รายงานวิจัยฉบับสมบูรณ์

โครงการพยาธิสรีรวิทยาของโรคที่พบบ่อยในประเทศไทย

PATHOPHYSIOLOGY OF DISEASES PREVALENT IN THAILAND

โดย นายแพทย์ปรีดา มาลาสิทธิ์ และคณะ

กันยายน 2542

สัญญาเลขที่ RTA/04/2539

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

กลุ่มวิจัยฯ ขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย ที่ให้การสนับสนุนเครือข่ายซึ่งร่วมทำวิจัยศึกษาชีววิทยาการแพทย์พื้นฐานของกลุ่มโรคที่พบบ่อยในประเทศ ขอขอบคุณคณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ที่ให้การสนับสนุนหน่วยอณูชีววิทยาการแพทย์ สถานส่งเสริมการวิจัย ได้มีโอกาสใช้ศักยภาพของนักวิจัย ห้องปฏิบัติการ ตลอดจนเครือข่ายงานวิจัยของคณะฯ ทำให้งานวิจัยดำเนินไปอย่างมีประสิทธิภาพ ขอขอบคุณผู้ร่วมวิจัยทุกท่านที่มีฉันทะและวิริยะร่วมมือช่วยเหลือในการศึกษาวิจัยและถ่ายทอดความรู้ให้กับนักศึกษาในโครงการต่างๆ ทำให้เครือข่ายขยายตัวอย่างมีประสิทธิภาพ

อนึ่ง งานทั้งหมดจะสำเร็จมิได้หากไม่ได้รับความร่วมมือจากคณะแพทย์และพยาบาลที่ดูแลกลุ่มผู้ป่วยไข้เลือดออก กลุ่มผู้ป่วยโรคไตเสียสมรรถภาพในการขับกรด (Renal Tubular Acidosis) และกลุ่มอื่นๆ ในโรงพยาบาล จ.ขอนแก่น จ.นครพนม และ จ. อุบลราชธานี

คณะผู้วิจัยขอขอบคุณทุกท่านที่ให้ความร่วมมือช่วยเหลือทำให้งานวิจัยนี้ดำเนินลุล่วงไปได้ด้วยดี

พฤศจิกายน 2542

1. ABSTRACT

Project Code: RTA 04/2539

Project Title: PATHOPHYSIOLOGY OF DISEASES PREVALENT IN THAILAND.

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Project Period: 1996-1999

OBJECTIVES

To operate a network of bio-medical research aiming at the study of the pathogenic mechanisms of diseases prevalent in the country. Three areas include:

1. Pathophysiology of a group of prevalent diseases in the Northeast of Thailand: Renal Tubular Acidosis, Lai-Tai, and Renal Stone.
2. Dengue virus infection and a newly discovered Densonucleosis viruses in indigenous mosquitoes.
3. Pathophysiology and complications associated with Thalassemia

HIGHLIGHTS OF THE RESULTS:

1) Discovery of a new genetic mechanism causing distal renal tubular acidosis found in group of children in Songkla and Khonkaen; related to the anion exchanger 1 (AE1) gene mutations with and without association with Southeast Asian Ovalocytosis (SAO).
2) Establishment of families with adult type of endemic renal tubular acidosis (eRTA) in Khonkaen and Ubolrajathanee ready for the identification of gene(s) responsible for the pathogenesis of one of the common endemic diseases - eRTA, either by candidate gene or genome screening techniques.
3) Discovery of a defective protein, nephrocalcin, that might contribute to the pathogenesis of renal stone in Isan.
4) Delineate a new mechanism that might play a key role in complement activation and generation of phlogistic cytokines and chemokines responsible for the vascular leakage in dengue hemorrhagic fever.
5) Describe the molecular evolution of dengue serotype 2 circulated in Bangkok and establish technology for molecular subtyping of the viruses.
6) Discovery of a new mechanism initiated by defective red cells from thalassemia patients, the generation of erythrocytic microvesicles, that might be the key factor leading to the increased susceptibility to infection in these patients.
7) Define renal function abnormalities in patients with thalassemia.

SIGNIFICANCE:

A network of scientists conducting basic bio-medical research on medical problems prevalent in the country and the region has been established. The program has resulted, at the time of report, in more than 10 publications, all in international journals; the program has assisted in establishing a network of efficient academic programs producing graduate students, a network of international research and academic collaborations, and a number of field sites with patients' databases. Most important of all, the group has established keystones in the fields of particular problems that would eventually lead to better understanding of the pathogenic mechanisms, more publications and eventual diagnoses, treatments and prevention of a group of diseases affecting large number of population.

KEYWORDS: dengue hemorrhagic fever, renal tubular acidosis, renal stone, Thalassemia

1.บทคัดย่อ

รหัสโครงการ	RTA 04/2539
ชื่อโครงการ	พยาธิสรีรวิทยาของโรคที่พบบ่อยในประเทศ
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วัตถุประสงค์

เพื่อสร้างเครือข่ายการวิจัยทางชีววิทยาการแพทย์ โดยมุ่งศึกษากลไกพยาธิกำเนิดของโรคที่พบบ่อยในประเทศ ซึ่งครอบคลุม 3 ขอบข่าย ได้แก่

1. พยาธิกำเนิดของกลุ่มโรคที่พบบ่อยในภาคตะวันออกเฉียงเหนือของประเทศไทย กล่าวคือ โรคไตเสียสมรรถภาพในการขับกรด โรคไหลตาย และโรคนิ่วในไต
2. การติดเชื้อไวรัสไข้เลือดออกและไวรัสเดนโซ่ ในยุง
3. พยาธิกำเนิดของภาวะแทรกซ้อนของโรคธาลัสซีเมีย

ผลการศึกษาวิจัยที่สำคัญ

1. ค้นพบกลไกทางพันธุกรรมแบบใหม่ที่เป็นสาเหตุของภาวะไตเสียสมรรถภาพ (RTA) ในการขับกรด ชนิดที่พบในกลุ่มคนไข้เด็ก ในจ.สงขลาและ จ.ขอนแก่น ความผิดปกติของยีน AE 1 เกิดร่วมกับหรืออิสระจากภาวะเม็ดเลือดแดงผิดปกติ Southeast Asian Ovalocytosis (SAO)
2. สามารถรวบรวมกลุ่มผู้ป่วยและครอบครัวที่เป็นโรค RTA ในภาคอีสาน (จ.ขอนแก่นและ จ.อุบลราชธานี) พร้อมที่จะศึกษาหายีน ที่เป็นสาเหตุของโรค โดยใช้เทคนิควิธี candidate gene หรือ genome screening
3. ค้นพบความผิดปกติของโปรตีน nephrocalcin ในปัสสาวะ ซึ่งอาจเป็นสาเหตุของนิ่วในไตของคนอีสาน
4. ค้นพบกลไกใหม่ ซึ่งอาจจะมีบทบาทที่สำคัญในกระบวนการกระตุ้นการทำงานของ complement และสร้าง cytokines กับ chemokines ที่ทำให้เกิดการรั่วของเลือดในโรคไข้เลือดออก
5. ทราบถึงวิวัฒนาการระดับอณูของเชื้อไวรัสไข้เลือดออก serotype 2 ที่แพร่กระจายในกรุงเทพฯ และประสบความสำเร็จในการพัฒนาเทคนิคสำหรับ molecular subtyping ของไวรัส
6. พบกลไกใหม่ซึ่งเกิดจากความผิดปกติของเม็ดเลือดแดงของผู้ป่วยโรคธาลัสซีเมีย เม็ดเลือดแดงของคนไข้จะสร้าง erythrocytic microvesicles ที่มีคุณสมบัติกระตุ้นเอนไซม์หลายชนิดซึ่งอาจเป็นปัจจัยสำคัญที่ทำให้ผู้ป่วยมีความไวต่อการติดเชื้อมากขึ้น
7. สามารถสร้างฐานความรู้ที่เกี่ยวข้องกับความผิดปกติของระบบการทำงานของไตในผู้ป่วยธาลัสซีเมีย

ความสำคัญ

คณะผู้วิจัยได้ประสบความสำเร็จในการสร้างเครือข่ายของนักวิจัยวิทยาศาสตร์การแพทย์พื้นฐานที่ร่วมกันศึกษาพยาธิกำเนิดของโรคต่างๆ ที่เป็นปัญหาและพบมากในประเทศ นอกจากนี้ยังมีผลงานลงตีพิมพ์ในวารสารต่างประเทศมากกว่า 10 เรื่อง กอรปกับได้ช่วยเชื่อมโยงหลักสูตรวิทยาศาสตร์การแพทย์พื้นฐานระดับปริญญาโทและเอก และได้ผลิตบัณฑิตหลายรุ่น ที่สำคัญคือการค้นพบข้อมูลใหม่ๆ ซึ่งสามารถนำไปประยุกต์ใช้ อันจะมีผลกระทบโดยตรงต่อการวินิจฉัยป้องกันและรักษาโรคเหล่านั้นให้ดียิ่งขึ้นในอนาคต

คำสำคัญ

Dengue hemorrhagic fever	โรคไข้เลือดออก
Renal tubular acidosis	ภาวะไตเสียสมรรถภาพในการขับกรด
Renal stone	นิ่วในไต
Thalassemia	โรคธาลัสซีเมีย

2. EXECUTIVE SUMMARY

2.1. OBJECTIVES

To set up an efficient network of research collaboration, focusing on diseases prevalent in the country and the region, aiming at obtaining data that enhance the understanding of the pathogenetic mechanisms which eventually would lead to better diagnosis, treatment and prevention of the diseases. The research activity will be used as a base from which efficient academic activity can be carried out and a network facility of clinical research established. The key areas are:

1. Pathogenic mechanisms of a group of prevalent diseases: Renal Tubular Acidosis, Lai-Tai, and Renal Stone.
2. Dengue virus infection and a newly discovered Densonucleosis viruses in indigenous mosquitoes.
3. Pathophysiology and complications associated with Thalassemia

2.2. SUMMARY OF PROJECTS, BREAKTHROUGHS AND ACHIEVEMENTS IN EACH KEY TOPIC

a. Pathogenic mechanisms of a group of prevalent diseases:

i. Relationships between the AE1 or band 3 gene, Southeast Asian Ovalocytosis and RTA; and the pathogenesis of endemic renal tubular acidosis (eRTA) prevalent in Isan

Our team has discovered a set of new genetic mutations of a gene – the *AE1* or Anion Exchanger I gene, that cause distal renal tubular acidosis, in patients with and without association with a relative common erythrocytic condition, particularly in the south of Thailand – the Southeast Asian Ovalocytosis (SAO). It is known that the mutation of the *AE1* gene is responsible for the SAO, but our findings further extend the pathogenic role of the gene to include RTA. The novelty of our findings is the description of a recessive type of RTA caused by compound heterozygosity of the mutated gene. One scientific paper (*Am J Kidney Dis* 1999;33(6):1147-52) and another (*Kidney International*) has been accepted for publication. Another manuscript is in preparation.

The findings are highly significant. Not only the mutated *AE1* is found in the south, but similar patients are also found in Isan – an area believed to be free of SAO. At least two mutations had been found to be associated with RTA, it is therefore expected that more mutations might be found. These findings have opened up new possibility of investigating the pathogenic mechanisms leading to red cell and renal tubular defects, and the structural/functional relationships of the defective proteins and their interactions with other proteins. Lastly, if these gene defects are found in near future to be polymorphic, what are the factors that are responsible for the high frequency. Do these defective genes convey any advantage to the hosts, or is malaria re-

sponsible for the high frequency, are two obvious questions that need more investigations.

As for the problem of eRTA, we have established families with adult type of eRTA in Ubolrajathanee and Khonkaen ready for the identification of gene(s) responsible for the pathogenesis, by the technique of candidate gene or genome screening. This will serve as a base of future study of this group of endemic diseases.

ii. Role of nephrocalcin in the pathogenesis of renal stone in Isan.

Nephrocalcin is a urinary acidic glycoprotein with a strong inhibitory activity for calcium oxalate crystal formation in urine. Abnormal isoforms had been identified in patients with recurrent stones in the U.S.A.. We have concluded a project investigating the characteristics of nephrocalcin, in urine of a group of patients in Ubolrajathanee. In essence, we have found that the phenotypes of the nephrocalcin isolated from patients who had renal stone and/or renal tubular acidosis were of the phenotypes found associated with stone former, and they enhanced the formation of crystals, rather than prevention in *in vitro*. These findings are significant since it is the first time that a pathogenic factor has been identified to be associated with renal stone, one of the commonest surgical condition prevalent in Isan, causing considerable morbidity to young adult population.

Current ongoing investigations include the development of specific antibodies, including monoclonals, against the protein which could be used in diagnostic tests that can identify the different phenotypes of the proteins in urine of patients and to identify the gene encoding the proteins. The gene for this protein has not yet been identified.

b. Dengue virus infection and densonucleosis viruses in mosquitoes

i. New mechanisms in the pathogenesis of shock and leakage in dengue hemorrhagic fever and/or dengue shock syndrome (DHF/DSS)

Complement activation is one of the hallmarks of DHF/DSS and its degree of activation correlates with the degree of shock and leakage, suggesting that the process might be involved in its pathogenesis. Mechanism involved in the activation is still unknown. We have completed a study (*J Immunol* 1998;161(11):6338-46.) showing that dengue infected cells expressed dengue antigens on their surfaces and in the presence of specific antibody, activated complement via both pathways. We also showed that human endothelial cells are permissive to the dengue virus; the infection leads to production of phlogistic chemokines and cytokines and eventually to apoptosis. We have also demonstrated that large amount of chemokines and cytokines are produced in both the plasma and pleural fluid of severe cases of DHF. Complement activation, chemokine induction, and apoptosis may act in concert to cause fulminant vascular leakage in DHF.

We have also shown that human monocytes (manuscript supplied), keratinocytes, fibroblasts are also permissive to dengue virus infection and the infection, in case of monocytes, will trigger the release of chemokines and cytokines and induce apoptosis.

The data have opened up new research possibilities, areas and research questions such as how dengue virus switch on the production of chemokines/cytokines and apoptosis, do these processes depends on the virus – ie. Virus virulence, does the process happen in the pleural and peritoneal cavities which are key sites of leakage in DHF. These are some of the investigations currently carried out by our team and collaborators.

ii. Dengue viral subtypes and the evolution of the viruses in Bangkok.

One of our key investigators in the network, Dr Nopporn Sittisombut, had devised a system that enable the detailed sub-typing of dengue 2 viruses (manuscript supplied). The team had applied the technology to study the evolution of dengue virus serotype 2 circulating in Bangkok from the year 1980-1994. The results indicated that subtype IIIa predominated over subtype IIIb during both 7-year periods. Yearly comparison indicated that dominance of subtype IIIa was maintained for upto ten consecutive years (1980-1989) with some fluctuations, and the subtype IIIb was not detected after 1992. The findings reveal contrasting long-term changes which can occur in dengue virus sub-populations in nature and are consistent with the proposed occurrence of genetic bottleneck of dengue serotype 2 viruses in Bangkok.

iii. Densonucleosis virus or densovirus (DV) in indigenous mosquitoes

Our group had been able to set up facilities to identify and culture group of densoviruses. We have successfully isolated viruses from *Aedes* cell lines and mosquitoes found in indigenous mosquitoes in and around Bangkok. The virus – densovirus, is a small, spherical, non-enveloped, single-stranded DNA, icosahedral virus within the *Parvovirus* family. The viruses are known to specifically infect mosquitoes of the genera *Aedes, Culex* and *Culiseta*. The findings are significant since the viruses can potentially be used for biological mosquito control, biotechnological production of proteins and peptides and introduction of genetic materials to mosquitoes.

c. Thalassemia

i. Autoantibodies to thalassemic red cells and surface complement activation by erythrocytes from patients with beta- thalassaemia/HbE disease.

