

PREVENTION AND TREATMENT OF MILITARY IMPORTANT
DISEASES IN THE TROPICS

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1. Dengue Hemorrhagic Fever at Bangkok Children's Hospital, 1983

PROBLEM : To determine the incidence of dengue hemorrhagic fever (DHF) and the serotype of dengue virus causing DHF in patients at Bangkok Children's Hospital (BCH).

BACKGROUND : For 21 years this laboratory has maintained a collaboration with the clinicians at BCH. During this time the laboratory has developed its capability for isolation of dengue viruses, while the clinicians at BCH have refined their diagnostic and therapeutic skills. At the present time, serologic evidence for dengue infection is found in about 90% of the cases diagnosed as DHF by the BCH clinicians.

A total of 4064 patients have been diagnosed as having DHF (Table 1). About 20% of these have yielded a dengue virus isolate. Overall, dengue 2 was the most frequent isolate, and dengue 4 was a relatively rare isolate. Dengue 1 and 3 were isolated with intermediate frequency.

During the period of surveillance, annual cycles have occurred, with the highest mean number of cases occurring in August, in which an average number of 40 DHF cases were admitted. The largest single month was July, 1980, in which 131 admissions occurred. In that year, 788 cases occurred, from which 240 dengue isolates were obtained. Since then DHF activity has been less.

Current Data : In 1983, 252 cases have occurred (Table 1) marking the year as the third mild one in a row for DHF. As usual, August was the largest month, with 63 cases occurring. Twenty-four dengue isolates have been isolated thus far from the 68 specimens which have been processed thus far, for an isolation rate of 35%. Of these isolates, the largest number were dengue 2.

Table 1. Dengue isolates from DHF patients, 1962 - October, 1983

Year	Total Cases	Den Isol	Den				%				Major Type
			D1	D2	D3	D4	D1	D2	D3	D4	
1962	148	50	17	23	9	1	34	46	18	2	D 2
1963	156	35	8	10	17	0	23	29	49	0	D 3
1964	333	105	29	53	20	3	28	50	19	3	D 2
1965	88	12	0	8	3	1	0	67	25	8	D 2
1966	55	10	7	1	0	2	70	10	0	20	D 1
1973	135	22	5	13	4	0	23	59	18	0	D 2
1974	151	21	8	7	6	0	38	33	29	0	D 1
1975	399	14	1	8	5	0	7	57	36	0	D 2
1976	176	9	0	6	1	2	0	67	11	22	D 2
1977	495	66	0	37	10	19	0	56	15	29	D 2
1978	185	33	0	28	1	4	0	85	3	12	D 2
1979	301	61	2	58	0	1	3	95	0	2	D 2
1980	788	240	50	174	14	2	21	73	6	1	D 2
1981	196	36	11	21	3	1	31	58	8	3	D 2
1982	206	25	4	17	1	3	16	68	4	12	D 2
1983	252*	24	5	10	5	4	21	42	21	17	D 2
Total	4064	763	147	474	99	43	19%	62%	13%	6%	

* 68 Specimens processed through October

CONCLUSIONS :

1. Dengue 2 continues to be isolated most frequently from DHF patients.
2. An increase in DHF activity in the next year or two is expected, as it is unusual to have 4 consecutive low years.

RECOMMENDATIONS :

1. This extremely valuable study of the etiology of DHF in Bangkok represents the most extensive longitudinal study of the etiology of DHF in existence.
2. This study should be continued, with modifications appropriate for the expected increase in DHF activity.

2. Epidemic Peripheral Neuropathy Associated with Dengue

PROBLEM : In January 1983 the epidemiology service at the refugee holding center at Panat Nikom, Thailand, reported an epidemic of peripheral neuropathy. During the preceding two months 25 patients had presented to the camp health service with signs of severe combined motor and sensory neuropathy. Many of the patients related a history that the illness had begun abruptly with a fever and rash followed in a few days by the rapid onset of neuropathic symptoms. Because dengue was considered among the possible causes of the epidemic, AFRIMS was consulted.

PROGRESS : Baseline rates of peripheral neuropathy at the camp were estimated to be 1 or 2 cases per month, presumably due to nutritional deficiency. Thirty cases were identified with a history of neuropathic symptoms within the past two months, 17 of whom also gave a history that the illness began with a fever. Males outnumbered females 22 to 8. Twenty-four cases were between the ages of 15 and 44. Twenty-five cases were examined by a neurologist in February and 17 of those examined had persistent objective evidence of neurological involvement. At that time six of the cases were admitted to Bangkok General Hospital for intensive evaluation. All had normal physical examinations and routine laboratory studies except for neuropathy. Nerve conduction studies showed impairment in five of the six cases studied. Sural nerve biopsies in these five cases were non-diagnostic, and no evidence of inflammation was found. CSF was normal in all six cases. Screening for toxic metals in blood and hair was negative. Serum specimens were obtained from 21 neuropathy cases, 91 healthy young adult household cohabitants of neuropathy cases, and 92 randomly selected young adult camp residents. Sera were tested for elevated dengue antibodies by HAI (titer > 160) and IgG antibody capture immunoassay (absorbance > .90). Sera from 11 of 13 cases of neuropathy with fever had evidence of recent flavivirus infection, whereas only 1 of 8 cases of neuropathy without fever ($p = .002$) and 11 of 81 random controls ($p < 10^{-6}$) did. Household contacts of febrile cases had a higher prevalence of high flavivirus antibodies than did contacts of afebrile cases or random controls ($p < .001$ for both). Isolations were not attempted from case sera as no specimens were available from acute cases. Isolation attempts from all five sural nerve specimens were negative. Two viruses were isolated from *Aedes aegypti* collected in case houses; both were dengue type 1. The pathogenetic relationship between the dengue infections and neuropathy in these cases is not clear.

