

Pathogenesis of Dengue Shock Syndrome

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PURPOSE: To determine if dengue shock syndrome is due to immunopathologic processes.

BACKGROUND: Dengue hemorrhagic fever (DHF) can be defined as a febrile illness due to dengue infection with associated hemorrhagic manifestations. The dengue shock syndrome (DSS) is considered to be a severe form of DHF differentiated by shock as manifested by a pulse pressure of less than 20 mm Hg or a fall in systolic blood pressure below 90 mm Hg.

The course of a patient with DSS typically consists of an early febrile phase lasting 3 to 6 days followed by a shock phase of a few hours to three days. The onset of shock is often preceded by lethargy and abdominal pain and is usually abrupt. Prior to the onset of shock, the hematocrit rises and the concentration of serum proteins falls. The decreased plasma volume is apparently due to a sudden increase in vascular permeability with resultant loss of plasma from the intravascular compartment. In most cases, early and effective replacement of plasma volume by infusion of colloids results in a favorable outcome.

All 4 dengue serotypes have been associated with DSS. The presence of 2 or more dengue serotypes and rapid dengue transmission in a locality appear to be the basic epidemiologic conditions essential to the occurrence of DHF and DSS. Epidemiologic evidence indicates that DSS is associated only with secondary dengue infections which result in an anamnestic antibody response with very high titers of effective complement fixing IgG antibody appearing early in the course of infection when virus or viral antigens may also be present *in vivo*. A marked depression of B1 c/a levels has been documented during the shock phase of illness, suggesting that the third component of complement may be consumed in DSS. These observations lead to the hypothesis that DSS may be immunologically mediated and that physiologically active products produced *in vivo* from activation of the complement system such as the anaphylatoxins, C3a and C5a, may be the means by which increased vascular permeability and shock are produced.

To test this hypothesis, a joint study sponsored by the WHO was undertaken in Bangkok from July—Oct 1971. Briefly an attempt was made to study at least 20 well characterized patients with DSS for

1. Immunohistochemical evidence of immune complexes in biopsy material and peripheral blood leukocytes
2. circulating immune complexes
3. immunologically induced consumption of complement components
4. evaluation of the coagulation and fibrinolytic systems.

This report will concentrate principally upon the virologic data obtained at SMRL and the complement data obtained at Scripps Institute. Data obtained from the other collaborating laboratories is not detailed but used only to emphasize major findings.

METHODS: Study patients were diagnosed as having DHF by clinicians; patients studied were in shock or felt likely to develop shock, but some non-shock patients were included for comparative purposes. A standard chart for pertinent signs, symptoms, clinical laboratory findings, and treatment was instituted and maintained at each of the 4 hospitals. At the conclusion of the study, the clinical charts were reviewed and the following criteria were used to grade patients studied into 4 groups based on severity of illness.

GRADE I: Fever accompanied by non-specific constitutional symptoms; the only hemorrhagic manifestation is a positive tourniquet test.

GRADE II: Fever and skin hemorrhage or other bleeding such as epistaxis or gingival hemorrhage.

GRADE III: Circulatory failure manifested by rapid, weak pulse with narrowing of pulse pressure (≤ 20 mm Hg) or hypotension (systolic pressure < 90 mm Hg).

GRADE IV: Moribund patients with undetectable blood pressure or pulse.

Techniques for viral isolation, identification of isolates, and serologic tests have been described in previous annual reports. Viral isolation attempts were made on initial serum samples obtained from all study patients. In hemagglutination-inhibition (HI) tests, antigen prepared from dengue-1 (Hawaii), dengue-2 (New Guinea C), dengue-3 (H-87), dengue-4 (H-241), Japanese encephalitis (Nakayama), and Chikungunya (Ross) viruses was used. Acetone extracted sera were tested against 8 units of antigen. All sera from individual patients were tested simultaneously.

