INTERACTION OF LDL AND PLATELETS IN ISCHAEMIC AND ISCHAEMIC RISK SUBJECTS

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ABSTRACT

Platelets play a vital role in the progression of atherosclerosis and thrombosis, a major cause of death worldwide. Platelets are activated by many triggers like elevated LDL in blood resulting in aggregation and formation of plaque. The purpose of this study was to investigate the effect of LDL and signal transduction inhibitor on the activation of platelets in Ischaemic risk subjects. Platelets from IHD and hyperlipidemic subjects were hypersensitive to ADP, as higher levels of platelet aggregation were observed in these groups. LDL from IHD hyperlipidemic subjects was more effective in activating platelets from any other group. Ox-LDL was more effective in activating platelets than native-LDL as monitored by level of platelet aggregation induced by PAF and thrombin. Calcium channel blocker, nifedipine and verapamil inhibited platelet aggregation at micromolar level. Protein kinase inhibitor, staurosporine was effective in inhibiting ADP induced aggregation at nanomolar level.

KEYWORDS

LDL, ox-LDL, Calcium, Platelet, Platelet aggregation

INTRODUCTION

Platelets are known to be involved in atherogenesis and its complications, and plasma lipoproteins have been shown to affect platelet activity, both *in vitro* and *in vivo*. Low-density lipoproteins (LDL) have been reported to possess platelet activating activities such as the enhancement of platelet sensitivity to stimuli (1-4) and the induction of platelet aggregation (5-6). Oxidative modification dramatically changes the biological properties of LDL, converting them into a form that is both atherogenic and pro-thrombotic (7-8).

A few reports have described the synergistic enhancement of platelet responses by LDL particles. LDL enhanced fibrinogen binding in response to, aggregation (4), thromboxane A₂ formation and serotonin secretion by thrombin (2,9); and platelet responsiveness to epinephrine and Ca²⁺ -ionophore (1). Acting as an independent agonist, very low concentrations of LDL (10 mg/L) induced a rise in cytosolic Ca²⁺ and inositol phosphate turnover (10-11), physiological concentrations induced changes in shape (0.25 g/L to 0.5 g/L) and aggregation (>0.75 g/L) (6), and at concentrations >3 g/L, LDL triggered phosphorylation of pleckstrin, the 47-kDa substrate of protein kinase C (PKC) (5).

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Two complimentary mechanisms responsible for platelet hyperreactivity in hypercholesteromic subjects have been proposed (5,12). One involves alterations in the lipid composition of platelet membranes, and characteristic increases in cholesterol and arachidonic acid content (12). The second mechanism involves LDL recognition by specific platelet receptors that may regulate platelet activation and aggregation induced by these particles (5). Using ligand binding assays it has been shown that platelet LDL receptors present biochemical, genetic, and immunological characteristics that are different from those of classical LDL receptors of nucleated cells and glycoprotein IV or CD36 is related to the binding process (13). However, many aspects of the interaction between LDL and their platelet receptors still not well understood.

In the present investigation, we have studied the effect of various agonist on the activation of platelets from IHD risk subjects. We have also demonstrated that platelets are activated to different extent depending on their origin and there is both change in lipid composition and involvement of Ca²⁺ dependent activation.

MATERIALS AND METHOD

Selection of Subjects

The study was conducted in four groups of human subjects as outlined below: Group I: Healthy Control subjects, Group II: Hyperlipidemic control subjects (Atherogenic index more than 5.0, TG more than 160 mg/dl) Group III: IHD cases with normal lipid status.

Group IV: IHD cases with hyperlipidemic status. Age and sex matched control subjects were selected either from the relatives of the patients or from hospital staff with their consent. These cases were thoroughly screened with regards to IHD, diabetes mellitus, hypertension or for any other significant illness. IHD and IHD hyperlipidemic subjects were selected from indoor and outdoor patients attending G.B. Pant Hospital. In these two groups patients were also screened for any associated diseases like diabetes mellitus, liver disorders and other chronic diseases or any drug medication affecting the study and such patients were excluded.

