POST-INOCULATION CHANGES IN ENZYME ACTIVITY OF *AEDES AEGYPTI* INFECTED WITH CHIKUNGUNYA VIRUS

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Summary. – Levels of acetylcholinesterase, non-specific esterases, glutathione-S-transferase and glucose-6-phosphate dehydrogenase in *Aedes aegypti* (L.) mosquitoes inoculated intrathoracally with Chikungunya virus were elevated, as compared to uninoculated control insects. A number of these enzymes are important in the insects defence mechanism against xenobiotics, such as pesticides. Malathion bioassays indicated a reduction in the susceptibility of experimentally injected insects with virus or virus-free inoculum, compared to non-inoculated controls. However, insects which were mock-inoculated (injected with no inoculum) showed a similar reduction in susceptibility suggesting that the observed effect was due to the mobilization of a defence reaction in the mosquitoes in response to injury during inoculation.

Key words: *Aedes aegypti*; insecticides; Chikungunya virus; glutathione-S-transferase; glucose-6-phosphate dehydrogenase; esterase

Introduction

For arbovirus vector control purposes it is of interest to explore possible relationships between susceptibility to arboviruses and insecticides in mosquitoes. In a recent study, Rawlins *et al.* (1988) found that Dengue-1 virus had no significant effect on the susceptibility of *Aedes albopictus* (Skuse) to Malathion. The possible relationships between protozoal infections in mosquitoes and their susceptibility to insecticides has been studied by Micks and Ferguson (1961), Prasittisuk and Curtis (1982), Spencer and Olson (1982) and Mourya and Soman (1987), while the effect of *Ascogregarina* on susceptibility of *Culex bitaeniorhynchus* to Japanese encephalitis infection was reported by Mourya and Soman (1985).

The present studies were undertaken to examine whether (1) the alphavirus Chikungunya (CHIK) in the vector *Ae. aegypti* (L.) affects its susceptibility to insecticides, and (2) the effects of infection on the underlying enzymes potentiate the resistance of the vector to insecticides.

Materials and Methods

Mosquitoes. Stocks of *Ae. aegypti* mosquitoes were maintained at 28 ± 2 °C and 80% relative humidity. Larvae were fed on yeast powder. Adults had continuous access to a diet of 10% glucose solution and were regularly given access to guinea pigs for blood feeding. For experimental purposes, adult mosquitoes (males and females) were maintained as above but were not allowed to feed blood. The following strains of *Ae. aegypti* were used: (a) Indian strain, originating from the colony maintained over 25 years at the National Institute of Virology, Pune, India, and (b) Bangkok strain, established in 1987 at the London School of Hygiene and Tropical Medicine, UK, from larvae collected from water storage containers in Bangkok, Thailand.

Virus. The following two strains of CHIK virus were employed: (a) Calcutta strain (634029), isolated from a febrile human case during an epidemic in Calcutta, India in 1963; stocks from the 7th mouse brain passage level were used. (b) Egyptian strain (E103), stocks from the 4th mouse brain passage level were used.

Working stocks of CHIK virus strains were prepared by inoculating 100 µl of a 10^{-2} dilution of original stock virus to monolayers

of C6/36 *Ae. albopictus* cell cultures in 25 cm^2 plastic flasks (Igarashi, 1979). The virus was adsorbed for 1 hr at room temperature on a rocking table. After washing the cells 3 times with maintenance medium (Liebovitz L-15 supplemented with 10% tryptose phosphate broth and 2% foetal bovine serum), 5 ml of fresh medium was added and cells were then incubated at 28 °C. Supernatant was harvested 3 days post infection (p.i.) and stored in 200 µl aliquots at -70 °C. Stock virus titers were $10^{7.4}$ PFU/ml for Calcutta strain and $10^{8.3}$ PFU/ml for Egyptian strain.

Infection of mosquitoes. Five day-old mosquitoes were infected with CHIK virus by intrathoracic inoculation using the method described by Rosen and Gubler (1974). Each mosquito received approx. 3 – 4 PFU in 0.2 µl inoculum. In a separate experiment, mosquitoes were infected orally by feeding through a sausage skin membrane on reconstituted blood and virus mixture (Leake, 1984). In enzyme assays and bioassays mosquitoes were used from 5 days p.i. (age 10 days) to 15 days p.i. (age 20 days). Mosquitoes stored at -70 °C on each day p.i. were subsequently assayed for virus by plaque assay. As controls for insecticide bioassays, mosquitoes were injected with an equivalent quantity of L-15 medium without CHIK virus (mock-infected controls). Similarly, another batch of mosquitoes was mock-inoculated i.e. simply received a prick of inoculation needle.

