome film[2] (ASA rating of 160) were used to record FAT results. Exposure times ranged from 15 to 45 minutes.

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[1] E. Leitz, Wetzlar, Germany.

[2] Kodak, Rochester, N. Y.

RESULTS

Comparative propagation of VEE virus

Despite repeated attempts neither cytopathic effect (CPE) or plaquing was obtained with the L cell line. Up to 5.0×10^5 $SMICLD_{50}$ of the GJ9-1BJ strain of VEE virus were used in combination with a moist 37C 5% CO_2 incubation system as employed by Hardy and Brown (60). In a similar fashion, repeated attempts failed to produce either CPE or plaquing in goat kidney, human amnion (FL), bovine turbinate, and horse kidney cell cultures.

The Vero African green monkey, duck embryo, and BHK-21 cell cultures were determined to be of approximately equal sensitivity in the propagation of virulent VEE virus. Cytopathic effects (CPE) were not readily apparent at 24 hours postinoculation under agar overlay. By 48 hours postinoculation CPE were evident and were somewhat different for each of the 3 cell cultures (Table 7).

The CPE of the Vero cell line consisted of 70 to 90 percent cytolysis of the plaque regions with rounding up of the remaining cells. Plaques became more difficult to detect between the 146th and 150th cell passages and the cells essentially lost their sensitivity to VEE-virus-induced CPE after 150 to 155 passages.

Duck embryo cells seemed to be more resistant than the other 2 cell types to VEE-induced cytolysis. As few as 5 percent and a maximum of 60 percent of the cells within a plaque were lysed due to virus replication with 20 to 95 percent of the remaining cells rounding up. Rounded up and morphologically normal cells also had very fine cytoplasmic vacuoles

Table 7. Susceptibility of different cell types to GJ9-1BJ VEE virus

Cell culture	Mean titer cell culture[a]	Suckling mouse $ICLD_{50}$	Cytopathic effects[b]	Mean plaque diameter[c]
Vero	6.4×10^8	5×10^{10}	48 hr, 70-90%	3.7mm
Duck embryo	2.7×10^8	5×10^{10}	48 hr, 5-60%	2.7mm
L cell	Negative	5×10^{10}	------	------
Goat kidney	Negative	9.1×10^{10}	------	------
Human amnion	Negative	9.1×10^{10}	------	------
Bovine turbinate	Negative	9.1×10^{10}	------	------
BHK-21	4.3×10^8	5×10^{10}	48 hr, 30-75%	3.3mm
Horse kidney	Negative	9.1×10^{10}	------	------

[a] PFU/ml.

[b] Hours postinoculation, % cells affected in the plaques.

[c] Determined during final 72 hr postinoculation plaque count.

present.

The overall cytopathic effect on BHK-21 cells was not as marked as that seen in Vero cells. Cytolysis varied from 10 to 45 percent of the plaque cell population with 5 to 75 percent rounding up. Cytoplasmic vacuolization was more marked in BHK-21 cells than that observed in duck embryo cells and was usually observed in greater than 30 percent of the plaque cells.

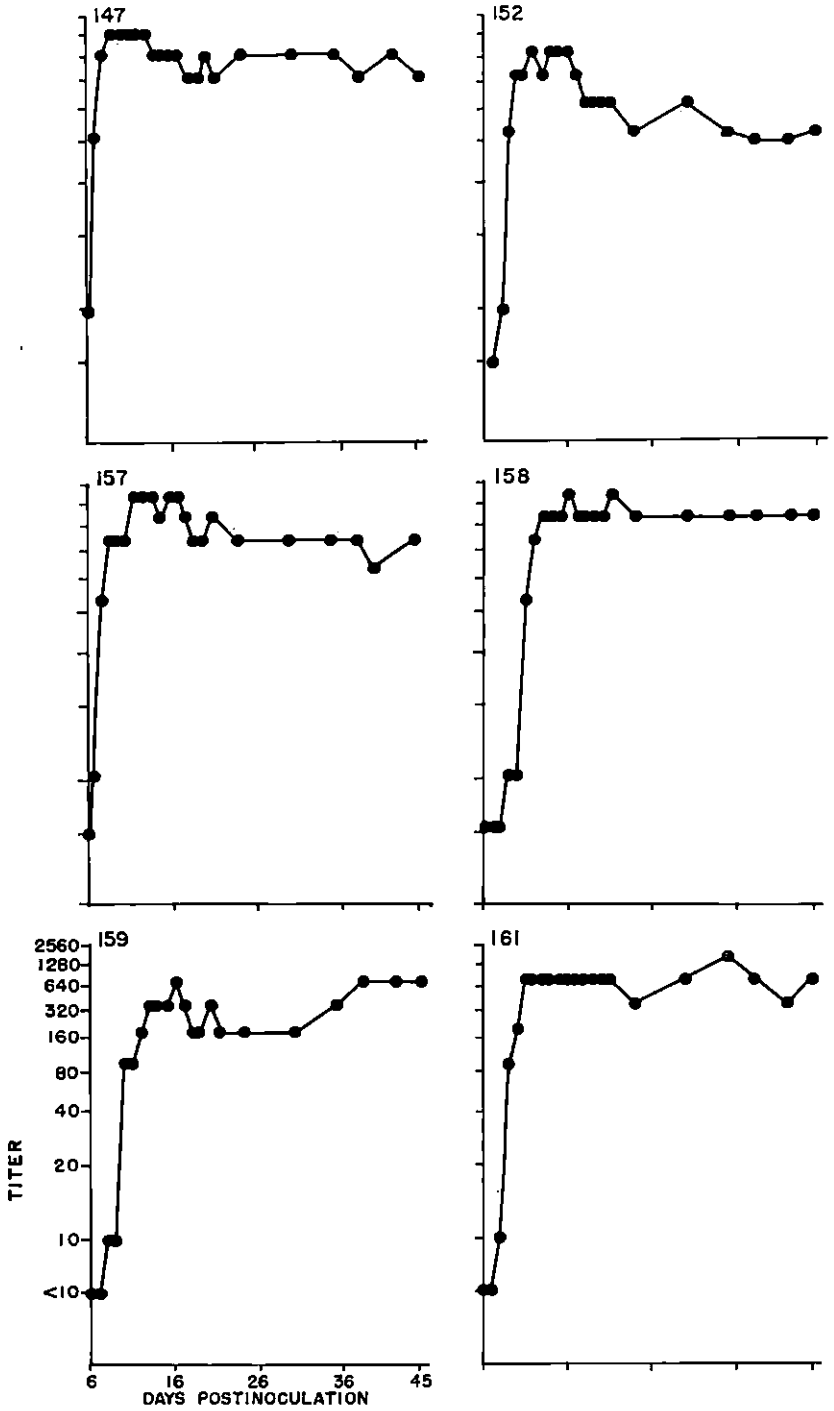
The goat as a sentinel

Serum neutralization (SN, TC-83 VEE virus) antibody titers of 1:10 or greater were detected in 1 of the 6 infected goats at 6 days postinoculation (DPI), 2 goats at 7 DPI, 5 goats at 8 DPI, and all goats by 9 DPI. The SN titers in 5 of the 6 goats rose very rapidly and by the second day of response had increased from 1:10 to 1:100 (Figure 1).

