

Evaluation of the Plasma Kinin System in Dengue Hemorrhagic Fever

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INTRODUCTION: Dengue virus infections represent an important and growing public health problem in the tropics and subtropics. The pathogenesis of the severe and life-threatening manifestations found in Asia, dengue hemorrhagic fever (DHF) with and without shock, is incompletely understood. Clinical and postmortem observations suggest that pharmacologically active mediators may play a role in causing the hypovolemia and shock that occur in severe DHF. The complement system is involved and anaphylatoxins may mediate the rapid changes in vascular permeability which lead to vascular collapse.

There is circumstantial evidence that the plasma kinin system may also participate in DHF. Increased vascular permeability with hypoproteinemia and circulatory collapse are central pathophysiological events in DHF. Plasma kinins are potent endogenous mediators of increased vascular permeability and the infusion of bradykinin into man produces systemic arteriolar dilatation, decreased peripheral vascular resistance, and drop in arterial blood pressure. Bradykinin activity is elevated in early stages of endotoxin shock in monkeys. Finally the complement and fibrinolytic systems are activated in DHF, and these 2 systems are functionally interrelated with the plasma kinin system.

The development of a sensitive radioimmunoassay for bradykinin and enzymatic assays for the bradykinin activator system, which includes prekallikrein (C1 esterase) inhibitor has recently helped clarify the dynamics of the plasma kinin system by permitting simultaneous and accurate measurements of its major components. We report here sequential measurements of complement (C3) and 4 kinin system components—factor XII (Hageman), bradykinin, prekallikrein and kallikrein inhibitor—in 18 patients hospitalized with DHF, 11 of whom developed shock.

METHODS: Dengue Hemorrhagic Fever Patients: All patients were admitted to the hemorrhagic fever ward of Bangkok Children's Hospital during July 1972. Seven patients were diagnosed as having DHF without shock and 11 patients as having DHF with shock (DSS) by criteria previously reported. Shock occurred on the day of admission (7 patients), hospital day 2 (2 patients), day 3 (1 patient) and day 5 (1 patient).

Fever Control Patients: Eight hospital in-patients having acute febrile illnesses not associated with dengue infection comprised the fever control (FC) group. Seven had been admitted with a presumptive diagnosis of DHF which was not tenable on the basis of their subsequent clinical course and diagnostic tests. The eighth patient developed a fever and a rash during treatment of tuberculous meningitis.

Therapy: Dengue and FC patients received intravenous glucose—lactate—Ringer's solution for dehydration or shock, chloral hydrate for agitation, acetaminophen for hyperpyrexia, and Vitamin C. Plasma was given to 4 DSS patients and blood transfusions to one FC patient.

Non-Fever Control Subjects: Eighteen afebrile Thai children were bled once for factor XII, bradykinin, prekallikrein, and kallikrein inhibitor assays; 2 children were healthy and 16 were convalescing from surgery or acute or chronic medical conditions.

Experimental Design. Venous blood specimens from all 26 febrile patients studied were obtained on hospital days 1 (admission) 2, 4, and 6 if not previously discharged. Convalescent samples were obtained from

14 of these patients on follow-up visits 13 to 20 days after onset of their illness. All patients were bled 2 to 6 times. At least one blood specimen was drawn from most DSS patients during, or within 1-2 hours of vascular collapse; one patient was bled one day before and after the day of shock (Fig. 2).

The following laboratory tests were performed on most venous blood specimens obtained from febrile patients. Hematocrit (expressed as percent of discharge Hct), leukocyte count (per mm^3) and platelet count ($\times 10^3$ per mm^3) were done by standard methods. Serum C3 concentrations (mg/100 ml) were determined by radial immunodiffusion using agar plates commercially obtained (Hyland Laboratory, Los Angeles, Ca.); samples from individual patients were tested simultaneously and in duplicate. Plasma bradykinin concentrations (nanograms/ml) were determined by radioimmunoassay. The lower range of sensitivity of the radioimmunoassay ranged between .31-.67 ng/ml; measurements in this range are expressed as .67 ng/ml in subsequent analysis. The activities of plasma prekallikrein (micromoles/ml/hr of tosyl arginine methyl ester hydrolysed) and kallikrein inhibitor (inhibitor units) were determined by enzymatic assay. Plasma factor XII activity (percent of Boston standard) was determined by a modification of the activated partial thromboplastin time using congenitally deficient plasma.

