ANANDA NISALAK*
SCOTT B. HALSTEAD**
PRICHA SINGHARAJ***
SUCHINDA UDOMSAKDI†
SYLVANUS W. NYE‡
KAMOLWAT VINIJCHAIKUL¶

Virology and Pathology Departments, U.S. Army—SEATO Medical Research Laboratory, Faculty of Public Health and Department of Pathology, Women's and Children's Hospital, Bangkok, Thailand

OBSERVATIONS RELATED TO PATHOGENESIS OF DENGUE HEMORRHAGIC FEVER. III. VIROLOGIC STUDIES OF FATAL DISEASE

Virologic study of fatal dengue hemorrhagic fever (DHF) is a problem of special importance to an understanding of the pathogenesis of this disease. As opposed to experience in patients surviving DHF, virus isolations from fatal cases are extremely rare, three having so far been reported in the literature.[1-3] This report summarizes studies on autopsy materials submitted to the SEATO Medical Research Virology Department during the period 1962-64.

MATERIALS AND METHODS

Clinical materials. Autopsy specimens for virus isolation were collected at the Children's, Police, Wachira, Prapinklao, Pramongutklao and Siriraj Hospitals in Bangkok and Thonburi from April, 1962, through December, 1964. After the patient expired, the body was usually moved into a refrigerator until autopsy permission. The interval between death and autopsy was measured in the first 46 study cases and is shown in Table 1. These cases were all autopsied at the Children's Hospital. Organ sections were placed in separate containers and transferred to the Virus Laboratory on wet ice. They were then frozen and stored at −70°C.

Venous blood was obtained from some patients with hemorrhagic fever who subsequently died. Collection of these specimens has been described.[3] In other instances heart blood was drawn immediately after the patient expired, placed at 4°C. and brought to the laboratory. If not already hemolysed, the red cells were separated from serum. Serum was quick frozen in a dry-ice-alcohol bath and stored at −70°C. until tested.

Twenty liver specimens were obtained immediately after death by use of Vim-Silverman needle. These tissues were immediately brought to the laboratory on wet ice.

---

* Sr. Researcher, U. S. Army-SEATO Lab., Bangkok.
** Formerly, Chief, Virology Department. Direct reprint requests to Dr. Halstead. Present address: Section of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, 3675 Kilauea Ave., Honolulu, Hawaii 96816.
*** Jr. Lecturer, Faculty of Public Health, Bangkok.
† Lecturer, Faculty of Public Health, Bangkok.
‡ Formerly, Chief, Pathology Department. U.S. Army SEATO Medical Research Laboratory. Present address: Dept. Pathology, Univ. North Carolina School of Medicine, Chapel Hill, North Carolina.
¶ Pathologist, Women's Hospital, Bangkok.
Received for publication 16 August 1969.

TABLE 1. INTERVAL BETWEEN THE TIME OF DEATH AND AUTOPSY EXAMINATION IN 46 HEMORRHAGIC FEVER DEATHS STUDIED IN 1962-63, BANGKOK

Death interval until autopsy (hours)	*No. of cases*	*Viruses isolated*
Within 6	2	0
6-12	7	0
12-24	28	0
24-36	3	0
36-48	3	0
48-72	1	0
Over 72	2	0

Autopsy materials were obtained from 27 patients dying of causes other than hemorrhagic fever for use in control studies. Final pathologic diagnosis in these cases, with age and sex of patients shown in parenthesis, are as follows: purulent meningitis (5mo.F, 1M, 2F, 7M), poliomyelitis (5mo.F, 7mo.M, 4F, 6M), enteritis (8mo.M), lobar pneumonia (2F), encephalitis (2M,2M,3M,3M,5F,6F,13M), heart failure (1F), acute bronchitis (3F), diphtheria (3M), aplastic anemia (16F), drug poisoning (1F), trauma (35M), scleroderma (60F), carcinoma of vulva (29F), carcinoma of gall bladder (57M), uremia (55M). In 16 cases heart blood was included with autopsy organs.

Preparation and inoculation of specimens. Each organ was weighed, ground with purified alundum and diluted 1:10 with 0.75% bovine albumin phosphate buffered saline (BAPS). Each milliliter contained 500 units of penicillin and kanamycin, and 500 micrograms of streptomycin. The suspension was centrifuged at 10,000 rpm for 10 minutes in Servall SS-34 rotor and the supernatant used for isolation attempts. In 1962 and 1963, serum from heart or venous blood was diluted 1:4 in 0.75% BAPS. In 1964, dilutions of 1:5, 1:10, 1:100 and 1:1000 were used in a limited number of specimens. Sera in these cases were tested at 1:4, 1:10, 1:100 and 1:1000. Red blood cells were washed in 1000 volumes of saline and inoculated into the laboratory host in a 1:10 suspension.