For immunochemical quantitation of complement proteins, the single radial immunodiffusion method was used. Monospecific antiserum to human complement proteins (C1q, C1s, C3, C4, C5, C6, C8, C9, and C3 Proactivator) was incorporated at a previously determined, optimal concentration into 1.5% agarose gel, containing isotonic buffer, pH 8.0 and 0.01 M EDTA. Seven μ l of 3 different test serum dilutions were applied into 3 mm wells and the diameter of the precipitin rings was measured after 24 hr. at room temperature. Each immunoplate received 5 different dilutions of a standard serum. The absolute amount of a complement protein in a given test serum was determined graphically using an r^2 vs concentration of complement. Proteins in the standard serum had previously been quantitated in La Jolla using highly purified proteins as reference substances. Transferrin was assayed with reagents made available by Dr. Ursula Muller-Eberhard. All assay reagents and the 10 different types of immunoplates were prepared in La Jolla. Complement determinations were performed in Bangkok and at La Jolla by Dr. Muller-Eberhard and associates.

Quantitation of the levels of clottable fibrinogen and split products of fibrinogen plus fibrin was done by a radial diffusion method utilizing antiserum to fibrinogen.

The values in μ g/ml of the various proteins in the normal serum pool used in this study are as follows: C3 1500, C4 400, C5 75, C6 60, C3 PA 230, transferrin 2500, and fibrinogen 1720. These values were quite similar to mean serum protein levels determined in 30 Thai children, and were used as normal levels in the following analysis.

RESULTS: The original study comprised 94 patients. Of these, 55 individuals who were adequately studied and had isolation or serologic evidence of recent dengue virus were selected to form the group of patients upon which this report is based. All patients with grade III and IV disease initially studied are included in this analysis. Five of these patients were grade I, 14 grade II, 23 grade III, and 13 grade IV. Pertinent signs, symptoms, and clinical laboratory findings of these patients are summarized in Table I. The clinical symptomatology of patients studied were similar to those described in previous outbreaks. Of the 4 major

manifestations of DHF, fever and hepatomegaly were found consistently in patients of all 4 grades of disease. Hemorrhagic manifestations were found in all grades of disease but Grade I (by definition), but severe bleeding—melena and or hematemesis—was observed only in shock cases. The last manifestation of DHF, shock, was limited (by definition) to Grade III and IV. Hemoconcentration (a high hematocrit falling by at least 20% on recovery) was observed in all grades of disease, but most frequently in Grade III patients. The lower incidence of hemoconcentration in Grade IV patients probably reflects blood loss from gastrointestinal hemorrhage.

Patients were considered to have had dengue infections if 4-fold rises in antibody titer to at least 2 of the group B antigens were found between acute and convalescent serum or if convalescent antibody titers to at least 2 antigens equalled or exceeded 1:640. Forty-five of the 55 study patients had diagnostic rises in antibody titer consistent with dengue infection and an additional 8 had high, fixed titers suggestive of recent dengue infection. Convalescent serum was not obtainable on 2 patients who died with symptoms suggestive of DSS.

An attempt was made to determine what proportion of the children studied had primary or secondary dengue infections. Since most patients were studied at least 4 days after the onset of illness, determination of type of infection could not be assessed by presence or lack of antibody in acute serum, but was assessed in this study by the magnitude of HI antibody titers in convalescent serum. Patients with HI antibody titers of $\geq 1:640$ to at least 2 dengue antigens were considered to have secondary infections, while those with convalescent antibody of $< 1:640$ were considered to have primary dengue infections. By these criteria, 51 of the 53 patients from whom convalescent serum were tested had secondary infections, as shown in Table 2. The 2 patients with primary infections were both infants (6 months of age or less) whose mothers possessed dengue HI antibody.