We have redefined the presence of autoantibodies to red cells from patients with beta-thalassemia/HbE disease and have found that apart from the presence of large number of antibody molecules on their surfaces, we have also found complement components, including poly-C9, associated with the circulating cells. These results constitute the first demonstration that circulating diseased erythrocytes may carry low numbers of potentially cytolytic C5b-9 complement complexes which may be partly responsible for the known ionic disturbances found in thalassaemic cells. Both bound C3 and C5b-9 could promote removal of diseased cells in the reticuloendothelial system.

ii. Vesiculation of thalassemic red cells: possible mechanism leading to complement activation and thrombo-embolic complications.

We have discovered (manuscript supplied) a novel mechanism that would explain why chronic complement activation, susceptibility to broad range of infections and thrombo-embolic complications occur in thalassemic patients. We have demonstrated that red cells from these patients "vesiculate" from their surfaces, producing small vesicles containing hemoglobin into the circulation. These vesicles had surfaces that efficiently activate the complement via the alternative pathway and were strongly pro-coagulant, consuming coagulation proteins. Chronic complement activation would result in low levels of complement components, and a state of acquired complement deficiency, hence the increased susceptibility to infection. The finding is one of the important missing links between the abnormal hemoglobin synthesis and a group of clinical abnormalities that cause serious morbidity and mortality in patients with thalassemia.

iii. Renal defects in patients with thalassemia.

We have defined (*Nephron* 1998;78(2):156-61, *Pediatr Nephrol* 1998;12(4):280-3) renal functional abnormalities in both adult (95 patients) and pediatric (104 patients) beta thalassemia patients and found that proximal tubular defect is the common functional abnormality. No glomerular defect was found. Severity of the abnormalities correlated with the degree of anemia and were least severe in patients on hypertransfusion and desferrioxamine therapy. This suggested that the damage might be caused by anemia and increased oxidation induced by excess iron deposits.

d. Graduate studies

From 2539 to 2542 (1996-9) the Medical Molecular Biology Unit has enrolled 16 new master and 4 Ph.D. students. 5 M.Sc. and 2 Ph.D. students graduated during the period. Other 2 students (1 M.Sc.- Miss Rungtawan with Dr Nopporn, and 1 Ph.D – Dr Peti with Prof. Prapon) graduated from collaborators in the network.

e. The Network

The support has strengthened the research activity and graduate studies at the Medical Molecular Biology Unit (MMBU), Office for Research & Development, at the Faculty of Medicine, Siriraj Hospital, Mahidol University. Within the Medical Faculty, it has encouraged research groups including: the Renal Units, Dept. of Medicine, and Pediatrics. The MMBU also functions as the Medical Biotechnology Unit of the National Center for Genetic Engineering and Biotechnology (BIOTEC) and collaborations with the BIOTEC staffs within the unit also are enhanced.

Within the Mahidol University, collaborations with the Dept. of Biochemistry and the Dept. of Biology, the Faculty of Science and the Dept. of Entomology, Faculty of Tropical Medicine, has resulted in an expanded work on RTA, Thalassemia and densonucleosis viruses. The network has expanded to include sites in provincial universities and hospitals including: the Dept. of Microbiology, Faculty of Medicine, Chiang Mai University, the Dept. of Pedi-

atrics, Khon Kaen University, Khon Kaen Hospital, Ubolrajathanee Hospital (Salprasit Prasong), Nakorn Panom Hospital, and Prince Songkla University (Depts. of Medicine, Pediatrics and Pathology).

International collaborations include: the Institute for Medical Microbiology and Hygiene, the Johannes Gutenberg University of Mainz, Germany; Department of Medicine, University of Chicago, U.S.A.; the department of Virology I, the National Institute of Infectious Diseases (NIID), Tokyo, Japan and the MRC Human Immunology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford University, Oxford, U.K..

3. PROJECTS, RESULTS, SIGNIFICANCE

3.1. Pathogenic mechanisms of a group of prevalent diseases:

3.1.1. Relationships between the AE1 or band 3 gene, Southeast Asian Ovalocytosis and RTA; and the pathogenesis of endemic renal tubular acidosis (eRTA) prevalent in Isan

Background

The Renal Unit of the Dept. of Medicine, Siriraj Hospital, under the guidance of Prof. Sanga Nilwarangkur and Prof Sumalee Nimmannit, has been conducting studies on the clinical manifestations, natural evolution, and pathogenesis of a group of endemic renal tubular acidosis (eRTA) prevalent in Isan since 2527(1984).[1-4] A group of patients who lived around Ubolrajathanee with distal renal tubular acidosis (dRTA) had been extensively studied and reported.[2] Acidification failure had also been found to be common in villagers around Khon Kaen and the prevalence was found to be around 3%.[4] In the same area, endemic hypokalemic periodic paralysis, sudden unexplained nocturnal death syndrome (SUNDS) and renal stone were also found to be common. Pathogenesis of the eRTA is not known but potassium deficiency from low nutritional intake has been proposed as one of the possible mechanisms.[3] Some patients with eRTA had been found to occur in families with varied pattern of inheritance.[2]

The Studies

$Cl^-/HCO3^-$ exchangers or band 3 protein that are expressed both in the erythrocytes and in the acid-secreting intercalated cells of the kidney is encoded by the *AE1* gene. Mutations of the *AE1* gene were found to be associated with dominant type of dRTA.[5-7] Since one of the mutations of the *AE1* gene is commonly found in the Southeast Asian and Melanesian region, causing a purely erythrocytic disease – the Southeast Asian Ovalocytosis (SAO), we therefore started to investigate patients with SAO in Prince Songkla University. Collaborating with the Departments of Medicine and Pediatrics of the Faculty of Medicine, we studied 20 individuals with SAO and two subjects including their families, with both SAO and dRTA. Renal acidification in the 20 individuals with SAO and in the parents of the two families was normal. However, the two clinically affected individuals with SAO and dRTA had compound heterozygosity of 27-bp deletion in exon 11 and missense mutation G701D resulting from CGG→CAG substitution in exon 17 of the *AE1* gene. Red cells of the two subjects with dRTA and SAO and the family members with SAO showed ~40% reduction in sulfate influx. These findings suggest that compound heterozygosity of abnormal *AE1* genes cause autosomal recessive dRTA in

SAO. This study has been accepted for publication in the *Kidney International.*

The team has also studied patients with dRTA in Songkla and defined their clinical manifestations.[8] Dr. Peti Thuwachit, a Ph.D. student attached to Prof. Prapon Wilairat, working together with our group, has also found another combination of SAO an dRTA and a new missense mutation A602H in exon 15. The study is a part of Dr Peti's Ph.D. thesis. Dr Peti graduated in 2542 (1999) and a manuscript is in preparation.

Patients with dRTA found associated with SAO in Songkla could be separated into two broad groups, one manifested early during childhood and other whose onset occurred during adulthood. The former had as recessive inheritance and showed strong association with mutations of the *AE1* gene. So far, no AE1 abnormality other than those known to be associated with the SAO has been found in the adult type.

The role of the AE1 gene in the pathogenesis of eRTA in Isan has been investigated. Collaborating with Dr Sukachart Kerdphol of the Dept. of Pediatrics, Khonkaen University, who had studied families of pediatric patients with dRTA, we are investigating the AE1 abnormalities in this group of patients. 7 families with RTA were found. Preliminary data showed two families with three patients with homozygous G701 mutations, similar to the patients reported by Dr. Worawan Tanphaichitr *et al.*.[9] Detail clinical studies of these patients are currently being conducted aiming at further defining the clinical manifestations of the homozygous G701D mutation. Dr.Worawan Tanphaichitr of the Dept. of Pediatrics, Siriraj Hospital, collaborating with Dr. Chairat Shayakul of the renal unit and Prof. Seth Alper of the Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center, MA, U.S.A., had reported two pediatric patients with homozygous G701D. The patients, who were also homozygous HbE/E, had dRTA and mild anemia with xerocytic type of hemolytic anemia. Using *in vitro* expression of the mutated cDNA and wild-type cRNA in *Xenopus* oocytes, they have demonstrated a loss-of-function of the AE1 protein that can be rescued by co-expression of the erythroid chaperonin protein, glycophorin A. It was not known how much erythrocytic manifestations was contributed by the G791D since the patients also had abnormal hemoglobin.

Relevance and Significance

It is generally believed that SAO is a condition prevalent in the southern part of the country and is relatively rare in Isan. The fact that patients with abnormal AE1 genes were found in the Khon Kaen area in our study, this has brought up important questions related to Isan: how frequent are these abnormalities in the area, is the SAO more prevalent than what we believed, are these mutations polymorphic, what advantage that these heterozygosity conveys? It is therefore imperative that a study of polymorphism of the AE1 mutations in population of Isan and the South should be conducted. Our study has opened another area of investigations relevant to health problems of the country.

Our studies of dRTA associated with the AE1 mutations are in fact a tip of the iceberg of a much larger problem of adult form of eRTA found endemic in Isan. Sumalee Nimmannit *et al.* had reported that 2.8% of the population in 5 villages within the Khon Kaen province failed to acidify their urine after acid loading.(4) So far we still have not been able to define the genetic abnormalities associated with the eRTA but the studied population will serve as the best research field from which both clinical and molecular genetics studies can be efficiently conducted. Together with the databases of a number of large families with well defined patients with dRTA in Ubolrajathanee and other well defined adult patients with spectrum of RTA, we plan to systematically conduct studies aiming at unravelling the pathogenic mechanisms leading to these important public health problems. We are confident that the studies will lead to better diagnosis, treatment and prevention of these prevalent conditions.

Future Research

Our current investigation and future plan include the systematic studies of the polymorphism of the mutated *AE1* genes in the populations of different part of the country, the new mutations associated with erythrocytic and renal tubular acidosis, the relationships between clinical phenotypes and molecular genotypes, in vitro studies of the molecular functional-structural relationships, chaperoning or other proteins that might influence the in vivo expression of the proteins.

Applications of modern molecular genetics to the established population, families of patients in Isan and also in Songkla will be emphasised. We plan to build a collaborative network, including the basic scientist teams from the Faculty of Science (Biochemistry- Prof Prapon Wilairat, Physiology – Dr Samaisuk Sopasan) and the Faculty of Medicine, Ramathibodi Hospital (Prof. Rajata Rajatanavin), each working in defined area, sharing common goals – better understanding the pathogenetic process and better care for patients and ultimately prevention.

3.1.2. Role of nephrocalcin in the pathogenesis of renal stone in Isan.

Background and the Study

In 1978, Prof. Yasushi Nakagawa of the Dept. of Biochemistry, University of Chicago had isolated and characterized a urinary acidic glycoprotein and showed that the protein is amongst one of the strongest inhibitors of calcium oxalate crystal formation in urine.(10) Abnormal isoforms had been identified in patients with recurrent stones in the U.S.A..(11) Collaborating with Prof. Nakagawa, Dr. Vipada Chaovakul at the Ubolrajathanee Hospital and the renal unit, Siriraj Hospital, a project investigating the characteristics of nephrocalcin in urine samples from a group of patients with renal stone (with and/or without renal tubular acidosis) in Ubolrajathanee has been concluded. A manuscript has been enclosed with this report.

Urine nephrocalcin isolated from 15 patients with RTA and/or renal stone from Ubolrajathanee hospital have been found to have isoforms similar to

found in "stone formers" as previously defined.[11] The isoforms have been isolated and tested against an *in vitro* system to measure their ability to inhibit calcium oxalate crystal growth. Some of the isoforms isolated from 4 patients showed the ability to accelerate the crystal formation rather than the usual inhibition; the isoforms also showed weaker binding capacity to calcium oxalate monohydrate as indicated by significantly lower dissociation constant to the crystal. Amino acid analysis showed different composition, with less aspartic acid, higher amount of hydrophobic amino acids Ile, Leu, and Val. And hydroxy amino acid residues (Ser and Thr). The reason for these amino acid composition difference is not known but it either indicates genetic factor(s) or phenotypic changes of the protein from some still unknown environmental factors.

Significance and Future Research

The work at this phase is still preliminary. This is the first time that a pathological factor has been identified to be associated with renal stone, a condition of public health importance prevalent in Isan. It remains to be proven that the defective protein is the major cause of prevalent renal stone. But the work has opened a still unexplored field of urinary protein inhibitors as the key contributory factor to the pathogenesis of renal stone. Attempts of finding pathogenic factors in the past had been disappointing since most of the researches focused on the roles of increased urinary excretion of calcium and/or magnesium, which have been proven to be uncommon in Isan.[1] More research should be carried out to investigate the relationships between the protein phenotypes and the frequency or susceptibility to stone formation. Technology has to be developed to simplify the phenotyping of the protein. The following is the summary of the plan:

- Production of nephrocalcin specific monoclonal antibodies. This would facilitate the phenotyping process of the proteins and allow the gene encoding the protein to be identified. This will be carried out collaboratively with Prof Y Nakagawa and Dr Watchara Kasinrerk of the Dept. of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University.
- Development of phenotyping technique of nephrocalcin by the combination of isoelectric focusing technique and western blot analysis, using the monoclonal antibodies. Application of the technique to study the association between renal stone and nephrocalcin phenotypes.
- Cloning the nephrocalcin gene.

Current and future investigations in this theme of research will be conducted with collaborations from two main laboratories and two clinical sites: The Dept. of Biochemistry, University of Chicago (Prof. Y Nakagawa), Dr. Watchara Kasinrerk, Chiang Mai University, Ubolrajathanee and Khon Kaen Hospitals (Dr. Vipada Chaowakul and Dr. Wattanachai Susaengrat). One PhD/MD student will take on this project (Mr Somchai Chutipongtanate).

3.2. Dengue virus infection

3.2.1. New mechanisms in the pathogenesis of shock and leakage in dengue hemorrhagic fever and/or dengue shock syndrome (DHF/DSS)

Background and the Studies

Clinical hallmarks that distinguish dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) from the less severe form of dengue fever (DF) are vascular leakage and hemorrhagic diathesis. DHF/DSS occurs almost exclusively in patients experiencing secondary immune response suggesting an immunological basis for the pathogenesis. Vascular leakage occurs predominantly in serous spaces, particularly in pleural and peritoneal cavities. Pathogenic mechanisms responsible for the leakage and hemorrhagic diathesis were not known. We have completed a study showing that dengue infected cells expressed dengue antigens on their surfaces and in the presence of specific antibody, activated complement via both pathways.[12] We also showed that human endothelial cells are permissive to the dengue virus; the infection leads to production of phlogistic chemokines and cytokines and eventually to apoptosis. We have also demonstrated that large amount of chemokines and cytokines are produced in both the plasma and pleural fluid of severe cases of DHF. Complement activation, chemokine induction, and apoptosis may act in concert to cause fulminant vascular leakage in DHF. Dengue virus infection was accompanied by the activation of NF-κB. Pretreatment of endothelial cells with pyrrolidinedithiocarbamate, an inhibitor of NF-κB activation, prevent apoptosis but not infection.