Virus recovery hosts. One day-old albino Swiss mice were inoculated intracerebrally and intraperitoneally in doses of 0.01 ml. and 0.02 ml., respectively. Following inoculation, one of two techniques was employed to detect dengue viruses: 1) three blind passages at 10 day intervals observing mice for sickness or death,[3] 2) challenge of a portion of inoculated mice at 14 days with 50-100 LD_{50} of weanling mouse pathogenic dengue 2 virus.[4*]

Tissue cultures. BS-C-1, a stable line of African grivet monkey kidney cells, was used for dengue virus isolation and neutralization tests. Techniques for isolation and recovery of virus have been described.[5] Enterovirus recovery attempts were made by inoculating organ suspensions in primary *Macaca irus* kidney cells. Preparation of these cells has been described.[3]

* The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

TABLE 2. MONTH AND YEAR OF DEATH OF 169 PATIENTS WITH FINAL DIAGNOSIS OF HEMORRHAGIC FEVER AND SPECIMENS TESTED FOR VIRUS ISOLATION

Year	*Total*	*Jan.*	*Feb.*	*Mar.*	*Apr.*	*May*	*June*	*July*	*Aug.*	*Sept.*	*Oct.*	*Nov.*	*Dec.*
1962	58	0	0	1	4	4	7	9	8	16	5	4	0
1963	23	0	0	0	1	4	0	3	5	8	1	1	0
1964	88	1	1	9	10	17	12	16	12	6	2	2	0
Totals	169	1	1	10	15	25	19	28	25	30	8	7	0

TABLE 3. NUMBER OF DAYS AFTER ONSET OF FEVER BEFORE DEATH OCCURRED IN 169 PATIENTS WITH A FINAL DIAGNOSIS OF HEMORRHAGIC FEVER AND SPECIMENS TESTED FOR VIRUS RECOVERY. BANGKOK, 1962-64

		Day of death after onset of fever										
Year	*Total*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Other*
1962	58	—	2	10	17	16	8	3	—	—	—	(11)-1, (12)-1
1963	23	2	1	7	6	4	2	1	—	—	—	—
1964	88	1	7	15	26	17	10	5	1	1	1	(18)-1, (?) -3
Totals	169	3	10	32	49	37	20	9	1	1	1	6

TABLE 4. AGE IN YEARS OF PATIENTS DYING WITH FINAL DIAGNOSIS OF HEMORRHAGIC FEVER AND SPECIMENS TESTED FOR VIRUS ISOLATION. BANGKOK, 1962-64

		Age (years)																	
Year	*Total*	*<1*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15-19*	*20-25*	*?*
1962	58	9	4	6	5	7	11	4	2	6	1		1	1				1	
1963	23	4	2	2	4	3	4	1		2								1	
1964	88	11	6	11	12	11	13	4	4	3	1	2		2	1	2	2	1	2
Totals	169	24	12	19	21	21	28	9	6	11	2	2	1	3	1	2	2	3	2

Virus identification. Identification of isolated agents was by tissue culture neutralization, complement-fixation test of immune serum produced to isolates or plaque reduction test. Details of these procedures have been described.[6]

Serology. Hemagglutination-inhibition (HI) tests were performed by methods described.[5]

RESULTS

Characteristics of population studied

Tables 2-5 describe various characteristics of the population of fatally ill patients with final clinical or pathological diagnosis of hemorrhagic fever. The distribution of cases by month shown in Table 2 shows the seasonal variation and coincides with the period of hemorrhagic fever hospitalization.[7] The distribution of 169 deaths by day of illness shown in Table 3 resembles data for the day of hospital admission of hemorrhagic fever patients at Children's Hospital and the Clinical Research Center Study.[8] Study of individual cases indicates that death in hemorrhagic fever usually occurs within 36 hours of admission. This is suggested by the similarity of day of hospitalization and day of death curves. Age, sex, and ethnic distribution of cases studied are shown in Tables 4 and 5. These resemble the distribution of the same groups in total hemorrhagic fever patients as well as fatal cases reported from Bangkok and Thonburi hospitals in 1962-4.[7] In 72 cases from Children's Hospital clinical findings, gross and microscopic autopsy protocols were reviewed by one of us (KV); an additional 68 autopsy protocols by two of us (SBH, SWN). Clinical and autopsy findings in every case included were compatible with those reported for dengue hemorrhagic fever.[8]

TABLE 5. SEX AND ETHNIC GROUP OF 169 PATIENTS DYING OF CLINICAL HEMORRHAGIC FEVER WITH SPECIMENS TESTED FOR VIRUS ISOLATION. BANGKOK, 1962-64

Year	*Male*	*Female*	*Unknown*	*Thai*	*Chinese*	*Unknown*
1962	26	32		37	21	
1963	8	15		20	3	
1964	42	43	3	66	20	2
Totals	76	90	3	123	44	2