Serum-neutralization testing of the sera with GJ9-1BJ VEE virus did not detect a serological response until 8 DPI in 1 goat. By 10 DPI 5 of the 6 goats had 1:10 titers. The 6th goat seroconverted by 15 DPI, was negative at 1:10 for the next 3 days, and regained a 1:10 titer at 19 DPI. The 5 goats with 1:10 titers at 10 DPI responded in a serologically uniform manner for the remainder of the 45 day monitoring period (Figure 2). Maximal SN response with GJ9-1BJ virus was 1:100 in 1 goat as compared with TC-83 virus SN titers of 1:1600 in 3 goats, and 1:800 in the other 3 goats of the group (Figure 1).

Hemagglutination-inhibition (HI) titers did not appear until 7 DPI (1 goat). The maximal HI response was 1:10,240 in 1 goat but another

Figure 1. Sentinel goat study. TC-83 SN antibody titers of goats 147, 152, 157, 158, 159, and 161 between days 6 and 45 postinoculation. Titers listed were the highest serum dilution that produced at least 90% plaque reduction. See Appendix for specific titers.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TITER
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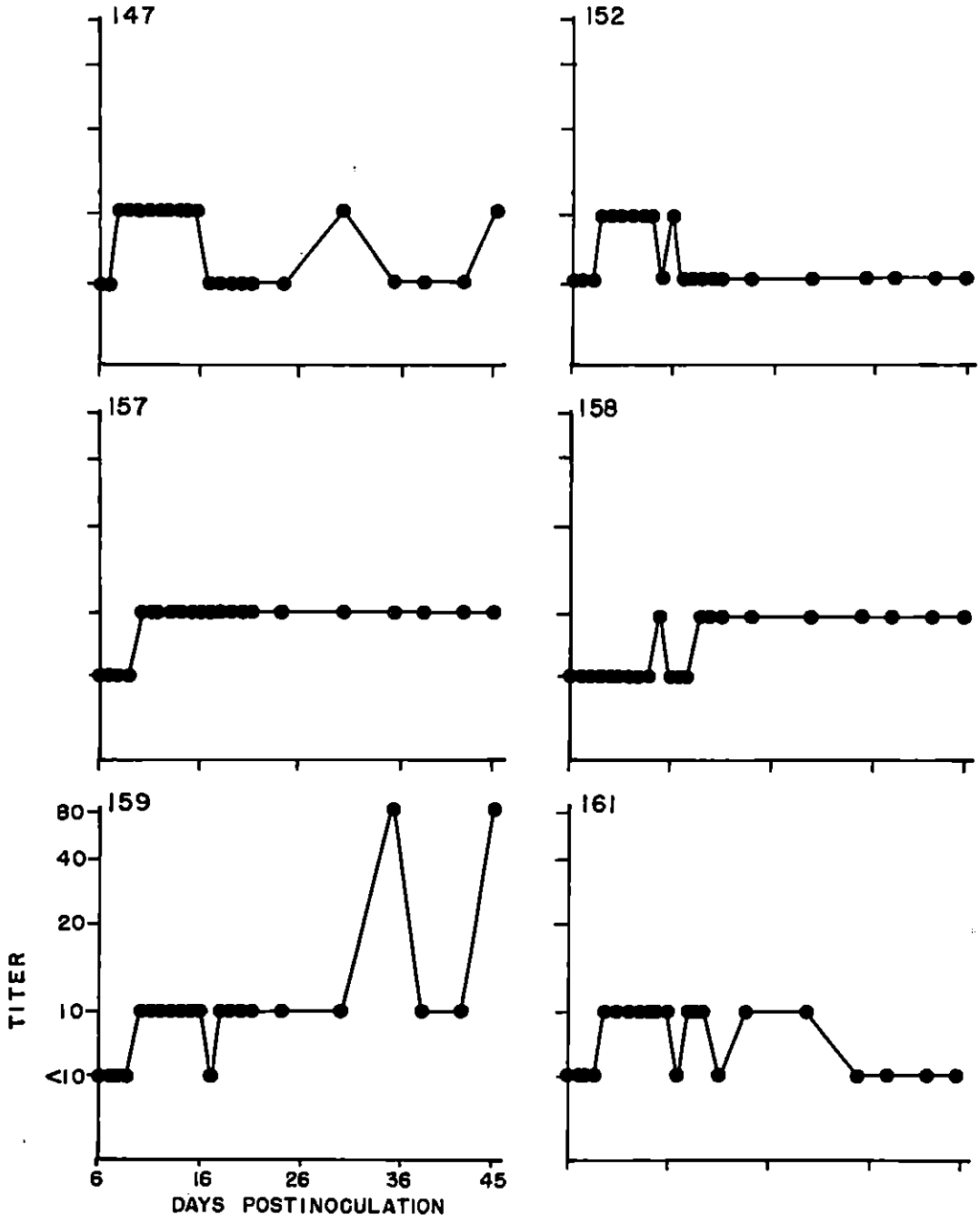
DAYS POSTINOCULATION

Figure 2. Sentinel goat study. GJ9-1BJ SN antibody titers of goats 147, 152, 157, 158, 159, and 161 between days 6 and 45 postinoculation. Titers listed were the highest serum dilution that produced at least 90% plaque reduction. See Appendix for specific titers.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DAYS POSTINOCULATION

goat did not exceed a titer of 1:40 during the observation period (Figure 3). The 2 contact transmission goats did not display detectable VEE viral antibodies by the 3 serological assay methods employed in this study. (See Appendix for titers by HI and SN).

The goat as a silent amplifier of IB VEE virus

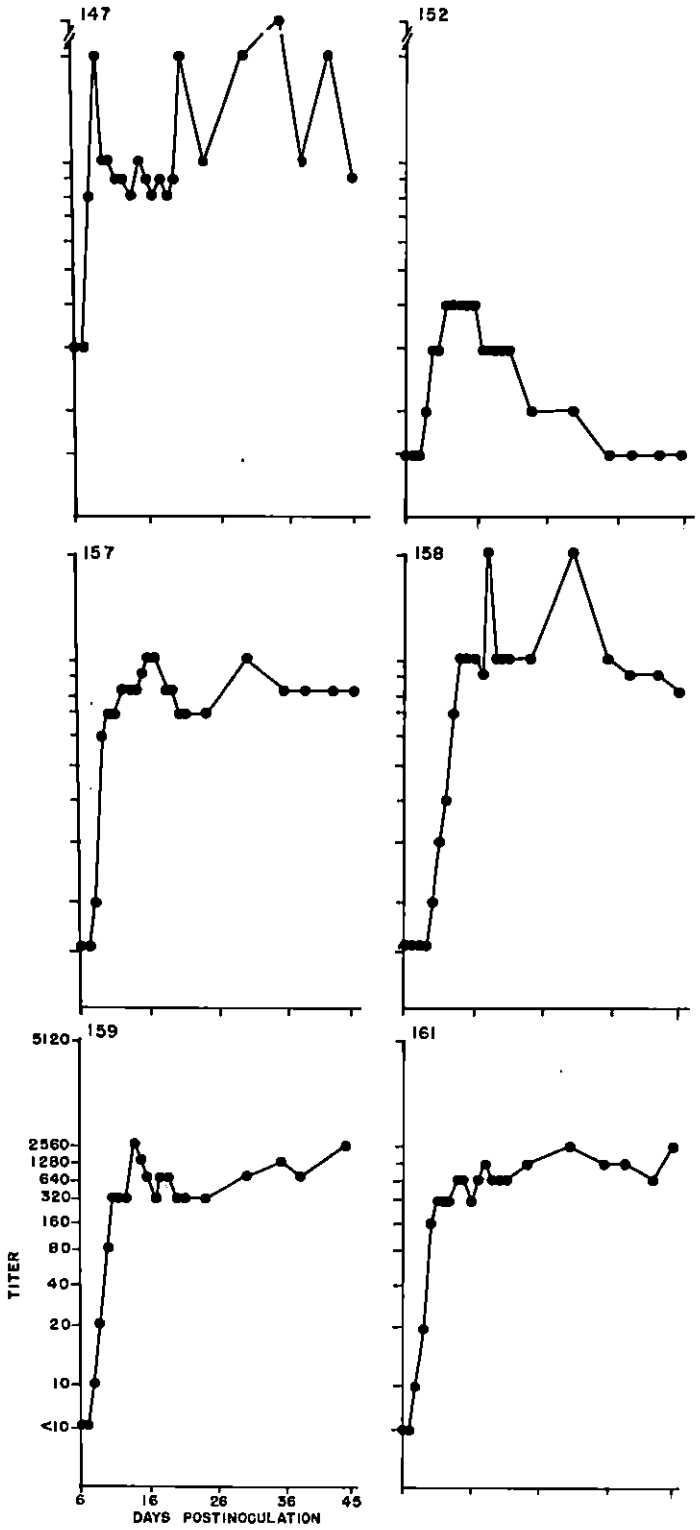
Viremia was not detected by the plaque assay technique using BHK-21 cells. By suckling mouse inoculation 5 of the inoculated goats were found to be viremic for 1 to 3 days, viremia commencing between the 1st and 5th days postinoculation. One of these goats was viremic from day 1 to day 3 postinoculation, another from day 2 to day 4, and a 3rd from day 3 to day 4. One goat was viremic on days 3 and 5, and one goat was viremic only on day 4. Peak viremia did not exceed 4.08 $\log_{10}$ $SMICLD_{50}$ per ml of serum. Respective $SMICLD_{50}$/ml of the sera are listed in Table 8. Oral, nasal, and genitourinary swabs obtained from day zero through day 7 were negative for VEE virus by suckling mouse inoculation.