Collection of Blood Sample and Isolation of Plasma

All subjects were instructed to observe an overnight fast for 12 hr prior to the venipuncture. The sample was collected after making the subject sit in sitting position quietly for 5 min to avoid rapid transient decrease in triglycerides due to stress. The sample was transferred to a glass tube after removing the needle from the syringe to prevent hemolysis. About 10 ml of blood was collected aseptically in heparinized syringe for LDL studies. Blood samples were centrifuged (Remi R-8 Lab Centrifuge) at 3000 rpm (1000g) for 5 minutes to separate plasma as supernatant for further analysis.

Measurement of Lipid Profile

Total plasma cholesterol, triglycerides and high-density lipoprotein (HDL)-cholesterol were analyzed using Olympus Auto-Analyzer analytical system and represented as mg/dl. Total HDL-cholesterol was isolated from plasma according to Burnstein *et al.* (14). LDL-cholesterol was calculated according to Friedwald's formula [LDL-cholesterol = Total Cholesterol - HDL-Cholesterol - TG/5].

Isolation of LDL

LDL was isolated from plasma by density gradient ultracentrifugation (15); all the solutions contain 1 mM EDTA to prevent oxidation. The ultracentrifugation was carried out at 250,000g (SW-28 rotor, Beckman L7-80 ultracentrifuge) for 2 hr at 10°C. Fractions were collected from the top of the tube by a syringe. Total content of tubes were fractionated into eight fractions. Each fraction was analyzed for total cholesterol, proteins, triglycerides and HDL/LDL-cholesterol. The amount of protein content in LDL samples was estimated using Bradford's reagent. Purity of LDL containing fractions was assessed by SDS-polyacrylamide gel electrophoresis. Pooled LDL fractions were dialysed overnight at 4°C in PBS against two changes. LDL content was concentrated by dialysing against PEG 8000 and used for further analysis.

Oxidation of LDL

In vitro oxidation of LDL was carried out by incubating it in the presence of 2.5 μ M or 7.5 μ M CuSO₄ at 37°C for 0-24 hr. The oxidation was stopped by the addition of 1mM EDTA and freezing the samples.

Isolation of platelets

Platelet rich plasma (PRP) was prepared by centrifuging the citrated blood at 250g at room temperature for 10 min. The required amount of PRP was removed from top. Then the sample was further centrifuged at 1000g at room temperature for 10 min for the platelets to sediment. Then the plasma was again removed from the top, which was platelet poor plasma (PPP).

Platelets were isolated by centrifugation of PRP at 1,000g for 10 min and pellet was immediately mixed 1:1 with a 0.1 M citrate buffer pH 6.0. Resuspended mixture was centrifuged again at 1000g for 10 min. The pellet was washed once with Tyrode's buffer (136 mM NaCl, 2.7 mM KCl, 3.3 mM NaH₂PO₄, 1 mM MgCl₂, 5 mM dextrose, pH 7.35), without disrupting the pellet to remove citrate, plasma proteins and Ca²⁺. The platelets were resuspended in Tyrode's buffer to give 2X10⁸ platelets/ml. Platelets were counted using Neubauer counting chamber. Platelet lipids, cholesterol and triglycerides were monitored as described previously for plasma.

Platelet Aggregation

Effect of various agonists and antagonists on the activation of platelets was studied by monitoring the extent of platelet aggregation caused by these agents. Platelet aggregation was studied employing aggregometer (Chronolog Corp., USA). For aggregation studies we used 500 μ l (10⁸ platelets) sample and to stir continuously magnetic stir bar added to cuvette. After the cuvettes were placed in the covered heating block (at 37°C), the stirrer was started, recorder pen lowered and the chart drive was switched ON. The results were expressed as percent light transmission (16).

