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Hemorrhagic Disease in Rodents Caused by Chikungunya Virus. 1. Studies of Hemostasis. (30201)

HARVEY J. WEISS,* SCOTT B. HALSTEAD AND SUDIE B. RUSS
(Introduced by W. H. Crosby)
Departments of Hematology and Virus Diseases, Walter Reed Army Institute of Research, Washington, D. C.

It has been shown that chikungunya virus (CHIK), isolated from a Thai child with mild hemorrhagic fever, produced a fatal hemorrhagic disease when inoculated into suckling rodents(1). Following a dose-related, asymptomatic incubation period of 2-5 days, the animals rapidly became ill, as manifested by sluggishness, poor feeding, cyanosis, decrease in skin temperature, cardiac enlargement and death. A significant proportion of animals develop grossly visible hemorrhages into the gastrointestinal tract and, to a lesser extent, into the bladder, joints and skin. Histologically, there are intraalveolar hemorrhages, focal skeletal muscle and fat pad necrosis and a diffuse enteropathy characterized by a decrease in goblet cells, thickening and blunting of villi in the small intestine, crypt formation, basophilia of the epithelial cytoplasm and marked congestion of the intestinal blood vessels in the lamina propria, submucosa and serosa.

The results of studies on the hemostatic abnormalities on these animals are presented here. Attempts were also made to modify the occurrence of hemorrhage by pretreating animals with various drugs.

* Present address: Dept. of Hematology, Mt. Sinai Hospital, New York.

Materials and methods. Hosts. Litters of eight or nine 24-36-hour-old suckling mice, Walter Reed Bagg strain, or suckling Wistar rats were used. Age of suckling animals was determined by noting births at 6-hour intervals.

Virus. KLA-16 strain of CHIK was used; it produced a high percentage of hemorrhage. Mice were infected with 10-1000 LD_{50} of virus obtained after 4-6 mouse passages (sm 4, 5, 6). Rats were infected with virus which had been passaged 3 times in mice and once or twice in suckling rats (sm3r1 or sm3r2).

Observation of hemorrhage. Between 60-96 hours following intracerebral inoculation of 0.01 ml of virus, rodents were observed at 6-hour intervals. Such frequent observation was necessary to detect presence of sick animals before they were destroyed by mothers. Animals with grossly visible gastrointestinal hemorrhage were recorded and removed from the cage. Occurrence of hemorrhage was expressed as the number with gastrointestinal hemorrhage divided by the total infected and surviving on the second day following inoculation. In experiments in which length of survival was of importance, the animals with hemorrhage were not removed but observed

TABLE I. Studies on Suckling Mice Infected with Chikungunya Virus (sm 2 and 3).*

	Control		Infected	
	No. studied		No. studied	
Platelets per mm³ ($\times 10^{-3}$)	10	(922)±(66)†	13	(228)±(36)
Bleeding time (sec)	12	(64)±(9)	10	(274)±(43)
Clotting " "	11	(76)±(10)	10	(322)±(38)

* Studies were performed on third day of infection. † Standard error of mean.

TABLE II. Studies of Hemostasis in Suckling Rats Infected with Chikungunya Virus.

	Control rats		Infected hemorrhagic rats	
	No. studied		No. studied	
Platelets per mm³ ($\times 10^{-3}$)	31	(526)±(46)*	26	(84)±(13)
Bleeding time (sec)	38	(77)±(5)	25	(366)±(31)
Clotting " "	64	(90)±(6)	51	(168)±(12)
10% Prothrombin (sec)	3	(35)±(.2)	13	(47)±(5)
Factor V "	37	(13.5)±(.1)	23	(17)±(.4)
Factor VII + X "	25	(20.1)±(.5)	13	(30.4)±(2.7)
Fibrinogen (mg %)	12	(173)±(9)	11	(194)±(33)

* Standard error of mean.

until they died. Virus titrations were calculated by the method of Reed and Muench(2).

Evaluation of drug effect on the hemorrhagic syndrome. One-half of each litter was inoculated with the experimental drug and the remainder were controls which were given saline. This technique is referred to as "split litters." To identify control mice, 3-4 mm of tail was removed approximately 24 hours before the experiment was started.