Virus recovery attempts

Specific organs tested are listed by year of collection in Table 6. Virus recoveries are designated. A variety of different techniques were used in

TABLE 6. SPECIMENS FROM PATIENTS DYING OF CLINICAL HEMORRHAGIC FEVER, AND TESTED FOR DENGUE VIRUS. BANGKOK, 1962-64

Specimen	*1962*	*1963*	*1964*	*Totals*
Heartblood, serum plasma	41[a]	2	60[b]	103
Liver	15	20	37	72
Liver biopsy			20	20
Spleen	14	19	32	65
Kidney	13	15	31	59
Heart	8	13	32	53
Lung	6	12	30	48
Brain	12	4	24	40
Adrenal	2		22	24
Pancreas		3	3	6
Stomach		1	6	7
Small intestine			19	19
Thymus		3	15	18
Lymph node			22[c]	22
Bone marrow			21[d]	21
Thyroid			13	13
Muscle			21	21
Skin			15	15

a. One dengue 1 virus recovered.

b. Three dengue 2 viruses recovered, one untyped dengue virus recovered.

c. One dengue 2 virus recovered.

d. One dengue 2 virus recovered in patient with dengue 2 virus recovered in heartblood (b).

virus recovery attempts. Efforts were also made to demonstrate the presence of viral antigen or of dengue neutralizing substances in autopsy materials. A dengue 2 strain was isolated from a lymph node suspension from a two-month old Thai male dying on the fourth day of a febrile disease characterized by vomiting, diarrhea and a late-appearing maculopapular rash, and a dengue 2 strain recovered from bone marrow suspension from a four-year old Thai female with shock syndrome. Serum from this same patient also contained dengue 2 virus. Table 7 summarizes the methods employed and the specimens tested. Most of the 1962 and 1963 autopsy materials were tested in suckling mice by the three blind passage method. Early in 1964 major viscera and heart bloods from 12 patients at log dilutions (undiluted through 10-3) were inoculated simultaneously into suckling mice and BS-C-1 cells. Three dengue viruses were recovered at a 1:10 dilution of serum in suckling mice, but not from 1:4 diluted serum; one of three of these sera yielded a virus from 1:100 dilution on BS-C-1 cell

TABLE 7. TEST PROCEDURES USED IN VIRUS RECOVERY ATTEMPTS ON SPECIMENS FROM PATIENTS DYING OF HEMORRHAGIC FEVER. BANGKOK, 1962-64

Specimen	*3 passage in sm**	*sm challenge only*	*BS-C-1† & 3 sm passage undil., 1:10, 1:100, 1:1000*	*BS-C-1 sm challenge*	*BS-C 1 only*	*Primary* Macaca irus *kidney cells*
RBC-WBC			12			
Serum or plasma	60[a]	32	12[b]	11[c]		11
Liver	38	27	12			14
Liver biopsy		4			16	
Spleen	36	23	11			15
Kidney	31	22	11			12
Heart	32	21	12			14
Lung	24	20	12			12
Brain	19	21	11			11
Adrenal	4	20	11			16
Pancreas	5	1				1
Stomach	6	1				
Small intestine	0	21				14
Thymus	4	14				9
Lymph node	0	22[d]				18
Bone marrow	1	20[e]				14
Thyroid	0	13				7
Muscle	0	21				16
Skin	0	15				10

* sm = suckling mice.

† BS-C-1 = Continuous African green monkey kidney cells.

a. Dengue 1 (strain 3156-62) recovered in 2nd s.m. passage from a 2 yr. old Thai male (HFI 62) from a 1:4 serum dilution obtained on 2nd day after onset of fever.

b. Dengue 2 (strain 10886) recovered from serum obtained on 4th day of illness (immediately after death) from a 5-yr. old Thai male (TH 363). Virus recovered after 1:10 dilution in s.m., and 1:100 dilution in BS-C-1 cells.

Untyped virus (strain 11127) recovered from serum obtained on 7th day of illness in a 7-yr. old Chinese male (TH 408). Agent recovered at 1:10 serum dilution in s.m.

Dengue 2 (strain 11194) recovered from serum obtained on the 3rd day of illness (immediately after death) of a 14-yr. old Thai male (TH 418). Virus recovered at a 1:10 serum dilution in s.m.

c. Dengue 2 (strain 15632) isolated from serum obtained on 2nd illness day in a 4-yr. old Thai female (PUO 269). Virus recovered at 1:10 dilution in BS-C-1 cells.

d. Dengue 2 (strain 15646) isolated from bone marrow obtained on 3rd illness day from pt. PUO 269 (see c.). Virus recovered at 1:10 dilution in BS-C-1 cells.

e. Dengue 2 (14444) isolated from lymph node obtained on 4th illness day from a 2-month old Thai male (TH 883). Virus isolated at a 1:10 suspension in s.m.