In addition we have also demonstrated that human keratinocytes were shown to be highly susceptible to infection by dengue virus. Infection also led to the production of infectious virions and inflammatory cytokines, including TNF-α, GMCSF, MCP-1, IL-1α, IL-6, and IL-8. In comparison to human skin fibroblasts, dengue virus-mediated cytotoxicity in terms of cell death was not observed in keratinocytes, while infection of fibroblasts progressively led to the death within only 24 hours. Keratinocytes therefore are likely to be a reservoir for dengue virus replication in the skin after inoculation by infected mosquito (manuscript in preparation).

Dengue infection of monocytes has also been investigated.(manuscript supplied) Infection of monocytes led to early surface expression of non-structural protein (NS1) followed by the envelope protein. This is in contrast with the infection in mosquito cells (C6/36) only NS1 was expressed . In the presence of anti-dengue antibodies, DV infected monocytes activated complement efficiently via both classical and alternative pathways and resulted in deposition of non-lytic C5b-9 complexes on their surfaces and soluble SC5b-9 in the media. Degree of activation correlated with the amount of the antibody. Results of the present study have provided addi-

tional information on the possible mechanisms responsible for active complement activation as found in DHF/DSS.

Significance and Future Research

Our investigations centre on the theme of the mechanisms of shock, leakage and the interactions of the dengue virus with the infected cells. Our findings had opened new areas of research and also the continual basic research into viral pathogenesis has resulted in technology spin offs into diagnostic tests and other technology developments. The following is the summary:

- It is possible that the processes of endothelial damage might happen in vivo at few special sites mainly the pleural and peritoneal spaces. This needs further confirmation by systematic pathological study – identification of virus or viral products and genes, expression of cytokines and chemokines in autopsied or necropsied tissues by using dengue specific monoclonal antibodies and specific oligonucleotide or peptide nucleic acid probes. The kinetics of phlogistic cytokines in pleural and/or ascitic fluids will also be studied. These investigations are currently being conducted in our laboratory.
- Our laboratory, collaborating with Dr. Vincent Deubel, Pasteur Institute, Paris, will investigate the characteristics and roles of the dengue NS1 protein. The followings are the key aims: complement activating capability of the dengue NS1 protein and its correlation with disease severity, development of technology to detect the protein in patients plasma aiming at using the technology in diagnostic and prognostic tests.
- Collaborating with Prof. Ichiro Kurane of the National Institute of Infectious Diseases, Tokyo, we shall identify dengue peptides recognized by T memory cells in Thais experiencing recent dengue infection. This will give essential data that will be applicable to the design of next generation of dengue vaccine.
- Collaborating with Prof. Adrew McMichael and one of our staffs, Dr Juthathip Mongkolsapaya at the Institute of Molecular Medicine, John Ratcliffe Hospital, Oxford, U.K., a project investigating the molecular mechanisms leading to dengue virus induced apoptosis will be set up and one of the Kanjanapisek students, Miss Wanwisa Dejnirattisai will join the team in Oxford for this investigation.

3.2.2. Viral subtypes and the evolution of dengue virus in Bangkok.

Background and the Research

Nothing is known about the evolution of the dengue viruses circulation in Bangkok since the first outbreak in the sixties. One of our key investigators in the network, Dr Nopporn Sittisombut, had devised a system that enable the

detailed sub-typing of dengue 2 viruses,[13] and applied the technique to study the evolution of dengue virus serotype 2 circulating in Bangkok from the year 1980-1994 (manuscript supplied). During the period subtype IIIa predominated over subtype IIIb; dominance of subtype IIIa was maintained for upto ten consecutive years (1980-1989) with some fluctuations. Within the subtype IIIa, two E protein variants, E346 Tyr and E346 His, co-circulated during 1980-1984 with wide fluctuations. From 1985 onward, E346 Tyr variant rapidly replaced the E346 His counterpart leading to the latter's absence during 1987-1994. Changes of the E346 variants correlated with decline of serotype 2 proportion among dengue isolates derived mainly from hospitalised cases. Thus, two subtypes of dengue serotype 2 co-circulated in Bangkok for at least a decade despite continued dominance of one subtype over the other. Within the dominant subtype, drastic change in the relative abundance of E protein variants leading to complete replacement of a variant took place within a few years period. These contrasting long-term changes of dengue serotype 2 subpopulations are consistent with the proposed occurrence of genetic bottleneck of subtype IIIa in Bangkok between 1980-1987.

Significance and Future Research

The basic technology developed by Dr. Nopporn can be the prototype of similar tests designed for other dengue serotypes, suitable to use to study to subtypes evolution of the viruses circulating in Thailand. The understanding of the evolution of the viruses and also the association of the subtypes with clinical severity will give a important insights into how the viruses spread and changes and also serve as a basis to investigate the role of virus virulence in the pathogenesis of DHF. Current and future activities include the transfer of technology to other laboratories, in particular the Arbovirus Unit of the Dept. of Medical Sciences (Thai NIH), the Ministry of Public Health and apply to the virus samples systematically collected from peripheral hospitals.

i. Other dengue virus research projects– developments of diagnostics (activities funded by BIOTEC.

Background and Current Research Activities

Our basic research into the pathogenesis of dengue virus has given us opportunity to develop applications and tools useful for dengue diagnosis and in near future – prognosis.

We have developed an efficient RT-PCR technique for the detection and identification of the dengue virus gene segments in biological specimens and have evaluated the test against three other techniques (Henchal, Morita, Lanciotti). A manuscript is included with this report. The test showed that our and Lanciotti's test are the methods of choice. Our test has been the most sensitive (80%) and 100% specific.

Dr Nopporn Sittisombut and Dr Pathai Yenchitsomanus are currently developing a quantitative RT-PCR method to quantify the amount of dengue virus RNA in suspension, employing the ABI PRISM 7700 sequence detection system (Perkin Elmers). The developed method will be compared against the NASBA method from Organon Technika and the latter company has ex-

pressed interest in supplying us with the kit and the detection system for the purpose of comparison.

Collaborating with a Thai private biotechnology company, the laboratory has supplied purified recombinant dengue envelope antigens (produced from E coli) and appropriate monoclonal antibodies for the production of a rapid diagnostic test (for dengue specific IgG and IgM antibodies). It is expected that a prototype of rapid diagnostic test can be developed within a period of one year 2542-3 (1999-2000). We have also developed a Dot Enzyme Immuno Assay (DEIA) for the detection of IgG anti-dengue antibody, and have established a field test at Nakorn Panom hospital. Preliminary results indicated that the kit is well accepted by technicians at the hospital and the efficacy is good.

Complete analysis of the DEIA against a panel of sera collected from Na-korn Panom Hospital have been completed. The sera were from patients admitted into the hospital, suspected to have dengue hemorrhagic fever and/or dengue shock syndrome. In most cases, two sera were collected from each patients, one collected at the time of admission and other at the time of discharge; average length of stay is 2 to 5 days. Total 306 serum samples are analysed and the results of DEIA were compared with standard HI and ELISA. The test was shown to give 92-93% sensitivity and 80-82% specificity when compared with HI and IgG-ELISA respectively. Further analysis revealed that all of the samples given "false positive" when compared with either HI or ELISA (thus resulted in low specificity) were the "first" samples taken from patients whose "second" or later (at the time of discharge) samples were shown to be positive. This indicated that the DEIA's positives were indeed real positive. In other words, DEIA could detect IgG antibodies earlier in the course of disease, much more sensitive than the conventional HI or IgG-ELISA. A patent has been applied (by BIOTEC) for the production procedure of the E coli recombinant dengue envelope proteins and a manuscript titled "Production of recombinant antigens suitable for dengue diagnosis" by Puttikhunt C, Pattanakitsakul S, Yenchitsomanus P, Malasit P., is now in preparation and part of the data was presented at the Siriraj Scientific Congress on March 8-12 and the BIOTEC annual meeting, March 1999.

Present gold standard test for detecting IgG and IgM anti-dengue antibody is an ELISA technique which depends on using suckling mouse brain dengue antigens and a elaborate system of treatments of patients' sera. The technique has generally been regarded as a gold standard from most laboratories. But the technique is difficult to set up and the ELISA plates have to be pre-treated by non-immune sera, an additional chore added to the long list of procedures. Collaborating with the Arbovirus unit of the Department of Medical Sciences, the Ministry of Public Health (Dr Sutharee Rojanasuphot et al.) and Dr Ananda Nisalak of AFRIMS, a new ELISA technique has been developed. The technique was based on the use of dengue antigens derived from cell culture and a monoclonal antibody. No special treatment of the patients' sera is needed and the test gives very low background and has proven to be sensitive and easy to be set up. Up to the time of this report, we are testing a set of coded sera (with known anti-dengue titre) from AFRIMS (courtesy of

Dr Ananda Nisalak) is being tested. Preliminary data showed good correlation with the current ELISA (both IgG and IgM) and the test has shown to be more sensitive. Final report of the test and a technical paper describing the technique are in preparation. We have already extended the test to include the detection of Japanese B encephalitis. The techniques have good potential to be developed into fully automated methods and commercially viable tests. Further developments include: installation of the test at the Department of Medical Sciences, at the Ministry of Health, modify and automate the tests, distribute the test to provincial hospital laboratories and commercial development (by the NIH). The key strategy is to provide tests that can be distributed countrywide, easy to produce and maintain. This will alter the current highly centralised system of dengue diagnosis. A manuscript describing the ELISA is in preparation. This latter part of developments (rapid tests, recombinant antigens, DEIA and ELISA) is funded by BIOTEC.

Future Research & Developments

- The laboratory and network will encourage developments of dengue diagnostic tests collaboratively with private sector, aiming at assisting the establishment of professional Thai diagnostic companies. The basic developments included production of recombinant dengue antigens with suitable quality and quantity for diagnostic tests, monoclonal antibodies to different dengue proteins (E, NS1, preM and others)
- Development of Peptide Nucleic Acid Probes (PNA) for hybridisation technology.

  Collaborating with DAKO A/S (Copenhagen, Denmark – Dr. Sven Godtfredsen, Executive Vice President) we will develop PNA probes that can specifically hybridise to complementary DNA or RNA sequences in tissues, using dengue and densonucleosis viruses as prototype models. PNA has many advantages over nucleic acid probes with higher fidelity, specificity; hybridisation procedures are much simpler. Preliminary data are very encouraging.
- Research using the full length infectious molecular clones of dengue virus type 2 strain 16681 by Dr Nopporn Sittisombut

  Two full-length molecular clones of dengue virus type 2 strain 16681 were generated in Dr Nopporn's laboratory in 1998 (funded by TRF). It is planned that the laboratory will concentrate on the study of the characteristics of artificially mutated clones, focusing on two major sites – the preM-M junction and the domain III of the envelope genes. The knowledge will be essential to the developments of new generation of dengue vaccines in future and also to the understanding of the pathogenesis of dengue virus.
- Production of monoclonal antibodies via DNA immunization

  Collaborating with Dr Watchara Kasinrerk, a project aiming at producing a group of monoclonal antibodies reactive to the majority of dengue proteins (prM-M, E, C, NS1, NS2-3 complexes, NS4 and NS5) will be established. The antibodies will be produced by

mainly DNA immunization, using specially designed plasmids containing corresponding segments of dengue virus genes. Majority of the antibodies are not currently available and will be most useful in dissecting the pathophysiology of the dengue viruses in future and also in the application into new generation of dengue virus diagnosis.

3.2.3. Densonucleosis virus or densovirus (DV) in indigenous mosquitoes

Background and the Current Research Activities

Densonucleosis virus or densovirus (DV) is a small spherical, non-enveloped, single stranded DNA icosahedral virus within the *Parvovirus* family. The viruses in the genus *Brevidensovirus* of the subfamily *Densovirinae* are found exclusively in mosquitoes of the genera *Aedes, Culex and Culiseta* – those known to carry dengue, Japanese Encephalitis and yellow fever viruses.[14, 15] Collaborating with Dr. Pattamaporn Kittayapong of the dept. of Biology, the Faculty of Science, Mahidol University, we have studied (unpublished) the genome of a densovirus given by her and found that the virus has similar genomic sequence to the DV isolated from C6/36 *A albopictus* cell line by Jousset and Boublik in 1993.[16] We have also successfully cultured the virus in our laboratory and set up PCR techniques capable of amplifying both the non-structural and also the structural VP parts of the genome. Currently 90% of the genome is cloned and the cloning should be completed within a short time.

Concurrently, collaborating with Miss. Supatra and Mr. Chamnarn Apiwathnasorn of the Dept. of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, we have started to systematically identify, isolate and culture the viruses from larvae and also adult mosquitoes collected within the metropolitan Bangkok and the surrounding provinces. The aims are to survey the prevalence of the virus in common mosquitoes and also to obtain viruses for further studies. Two novel viruses from *Culex* and *Aedes* mosquitoes has been isolated and characterized. Preliminary data showed that their sequences are distinct from those reported.[17-20]

Significance and Future Research

The key reasons why it is essential to study these viruses are as follow: the virus naturally infected mosquitoes that carry viruses causing two major public health diseases prevalent in the country and the region - dengue, and Japanese encephalitis viruses. The diseases are now becoming world wide and have been classified as emerging diseases due to their rapid spreading. It is therefore important to know how the viruses interact in natural environment and how widespread are the infections. The facts that the viruses were known to be toxic to mosquito larvae and anomalously the viruses are found not causing any pathogenic effects to mosquitoes cell lines[16] are paradoxes that potentially could be beneficial to the control of the vectors. Potential role as a potent biological larvicide is obvious since parvovirus is known to be robust,

survive well in open environment and infected varieties of vectors. On the other hand, the virus, in some still obscure way, remain dormant within insect cells and could be propagated in culture without causing any deteriorating effects to the cells. This latter property hints that in some circumstances, the virus could co-exist with the insects and might interfere with infections of both dengue and Japanese encephalitis viruses. These are the topics to be investigated by our team. Attempts will be made to pool together group of entomologists in the country, co-ordinating with the BRT project of BIOTEC/TRF, to work toward a common goal to understand these viruses and explore the potential of using them as biological tools.

3.3. Thalassemia

3.3.1. Autoantibodies to thalassemic red cells and surface complement activation by erythrocytes from patients with beta- thalassaemia/HbE disease.

We have extended the research findings pioneered by the late Prof. Mongkol Kruatrachu [21, 22]that red cells from patients with beta thalassemia hemoglobin E (βThal/HbE) had antibodies on their red cell surfaces. We have demonstrated that apart from the presence of antibody molecules, complement components, including C3 and poly-C9, were bound onto their surfaces.[23] These results constitute the first demonstration that circulating affected erythrocytes may carry low numbers of potentially cytolytic C5b-9 complement complexes which may be partly responsible for the known ionic disturbances found in thalassaemic cells. Both bound C3 and C5b-9 could promote removal of diseased cells in the reticuloendothelial system. This investigation, together with our findings that complement components of both the alternative and classical pathways in βThal/HbE were low, led us to further investigate the mechanisms of complement activation as described below.