We studied the effect of various agonists, ADP, thrombin, PAF, calcium ionophore (A23187), LDL and antagonists like, nifedipine, verapamil and staurosporine on platelet aggregation from different subject groups.

RESULTS AND DISCUSSION

Platelets circulate as non-nucleated discs and consists of a trilaminar phospholipoprotein membrane, three types of granules and an irregular internal network of canaliculi, through which the granule contents can be released on to the platelet surface.

When activated, platelets immediately change shape,

Table 1. Platelet aggregation (%) in subjects of different groups. Platelets were incubated with LDL for 30 min. and then 8 μ M ADP added to obtain maximum aggregation. Results are represented as mean $\pm$ SD.

Subjects n=50	Platelet aggregation (%)
Normal	37.80 $\pm$ 6.20
Hyperlipidemic	51.15 $\pm$ 2.68
IHD	57.83 $\pm$ 7.11
IHD Hyperlipidemic	69.30 $\pm$ 12.42
The difference between Normal vs IHD and Hyperlipidemic vs IHD hyperlipidemic is significant P<0.001.	

Table 2. Effect of platelet activating factor (PAF) on platelet aggregation from normolipidemic control subjects (n=4). Washed platelets were incubated with different concentrations of PAF and then platelet aggregation measured.

PAF (nM)	Platelet aggregation (%)
0	20.00
1	21.25
5	35.00
10	43.75

Table 3. Effect of different concentration of calcium ionophore (A23187) on ADP induced platelet aggregation. Washed platelets were incubated with sub-optimal concentration of ADP (4 μ M) and then stimulated by different concentrations of ionophore A 23187.

ADP (4 μ M) + Ionophore (μ M)	Platelet aggregation (%)
0	5
12.50	10
27.50	20
35.00	50
43.75	53.75

losing their discoid shape, and form tiny spheres with numerous projecting pseudopods. After adhesion of a single layer of platelets to the damaged vascular endothelium platelets stick to one another and form aggregates. Certain substances react with specific platelet membrane surface receptors and initiate platelet aggregation and further activation. These

include exposed collagen fibers, ADP, adrenalin, serotonin, thrombin, and certain arachidonic acid metabolites, including thromboxane A_2 .

The turbidometric method for quantifying *in vitro* platelet aggregation is the most commonly used method (17). Low concentrations of thrombin, collagen and ADP result in the formation discocochinocytes. The aggregation profiles obtained from an aggregometer show an initial decrease in percent light transmission (%T) followed by an increase in transmission as the platelet aggregate. The initial decrease has been correlated with increases in the proportion of echinocytes in the platelet suspension.

In the present investigation, we have employed this effective tool, platelet aggregation, as a measure of platelet activity in response to various agonists and antagonists in normolipidemic control, established IHD patient and high risk group subjects (hyperlipidemic).

ADP Stimulated Platelet Aggregation

Platelet aggregation was monitored in response to different concentrations of ADP to find out effective concentration. ADP at 1-2 μ M resulted in 6.25% aggregation only, thereafter with increase in ADP concentration there was a corresponding increase in the platelet aggregation. We observed 10 μ M as saturable concentration of ADP. There was no significant difference between platelet aggregation in response to 8 μ M and 10 μ M ADP (Fig. 1). For monitoring, the extent of platelet activity in different groups, we used 8 μ M ADP and for interaction studies we used 4 mM ADP, as it was sub-optimal concentration.

Our results from different groups (normolipidemic control, hyperlipidemic, IHD and IHD hyperlipidemic) show that platelets from patients (IHD) and high risk groups (hyperlipidemic) are more sensitive than platelets from normolipidemic control subjects in response to 8 μ M ADP (Table 1). There was no difference between the aggregation pattern in males and female subjects of these groups (data not shown). Our results demonstrated that platelets from IHD and high risk groups were more sensitive when tested *in vitro* for aggregation. These observations are in contrast to those reported earlier (18), they did not observed any significant difference in platelet aggregation between IHD patient and normolipidemic control subject, though majority of workers have observed differences in platelet aggregation (19-21). Platelets from patients with familial hypercholesterolemia (FH), a condition in which there is early rapid generation of atherosclerosis have been found to be hypersensitive to aggregation and total nucleotide release (22).

