Isolation of Venezuelan Equine Encephalomyelitis Virus by Bone Marrow Culture. (29555)

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Infection in humans and monkeys with the Trinidad strain of Venezuelan equine encephalomyelitis (VEE) virus is typically a febrile disease of 2 to 7 days duration. Virus usually appears in the blood during the first 24 hours of infection, rapidly achieves high titer, and soon disappears, rarely being demonstrable after the fourth or fifth day. Shortly after the end of the viremic phase, hemagglutination-inhibiting (HI) and neutralizing antibodies appear in the peripheral blood. The relatively brief period during which virus circulates limits the ability to isolate and identify the agent, a difficulty common to many viral infections. The present study was undertaken in an effort to overcome this problem. Virus content of bone marrow was measured during the course of VEE infection to determine if virus persisted in this tissue beyond the viremic period at a time when antibody was present, and to investigate the usefulness of *in vitro* culture of bone marrow as a source of virus and as a susceptible tissue for virus propagation.

Materials and methods. Virus strains. Virulent VEE virus was the egg-adapted Trinidad strain(1). The attenuated strain was the 80th guinea pig heart cell tissue culture passage of the Trinidad strain described by Berge *et al*(2).

Diluent and nutrient media. Virus diluent consisted of phosphate buffered saline, pH 7.4(3) containing 0.5% normal rabbit serum. Nutrient medium was 85% Eagle's medium (4) and 15% horse serum, containing 200 units/ml of penicillin and 50 μg/ml of streptomycin.

Test animals. Healthy young adult monkeys *(Macaca mulatta)* were obtained from the Animal Farm, U. S. Army Biological Laboratories, Fort Detrick, Md.

Weanling white mice, CD-1 strain, approximately 3 weeks of age, were obtained from Charles River Breeding Laboratories, Brookline, Mass.

Virus assay. Blood, bone marrow and tissue culture fluids were tested for viral content by intraperitoneal inoculation of each of 6 mice with 0.3 ml, a method previously shown to be as sensitive as tissue culture(5). Where titrations were performed, results are expressed as mouse intraperitoneal median lethal doses ($MIPLD_{50}$) for virulent virus, and mouse intraperitoneal median infectious doses ($MIPID_{50}$) for attenuated virus. Infection by virulent virus in mice was manifested by paralysis and death during a 10-day observation period. Virulent VEE virus was identified by employing mice specifically immunized with attenuated VEE virus; such mice survived challenge with materials which were lethal for non-immunized controls. Attenuated virus was detected and identified by survival of mice after challenge with virulent VEE virus 14 days after inoculation of test material.

Hemagglutination-inhibition test. The hemaglutination-inhibition test procedure was that of Clarke and Casals(6).

Marrow culture. A 1:10 dilution of each marrow sample was made with nutrient media and inoculated into a series of tissue culture flasks; half contained a confluent sheet of L cells (stock culture was obtained from Virus and Rickettsia Division, U. S. Army Biological Laboratories, Fort Detrick, Md.). Thus, 2 cultural systems were established; one containing marrow cells alone and the other marrow cells in combination with L cells. All cultures were maintained at 37°C in a stationary position. Cultures were observed daily and supernatant fluids changed at frequent intervals. Aliquots of the fluids were stored at —60°C for subsequent testing in mice.

In some instances, samples of peripheral blood obtained from monkeys at the time of

TABLE I. Presence of VEE Virus in Monkey Blood and Bone Marrow as Determined by Mouse Inoculation.

Specimen	No. positive/No. specimens tested Day of infection 1	2	3	4	5	6	7
Blood	2/2	2/2	6/6	6/6	2/8	0/7	0/8
Bone marrow	2/2	2/2	4/4	1/1	1/5	0/5	0/8

marrow aspiration were inoculated into L cell cultures. Cultures were observed for cytopathogenic effect (CPE) and fluids removed for testing in mice.

Infection and sampling. Monkeys were infected by subcutaneous inoculation with approximately 1000 $MIPLD_{50}$ of virulent VEE virus. Sera obtained prior to and at intervals following inoculation were tested for presence of HI antibody; base-line sera were negative in all instances. Heparinized venous blood and samples of bone marrow, aspirated from the posterior iliac crest under pentothal anesthesia, were obtained during the course of infection.

Results. VEE infection in monkeys following subcutaneous inoculation of 1000 $MIPLD_{50}$ of the virulent virus was accompanied regularly by viremia (Table I). By testing in mice virus was demonstrated in all blood and bone marrow samples obtained during the first 4 days of infection, but none after day 5.

In 5 of 12 monkeys, however, bone marrow culture resulted in isolation of virus in one or both cultural systems, relatively late in the course of infection, after viremia had subsided (Table II). With the exception of the day 5 marrow from monkey No. 10A-9, direct mouse inoculation of the marrow samples failed to demonstrate virus and all corresponding peripheral blood samples were negative when tested in mice and L cell cultures.