Virus plaque assay was performed using Vero cell cultures in 96-well microplates. Mosquitoes were triturated individually in 200 µl of L-15 medium, and supernatant fluid obtained after 10 mins of centrifugation at 10,000 rpm, or cell culture supernatants were diluted ten-fold and added to 96-well plates followed by Vero cell suspension and carboxymethylcellulose overlay. Plates were stained after 2 days of incubation at 37 °C to reveal plaques. Virus titers were calculated as log PFU per mosquito.

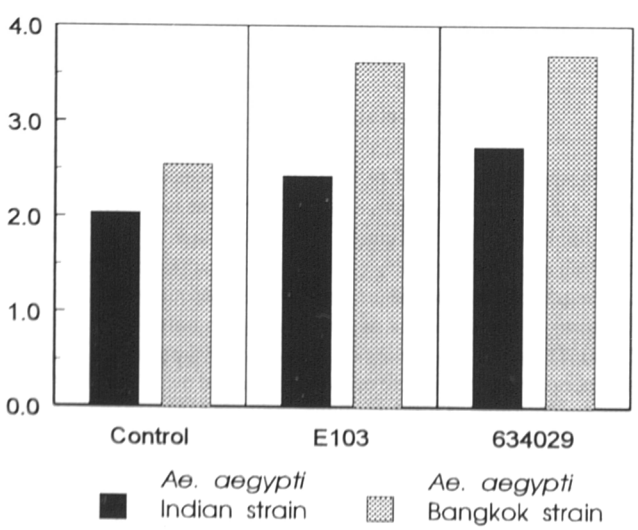

Fig. 1
Effect of Chikungunya virus infection by intrathoracic route on acetylcholinesterase activity in Indian and Bangkok strains of *Ae. aegypti* mosquitoes
Ordinate: enzyme activity per mg protein. E103: Egyptian strain of Chikungunya virus; 634029: Calcutta strain of Chikungunya virus. Control: non-infected mosquitoes.

Insecticide bioassays, enzyme assays and protein assays were done as described by Mourya *et al.* (1993).

Results

Intrathoracic inoculation of *Ae. aegypti* produced higher levels of CHIK virus titer (Table 1) and 100% infection rate in *Ae. aegypti* of both strains, compared to oral infection (Table 2). Virus titers were consistently high from 2 – 4 days p.i. onwards (Table 1). Insecticide bioassays and en-

Table 1. Virus titers in mosquitoes *Ae. aegypti* inoculated intrathoracally with Chikungunya virus

Days p.i.	Virus titer (log PFU/mosquito) *Ae. aegypti*			
	Indian strain		Bangkok strain	
	Virus strain			
	Calcutta	Egypt	Calcutta	Egypt
1	2.61	3.02	3.48	4.87
2	4.86	4.66	5.81	7.03
3	3.87	5.14	5.11	6.46
4	5.15	6.54	5.73	6.91
5	5.27	7.14	5.11	7.06
6	5.48	5.80	5.50	5.41
7	5.96	6.05	5.91	6.29
8	5.42	5.95	5.29	5.98
9	6.17	7.12	6.24	7.19
10	6.22	7.05	4.91	6.53

Virus titers are means from groups of 5 mosquitoes.

Table 2. Virus titers in mosquitoes *Ae. aegypti* infected orally with Chikungunya virus

Days p.i.	Virus titer (log PFU/mosquito) *Ae. aegypti*			
	Indian strain		Bangkok strain	
	Virus strain			
	Calcutta	Egypt	Calcutta	Egypt
3	nil (0/5)	3.11 (3/5)	nil (0/5)	nil (0/5)
6	3.74 (2/5)	5.33 (2/5)	nil (0/5)	6.24 (1/5)
9	nil (0/5)	3.45 (2/5)	nil (0/5)	nil (0/5)
12	nil (0/5)	5.16 (2/5)	3.25 (2/5)	4.54 (1/5)
15	3.72 (2/5)	5.24 (1/5)	3.84 (1/5)	5.64 (1/5)
18	5.89 (2/5)	6.64 (1/5)	6.24 (1/5)	5.94 (2/5)

Virus titers are means from groups of 5 mosquitoes. In parentheses the ratio of positive to total number of mosquitoes inoculated.

zyme assays were performed on mosquitoes that had been infected intrathoracally 5 days earlier. There was a slight increase in the levels of acetylcholinesterase (Fig. 1), glutathione-S-transferase and glucose-6-phosphate dehydrogenase (Fig. 2) in virus-infected mosquitoes. The increase in the levels of the general esterase activity with alpha- and beta-naphtylacetate (esterase-A, esterase-B, Fig. 3) was noticeable from 5 to 15 days p.i.