FUTURE OBJECTIVE :

(1) Dengue should be considered among the possible causes of acute peripheral neuropathy with fever.

3. In Vitro Dengue Antibody Synthesis by Patient Peripheral Blood Mononuclear Leukocytes

PROBLEM : There are no currently available methods for the rapid diagnosis of dengue virus infections. In our previous attempts to develop a reliable method, we have taken two conventional approaches : first, to detect virus specific antigens in blood or other clinical specimens, and second, to detect

virus-specific early antibody (IgM) in serum. Neither of these approaches has proven fully satisfactory. Relatively low levels of virus and presumably virus-specific antigen circulate in the blood during acute dengue (as compared to some other virus diseases such as hepatitis B), and of what little there is, it is often blocked by the patient's own antibodies, especially in secondary infections. Our efforts at detection of virus-specific early antibody in patient sera were partially successful in that a rapid and specific solid phase antibody capture immunoassay was devised. However, the diagnostic sensitivity of the assay was entirely dependent upon the time after the onset of illness that the test serum sample was obtained. In most patients with primary dengue, IgM anti-dengue activity could not be detected in serum obtained at the time of admission, not until a few days later. In our preliminary studies with the antibody capture immunoassay, we noted that this type of assay is especially powerful in the detection of virus-specific antibodies in biological fluids with low total antibody content but in which the specific activity of that antibody is high. We reasoned that one such biological fluid with a low total Ig content but a high specific activity was recently synthesized antibody by leukocytes from acutely infected dengue patients.

During the last half of 1982 and early in 1983, we obtained peripheral blood mononuclear leukocytes (PBML) from 108 patients hospitalized with a provisional clinical diagnosis of DHF at Children's Hospital. Clinical, serological, and virological evaluation of the patients was according to standard AFRIMS-BCH protocol (see DHF-PUO 1982). Seven of these cases were subsequently proven to not be due to dengue, while 101 were dengue related. Eleven of the 101 showed a primary seroresponse pattern, and 90 a secondary seroresponse pattern. Of the secondary cases, 68 had diagnostically high levels of dengue HAI antibodies ($> 1:1280$ to at least on antigen) in the acute serum sample, while 22 had relatively low (non-diagnostic) levels of HAI antibodies in the acute serum. Ficoll-hypaque purified PBML were washed extensively and the cells were dispensed into wells of a 96 well polystyrene "U" bottom plate in which specific wells had been previously sensitized with goat anti-mu or anti-gamma human immunoglobulin heavy chain specific anti-sera. One hundred microliters of cells were added to sensitized wells at concentrations of 10^6 , 10^5 , and 10^4 cells per milliliter. No specific antigens or mitogens were added. Equal aliquots were cultured as intact cells without inhibitors, intact cells with cycloheximide (2.5 micrograms per milliliter), or as cells which were disrupted by one cycle of rapid freezing and thawing. Patient sera and control sera containing known dengue antibodies were tested on each plate. Plates were incubated overnight at 37°C . in 5% CO_2 , then washed. "Captured" IgG or IgM was detected by stepwise addition of dengue type two suckling mouse brain antigen, hyperimmune anti-flavivirus IgG conjugated to horse-radish peroxidase, and substrate, as would normally be done in our standard antibody capture immunoassay procedure for serum. A positive result was defined as an absorbance value of >2 times the absorbance value obtained using the known negative control serum at a 1:100 dilution. Only in the last 66 cases studied was the activity of the disrupted PBML studied. Activity detected in the disrupted cell aliquot was taken to be due to preformed intracellular immunoglobulin. Results are summarized in the following tables.

<u>Patient Group</u>	<u># Tested</u>	<u>M Synth</u>	<u>G Synth</u>	<u>M or G Synth</u>
Not dengue	7	0	0	0
Primary	11	7	1	7
Secondary (low)	22	3	17	17
Secondary (high)	68	22	68	68

<u>Patient Group</u>	<u># Tested</u>	<u>Intracell</u>		<u>Intracell</u>
		<u>M</u>	<u>G</u>	<u>M or G</u>
Not dengue	4	0	0	0
Primary	6	5	1	5
Secondary (low)	11	1	8	8
Secondary (high)	45	7	45	45

These results demonstrate that during acute dengue infections there are cells circulating in the peripheral blood that are actively synthesizing dengue antibodies.