A dengue virus was isolated from the acute serum specimen of 9 of the 55 study patients; 8 isolates were dengue 2 and one isolate dengue 1. In addition an isolate of dengue 3 and isolates of chikungunya virus were obtained in 1970 from Bangkok children not included in the study. An isolation of dengue virus from study patients correlated with time after onset of disease the patient was initially studied (7 of the 9 patients with dengue isolates were studied on or before the fourth day of disease) and with the dengue 2 HI antibody titer in the serum used for isolation (Table 3). Five of the 9 children with dengue isolates had virus present with dengue antibody in early serum.

One hypothesis that could be tested in the study was whether there was a difference in HI antibody response between children with shock and children without shock. Shown in the figure on page 85 are \log_{10} geometric mean dengue 2 HI antibody titers and 95% confidence limits by day after onset of illness for both categories of patients. There is no significant difference in HI antibody titer between shock and non-shock patients at any time after onset of illness.

Since no differences in antibody titers were found between the 2 groups, it is permissible to use HI titers of patients with or without shock in order to describe antibody patterns of either group. Shown in the figure on pages 87 are geometric mean dengue 2 HI antibody titers for patients with serum tested on day 3 or 4 and day 5 or 6 and day 7 or 8. Similarly shown are titers for patients with serum tested on day 4 or 5 and day 6 or 7, and day 8 or 9. Between 4 to 7 days after onset dengue antibody titers rose geometrically. It is precisely during this time after infection when shock developed in the majority of severely ill children in this study.

Sequential levels of complement components and transferrin on 4 representative patients are shown in the figure on page 86 supplied through the courtesy of Dr. Muller-Eberhard. Patient #26 (Grade IV) was a 6 month old Thai female admitted on the 4th disease day in shock and passing melena. On examination she was unconscious, cyanotic, and unresponsive to pain with a pulse exceeding 180/min, unobtainable blood pressure, scattered skin petechiae, and a liver palpable 2 cm below the RCM. Initial laboratory findings included a Hct of 24%, WBC of 19,300, and a platelet count of 27,000/mm³. Shock responded

to I.V. infusions of fluids and blood within 6 hours of admission. The platelet count remained low until the 7th disease day when it rose to 221,000/mm³. Dengue 2 virus was isolated from serum obtained on admission. Dengue 2 HI antibody titer was <1:20 on admission and 1:160 7 days later. This is the only child with a primary dengue infection who developed shock; the infant's mother had serum HI antibody to all 4 dengue serotypes. This child exhibited very low C3 and C4 levels and less depressed C3 PA and C5 levels until the 10th day of disease. Between day 10 and day 21, a recovery of all complement proteins to normal or supranormal levels was noted. Transferrin levels were relatively stable throughout.

Patient #33 (Grade IV) developed shock while hospitalized on the 6th disease day. Dengue 2 virus was isolated from serum on admission (DD4) in which no dengue HI antibody was detectable at 1:20 dilution. Dengue antibody at a level of 1:320-1:640 was present on the day of shock and rose to between 1:5120- \geq 1:20480 12 days after the onset of illness. The child recovered from shock on DD9. On day 5, before shock was manifest, C3 and C3PA levels were 70-75% normal, C5 was 50% normal, and C4 supranormal (150%). With onset of shock C3 and C4 fell, whereas C3PA was little effected. Transferrin remained between 80-90% normal throughout the preshock and shock period falling 70% normal after shock. By day 12, values of all complement components were approaching normal levels.

Patient #13 (Grade III) was admitted on the 6th DD with a blood pressure of 50/0, cold and clammy skin with occasional petechiae, a Hct of 45%, WBC of 20,200, and decreased platelets on smear. Dengue virus was not isolated from her initial serum which contained HI antibody to the 4 dengue serotypes at levels of 1:320-1:1280. A greater than 4-fold rise in antibody to all dengue serotypes was demonstrated between acute and convalescent serum. Shock responded to infusions of plasma within 4 hours of admission. This child shows very low C3, C4, and C5 levels following the onset of shock with C3PA less severely affected. Transferrin remains between 80-90% normal with a late rise. C3, C3PA, and C5 levels rose to about 70% or normal by day 12 when C4 was still markedly low.

