THE ROLE OF HEMAGGLUTININS IN THE MIDGUT EXTRACTS OF TWO LINES OF *AEDES AEGYPTI* IN THEIR SUSCEPTIBILITY TO DENGUE-2 VIRUS

P.V. BARDE[1], M.I. KHAN[2], M.D. GOKHALE[1], A.C. MISHRA[1], D.T. MOURYA[1*]

[1]National Institute of Virology, 20-A, Dr. Ambedkar Road, Pune, India; [2]Division of Biochemistry, National Chemical Laboratory, Pashan Road, Pune 411 008, India

Received February 25, 2003; accepted May 25, 2004

Summary. – Hemagglutinin activity (HA) was studied in the midgut extracts from highly (h) and lowly (l) susceptible strains of *Aedes aegypti* mosquitoes to Dengue-2 virus (DEN-2). HA in the midgut extracts from these two isofemale strains of mosquitoes was high in (l) as compared to (h) mosquitoes. HA was found to be higher with chicken red blood cells (RBCs) than with rabbit and human RBCs of O group. Larval midgut extracts showed higher activity than those from adult female mosquitoes. Exposure of midgut extracts to 100°C for 10 mins destroyed the activity. The activity was observed between pH 6 and pH 10. HA in midgut extracts was also studied using twenty different carbohydrates; five of them showed an inhibition of HA. The inhibitory carbohydrates, when incorporated into DEN-2-infected bloodmeal, showed a reduction in the susceptibility of mosquitoes to the virus as compared to the control ones fed on the virus alone. Similarly, when these carbohydrates were incorporated in the DEN-2-infected inoculum, the inoculated mosquitoes showed a reduction in the susceptibility to the virus. HA in the virus-infected midgut extracts was higher than that in the uninfected controls. These results suggest that the presence of HA in the midgut may be one of the factors that affect the susceptibility of *Ae. aegypti* mosquitoes to DEN-2.

Key words: Aedes aegypti; carbohydrates; dengue; Dengue-2 virus; hemagglutinin; mosquito

Introduction

Dengue (DEN) is a serious threat to the public health in tropical and subtropical countries. *Ae. aegypti* is the principal vector of this virus in urban and rural areas. Mosquito populations, in general, exhibit variation in their susceptibility to DEN viruses. Studies have shown that the susceptibility of mosquitoes to flaviviruses is genetically determined, polygenic and recessive (Miller and Mitchell, 1991). There are various intrinsic and extrinsic factors that influence the susceptibility of the mosquito to the virus (Hardy *et al.,* 1983). Among these the mesentronal barrier is considered to play a key role in determining the susceptibility of mosquitoes to viruses (Leake, 1992; Mourya *et al.,* 1994). It has been reported that the mechanism of refractoriness in this species of mosquitoes to filarial worm is due to six polypeptides, which are induced after sucking an infected bloodmeal (Wattam and Christensen, 1992). In our earlier study, Chikungunya virus putative receptors were found in the brush border membrane of midgut of both larval and adult mosquitoes and their presence in higher number has been considered an important factor of susceptibility to this virus (Mourya *et al.,* 1998).

*Corresponding author. The address for correspondence: Microbial Containment Complex, Sus Road, Pashan, Pune 411021, India. E-mail: mouryadt@vsnl.net; fax: +9120-25893640.

Abbreviations: DEN-2 = Dengue-2 virus; DEN (h) = highly susceptible to DEN-2; DEN (l) = lowly susceptible to DEN-2; HA = hemagglutination, hemagglutinin activity; HAI = hemagglutination inhibition; i.c. = intracerebral; i.t.= intrathoracic; PMSF = phenylmethylsulfonyl fluoride; PBS =phosphate-buffered saline; RBC = red blood cell; TBS = Tris-buffered saline

The studies by Nayar and Knight (1997) have shown that hemagglutinins also play an important role in determining the susceptibility of mosquitoes to the filarial worm *Brugia malayi*.

The present study was undertaken to determine (i) the association of HA with the susceptibility of *Ae. aegypti* mosquitoes to DEN viruses and (ii) the role of HA inhibitory carbohydrates in this susceptibility.