Signaling Components During Platelet Aggregation

It is known that Ca^{2+} act as second messenger in most of signal transduction pathways in animals. In order to study the signal transduction mechanism during the activation of platelets with ADP and LDL we studied effect of pharmacological inhibitors and other agonists and antagonists on platelet aggregation and Ca^{2+} . Platelet activating factor (PAF) has been recognized as a potent mediator of several types of inflammation and shock. PAF is produced by and released from a wide variety of cells, and it stimulates different targets cells in many organs (23). Recent studies have demonstrated that PAF antagonists provide significant protective effects against the damage induced by ischemia that is followed by reperfusion in brain, kidney, and gastrointestinal tract, and it has been suggested that PAF plays an important role in such damage (24). In addition, PAF antagonists have been shown to reduce the incidence of ischemia-induced arrhythmias (25-26).

The purpose of the present study was to study sensitivity of platelets from different groups. We used various concentrations of PAF to find out the effective concentration (Table 2). It was observed that with increasing the concentration from 1-10 nM there was linear increase in platelet aggregation. With 10 nM PAF we obtained 43.75% platelet aggregation, which was maximum in normolipidemic control subjects. With PAF we observed more aggregation of platelets than with ADP alone. With 5 nM PAF 35% aggregation was observed. About 50% aggregation of maximum was obtained in 1 nM PAF. Similar observations have been made earlier (27-29). Beaumont *et al.* (29) reported 63% aggregation with 69 nM PAF and 60.2% with 17 nM PAF. The difference in extent of aggregation could be because these workers have used PRP whereas we have used washed platelets. Some of the factors in serum might enhance the action of PAF and also they used higher concentration of PAF, in our hands 10nM was saturating level.

We further investigated the effect of calcium ionophore (A23187), calcium channel blockers verapamil and nifedipine, and protein kinase inhibitor-staurosporine on platelet aggregation induced by ADP in order to establish the presence of various components of signaling mechanism. Addition of ionophore resulted in the increase in aggregation over the aggregation obtained with sub-optimal concentration of ADP. With 50 μM ionophore, we obtained much higher level of platelet aggregation compared to aggregation obtained after saturable level of ADP (Table 3). The increase in aggregation was in proportion to the concentration of ionophore used indicating that increase in Ca^{2+} results in increased aggregation. Observations made by Han *et al* (30), also suggests the role of Ca^{2+} and induction of aggregation by ionophore.

Table 4. Platelet aggregation in response to increasing concentrations of nifedipine. Washed platelets were incubated with nifedipine and then stimulated by adding ADP.

Nifedipine (μM) + ADP (10 μM)	Platelet aggregation (%)
0	38.0
50	30.0
100	27.5
200	20.0
500	13.75

Table 5. Platelet aggregation in response to increasing concentrations of verapamil. Washed platelets were incubated with verapamil and then stimulated by adding ADP.

Verapamil (μM) + ADP (10 μM)	Platelet aggregation (%)
0	38.0
100	35.0
200	25.25
500	6.25

Table 6. Effect of different concentration of Staurosporine on ADP induced platelet aggregation. Washed platelets were incubated with staurosporine and then stimulated by adding ADP.

Staurosporine (nM) + ADP (10 μM)	Platelet aggregation (%)
0	38.00
5	35.00
10	21.25
20	6.25

Figure 1. Platelet aggregation in response to increasing concentrations of ADP.

Figure 2. Effect of native and oxidized LDL (100 µg/ml) on platelet aggregation. Aggregation was stimulated by the addition of 1U/ml thrombin to the preparation. Washed platelets were incubated with N-LDL and Ox-LDL for 30 min prior to the addition of thrombin at 37°C.