The blood obtained for bradykinin, prekallikrein (PK), kallikrein inhibitor (KI) and factor XII assays was processed within 10 minutes of being drawn using plastic equipment. Blood for PK, KI and factor XII assays was added to chilled tubes containing 3.8% sodium citrate and the plasma separated and frozen at -20°C . Blood for bradykinin assay was added to tubes containing sodium EDTA and hexadimethrine, the plasma separated, precipitated with trichloroacetic acid, and frozen. After coding, the plasma was shipped in dry ice to Boston where assays were performed on freshly thawed plasma between 2 and 5 months after the blood was collected.

Serological tests: Admission and convalescent sera were obtained from the 26 febrile patients and tested simultaneously by the hemagglutination-inhibition test in microtiter plates against 8 units of dengue serotypes 1-4 and chikungunya virus antigens. Patients were considered to have had recent dengue infections if 4-fold or greater rises in antibody titers against dengue antigens were found in paired sera, or if the anti-dengue titers were elevated and fixed ($\geq 1:640$). We did not attempt to isolate virus from these patients, but dengue 2 virus was recovered from children in Bangkok during the 1972 DHF epidemic season.

RESULTS: All DHF and DSS patients had serologic evidence of recent secondary dengue infections; no FC patients had evidence of recent dengue or chikungunya virus infections.

Laboratory values for the 4 groups of patients are listed in Table 1. In order to illustrate differences found between clinical groups, the admission and lowest or highest values, selected from multiple bleedings, are shown as the mean \pm S.D. The NFC values are compared with the FC admission values, and FC with DHF and DSS values; probability levels are shown (Two-tailed Student's t-test). A comparison of the FC and NFC subjects revealed significantly lower prekallikrein activity in FC patients. In contrast to fever controls, dengue patients (especially the DSS group) had greater hemoconcentration, significantly lower platelet counts, and lower C3, factor XII, and prekallikrein levels on admission and/or during the acute illness. Bradykinin and kallikrein inhibitor values were similar in all clinical groups.

The temporal profiles of platelet, C3, and kinin components in DSS patients are illustrated in Figures 1a-1f. The admission values of FC patients were selected for analysis because they are generally the lowest of repeated measurements in this group. Fig. 1a illustrates the normal C3 concentration before shock, the low levels on the day of shock, and the progressively higher concentrations during convalescence; a similar pattern exists for platelet counts (Fig. 1b). Prekallikrein (Fig. 1c) and factor XII (Fig. 1d) activities are significantly depressed before the shock day, and higher afterwards. Kallikrein inhibitor activity (Fig. 1e) shows a tendency toward low values before shock, normal levels during and for 3 days after shock, and low levels again during late convalescence; the values are widely scattered, however, and differences are not statistically significant. Bradykinin concentrations are not significantly elevated at any time in DSS patients (Fig. 1f).

Changes in laboratory values are illustrated in more detail by a patient (Fig. 2) who developed shock 3 days after admission, and therefore provided more than 1 pre-shock blood sample. The pre-shock period is characterized by a rising hematocrit and by a falling platelet count and C3 concentration. By contrast, factor XII and prekallikrein activity was low but rising when shock occurred, and normal bradykinin concentration did not change. Kallikrein inhibitor activity fluctuated widely. Similar changes were observed in another patient who developed shock 5 days after admission.

DISCUSSION: This study was designed to determine whether the plasma kinin system is activated in dengue hemorrhagic fever. In order to confirm the plausibility of bradykinin participation in DHF, we attempted to show a temporal relationship between clinical shock and elevated levels of plasma bradykinin, or of kinin system component activation. The plasma kinin system is thought to be activated sequentially as shown in Figure 3. Evidence of full activation of the kinin system should include the simultaneous demonstration of factor XII, prekallikrein, and kallikrein inhibitor depletion, and formation of bradykinin.