Materials and Methods

Mosquitoes. The selection procedure for establishing DEN (h) and DEN (l) isofemale strains of *Ae. aegypti* has been described earlier (Mourya *et al.,* 2001). All the mosquitoes were reared in an insectary maintained at 28 + 2°C and 70–80% RH.

Viruses. DEN-2 Jammu strain used in the present study was isolated from a DEN hemorrhagic fever patient from Jammu, India. A stock virus was prepared in infant mice by intracerebral (i.c.) route and used at the 8th mouse passage level.

Infection of mosquitoes through membrane. Dilution of the virus was made in defibrinated white leghorn chicken blood. Four- to five-day-old female mosquitoes were fed on a DEN-2-infected blood through Parafilm (American National Can Co., USA) as described by Harada *et al.* (1996). First a stock virus was prepared, which was distributed into feeding cups containing different carbohydrates to feed different mosquito strains simultaneously.

Infection of mosquitoes by (intrathoracic) i.t. route. Four to five-day-old female mosquitoes of either strain were inoculated i.t. with 126 ID_{50} (titrated in mice) in 0.2 µl, following the method described by Rosen and Gubler (1974). First a stock virus inoculum was prepared which was distributed into tubes containing different carbohydrates.

Mosquito midgut extracts. Adult or larval midguts of either strain were dissected in phosphate-buffered saline (PBS) with 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) on ice and stored at -20°C until used. Before use the guts were triturated in PBS with 1 mmol/l PMSF and 0.1% Triton X-100 the grinding buffer, using 1 µl of the diluent per midgut. For the HA test the homogenate was centrifuged at 6,000 r.p.m. for 10 mins and the supernatant was used. Proteins were assayed in the batches from either mosquito strain before testing and the protein concentration in the batches was made equal by dilution with PBS.

Preparation of RBCs. Blood from human volunteers, rabbits and chickens was collected in the Alsever's solution. The RBCs were pelleted and washed with 10 mmol/l Tris-buffered saline pH 7.2 (TBS) The 0.5% RBCs solution was made and used for determining HA.

HA titration. Serial twofold dilutions (from 1:2 to 1:512) of midgut extracts in 25 µl were prepared using the grinding buffer and round-bottom microwell plates (Nunc), as described by Nayar and Knight (1997). Twenty-five µl of washed RBC suspension was then added per well. The plates were incubated at room temperature (28 ± 2°C) for 1 hr in humid conditions. The HA titer was read by binocular microscope. Five replicates of each sample were titrated. The HA titer was expressed as the reciprocal of the lowest dilution still causing HA.

HAI titration, effect of carbohydrates. Twenty different carbohydrates (Sigma) were tested in this study. To 25 µl of extracts 25 µl of serial twofold dilutions of carbohydrates (up to 1:256) was added. The plates were incubated at what temperature for 1 hr and 50 µl of 0.5% RBCs per well was added. The plates were incubated at room temperature for 1 hr and HAI was recorded.

Effect of age. The larval or adult midguts were dissected at different intervals and stored at -70°C until used. The extracts were prepared just before the use as described above.

Effect of temperature. The midgut extract from an aliquot from either mosquito strain was distributed to six batches. These batches were then exposed to different temperatures and the HI titer was assayed.

Effect of pH on midgut extracts was studied by adding TBS of pH ranging from 2 to 10 to extracts in the ratio of 1:1. The samples were kept at room temperature for 30 mins before the HA titer was assayed.

Effect of carbohydrates on virus multiplication. The carbohydrates, which inhibited HA in midgut extracts, were used either alone or in combination.

A. *Effect on virus multiplication in i.t. infected mosquitoes.* Mosquitoes were inoculated i.t. with carbohydrates (0.2 mol/l) and the virus diluted in PBS containing 0.75% bovine albumine. Control mosquitoes were of various kinds: mosquitoes inoculated with virus alone, mock-inoculated mosquitoes, and uninoculated mosquitoes. Mosquitoes were incubated for 12 days and the antigen was detected in the head squashes using the IFA technique. Midguts of some positive and negative mosquitoes were pooled separately and assayed for the HA titer.