inoculation. No other viruses were recovered at 1:100 or 1:1000 dilutions in either host system. As a result of these studies all subsequent virus isolation attempts in tissue culture and suckling mice from serum or plasma were carried out at a 1:10 dilution. During the latter half of 1964, specimens from 43 fatally ill hemorrhagic fever patients were processed in suckling mice by the dengue 2 challenge technique. Eleven heart bloods from these patients were processed in BS-C-1 cells with recovery of a dengue type 2 virus from a four-year old Thai female who died within 12 hours of admission to the CRC Study ward on the second day of illness. This case has been described elsewhere.[9,10]

In 1964, 20 liver biopsies obtained immediately after death were trypsinized and inoculated onto BS-C-1 monolayers and into tubes containing BS-C-1 growth medium. In a few instances viable but sparse growth of fibroblast-like cells was obtained on primary explant. No cytopathic effect was noted in primary cultures. BS-C-1 cells inoculated with liver cells did not resist challenge with a cytopathic virus.

Enterovirus isolation attempts in primary monkey kidney (*Macaca irus*) cells were made on specimens from 22 patients. No enteroviruses were recovered. However, two agents were isolated in suckling mice that produced gross and microscopic lesions morphologically identical to those caused by coxsackie A viruses. These agents were recovered from heart tissue from a three-year old Thai female dying on the third day of disease and from the lung of a five-year old Chinese female dying on the fifth day of disease.

Attempt to demonstrate dengue antigen in organs

To determine if inactivated dengue antigen was present in autopsy organs, the liver, heart, lung, kidney, spleen, adrenal, intestine, and brain suspensions from a four-year old Thai male were diluted 1:10 in saline and inoculated in 0.01-0.02 ml. amounts intracerebrally and intraperitoneally in weanling mice three times at weekly intervals. Ten days after the last inoculation, mice were bled and serum tested for hemagglutination-inhibition antibodies to dengue 1 and Japanese encephalitis antigens. There was no detectable HI antibody at a 1:10 or greater dilution in any mouse serum.

Neutralizing substances in autopsy tissue suspensions

Following these studies, efforts were made to determine whether anti-dengue substances were present in organ suspensions. Supernatants of organ suspensions, prepared as described for virus isolation attempts, were diluted to 1:10 or higher in 0.75% BAPS. To these, serial tenfold dilutions of prototype dengue viruses types 1-4 were added and the mixtures incubated at 37°C. for one hour. Virus-organ suspensions were then inoculated in BS-C-1 tubes and tested for presence of interfering agents by the CVR

TABLE 8. NEUTRALIZING SUBSTANCES IN AUTOPSY ORGANS OR HEARTBLOOD FROM PATIENTS DYING WITH CLINICAL HEMORRHAGIC FEVER

Case No.	*Log neutralization index 1.7 or greater vs. indicated dengue strain**										
	TH 406	*TH 409*	*TH 596*	*TH 616*	*PUO 296*	*TH 1132*	*TH 966*	*TH 985*	*TH 973*	*TH 1106*	*TH 1110*
Age, sex	6 F	2 F	2 M	5 F	4 F	5/12 M	2 M	5 F	3 M	8 F	1 M
Day of death	5	4	4	5	3	5	5	7	5	3	4
Dilution tested	as indicated	as indicated	1:20	1:20	1:20	1:20	1:20	1:20	1:20	1:20	1:20
Specimen blood	(1:10) 1,2,3,4**	(1:10) 1,2,3,4			1,2,3,4	1,2,3,4	2				
Liver	(1:50) 1	(1:50) —	3	1,2,3,4	—	1	—	—	1	—	—
Spleen			—	1,2,3,4	1			1,2,3,4	1,2	—	—
Kidney			—	—	1	—	—	1,2,3,4	1,2	—	—
Heart			—	—	—	—		3	1	—	—
Lung			—	—	—			—	1	—	—
Brain					—	—	—		1	—	—
Adrenal					1			2	1,2,3	—	—
Pancreas											
Stomach											
Sm. intestine					—	—				—	—
Thymus								2,3	1		
Lymph node					—	—		2	1,2,3	—	—
Bone marrow									1		
Thyroid											
Muscle					—			1,2	1	—	—
Skin					—					—	—

* blank = not tested.
— = LN1 $\leq$ 1.6.
** Numbers 1-4 in rest of table indicate dengue type.

technique. Table 8 shows that of 11 autopsies so studied, significant anti-dengue neutralizing substances (LNI$\geqslant$1.7) were found in one or more organs in seven cases.