Figure 3. Effect of LDL (100 µg/ml) from different groups on PAF induced platelet aggregation. LDL was isolated from different subject groups and incubated with platelets from control subjects. Platelet aggregation was induced by adding PAF to achieve maximum aggregation (47.50% in this representative experiment).

It was found that calcium-channel blocker, verapamil and nifedipine inhibited, ADP induced platelet aggregation, *in vitro* indicating that Ca^{2+} level changes during platelet aggregation. We observed that minimum concentration of nifedipine that inhibited aggregation by threshold concentration of ADP was 50-200 µM (Table 4). The minimum concentration of verapamil that inhibited aggregation varied from 100-300 µM (Table 5). Our results indicate that higher concentration of verapamil are required compared to nifedipine to inhibit ADP induced platelet aggregation. Han *et al.* (30) observed that in contrast to nifedipine, verapamil failed to inhibit arachidonic acid induced aggregation at concentration as high as 550 µM, but for epinephrine induced aggregation inhibition by verapamil could be observed at very low concentration compared to nifedipine (31-32). Low concentrations of verapamil (25-500 µM) have been shown to block the transport of extracellular calcium ions into platelets in response to epinephrine. Subsequent intracellular calcium mobilization in response to epinephrine is also inhibited when calcium entry is blocked by verapamil (33). Dandona *et al.* (34) have shown that verapamil inhibit platelet shape change in the absence of extracellular calcium. This would suggest that verapamil has an inhibitory effect on intracellular calcium mobilization.

We also studied the effect of staurosporine, protein kinase C inhibitor on platelet aggregations. It is evident from the data that protein kinase C plays a role in ADP induced platelet aggregation (Table 6). It is possible that the mobilization of intracellular ionized calcium contributes to the activation of PKC which requires calcium. Oda *et al.* (35) have shown that protein tyrosine phosphorylation is involved in the shear stress-induced platelet aggregation, which could be inhibited by staurosporine. Dandona *et al.* (34) have shown that H-9, specific inhibitor of protein kinase C, and staurosporine inhibited platelet shape change, which represents the initial phase of platelet activation. Our results support these observations and show that calcium, calmodulin and protein kinase C are involved in ADP dependent platelet aggregation.

LDL and Platelet Interaction

Platelets and lipoproteins are intimately involved in the pathogenesis of a wide variety of disease including atherosclerosis, thrombosis, and coronary heart disease. Evidence accumulated over the years suggests the possibility of a direct relationship between plasma lipoproteins and the hemostatic function of platelets. A number of studies demonstrated that native LDL enhanced the platelet sensitivity to stimulation and induced platelet activation (3,5,6,22,36-38). The native LDL contains large amounts of unsaturated fatty acids, which are oxidized easily in the presence of oxygen (39). In various

biological functions oxidized LDL behaved differently from native LDL (40-41). Oxidized LDL was believed to be an important contributor to endothelial cytodamage and atherogenesis. It has been observed that, when platelets from normolipidemic subjects were incubated with 1 U/ml thrombin, it resulted in the platelet aggregation to the tune of 40%. On the other hand, when we incubated these platelets with 100 µg/ml N-LDL along with 1.7 mM CaCl₂ for 30 min and then added thrombin, it enhanced platelet aggregation to 52.5% (Fig. 2). Further, when platelets were incubated with 100 µg/ml Ox-LDL, it was observed that thrombin induced aggregation was increased upto 72.5%. These results suggest that LDL renders sensitivity for thrombin induced aggregation in the platelets, further, Ox-LDL is more effective in making platelets sensitive to thrombin.