B. *Effect on virus multiplication in orally infected mosquitoes.* Batches of mosquitoes were infected orally as described above. A batch fed on blood-virus mixture was used as a control, while a test batch was fed on blood, virus and carbohydrates (0.2 mol/l). These mosquitoes were incubated for 12 days and the HA titer was assayed as above. Midguts of some of the positive mosquitoes were used for HA titer assay. Similarly, midguts of individual positive and negative mosquitoes were pooled, respectively, and used for HA titer assay.

Results

HA in midgut extracts

HA was found in midgut extracts from either strain of *Ae. aegypti* mosquitoes. When the HA titer was assayed with chicken RBCs, it was 4-fold higher as compared to rabbit and 8-fold higher as compared to human "O" RBCs. Therefore in further studies chicken RBCs were used. It was interesting to note that the activity was always 2–4 fold higher in the case of midgut extracts from the DEN (l) strain as compared to those from the DEN (h) strain mosquitoes. Similarly, the larval midgut also showed higher activity as compared to the adult midgut. The freshly emerged female mosquitoes had lower activity than the larvae. After emergence there was a diclining trend of the HA titer (Table 1). There was minimal difference in HA titers between fresh midgut

Table 1. Effect of age and strain of mosquitoes on HA

Mosquito stage	HA titer	
	Den (l)	Den (h)
IV instar larva	64	16
0-day-old female	32	8
2-day-old female	16	4
4-day-old female	8	4
6-day-old female	8	8
8-day-old female	8	4
10-day-old female	16	4
14-day-old female	8	2

For the abbreviations see their list on the front page.

Table 2. Effect of temperature on HA in larval midgut extracts

Temperature (°C)	Storage time	HA titer	
		DEN (l)	DEN (h)
-20	Till use	256	64
4	30 mins	128	64
Room temperature	Till use	64	16
37	30 mins	128	32–64
60	10 mins	32–64	16–32
60	30 mins	4–8	2–4
100	10 mins	–	–

Table 3. Effect of pH on HA in larval midgut extracts

pH	HA titer	
	DEN (l)	DEN (h)
2	0	0
4	0–2	0
6	32	8
7	128	8–32
8	128	32
10	32–64	16

For the abbreviation see their list on the front page.

Table 4. Inhibition of HA of midgut extracts of *Aedes aegypti* mosquitoes by various carbohydrates

No.	Carbohydrate (mol/l)	Inhibition of HA[a]					
		1[b]	2	3	4	5	6
1	Glucose (0.5)	–	+	+	+	+	+
2	Galactose (0.2)	+	+	+	+	+	+
3	Gla NAC (0.2)	–	–	±	+	+	+
4	Glu NAC (0.1)	+	+	+	+	+	+
5	Mannose (0.2)	+	+	+	+	+	+
6	a-methyl mannose (0.5)	+	+	+	+	+	+
7	b-methyl glucose (0.5)	+	+	+	+	+	+
8	Mannosamine hydrochloride (0.1)	+	+	+	+	+	+
9	Lactose (0.1)	+	+	+	+	+	+
10	Ribose (0.5)	+	+	+	+	+	+
11	Rahamannose (0.5)	+	+	+	+	+	+
12	Rathose (0.1)	+	+	+	+	+	+
13	Aribinose (0.1)	+	+	+	+	+	+
14	Xylose (0.5)	–	–	±	+	+	+
15	Mannose-9-glycoprotein (10 mg/ml)	–	–	–	–	–	–
16	Fucose (0.2)	–	–	±	+	+	+
17	Fibrinogen (10 mg/ml)	+	+	+	+	+	+
18	Sialic acid (10 mg /ml)	–	–	±	+	+	+
19	Glucosamine (0.1)	–	–	–	–	–	–
20	Maltose (0.1)	+	+	+	+	+	+

[a]Degree of inhibition as compared to positive controls: (–) = a higher than twofold reduction of the HA titer; (±) = twofold reduction of the HA titer; (+) = no reduction of the HA titer.
[b]1–6 show further twofold dilutions of the carbohydrates.