Two DHF patients had dengue neutralizing substances in serum, but not in tissue suspensions; only a few organs were tested in these cases, however. Some organ suspensions from several of the tested cases neutralized only one dengue virus while serum from the same patient was broadly reactive. This phenomenon was most extensively documented in case PUO 269. Dengue type 2 viruses were isolated from venous blood obtained on the second day of illness and from bone marrow obtained at autopsy after the child had died on her third illness day. Serum obtained at death significantly neutralized dengue viruses types 1-4; supernatants of spleen, kidney, and adrenal suspensions neutralized significant amounts of only dengue 1 virus. In the studied cases, neutralizing activity was found most frequently in spleen, but often in adrenal, thymus, liver, abdominal lymph nodes and kidney.

The nature of dengue neutralizing substance was studied in the liver suspension from case TH 406. This suspension, at a 1:50 dilution, neutralized 2.5 logs of dengue type 1, but no significant amount of polio 1, Coxsackie B 4, Sindbis or dengue type 2-4 viruses. The same suspension, when incubated for 12 hours at 37°C. on BS-C-1 cell sheets, failed to protect these cells against challenge by approximately 100 TCD_{50} or InD_{50} of polio 1, Coxsackie B 4, Sindbis, dengue 1, or dengue 2 viruses. That neutralization of dengue viruses was not a general property of human organ homogenates was evidenced by the failure of many suspensions of various organs to neutralize dengue viruses and the absence of significant neutralizing activity in a 1:20 dilution of liver, spleen, kidney, or brain suspensions from a six-year old Thai female dying of a chronic neurological disease.

Antibody response in fatal hemorrhagic fever

Because of failure to isolate dengue viruses from patients dying of hemorrhagic fever, for diagnostic purposes, attempts were made to demonstrate presence of dengue antibodies in serum in as many cases as possible. Serums adequate for testing were available for 101 fatal cases. In some of the remaining cases, HI antibody content of spleen suspensions were investigated. A 20% spleen suspension was made in borate saline, pH9.0. The suspension was clarified by centrifugation at 10,000 rpm for 10 minutes in a Servall centrifuge. The supernate was extracted with kaolin and goose red cell adsorbed precisely as for serum. In the test, initial dilution of spleen suspensions was calculated on the basis of original tissue weight. Serial twofold dilutions were made, and HI tests conducted in the usual fashion. For 17

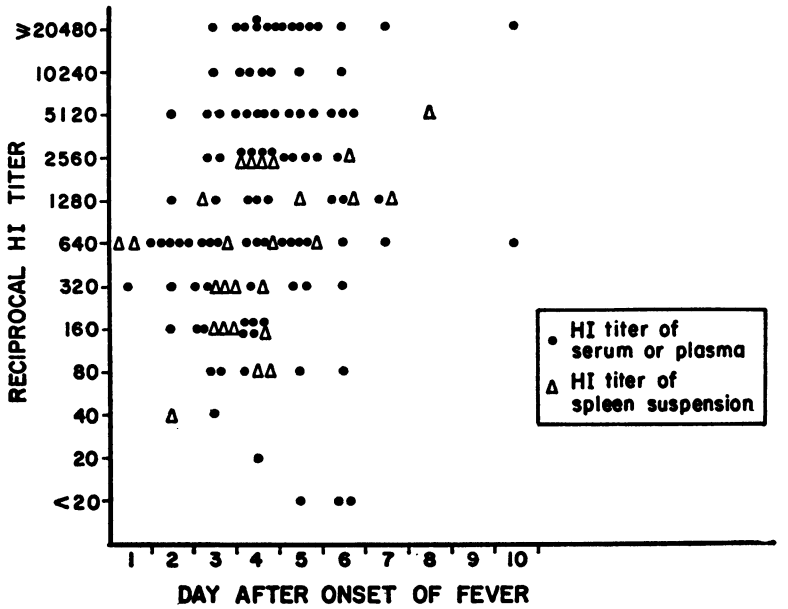

FIG. 1. Distribution of dengue type 1 HI antibody by day of illness in serum, plasma or spleen suspensions from 125 patients, ages 1-21, dying of clinical hemorrhagic fever. Bangkok, 1962-1964.