Clinical evidence of disease in the domestic goat was lacking (Figure 4) during the 21-day observation period with one exception. Goat number 159 displayed an increased body temperature from day 2 through day 10, which peaked on day 5 at 41.1C (105.9F). The goat's normal body temperature was 39.1C (102.3F) as determined from baseline data (Table 9).

VEE antiserum production

After intravenous challenge of the 3 groups of goats at 50 days post-inoculation, the maximum antibody titers as determined by the 3 tests were

Figure 3. Sentinel goat study. HI antibody titers of goats 147, 152, 157, 158, 159 and 161 between days 6 and 45 postinoculation. See Appendix for specific titers.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TITER
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16
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36
45
DAYS POSTINOCULATION

Table 8. Virus titers in serum of goats inoculated with GJ9-1BJ VEE virus

Goat number	Days Postinoculation	Magnitude of Viremia (log_{10} SMICLD$_{50}$/ml serum)
147	1	4.08
	2	2.2
	3	2.2
152	0-14	0.0
157	3	<1.7
	4	<1.7
158	4	1.95
159	2	<1.7
	3	3.27
	4	<1.7
161	3	2.36
	5	<1.7

Table 9. Body temperatures of goat number 159 inoculated with IB VEE virus

Days postinoculation	Body temperature	
	C	F
0	39.2	102.5
1	38.9	102.1
2	39.1	102.4
3	39.6	103.2
4	40.7	105.2
5	41.1	105.9
6	40.2	104.4
7	40.2	104.3
8	40.3	104.6
9	40.9	105.3
10	40.1	104.2
11	38.7	101.6
12	38.9	102.1
Normal body temperature	39.1	102.3

1:51,200 (SN, TC-83, 7 of 12 goats), 1:1280 (SN, GJ9-1BJ 2 of 12 goats), and 1:64,000 (HI, 5 of 12 goats). See Appendix for listing of titers from zero through 90 days postinoculation. Serum from goat number 152 exanguinated at 72 DPI was selected for conjugation on the basis of a GJ9-1BJ SN titer of 1:1000.

Conjugate evaluation

After the QAE-Sephadex conjugate fractions had been concentrated by pressure dialysis they were examined for specific fluorescence on TC-83 VEE virus-infected BHK-21 cells at 24 and 48 hours postinoculation. Only that fraction obtained by addition of 0.2 M NaCl demonstrated specific fluorescence. The fluorescence was finely granular in appearance and occurred only in the cytoplasm of infected cells (Figures 8, 10, and 11).

Specificity of staining was evaluated by 2 methods. Baby hamster kidney (BHK-21) cells were infected with approximately 10^3 PFU per ml of eastern equine encephalomyelitis or western equine encephalomyelitis viruses. At 24 hours postinoculation the infected cell cultures were fixed in acetone at 4C and were examined 1 day later with the conjugate. Cross-staining resulting in unwanted specific fluorescence did not occur (Figures 12 and 13).

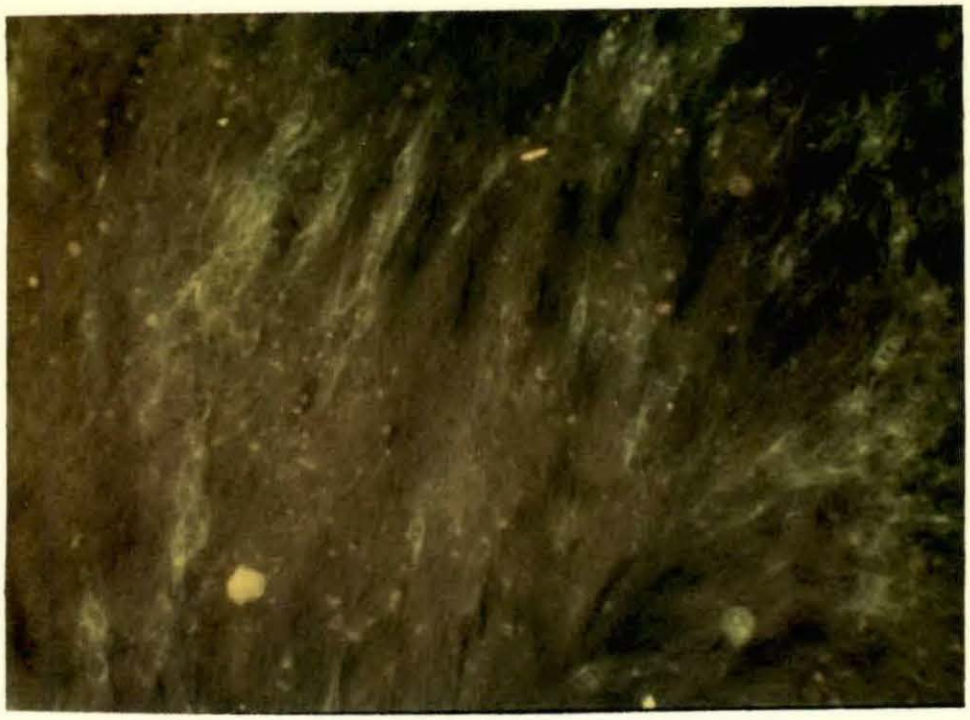
The second conjugate specificity parameter was the staining of normal BHK-21 cells 24 hours after they had been subjected to the same regimen as that employed for infecting cells. Nonspecific staining of normal cells was not observed (Figure 5).