We found significant depression of only 2 of the 4 kinin system components—prekallikrein and factor XII. Depressed enzymatic activity of these 2 serum proteins could result from at least 3 different mechanisms: sequestration of the protein extravascularly, depressed synthesis, or an increased rate of activation and consumption. ¹²⁵I-labelled albumin (mol. wt. = 49,000) crosses altered blood vessels during the shock phase of DHF, and is sequestered extravascularly. Depressed serum concentrations of transferrin (mol. wt. = 90,000) are also found during the shock phase. It seems likely that at least part of the consistently depressed activity of prekallikrein (mol. wt. = 127,000) and Hageman factor (mol. wt. = 110,000) is due to their extravasation preceding shock. A point against extravasation, however, is that kallikrein inhibitor (MW = 50,000) was not similarly depressed before shock.

Decreased synthesis of prekallikrein and factor XII by a damaged liver may be another cause for the depressed activity of these proteins. Abnormal liver function and liver necrosis unrelated to shock have been described in DHF. Prekallikrein levels, in particular, are a very sensitive index of liver dysfunction. Kallikrein inhibitor, although synthesized by the liver, is not very sensitive to hepatic dysfunction and remains normal in severe liver disease.

The third possibility, increased consumption of these two proteins as a consequence of their activation, is judged unlikely in view of the normal levels of kallikrein inhibitor (KI) and bradykinin found. The depletion of KI as it forms an inactive stoichiometric complex with kallikrein is a uniform finding in plasma prekallikrein activation. In DHF patients studied, there was no consistent depletion of KI at any stage of illness, suggesting that conversion of prekallikrein to kallikrein did not occur. The observed variations of KI activity during DHF could be ascribed to KI interacting with plasma enzymes other than kallikrein, such as C1 esterase, factors XI and XII, plasmin and thrombin. A second argument against consumption of prekallikrein and factor XII are the normal bradykinin concentrations found during DHF. This implies that prebradykinin was not converted to bradykinin by kallikrein, and thus that prekallikrein was not converted to kallikrein. Admittedly, if bradykinin was generated, it may have been efficiently and rapidly removed by controlling kinase systems after its formation; the half-life of bradykinin *in vivo* is less than 30 seconds. Alternatively, bradykinin may have been activated but operative peripherally and not detected in venous blood.

Although the foregoing evidence against prekallikrein and factor XII activation in DHF is indirect, it does suggest that if consumption does occur, pathways other than those presently considered important for bradykinin activation are operative. For example, the complement and fibrinolytic systems are activated in DHF, and factor XII may participate in this process. The low values of prekallikrein in fever control patients implies that mechanisms associated with febrile disease *per se* are in part responsible for the low levels in DHF; prekallikrein metabolic turnover studies should help clarify these mechanisms.

Clinical shock appeared to be more closely related temporally to changing levels of C3 and platelet counts than to levels of prekallikrein and factor XII. Two patients had lower prekallikrein and factor XII values on admission than during shock, which occurred 3 and 5 days later. If the assumption holds that the

observed low levels of two of the kinin components are due to consumption, and that maximum bradykinin activation is required for the development of shock, then the time course of activation seems poorly correlated temporally to the onset of vascular collapse in these two patients.

By contrast C3 concentrations were normal on admission in these two patients, but began to decline several days before shock and reached lowest levels 1-2 days afterwards. Platelet counts tended to parallel the changing C3 concentration. Complement (C3) activation may result in generation of the vasoactive complement peptides C3a and C5a followed by rapid removal of activated C3 from the circulation. The sequence of increased vascular permeability resulting in hypoproteinemia, hypovolemia, and vascular collapse in DSS may be mediated totally or in part by vasoactive complement peptides generated prior to shock.

CONCLUSIONS: In order to explore the role of the plasma kinin system in the pathogenesis of dengue hemorrhagic fever (DHF), bradykinin, prekallikrein, kallikrein, kallikrein inhibitor, factor XII (Hageman), and serum complement (C3) were measured simultaneously during the acute and convalescent stages of illness in 7 children with DHF without shock, in 11 with dengue shock syndrome (DSS), and in 8 patients with acute febrile illnesses other than dengue (FC). Prekallikrein, factor XII and C3 levels were significantly lower in both dengue patient groups compared to FC patients, with the lowest mean levels found in DSS. However, bradykinin concentrations were not elevated and mean kallikrein inhibitor activity levels were not depressed in dengue patients. Two DSS patients studied at least 2 days before onset of shock had falling C3 levels which were more closely related temporally to the onset of shock than were their rising levels of prekallikrein. The results fail to provide convincing evidence for full activation of the plasma kinin system leading to free bradykinin or a significant role for bradykinin in the immunopathogenesis of DHF; results do re-focus attention on complement as a potentially important pharmacological mediator of dengue shock syndrome.