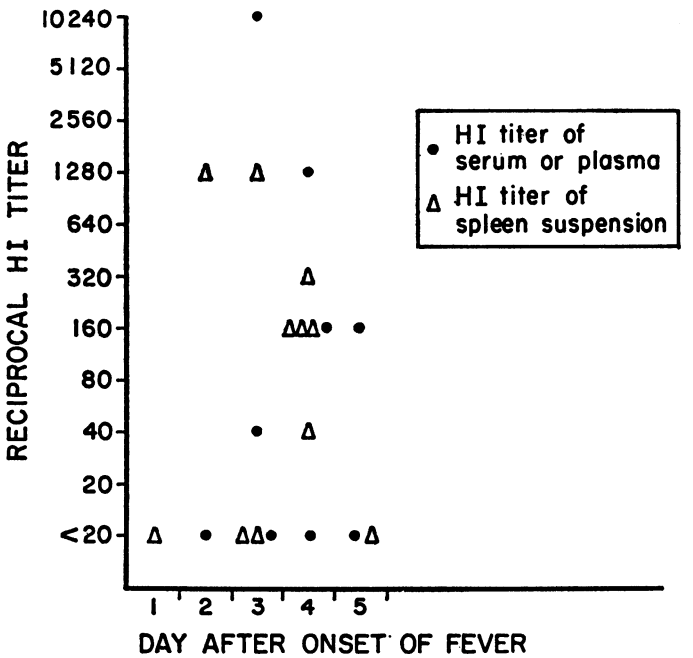

FIG. 2. Distribution of dengue type 1 HI antibody by day of illness in sera, plasma, or spleen suspensions from 20 patients, ages less than 12 months, dying of clinical hemorrhagic fever. Bangkok, 1962-1964.

patients, spleen suspensions and serum were available allowing comparison of HI titers. In 15 cases, antibody levels in the two tissues were within two-fold of each other. Figure 1 shows the HI titers observed in 99 sera and in spleen suspensions from 26 other fatal hemorrhagic fever cases; all of these specimens were obtained from children one year old or over.

Because children less than one year old who survive DHF frequently have a primary dengue antibody response, it was of interest to determine the type of antibody response found in patients dying of HF in this age group. As Figure 2 shows, 8 of 20 patients in this group had no detectable HI antibody at a dilution of 1:20 of serum or spleen suspension. Two patients had antibody at low levels. Ten patients had antibody at levels suggesting secondary antibody responses. The age of patients with highest titers is as follows; 5 mo.-1:1280; 6 mo.-1:1280; 7 mo.1:1280 and 1:10,240. Antibody responses in children 12-24 months old are illustrated in Figure 3. These resembled the secondary antibody responses seen in children in older age groups and shown in Figure 1. Antibody titers in fatal hemorrhagic fever cases over the age of one year were quite different from those observed in a small group of controls (Fig. 4). Extensive HI antibody data for the normal Bangkok population have been published.[8,7]

Data presented elsewhere show that mean HI titers in sera from fatal hemorrhagic fever cases were lower at every interval after onset of disease than serum titers in patients who survived DHF.[10] The significance of this observation is discussed separately.[11]

DISCUSSION

This paper adds to published reports the recovery of seven dengue viruses from six patients dying of DHF. Of 169 cases with one or more tissues tested for virus content, in only two instances were viruses recovered from tissues other than blood. Despite these isolations, the general experience was that dengue virus could not be isolated from sera or a wide variety of organs tested by many different virus isolation techniques. Interpretation of the failure to isolate dengue viruses, particularly from organs, is difficult to assess since the site of dengue virus replication in human beings is unknown. Organs from which dengue has been isolated are: dengue 4 from liver,[1] dengue 3 from heart[3] and dengue 2 from lung[3] and in our study, dengue 2 viruses from bone marrow and lymph node. We have also recovered dengue viruses from bone marrow suspensions in several DHF fever patients who survived their disease.[12] Virus recovery rates in these cases were not higher than from venous blood. The reported isolations of dengue viruses in organ suspensions do not constitute evidence of virus replication in those tissues, especially since all viruses isolated were from

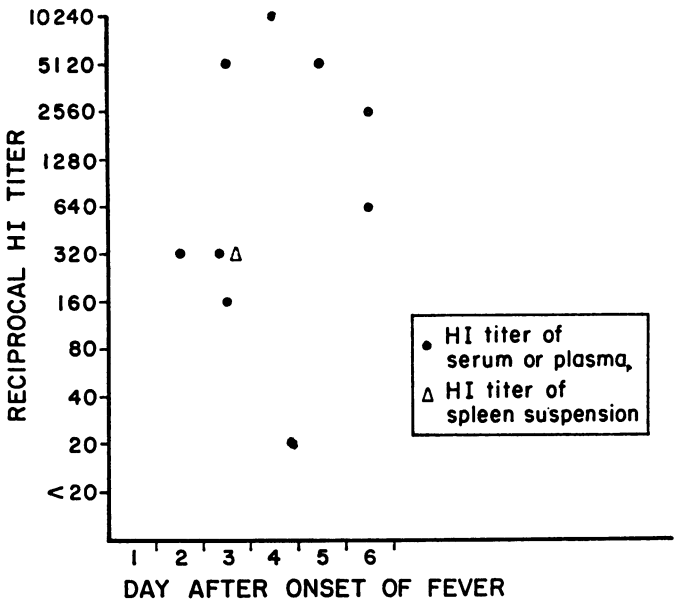

FIG. 3. Distribution of dengue type 1 HI antibody by day of illness in sera, plasmas or spleen suspensions from nine children, ages 12-24 months, dying of clinical hemorrhagic fever. Bangkok, 1962-1964.