Patient #41 (Grade II) is a 10 year old male admitted on the 4th day of illness with fever, anorexia, and abdominal pain. On admission, he had scattered petechiae, a liver palpable 3 cm below the RCM, and a Hct of 50%. Three days later his Hct was 40%, WBC 4,150, and platelet count 14,000/mm³. No evidence of shock or further bleeding manifestations developed during his hospitalization. Dengue virus was not isolated from serum obtained on the 6th disease day which had HI antibody to all dengue serotypes at levels greater than 1:20,480. The pattern of complement protein changes was mildly abnormal with C3, C4, C5, C3PA, and transferrin initially being 50-90% of normal on admission; by day 10 all values fell within 84-153% of normal.

Table 4 shows the proportion of shock patients with greater than 50% reduction in the complement proteins C3, C4, C5 and C3PA and transferrin on days after onset of shock. Between 60-82% of shock patients had significant C3 reduction on the day of or 2 days after onset of shock. On the other hand, 50% reduction in the non-complement protein transferrin were uncommon in shock patients, suggesting that reductions in C3 were due to consumption rather than inhibition of synthesis or extravasation from the vascular compartment. Significant depression of C5 and C4 levels were evident in 30-50% of shock patients and of C3PA levels in 15-30% of shock patients during the shock or immediate post shock period. In more convalescent samples obtained 6 days after the onset of shock, no patients had greater than 50% reductions of C3, C5, or C3PA, but about 25% of patients still had C4 reduction. Thus significant depression of serum complement protein levels in DSS patients was temporarily related to the shock or immediate post shock phase of illness.

Table 5 shows the proportion of patients with greater than 33% reductions in various blood proteins and platelets on the day of onset of shock and 2 subsequent days in patients developing or not developing shock. Of shock patients, 100% showed depression of C3, 89% of C5, 77% of fibrinogen, 72% of C4, and 67% of C3PA. However, 56% of shock patients showed similar reductions of transferrin levels. Consequently, depression of complement proteins or fibrinogen at this level may be caused by mechanisms other than specific consumption—i.e. by failure of synthesis or by extravasation of these proteins from the vascular space across endothelium of increased permeability.

Table 6 shows the proportion of patients with greater than 50% reductions of the same blood components. At this level, relatively few shock patients (11%) had depressed transferrin concentrations; thus greater than 50% depression of complement proteins were likely to reflect specific consumption. Of shock patients, 89% had depressed C3, 51% depressed C5, 58% depressed C4, and 33% depressed C3PA concentrations in serum obtained on the day of or 2 days subsequent to shock. Although 44% of children not developing shock had depressed C3 levels, significant depression of C5, C4, and C3PA were unusual in these patients with clinically milder illness.

DISCUSSION: The primary question posed in this study was whether the shock syndrome accompanying dengue was due to underlying immunopathological processes. All but 2 of the study patients had secondary dengue infections which are characterized by an early (4–5 days after onset of illness) and geometric rise in dengue antibody. The 2 exceptions were infants who may well have had circulating maternally acquired dengue antibody at the time of infection. Thus antibody was present at infection or mobilized early in infection in these patients at a time when dengue virus or non-infectious viral antigens may be present. The presence of dengue virus in serum containing detectable quantities of reactive antibody was shown in 5 of the 9 viremic patients and fulfills in vivo conditions for immunocomplex formation in these patients. Attempts to demonstrate circulating immunocomplexes by precipitation with Clq and monoclonal Rheumatoid factor were made in this study; the specificity of precipitins found as immune complexes remains questionable and must be sought by more specific and sensitive techniques in future studies. The virologic data however provide reasonable circumstantial evidence for the formation of immune complexes in this disease. It seems likely that these immune complexes could fix complement in vivo.