Table 5. Effect of carbohydrates on the virus multiplication in mosquitoes infected i.t. and orally

	Infected i.t.[a]	Infected orally[b]
Virus control	41/50 (82%)[c]	16/36 (44%)
Virus + Gal NAC	39/60 (65%)	14/36 (36%)
Virus + fucose	36/57 (63%)	11/36 (30%)
Virus + xylose	36/72 (50%)	13/36 (35%)

[a]The virus dose of $10^{1.2}$ MID_{50}/0.2 µl.
[b]The post feeding titer of $10^{4.1}$ LD_{50}/0.02ml in infant mice infected by i.c. route.
[c]The number of positive head squashes/the number of head squashes examined (percentage) on day 12 post inoculation.

extracts and extracts stored at -20°C and at 4°C for 60 mins. When the extracts were exposed to 60°C for 30 mins there was a sharp decline in the HA titer. However, after exposure to 100°C for 10 mins the extracts lost their HA titer completely (Table 2). pH 4 or lower values had a similar effect. The HA titer was optimal at pH 7–8, but an increase from 9 to 10 resulted in a decrease (Table 3). The HAI test showed that five carbohydrates inhibited HA in midgut extracts of mosquitoeas (Table 4). Some carbohydrates, namely N-acetyl-galactosamine (Gal NAC), xylose, fucose, sialic acid and glucosamine showed a twofold decrease in the HA titer.

Effect of HA and HA inhibiting carbohydrates on virus replication

Of the five carbohydrates, which showed various degree of inhibition of HA, three, namely xylose, fucose and Gal NAC, when inoculated i.t. or orally fed along with the virus in mosquitoes, reduced the positivity of head squashes as compared to controls. This difference was not appreciable in the case of orally infected mosquitoes. However, when the mosquitoes were infected by i.t. route, these carbohydrates showed noticeable differences (Table 5). A difference in the HA was noticed in the two strains of

mosquitoes. However, each strain comprised of different proportions of susceptible and refractory mosquitoes. Hence, to determine HA in the midgut extracts of DEN-2-positive mosquitoes, four batches of mosquitoes were compared; one batch inoculated with DEN-2 and three control batches. It was also observed that the midgut extracts of DEN-2-positive mosquitoes after 14 days of virus inoculation showed a fourfold increase of the HA titer as compared to the diluent-inoculated, mock inoculated and uninoculated batches of mosquitoes.

Discussion

Hemagglutinins are well demonstrated in mosquitoes and various other insects. Even mosquito cell lines are also known to show HA (Ghosh and Bhat, 1971). HA is present in newly emerged females, which show a declining trend of this activity as they grow up. A reduction of the HA titer in the extracts heated to higher temperatures suggests that HA is thermolabile. These results are in accord with those obtained earlier by other workers (Nayar and Knight, 1997; Mohamed and Ingram, 1994; Ingram and Molyneux, 1993). Similarly, the optimum pH range for the HA in midgut extracts is between 6.0 and 10.0 in accord with the data on other insects (Ingram and Molyneux, 1993). However, our results suggest that the HA in midgut extracts was optimal at pH 8.0. The decrease in HA at low pH was expected since the pH in the midgut is alkaline. The higher HA in larvae than in adults is interesting. We feel that it may be due to higher protein content in larvae than in adults.

Complete or partial inhibition of HA by various carbohydrates with sheep and human RBCs has been demonstrated (Nayar and Knight, 1997; Nayar and Sehgal, 1993). When midgut extracts were studied in the present work for the HAI activity with chicken RBCs, the HA was inhibited by fucose, xylose, glucosamine, sialic acid and N-acetyl glucosamine.

It has been suggested that haemagglutinins may act as anti-bacterial lectins (James and Rossignol, 1991), or that perhaps they may allow a freer movement of filarial worms in the midgut and may facilitate their penetration from the midgut into the hemocoel (Nayar and Knight, 1995a). Differences in the HA in the salivary glands of susceptible and refractory strains of mosquitoes have been reported by Nayar and Knight (1997). The above studies suggest that carbohydrate inhibitors of various specificities are present in the hemolymph or body fluid of different species of *Diptera*. Similarly, it has also been reported that an injury response as injection of saline or carbohydrates into mosquitoes enhances the parasite encapsulation process (Nayar and Knight, 1995b). Ogura (1986) has shown that injection of a mixture of sugars into *Armigeres subalbatus* reduces the injury response of the parasite due to inhibition of lectin binding which results in reduction of the injury response of microfilariae.