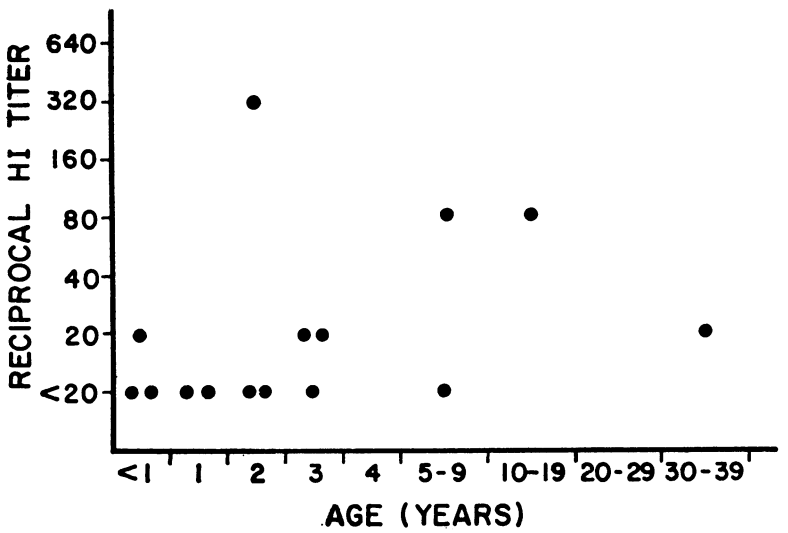

FIG. 4. Distribution of dengue type 1 HI antibody by age in serum from 15 patients dying of diseases other than hemorrhagic fever. Bangkok, 1962-1964.

different organs. Recovered viruses could have resulted from contamination of organ by viremic blood or lymph.

It may be argued that the absence of virus at death indicates postmortem virus destruction. Against this argument are the observations that virus recovery rates in blood specimens obtained before death (1/20) and after death (3/99) were similar. Virus was not recovered from immediate postmortem liver biopsies or in autopsies performed soon after death. Further, in a single case it was not possible to demonstrate development of dengue antibodies in mice immunized with organ suspensions. Immunofluorescent studies of human autopsy organs[13] and skin biopsies[14] from surviving patients similarly have been generally negative. Attempts to dissociate virus-antibody complexes by dilution or dissociation of virus from antibody by use of fluorocarbons[19] were unsuccessful. These efforts do not constitute rigorous exclusion of the presence of either primary or secondary infecting dengue viruses in tissues of fatal DHF cases. Successful demonstration of dengue virus in tissue may provide important information concerning the pathogenetic mechanisms of DHF. This goal should be pursued with more sophisticated techniques.

The detection of significant amounts of apparently specific dengue neutarlizing substances at 1:20 or higher dilutions of organ suspensions may explain the low rate of recovery of dengue viruses from various tissues. It was curious that in several cases monotypic anti-dengue activity was present in more than one organ suspension while serum neutralized all four virus types. It may be expected that a considerable dilution of serum is effected both by tissue itself and during subsequent dilution of preparations in the laboratory. The antibody detected may, therefore, be the dengue serum antibody with the highest concentration or highest reactivity. The isolation of a dengue type 2 virus from one case in which dengue 1 neutralizing activity was found in organ suspensions suggests the possibility that this patient had a dengue type 1 infection before her final illness. Her dengue 2 infection may have stimulated the production of high titered dengue 1 antibody as would be expected by the "doctrine of original antigenic sin." As a second explanation of the observed discrepancy in antibody specificity in serum and tissue suspension, we suggest that dengue neutralizing antibodies may become associated with and possibly concentrated in tissues following dengue virus infection and persist in tissue sites for long periods. As discussed elsewhere[11] heterologous dengue antibody may mediate cytotoxic reactions during secondary dengue infections. If so, it will be important to determine the antibody immunoglobulin types produced, the fate and distribution of these following primary and secondary dengue infections.

The lack of demonstrable etiologic agent in most of these cases should not be interpreted as weakening the evidence that all or nearly all patients in this series died during the course of a dengue infection. The clinical illnesses in the reported patients have been reviewed and resemble surviving documented cases of DHF. Autopsy findings were reviewed and were consistent with the diagnosis. This includes the eight infants less than 12-months old from whose organs we were unable to recover virus and whose sera contained no detectable dengue 1 antibody at 1:20 dilution. The seasonal distribution of cases, age, sex, and ethnic distribution, and day of death all resemble distributions in patients admitted during the same period who had virologically confirmed DHF. Finally, all but a very small proportion of these patients had dengue HI antibody in serum or spleen suspension. This occurred at titers and a higher frequency than would be expected in a normal group of the same age and sex composition.