Associated with the increase in enzyme activity was a reduction in the susceptibility of the infected mosquitoes to the organophosphate Malathion as measured by bioassay (Table 3), but not the carbamate Propoxur or the pyrethroid Permethrin. Mosquitoes were exposed to accurate descriminating doses (10 – 20 mins) rather than 1 hr exposure, which gave 100% mortality in all the insect batches. Mosquitoes that had been subjected to mock-inoculation, also showed an equivalent reduction in Malathion sensitivity (Table 3).

Table 3. Effect of Chikungunya virus infection on the susceptibility of *Ae. aegypti* mosquitoes to Malathion, Propoxur and Permethrin

Insecticide	Mortality of mosquitoes					
	Mock-infected		Mock-inoculated		Virus-infected	
	Exposure time (mins)					
	10	20	10	20	10	20
	Bangkok strain					
Malathion	34% (17/50)	74% (37/50)	26% (13/50)	50% (25/50)	24% (12/50)	49% (24/44)
	Indian strain					
	26% (26/101)	62% (63/101)	29% (28/97)	53% (59/101)	24% (24/101)	45% (45/101)
	Bangkok strain					
Propoxur	54% (13/24)	72% (18/25)	56% (14/25)	80% (12/15)	60% (9/15)	71% (10/14)
Permethrin	84% (26/31)	100% (17/17)	80% (24/30)	100% (12/12)	80% (20/25)	100% (20/20)