3.3.2. Vesiculation of thalassemic red cells: possible mechanism leading to complement activation and thrombo-embolic complications.

Background, the Studies and their Significance

The primary defect in thalassemia syndromes is reduced or absent production of one or more globin chains of the hemoglobin tetramer, which results in a relative excess of the unpaired chain. The excess unmatched globin chains precipitate and incur damage to the cell. The underlying mechanisms are not well understood, but oxidative membrane damage is believed to be of main importance. Thus, concentrations of polyunsaturated fatty acids are regularly decreased, and membrane protein thiols are reduced as a reflection of their pathological oxidation. Intracellular $Ca2^{+}$ levels are increased, which may be a consequence of enhanced $Ca2^{+}$ flux across the damaged membrane. Further to these findings, little is known on pathological cell biology of the diseased erythrocytes. Moreover, an explanatory model does not exist to account for the systemic afflictions, in particular of the coagulation and complement system, that are characteristic of thalassemia patients. In particular, it

has remained an enigma why a hypercoagulable state exists in these patients, leading to increased incidents of thrombotic events. Further, thalassemic patients have chronically low complement levels, a finding that also lacks a satisfactory explanation.

Membrane vesiculation is a physiological process that occurs during cell ageing. Thereby, small lipid vesicles are released from the cells. Enhanced vesiculation can be artificially induced in normal erythrocytes by treating the cells with Ca2+ ionophore, or by subjecting cells to attack by permeabilizing agents such as complement. Hence, we hypothesised that the thalassemic red cells might exhibit an enhanced tendency to vesiculate.

Indeed our study has demonstrate that that RBC vesicles exist *in vivo,* that their concentrations are elevated in patients with thalassemia, and that thalassemic cells exhibit abnormally high vesiculation rates. It is shown that vesicle formation can be induced *in vitro*, and that the isolated vesicles activate both the complement and the coagulation system. A concept is advanced in which major clinical findings in thalassemic patients may be traced to the pathological tendency of their RBC to vesiculate. In particular, a simple explanation is offered for the characteristically low complement levels, which predispose these patients to infections, and for the hypercoagulable state that is often manifested in thrombotic complications. The finding is one of the important missing links between the abnormal hemoglobin synthesis and a group of clinical abnormalities that cause serious morbidity and mortality in patients with thalassemia.

Future Research

Current and future investigations will focus on the unravelling of the pathogenetic processes responsible for the "CYTO-IMMUNO-PATHO-COAGULOPHATHY", as coined by Professor Prawase Wasi, found associated with different phenotypes of thalassemia. One of our current research topics carried out by an PhD/MD students is to ask the question how the vesicles generated from thalassemic red cells will affect the polymorphs, monocyte/macrophages and the coagulation system. We would collaborate in a long term cohort studies to look into the effects of different chemical agents, in particular antioxidants, and chelating agents on these parameters, hoping that optimal treatments that can arrest or change the deteriorating effects could be found. The key theme of the future investigations that applies well to the study of thalassemia is to look into the affects of the hemoglobin abnormalities on the functions of the "Innate Immunity" systems – a new exciting field in bio-medical research that might give answers to why thalassemic patients are cachectic, more susceptible to infections, having progressive end organ diseases affecting heart, kidney, lung, blood vessels and others; and why some patients had more severer phenotypes having the same genotypes of thalassemia.

3.3.3. Renal defects in patients with thalassemia.

Background and the Research

We have defined[24, 25] renal functional abnormalities in both adult (95 patients) and pediatric (104 patients) beta thalassemia patients and found that proximal tubular defect is the common functional abnormality. In contrast to general beliefs no glomerular defect was found. In pediatric patients, significantly higher levels of proteinuria and low molecular weight proteinuria were found in all patients compared with normal children. Aminoaciduria was detected in one-third of patients. Thalassemic patients had significantly lower morning urine osmolarity, higher urine N-acetyl- beta-D-glucosaminidase and malondialdehyde (MDA, an indicator of lipid peroxidation). Patients with severe anemia had significantly higher low- molecular weight proteinuria and MDA, and lower urine osmolarity than those with moderate anemia. Our data confirmed the high frequency of renal abnormalities in beta-thalassemia patients and indicated some degree of proximal tubular dysfunction. Damage was mildest in patients on hypertransfusion and desferrioxamine therapy. This suggested that the damage might be caused by anemia and increased oxidation induced by excess iron deposits.

Ongoing and Future Research

Cohort studies of patients receiving different dosages of chelating agents are currently being conducted. The main aims are to define optimal dosage of the chelating agents that would lessen the degree or prevent tubular damage. There is a very good chance that the prevention can be achieved by using sub-chelating dosage of the drugs. This will be beneficial to larger number of population since most of the chelating agents are expensive. We believe that the tubulo-interstitial damage is part of the patho-physiological processes initiated by the abnormal hemoglobin synthesis and the understanding of the underlying processes will serve as a basis from which optimal and efficient treatments can be given.

4. GRADUATE STUDIES

Graduate students participated in the network and their research topics

PROJECTS	GRADUATE STUDENTS & DEGREE	DATE OF GRADUATION
Molecular epidemiology of dengue viruses°°°	Miss Rungtawan Sriburi, for a M.Sc. (Chiangmai, Microbiology)	2540 1997
Infection of dengue virus in different cell types: expression of antigens and complement activation	Miss Panisadee Avirutnan, for PhD (Mahidol, Microbiology, PhD/MD pgm)	2541 1998
Microvesicles in Thalassemia*	Mr. Prapat Suriyaphol, for a Ph.D. (Mahidol, Biochemistry, PhD/MD pgm)	July 2541 1998
Dengue recombinant envelope proteins°°	Miss Supa Srisaad, M.Sc. (KMIT, Lardkrabang)	Apr 2542 1999
Dengue recombinant non-structural antigens°°	Miss Thanapan Prommoon M.Sc. (KMIT, Larkrabang)	late 2542 1999
Molecular Cloning of densonucleosis virus°	Miss Kusavadee Ek-U M.Sc. (Mahidol, Immunology)	late2542 1999
AE1 gene, RTA and ovalocytosis***	Miss Thitima Tungsirikul M.Sc. (Mahidol, Immunology)	mid 2542 1999
Analysis of Reg Gene in Thai patients with fibrocalculous pancreatic diabetes.***	Miss Wathip Boonyasrisawat (Fac Med, Siriraj, Mahidol U)	2542 1999
AE1 gene, RTA and ovalocytosis**	Dr Peti Thuwajit, M.D. for a Ph.D. (Mahidol University, Biochemistry)	2542 1999
Factor VIII gene in Thai hemophiliacs***	Miss Prapapon Thanutrkul (Fac Med, Siriraj, Mahidol U)	2542 1999
Densonucleosis virus: Production of antibodies to different gene products°	Miss Kopporn Boonnag, M.Sc Immunol (Fac Med, Siriraj, Mahidol U)	2543 2000
Genetic diversity of *A aegypti* densonucleosis virus°	Miss Ubonwan Jotekratote, MSc (Fac Med, Siriraj, Mahidol U)	2543 2000
Analysis of Rev1A and 1B genes in insulin dependent diabetes mellitus***	Miss Pinya Poonsawasdi (Fac Med, Siriraj, Mahidol U)	2543 2000
Characterization of nephrocalcin*	Mr Somchai Chutipongtanate, PhD (Fac Med, Siriraj, Mahidol U, PhD/MD)	2546-7 2003-4
Identification and isolation of densonucleosis virus from mosquito larvae°	Miss Piangpim Burivong (Fac Med, Siriraj, Mahidol U)	2544 2001
AE1 gene, RTA and ovalocytosis***	Miss Kanchana Dokladda, Ph.D (Mahidol University, Immunology) Kanjanaphisek Grant	2545-6 2002-3
T cell dengue peptide mapping in Thai*	Miss Wanwisa Dejnirattisai, PhD (Fac Med, Siriraj, Mahidol U)	2545-6 2002-3
Microvesiculation of red cells in Thalassemia*	Mr Supamit Ukarapong, PhD (Fac Med, Siriraj, Mahidol U, PhD/MD)	2546-7 2003-4

*Supervised by P Malasit
** Supervised by Prapon Wilairat, *** by Pathai Yenchitsomanus.
° Supervised by Dr Sa-nga Pattanakitsakul, °°by Dr Chunya Puttikhunt, °°°by Dr Nopporn Sittisombut

Number of graduate students enrolled into the unit

	2535 1992	2536 1993	2537 1994	2538 1995	2539 1996	2540 1997	2541 1998	2542 1999	**Total**
M.Sc.	1*	1*	1*	1	5**	4	4	3	**20**
Ph.D.	2*		1				2	2	**7**

* Graduated before 1999, ** graduated in 1999.

Number of Students Graduated During 2539-42 (1996-9)
Total 5 master degree (Roonruang, Sirina, Sumalee, Supa, Wathip) and 2 Ph.D. (Prapat, Panisadee) students graduated during 2539-42(1996-9) from the Medical Molecular Biology Unit, Siriraj Hospital; 1 MSc. And 1 Ph.D. student graduated from the network collaboration (Miss Rungtawan with Dr Nopporn and Dr Peti with Prof. Prapon).

Names of the graduate students working in the Medical Molecular Biology Unit (2538-42)

Master Degree (Year of enrolment and supervisors' names in parentheses)

1. Prapaporn Thanutrkul 2538 (Dr. Pathai)
2. Wathip Boonyasrisawat 2539 (Dr. Pathai) graduated 2542
3. Thitima Tungsirikul 2539 (Dr. Pathai)
4. Kusavadee Ek-U 2539 (Dr. Sa-nga)
5. Supa Srisaad 2539 (Dr. Prida, Dr. Chunya), grad 2542
6. Thanapan Prommoon 2539 (Dr. Chunya)
7. Pinya Poonsawasdi 2540 (Dr. Pathai)
8. Kopporn Boonnag 2540 (Dr. Sa-nga)
9. Worawuth Chinchang 2540 (Dr. Pathai)
10. Ubonwan Jolkrathok 2540 (Dr. Sa-nga)
11. Korakot Sarasith 2541 (Dr. Prida) resigned 2542
12. Pinit Sripathuras 2541 (Dr. Prida) switched to MD course
13. Piangpim Burivongs 2541 (Dr. Sa-nga)
14. Saranya Kittanakom 2541 (Dr. Pathai)
15. Nuanpan Khemnu 2542 (Dr. Chunya)
16. Duangkamol Bunditvorapoom 2542 (Dr. Pathai)
17. Kusuma Vathawornanant 2542 (Dr. Sa-nga)

Ph.D.

1. Wanna Thongnopkul 2537 (Dr Pathai)
2. Wanwisa Dejniwat* 2541 (Dr Prida)
3. Kanchana Dokladda* 2541 (Dr Pathai)
4. Mr Somchai Chutipongtanate** 2542 (Dr Prida)
5. Mr Supamit Ukarapong** 2542 (Dr Prida)

* Kanjanaphisek (Golden Jubilee Research Grant)
** PhD/MD China Medical Board Program, Mahidol University

5. RESEARCH NETWORK

THE NETWORK

Principal Investigator: Prida Malasit, M.D., F.R.C.P..
Medical Molecular Biology Unit (MMBU),
Office for Research & Development
Siriraj Hospital, Faculty of Medicine
Mahidol University

Key Collaborators/Network:

Dr. Pathai Yenchitsomanus, Ph.D.	MMBU, Siriraj Hospital, Mahidol U.
Dr. Sa-gna Pattanakitsakul, Ph.D.	MMBU, Siriraj Hospital, Mahidol U.
Dr. Somkiat Vasuwattakul, M.D.	Medicine, Siriraj Hospital, Mahidol U.
Dr. Prapon Wilairat, Ph.D.	Biochemistry, Fac. Science, Mahidol U.
Dr. Nopporn Sittisombut, M.D., Ph.D.	Microbiology, Chiang Mai University
Miss Suntharee Rojanasuphot, B.Sc.	Dept. Medical Sciences, Min. Pub. Hlth
Dr. Vipada Chaovakul, M.D.	Medicine, Ubolrajathanee Hospital
Dr Sukachat Kerdphol, M.D.	Pediatrics, Khonkaen University
Dr. Wattanachai Susaengrat, M.D.	Medicine, Khonkaen Hospital
Dr Sirijit Wasanawattan, M.D.	Pediatrics, Khonkaen Hospital
Dr Charoen Kaitwatcharachai, M.D.	Medicine, Prince of Songkla University
Dr. Vichai Laosombut, M.D.	Pediatrics, Prince of Songkla University
Miss Supatra Thongrungkiat	Entomology, Faculty of Tropical Medicine
Mr. Chamnarn Apiwathnasorn	Entomology, Faculty of Tropical Medicine
Dr Chunya Puttikhunt	Medical Biotechnology Unit, c/o Medical Mol. Biol.Unit, BIOTEC.

International Collaborators

Dr. Sucharit Bhakdi, M.D.	Institute for Medical Microbiology & Hygiene, Mainz University, Germany
Dr. Yasushi Nakagawa, Ph.D.	Medicine, University of Chicago, U.S.A.
Dr. Ichiro Kurane, M.D., Ph.D.	National Institute for Infectious Disease, Tokyo, Japan
Dr Andrew McMichael	Institute of Molecular Medicine, Oxford University, U.K..

