LETTER-TO-THE EDITOR

EFFECT OF ANTICOAGULANTS ON THE SUSCEPTIBILITY OF *AEDES AEGYPTI* MOSQUITOES TO DENGUE VIRUS INFECTION

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Various theories regarding hemorrhagic manifestations of dengue fever have been put forward, which include sequential infection with different virus serotypes, virus strains and several host factors like cytokines and cytotoxic factors, etc. (*1*). The role of *Aedes aegypti* mosquito vector has not been seriously considered in the selective and gradual increase of dengue hemorrhagic fever (DHF) cases.

The present study was performed with a view that in DHF cases, due to blood coagulation defects, the increased blood-clotting time in the midgut of mosquitoes may cause an increase in the infectivity of mosquitoes under these conditions as compared to mosquitoes feeding on classical dengue cases. Since also a higher viremia is associated with DHF cases, the increased blood clotting time in the midgut may have a synergistic effect and may increase the opportunity of virus particles binding to virus-specific receptors present on the gut epithelial cells. Thus, chances of mosquitoes feeding on a DHF patient would result in more infective mosquitoes as compared to those feeding on a classical dengue patient. Therefore, the probability of mosquitoes to pick up and transmit the virus strains responsible for causing DHF would likely be greater than that of mosquitoes to pick up the virus stains causing a classical dengue. The present communication therefore reports data on the effect of anticoagulants on the susceptibility of *A. aegypti* mosquitoes to dengue virus infection.

Dengue-2 virus isolated from a patient suffering from DHF at Jammu, India was used. Four-to-five-day old female mosquitoes bred in our laboratory were orally infected through an artificial membrane, Parafilm (American National Can Co., USA) (*2*). Different concentrations of commercially available anticoagulant heparin sulphate of pharmaceutical grade of heparin sulphate) and EDTA (Sigma) were added to feeding suspensions prepared from the mixture of white leghorn fowl blood and dengue virus. Since in the controls it was impossible to feed mosquitoes through artificial membrane without using anticoagulant, mosquitoes were fed on defibrinated chicken white leghorn fowl blood containing the standard virus dose. Post feeding virus titers in the mosquito feeding suspensions were estimated in mice by intracerebral route of virus inoculation (*3*). The orally infected mosquitoes were maintained at 28°C±2°C and relative humidity of 80±5%. On day 10 post infection the mosquito positivity was determined by indirect immuno-fluorescence test on head squashes as described earlier (*4*).

The results showed that heparin had an adverse effect on the susceptibility of mosquitoes to dengue virus infection, which was dose dependent too. Recently, heparin has been shown to compete with virus receptors in mammalian cells (*5*), however, there are no reports on the presence of heparin sulphate dengue-specific receptors in mosquitoes. This could

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Abbreviations: DHF = dengue hemorrhagic fever

possibly be an additional factor for a higher infectivity of the mosquitoes fed on higher concentrations of heparin. However, when similar experiments were performed using EDTA in the blood to reduce the blood clotting time, the results showed that the positivity of mosquitoes for dengue virus was lower when fed on a blood-virus mixture containing EDTA as compared to the mosquitoes fed on heparinized blood-virus mixture (see the table). The batches of mosquitoes fed on higher concentrations of heparin and EDTA showed a significant difference in positivity as compared with the batches fed on defibrinated blood. The mosquitoes fed on a defibrinated blood-virus mixture showed the positivity comparable with that of the mosquitoes fed on lower doses of heparin. Since it was not possible to feed mosquitoes on the blood without using anticoagulants, no proper control could be included in these experiments which would enable to demonstrate directly the effect of anticoagulants. It is very difficult to simulate natural conditions in the laboratory. The effect of anticoagulants under study could be evaluated if batches of mosquitoes were fed directly on classical dengue fever and DHF cases under identical conditions at the time of epidemics. However, due to medical ethics and practical considerations, this is not possible.