It has been suggested that the HA in the hemolymph may be involved in controlling the activation of defense mechanisms, such as activation of the PpO, which causes encapsulation and melanization (Ogura, 1986; Nayar and Knight, 1996, 1997). These studies have shown that endogenous HA present in the hemocoel react with the carbohydrate moieties present in the parasite soon after they are ingested by the mosquito from the infected host, reach the hemocoel and form a capsule around microfilarial sheaths. The mosquito then recognizes these encapsulated microfilarial sheaths as non-selves, which then activate the PpO cascade.

Our results show that when a HA inhibiting carbohydrate was inoculated or was fed orally with the virus, there was a reduction in the infectivity rate. In the case of inoculation the reduction amounted to 15–30%, while in the case of oral feeding it was less marked. The lower difference observed in the case of orally fed mosquitoes could be due to the low amount of sugars used as compared to the size of blood meal for mosquito or due to various interacting factors inside the midgut (e.g. the action of several enzymes). It is very difficult to understand the exact mechanism of action of these sugars in the midgut, but it is possible that these sugars might have competed with virus binding receptors, which are also glycosylated. It is also possible that the virus and sugar could form a complex of a low affinity for receptors.

Initial steps of entry of virus into the cell are adsorption and penetration. The envelope protein of flaviviruses is responsible for binding to receptors; it contains two N-linked glycosylation sites. It is suggested that mannose residues located on virion glycoproteins contribute to binding and penetration of the virus into cell. The treatment of viruses or cells with the lectin concanavalin A causes inhibitory effect on infection process due to binding of lectin to mannose residues in viral protein. Similarly, D-(+)-mannose or α-methyl-D-mannoside has been shown to cause a similar inhibitory effect (Hung *et al*., 1999).

The higher HA in midguts of confirmed positive mosquitoes could be due to (i) other defensive mechanisms like increase in certain enzymes like esterases (Mourya *et al*., 1995), (ii) presence of large amount of viral antigen or (iii) a combined effect.

Detailed studies on individual purified lectins are necessary for understanding the exact relationship of HA to infectivity. Our studies on the role of hemagglutinins and their carbohydrate inhibitors in the susceptibility of *Ae. aegypti* to DEN-2 indicate differences in the effects of individual carbohydrates. Recently, it has also been shown that the DEN-2 entry through adsorption and penetration

leading to infection is accomplished within 2 hrs and that carbohydrate residues may contribute to this stage of virus infection of mosquito cells (Hung *et al*., 1999). These *in vitro* studies have shown that about 20 mins is adequate time for binding of the virus to the receptors. The data presented in our study indicate a possible virus-carbohydrate interaction, which may affect the dynamics of virus infection at the midgut level.

Acknowledgements. Our sincere thanks are due to Prof. J.K. Nayar, Florida Medical Entomological Laboratory, University of Florida, USA for his critical comments and suggestions in preparing this manuscript. Technical help rendered by Mr. C.B. Lande during this study is also gratefully acknowledged.

References

Ghosh SN, Bhat UKM (1971): Haemagglutinating activity in normal culture fluid of *Aedes albopictus* cell lines. *Curr. Sci.* **40,** 354–355.

Harada M, Matsuoka H, Suguri SA (1996): Convenient mosquito membrane feeding method. *Med. Entomol. Zool.* **47**, 103–105.

Hardy JL, Honk EJ, Kramer LD, Reeves WC (1983): Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Ann. Res. Entomol*. **28**, 229–262.

Hung S-L, Lee P-L, Chen H-W, Chen L-K, Kao C-L, King C-C (1999): Analysis of the steps involved in dengue virus entry into host cells. *Virology* **257**, 156–167.

Ingram GA, Molyneux DH (1993): Comparative study of haemagglutination activity in the hemolymph of three tsetse fly *Glossina* species. *Comp. Biochem. Physiol*. **106**, 563–573.