Table 1. Comparison of Laboratory Values Obtained for Patients with Dengue Hemorrhagic Fever, Dengue Shock Syndrome and for Fever and Non-Fever Controls

Measurement ^x	Non-Fever Control (18 pts)	Fever Control (8 pts)	Dengue Hemorrhagic Fever (7 pts)	Dengue Shock Syndrome (11 pts)
Peripheral Leukocyte count: Admission	—	5900±2,700	6800±5900	8,800±5,600
Hemo — concen- tration:	Admission	98±14	123±34	127±30(a)
	Maximum	105±3	125±33	137±21(b)
Platelet count:	Admission	233±80	99±73(b)	96±169(a)
	Lowest	190±89	71±78(a)	31±11(b)
C3:	Admission	108±31	70±13(b)	86±47
	Lowest	102±28	66±13(b)	61±21(b)
Factor XII:	Admission	84±36	117±43	59±43(a)
	Lowest	—	94±36	46±30(b)
Prekalli — krein:	Admission	91±16	74±15(c)	41±18(b)
	Lowest	—	72±15	37±16(b)
Kallikrein Inhibitor:	Admission	0.99±0.22	1.00±0.25	0.85±0.37
	Lowest	—	0.75±0.24	0.64±0.30
Bradykinin:	Admission	1.52±1.42	2.39±3.55	1.06±0.70
	Highest	—	2.71±3.50	1.92±1.67

^x all values = mean ±S.D.; units of measurement defined in text; (a) P<.05 compared to FC; (b) P<.01 compared to FC; (c) P<.05 compared to NFC; other differences not significant (P>.05).