Figure 4. Absence of clinical signs in a domestic goat three days after infection with the GJ9-1BJ strain of Venezuelan equine encephalomyelitis (VEE) virus.

Figure 5. VEE conjugate applied to normal BHK-21 cell monolayer. Note absence of specific fluorescence. 125x

Figure 6. VEE conjugate applied to GJ9-1BJ VEE-virus-infected BHK-21 cell monolayer at 24 hours postinoculation. Note specific perinuclear and cytoplasmic fluoresence. 125x

Figure 7. Normal conjugate applied to GJ9-1BJ VEE-virus-infected BHK-21 cell monolayer at 24 hours postinoculation. Note absence of specific fluorescence. 125x



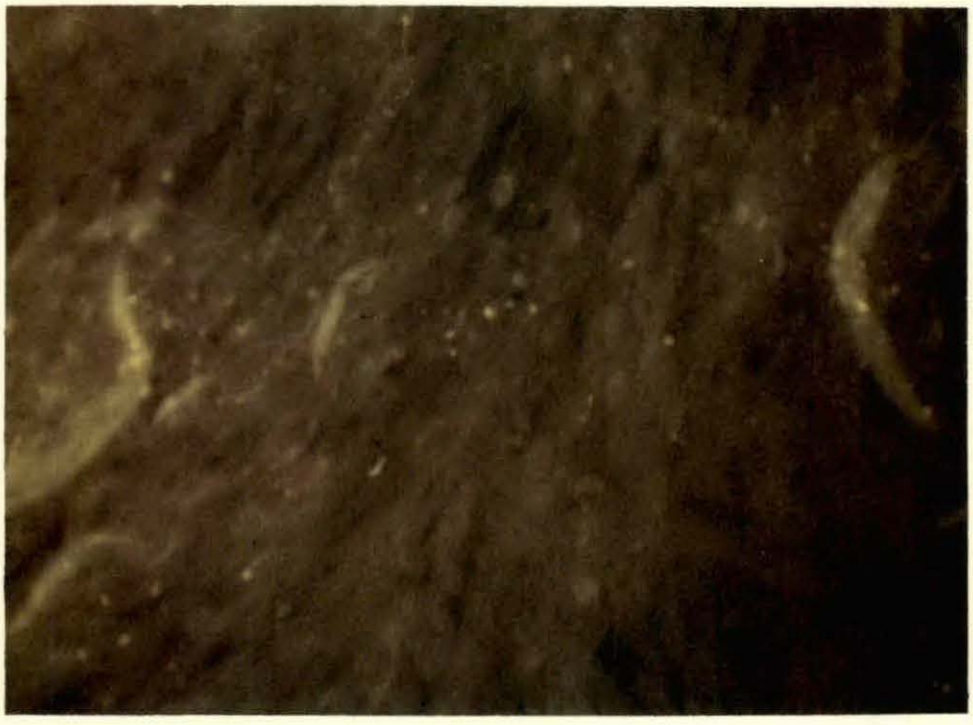


Figure 8. VEE conjugate applied to TC-83-VEE-virus-infected BHK-21 cell monolayer at 24 hours postinoculation. Note specific, granular perinuclear and cytoplasmic fluorescence. 500x

Figure 9. VEE conjugate applied to GJ9-1BJ-VEE-virus-infected BHK-21 cell monolayer at 24 hours postinoculation. Note specific, granular perinuclear fluorescence. 1188x

Figure 10. VEE conjugate applied to TC-83-VEE-virus-infected BHK-21 cell monolayer at 48 hours postinoculation. Note specific cytoplasmic fluorescence of rounded up cells. 125x

Figure 11. VEE conjugate applied to TC-83-VEE-virus-infected BHK-21 cell monolayer at 48 hours postinoculation. Note specific, granular cytoplasmic fluorescence of rounded up cells. 500x

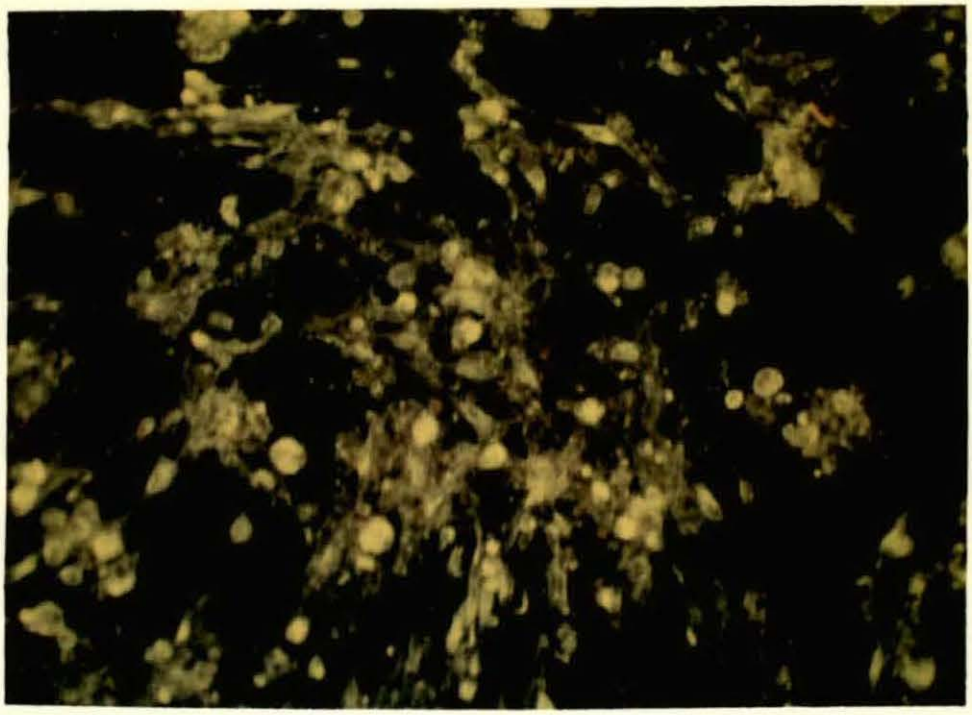
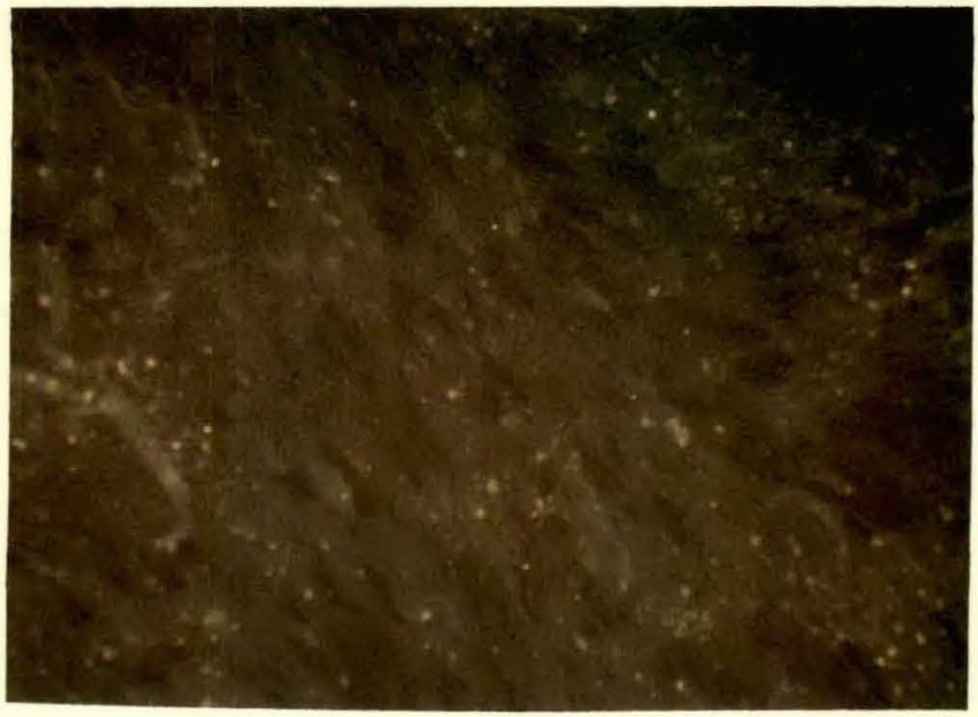