FUTURE OBJECTIVES :

1. This unique approach for the rapid diagnosis of acute dengue infections should be developed further. Attempts should be made to demonstrate specific antibody containing cells by staining of washed viable cells in suspension and staining intact but fixed cells. Ideally these intact and viable or fixed cells could be precisely quantitated through the use of a fluorescent activated cell sorter.
2. PBML obtained from the acute blood of DHF patients should be used as fusion partners for the development of human anti-dengue monoclonal antibodies.
3. The general applicability of this diagnostic approach for other infectious diseases should be evaluated.
4. Development of Dengue Type-Specific Serologic Assays Using Mouse Monoclonal Antibodies

PROBLEM : Existing serological methods for determining the infecting virus type in dengue virus infections are based on the neutralization of virus growth in cell cultures or in inoculated animals. These methods are time consuming, relatively expensive, and require expertly trained technical staff for their performance. We hypothesized that a new assay for sero-type specific antibodies in human sera could be developed using type-specific murine monoclonal antibodies. We envisioned testing human sera for their ability to block the attachment of a labelled mouse monoclonal antibody to its epitope on the homologous dengue virus surface glycoprotein (V3).

PROGRESS : One hundred and forty-two dengue antigen-reactive but otherwise largely uncharacterized monoclonal antibodies in the form of cell culture supernatant fluids (CCF's) were shipped from WRAIR to AFRIMS. CCF's were screened by antibody capture radioimmunoassay or enzyme-linked immunoassay (ACRIA or ACELISA) as follows : (1) the plastic solid phase was sensitized with goat anti-mouse IgG (2) CCF at a 1:10 dilution was added to saturate all binding sites on the solid phase (3) dengue antigen in the form of supernatant fluid of cultures of infected C6/36 cells was added (4) bound antigen was detected with iodine-125 or peroxidase labelled human convalescent DHF IgG. Binding of an antigen to the fixed CCF was quantitated as the "relative antigen binding activity" (RABA). The monoclonal chosen as a reference was one which reacted well with all four dengue serotypes as well as with Japanese encephalitis antigen, AD2-4G2. By definition the RABA of 4G2 was 100% for all antigens. An initial trial was performed comparing the binding spectrum of monoclonal CCF's by ACRIA at AFRIMS to that obtained by IFA at WRAIR using a panel of 21 CCF's of various specificities. The concordance was excellent, except that one type specific monoclonal antibody (AD1-15F3) as determined by IFA was unreactive by ACRIA. This monoclonal has subsequently been shown to react with a non-structural protein (not with V3). Based on an initial series of assays using Den 1, 2, 3, 4, JE, and uninfected control antigens, the 142 monoclonal CCF's were characterized as follows : flavivirus group, 27; dengue complex, 07; dengue subcomplex (more than on type but not all four), 13; type one, 34; type two, 9; type three, 4; type four, 10; non-reactive, 38. Next the bank of CCF's was screened against other flaviviruses known to be found in Thailand, Wesselsbron, Langat, and Tembusu. All of the 27 CCF's previously designated as flavivirus group reactive also reacted with all three of these virus antigens. Surprisingly, some of the previously designated "complex reactive", "subcomplex reactive", and "type specific" CCF's also reacted with some of these antigens. For example, the "dengue complex reactive" monoclonal AD3-1B7 was found to bind both Tembusu and Wesselsbron, but not Langat.

Next, a series of blocking experiments was conducted to determine if those monoclonal antibodies that shared a similar binding spectrum cross blocked one another. Based on the results of the binding experiments, mouse ascitic fluids (MAF's) were prepared at WRAIR for certain clones. In the first series of blocking experiments using labelled AD2-4G2, most flavivirus group reactive CCF's blocked, but some did not, suggesting the presence of at least two flavivirus group specific epitopes on V3. A surprise finding was that some of the CCF's caused increased binding of the labelled AD2-4G2 to antigen. Two monoclonal antibodies were found that were especially strong "promoters" of AD2-4G2 binding : the "dengue complex" reactive AD3-1B7 and the "dengue two specific" AD2-2H3. Five to 10 fold more labelled 4G2 is bound to Dengue 2 in the presence of either of these monoclonal antibodies. The discovery of the phenomenon of "promotion" of binding of one monoclonal antibody by another greatly complicates the prospects for developing a type specific serologic assay based on the blocking of monoclonal antibody attachment to antigen by test human sera, for it suggests that the dengue V3 protein is not a fixed structure. Instead, binding of non-specific antibodies at a remote site may alter the structure of a type specific epitope. Additional cross-blocking experiments are underway to develop a more complete epitopic map of the dengue V3 and to define more precisely the nature of the "promotion" phenomenon.