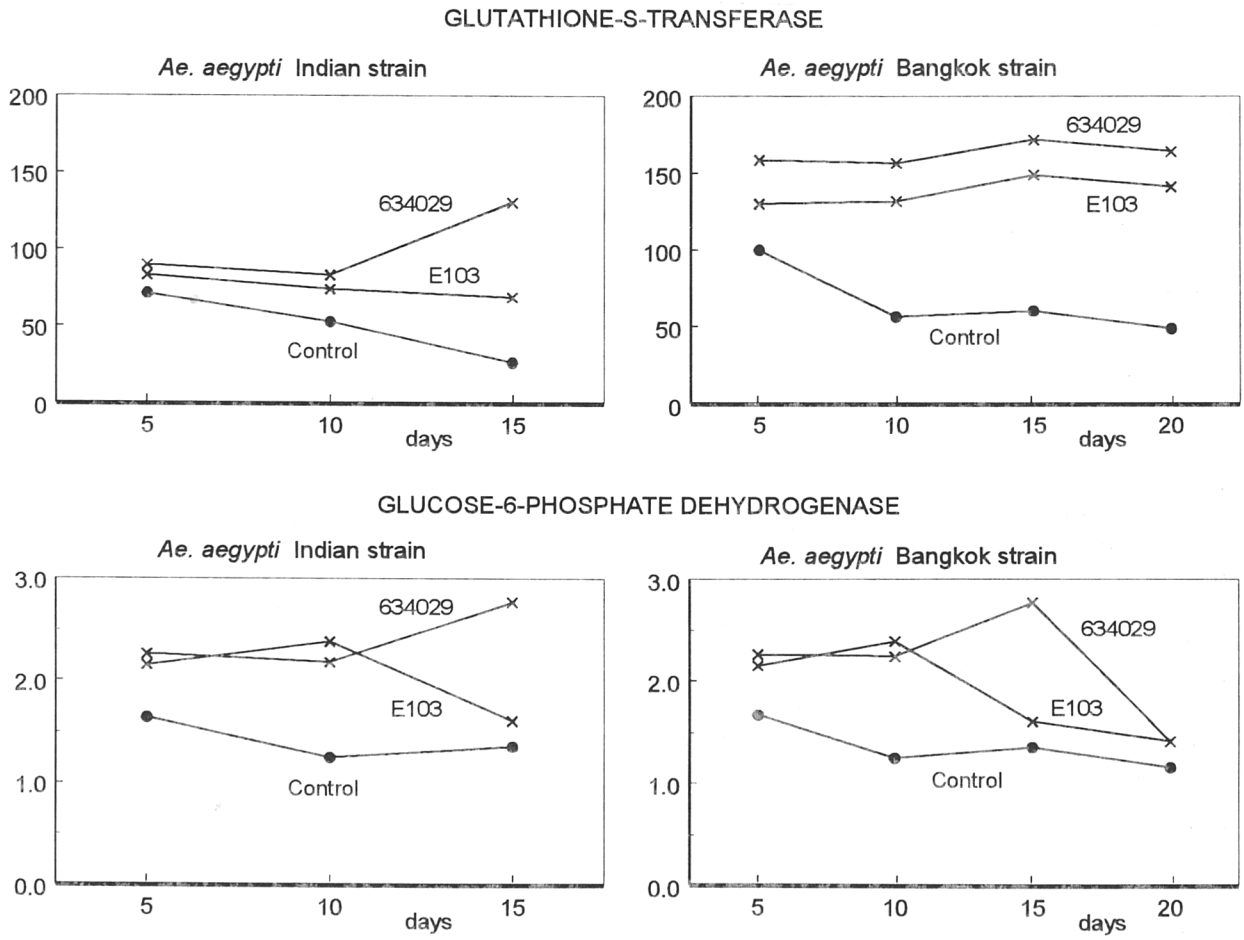

Fig. 2

Effect of Chikungunya virus infection by intrathoracic route on glutathione-S-transferase and glucose-6-phosphate dehydrogenase activities in Indian and Bangkok strains of *Ae. aegypti* mosquitoes

Abscissa: age of mosquitoes. For the rest of legend see Fig. 1.

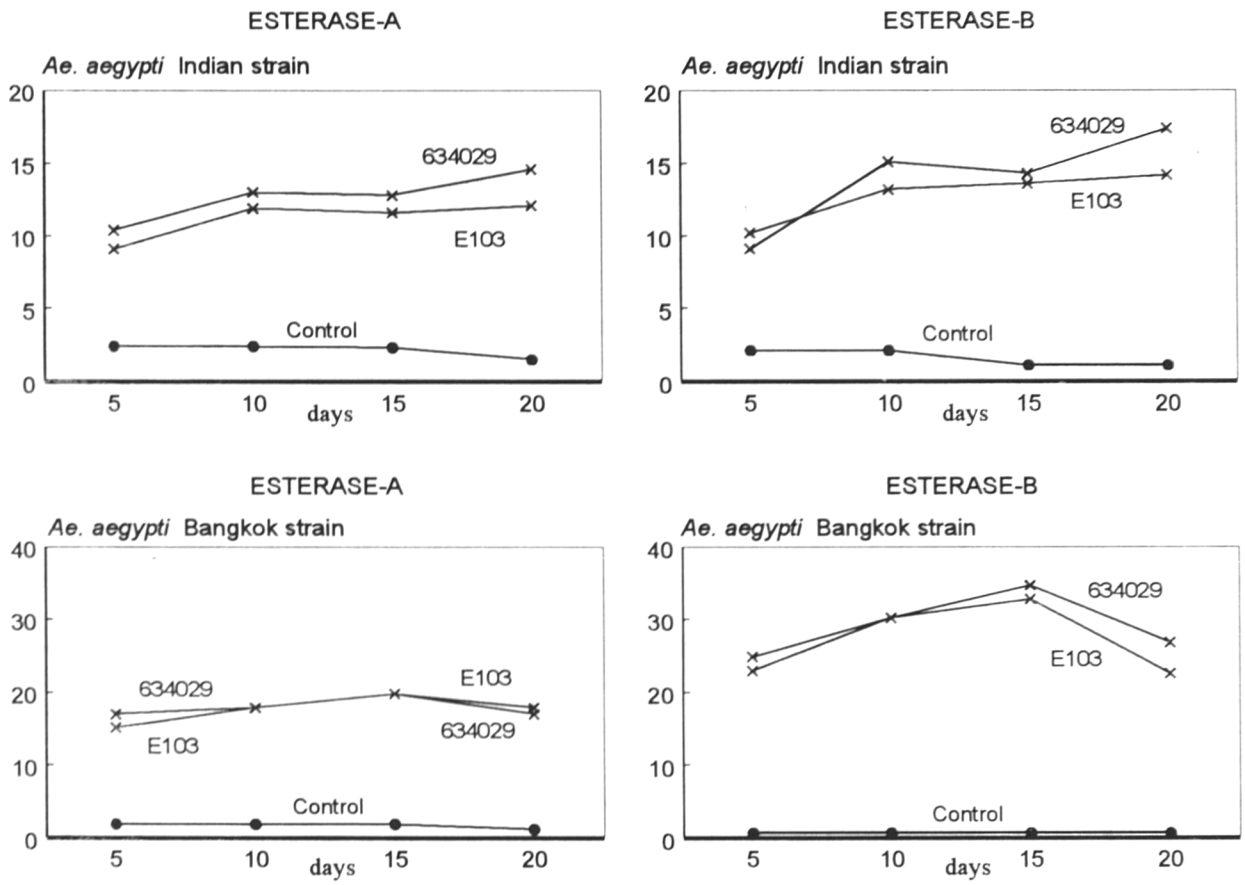

Fig. 3

Effect of Chikungunya virus infection by intrathoracic route on esterase-A and esterase-B activities in Indian and Bangkok strains of *Ae. aegypti* mosquitoes. For the legend see Fig. 2.