Quantitation of virus in tissue culture fluids was carried out in some instances (Fig. 1). In the culture system employing marrow cells alone, virus not only persisted but multiplied to relatively high titer. In general, marrow cells obtained from monkeys early in the infection survived *in vitro* for only a few days, and inoculation of these early specimens containing virus in high titer into L cell cultures resulted in gross CPE in 48 to 72 hours. Marrow cells obtained after day 4 of infection could, with few exceptions, be

TABLE II. VEE Virus Content of Tissue Culture Fluid as Determined by Mouse Inoculation.

Monkey No.	Day of infection	Hemagglutination-inhibition antibody titer	Tissue culture fluid Day of incubation	Marrow culture	Marrow - L cell culture
C-20	7	1:80	1	—	+
			2	—	+
			3	—	+
			4	+	+
			7	+	+
B-336	6	Not done	1	—	—
			4	—	+
	7	1:80	3	+	—
			5	+	—
N-11	6	Not done	1	+	+
			5	+	+
			7	+	+
7A-11	6	1:80	1	+	+
			2	—	+
			4	—	+
			5	—	+
10A-9	5	1:10	1	+	Not done
			2	+	
			3	+	
			4	+	

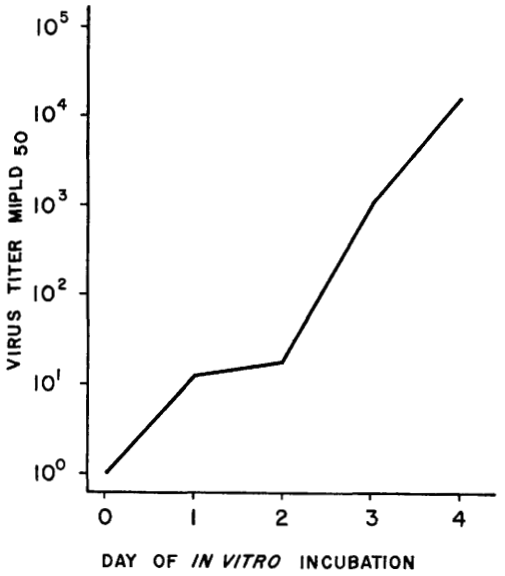

FIG. 1. VEE virus content of supernatant fluid from monkey No. 10A-9 marrow culture.

maintained *in vitro* for at least 1 week. In some instances cultures of marrow cells alone or in combination with L cells remained viable for almost a month; virus was produced continuously during this period. Such chronically infected cultures were noteworthy in that VEE virus usually produces marked CPE and death of L cells in 48 to 72 hours. Mouse inoculation of virus harvested from these long term cultures demonstrated no viral attenuation.

A portion of each of the marrow samples obtained after day 5 of infection was subjected to 1 or 2 freeze-thaw cycles to disrupt cells prior to inoculation into L cell culture. Experience with the virus in this laboratory has shown it to be stable upon such treatment. Supernatant fluids of cultures inoculated with these materials contained no virus. Thus it would appear that viable marrow cells were required for virus multiplication to occur, in spite of the fact that L cells are known to be highly susceptible to infection with VEE virus.

The investigative use of VEE virus as an antineoplastic agent in man, reported by Tigertt and coworkers(7), afforded the opportunity to extend these studies to a small series of patients. A single bone marrow specimen was obtained from each of 6 patients with lymphomatous disease 8 to 13 days after subcutaneous inoculation of the attenuated strain. Direct mouse inoculation failed to demonstrate virus in any of these specimens. In one instance, however, *in vitro* incubation of a marrow sample obtained on postinoculation day 8 resulted in multiplication and demonstration of the virus. Supernatant fluids removed from the marrow culture after 24 and 48 hours of *in vitro* incubation protected mice against challenge with virulent virus. The fluids were of low virus titer, containing slightly greater than 100 $MIPID_{50}$ per ml at 48 hours. The cultures consisted of marrow cells only; the marrow-L cell monolayer was not employed. At a later date the same marrow sample was inoculated into an L cell culture after prior freeze-thaw cycles; no virus was recovered.

As objective evidence of tumor regression was noted in some patients infected with the attenuated strain of virus and in the hope that the Trinidad strain would exert a more profound antitumor effect, 2 patients received this virus. Both had far-advanced Hodgkin's disease which was progressive despite all other forms of therapy. Both tolerated the infection well and neither exhibited evidence of encephalitis. In one, a dramatic, though short-lived, remission of the Hodgkin's disease occurred. Bone marrow aspirates and samples of peripheral blood were obtained from this patient on postinoculation days 2, 6, 10 and 18 and tested for virus content (Table III). Day 2 and day 6 marrow contained virus in lower titer than the corresponding peripheral blood. On days 10 and 18 both blood and bone marrow were negative upon direct mouse inoculation.