FUTURE OBJECTIVES :

1. Development of type-specific serologic assays should continue as a high priority effort. Alternative approaches using monoclonal antibodies should be explored, such as the definition of those clones that react with "linear structure defined" epitopes and the preparation of oligopeptide antigens.

2. Monoclonal antibodies should be raised against virus strains native to Thailand, in an effort to develop strain, rather than type specific assays, for use in epidemiological studies.

5. Arachadonic Acid Metabolites as Mediators of the Increased Vascular Permeability of Dengue Hemorrhagic Fever

PROBLEM : Dengue hemorrhagic fever is characterized by fever, thrombocytopenia, increased vascular permeability (as evidenced by an elevated hematocrit), and variable degrees of bleeding ranging from a positive tourniquet test to overt major gastrointestinal hemorrhage. In most patients the major life-threatening pathophysiological event is an abrupt increase in the vascular permeability at the time of the onset of the shock. No systematic study of the potential mediators of this increase in vascular permeability have been made. Since the macrophage and other phagocytic cells of the reticuloendothelial system are the main cells infected during DHF, and since at least some macrophage lysosomal enzymes such as acid phosphatase are released into the plasma during acute DHF, a reasonable hypothesis is that the vasoactive mediators of shock in DHF are synthesized and released by macrophages. Recently Austen and colleagues developed a radioimmunoassay for the detection and quantitation of leukotrienes. Leukotrienes, like prostaglandins, are metabolites of arachidonic acid, and they have been shown to be potent mediators of immediate type hypersensitivity reactions, especially vascular permeability. We therefore set out to measure plasma and leukocyte levels of leukotrienes and other vasoactive arachidonic acid metabolites in DHF.

PROGRESS : Ten milliliters of acid-citrate-dextrose anticoagulated blood were obtained on hospital day 1, day 2, and day 14 from six patients with clinical diagnoses of DHF with shock at Children's Hospital. Plasma was precipitated with cold ethanol and the supernatant immediately frozen. Peripheral blood mononuclear leukocytes were obtained by Ficoll-hypaque fractionation, washed, divided into four aliquots, and two aliquots each incubated for 20 minutes or 2 hours. The entire cultures were then snap frozen in liquid nitrogen. Plasma and cell cultures were transported to Boston for testing. To date all plasmas have been tested by RIA for leukotrienes C4 and B4, with negative results. RIAs of the leukocyte samples are not complete. Selected samples will be fractionated by high-performance liquid chromatography to test for immunoreactive leukotrienes.

FUTURE OBJECTIVES :

1. A systematic and integrated search for possible mediators of the increased vascular permeability in DHF should be undertaken. All potential mediators should be sought and carefully measured, such as histamine, kinins,

and complement factors, as well as arachadonic acid metabolites.

2. Clinical specimens obtained directly from patients should be inoculated directly into appropriate bioassay systems (such as the hamster cheek pouch) in an effort to detect circulating mediators.

6. Development of a Sub-Human Primate Model for Dengue Hemorrhagic Fever

PROBLEM : Many of the fundamental problems concerning the pathogenesis of Dengue Hemorrhagic Fever, such as the role of enhancing antibodies, the requirement for specific sequences of serotypes, the types of cells infected in vivo, and the nature and source of the mediators of increased vascular permeability, could be resolved if a reliable animal model of DHF were available. Attempts to develop such a model in the past have been totally unsuccessful. Our recent epidemiologic studies of DHF in Thai infants under one year of age, taken together with supporting laboratory data, have suggested a critical role for antibody-dependent enhancement of virus growth in cells with Fc receptors. We therefore sought to develop such a model for infant DHF based on this current epidemiological and laboratory information.

PROGRESS : Over a series of seven experiments, total of 44 primates were tested : 17 were control animals which received either no virus inoculation or no pre-treatment with dengue immune serum, while 27 received some combination of serum and virus that might be expected to produce enhanced virus growth in vivo. The virus strain Den 2/D80-616 was used throughout. This virus was selected because it had caused a fatal illness in a human infant and showed good "enhancability" in vitro. Five different antibody preparations were tested in the model : (1) Serum 2734/80, obtained from the mother of case D80-616 at the time the infant was admitted to the hospital, (2) serum 1799/81, obtained from the same woman approximately 18 months later, (3) serum 0653/82, obtained from the mother of another infant DHF case about two years after the death of the child, (4) mouse monoclonal antibody AD2-4g2, which reacts with a flavivirus group determinant on the surface glycoprotein (V3) of the dengue virion, and (5) Rhesus monkey monotypic Dengue 1 immune serum E-87. In all series the test primates were inoculated with a dose of serum which was calculated to give a dilution of antibody in the extracellular fluid space which would give maximum enhancement of growth of strain D80-616. Of the 27 test animals only one animal died : infant Rhesus monkey DA-9 died with profound thrombocytopenia, diffuse internal and external hemorrhages, and sustained viremia. At necropsy this animal was found to have a single large tuberculous mediastinal node, with absolutely no evidence of disseminated disease elsewhere. Other than diffuse hemorrhages, little pathology was found. Dengue virus was isolated from liver, spleen, lymph nodes, and thymus. Another animal in the same trial (DA-4) also developed sustained thrombocytopenia but did not die. Both DA-9 and DA-4 had been inoculated with serum 1799/81. Attempts to reproduce this experiment using the same serum in tuberculin skin test positive animals were unsuccessful. None of the other experimental animals in the other trials developed signs of DHF-like disease, although occasional low platelet counts were seen.