Figure 12. VEE conjugate applied to western equine encephalomyelitis virus-infected BHK-21 cell monolayer at 24 hours postinoculation. Note absence of cross-staining. 125x

Figure 13. VEE conjugate applied to eastern equine encephalomyelitis virus-infected BHK-21 cell monolayer at 24 hours postinoculation. Note absence of cross-staining. 125x



When the directly-tagged conjugate preparation was used to stain TC-83 virus-infected BHK-21 cells marked nonspecific staining was observed. Dilution of the conjugate beyond 1:8 was not feasible due to markedly diminished specific staining and at the 1:8 dilution the level of nonspecific fluorescence was not tolerable. On that basis further fluorescent antibody studies on VEE virus were done with QAE-Sephadex-treated conjugate.

The normal goat serum conjugate was prepared from Sephadex G-200 fractionated IgG which was tagged with FITC and purified by QAE-Sephadex column chromatography. The working dilution of 1:4 was derived by comparison of background fluorescence of both the normal and VEE conjugates when VEE virus-infected and normal BHK-21 cell monolayers were stained (Figures 5, 6, and 7). This conjugate was then employed as a specificity of staining control when conducting fluorescent antibody tests (FAT) on VEE virus-infected pony tissue suspensions.

Comparison of sensitivity of FAT to suckling mouse inoculation for virus identification

Initially the 2 stock virus preparations employed in this study were assayed by the fluorescent antibody test and intracerebral inoculation of suckling mice. Fluorescent antibody assay in BHK-21 cells was found to have approximately 1.0 $\log_{10}$ per ml greater sensitivity.

When 20 viremic pony tissues were tested by the 2 systems suckling mice were the most sensitive virus detection system. The threshold of

sensitivity of the fluorescent antibody test appeared to be between 1.6 and 2.0 $\log_{10}$ $SMICLD_{50}$/ml of 10 percent pony tissue suspension. By FAT all tissue suspensions were positive by 24 hours postinoculation with the exception of 4 of 6 tissues in the 2.0 to 2.8 $\log_{10}$ $SMICLD_{50}$/ml virus titer range which were positive at 48 hours postinoculation. Two tissues with a titer of 1.6 $\log_{10}$ $SMICLD_{50}$/ml were negative at 24 and 48 hours postinoculation by the fluorescent antibody test (Table 10).

Table 10. Virus detection by the fluorescent antibody test (FAT) and by intracerebral inoculation of suckling mice

Specimen	Pony number[a]	FAT[b]	Hours postinoculation	SMICLD$_{50}$[b]
TC-83 virus		> 9.0	24	8.7
10% suckling mouse brain GJ9-1BJ virus		≥10.0	24	9.5
Spleen	18	Pos.	48	2.1
Tonsil	18	Pos.	24	2.1
Renal and splenic lymph nodes	19	Pos.	24	7.1
Adrenal	19	Pos.	24	6.3
Salivary gland	19	Pos.	24	6.0
Liver	19	Pos.	24	6.7
Spleen	522	Pos.	24	3.5
Adrenal	522	Pos.	48	2.8
Mesenteric lymph node	522	Pos.	24	5.7
Liver	522	Pos.	48	3.6
Thymus	522	Pos.	24	5.2
Renal lymph node	522	Pos.	24	3.8
Pancreas	522	Pos.	24	4.0
Lung	524	Neg.	48	1.6
Spleen	524	Pos.	24	2.2
Thymus	526	Pos.	24	5.3
Mesenteric lymph node	526	Pos.	24	6.8
Cerebellum	529	Neg.	48	1.6
Prescapular lymph node	530	Pos.	24	4.3
Spleen	530	Pos.	48	2.0

[a]Ten percent tissue suspension in 0.75% BAPBS from ponies inoculated with 1000 SMICLD$_{50}$/ml GJ9-1BJ VEE virus intradermally.

[b]Log_{10} per ml of inoculum; positive or negative (FAT).

DISCUSSION

The baby hamster kidney (BHK-21) cell line has been proven superior to either primary duck embryo or Vero African green monkey cell lines for titration of Venezuelan equine encephalomyelitis (VEE) virus. The Vero cell line is slightly more sensitive for the titration of the GJ9-1BJ strain of VEE virus but it lacks the potential for continuous serial passage that the BHK-21 line has as supplied from the American Type Culture Collection. Baby hamster kidney cells have been passaged in excess of 200 times at the National Animal Disease Laboratory, Ames, Iowa, without loss of sensitivity for VEE virus. In contrast, the Vero cell line is supplied at the 122nd passage level and has been found in this laboratory to lose its susceptibility to VEE virus between the 150th and 155th passage level. Earley, et al. (34) also did not find Vero cells to be sufficiently susceptible after 30 serial passages. Thus, the time saved by not having to restart the cell line from the 122nd passage level every three months more than justifies the choice of the BHK-21 cell line over the Vero cell line.

Primary duck embryo cell cultures are slightly less sensitive for the detection of the epizoodemic strain of VEE virus than BHK-21 cells. The mean VEE plaque diameter in the duck cells is also 1.0 mm less than in BHK-21 cells which makes enumeration of plaque-forming units more difficult. The BHK-21 cell line is thus the cell culture of choice of the 8 evaluated in this study.

Experimental inoculation of the goat with the minimal exposure of 1000 $SMICLD_{50}$, an average mosquito inoculum, revealed the goat to be an excellent sentinel for VEE virus activity. The 6 inoculated goats developed titers greater than 1:10 as determined by hemagglutionation-inhibition (HI) and serum neutralization (SN) tests. Both persisted in excess of 40 days. In view of these findings the investigation of Dickerman, et al. (33) would seem to have provided questionable information on the bovine as a possible sentinel species. In that study 8 native HI and SN seronegative Colombian bovines were inoculated with a great excess of IB VEE virus, 7.1 $\log_{10}$ primary chicken embryo cell plaque-forming units (CEC-PFU), to "insure establishment of infection." The 7.1 $\log_{10}$ CEC-PFU of virus would be roughly equivalent to 9 $\log_{10}$ $SMICLD_{50}$. Considering an average mosquito inoculum of 1000 $SMICLD_{50}$, approximately 1,000,000 average IB VEE viremic mosquitoes would have had to feed upon a single animal over a given period. In view of the information obtained herein with another domestic ruminant species, the goat, it would appear necessary to repeat that study with a lower inoculum of IB VEE virus.