SUMMARY

Tissues from 169 patients dying of hemorrhagic fever in Thailand in 1962-4 were studied for virus recovery or content of dengue virus neutralizing substances. That cases studied were dengue hemorrhagic fever is evidenced by the following: 1) the day after onset of illness of hospitalization, month of hospitalization and age, sex, and ethnic group distribution were similar in fatal cases to those in patients who survived confirmed dengue hemorrhagic fever, 2) autopsy protocols were reviewed in 140 instances—all cases were consistent with the reported spectrum of pathologic findings in Thai hemorrhagic fever and 3) serum or spleen suspensions from 137/147 fatal cases contained HI antibody to dengue viruses. In 103 isolation attempts from serum, only four dengue viruses were recovered, three dengue type 2, one dengue type 1 and one untyped dengue-like virus. Isolation attempts from 523 organs from 98 patients yielded one dengue type 2 virus from bone marrow and one dengue type 2 virus from lymph node. Efforts to increase virus recoveries or demonstrate antigen in tissues included dilution of starting materials, use of suckling mice and tissue culture as recovery systems, use of explants from fresh postmortem liver biopsies as a virus recovery system and use of organ suspensions as immunogens and measurement of development of dengue antibody in mice. These efforts were uniformly unproductive. A reason for these results may be the presence of dengue neutralizing substances in suspensions of autopsy organs. In several instances organ suspensions neutralized only one dengue virus type while serum from the same patient had broadly heterotypic neutralizing activity.

ACKNOWLEDGMENTS

We express our appreciation to the staff of the Virology Department for technical assistance. We are indebted to Dr. Dhira S. Comer, pathologist, U.S. Army—SEATO Laboratory, as well as to numerous other pathologists and clinicians at Wachira, Police, Pramongutklao, Siriraj, Prapinklao and Bangkok Sanitarium hospitals for providing some of the autopsy materials studied.

REFERENCES

1. Dasaneyavaja, A. and Charansri, U.: First known isolation of a dengue virus from other human source than blood. *SEATO Medical Research Monograph No. 2. Symposium on Hemorrhagic Fever. 10-11 August, 1961,* Bangkok, Thailand, 1962, p. 61.
2. Chan, Y. C., Lim, K. A., and Ho, B. C.: Recent epidemics of hemorrhagic fever in Singapore. *Jap. J. med. Sci. Biol.,* 1967, *20,* 81-88.
3. Nimmannitya, S., Halstead, S. B., Cohen, S. N., and Margiotta, M. R.: Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. *Amer. J. trop. Med. Hyg.* (in press).
4. Hammon, W. McD. and Sather, G. E.: Virologic findings in the 1960 hemorrhagic fever epidemic (dengue) in Thailand. *Amer. J. trop. Med. Hyg.,* 1964, *13,* 629-641.
5. Halstead, S. B., Sukhavachana, P., and Nisalak, A.: Assay of mouse adapted dengue viruses in mammalian cell cultures by an interference method. *Proc. Soc. exp. Biol. (N.Y.),* 1964, *115,* 1062-1068.
6. Halstead, S. B., Udomsakdi, S., Simasthien, P., Singharaj, P., Sukhavachana, P., and Nisalak, A.: Observations related to pathogenesis of dengue hemorrhagic fever. I. Experience with classification of dengue viruses. *Yale J. Biol. Med.,* this issue.
7. Halstead, S. B., Scanlon, J. E., Umpaivit, P., and Udomsakdi, S.: Dengue and chikungunya virus infection in man in Thailand, 1962-1964. IV. Epidemiological studies in the Bangkok metropolitan area. *Amer. J. trop. Med. Hyg.* (in press).
8. Bhamarapravati, N., Toochinda, P., and Boonyapaknavik, V.: Pathology of Thailand hemorrhagic fever: a study of 100 autopsy cases. *Ann. trop. Med. Parasit.,* 1967, *61,* 500-510.
9. Cohen, S. N. and Halstead, S. B.: Shock associated with dengue infection. I. The clinical and physiologic manifestations of dengue hemorrhagic fever in Thailand, 1964. *J. Pediat.,* 1966, *68,* 448-456.
10. Halstead, S. B., Nimmannitya, S., and Cohen, S. N.: Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J. Biol. Med.,* this issue.
11. Halstead, S. B.: Observations related to pathogenesis of dengue hemorrhagic fever. VI. Hypotheses and discussion. *Yale J. Biol. Med.,* this issue.
12. Halstead, S. B. and Nisalak, A.: Unpublished observations.
13. Bhamarapravati, N. and Boonyapaknavik, V.: Pathogenetic studies on Thai hemorrhagic fever: Immunofluorescent localization of dengue virus in human tissue. *Bull. Wld Hlth Org.,* 1966, *35,* 50-51.
14. Bhamarapravati, N.: Personal communication. Address: Department of Pathology, Siriraj Hospital, Dhonburi, Thailand.