FUTURE OBJECTIVES :

1. Attempts to produce a model of DHF should continue.
2. Possible strategies include duplication of the human situation as closely as possible by immunizing female primates before pregnancy so that the infants are rendered passively immune through transplacental antibodies, or by inoculating primates with carefully selected monoclonal antibodies with especially strong enhancing properties.
3. In future models the state of activation of the reticuloendothelial system must be carefully controlled.
7. In Vitro Antibody Synthesis by Leukocytes Obtained from the Blood and Cerebrospinal Fluid of Patients with Acute Japanese Encephalitis

PROBLEM : In the course of development of the antibody capture assay for the rapid diagnosis of Japanese encephalitis virus infections through the detection of virus specific antibodies in the cerebrospinal fluid, we noted that the activity of anti-JE IgM and IgG in CSF was greater than could be accounted for by passive diffusion alone. The assumption has previously been made that antibodies are synthesized in the human CNS during encephalitis, but this has never been directly proven. Because we have been able to detect antibody synthesis by peripheral blood mononuclear leukocytes (PBML) obtained from the blood of patients with dengue infections, we set out to test the hypothesis that circulating mononuclear leukocytes actively synthesize JE antibodies in the peripheral blood and in the CNS during acute JE infections of humans.

PROGRESS : All patients admitted to the Kampanghet, Thailand Provincial Hospital during the 1982 JE season with a clinical suspicion of infection of the CNS were studied. PBML and CSF leukocytes were extensively washed and cultured for 72 hours without specific antigenic or mitogenic stimulation, and the culture supernatant fluids tested for JE IgM and IgG by antibody capture immunoassay. Sixteen cases were subsequently proven to be due to JE by conventional serologic techniques and 12 were proven to be not due to JE. None of the cultures of cells from the blood or CSF of control cases (not JE) showed any synthesis of JE antibodies. Admission PBML from JE cases showed IgM and IgG synthesis in 9 and 14 cases, respectively. CSF cells from 4 cases showed in vitro synthesis of IgG and one also showed synthesis of IgM. Cases in which JE antibody synthesis by CSF leukocytes could be demonstrated tended to be older males with more mental status impairment, higher CSF leukocyte counts, and greater and broader serum seroresponse patterns than did JE cases in which synthesis by CSF leukocytes could not be demonstrated.

FUTURE OBJECTIVES :

1. Techniques should be developed for the histochemical and/or immunofluorescent identification and quantitation of JE specific antibody producing cells in brain and CSF.

2. Circulating PBML and CSF leukocytes from acutely ill JE patients may be suitable "fusion partners" for the development of JE specific human monoclonal antibodies.

8. Isolations of Japanese Encephalitis Virus from Kamphangphet, Thailand, 1982

PROBLEM : To isolate strains of Japanese encephalitis virus from a number of human, pig, and mosquito sources in an epidemic area for comparisons of molecular characteristics.

PROGRESS : During the rainy season of 1982, a prospective surveillance of encephalitis in a province about 200 kilometers north of Bangkok was established. All cases of encephalitis seen at the hospital were examined and tested using the MAC ELISA for evidence of IgM anti-JE virus antibody in the CSF. Homes of selected confirmed cases were visited, and mosquito traps put out. JE seronegative sentinel pigs were placed in the vicinity of several of these homes. Mosquito collections and pig bleedings were performed every third night, on the average, during the period of greatest transmission of JE virus. Pig sera were tested for the appearance of JE antibody and virus sought in specimens collected 4 to 7 days before the seroconversion, since pigs would have been most likely to be viremic at that time. Mosquito collections were sorted and speciated. Pools of 200 mosquitoes were analyzed by using an ELISA for the presence of JE antigen and cultured by inoculation of C6/36 *Aedes albopictus* cells. Initially, ELISA and culture results were compared, but because of the large number of pools, ELISA was subsequently relied upon for screening.

RESULTS : Five hundred and six mosquito collections were performed. One hundred and eleven of these, containing 1,759 pools, (16 pools per trap) with approximately 350,000 mosquitoes have been sorted. Virus isolation from 70 of the 111 counted traps has been completed. 54 viruses have been isolated (Table 1), almost exclusively from dry ice baited light traps.