However, sufficient numbers of goats must be available to the potential vector to be able to detect VEE virus activity in an area. Hayes, et al. (61) have demonstrated in a moquito host preference study in Hale County, Texas, that the predominant feeding habit of mosquitoes of the genera Aedes, Anopheles, Culiseta, and Psorophora is to feed upon mammalian species. Sudia (115) has stated that only Psorophora confinnis and Aedes taeniorhynchus have been proven as possible vectors

of the IB strain of VEE virus. He has also reported that 83 virus isolates compatible with VEE were obtained from Psorophora confinnis during the Texas epizoodemic (117). The bionomics of P. confinnis have been shown to be associated with temporary fresh water pools located in sunny, grassy areas (115). Such areas can often be found in livestock pastures. Therefore, in regions where large goat populations are present the likelihood of the goat being a sentinel of epizoodemic VEE virus activity would appear to be very good.

Viremic goats on the other hand, do not offer a source of silent amplification of epizoodemic VEE virus according to the results of this study. Goat viremia did not exceed 4.08 $\log_{10}$ $SMICLD_{50}$/ml, with a mean peak viremia of 2.67 $\log_{10}$ $SMICLD_{50}$/ml. The average viremia required to infect vector species of mosquitoes has been stated by Chamberlain (14, 16) to be 5.0 $\log_{10}$ $SMICLD_{50}$/ml. Experimentally the infection threshold of Psorophora confinnis has been found to be 4.9 to 5.2 $\log_{10}$ $SMICLD_{50}$/ml (115). Consequently, the goat is not likely to be an amplification mechanism of epizoodemic VEE virus.

Due to the excellent serologic response of the goats inoculated as sentinels they were hyperimmunized in an attempt to produce a more specific VEE antiserum than that which was likely produced by the goats given multiple virus injections to stimulate antibody formation. The serological response of the 4 antiserum goats was no better than that of the goats of the sentinel study given only one injection of virus. The serum of goat number 152 was chosen for conjugate preparation because of its high antibody titer as determined by the GJ9-1BJ SN test. Serological

testing by the other two techniques, HI and TC-83 SN later revealed an obvious lack of correlation of serum titers. The most plausible explanation of such differences is the presence of residual infectivity representing nonneutralized virus. Several workers have reported a similar phenomenon of nonneutralized virus in other virus-serum neutralization test systems. Ashe and Notkins (5), Bradish, et al. (10), and Wallis and Melnick (127) have theorized residual infectivity to be due to the formation of infectious virus antibody complexes which resist further neutralization by antibody. Lafferty (78) used kinetic serum neutralization testing to show that residual infectivity was due to lack of antibody avidity. Fazekas de St. Groth, et al. (38) set forth the concept that residual infectivity was due to dissociation of the virus-antibody complex. Through extensive kinetic serum neutralization testing with VEE virus Hahon (58,59) has found that residual infectivity of VEE virus is not due to dissociation of noninfective antigen-antibody complexes, to the presence of a genetically stable persistent viral subpopulation, or to the presence of free infectious ribonucleic acid in the virus preparation used for the serum neutralization test. His experiments with TC-83 and Trinidad IA strains of VEE virus (58) have clearly demonstrated 2 factors contributing to VEE virus SN residual infectivity. One factor is the formation of viral aggregates of the virus preparation employed in the test system resulting in incomplete neutralization. Hahon's main concept was, however, that residual infectivity was due to the presence of sensitized VEE virions that were not neutralized

by the antiserum employed in the SN test. Supporting evidence for such a theory was obtained when neutralized suspensions were incubated at 35C for 1 hour with a 1:10 dilution of anti-IgG serum. Addition of the anti-IgG serum to a neutralized suspension yet containing 5.4 $\log_{10}$ units of residual virulent IA VEE virus infectivity further reduced viral infectivity of the suspension by an additional 3.7 $\log_{10}$ units. Similar work has also been done with lactic dehydrogenase virus and poliovirus (73,91,92).

A third component of Hahon's study was the determination of residual infectivity of Trinidad IA VEE virus as compared to the TC-83 attenuated strain. The percentage of original infectivity remaining after serum neutralization with a 1:50 dilution of monkey VEE antiserum to Trinidad IA VEE virus was approximately 20% as compared to 2.2% residual activity for TC-83 VEE virus after 15 minutes incubation at 35C. Clearly, the low titers obtained with the GJ9-1BJ SN test as compared to those of the TC-83 SN tests would appear to have been the result of residual infectivity of the virulent IB strain of virus. The minor amount of residual infectivity of the TC-83 SN test system made it possible to obtain good correlation with the VEE HI test. The information subsequently derived from the TC-83 SN and VEE HI tests indicated that several goats could have served as a source for high-titered antisera for conjugation.

The fluorescent antibody conjugate prepared from the serum harvested from goat number 152 22 days after intravenous challenge with 25,000

$SMICLD_{50}$ of GJ9-1BJ VEE virus was determined to be a highly specific preparation. Although antigenic interrelationships with eastern equine encephalomyelitis (EEE) and western equine encephalomyelitis (WEE) viruses were detected by EEE and WEE HI tests, they could not be demonstrated with the fluorescent antibody test.

Pooled normal goat serum was used to prepare a normal serum conjugate for use as a VEE conjugate specificity of staining control. When the VEE conjugate was used on the BHK-21 cell line in a fluorescent antibody test, as little virus as 2.1 $\log_{10}$ $SMICLD_{50}$/0.1 ml inoculum from GJ9-1BJ VEE-virus-infected pony tissue suspensions could be detected. If the Leighton tube inoculum would have been increased to 1.0 ml of tissue suspension the sensitivity of the VEE fluorescent antibody test (FAT) would have likely been within 1 $\log_{10}$ dilution of that of intracerebral inoculation of 2- to 4-day-old suckling mice. The smaller inoculum was chosen for use in FAT to make it a more rapid diagnostic test. If the larger inoculum had been employed at least two washings with tissue culture medium would have been required to remove toxic tissue components from the BHK-21 monolayer after the 1 hour virus adsorption period. An additional factor to be considered was that of personnel safety. The use of a smaller inoculum with no washing of monolayers decreased the possibility of aerosol exposure to laboratory personnel.

SUMMARY

The baby hamster kidney (BHK-21) cell line was shown to be superior to either primary duck embryo cells or Vero African green monkey cells as a host system for propagation of the epizoodemic strain, GJ9-1BJ, of Venezuelan equine encephalomyelitis (VEE) virus. For that reason the BHK-21 cell line was chosen for evaluation of VEE fluorescent-antibody conjugates.

Surveillance of possible VEE virus activity is of critical importance in avoidance of major epizoodemics. The goat has been evaluated as a sentinel for IB VEE virus activity in this study and was found to be a very sensitive sentinel animal. Six goats of mixed sex from 4 to 18 months of age when exposed to a single average mosquito inoculum of VEE virus began to seroconvert within 6 days. Serological evaluation using the TC-83 and GJ9-1BJ serum neutralization tests and the VEE hemagglutination-inhibition test showed that the 6 goats were seropositive for VEE antibody for 49 days. A minimum of VEE virus thus stimulated a very adequate serological response in the goat.

Following challenge of the sentinel goats with 25,000 $SMICLD_{50}$ of the GJ9-1BJ strain of VEE virus a suitable serum for conjugation was obtained. The VEE antiserum and normal goat serums were conjugated and evaluated for staining specificity in the BHK-21 cell line. No cross-staining of the VEE conjugate with eastern equine encephalomyelitis virus or western equine encephalomyelitis virus was observed despite a 1:10 cross reaction for both viruses as detected by EEE and WEE hemagglutination-inhibition testing of the same serum.