Significant depression of complement components during or shortly after the shock phase was evident in all shock patients studied. Greater than 1/3 reductions in C3 were found in all patients and similar reductions in C5, C4, and C3 PA were found in 67–89% of children with DSS. However since similar depression of transferrin, a non-complement protein not consumed in immunologic reactions, was found in over 50% of such patients, a 1/3 depression in complement protein concentration may not necessarily reflect immunologic consumption in these patients. Only a small proportion of shock patients (11%) had greater than 50% depression of transferrin levels during shock; thus a 50% reduction of complement proteins would suggest specific immunologic activation of the complement system. Since 89% of shock cases had 50% depression in C3 levels and over 50% had similar depression in C4 and C5 levels, specific activation and consumption of these complement proteins is likely in DSS patients. Significant depression of complement proteins was found generally only in the immediate shock phase of disease and was uncommon 3 or more days after the onset of shock. A much smaller proportion of non-shock patients had 50% depression of individual complement proteins than shock patients, suggesting a correlation between shock and depressed complement concentrations.

Several other points accrue from the complement data. First, most shock patients showed depressed C4 and C3PA levels, suggesting that the complement system was activated in vivo through both of the 2 known mechanisms, classical (involving the C1 proteins and C4 and C2) and through the recently described C3PA system. Secondly, almost all shock patients had evidence of specific consumption of C3 and C5. Specific activation of C3 and C5 implies the enzymatic liberation in vivo of 2 biologically active anaphylatoxins, C3a and C5a, which are the most potent mediators of increased vascular permeability known. Data from this study suggests that the increased vascular permeability responsible for shock in these DSS patients could be mediated by these anaphylatoxins released as a result of in vivo immunological activation of the complement system.

Further studies will be required to test this hypothesis. Indeed studies to determine the decay of radioactively labelled C3 in DSS patients, to determine if C3a and C5a can be detected in shock phase serum, and to determine the role of serum inhibitors of activated complement components (such as C3a and C5a) are planned in the coming year.

Table 1.
Summary of clinical signs and laboratory findings in 55 study patients

Findings	Severity of Illness			
	Grade I (5) %	Grade II (14) %	Grade III (23) %	Grade IV (13) %
Fever	100	100	100	100
Hepatomegaly (2–5 cm)	100 (4/4)	91 (10/11)	100 (20/20)	100 (13/13)
Positive tourniquet test	50 (1/2)	92 (11/12)	84 (16/19)	62 (5/8)
Platelet count				
<50,000/mm ³	60 (3/5)	54 (7/13)	85 (17/20)	92 (12/13)
Petechiae	0	100 (12/12)	52 (12/23)	69 (9/13)
Epistaxis	0	0	17 (4/23)	8 (1/13)
Hematemesis/melena	0	0	13 (3/23)	69 (9/13)
Hemoconcentration (≥ 20% increased hematocrit)	60 (3/5)	71 (10/14)	91 (21/23)	69 (9/23)

Table 2.
Proportion of DHF Patients Studied with Primary or Secondary Dengue Infections

Grade Disease	Primary Dengue Infection (conv. titer <640) (No.)	Secondary Dengue Infection (conv. titer ≥ 640) (No.)	Unclassifiable (No.)
I and II	1	18	0
III	0	23	0
IV	1	10	2
Totals	2*	51	2**

* Both infants <6 months age

** Both fatalities, convalescent serum not available

Table 3.
Dengue Isolation in Relationship to Initial Dengue 2 HI Titer in DHF Patients

<u>Dengue 2 HI Titer</u>	<u>No. of Patients</u>	<u>No. with Isolates</u>
≤ 80	16	9 (56%)
≥ 160	39	0

Table 4.