James AA, Rossignol PA (1991): Mosquito salivary glands: Parasitological and molecular aspects. *Parasitology Today* **7,** 267–271.

Leake CJ (1992): Arbovirus-mosquito interaction and virus specificity. *Parasitology Today* **8**, 123–128.

Miller BR, Mitchell CJ (1991): Genetic selection of a flavivirus-refractory strain of the yellow fever mosquito *Aedes aegypti*. *Am. J. Trop. Med. Hyg*. **45**, 399–407.

Mohamed HA, Ingram GA (1994): Effects of physicochemical treatments on haemaggluination activity of *Anopheles gambiae* hemolymph and midgut extract. *Med. Vet. Entomol*. **8,** 8–14.

Mourya DT, Gokhale MD, Basu A, Barde PV, Sapkal GN, Padbidri VS, Gore MM (2001): Horizontal and vertical transmission of dengue-2 virus in highly and lowly susceptible strains of *Aedes aegypti* mosquitoes. *Acta Virol.* **45**, 67–71.

Mourya DT, Gokhale MD, Malunjkar AS, Bhat HR, Banerjee K (1994): Inheritance of oral susceptibility of *Aedes aegypti* to Chikungunya virus. *Am. J. Trop. Med. Hyg*. **51**, 295–300.

Mourya DT, Hemingway J, Leake CJ (1995): Post-inoculation changes in enzyme activity of *Aedes aegypti* infected with Chikungunya virus. *Acta Virol.* **39**, 31–35.

Mourya DT, Ranadive SN, Gokhale MD, Barde PV, Padbidri VS, Banerjee K (1998): Putative chikungunya virus-specific receptors on the midgut brush border membrane of *Aedes aegypti* mosquito. *Indian J. Med. Res*. **107**, 10–14.

Nayar JK, Knight JW (1995a): Comparison of migration and encapsulation of *Brugia malayi* microfilariae from the midgut to the haemocoel between *Anopheles quadrimaculatus* and *Aedes aegypti*. *J. Invertebr. Pathol.* **65**, 295–299.

Nayar JK, Knight JW (1996): Cytochemical localization of lectin-binding to intracellularly melanized first stage larvae of *Brugia malayi* (Buckley) (Nematoda: Filarioidea) in *Anopheles quadrimaculatus* (Say) (Diptera: Culicidae). *Int. J. Insect Morphol. Embryol*. **25**, 331–339.

Nayar JK, Knight JW (1997): Haemagglutinins in *Anopheles quadrimaculatus* strains susceptible and refractory to *Brugia malayi*, and their role in the immune response to filarial parasites. *Comp. Biochem. Physiol*. **116**, 109–117.

Nayar JK, Knight JW (1995b): Wounding increases intracellular encapsulation (melanization) of developing *Brugia malayi* (Nematoda: Filarioidea) larvae in thoracic muscles of *Anopheles quadrimaculatus*. *Comp. Biochem. Physiol*. **112**, 553–557.

Nayar JK, Sehgal SS (1993): Haemagglutinating activity in tissue extracts of strains of *Anopheles quadrimaculatus* and *Aedes aegypti* susceptible and refractory to *Brugia malayi*. In Borovsky D, Spielrnan A (Eds): *Third Symposium Proceedings of Host Regulated Developmental Mechanisms in Vector Arthropods. Florida Medical Entomology Laboratory*. University of Florida, Vero Beach, USA, pp. 277–287.

Ogura N (1986): Haemagglutinating activity and melanin deposition on microfilariae of *Brugia pahangi* and B. malayi in the mosquito, *Armigeres subalbatus*. *Japan J. Parasitol*. **35**, 542–549.

Rosen L, Gubler DJ (1974): The use of mosquitoes to detect and propagate dengue, viruses. *Am. J. Trop. Med. Hyg*. **23**, 1153–1160.

Wattam AR, Christensen BM (1992): Induced polypeptides associated with filarial worm refractoriness in *Aedes aegypti*. *Proc Natl. Acad. Sci. USA* **80**, 6502–6505.