Table 1. JE virus isolations, Kamphangphet, 1982.
(as of 20 October, 1983)

<u>Number</u>	<u>Virus</u>	<u>Method of Confirmation Sources</u>	
22	JEV	Neutralization	Mosquito
5	JEV	IFA (neut pending)	Mosquito
13	Tembusu	Neutralization	Mosquito
6	Flavivirus	IFA (neut pending)	Mosquito
7	Other	6 sent to YARU for identification	Mosquito
4	JEV	IFA	Pig serum
1	JEV	KFA	Human brain

Of 26 JE virus isolates analyzed thus far, 19 were identified by ELISA antigen detection on the mosquito pool (Sensitivity = 73%), while 7 strains came from ELISA negative pools. The mean ELISA OD for ELISA positive, culture positive pools (defined as : $OD > 2 \times$ (Control negative mosquitoes)) was 0.137, as compared to a mean of 0.025 for control pools. The mean of culture positive, ELISA negative pools was 0.05, as compared with a mean of 0.04 for the control negatives, confirming that the ELISA method does not distinguish all positive pools.

Of 21 sentinal pigs bled serieally, HAI rises indicated infection in 10. JE virus was isolated from 4 of these. One isolate was obtained from a human brain.

WORK REMAINING : Many ELISA positive pools remain to culture on tissue culture. Three of the JE strains have been sent to USAMRIID for fingerprinting. Mosquito sorting should be completed within 2 months, so ecologic variables can be analyzed.

CONCLUSIONS :

1. Mosquito and pig collections in epidemic areas are productive sources of JE virus.
2. The ELISA for screening pools of mosquitoes is somewhat insensitive, but considerably less time consuming than the culture method. Its use is appropriate where large numbers of specimens require screening.
3. Large amounts of JE virus circulate among the vectors and amplifier hosts in the northern part of Thailand during epidemics of encephalitis.

FUTURE OBJECTIVES : Analysis of the mosquito and pig collections has proved fruitful in that all goals for isolation of virus strains were met. However, the unanticipated large number of mosquitoes has resulted in an extremely larged work load for the laboratory. Analysis of a sample of the pools may be necessary. After analysis of the data, this study will be complete.

9. Development of IgM Anti-Rabies Antibody in the CSF of Humans with Rabies

PROBLEM : To test the hypothesis that formation of antiviral IgM antibody can be detected in the CSF of patients with rabies by applying principals of learned in the study of Japanese encephalitis.

BACKGROUND : This laboratory has reported the development of an IgM capture method of detecting virus specific antibody in the CSF of patients with encephalitis due to Japanese encephalitis virus (Burke, D.S., et al. Antibody capture immunoassay detection of Japanese encephalitis virus immunoglobulin M and G antibodies in cerebrospinal fluid. J. Clin. Microbiol., pp 1034-1043, (1982).). A similar assay, using anti-human mu chain, test CSF or serum, inactivated rabies virus in the form of Merieux rabies vaccine, and I125 labeled rabbit anti-rabies antibody was developed. We previously reported that

this test allowed detection of IgM antibody in the serum of many individuals receiving pre-exposure rabies prophylaxis. More recently, we have evaluated the test in several patients with confirmed rabies encephalitis.

PROGRESS : To date, three patients with rabies encephalitis have been tested. Serial CSF and Serum specimens were collected and tested in the rabies MAC RIA and neutralization assays.

The first patients developed no detectable antibody of any type in their CSF prior to death. However, the third patient demonstrated a dramatic rise in the CSF and serum IgM just prior to death. The specimens which contained the IgM antibody were the only ones in the three patients in which specific neutralizing antibody was detected (Table 1).

Table 1. Anti-rabies antibody in serial specimens of a patient with confirmed rabies.

Date	Days to Death	CSF p/n	Serum p/n	Rabies neut	IgM peak
3/12	12	.9	1.4	-	
3/13	11	1.2	1.1	-	
3/15	9	.9	1.0	-	-
3/17	7	1.0	1.4	-	
3/19	5	.9	1.5	-	
3/20	4	1.4	1.5	-	
3/21	3	1.0	1.6	-	
3/22	2	1.3	2.1	+	
3/23	1	1.9	2.5	+	+
3/24	0	1.9	3.1	+	

Sucrose density gradient fractionation of the serum and CSF samples from this patient demonstrated the appearance of a 19 s peak in the antibody in the late serum and CSF specimens, confirming the presence of IgM antibody in these specimens.

In contrast to the regular appearance of virus specific anti JE antibody in Japanese encephalitis, the occurrence of IgM anti-rabies antibody in the serum and CSF of patients with rabies was seen in only one of three patients studied. The test results were slightly more positive in the serum specimens, suggesting the possibility that diffusion of antibody could account for the CSF positivity. The rise in the counts in this test near the time of the patient's death suggests that the antibody was not effective in halting the progress of the disease.

CONCLUSIONS : IgM antibody may appear in the CSF of rabies patients, but does not seem to do so in all cases.