รายชื่อผู้ทำงานในโครงการ

ชื่อ-นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	หน่วย/ภาควิชา	คณะ	มหาวิทยาลัย /สถาบัน		
โครงการการศึกษาโรค Renal Tubular Acidosis (RTA)							
คณะแพทยศาสตร์ศิริราชพยาบาล							
1. ผศ.ดร.เพทาย เย็นจิตโสมนัส	ผู้ช่วยศาสตราจารย์	ผู้ช่วยศาสตราจารย์	หน่วยอณูชีววิทยาการแพทย์	บัณฑิตวิทยาลัย	ม.มหิดล	หัวหน้าโครงการร่วมกับ นพ.สมเกียรติ วสุวัฏกุล	1.หัวหน้าโครงการร่วมกับนพ.สมเกียรติ วสุสัฏกุล ในทุนเฉลิมพระเกียรติ คณะแพทยศาสตร์ศิริราชพยาบาล ในการศึกษาพยาธิกำเนิดของโรค RTA 2.หัวหน้าโครงการศึกษาและพัฒนาวิธีตรวจวินิจฉัยโรคธาลัสซีเมีย จากทุนสนับสนุนของศูนย์พันธุวิศวกรรมฯ 3.หัวหน้าโครงการ quantitation of dengue virus โดยวิธี PCR/ELISA
2. นพ.สมเกียรติ วสุวัฏกุล	อาจารย์	อาจารย์	หน่วยโรคไต/อายุรศาสตร์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	หัวหน้าโครงการร่วมกับ ผศ.ดร.เพทาย เย็นจิตโสมนัส	หัวหน้าโครงการร่วมกับ ผศ.ดร.เพทาย เย็นจิตโสมนัส ในทุนเฉลิมพระเกียรติ คณะแพทยศาสตร์ศิริราชพยาบาล ในการศึกษาพยาธิกำเนิดของโรค RTA
3. น.ส.หนึ่งหทัย สวัสดี	นักวิทยาศาสตร์ (วท.บ.)	นักวิทยาศาสตร์ (วท.บ.)	หน่วยอณูชีววิทยาการแพทย์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	นักวิทยาศาสตร์ในโครงการ (หนักงานมหาวิทยาลัย)	นักวิทยาศาสตร์ในโครงการ (พนักงานมหาวิทยาลัย)
4. น.ส.สุมาลี โอหารวิรุห์	นักวิทยาศาสตร์ (วท.ม.)	นักวิทยาศาสตร์ (วท.ม.)	หน่วยอณูชีววิทยาการแพทย์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	นักวิทยาศาสตร์ในโครงการ (ลูกจ้างในโครงการ)	นักวิทยาศาสตร์ในโครงการ (ลูกจ้างในโครงการ)
5. น.ส.กาญจนา คอกลัดคา	นักศึกษาปริญญาเอก	นักศึกษาปริญญาเอก		บัณฑิตวิทยาลัย	ม.มหิดล	นักศึกษาปริญญาเอกโครงการกาญจนาภิเษก	ลาออกเนื่องจากได้รับทุน กพ.ศึกษาต่อเรื่องเดียวกันที่ ม.บริสตอล ประเทศอังกฤษ
6. น.ส.ศรัณยา กิจธนาคม	นักศึกษาปริญญาเอก	นักศึกษาปริญญาเอก		บัณฑิตวิทยาลัย	ม มหิดล	นักศึกษาปริญญาเอกโครงการกาญจนาภิเษก	นักศึกษาปริญญาเอกโครงการกาญจนาภิเษก
7. น.ส.ฐิติมา ตั้งศิริกุล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท		บัณฑิตวิทยาลัย	ม.มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท

ชื่อ-นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	หน่วย/ภาควิชา	คณะ	มหาวิทยาลัย /สถาบัน		
โรงพยาบาลและม.ขอนแก่น							
1. นพ.วัฒนชัย สุแสงรัตน์	นายแพทย์	นายแพทย์	อายุรกรรม	รพ.ศูนย์ขอนแก่น	กระทรวงสาธารณสุข	ผู้ร่วมวิจัยในโครงการฯ	ผู้ร่วมวิจัยในโครงการฯ
2. รศ.นพ.สุขชาติ เกิดผล	รองศาสตราจารย์	รองศาสตราจารย์	กุมารเวชศาสตร์	คณะแพทยศาสตร์	ม.ขอนแก่น	ผู้ร่วมวิจัยในโครงการฯ	ผู้ร่วมวิจัยในโครงการฯ
อุบลราชธานี							
3. พญ.วิภาดา เชาวกุล	แพทย์หญิง	แพทย์หญิง	อายุรกรรม	รพ.สรรพสิทธิประสงค์	กระทรวงสาธารณสุข	ผู้ร่วมวิจัยในโครงการฯ	ผู้ร่วมวิจัยในโครงการฯ
ม.สงขลานครินทร์							
4. นพ.เจริญ เกียรติวัชรชัย	อาจารย์	อาจารย์	อายุรศาสตร์	คณะแพทยศาสตร์	ม.สงขลานครินทร์	ผู้ร่วมวิจัยในโครงการฯ	ผู้ร่วมวิจัยในโครงการฯ
โครงการ thalassemia							
1. ศ.พญ.ลีนา องอาจยุทธ	ศาสตราจารย์	ศาสตราจารย์	อายุรศาสตร์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	หัวหน้าโครงการการศึกษาภาวะแทรกซ้อนทางไตคนไข้ธาลัสซีเมียในผู้ใหญ่	หัวหน้าโครงการการศึกษาภาวะแทรกซ้อนทางไตคนไข้ธาลัสซีเมียในผู้ใหญ่
2. รศ.พญ.อัจฉรา สมบูรณานนท์	ผู้ช่วยศาสตราจารย์	รองศาสตราจารย์	กุมารเวชศาสตร์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	1.หัวหน้าโครงการการศึกษาภาวะแทรกซ้อนทางไตคนไข้ธาลัสซีเมียในผู้ป่วยเด็ก 2. หัวหน้าโครงการการศึกษา RTA ในผู้ป่วยเด็ก	1. หัวหน้าโครงการการศึกษาภาวะแทรกซ้อนทางไตคนไข้ธาลัสซีเมียในผู้ป่วยเด็ก 2. หัวหน้าโครงการการศึกษา RTA ในผู้ป่วยเด็ก
3. ดร.ประพัฒน์ สุริยผล	นักศึกษาแพทย์	อาจารย์	หน่วยอณูชีววิทยาการแพทย์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	นักศึกษาปริญญาเอกโครงการ Ph.D- M.D. ม.มหิดล	บรรจุเป็นอาจารย์หน่วยอณูชีววิทยาการแพทย์(ศิริราช) กำลังศึกษา post-doctorate ในประเทศเยอรมันนี จากทุนคณะแพทย์ฯ และรัฐบาลเยอรมัน เป็นเวลา 2 ปี
4. นศพ.ศุภมิตร อัครพงศ์	นักศึกษาแพทย์	นักศึกษาแพทย์		บัณฑิตวิทยาลัย	ม.มหิดล	นักศึกษาปริญญาเอกโครงการ Ph.D- M.D. ม.มหิดล	นักศึกษาปริญญาเอกโครงการ Ph.D- M.D. ม.มหิดล

ชื่อ-นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
โครงการศึกษานิ่วในไตและโปรตีน nephrocalcin ร่วมกับมหาวิทยาลัยซิคาโก							
1. นศพ.สมชาย ชุติพงศ์ธเนศ	นักศึกษาแพทย์	นักศึกษาแพทย์		คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	นักศึกษาปริญญาเอกโครงการ Ph.D- M.D.ม.มหิดล	นักศึกษาปริญญาเอกโครงการ Ph.D- M.D. ม.มหิดล
โครงการศึกษาไข้เลือดออก							
1. รศ.นพ.นพพร สิทธิสมบัติ	รองศาสตราจารย์	รองศาสตราจารย์	จุลชีววิทยา	คณะแพทยศาสตร์	ม.เชียงใหม่	1 หัวหน้าโครงการ molecular typing of dengue virus 2 หัวหน้าโครงการ quantitation of dengue virus by PCR	1. หัวหน้าโครงการ molecular typing of dengue virus 2. หัวหน้าโครงการ quantitation of dengue virus by PCR
2. น.ส.สุนทรี โรจนสุพจน์	ผู้เชี่ยวชาญพิเศษ	ผู้เชี่ยวชาญพิเศษ	ฝ่ายอาโบไวรัส	กรมวิทยาศาสตร์การแพทย์	ก.สาธารณสุข	ผู้ร่วมโครงการการวินิจฉัยและระบาควิทยาของไข้เลือดออกร่วมกับหน่วยอาร์บไวรัส	ผู้ร่วมโครงการการวินิจฉัยและระบาควิทยาของไข้เลือดออกร่วมกับหน่วยอาร์บไวรัส
3. ดร.ชัญญา ทุทธิขันธ์	นักวิจัย	นักวิจัย	BIOTEC	BIOTEC	NSTDA	ดูแลโครงการเทคโนโลยีการวินิจฉัยไข้เลือดออก	ดูแลโครงการเทคโนโลยีวินิจฉัยไข้เลือดออก
4. นพ.สมชาย เชื้อนานนท์	นายแพทย์	นายแพทย์	กองกุมารเวชกรรม	รพ.นครพนม	ก.สาธารณสุข	ผู้ร่วมโครงการการวินิจฉัยและระบาควิทยาของไข้เลือดออก	ผู้ร่วมโครงการการวินิจฉัยและระบาควิทยาของไข้เลือดออก
5. นายพิชัย ทองธราดล	นักวิทยาศาสตร์ (วท.บ.)	นักวิทยาศาสตร์	กองพิสูจน์	รพ.นครพนม	ก.สาธารณสุข	ผู้ร่วมโครงการการวินิจฉัยและระบาควิทยาของไข้เลือดออก	ผู้ร่วมโครงการการวินิจฉัยและระบาควิทยาของไข้เลือดออก
6. พญ.ศิริจิตต์ วาสนะวัฒน์	แพทย์หญิง	แพทย์หญิง	กุมารเวชกรรม	รพ.ศูนย์ขอนแก่น	ก.สาธารณสุข	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก
7. น.ส.ปัญจพร ทุทธเทศก์	นักวิทยาศาสตร์ (วท.ม.)	นักวิทยาศาสตร์	หน่วยอณูชีววิทยาการแพทย์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก
8. น.ส.อรุณรุ่ง ใจรังษี	นักวิทยาศาสตร์ (วท.บ.)	นักวิทยาศาสตร์	หน่วยอณูชีววิทยาการแพทย์	คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก
9. น.ส.นวลพรรณ เข็มหนู	นักวิทยาศาสตร์ (วท.บ.)	นักศึกษาปริญญาโท	หน่วยอณูชีววิทยาการแพทย์	บัณฑิตวิทยาลัย	ม.มหิดล	ผู้ร่วมโครงการการพยาธิกำเนิดของโรคไข้เลือดออก	กำลังศึกษาต่อปริญญาโท ม.มหิดล เป็นนักศึกษาทุน TGIST (สวทช)
10. ดร.ปนิษฎ์ อวิรุทธ์นันท์	นักศึกษาแพทย์	นักศึกษาแพทย์		บัณฑิตวิทยาลัย	ม.มหิดล	นักศึกษาโครงการ M.D.-Ph.D.	จบการศึกษา Ph.D. แล้ว กำลังศึกษาต่อหบ. และจะบรรจุเป็นอาจารย์ในหน่วยอณูชีววิทยาการแพทย์และศึกษาต่อ post doctorate
11. น.ส.ธนพรรณ พร้อมมูล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท		บัณฑิตวิทยาลัย	สถาบันพระจอมเกล้า (ลาดกระบัง)	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
12. น.ส.วันวิสา เคชนิรัติศัย	นักศึกษาปริญญาเอกกาญจนาภิเษก	นักศึกษาปริญญาเอกกาญจนาภิเษก		บัณฑิตวิทยาลัย	ม.มหิดล	นักศึกษาปริญญาเอกกาญจนาภิเษก	นักศึกษาปริญญาเอกกาญจนาภิเษก

ชื่อ-นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
โครงการศึกษา densovirus ในยุง							
1. ผศ.ดร.สง่า พัฒนากิจสกุล	อาจารย์	ผู้ช่วยศาสตราจารย์	หน่วยอณูชีววิทยาการแพทย์	บัณฑิตวิทยาลัย	ม.มหิดล	หัวหน้าโครงการศึกษา densovirus ในยุง	หัวหน้าโครงการศึกษา densovirus ในยุง
2. ผศ.สุพัตรา ทองรุ่งเกียรติ	ผู้ช่วยศาสตราจารย์	ผู้ช่วยศาสตราจารย์	กีฏวิทยาการแพทย์	คณะเวชศาสตร์เขตร้อน	ม.มหิดล	ผู้ร่วมโครงการศึกษา densovirus ในยุง	ผู้ร่วมโครงการศึกษา densovirus ในยุง
3. ผศ.ดร.ปัทมาภรณ์ กฤดยพงศ์	ผู้ช่วยศาสตราจารย์	ผู้ช่วยศาสตราจารย์	ชีววิทยา	คณะวิทยาศาสตร์	ม.มหิดล	ผู้ร่วมโครงการศึกษา densovirus ในยุง	ผู้ร่วมโครงการศึกษา densovirus ในยุง
4. น.ส.กุสาวดี เอกอุ	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท		บัณฑิตวิทยาลัย	ม.มหิดล	ผู้ร่วมโครงการศึกษา densovirus ในยุง	ผู้ร่วมโครงการศึกษา densovirus ในยุง
5. น.ส.อุบลวรรณ โจทก์กระโทก	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท		บัณฑิตวิทยาลัย	ม.มหิดล	ผู้ร่วมโครงการศึกษา densovirus ในยุง	ผู้ร่วมโครงการศึกษา densovirus ในยุง
6. น.ส.เพียงพิมพ์ บุรีวงศ์	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท		คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
7. น.ส.กุสุมา เอื้อถาวรอนันต์	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท		คณะแพทยศาสตร์ศิริราชพยาบาล	ม.มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท

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7. RESEARCH OUTPUT

7.1. INTERNATIONAL PUBLICATIONS

1. Nademanee K, Veerakul G, Nimmannit S, Chaowakul V, Bhuripanyo K, Likittanasombat K, **Malasit P**, *et al.* Arrhythmogenic marker for the sudden unexplained death syndrome in Thai men. Circulation 1997;96(8):2595-600.

2. **Malasit P**, Mahasorn W, Mongkolsapaya J, Singhathong B, Fucharoen S, Wasi P, et al. Presence of immunoglobulins, C3 and cytolytic C5b-9 complement components on the surface of erythrocytes from patients with beta- Thalassemia/HbE disease. *Br J Haematol* 1997;96(3):507-13.

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9. Boonyos Raengsakulrach, Nisalak A, Maneekarn N, Yenchitsomanus P, **Malasit P**, Sittisombut N, Limsomwong C, Jairungsri A, Thirawuth V, Green S, Kalayanarooj S, Suntayakorn S, Vaughn DW. Comparison of the efficacy of four RT-PCR tests for the detection of dengue virus. (Manuscript supplied)

10. Avirutnan P, **Malasit P**, Bhakdi S. Expression of dengue viral antigens results in complement activation on the surface of dengue infected monocytes. (Manuscript supplied)

11. Rungtawan Sriburi, Sirilak Boonres, Boonyos Ruengsakulrach, Pathai Yenchitsomanus, Niwat Maneekarn, Siripen Kulayanarooj, Suchitra Nimmannitya, David Vaughn, Prida Malasit, Nopporn Sittisombut, Ananda Nisalak. Contrasting changes of dengue serotype 2 virus subtypes and E protein variants during a 15-year period (1980-1994) in Bangkok, Thailand. (Manuscript supplied)

12. Yasushi Nakagawa, Carbalho M, **Malasit P**, Nimmannit S, Chaowagul V and Nilwarangur S. Kidney stone inhibitors in patients with renal stone and renal tubular acidosis in the northeast of Thailand. (Manuscript supplied)

7.2. LOCAL PRESENTATIONS

1
Presented at the *7th Scientific Annual Meeting of the Virological Association (Thailand) on the 15 December 1997*
Predominance of subtype IIIa amongst dengue serotype 2 viruses in Bangkok during 1987-1994
Rungtawan Sriburi, Boonres S, Maneekarn N, Raengsakulrach B, Yenchitsomanus P, Kalayanarooj S, Nisalak A, Vaughn D, **Malasit P**, Sittisombut N.

2
Presentation at the XIV *Annual Meeting of the Royal College of Physicians of Thailand* 23 April 1998, Cha-am
Distal renal tubular acidosis (dRTA) in a patient with Southeast Asian Hereditary Ovalocytosis (SAO): Role of urine pCO_2 as a tool to detect AE1 abnormality.
Charoen Kaiwatcharachai, S Vasuvattakul, Yenchitsomanus Y, Thuwajit P, **P Malasit**, D Chuawatana, S Mingkum, Halperin ML, and S Nimmannit.