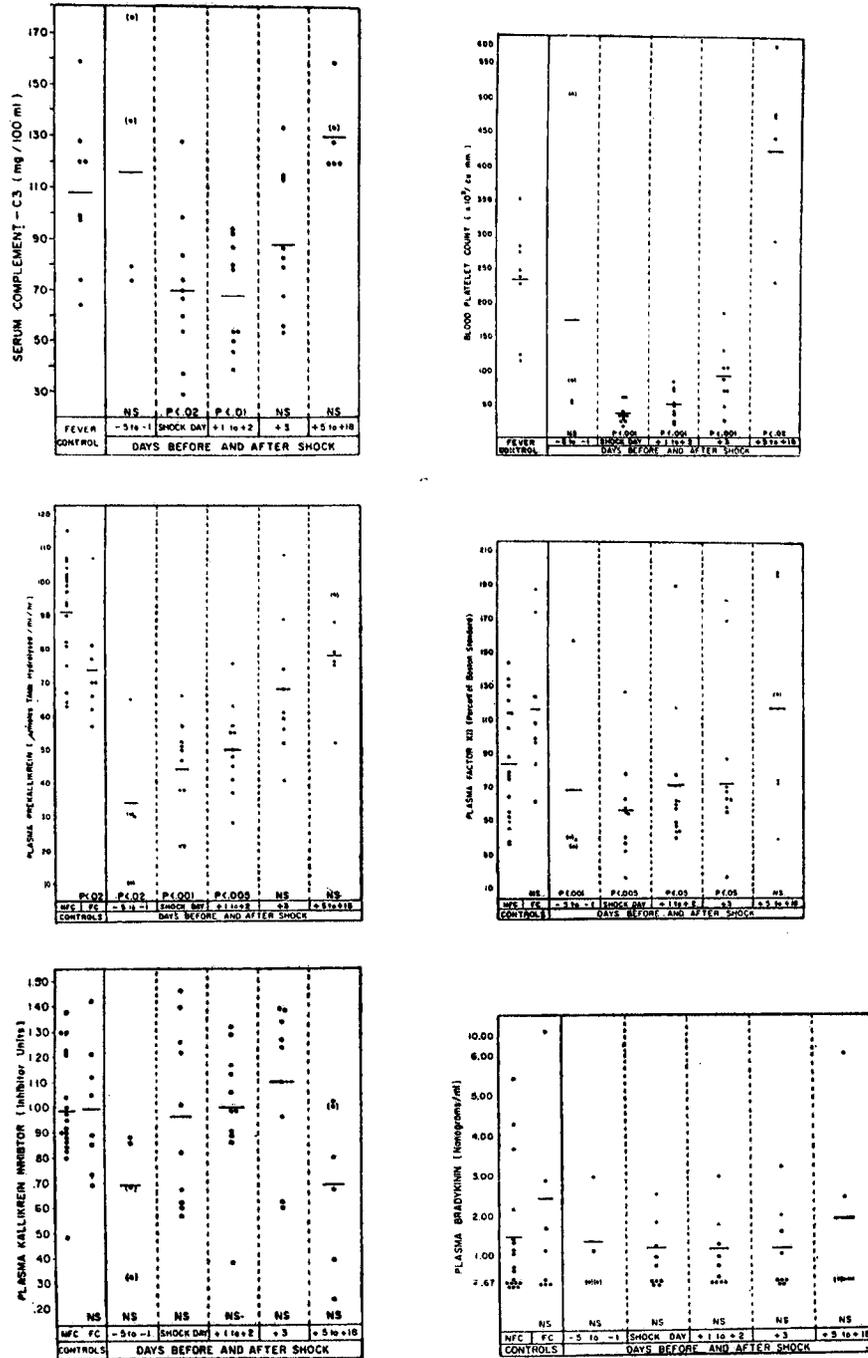


Figure 1a - 1f

Temporal profiles of C3, platelet and kinn components in patients with dengue shock syndrome (DSS). Non-fever controls (NFC) are single determinations, fever controls (FC) are admission values, and each point for DSS patients represents a single measure, except for points in parenthesis which represent the average of 2 or more determinations on the same individual in a given time period. Mean values are indicated by bars. Significance limits are calculated from a comparison of FC and NFC, and FC and DSS (Student's T test, two-tailed); NS=not significant ($P > .05$).