FUTURE OBJECTIVES : This test provides an assay that may facilitate an understanding of the pathogenesis and treatment of rabies. However, the test gives low levels of positive results even when it is positive. Although it has limited promise as a diagnostic test, further evaluations of clinical specimens should be done.

10. Detection of Rabies Immunoglobulin in Serum and Cerebrospinal Fluid of Quarantined Dogs by IgM Antibody Capture RIA

PROBLEM : Rabies is an important zoonotic disease in Thailand. Presumptive diagnosis may be made on clinical signs but confirmation is not made until the dog dies or is euthanized and specific fluorescent antibody staining of brain tissue for rabies virus antigen is completed. An antemortum diagnostic technique would be valuable to permit initiation of prophylactic therapy of the patient without euthanizing the dog or waiting until death occurs.

OBJECTIVES : To determine if rabies infection can be diagnosed in the living animal by detecting rabies specific IgM in the serum or cerebrospinal fluid.

PROGRESS : Serum & CSF samples were obtained from thirty-seven dogs that were held in rabies quarantine at the Thai Red Cross Society. Twenty-four of these dogs developed typical clinical signs of rabies and died from one to three days after entering quarantine. Mouse inoculation of hippocampus from the brains of these dogs resulted in death in 21 of 24 of the injected litters and brain smears of these mice were all positive by FA for rabies. Six of the rabid dogs had rabies virus in their CSF, which was demonstrated by mouse inoculation.

Thirteen of the quarantined dogs did not have rabies and all but one survived. This dog died of a canine distemper-like illness but the specific cause of death was not determined. An immunoglobulin M-antibody capture radioimmunoassay (MACRIA) was performed on each serum and CSF sample. Significantly higher counts per minute (cpm) were found in the CSF & serum of the 21 dogs with proven rabies when they were compared with the 13 non rabid dogs. The three dogs that died following a clinical rabies illness had negative mouse inoculation tests but significantly higher CSF and serum MACRIA counts than the non-rabid dogs. The ratio of the CSF : serum cpm in known rabid and clinically rabid dogs was 1.19 - 1.20 while this ratio in the non-rabid dogs was 0.71. When sucrose density gradient separation of the rabid dog serum was tested the major count peak was in the early fractions where IgM would be expected to appear.

FUTURE OBJECTIVES :

1. Test sequential samples of experimentally infected dogs to determine how early after infection rabies IgM can be detected.
2. Determine effects of vaccination with live virus on serum and CSF IgM.

11. Identification of Enterotoxigenic *Escherichia coli* in Patients with Diarrhea in Asia with Three Enterotoxin Gene Probes

OBJECTIVE : To apply the DNA hybridization assay in identifying ETEC in Asia.

PROGRESS : Nine hundred and eighty-four enterotoxigenic *Escherichia coli* (ETEC) and 733 non-ETEC isolated from patients with diarrhea in Asia (one isolate/patient) were examined for homology with radiolabelled fragments of DNA encoding heat-labile toxin (LT), or heat-stable toxin of porcine origin (ST-P) or of human origin (ST-H). Two hundred and forty-six ETEC that produced LT and ST as determined by the Y-1 adrenal and suckling mouse assays were homologous with the LT probe. Of these 246 LTST ETEC 156 (63%) were homologous with the ST-H, 46 (19%) with the ST-P, and 44 (18%) with both probes. Four hundred and one LT ETEC were homologous with the LT probe. Of 337 ST ETEC identified by the suckling mouse assay, 244 (72%) were homologous with the ST-H, 84 (25%) with the ST-P, and nine (3%) with both probes. None of the 733 isolates that were non-enterotoxigenic as determined by the Y-1 adrenal and suckling mouse assays were homologous with genes coding for enterotoxin.

Four isolates (not included among the 984 ETEC examined) that were initially considered to produce LT because sterile culture supernatants produced rounding of Y-1 adrenal cells were not homologous with the LT probe. Sterile culture supernatants of these four isolates caused rounding after eight hours and subsequent destruction after 24 hours of Y-1 adrenal tissue cultures. This effect was not inhibited by convalescent human cholera antiserum, Swiss Serum Institute cholera antitoxin, or antiserum to purified LT. These isolates were also negative in the Biken test previously used to identify LT producing *E. coli*. The DNA hybridization technique with three enterotoxin gene probes is a specific technique to identify ETEC in a large number of specimens in Asia.

FUTURE OBJECTIVE : The DNA hybridization assay will be used to examine for enterotoxigenicity from Africa, the Near East, the Philippines, Indonesia, and Peru. Improvements in the test to examine specimens directly without testing individual *E. coli* isolates is planned.

12. Absence of Nucleotide Sequence Homology Between Genes for *Vibrio cholerae* Toxin and *Vibrio fluvialis*

PROBLEM : *Vibrio fluvialis* have been isolated from patients with diarrhea and from a variety of environmental sources in various parts of Asia. The enteropathogenicity of this organism has not, however, been clearly defined.