3
Presentation at the *Siriraj Annual Meeting*, March 8-12
Abstract F42: **Chemokines in pleural fluids of fatal cases of dengue hemorrhagic fever/ DENGUE SHOCK SYNDROME (DHF/DSS).**
Prida Malasit, Panisadee Avirutnan, Sirijitt Vasanawathana, Kulkanya Chokephaibulkit, Aroonroong Jairungsri, Pathai Yenchitsomanus, Matthias Husmann, and Sucharit Bhakdi.

4
Abstract F41: **Dot enzyme immunoassay (DEIA) for the diagnosis of dengue virus infection using recombinant dengue envelope protein antigens**
Chunya Puttikhunt, Nuanpan Khemnu, Sa-nga Pattanakitsakul, Pathai Yenchitsomanus, Supa Srisa-ad, Somchai Chuananon, Areerat Srijuggravalvong, Suntharee Rojanasuphot, **Prida Malasit.**

5
Abstract to the *7th International Conference on Thalassemia and the Hemoglobinopathies (the 9th Thalassemia Parent and Thalassemics International Conference. 31st May-4th June 1999 at the Imperial Queen's Park Hotel, Bangkok.*
Investigations into the pathogenic mechanisms responsible for the increased susceptibility to infections in thalassemic patients
Prapat Suriyaphol, **Prida Malasit**, Prapon Wilairat, Suthat Fucharoen, Sucharit Bhakdi

6

ANNUAL PRESENTATION
TRF Senior Research Scholar Groups
Tuesday 2nd March- 2542
At the Pathology Lecture Theatre, 8th fl. Adulyadejvikrom Building
Siriraj Hospital.

I PREVALENT DISEASES IN ISAN AND SONGKLA
Renal Tubular Acidosis (RTA) and Southeast Asian Ovalocytosis (SAO)

Overview of Renal Tubular Acidosis in Isan	Sumalee Nimmannit
Family pattern of endemic renal tubular acidosis (EnRTA)	
Distal renal tubular acidosis and Southeast Asian ovalocytosis (SAO)	Somkiat Vasuvattakul
Association between the molecular defects of the AE1 and dRTA in Songkla: compound heterozygosity as the cause of dRTA	Pathai Yenchitsomanus
Abnormal sulphate test in Southeast Asian Ovalocytosis (SAO) with and without dRTA: preliminary results from five families in Songkla	Peti Thuwajit, Prapon Wilairat
Erythrocyte sodium-lithium counter transport and dRTA	Kriengsak Vareesangthip
A hypothesis: Abnormal AE1 gene, malaria and distal renal tubular acidosis	Prapon Wilairat

II DENGUE VIRUS:
PATHOGENESIS, DIAGNOSIS AND MOLECULAR EPIDEMIOLOGY

Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis	Panisadee Avirutnan
Evolution of dengue 2 subtypes in Bangkok within a span of 15 years (1980-1994)	Nopporn Sittisombut
Recombinant dengue envelope proteins for dengue diagnosis	Chunya Puttikhunt

III PATHOPHYSIOLOGY OF THALASSEMIA

Red cell vesiculation: Its role in the pathogenesis of increased susceptibility to infection in patients with Thalassemia	Prapat Suriyaphol

IV DENSONUCLEOSIS VIRUS

Molecular cloning of densonucleosis virus	Sa-nga Pattanakitsakul

V MOLECULAR GENETICS

Molecular genetics of Thai hemophilia patients	Pathai Yenchitsoma

7.3. INTERNATIONAL PRESENTATIONS

1
Presentation at the XVI *International Congress of Nephrology* 25-29 May 1997, Sydney, Australia
Distal renal tubular acidosis (dRTA) in a patient with Southeast Asian Hereditary Ovalocytosis (SAO)
Charoen Kaiwatcharachai, S Vasuvattakul, **P Malasit**, D Chuawatana, S Mingkum, and S Nimmannit.

2
Presentation at the XVI *International Congress of Nephrology* 25-29 May 1997, Sydney, Australia
Indirect evidence of increasing ammonium (NH_4^+) production in the villagers with potassium (K) deficiency in the northeast of Thailand.
Somkiat Vasuvattakul, W Susaengrat, S Nimmannit, **P Malasit**, Ong-ajyooth S, D Chuawatana, S Nilwarangkur.

3
Presentation at the 31st Annual Meeting of the *American Society of Nephrology* 25-28 October 1998, Pennsylvania, U.S.A..
Distal renal tubular acidosis in Southeast Asian Ovalocytosis is associated with compound heterozygosity for the mutations in the Anionic Exchanger 1.
Somkiat Vasuvattakul, Yenchitsomanus P, Vachuanichsanong P, Thuwajit P, Kaitwatcharachai C, Laosombut V, **Malasit P**, Wilairat P, Nimmannit S.

4
Presented at *the "Progress in Clinical Virology IV" Meeting of the European Society for Clinical Virology, Hamburg, September 1998*
An Integrated Hypothesis of Dengue Shock Syndrome.
Panisadee Avirutnan, **Malasit P,** Bhakdi S, Husmann M.

5
Presentation at the Xith International Congress of Virology, 9-13 Aug. 1999 at the Sydney Convention Center, Darling Harbour, Sydney, Australia.
ON THE PATHOGENESIS OF DENGUE SHOCK SYNDROME (DSS)
Panisadee Avirutnant, **Prida Malasit**, Sucharit Bhakdi, Matthias Husmann

6
Presentation at the 32nd Annual Meeting of the American Society of Nephrology, Nov. 5-8, 1999 at the Miami Beach Convention Center, Miami, FL., USA..
Compound heterozygosity of AE1 genes cause recessive distal tubular acidosis in Southeast Asian Ovalocytosis.
Vasuvattakul S, Yenchitsomanus P, Thuwajit P, Kaitwatcharachai C, Vachuanichsanong P, Laosombat V, **Malasit P**, Wilairat P, Nimmannit S.

8. APPENDIX

8.1. REPRINTS AND MANUSCRIPTS

REPRINTS

1. *Circulation* 1997;96(8):2595-600.
2. *Br J Haematol* 1997;96(3):507-13.
3. *Pediatr Nephrol* 1998;12(4):280-3.
4. *Nephron* 1998;78(2):156-61.
5. *J Immunol* 1998;161(11):6338-46.
6. *Am J Kidney Dis* 1999;33(6):1147-52.
7. *Kidney International* (In press, Nov 1999).

MANUSCRIPTS

8. Suriyaphol P, ***et al.*** Red cell microvesicles in Thalassemia.
9. Boonyos Raengsakulrach, *et al.* Comparison of the efficacy of four RT-PCR tests for the detection of dengue virus.
10. Avirutnan P, ***et al.*** Expression of dengue viral antigens results in complement activation on the surface of dengue infected monocytes.
11. Rungtawan Sriburi, *et al.* Contrasting changes of dengue serotype 2 virus subtypes and E protein variants during a 15-year period (1980-1994) in Bangkok, Thailand.
12. Yasushi Nakagawa, *et al.* Kidney stone inhibitors in patients with renal stone and renal tubular acidosis in the northeast of Thailand.

Arrhythmogenic Marker for the Sudden Unexplained Death Syndrome in Thai Men

Koonlawee Nademanee, MD; Gumpanart Veerakul, MD; Sumalee Nimmannit, MD; Vipada Chaowakul, MD; Kiertijai Bhuripanyo, MD; Khanchit Likittanasombat, MD; Kriang Tunsanga, MD; Surachai Kuasirikul, MD; Prida Malasit, MD; Sudarat Tansupasawadikul, MD; Pyatat Tatsanavivat, MD

Background Between 1981 and 1988, the Centers for Disease Control and Prevention reported a very high incidence of sudden death among young male Southeast Asians who died unexpectedly during sleep. The pattern of death has long been prevalent in Southeast Asia. We carried out a study to identify the clinical markers for patients at high risk of developing sudden unexplained death syndrome (SUDS) and long-term outcomes.

Methods and Results We studied 27 Thai men (mean age, 39.7±11 years) referred because they had cardiac arrest due to ventricular fibrillation, usually occurring at night while asleep (n=17), or were suspected to have had symptoms similar to the clinical presentation of SUDS (n=10). We performed cardiac testing, including EPS and cardiac catheterization. The patients were then followed at ≈3-month intervals; our primary end points were death, ventricular fibrillation, or cardiac arrest. A distinct ECG abnormality divided our patients who had no structural heart disease (except 3 patients with mild left ventricular hypertrophy) into two groups: group 1 (n=16) patients had right bundle-branch block and ST-segment elevation in V_1 through V_3, and group 2 (n=11) had a normal ECG. Group 1 patients had well-defined electrophysiological abnormalities: group 1 had an abnormally prolonged His-Purkinje conduction time (HV interval, 63±11 versus 49±6 ms; $P=.007$). Group 1 had a higher incidence of inducible ventricular fibrillation (93% for group 1 versus 11% for group 2; $P=.0002$) and a positive signal-averaged ECG (92% for group 1 versus 11% for group 2; $P=.002$), which was associated with a higher incidence of ventricular fibrillation or death ($P=.047$). The life-table analysis showed that the group 1 patients had a much greater risk of dying suddenly ($P=.05$).

Conclusions Right bundle-branch block and precordial injury pattern in V_1 through V_3 is common in SUDS patients and represents an arrhythmogenic marker that identifies patients who face an inordinate risk of ventricular fibrillation or sudden death. (*Circulation*. 1997;96:2595-2600.)

Key Words • Thailand • bundle-branch block • death, sudden • fibrillation • ventricle

In the 1980s, the CDC reported that the number of young male Southeast Asian refugees in the United States who died suddenly and unexpectedly had risen dramatically.[1,2] The victims had all been active and apparently healthy before dying suddenly while sleeping at night. The time of death ranged between 10:00 PM and 8:00 AM in 82 documented cases. All the decedents, except 1, were men between the ages of 16 and 63 years (median, 32 years). This syndrome, which came to be known as SUDS, had been prevalent for many years in Asia, particularly in the Southeastern region.[3-8] The native populations of this part of the world have long been familiar with this pattern of death: SUDS was known by the name *Lai Tai* ("died during sleep") in northeast Thailand,[3-5,6] *Bangungut* ("moaning and dying during sleep") in the Philippines,[4] and *Pokkuri* ("sudden unexpected death at night") in Japan.[7,8] In Thailand, SUDS first attracted public attention when accounts were published of young male Thai construction workers in Singapore who died suddenly in 1990.[9] The pattern of death in these young Thai male workers was similar to that described in the CDC report. A subsequent epidemiological survey of young Thai men living along the northeastern border of Thailand abutting Laos and Cambodia found that the annual SUDS death rate was 26 to 38 per 100 000 men (range, 20 to 49 years old).[5,6] This figure confirmed that SUDS was the leading natural cause of death of young Thai men, and, as such, posed a critical medical and social threat to Thai society.

Although researchers had determined that VF was the rhythm leading to cardiac arrest or death[10-12]—a characteristic that placed SUDS in the constellation of idiopathic VF[13]—SUDS presented a particularly vexing problem for physicians. There were no precipitating factors or premonitory symptoms before death occurred. SUDS victims had no overt structural heart disease. The clinical markers that would identify patients at high risk for SUDS had not been discovered. We consequently carried out a study to determine the prevalence of cardiac abnormalities in patients who run a great risk of developing SUDS.

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Selected Abbreviations and Acronyms

CDC = Centers for Disease Control and Prevention
EPS = electrophysiological study
ICD = implantable cardiac defibrillator
LVH = left ventricular hypertrophy
MRI = magnetic resonance imaging
RBBB = right bundle-branch block
SAECG = signal-averaged ECG
SUDS = sudden unexplained death syndrome
VF = ventricular fibrillation
VT = ventricular tachycardia

prospectively evaluate their clinical outcome, and identify the clinical markers for SUDS.

Methods

Study Population

From January 1994 through September 1996, we prospectively studied 27 Thai men who were thought by referring physicians to have survived SUDS-like episodes and thus had been identified as possible *Lai Tai* patients. The protocol required the following inclusion criteria for the study patients: (1) SUDS victims were patients who had been apparently healthy before suddenly developing sudden cardiac arrest due to VF but had been successfully resuscitated, and (2) probable SUDS patients experienced symptoms that reflected the clinical presentation of SUDS[3,6]: agonal respiration, unresponsiveness after labored respiration during sleep, transient symptoms of distress (eg, moaning, thrashing, grimacing), and syncope or seizure-like symptoms. Probable SUDS patients did not have documented cardiac arrest or VF before being referred. We excluded patients who had structural heart disease or identifiable causes of VF causing cardiac arrests, such as prolonged QT syndrome, myocardial ischemia, or drug-induced life-threatening arrhythmias.

Cardiac Testing

All patients were transferred to our hospitals for a routine physical examination. Cardiac tests performed included ECG, EPS, exercise treadmill test, Holter monitoring, echocardiographic study, SAECG, and cardiac catheterization, including coronary angiography and left and right ventricular angiography. MRI of the heart was obtained if possible. The patients were followed in the outpatient clinics and were treated with amiodarone, propranolol, or an ICD according to the physician's discretion. The ICDs that were used were model CPI-P3 (Guidant-CPI, Inc) with a storage memory and shock E-gram (far-field electrogram), which allowed us to determine the precise rhythms before, during, and after defibrillation.

Stimulation Protocol for VT Induction

The stimulation protocol included ventricular stimulation at the right ventricular apex and three cycle length driving trains (normal sinus rhythm or 600-ms ventricular pacing, 500-ms ventricular pacing, and 400-ms ventricular pacing). If VT was not induced at the right ventricular apex, then the right ventricular outflow tract was used for induction in the same manner. Isoproterenol was not used for the induction. Induced arrhythmias were defined as (1) VF (≥300 bpm), (2) polymorphic VT (<300 bpm), or (3) monomorphic VT (<300 bpm). "Sustained" was defined as lasting 30 seconds or requiring earlier termination because of hemodynamic compromise. "Nonsustained" was defined as inducible arrhythmias of at least 15 beats but less than 30 seconds.

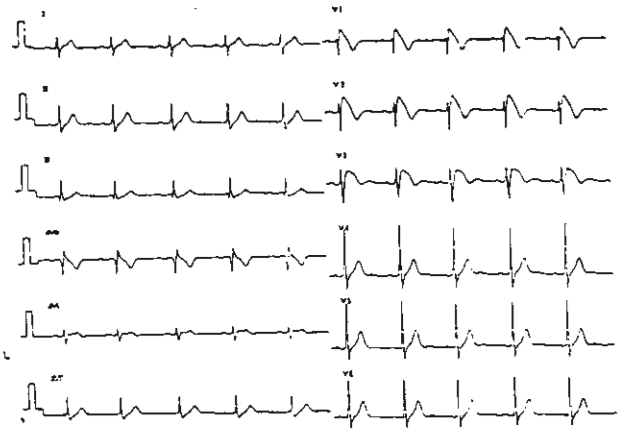

FIG 1. Typical ECG pattern of 1 group 1 patient showing the RBBB and precordial injury pattern demonstrated by ST-segment elevation in V_1 through V_3.