Proportion of Shock Patients with > 50% Reduction Indicated Serum Protein on Day after Onset of Shock

Day after onset of shock	% with 50% reduction in indicated serum protein					
	No. Studied	C ₃	C ₅	C ₄	C ₃ PA	Transferrin
0	27	17 (63%)	11 (41%)	10 (37%)	6 (22%)	2 (7%)
1	34	28 (82%)	10 (29%)	17 (50%)	10 (29%)	3 (9%)
2	27	16 (59%)	8 (30%)	11 (41%)	4 (15%)	0 (0%)
3	24	6 (25%)	1 (4%)	6 (25%)	4 (17%)	0 (0%)
4	21	1 (5%)	0 (0%)	4 (19%)	2 (10%)	0 (%)
≥ 6	17	0 (0%)	0 (0%)	4 (24%)	0 (0%)	0 (%)

Normal levels of serum proteins used for analysis:

C ₃	1500 ug/ml.	C ₃ PA	230 ug/ml.
C ₅	75 ug/ml.	Transferrin	2500 ug/ml.
C ₄	400 ug/ml.		

Table 5.
Proportion of Patients with > 33% reduction in indicated blood constituent
on day of onset of shock or the 2 subsequent days

<u>Serum Protein</u> <u>(or platelets)</u>	<u>Patients with shock</u>		<u>Patients without shock*</u>	
	<u>No. Studied</u>	<u>No. with 33% reduction (%)</u>	<u>No. Studied</u>	<u>No. with 33% reduction (%)</u>
C ₃	36	36 (100%)	18	14 (78%)
C ₅	35	31 (89%)	18	9 (50%)
C ₄	36	26 (72%)	18	9 (50%)
C ₃ PA	36	24 (67%)	18	2 (11%)
C ₆	35	21 (60%)	18	1 (6%)
Transferrin	36	20 (56%)	18	3 (17%)
Fibrinogen	35	27 (77%)	16	3 (19%)
Platelets	31	28 (90%)	12	9 (75%)

Normal levels of blood constituents used for analysis:

C ₃	1500 ug/ml	C ₆	75 ug/ml
C ₅	75 ug/ml	Transferrin	2500 ug/ml
C ₄	400 ug/ml	Fibrinogen	1720 ug/ml
C ₃ PA	230 ug/ml	Platelets	100,000/mm ³

* Disease day 5, 6, or 7 or disease day 6, 7, and 8 in patients admitted after disease day 5.

Table 6.
Proportion of Patients with > 50% reduction in indicated blood constituent on day
of onset of shock or the 2 subsequent days

<u>Serum Protein</u> <u>(or platelets)</u>	<u>Patients with Shock</u>		<u>Patients without Shock*</u>	
	<u>No. Studied</u>	<u>No. with 50% Reduction (%)</u>	<u>No. Studied</u>	<u>No. with 50% Reduction (%)</u>
C ₃	36	32 (89%)	18	8 (44%)
C ₅	35	18 (51%)	18	1 (6%)
C ₄	36	21 (58%)	18	1 (6%)
C ₃ PA	36	12 (33%)	18	0 (0%)
C ₆	35	7 (20%)	18	0 (0%)
Transferrin	36	4 (11%)	18	0 (0%)
Fibrinogen	35	10 (29%)	16	0 (0%)
Platelets	31	28 (90%)	12	7 (58%)

Normal levels of blood constituents used for analysis:

C ₃	1500 ug/ml	C ₅	75 ug/ml
C ₅	75 ug/ml	Transferrin	2500 ug/ml
C ₄	400 ug/ml	Fibrinogen	1720 ug/ml
C ₃ PA	230 ug/ml	Platelets	100,000/mm ²

* Disease day 5, 6, and 7 or disease day 6, 7, and 8 in patients admitted after disease day 5.