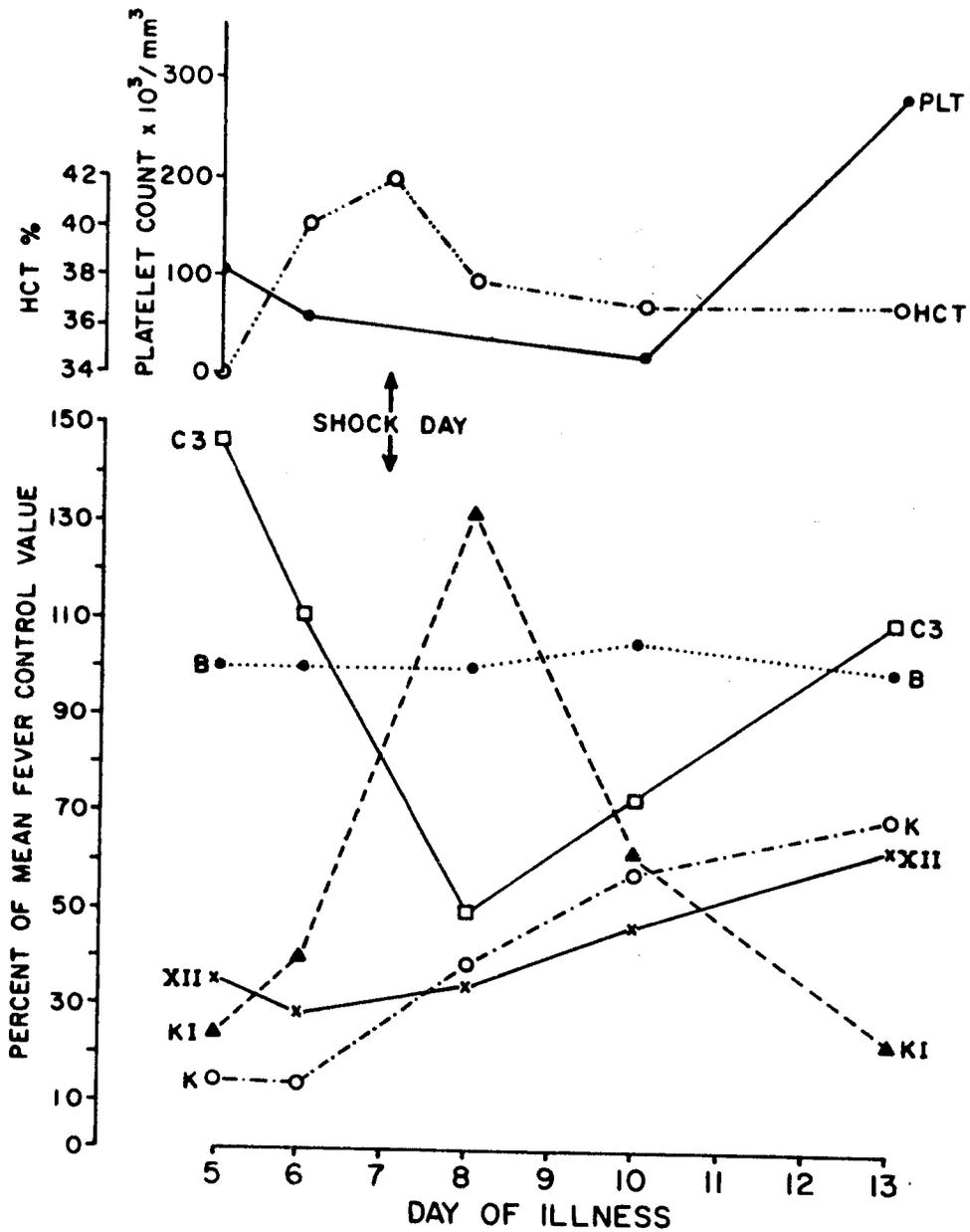


Figure 2. Changing Laboratory Values in a Patient with Dengue Shock Syndrome. PLT, platelet; Hct, percent hematocrit; C3, complement; B, bradykinin; KI, kallikrein inhibitor; k, prekallikrein; XII, Factor XII.

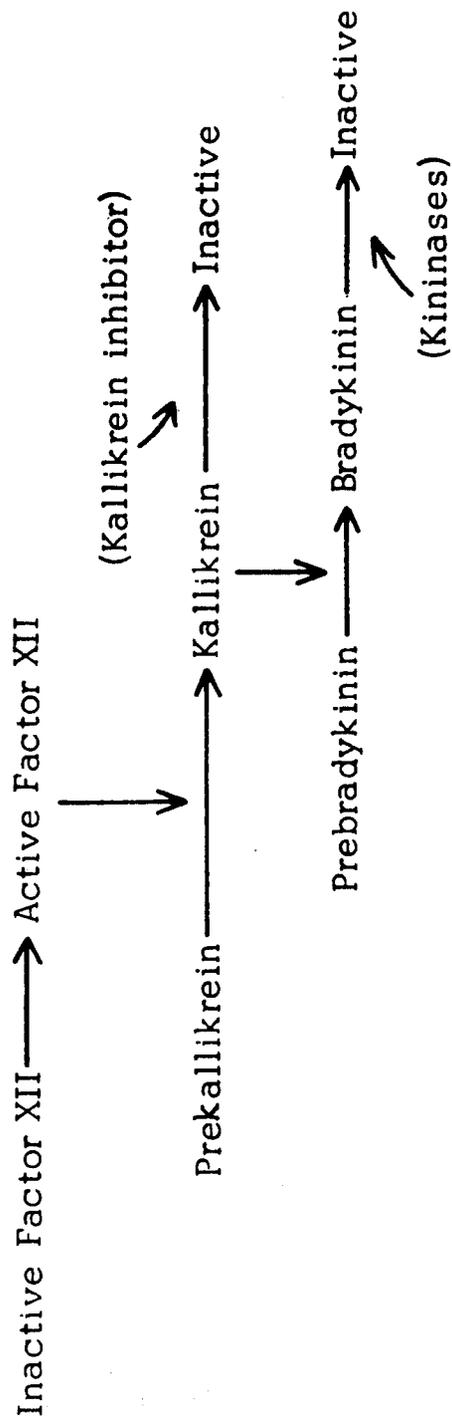


Figure 3. Pathway for Bradykinin Activation.