SAECG

SAECG was analyzed by the time domain method at the 40-Hz filter. Abnormal values are (1) total duration of the SAECG-QRS complex of >114 ms, (2) RMS voltage in the terminal 40 ms of the SAECG-QRS complex (v40) of <20 μV, and (3) duration of low-amplitude signals with 40 μV in the terminal portion of the SAECG-QRS complex of >38 ms. Our protocol required at least two of these three criteria for a positive SAECG.

Data and Statistical Analysis

Our primary variables for clinical outcomes during the follow-up period include the development of VF, cardiac arrest, or death. Two-sample *t* test was used to compare the differences in the mean values of the two groups, and a Kaplan-Meier life-table analysis was used to determine the differences in event-free survival rates between the two groups. The Fisher exact test was used for comparing the incidence of inducible VT/VF, positive SAECG, and clinical occurrence of VF or cardiac arrest between the two groups.

Results

We studied 27 male patients (mean age, 39.7±11 years), which included 17 SUDS survivors and 10 probable SUDS patients. All patients had normal cardiac function (mean left ventricular ejection fraction, 64±9%) and no evidence of structural heart disease. The mean interventricular septal and posterior wall thicknesses were 1.1±0.2 and 0.99±0.1 cm, respectively; only 3 patients had a small increase in the wall thickness (>1.1 cm), suggesting mild LVH. All patients had normal coronary arteries and a normal right ventricle. All patients had normal exercise treadmill tests. Five patients, all of whom were SUDS survivors, had normal cardiac MRI results.

Of the 17 SUDS survivors who developed cardiac arrest due to VF before the study, 12 developed VF while sleeping between 8:00 PM and 6:00 AM. Two patients suffered VF arrest while awake at 10:00 PM and 1:00 AM, respectively. The other 3 patients developed VF in the late afternoon. There was no evidence of identifiable secondary causes of VF. Four SUDS survivors and two probable SUDS patients had serum potassium levels of <3.5 mEq/L. All patients had their potassium level repleted to normal before undergoing EPS.

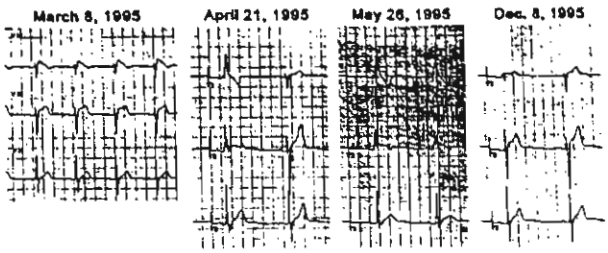

FIG 2. Dynamic changes in the ECG pattern waxing and waning from week to week. This 17-year-old patient had symptoms similar to the clinical presentation of SUDS but never had documented VT/VF. On March 8, 1995, the ECG in V_1 through V_3 showed the precordial injury pattern and possibly an incomplete RBBB. On April 21, 1995, the ECG showed an intermittent RBBB with slight ST-segment elevation in V_2. On May 26, 1995, the complete RBBB was evident with ST-segment elevation in V_1 and V_2. On December 8, 1995, the ECG pattern normalized, but the patient died suddenly at night 3 months later.

ECG and Electrophysiological Abnormalities

Sixteen of the 27 patients had unique ECG abnormalities that manifested as a RBBB with a significant precordial injury as demonstrated by ST-segment elevation in V_1 through V_3 (Fig 1). These ECG abnormalities are identical to those described by Brugada and Brugada in patients who have idiopathic VF.[14] However, the RBBB-like pattern in V_1 may not represent a true RBBB but have been caused instead by a marked J-junction elevation in V_1, which would produce the RBBB-like pattern. This configuration waxed and waned over time in several patients (Fig 2). In 6 patients, the abnormal ECG pattern normalized during exercise but reappeared after they stopped exercising during the recovery period.

Only 23 of the 27 patients underwent EPS; 2 patients died before undergoing EPS, and 2 refused to participate. Of the 23 patients undergoing EPS, 14 had inducible sustained VF necessitating cardioversion (Fig 3), and 9 patients had no inducible arrhythmias. SAECG was performed in 22 of the 23 patients undergoing EPS (1 patient did not undergo SAECG testing); 12 had a positive SAECG, and 10 had a negative SAECG.

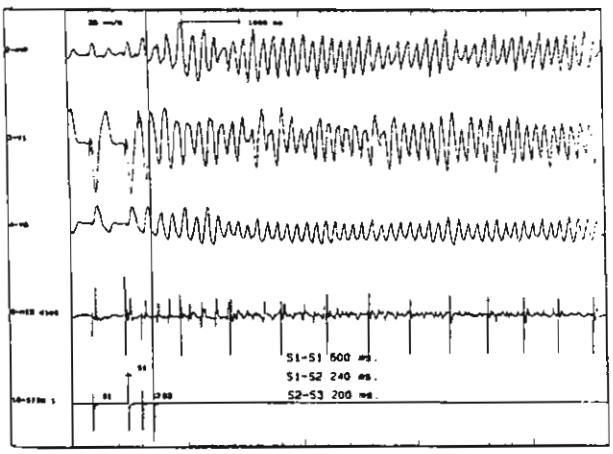

FIG 3. Polymorphic VT/VF induced by programmed electrical stimulation in 1 SUDS survivor who had RBBB and a precordial injury pattern in V_1 through V_3.

Characteristics of Study Patients: Group 1 vs Group 2

Variable	Group 1: Patients With RBBB With ST Elevation in V_{1-3}	Group 2: Patients With Normal ECG
No. of patients	16	11
Mean age, y	40±12	39±8
History of documented VF, n (%)	14 (88)*	3 (27)
Inducible VT/VF, n (%)	13 of 14 (93)†	1 of 9 (11)
Late potential on SAECG, n (%)	11 of 13 (92)‡	1 of 9 (11)
QT_c, s	0.45±0.03	0.42±0.03
AH, ms	91±10	85±9
HV, ms	63±11§	49±6
LVEF, %	64±10	64±9
Posterior wall thickness, cm	0.99±0.1	1±0.1

LVEF indicates left ventricular ejection fraction.
*P=.003; †P=.0002; ‡P=.002; §P=.007.

Clinical Differences Between Patients With and Without RBBB and Precordial Injury as Demonstrated by ST-Segment Elevation in V_1 Through V_3

The Table shows that the ECG abnormalities divided our patient population into two distinct groups: patients who had RBBB and a precordial injury pattern in V_1 through V_3 (group 1) and those who did not (group 2). The group 1 patients who had RBBB with a precordial injury pattern in V_1 through V_3 also had well-defined electrophysiological abnormalities. The group 1 patients had an abnormally prolonged His-Purkinje conduction time: the mean HV interval was 63±11 ms in the group 1 patients compared with 49±6 in the group 2 patients (P=.007). However, the AV nodal conduction time was normal in both groups: the mean AH intervals were 91±10 (group 1) and 85±9 ms (group 2) (not statistically different). Thirteen of the 14 group 1 patients had inducible VF (93%) compared with only 1 of the 9 group 2 patients (11%; relative risk, 8.35; P=.0002). Moreover, 92% of the group 1 patients had a positive SAECG (11 of the 13 patients) compared with 1 of the 9 group 2 patients (11%; relative risk, 7.62; P=.002). All these findings signified a primary electrical instability that represented an electrophysiological substrate for VF.

VF and Clinical Outcome

VF did occur more often in the group 1 patients. Those patients who displayed the SUDS markers—RBBB and precordial injury pattern in V_1 through V_3—developed VF more frequently than the group 2 patients. Fourteen of the 16 group 1 patients were SUDS survivors (87.5%) who survived an episode of VF compared with only 3 of the 11 (27%) group 2 patients who were SUDS survivors (relative risk, 3.2; P=.003). During the follow-up period (mean, 11.8±7 months; range, 3 to 25 months), 7 patients died suddenly and unexpectedly: 6 were group 1 patients (2 were on propranolol, 1 was on amiodarone, and the other 3 were not on any antiarrhythmics) and 1 was a group 2 patient (on propranolol at the time of death). A total of 10 of the 16 group 1 patients developed either sudden death (n=6) or VF as displayed on the shock E-gram of the ICD (n=3) or the ECG rhythm strip recorded in the emergency department (1 patient who survived a recurring episode) compared with only 2 of the 11 group 2 patients (1 had

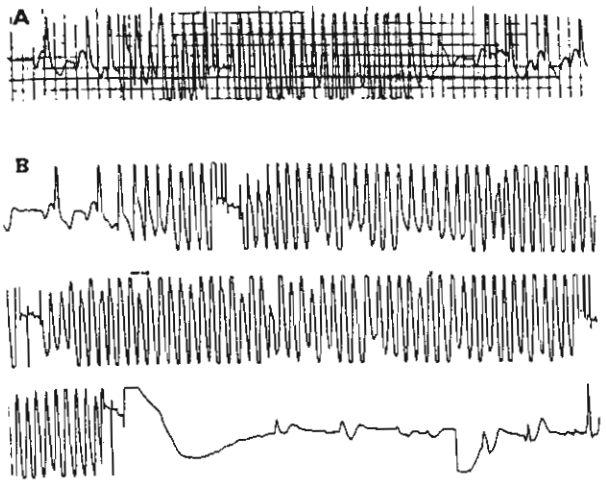

FIG 4. A, Spontaneous transient VF episode detected with a P3-CPI defibrillator. B, Continuous rhythm strip of the sustained episode that occurred after midnight while the patient was sleeping. The patient developed agonal respiration at this time and was successfully defibrillated by the device. The patient then regained consciousness but was not aware that the defibrillator had discharged.

sudden death and the other developed VF and was resuscitated; relative risk, 3.4; $P=.047$). All episodes occurred between 9:00 PM and 7:00 AM during sleep except for 2 patients: 1 had VF in the early afternoon at ≈2:00 PM and the other died suddenly at ≈10:00 AM while awake. The time of death or VF during the follow-up period is remarkably similar to the time of VF episodes observed before the study. One of the 2 group 1 patients who had only been identified as a probable SUDS patient died after having gone to sleep at 10:00 PM. This patient, who was only 17 years old, had not previously experienced VF, but he had manifested the clinical markers for SUDS (Fig 2).

Eight patients (6 group 1 and 2 group 2 patients) were treated with an ICD, 8 (5 group 1 and 3 group 2 patients) with propranolol, and 4 with amiodarone (2 group 1 and 2 group 2 patients), and 7 (3 group 1 patients and 4 group 2 patients) were not treated with antiarrhythmic therapy. Three patients who had received ICDs developed spontaneous VF during sleep; thus far, neither of the 2 group 2 patients with an ICD has experienced a defibrillation shock. An example of VF episodes as detected by the shock-E gram is shown in Fig 4. This patient, who had RBBB and a precordial injury pattern V_1 through V_3, had survived an episode of cardiac arrest before ICD implantation. Fig 4 shows a VF episode detected by the ICD. The first episode (Fig 4A) shows a nonsustained VF episode at ≈5:00 AM while he was asleep and asymptomatic. The second episode (Fig 4B) shows a sustained episode triggering the ICD discharge. This episode occurred 2 months later during sleep after midnight; the patient was found to have agonal respiration by his wife before the ICD discharged. In fact, the patient did not know that the ICD had discharged at that time until he came for a follow-up appointment. Similarly, the VF episodes detected by the ICD occurring during sleep in the other 2 SUDS survivors were associated with labored respiration and groaning. Moreover, their spouses were unable to arouse these patients until shortly after the defibrillation; 1 of the other patients

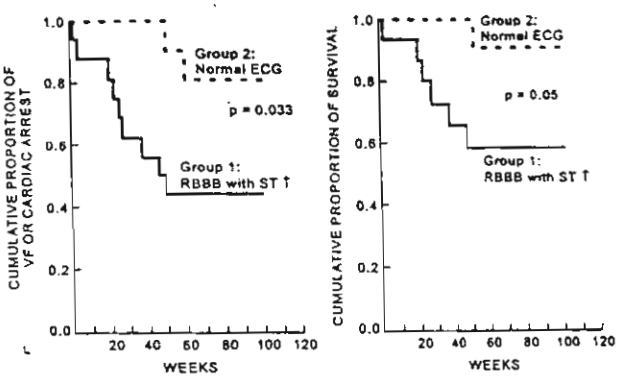

FIG 5. Kaplan-Meier plot showing high mortality rate due to sudden death and the high incidence of VF and cardiac arrest occurring in the patients who had RBBB and a precordial injury pattern in V_1 through V_3 (group 1) compared with those who did not have that clinical marker (group 2).

also did not know that the ICD had discharged. These symptoms and narratives vividly mirror those of SUDS victims described in the literature.

The VF episodes detected by the ICD emphasize two important points. The ICD confirmed that VF is the culprit rhythm causing SUDS. And the ICD effectively terminates the VF; it prevents both sudden death and the adverse consequences of cardiac arrest. A life-table analysis showing a comparison of the cumulative proportion of either VF or sudden death between the two groups is shown in Fig 5. The group 1 patients had a much higher mortality rate and incidence of VF recurrence.

Discussion

Our data demonstrate that SUDS patients face a significantly high incidence of recurrent VF, most of whom—especially SUDS survivors—manifest distinct ECG abnormalities. The group 1 patients had the RBBB pattern and a precordial injury pattern in V_1 through V_3; these ECG abnormalities correlated with a higher incidence of inducible and spontaneous VF as well as a higher death rate, electrophysiological abnormalities, and a positive SAECG. The group 2 patients did not manifest these ECG or electrophysiological abnormalities and had a significantly lower VF and death rate. All deaths in our study were sudden death; one can speculate that the death rate may have been higher if the 3 patients who had recurrent VF did not receive ICDs. These findings underscore the fact that the abnormal ECG pattern of the RBBB and precordial injury in V_1 through V_3 is an important arrhythmogenic marker for VF in our patient population.

These ECG abnormalities are identical to those described by Brugada and Brugada in their studies of idiopathic VF[14]; our patients share other similarities. Both groups have no organic heart disease, are composed almost exclusively of men, have a high incidence of inducible polymorphic VT degenerating to VF, and have a high morality rate.