Figure 1.
 Comparison of Dengue HI antibody titers, by day of illness in patients with and without shock

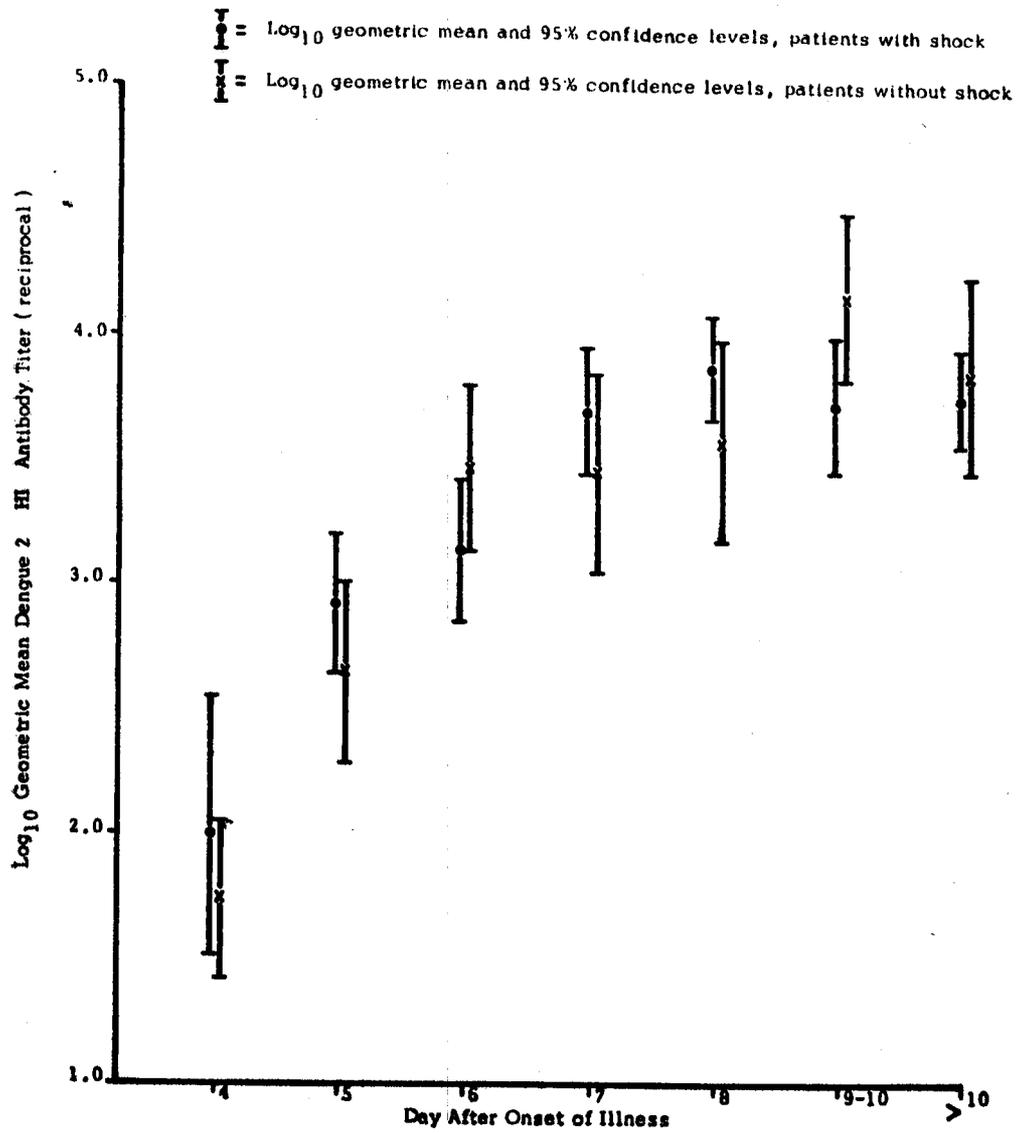


Figure 2.
Dengue HI antibody patterns in patients with shock, by day of illness