We also determined platelet response after incubating platelets with different concentration of LDL, and then inducing aggregation by PAF. Maximum level of aggregation was obtained with 10 nM PAF. Data indicate that as the concentration of LDL increased, and at 750 µg/ml concentration, even 1 nM PAF could bring about maximum aggregation (data not shown). This clearly indicate that serum lipid concentration influence the sensitivity of platelets to various agonists. When we incubated platelets with N-LDL (100 µg/ml) we could obtain the maximum aggregatory response with 5 nM PAF, and increasing the concentration beyond 10 nM PAF did not increased it further. Whereas, if the platelets were incubated with Ox-LDL (100 µg/ml) the effective concentration of PAF required to get maximum platelet aggregation was 3 nM and with 5 nM PAF, we did not observed further any change in aggregation level.

Further, the effect of LDL on platelet activation from different subject groups was studied. Here the experiment was designed to investigate the role of LDL in further enhancement of aggregation from different subject groups, i.e. LDL was isolated from all four groups and incubated along with the platelets isolated from normolipidemic control subjects. It was observed that in normolipidemic control subjects, 10 nM PAF was required to obtain maximum response in the platelet, in the absence of LDL. But when, platelets were incubated with 100 µg/ml LDL, prior to activation with PAF, concentration of PAF required to bring about maximum aggregation decreased to 5 nM. When platelets were incubated with LDL from hyperlipidemic subjects we could obtain maximum platelet response with 4 nM PAF and where LDL from IHD and IHD hyperlipidemic was used, the level of PAF required was 3 and 1 nM, respectively to obtain maximum response (Fig. 3). This could also be due to higher basal level of oxidized LDL in IHD and hyperlipidemic subjects, it corroborates with our earlier results from

these groups (39). More work need to be done to know the exact mechanisms of signal transduction induced by different agonists. It would be worth studying the level of activation in different subject groups to monitor and understand the etiopathology of cardiac diseases and atherosclerosis *per se*.

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serotypes. Infection with one dengue serotype provides lifelong immunity to that virus, but there is no cross protective immunity to the other serotypes. Thus persons living in a dengue-endemic area can get infected with all four serotypes during their lifetime.

The structure of many flaviviruses (including DEN virus) has been determined by using a combination of cryo-electron microscopy and fitting the known structure of glycoprotein-E into electron density map to a resolution of ~11Å. The virus core within a lipid bilayer has a less ordered structure than the external icosahedral scaffold of 90 glycoprotein-E dimers. The three E monomers per icosahedral asymmetric unit do not have quasi-equivalent symmetric environment. In addition, structures of immature dengue and yellow fever virus particles were determined to 16Å and 25Å resolution, respectively (Fuller *et al.*, 1995). The closely similar structures show 60 icosahedrally-organized, trimeric spikes on the particle surface. Each spike consists of three prM:E heterodimers, where E is an envelope glycoprotein and prM is the precursor to the membrane protein M. The pre-peptide components of the prM proteins in each spike cover the fusion peptides at the distal ends of the E glycoproteins (Wang *et al.*, 1999). Each heterodimer is associated with an E and a prM transmembrane density. These transmembrane densities represent either an EE or prM-prM antiparallel coil, due to which each protein spans the membrane twice. The carboxy terminus of each protein remains on the exterior of the viral membrane, consistent with the predicted membrane-spanning domains of the unprocessed polyprotein (Hung *et al.*, 1999). The structure suggests that flaviviruses employ a fusion mechanism in which the distal beta barrels of domain II of the glycoprotein-E are inserted into the cellular membrane.

TRANSMISSION AND INFECTION WITH DENGUE VIRUS

The primitive enzootic transmission cycle of dengue virus involves canopy-dwelling *Aedes* species mosquitoes and lower primates in the rain forests of Asia and Africa (Gubler *et al.*, 1988). A number of *Aedes* (*Stegomyia*) mosquito species may act as vector in these situations, including *Ae. aegypti*, *Ae. albopictus*, *Ae. polynesiensis*, and some members of *Ae. scutellaris* group. From a public health standpoint, the urban endemic/epidemic cycle is the most important transmission cycle. The viruses are maintained in an *Ae. aegypti*-human-*Ae. aegypti* cycle, with periodic epidemic occurring at 3 to 5 year intervals. Humans are infected with dengue virus by the bite of an infective *Aedes* mosquito (Gubler *et al.*, 1988) that prefers to lay its eggs in water filled household. Adult *Ae. aegypti* mosquitoes are unobtrusive, prefer to rest indoors and feed on humans during daylight hours. The female mosquitoes