The conjugate was assayed for sensitivity for the detection of VEE virus in cell cultures inoculated with equine tissue suspensions. The (BHK-21) cell line when combined with a conjugate highly specific for VEE virus has been proven to be nearly as sensitive as intracerebral inoculation of the suckling mouse for the detection or identification of VEE virus. In a diagnostic sense the test can now be applied to routine screening of VEE diagnostic specimens. All negative specimens should then be tested in suckling mice by the intracerebral route for isolation of VEE virus not detected by the fluorescent antibody test. The fluorescent antibody test would be of greatest value in the event of an another outbreak of epizoodemic VEE among the equidae of the United States. Large numbers of specimens likely to be positive for VEE virus would be encountered and a rapid, efficient technique such as the fluorescent antibody test would then be of critical importance.

APPENDIX

TRIS Buffered Saline pH 8.0

1. 0.5M TRIS:

   Dissolve 60.57 g TRIS and 4.0 g sodium azide (NaN_3) in 200 ml triple-distilled, deionized water and q.s. to 1000 ml.

2. 0.5M HCl:

   q.s. 42 ml of 11.9 M HCl to 1000 ml.

3. Buffer preparation:

   a. two parts TRIS (133.3 ml) and one part HCl (66.6 ml) are mixed.

   b. add 1.19 g of NaCl to 200 ml of TRIS buffered saline for a final NaCl concentration of 0.1M.

   c. add 4.0 g NaN_3 per liter of final buffer.

0.5 M Carbonate-Bicarbonate Buffer

1. Reagents required:

   a. 0.5 M solution of sodium bicarbonate ($NaHCO_3$)

   b. 0.5 M solution of sodium carbonate (Na_2CO_3)

2. Mix 50 volumes reagent a with 4.5 volumes reagent b (pH should be 9.0 when diluted 10 times in triple-distilled water).

0.1 M Phosphate-Buffered Saline

pH 7.2:

1. Prepare stock solutions a and b:

   a. 0.2 M monobasic sodium phosphate (NaH_2PO_4)
   Dissolve 27.8 g NaH_2PO_4 in 1000 ml distilled water.

   b. 0.2 M dibasic sodium phosphate (Na_2HPO_4)
   Dissolve 53.65 g $Na_2HPO_4 \cdot 7H_2O$ in 1000 ml distilled water.

2. Mix 28.0 ml of solution a with 72.0 ml of solution b. Dilute to a total of 200 ml with distilled water.

pH 6.8:

1. Prepare stock solutions a and b as shown.

2. Mix 51.0 ml of solution a with 49.0 ml of solution b. Dilute to a total of 200 ml with distilled water.

Dextrose-Gelatin-Veronal Buffer

1. Dissolve 0.58 g 5,5-diethyl barbiturate acid and 0.6 g gelatin in 250 ml distilled water by heating.

2. Combine the following reagents with solution 1:

Sodium 5,5-diethyl-barbiturate	0.38 g
$CaCl_2$ (anhydrous)	0.02 g
$MgSO_4 \cdot 7H_2O$	0.12 g
NaCl	8.50 g
Dextrose	10.00 g

3. q.s. to 1000 ml with distilled water.

0.2% Bovalbumin, pH 6.0 Borate Saline (BBS)

1. Prepare pH 9.0 borate saline as follows:

   a. mix 80 ml of 1.5 M NaCl, 100 ml of 0.5 M H_3BO_3, and 24.0 ml of 1.0 M NaOH.

   b. q.s. to 1000 ml with distilled water.

2. Add Cohn fraction V bovine serum albumin to borate saline (1) to a concentration of 0.4%.

3. Prepare 0.15 M NaCl-0.2 M Na_2HPO_4 as follows:

   a. mix 100 ml of 1.5 M NaCl and 100 ml of 2.0 M Na_2HPO_4

   b. q.s. to 1000 ml with distilled water.

4. Prepare 0.15 M NaCl-0.2 M NaH_2PO_4 as follows:

   a. mix 100 ml of 1.5 M NaCl and 100 ml of 2.0 M NaH_2PO_4

   b. q.s. to 1000 ml with distilled water.

5. Mix 11 ml of solution 3 with 89 ml of solution 4.

6. Mix equal volumes of solutions 2 and 5, check pH, and BBS is then ready for use.

Veronal Buffer (VBD)

1. Dissolve 42.5 g NaCl and 1.87 g sodium 5,5-diethyl-barbiturate in 700 ml distilled water.

2. Dissolve 2.875 g 5,5-diethyl barbiturate acid in 250 ml hot distilled water.

3. Dissolve 20.333 g $MgCl_2$ (1.0 M) and 4.411 g $CaCl_2$ (0.3 M) in 1000 ml distilled water.

4. 5 x VBD:

   a. Mix solutions 1 and 2 and cool to room temperature.

   b. Add 2.5 ml of solution 3 to a and q.s. to 1000 ml with distilled water.

   c. To use, dilute 1:5 and add 1.0 g gelatin per 1000 ml of final diluent.

Sentinel goat serological response - HI[a]

DPI[b]	Goat Number					
	147	152	157	158	159	161
0	<10	<10	<10	<10	<10	<10
1	<10	<10	<10	<10	<10	<10
2	<10	<10	<10	<10	<10	<10
3	<10	<10	<10	<10	<10	<10
4	<10	<10	<10	<10	<10	<10
5	<10	<10	<10	<10	<10	<10
6	<10	<10	<10	<10	<10	<10
7	20	<10	<10	<10	<10	<10
8	640	<10	10	<10	10	10
9	5120	10	160	<10	20	20
10	2560	20	320	10	80	160
11	2560	20	320	20	320	320
12	1280	40	640	40	320	320
13	1280	40	640	320	320	320
14	640	40	640	2560	2560	640
15	2560	40	1280	2560	1280	640
16	1280	40	2560	2560	640	320
17	640	20	2560	1280	320	640
18	1280	20	640	5120	640	1280
19	640	20	640	5120	640	1280
20	1280	20	320	2560	320	640
21	5120	20	320	2560	320	640
24	2560	10	320	2560	320	1280
30	5120	10	2560	5120	640	2560
35	10,240	<10	640	2560	1280	1280
38	2560	<10	640	1280	640	1280
42	5120	<10	640	1280	1280	640
45	1280	<10	640	640	2560	2560

[a]Reciprocal of highest serum dilution giving inhibition of hemagglutination.

[b]Day postinoculation.

Sentinel goat serological response - SN(GJ9-1BJ)[a]

DPI[b]	Goat Number					
	147	152	157	158	159	161
0	<10	<10	<10	<10	<10	<10
1	<10	<10	<10	<10	<10	<10
2	<10	<10	<10	<10	<10	<10
3	<10	<10	<10	<10	<10	<10
4	<10	<10	<10	<10	<10	<10
5	<10	<10	<10	<10	<10	<10
6	<10	<10	<10	<10	<10	<10
7	<10	<10	<10	<10	<10	<10
8	10	<10	<10	<10	<10	<10
9	10	10	<10	<10	<10	<10
10	10	10	10	<10	10	10
11	10	10	10	<10	10	10
12	10	10	10	<10	10	10
13	10	10	10	<10	10	10
14	10	10	10	<10	10	10
15	10	<10	10	10	10	10
16	10	10	10	<10	10	10
17	<10	<10	10	<10	<10	<10
18	<10	<10	10	<10	10	10
19	<10	<10	10	10	10	10
20	<10	<10	10	10	10	10
21	<10	<10	10	10	10	<10
24	<10	<10	10	10	10	10
30	10	<10	10	10	10	10
35	<10	<10	10	10	100	<10
38	<10	<10	10	10	10	<10
42	<10	<10	10	10	10	<10
45	10	<10	10	10	100	<10

[a]Reciprocal of highest serum dilution that produced at least 90% plaque reduction.