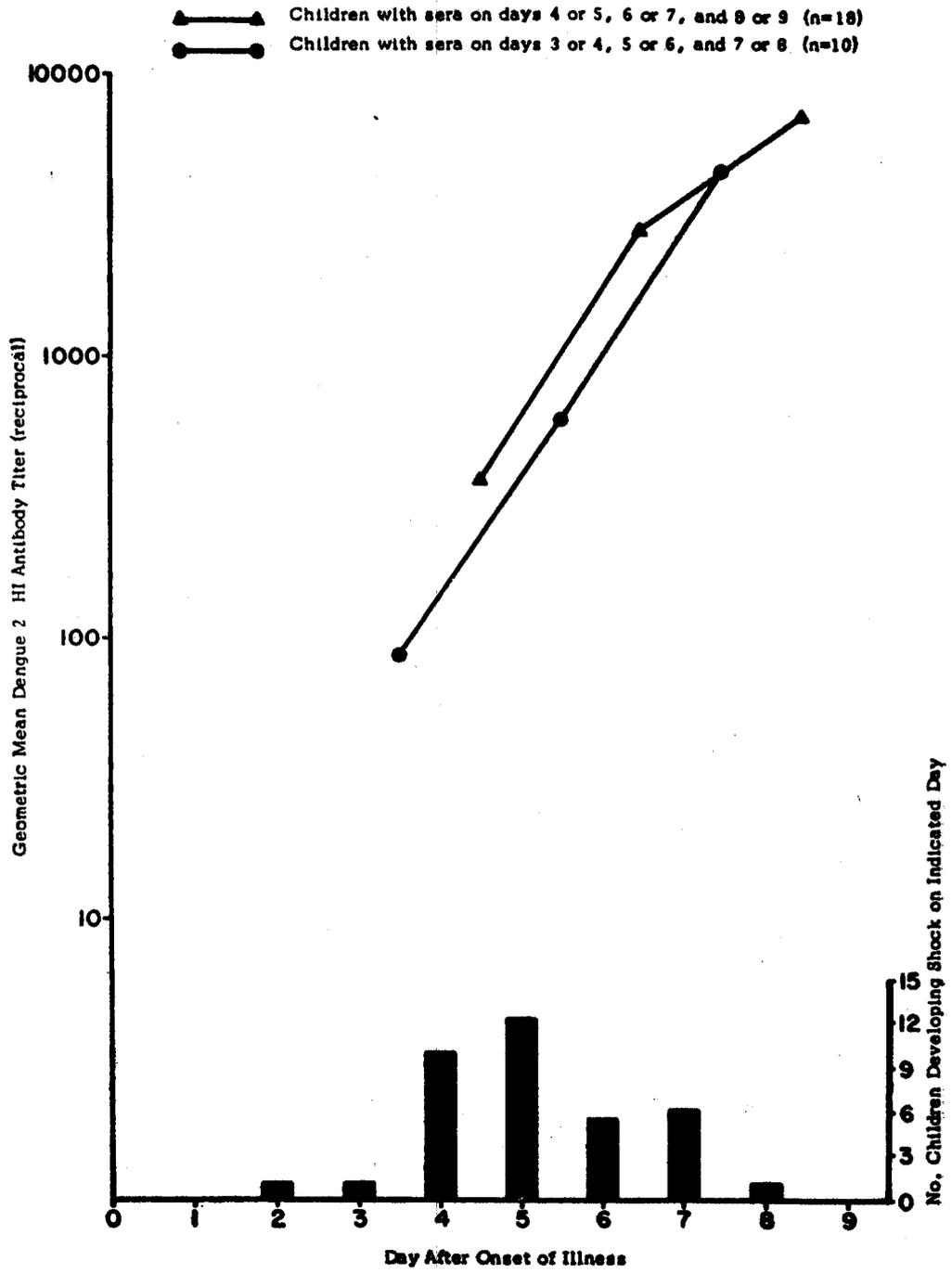


Figure 3.
Results of sequential determination of serum complement components
and transferrin, on 4 patients, by day of illness