often disrupt the feeding process at slightest movement and return to the same or a different person to continue feeding moments later. The *Ae. aegypti* females may thus feed on several persons during a single blood meal and transmit dengue virus to multiple persons within a short period of time (Gubler, 1979, 1981, 1984). This behavior makes *Ae. aegypti* an efficient epidemic vector.

Dengue fever (DF) is an acute febrile illness with chills, headache, retro-ocular pain, body ache and arthralgia in more than 90% of cases accompanied by nausea or vomiting and a maculopapular rash resembling measles that last 2-7 days in about 60% of cases. This is followed by sharp pain in the muscles and joints with sensations as if bones are breaking, that gave the name 'break bone fever' to DF. Another name, 'saddle back fever' has also been used because of regular temperature fluctuations in the patient. The symptoms last for about a week and death is uncommon. However, the co-infection with one of the other dengue viruses results in dengue hemorrhage fever (DHF), resulting in appearances of rashes on the face and extremities, and severe vomiting and shock ensues that may lead to death of the patient. WHO reported DF/DHF to be the second (after malaria) most important tropical infectious disease, with about 2.5 billion people living in high risk areas for dengue transmission. Each year about 50 to 100 million cases of DF recur that lead to 500,000 hospitalizations and 25,000 deaths (WHO, 1999).

The spread of viral infection depends on the transfer of the viral genome and accessory proteins from an infected cell to uninfected cells that requires a sequence of discrete events. Depending on the virus, these include virus assembly in the infected cell, release of infective virions from the infected cell into the extracellular space, attachment of the virus to receptor structures on the host cell surface, their internalization by endocytosis, penetration through a host cell membrane, uncoating of the viral genome, and its intra-cytoplasmic relocation to nucleus and/or other sites within the cell (Reviewed by Marsh and Helenius; 1989).

DENGUE VIRUS STRAIN VARIATION

Little is presently known about the role of virus-specified factors in the transmission and pathogenesis of dengue. Considerable microevolution of dengue virus strains found in studies employing monoclonal antibody analysis (Vazeille *et al.*, 1999), RNA finger printing (Lorenz *et al.*, 1984) or sequencing of selected regions of the genome. As variants with similar genetic structure have been found within a specific geographic location, the studies elucidating the movements of dengue viruses and source of epidemics suggest that genetic variation in virus strains may determine

virulence and explain the changing patterns of disease. All the four endemic dengue viruses have evolved independently from sylvatic progenitors by adapting to peridomestic-mosquito vectors and human reservoir hosts. The rise of urban civilizations associated with peridomestication of *Ae. aegypti* and *Ae. albopictus* mosquitoes provided the opportunity for adaptation and have resulted in the emergence of dengue in urban areas of tropics. This hypothesis predicts that endemic dengue virus strains are more efficient at infecting urban mosquitoes such as *Ae. aegypti* and *Ae. albopictus* with sylvatic versus urban strains. This hypothesis was tested by retrospectively examining evidence for adaptation of epidemic and endemic versus sylvatic strains of DEN-2 to *Ae. albopictus* and *Ae. aegypti*. Second-generation offsprings of mosquitoes from different geographic regions in America and Southeast Asia were tested for their susceptibility to epidemic/endemic and sylvatic DEN-2 isolates from West Africa, Southeast Asia and Oceania. Both *Aedes* species were found to be highly susceptible (upto 100% infected) to endemic/epidemic DEN-2 strains after ingesting artificial blood meals but significantly less susceptible (as low as 0%) to sylvatic DEN-2 strains. These results support the hypothesis that adaptation to peridomestic mosquito vectors mediates dengue emergence from sylvatic progenitor viruses. The four independent evolutionary dengue virus emergence events (DEN-1 to DEN-4) suggest that adaptation of dengue virus to new vectors and hosts occurred repeatedly from 300 to 1,500 years ago in Asia or Oceania and resulted in emergence and changing patterns of the disease (Holmes *et al.*, 2003).