Virus infected bloodmeal		Head squashes positive/tested					
		Replicate			Total	(average±)	P[c]
		1	2	3			
Feeding suspension titer[b]		3.1		3.1	3.1		
Defibrinated blood		4/24	3/24	5/24	12/72	(16.7±4.2)	
Heparin	625 IU/ml	12/24	10/24	13/24	35/72	(48.6±6.4)	0.0001
	250 IU/ml	7/24	8/24	10/24	25/72	(34.7±6.4)	0.0093
	50 IU/ml	6/24	4/24	7/24	17/24	(23 6±6.4)	0.2253
EDTA	68 IU/ml	9/24	10/24	9/24	28/72	(38 9±2.4)	0 0025
	34 IU/ml	5/24	7/24	7/24	19/72	(26.4±4.8)	0.1083
	17 IU/ml	3/24	3/24	5/24	11/72	(15.3±4.8)	0.7630

[a]Head squashes of mosquitoes tested for dengue antigen by indirect immunofluorescence on day 10 post infection.
[b]Virus titer log LD_{50}/0.02ml in infant mice by intracerebral route.
[c]Statistical analysis was performed using chi-square (Cochran's test for combination of 2 x 2 contingency tables) (*10*) for comparison of head squashes positivity in the Heparin and EDTA batches of mosquitoes. In each case values were compared with those obtained with defibrinated blood (control).

In the mosquito vectors several refractory mechanisms to filarial worms have been reported. One of these is the accelerated blood clotting in the midgut. When blood coagulates quickly the microfilariae are trapped in the blood clots and are prevented from penetrating the wall of the midgut (*6, 7*). Only the microfilariae present on the surface of the blood bolus have an opportunity to penetrate the midgut wall. Therefore the chance of microfilariae to cross the midgut increases when the parasitemia is higher. This physiological factor may also play an important role in dengue vectors. Blood-clotting time at room temperature for a normal person is 8–10 mins; however, when mosquitoes take a bloodmeal, due to the presence of the anticoagulant Apyrase in the saliva the blood clotting time increases in the midgut.

Recently, it has been shown that the binding and penetration of dengue virus in the mosquito C6/36 cell line is accomplished within 28 mins (*8*). The virus particles present on the outer surface of the infected blood bolus in the midgut only get opportunity to come into contact with epithelial cells and the receptors present on them. In due time owing to increasing concentration of digestive enzymes and secretion of the peritrophic membrane this process gradually declines.

The viremia of dengue virus in peripheral human blood varies from 10^3 to 10^8 LD_{50}/ml. It has been shown (*1*) that the infectivity of mosquitoes correlates with the titer of virus in the bloodmeal1. Blood bolus of a low virus titer (10^3 LD_{50}/ml) would contain comparatively fewer virus particles on the outer surface than that of a high virus titer (10^8 LD_{50}/ml). Firstly, it has also been suggested (*1*) that the increased viremia is one of the factors causing hemorrhage in DHF patients. Secondly, reports have suggested that coagulation defects occur in DHF cases (*9*). Mosquitoes feeding on a DHF patient will have longer blood-clotting time and as postulated above there would be a higher viremia in DHF patients. In this situation the blood bolus surface touching midgut epithelial cells would contain more virus particles, hence, this would increase the opportunity of virus particles binding to virus-specific receptors present on the gut cells.

These data show that the incorporation of anticoagulants in the bloodmeal increased the susceptibility of mosquitoes to dengue virus. The increase in the susceptibility was associated with the quantity of anticoagulants present in the bloodmeal for mosquitoes. Thus, it appears that an increased viremia in the human blood and a prolonged blood clotting time in the midgut of mosquitoes may have a synergistic effect.

The data presented here also suggest that the feeding on a DHF patient would result in more infective mosquitoes than that on a classical dengue patient. Besides all other factors, this vector factor also seems to have some epidemiological relevance for gradual increase in DHF in many areas. Therefore the possible role of *A. aegypti* in hemorrhagic manifestations encountered in dengue cases warrants serious considerations, especially with regard to the fact that experimental results obtained to date are weak, since it is very difficult to simulate natural conditions in the laboratory.

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