Discussion

Arboviruses from both the *Alphaviridae* (e.g. CHIK) and *Flaviviridae* families (e.g. Dengue-1, Rawlins *et al.*, 1988) have now been shown to have no significant effect on the susceptibility of *Ae. aegypti* mosquitoes to Malathion, as measured by laboratory bioassay.

However, mosquitoes injected with either CHIK virus or simply with L-15 medium showed reduced susceptibility and increased level of a number of enzymes. This prompted us to consider whether the response was traumatic or provoked by inoculation of foreign protein. Mosquitoes injected with CHIK virus, or mock-inoculated without the introduction of any material whatsoever, showed an equivalent degree of reduced susceptibility to Malathion. Increased enzyme levels therefore appear to be a defence response to injury. The expanding literature on insect responses to microorganisms (Lackie, 1988) recognizes 5 different classes of defensive peptides, namely lysosymes, cecropins, attacins, diptericins and defencins, but these have not yet been investigated in mosquitoes.

This is the first report on enzyme levels rising as a trauma-induced defensive response in mosquitoes. The greates rise was noted in general esterases, important enzymes for biochemical detoxication of foreign compounds such as insecticides. These enzymes initially hydrolyze foreign compounds, rendering them more polar for bio-degradation by other conjugating enzyme systems. The rise in level of general esterases is not sufficient to protect infected or mock-inoculated mosquitoes from diagnostic doses of organophosphates and carbamates. These findings are also to suggest that, if biochemical assays are performed on field collected *Ae. aegypti* mosquitoes and even if the test population consists of CHIK virus-infected mosquitoes, it will not show any false resistance, since the rise in the level of esterases in the infected mosquitoes is low, i.e. only 2- to 4-fold. It will be interesting to see whether the enzymes involved in such defense reaction are the same as those esterase enzymes which normally confer resistance to insecticides.

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References

Igarashi, A. (1979): Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and Chikungunya viruses. *J. Gen. Virol.* **40**, 531–544.

Lackie (1988): Immune mechanisms in insects. *Parasitol. Today* **4**, 98–105.

Leake, C.J. (1984): The vector competence of colonised *Aedes (Stegomyia) katherinensis* for dengue 2 virus. *Trans. R. Soc. Trop. Med. Hyg.* **78**, 829–832.

Micks, D. W., and Ferguson, M. J. (1961): Microorganisms associated with mosquitoes. III. Effect of reduction in the microbial flora of *Culex fatigans* Wiedeman on the susceptibility to *Plasmodium relictum* Grassi and Feletti. *J. Insect Pathol.* **3**, 244–248.

Mourya, D.T., and Soman, R.S. (1985): Effect of gregarine parasite, *Ascogregarina culicis* and tetracyclin on the susceptibility of *Culex bitaeniorhynchus* to JE virus. *Indian J. Med. Res.* **81**, 247–250.

Mourya, D.T., and Soman, R.S. (1987): Evaluation of the combined effect of certain insecticides and insect growth regulator with the gregarine parasites on *Aedes* mosquitoes. *Indian J. Parasitol.* **11**, 9–11.

Mourya, D.T., Hemingway, J., and Leake, C.J. (1993): Changes in enzyme titres with age in four geographical strains of *Aedes aegypti* and their association with insecticides resistance. *Med. Vet. Entomol.* **7**, 11–16.

Prasittisuk, C., and Curtis, C.F. (1982): Absence of effects of insecticides on susceptibility of anophelines to *Plasmodium yoelii nigeriensis*. *S.E. Asian J. Trop. Med. Pub. Hlth.* **13**, 127–132.

Rawlins, S.C., Eliason, D.A., Moore, C.G., and Campos, E.G. (1988): Effects of dengue-1 infection in *Aedes albopictus* on its susceptibility to malathion. *J. Am. Mosquito Contr. Assoc.* **4**, 372–373.

Rosen, L., and Gubler, D. (1974): The use of mosquitoes to detect and propagate dengue viruses. *Am. J. Trop. Med. Hyg.* **23**, 1153–1157.

Spencer, J.P., and Olson, J.K. (1982): Evaluation of the combined effects of methoprene and the protozoan parasite *Ascogregarina culicis* on *Aedes* mosquitoes. *Mosquito News* **42**, 384–390.