CELLULAR RECEPTORS FOR VIRUS

In order to cause infection, a virus must be able to bind to cell surface. The binding occurs through interaction between the surface proteins of virion and specific viral receptors on target cell membranes. Enveloped and non-enveloped viruses have multiple identical proteins on their surface and possess the inherent capacity to bind to receptors through multivalent interactions, providing them considerable versatility in terms of binding capabilities.

Virus binding has been primarily studied in tissue culture systems. A combination of factors including temperature, ionic strength, pH, composition of the medium and presence of serum play major role in initial binding of the virus to the cell (Holmberg-Holm, 1981). Techniques such as affinity isolation with whole virus or viral components, antireceptor antibodies or anti-idiotypic antibodies to the viral binding sites, chemical cross-linking, DNA transfection, and somatic cell hybrids have been used in attempts to identify specific binding components (reviewed by Sommerfelt and Marsh, 1988). However, few specific virus receptors have been unambiguously identified and still

fewer have been demonstrated to play a functional role in virus entry *in vivo*. Viruses have developed a variety of strategies to recognize and bind to host cells. They can use proteins, lipids, or oligosaccharides as receptors, however, many of these receptors are the proteins involved in ligand binding, endocytosis, and cell recognition. Based on the nature, the viruses can be classified into following groups.

A. High Specificity Binding/ Narrow Host Range:

Viruses such as human immunodeficiency virus (HIV-1 and HIV-2) and Epstein-Barr virus (EBV) bind to components expressed only on a limited number of cell types. In case of HIV, the virions bind to CD4 molecules expressed on helper/inducer T lymphocytes, cells of monocyte-macrophage lineage, and certain cells transfected with the CD4 gene (Dalglish *et al.*, 1986). Although CD4 binds to envelope glycoprotein of HIV-1, a second receptor is necessary for entry and infection. The T-cell tropic strains of HIV-1 use the coreceptor CXCR4, while the macrophage tropic strains use CCR5. Both are receptors for chemokines, and their normal ligands can block HIV infection of the cell.

B. High Specificity Binding/ Broad Host Range:

Orthomyxoviruses and paramyxoviruses bind with considerable specificity to sialic acid, often in distinct linkage configurations. The binding site for sialic acid in influenza virus HA is highly conserved depression in the HA1 subunit at the tip of the spike protein (Wilson *et al.*, 1991). Sialic acid is often the terminal saccharide residue on cell surface glycoproteins and glycolipids and the complex carbohydrates such as heparan sulfate proteoglycan may be involved in herpes simplex virus infection (Wu and Spear, 1995) and dengue viral infection (Wei *et al.*, 2003).

C. Broad Host Range: A number of viruses, such as the alphavirus, SFV, rhabdo virus and vesicular stomatitis virus (VSV) exhibit a broad host range in cell culture but do not appear to utilize carbohydrates as the binding component. SFV probably utilizes several cell surface proteins as receptor and can bind to MHC class1 antigens on human and murine lymphoblastoid cells (Helenius *et al.*, 1995). However, it can also infect cells that do not express MHC molecules (Oldstone *et al.*, 1980).

D. Different Viruses Using the Same Binding Sites:

Orthomyxoviruses and paramyxoviruses, which bind to sialic acid residues use same cellular receptors. Competition experiments have shown that other viruses may share these receptors also. Eighteen different retroviruses that infect cultured human cells, utilize eight distinct receptors for their binding. Though it remains unclear whether the viruses that share common receptors utilize the same epitopes or multiple epitopes are present for different viruses