No virus was recovered from L cell cultures inoculated with peripheral blood obtained on postinoculation days 10 and 18. HI antibody titers of day 10 and day 18 sera were respectively 1:40 and 1:10,240. The latter serum neutralized greater than 10^6 $MIPLD_{50}$ of VEE virus. Virus was recovered from both day 10 and day 18 marrow by marrow culture. Five days of *in vitro* incubation of the day 18 marrow were required

TABLE III. VEE Virus Content of Blood, Bone Marrow and Bone Marrow Culture Fluids —Patient C. D.

Post-inoculation day	Titer of blood*	Titer of marrow* prior to *in vitro* cultivation	Days of *in vitro* cultivation	Virus titer* of supernatant fluid: Marrow cells alone	Virus titer* of supernatant fluid: Marrow and L cells
2	8.5	5.1	1	3.5	4.9
			2	2.7	5.9
			3	1.8	5.0
			4	2.5	5.8
6	4.5	2.9	1	1.0	3.5
			2	1.0	6.9
			3	3.1	9.5
			4	4.1	8.7
10	Neg	Neg	1	.8	.8
			2	1.8	Neg
			3	4.1	"
			4	5.9	1.0
18	Neg	Neg	1	Neg	Neg
			2	"	"
			3	"	"
			4	"	"
			5	"	4.7
			6	"	7.5

* Log_{10} - $MIPLD_{50}$ per ml.

before virus was demonstrated. No CPE occurred in this culture, and the infected cells were maintained for more than one month. After repeated freeze-thaw cycles portions of these marrows (day 10 and 18) were incubated with L cells and, as was the case with the monkey marrows, no virus was recovered. This again indicates that marrow cells must remain viable *in vitro* to allow for virus multiplication.

Discussion. The appearance of antibody in peripheral blood coincides with the end of the viremic phase of VEE infection, as in most viral infections. It is now well known, however, that some viruses persist in tissues for prolonged periods in spite of high levels of serum antibody; examples include herpes simplex, hepatitis and adenoviruses in man, and psittacosis in birds(8). Specifically with VEE, virus can survive for months in the tissues of hibernating bats; at the end of hibernation, it achieves high titer in the blood (9).

Studies in various animal species during the course of infection with virulent VEE virus indicated it to be pantropic. In mice, virus was isolated from various tissues including brain, bone marrow, spleen and lymph nodes. The titer of virus in these tissues remained elevated as viremia subsided, suggesting that virus in the blood was more accessible to neutralizing antibody than was tissue virus(10).

In the guinea pig, massive necrosis of lymphocytes in lymph nodes and spleen and necrosis and depopulation of the bone marrow have been observed(11). The effects of infection with the attenuated strain of VEE virus on these tissues have been compared to those produced by X-irradiation(12). Administration of an attenuated strain of VEE virus to normal humans resulted in a reduction in total white cell count in peripheral blood, relative lymphocytosis and minimal thrombocytopenia. In patients with lymphomatous disease, vacuolated abnormal lymphocytes appeared in the peripheral blood on about the 7th postinoculation day; reticulocytopenia, lymphocytopenia, thrombocytopenia and granulocytopenia then followed in that order. Marrow panhypoplasia was present at the time of maximum blood changes. Complete recovery of bone marrow and circulating elements occurred in from 3 to 14 days(13).

In view of the demonstrated effect of VEE virus on the reticulo-endothelial system of various animal species and the hematologic

changes occurring in infected humans, it is reasonable that virus should persist in this tissue after the appearance of circulating antibody. Virus isolation from this tissue late in infection only after *in vitro* incubation of marrow cells suggests that the presence of virus within marrow cells *in vivo* was masked by antibody, but became manifest after antibody was removed from the cellular environment. The demonstration that marrow must remain viable *in vitro* to allow viral multiplication to occur, even in the system employing susceptible L cells, indicates that initial viral multiplication occurred within marrow cells rather than within L cells.

The advantages of the marrow culture are several. It allows for unmasking and isolation of VEE virus relatively late in the course of infection, at a time when circulating antibodies have appeared. Secondly, it provides a relatively simple tissue culture system, *i.e.*, in the cultural system in which marrow cells were incubated alone, the marrow served as both a source of virus and a susceptible tissue supporting virus multiplication under *in vitro* conditions. While not tested in other viral infections, this technique may be of assistance in isolation attempts, particularly in infections in which viremia is short-lived. In addition, this system may be of value in attempts to isolate agents for which susceptible host systems are not immediately known or available. If the virus multiplies in *in vitro* cultures of the host's own marrow, considerable quantities of infected fluid would be provided for subsequent testing. The method may have particular application under field conditions, where the capacity to preserve specimens in frozen state is not immediately available.

Summary. *In vitro* culture of bone marrow obtained relatively late in the course of disease in both man and monkeys has resulted in isolation of VEE virus not detected by conventional procedures. Viable marrow cells served as both the source of virus and as susceptible tissue supporting virus multiplication. It is suggested that this method may have application in the diagnosis of other viral diseases.

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