Studies of hemostasis. Bleeding time was determined as follows: tails were cut 2-3 mm from the body and the blood issuing from the cut surface was absorbed onto filter paper every 15-30 seconds until bleeding stopped. Animals were held firmly but without excessive pressure in the supine position for the procedure. *Clotting time in capillary tubes.* Blood was obtained from the jugular vein as follows: a small incision was made in the skin overlying the vein with a pair of iris scissors, the point of which was then inserted into the incision. The external jugular was cut and a stopwatch begun. Blood was allowed to "well up" and a 10 lambda capillary tube† (I.D. = 0.42 mm) was inserted into the center of the drop and filled by capillary action. The tube was carefully broken every 30-60 seconds and the segments were pulled apart. The first appearance of a fibrin strand of at least 2 mm in length was taken as the end point. Platelet counts were performed by the phase microscopy method of Brecher and Cronkite(3) on blood obtained by jugular puncture. Blood for determining clotting factors was obtained by jugular puncture from rats only. It was drawn up into a cooled siliconized tuberculin syringe, mixed with one-tenth volume of cooled 1.34% sodium oxalate in silicone-treated tubes and held in an ice water bath until centrifuged. An average of 0.3 ml of plasma was obtained from each rat. *One-stage prothrombin* time was measured on plasma diluted 1:10 with saline (10% prothrombin) by the method of Quick using rabbit brain thromboplastin. *Factors V* and *VII + X* were determined by the one-stage method of Owren(5). *Fibrinogen* was determined as clottable protein by adapting a micro method to the method described by Quick(4).

Results. Suckling mice. Mice infected with chikungunya sm 3-5 developed significant thrombocytopenia and prolongation of the bleeding and clotting time (Table I). In mice infected with Murray Valley encephalitis, Kyasanur Forest disease, Omsk hemorrhagic fever, Semliki Forest, Uruma, Eastern equine encephalitis, Western equine encephalitis and

† Microcaps, Drummond Scientific Co., Philadelphia, Pa.

TABLE III. Blood Chemistries in 4- to 5-Day-Old Rats.

Test	Normal			Chikungunya infected		
	No. tested	Avg	Range	No. tested	Avg	Range
Albumin	6	3.4 g/100 ml	(2.7 – 3.9)	6	3.8 g	(2.9 – 4.4)
Globulin	6	1.6 g	(1.2 – 2.1)	6	1.9	(1.2 – 2.4)
Thymol turbidity	13	3.1 units	(1.0 – 6.5)	16	1.5	(.2 – 2.8)
Transaminase (SGOT)	6	213.0	(160 –330)	6	1330	(1080 –2070)
Total bilirubin	6	.30	(.17– .58)	5	.73	(.30– 1.24)
Direct "	6	.07	(.01– .15)	5	.43	(.23– .92)
Alkaline phosphatase	4	125 K.A. units	(114–136 K.A. units)	5	165.6 K.A. units	(154–188 K.A. units)
Urea nitrogen	8	22.9	(19.2 – 25.7)	13	32.2	(25.2 – 41.6)

TABLE IV. Effect of Cortisone on Virus Titer and G.I. Hemorrhage in 1- to 2-Day-Old Mice Following Intracerebral Infection with Chikungunya Virus.

	Exp 1		Exp 2	
	Cortisone	No cortisone	Cortisone	No cortisone
Titer (neg $\log_{10}$/0.01 ml)	8.2	7.7	8.1	7.0
No. hemorrhagic mice/total No. infected	16/64	8/59	19/62	5/56

dengue 2 arthropod borne viruses the bleeding time, in general, was not prolonged. The clotting time was prolonged in all groups except those infected with Semliki Forest and dengue 2 virus.

Suckling rats. (Table II). Significant thrombocytopenia was seen in the majority of hemorrhagic animals. Bleeding time was greatly prolonged. An abnormal one-stage prothrombin time was observed in many of the animals and was due, at least in part, to decreases in factor V and factors VII + X. Clotting time was very slightly prolonged in most of the animals. Fibrinogen concentration was normal. Liver chemistries are shown in Table III.

Attempts to modify the hemorrhagic syndrome by pretreating mice with various drugs. Corticosteroids. Suckling mice were injected daily, for four days, with 0.25 ml of a solution containing 10 mg of hydrocortisone hemsuccinate‡ per kg body weight. Mice were infected on the day that steroid treatment was begun. As shown in Table IV, mice treated with corticosteroids became infected and died at higher virus dilution and had a higher incidence of hemorrhage than those treated with saline.

Heparin. In Experiment 1 twelve litters of mice were infected intracerebrally with 10 LD_{50} of CHIK sm 5 hemorrhagic line. Four of these litters received saline and four sets, containing two litters each, received different dosages of heparin. Heparin§ solutions were prepared by diluting a stock aqueous solution (containing 1000 U/ml), to 1:32, 1:64, 1:128 and 1:256, and each mouse received daily injections of 0.01 ml of heparin or saline. Injections were intraperitoneal for the first 2 days and then subcutaneous on the dorsum for the remainder of the experiment. The daily dosage of heparin for the mice receiving the 1:32 dilution, calculated for a 2.5 g mouse, was 1.25 mg per kg body weight. As shown in Table V, compared with the control group, the 1:32 dilution (1.25 mg per kg) seemed to offer some protection against hemorrhage. Experiments 2 and 3, using larger groups, were then performed. Mice were inoculated intracerebrally with 50 LD_{50} of CHIK sm 6C virus. One-half of each litter was inoculated over the dorsum with saline. The other mice in each litter were injected with 0.01 ml of heparin as shown in Table V. Hemorrhage was again less in the heparin treated groups. Combining the data from the three experiments, hemorrhage occurred in 39% of the saline

‡ Solu-cortef, Upjohn Co.