[b]Day postinoculation.

Sentinel goat serological response - SN(TC-83)[a]

DPI[b]	Goat Number					
	147	152	157	158	159	161
0	<10	<10	<10	<10	<10	<10
1	<10	<10	<10	<10	<10	<10
2	<10	<10	<10	<10	<10	<10
3	<10	<10	<10	<10	<10	<10
4	<10	<10	<10	<10	<10	<10
5	<10	<10	<10	<10	<10	<10
6	10	<10	<10	<10	<10	<10
7	100	<10	10	<10	<10	<10
8	800	10	100	<10	10	10
9	1600	100	400	10	10	100
10	1600	400	400	10	100	200
11	1600	400	400	100	100	800
12	1600	800	1600	400	200	800
13	1600	400	1600	800	400	800
14	800	800	1600	800	400	800
15	800	800	800	800	400	800
16	800	800	1600	1600	800	800
17	800	400	1600	800	400	800
18	400	200	800	800	200	800
19	400	200	400	800	200	800
20	200	200	400	800	400	800
21	400	200	800	1600	200	800
24	800	100	400	800	200	400
30	800	200	400	800	200	800
35	800	100	400	800	400	1600
38	400	100	400	800	800	800
42	800	100	200	800	800	400
45	400	100	400	800	800	800

[a]Reciprocal of highest serum dilution that produced at least 90% plaque reduction.

[b]Day postinoculation.

Antiserum production in the goat. Serological response.

Goat Number[a]	Day Postinoculation			
Hemmagglutination-inhibition[b]				
	0	21	30	42
149	<10	800	1600	800
150	<10	<10	400	400
155	<10	800	800	200
156	<10	200	200	100
Serum Neutralization (GJ9-1BJ)[c]				
149	<10	100	10	<10
150	<10	<10	10	10
155	<10	10	100	<10
156	<10	10	10	10
Serum Neutralization (TC-83)[c]				
149	<10	800	1600	800
150	<10	<10	400	400
155	<10	800	800	200
156	<10	200	200	100

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[a]Group 1 = 149, 150. Group 2 = 155, 156.

[b]Expressed as reciprocal of highest serum dilution giving inhibition of hemagglutination.

[c]Reciprocal of highest serum dilution that produced at least 90% plaque reduction.

Serological response following hyperimmunization - HI[a]

DPI[b]	Goat Number					
	147	149	150	152	155	156
49	640	320	160	<10	320	40
52	640			20		
56	32,000			32,000		
59	32,000	32,000	32,000	64,000	32,000	32,000
63				32,000	32,000	16,000
66	16,000			32,000		
70	4000	64,000	16,000	64,000	16,000	16,000
72		64,000	4000	16,000		
73	4000					
77	4000					
80	2000				4000	4000
84	2000					
87	2000					
90	2000				20	2000

DPI	Goat Number					
	157	158	159	160[c]	161	162
49	320	320	320	<10	320	<10
52	160	320	320	<10	160	<10
56	32,000	8000	64,000	10	64,000	<10
59	32,000	8000	64,000	640	16,000	<10
63	64,000	8000	32,000	1280	16,000	<10
66	32,000	8000	32,000	2560	16,000	<10
70	32,000	8000	8000	1000	16,000	<10
72						<10
73	16,000	4000		1280		<10
77	32,000	2000		5120		<10
80	16,000	2000		2560		<10
84	8000	2000		2560		<10
87				2560		<10
90	8000	2000		2560		<10

[a]Reciprocal of highest serum dilution giving inhibition of hemagglutination.

[b]Day postinoculation.

[c]40,000 $SMICLD_{50}$ goat esophagus origin GJ9-1BJ VEE virus administered intravenously on day 50.

Serological response following hyperimmunization - SN,GJ9-1BJ[a]

DPI[b]	Goat Number											
	147	149	150	152	155	156	157	158	159	160[c]	161	162
49	<10	10	100	<10	<10	10	10	10	100	<10	<10	<10
52	<10			80			<10	<10	100	<10	<10	<10
56	80			1280			320	1280	1280	100	160	<10
59	320	1280	640	1280	160	320	320	320	1280	640	640	<10
63	10	640	100	1280	100	320	100	160	1280	320	160	<10
66	40			1280			160	80	1280	160	320	<10
70	80	640	320	1280	40	160	20	80	640	100	100	<10
72		100	160	1000								
73	20						40	100		100		<10
77	40						80	100		100		<10
80	40				20	100	80	100		20		<10
84	40							40		40		<10
87	40							40		40		<10
90	40				20	80	80	40		10		<10

[a]Reciprocal of highest serum dilution that produced at least 90% plaque reduction.

[b]Day postinoculation.

[c]40,000 $SMICLD_{50}$ goat esophagus origin GJ9-1BJ VEE virus administered intravenously on day 50.

Serological response following hyperimmunization - SN TC-83[a]

DPI[b]	Goat Number					
	147	149	150	152	155	156
49	400	400	200	100	400	100
52	1600			200		
56	51,200			51,200		
59	51,200	51,200	12,800	25,600	51,200	12,800
63	51,200	51,200	12,800	12,800	51,200	12,800
66	51,200			12,800		
70	25,600	25,600	6400	12,800	12,800	12,800
72		51,200	12,800	12,800		
73	51,200					
77	25,600					
80	12,800				12,800	3200
84	12,800					
87	12,800					
90	12,800				12,800	1600

DPI	Goat Number					
	157	158	159	160[c]	161	162
49	200	400	800	10	400	<10
52	400	800	800	10	800	<10
56	51,200	51,200	51,200	800	51,200	<10
59	51,200	51,200	51,200	800	51,200	<10
63	51,200	51,200	51,200	800	51,200	<10
66	51,200	25,600	51,200	200	25,600	<10
70	12,800	6400	51,200	200	25,600	<10
72						<10
73	51,200	12,800		200		<10
77	25,600	6400		200		<10
80	12,800	12,800		200		<10
84	12,800	6400		200		<10
87		6400		200		<10
90	12,800	6400		200		<10

[a]Reciprocal of highest serum dilution that produced at least 90% plaque reduction. Final titer recorded on day of exanguination with serum harvest.

[b]Day postinoculation.

[c]40,000 $SMICLD_{50}$ goat esophagus origin GJ9-1BJ VEE virus administered intravenously on day 50.

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ACKNOWLEDGEMENTS

The author wishes to thank Doctor C. J. Maré for his counsel and guidance in the development and completion of this thesis.

This study was conducted with the guidance and general supervision of Doctor E. A. Carbrey.

The author is indebted to the late Doctor E. P. Pope who through his vision established this two-year advanced veterinary training program.

The author extends his appreciation to Doctor E. C. Sharman and the Emergency Programs staff who made this developmental program possible by their support.

Appreciation is expressed to Doctor J. E. Pearson and Messrs. G. Gustafson, D. Senne, and M. Snyder for their assistance in the effectuation of laboratory principles and techniques, and to Mister R. M. Glazier and his staff for preparation of the excellent graphs and photographs.