PROGRESS : *V. fluvialis* from different sources in Asia were examined in the DNA hybridization assay (1) to determine whether these organisms shared DNA sequence homology with cloned genes for *V. cholerae* toxin (2).

Ninety-two isolates of *V. fluvialis* isolated in Asia (Legend) were examined. None hybridized with radiolabelled genes coding for cholera toxin under stringent or more relaxed hybridization conditions (50% formamide at 65°C, or 25% formamide at 54.5°C) (3). The enteropathogenicity of this organism does not appear to be due to an enterotoxin whose structural genes show nucleotide sequence homology with *V. cholerae* toxin.

FUTURE OBJECTIVES : Study completed

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Legund : Source of 92 *Vibrio fluvalis* examined for DNA homology with cloned *Vibrio cholerae* toxin genes

<u>Source of Isolates</u>	<u>Country of Origin</u>	<u>No. of Isolates</u>
Patients with diarrhea	Japan+	17+
	Thailand	14
	Bangladesh	6
	Singapore	3
	India	2
Patients without diarrhea	Japan	6
	Thailand	4
Water	Thailand	12
Pigs	Thailand	7
Food	Thailand	6
Flies	Thailand	5
Fish	Thailand	3
	Thailand	3
Shellfish	Thailand	3
	Japan	1
Cows	Thailand	3
Total		92

+ Isolates from travelers from Southeast Asia on arrival at Osaka International airport. Isolates from Thailand were isolated during longitudinal studies of enteric disease in Soongnern, Thailand.

13. Flies as a Source of Enteric Pathogens in a Rural Village in Thailand

OBJECTIVE : To determine if flies are important disseminators of enteric pathogens in a rural village in Thailand.

PROGRESS : The village of Ban Pong in northeastern Thailand was studied from January through December 1981 to determine the importance of flies as sources of enteric pathogens. The number of flies, that were predominantly *Musca domestica*, increased in kitchens and animal pens in the hot dry spring at a time of the year when the incidence of diarrhea was highest in the village. Enterotoxigenic *Escherichia coli* (ETEC), *Shigella*, non-O1 *Vibrio cholerae* and *Vibrio fluvialis* were isolated from 69 percent of fly pools from yards, 38 percent from animal pens, 35 percent from bathrooms, and 8 percent from kitchens. ETEC were isolated from one fly pool in May and another in June when the incidence of ETEC infections was highest in the village. Flies often carry and presumably disseminate enteric pathogens in rural Thailand.

FUTURE OBJECTIVE : Study completed.

14. Immune Dysfunction in Malaria : A Biochemical Approach

PROBLEM : Hereditary deficiency of the purine enzyme, adenosine deaminase is associated with severe combined immunodeficiency disease (SCID) - a condition in which both T- and B-lymphocyte function is impaired. Partial restoration of lymphocyte function can be achieved in SCID patients by enzyme replacement therapy involving whole blood or packed RBC transfusion. It thus appears that the ADA in normal RBC is sufficient to correct in part the purinogenic defect in ADA deficiency lymphocytes. The precise mechanism for this effect is not understood although it may involve the role of the red cell mass in systemic adenosine metabolism in a way that influences purine metabolism in lymphocytes. Acute human malaria infection is characterized by immune suppression. In particular there is a decreased functional responsiveness of mononuclear cells - especially T-lymphocytes. It is also known that the intraerythrocytic malaria parasite produces major changes in the purine metabolism of the host red cell mass.

Adenosine deaminase (ADA) was shown to be greatly increased in acute *P. falciparum* malaria. The parasite ADA enzyme was isolated and characterized. A high level of ADA activity in the peripheral blood would be predicted to perturb purine homeostasis with consequences for host lymphocytes. A defect in cyclic nucleotide (cAMP) metabolism has been identified in mononuclear cells from patients with acute malaria. The defect involves reduced levels of endogenous cAMP and decreased adenylate cyclase activity. An adenosine receptor has been shown to be associated with adenylate cyclase on the surface of lymphocytes. When adenosine combines with the surface receptor adenylate cyclase is activated and cAMP is produced. In malaria there appears to be a defect in the coupling mechanism such that cAMP production is depressed. Work is currently underway to investigate the mechanism for the cAMP defect. Additionally work is being done to test whether the immunopotentiator, isoprinosine, can reverse the cAMP defect. Studies involving monoclonal antibodies are also underway to identify which specific lymphocyte subsets are

associated with the biochemical defect.

RECOMMENDATION : These studies suggest what may be a biochemical correlate of immune dysfunction in human malaria infection. The perturbation in erythrocyte adenosine metabolism caused by the malaria parasite may produce a defect in lymphocyte purine metabolism which renders this cell functionally defective. Work is currently underway to confirm these preliminary observations in a larger population study and to elucidate specific lymphocyte subsets. Specific emphasis is being given to studies on malaria lymphocyte adenosine metabolism, adenosine receptors and cyclic nucleotides.

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