§ Liquamine sodium, Organon, 100 units per mg.

TABLE V. Effect of Heparin on Occurrence of Hemorrhagic Enteritis in 1- to 2-Day-Old Infected Mice.

	Heparin treated			Saline treated	
Exp	Dosage, mg/kg	Total No. infected	No. with hemorrhage	Total No. infected	No. with hemorrhage
1	1.25 daily	14	2	34	16
2	1.25 T.I.D.	52	4	47	7
3	2.0 B.I.D.	37	16	37	23
Total		103	22	118	46

TABLE VI. Effect of Sterilizing Gut on Occurrence of Hemorrhagic Entertitis in Infected Mice (Split Litters).

	Antibiotic treated mice		Control mice	
	Developed hemorrhage	No hemorrhage	Developed hemorrhage	No hemorrhage
	20	8	17	10
Sterile gut	10/15	—	1/14	

treated animals and in 21% of those receiving heparin in a daily dose of 1.25-4 mg per kg. The results are highly significant ($p<0.01$).

Antibiotics. Attempts were made to sterilize the gut and tissues of infected suckling mice by treatment with neomycin-polymixin B orally and oxytetracycline parenterally. Six litters of mice were inoculated intracerebrally with 100 LD_{50} of sm 6C virus. Thirteen hours after infection, one-half of each litter was fed daily with 0.01 ml of a 1:20 dilution of neomycin-polymixin B‖ by blunt needle and tuberculin syringe. For a 2.5 g mouse this dose is equal to 10 mg per kg body weight neomycin and 10,000 units per kg of polymixin B. These mice also received daily 0.01 ml injections of a 1:10 dilution of oxytetracycline,¶ (10 mg per kg body weight). To determine if antibiotic treatment had sterilized the gut, the gastrointestinal tract of hemorrhagic mice from both the treated and untreated group was removed aseptically, opened and the contents streaked on blood agar pour plates. If no colonies were seen 48 hours after streaking, the gut content was considered sterile. As may be seen in Table VI, the gut in 66% of the treated group was sterile; the incidence of hemorrhage was essentially the same as the nontreated group.

‖ Daribiotic, S. E. Massengill Co., Bristol, Tenn.

¶ Liquamycin, Chas. Pfizer Co., New York.

Discussion. Whether by coincidence or not, hemostatic abnormalities in infant rodents infected with KLA-16 strain of Thailand chikungunya virus were essentially the same as found in children with Thai hemorrhagic fever caused by dengue virus(6). In both, there occurred a profound thrombocytopenia, prolonged bleeding time, variable depression of one-stage prothrombin time, and factor V and factors VII + X. In 13 out of 25 children, the clotting time in siliconized tubes was markedly prolonged. In the infected rodents, a prolonged capillary clotting time was a common finding. Fibrinogen concentration was normal. The prothrombin abnormalities in the children and infant rodents appeared to be related to abnormal liver function.

The significant abnormalities in platelets and clotting factors found in these rodents does not imply that these alone are responsible for the hemorrhages observed. Hemorrhage appears primarily in the gastrointestinal tract and could be due to vascular lesions in the gut rather than a generalized bleeding tendency. The infected animals appeared to be in severe and prolonged shock, a condition often associated with hemorrhagic enteritis(7,8). Lillehei, studying hemorrhagic shock in dogs, attributed this to bowel ischemia consequent to severe vasoconstriction(9). Hardaway *et al,* however, postulated the occurrence of intravascular clotting during pro-

longed shock, thereby producing ischemic necrosis due to occlusion of the microcirculation by microthrombi(8). In support of this theory was the finding that pretreating the animals with heparin reduced the incidence of hemorrhagic enteritis, similar to the findings in the present study with chikungunya virus. Hemorrhagic enteritis has also been observed following the single injection of large amounts of bacterial endotoxin into mice(10) and dogs(11,9). Again, pretreatment with heparin appeared to protect dogs against gastrointestinal hemorrhage. Mims tested the hypothesis that the hemorrhagic enteritis produced in mice by a variety of stimuli was due to absorption of bacterial endotoxin from the gut(12). His results, however, did not support this hypothesis. Similarly, we found that sterilizing the gut failed to decrease either death or hemorrhage in chikungunya infected mice. Finally, the protective effect of heparin as well as the deleterious effect of steroids are reminiscent of findings in the generalized Schwartzman reaction(13,14). It is difficult to implicate this type of reaction since characteristic fibrinoid necrosis in small vessels and bilateral renal cortical necrosis were not seen.

The data, therefore, do not support any single known mechanism as the cause of the hemorrhagic syndrome in these animals. The protective effect of heparin, as well as the thrombocytopenia and decrease in factor V, suggest that intravascular clotting, perhaps initiated by shock or tissue damage, may play a role. Plasma fibrinogen was normal, however, and microthrombi were not seen. Furthermore, the decrease in factor V was modest, and, together with decreases in factors VII + X, is consistent with the other abnormalities in liver function observed. Other mechanisms by which viral infections may produce thrombocytopenia are discussed in greater detail elsewhere(6).

Although similarities are evident in the hemorrhagic disease of rodents caused by chikungunya virus and a hemorrhagic disease of humans caused predominantly by dengue viruses, there is insufficient evidence at present to state that the syndrome in rodents is a biologic model of the human disease. Nor is it possible to ascribe general biologic hemorrhagic potential to chikungunya virus since hemorrhagic disease in rodents is also caused by other viruses, such as influenza(12, 15) Newcastle's disease(12,16) and ectromelia(12,17), which are not associated with human hemorrhagic syndromes. Nonetheless, elucidation of the mechanism of chikungunya hemorrhagic disease in rodents and the respective contribution of host and virus to the syndrome might be useful in achieving a better understanding of the pathogenesis of virus hemorrhagic fevers.

Summary. Suckling mice and rats develop a hemorrhagic syndrome when infected with a low mouse passage strain of Thailand chikungunya virus. Studies of hemostasis revealed thrombocytopenia, prolonged bleeding time, prolonged clotting time and decrease in prothrombin time and factor V and factors VII + X. Attempts to modify the occurrence of hemorrhage by pretreatment with corticosteroids or antibiotics were unsuccessful. Animals treated with heparin appeared to be somewhat protected against hemorrhage but not death.

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The First Isolations of Powassan Virus in New York State.* (30202)

ELINOR WHITNEY AND HUGO JAMNBACK (Introduced by Rachel Brown)
Division of Laboratories and Research, New York State Department of Health, and State Museum and Science Service, New York State Education Department, Albany

Group B arbovirus activity was demonstrated in New York State by the detection of antibodies closely related to Powassan (POW) virus in wild animal and in human sera(1). The initial isolation of POW(2) virus was from a child who lived in Canada approximately 200 miles north of the New York State border. Four years later, other POW strains were recovered from *Ixodes marxi* and from the blood of a *Tamiasciurus hudsonicus* (red squirrel) collected in the same vicinity(3). The United States strains of POW virus were isolated from *Dermacentor andersoni* collected in Colorado(4) and, more recently, in South Dakota(3). The presence of Group B arbovirus in New York State has now been established by the isolation of POW virus from wild animals and ticks in northern St. Lawrence County and from a fox in Broome County, 200 miles to the south.

Materials and methods. Under the supervision of one of us (Dr. Hugo Jamnback), mosquitoes and wild animals were live-trapped in the summer of 1964 at various sites on Barnhart Island in the St. Lawrence River. Barnhart Island is separated from the mainland by 2 locks and a canal. Animals and arthropods were also collected on or near dairy farms where arbovirus activity had previously been detected(5). Ectoparasites found on the animals were removed and placed in separate tubes. Blood was collected from the animals; serum and cells were separated; sections of brain, liver, spleen, and kidney were put into separate tightly capped plastic tubes. Mosquitoes were identified and separated as to species and put in plastic tubes. All samples were stored frozen at —20°C until shipped to the laboratory.

Unless otherwise noted, methods of shipment, subsequent storage, preparation of inocula, isolation technics, identification and serologic tests were those previously described(1,6).

In the neutralization (neut.) test for antigenic comparison of strains, 2-fold serial dilutions of serum (1:4-1:256) were mixed with a constant dose of virus.

Hemagglutination (HA) antigens were prepared by the sucrose-acetone method(7). Serum inhibitors were removed by acetone treatment. Hemagglutination-inhibition (HI) tests were done by the Takatsy-loop microtechnic(8) and disposable plates† were used.

Complement-fixation (CF) antigens were HA antigens inactivated by ultraviolet light for 30 minutes. The CF method of Kent and Fife(9) which employs 5 units of complement was used. Box titrations of antigen and sera were carried out.

The baby hamster kidney cell line designated BHK21(10) was obtained from Dr.

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† Cooke Engineering Co., Alexandria, Virginia.