Our patient population is also identical to the SUDS patients described in the CDC report.[1,2] In both our study and the CDC report, the patients were quite young and the event rate was significantly high. These findings are in line with the SUDS death rate of 26 to 38 per

100 000 reported in the study of the young Thai men (between 20 and 49 years old). The time of VF occurrence and sudden death in our population is reproducible when comparing the time that the episodes occurred before enrollment in the study with that during the follow-up period. Most VF episodes or sudden death in our patients occurred at night between 8:00 PM and 7:00 AM, suggesting that both the pattern and mechanism of death were similar in our patient population and that of the CDC.[1,2] The only difference between the CDC study population and our patient population is ethnicity: there were no Thais in the CDC population because the Thai people were not part of the refugee population coming to the United States at the end of the Vietnam War. However, a similar pattern of death occurred among the Thai construction workers who had come to Singapore.[9,15]

The abnormal ECG pattern of the RBBB and right precordial injury pattern in V_1 through V_3 can be dynamic, waxing, and waning over time (Fig 2). This observation becomes relevant in the case of the 3 SUDS survivors who had a normal ECG: it could signify either that the patients truly had no ECG abnormalities or that the recording was simply reflecting an ebbing phase of the cycle. Our finding that exercise normalized the ECG abnormalities suggests that sympathetic stimulation plays a major role in correcting the ECG abnormalities. Our data confirm those of Miyazaki et al,[16] who recently published a study about the autonomic influence on similar ECG patterns in 5 patients who had the Brugada syndrome. An isoproterenol infusion normalized the ECG pattern, a finding that parallels our observation that exercise normalized the ECG abnormalities. Our observations and those of Miyazaki et al support the hypothesis offered by Yan and Antzelevitch[17] that these ECG abnormalities were possibly caused by the outward shift in the ionic currents active at the end of phase 1 of the action potential. Increased I_{to} or decreased I_{Ca} may cause loss of the dome of phase 2 repolarization of the cardiac action potential in the right epicardium. Stimulation of I_{Ca} with a β-agonist could restore the lost dome; acetylcholine facilitated the loss of the action potential dome by suppressing I_{Ca}. The loss of the action potential dome in the epicardium, and not in the endocardium, would cause the precordial injury pattern, which may lead to phase 2 reentry- induced VF.[18] Whether this mechanism is operative in our patients remains speculative and deserves study.

All our patients had no structural heart disease, except for 3 patients who had mild LVH. However, the group 1 patients had an abnormally prolonged His-Purkinje conduction time associated with a positive SAECG. These two abnormalities are usually linked to structural changes in the heart and conduction system. A correlation can be made between our findings and those of Kirschner et al,[19] who performed autopsy studies of Laotian and Cambodian refugees in the United States.

The autopsy studies revealed conduction system anomalies, specifically, persistent fetal dispersion of the AV node and His bundle, in 14 of the 18 hearts examined of patients who had died suddenly during sleep. Whether fetal dispersion existed in our patients is not known, but if it did, it would account for the prolonged His-Purkinje conduction time. Persistent fetal dispersion causes sudden cardiac death in the young, as first described by James and Marshall.[20]

Clinical Implications

The poignant message that emerged from our study is that SUDS patients face a grave risk of VF recurrence and sudden cardiac death. Our finding is the first prospective study to demonstrate that SUDS patients who have idiopathic VF run an inordinate risk of sudden cardiac death and confirm the conclusion made by the Brugadas that patients with RBBB and ST-segment elevation in V_1 through V_3 face a grave prognosis. For many years, investigators viewed the phenomenon of sudden cardiac death in Southeast Asian men as a regional subset of the idiopathic VF population (separate and enigmatic), but the fact that patients from Europe who have idiopathic VF and SUDS patients from Asia share essentially the same ECG abnormalities, as well as other clinical characteristics, necessitates a reconsideration: The phenomenon of SUDS is not contained within one geographic region but instead spans across regions, from Southeast Asia (among SUDS patients) to Europe and North America (among patients with idiopathic VF).

Although current knowledge of these syndromes is still in its nascency and the best therapy for these patients has yet to be determined, our preliminary data suggest that the ICD may be logical treatment of choice. Meanwhile, we must educate physicians and cardiologists about these clinical markers and potential SUDS patients to avoid the devastation of young patients who may die prematurely.

Acknowledgments

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Presence of immunoglobulins, C3 and cytolytic C5b-9 complement components on the surface of erythrocytes from patients with β-thalassaemia/HbE disease

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Summary. The occurrence of IgG, IgM, IgA, C3 and C5b-9 complement complexes on erythrocytes from 43 patients with β-thalassaemia HbE disease was investigated. Indirect immunoradiometric assays using radioiodinated protein A were employed to quantify the individual components. We confirmed that circulating erythrocytes from thalassaemic patients contained elevated amounts of IgG, and small but significant amounts of C3. In addition, small but significant amounts of C5b-9 were detected. Levels of cell-bound IgG, C3 and C5b-9 were higher in splenectomized versus non-splenectomized patients. The presence of C5b-9 on circulating cells from five splenectomized patients was confirmed by an ELISA employing a monoclonal antibody specific for a C5b-9 neoantigen. When C5b-9 positive cells from two patients were solubilized with detergent and subjected to sucrose density gradient centrifugation, the terminal complexes sedimented as 25–40S macromolecules, thus behaving as membrane C5b-9 complexes. The presence of C8 and C9 in these high molecular weight fractions was directly demonstrated by Western blotting. These results constitute the first demonstration that circulating diseased erythrocytes may carry low numbers of potentially cytolytic C5b-9 complement complexes which may be partly responsible for the known ionic disturbances found in thalassaemic cells. Both bound C3 and C5b-9 could promote removal of diseased cells in the reticuloendothelial system.

Keywords: thalassaemia, complement, immunoglobulin, erythrocyte, autoantibody.

Thalassaemia is a group of inherited disorders in which there is a defect in the synthesis of one or more of the globin subunits of haemoglobin. In Thailand the common types are α and β thalassaemia, with heterozygote incidence in the population at 20–30% and 3–9% respectively (Fucharoen & Winichagoon, 1987; Wasi *et al*, 1969). Thalassaemia can occur alone or in association with abnormal haemoglobins such as haemoglobin E (HbE), and haemoglobin Constant Spring (Hb CS). The most common form of β thalassaemia in Thailand is the combination of β thalassaemia with abnormal haemoglobin E (βthal/HbE) (Wasi *et al*, 1969). In this condition, marked reduction in the synthesis of β chains results in the excess of unbound alpha chains which precipitate within the erythrocytes (Wickramasinghe *et al*, 1981). The imbalance of haemoglobin synthesis leads to many metabolic defects within the erythrocytes, resulting in shortened life-span and anaemia (Fucharoen *et al*, 1987). Although the primary molecular defect is located in the haemoglobin molecule, these cells are characterized by multiple plasma membrane abnormalities including markedly decreased sialic acid content (Kahane *et al*, 1978), and exhibit decreased deformability and osmotic fragility (Dacie, 1960). The causal relationships between these functional defects and the intracellular haemoglobin defect remain poorly understood. Kruatrachue *et al* (1980) reported a high incidence of Coombs positivity for IgG and complement in patients with thalassaemia. The mechanisms responsible for this finding have not yet been delineated, nor is it known whether bound complement fragments derive from genuine complement activation on the cell surface, or whether the molecules are passively adsorbed to the altered membrane. Many investigators have reported the appearance of

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autoimmune antibodies against erythrocyte antigens in β-thalassaemic patients (Galili *et al.* 1983); this might provide a mechanism for genuine complement activation on the cells.

In the present study we sought to determine whether thalassaemic erythrocytes might carry terminal C5b-9 complexes, since these molecules represent the most reliable markers of on-going complement activation. Using highly specific immunoassays, C5b-9 complexes were detected on circulating red cells in a large percentage of patients. Attention is drawn to the possibility that attachment of subhaemolytic quantities of these complexes to target cells may be partially responsible for the known intracellular ionic disturbances, including elevated Ca^{2+}, encountered in thalassaemic cells (Shalev *et al.* 1984), and for the high rates of spontaneous Na- and K-fluxes across the membranes (Nathan *et al.* 1969; Wiley, 1981). Complement activation on thalassaemic cells may represent a significant factor ultimately contributing to the shortened life-span and enhanced clearance of cells in the reticuloendothelial system.

MATERIALS AND METHODS

Patients and red cell samples. 43 patients with β thalassaemia/haemoglobin E disease were studied. The diagnosis was based on accepted criteria which include the characteristic clinical and haematological manifestations, and haemoglobin type determined by starch gel electrophoresis (Wasi *et al.* 1969).

10 ml blood in EDTA (10 mM) were obtained from each patient. The erythrocytes were collected by centrifugation and washed twice in isotonic phosphate buffer saline (PBS), pH 7·4. 50 μl of packed cells were diluted in PBS and the number of the cells determined in a Coulter cell counter. Red cells from normal subjects (17 laboratory personnel volunteers) were similarly collected and assayed simultaneously for the presence of immunoglobulins and complement components on their surface by the immunoradiometric assay.

Immuno-radiometric assay. A two-stage immuno-radiometric assay as reported by Salama *et al* (1985) was used to detect IgG, IgM, IgA, C3 and C5b-9 on the surface of red cells. Monospecific rabbit anti human IgG, -IgM, -IgA and C3c were obtained from Dakopatts, Copenhagen, Denmark. The antibodies were absorbed twice with pooled normal RBC (60 min at 37°C) and diluted in PBS. Affinity-purified rabbit anti-human C5b-9 IgG antibodies were prepared as described (Bhakdi & Muhly, 1983). Staphylococcus protein A was purchased from the Public Health Laboratory Service, Porton Down, U.K. Radio-iodination was carried out by the iodogen method (Pierce Chemical Co., Ill., U.S.A.) The specific activity of labelled protein A was 0·5 μCi/μg protein.

50 μl of washed packed red cells were incubated with 100 μl of 1:100 dilution of the unlabelled antibody (anti-human IgG, -IgM,-IgA, -C3c, C5b-9). After 30 min incubation at room temperature, the RBC were washed twice and resuspended in 100 μl of PBS. 10^5 cpm of ^{125}I protein A was added and incubated for 30 min at room temperature. Cells were washed twice and counted in a gamma counter. The background binding of ^{125}I protein A to the red cells without prior treatment with respective anti-immunoglobulin antibodies was subtracted from the total bound ^{125}I protein A count obtained. The binding of ^{125}I protein A was calculated as number of molecules of protein A bound per red cell.

The background binding of ^{125}I protein A was determined using RBC from 17 normal individuals. Their red cells were pre-incubated with corresponding antisera and the amount of bound ^{125}I protein A determined.

Other assays for C5b-9. Quantitation of the terminal membrane C5b-9 complex by ELISA was performed in a separate group of patients: five splenectomized, seven non-splenectomized patients with mild β thal/HbE and 10 normal individuals, as described (Hugo *et al.* 1987). 400 μl of 50% red cell suspensions were lysed with 5 mM phosphate buffer pH 8 and four cycles of rapid freeze/thaw in liquid nitrogen. The ghosts were washed with 5 mM phosphate buffer pH 8. Elution of membrane bound C5b-9 was achieved by solubilizing with 500 μl of 1% Triton X-100 (Sigma) in 1 mM EDTA pH 8 for 12 h at 4°C. The mixture was then incubated for 1 h at room temperature with 100 μl of 1% H_2O_2, to block endogenous peroxidase. 100 μl of the mixture was assayed.

Monoclonal anti C5b-9 (clone 3B1) (Hugo *et al.* 1985) was used to coat polystyrene micro-ELISA plates (Nunc F1 96-well plate, Roskilde, Denmark). Each well was coated with 100 μl of the purified monoclonal antibody at the concentration of 2–3 μg/ml in 0·05 M sodium bicarbonate buffer, pH 9·6, for 16–24 h at 4°C in a humid box. Affinity purified rabbit polyclonal antibody to C5b-9, biotinylated anti-rabbit IgG and streptavidin-biotinylated-horseradish peroxidase complexes (Amersham, Braunschweig) were used to detect the bound complexes with O-phenylenediamine (Sigma, Munich, Germany) as chromogen (Hugo *et al.* 1985).

Additionally, RBC membranes from two splenectomized patients which were positive for C5b-9 in the ELISA were solubilized with deoxycholate and centrifuged in sucrose density gradients; the methods employed for preparing and calibrating these gradients have been described in detail in earlier publications (Bhakdi & Tranum-Jensen, 1982, 1987). Fractions from the gradient were analysed by ELISA and by SDS-PAGE/immunoblotting using affinity-purified anti-C8 and anti-C9 rabbit IgG as described (Bhakdi & Tranum-Jensen, 1986).

Statistical analysis. All calculations were carried out on an Apple Macintosh with the StatView statistical package. Comparison of data between different groups was performed using unpaired Student's *t* test. A *P* value of $<0·05$ was regarded as significant.

RESULTS

Forty-three patients with β thalassaemia/HbE disease were studied. The mean and mode age were 28·2 and 26 years, respectively. 19 patients had undergone splenectomy 1 month to 28 years prior to study. The mean age of the splenectomized patients was 26·7 years. Red cells from 17 healthy adults were included as controls (mean age 27·1

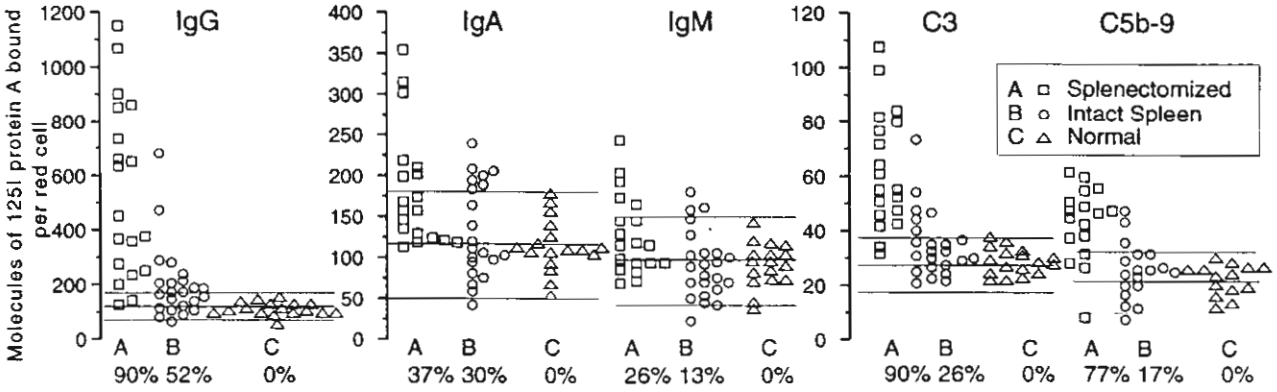

Fig 1. Scattergrams of the amount of ^{125}I protein A bound on the surface of erythrocytes pretreated with different monospecific antibodies. The amounts bound are expressed as the number of molecules of protein A per red cell. The horizontal bars depict the means and 2 standard deviations (95% confidence level) of the amount bound onto the red cells from normal individuals. The values underneath each category represent the percentage of patients whose values of the bound protein A were above 2 standard deviations of the means of the corresponding normal controls.

years). No patient had received any blood transfusion in the 3 months prior to the study.

The results of the ^{125}I protein A binding to red cells pretreated with specific antibody to IgG, IgM, IgA, C3 and C5b-9 are depicted in Fig 1. The amounts of immunoglobulins and complement components bound on the surface are expressed as the number of molecules of ^{125}I protein A per red cell. Thalassaemic patients (both splenectomized and intact spleen) showed significantly higher levels of bound IgG, IgA, C3 and C5b-9 than controls ($P = 0{\cdot}002$, $0{\cdot}014$, $0{\cdot}0004$ and $0{\cdot}0025$ respectively). Splenectomized patients showed significantly higher levels of bound IgG, C3 and C5b-9 (Fig 1, Table I) than patients with intact spleen and controls (all with $P < 0{\cdot}001$). Patients with an intact spleen had significantly increased IgG and C3 levels when compared to normal controls ($P = 0{\cdot}023$ and $0{\cdot}016$ respectively, Table I). The binding of C5b-9 in thalassaemia patients with intact spleens was not significantly different from controls ($P = 0{\cdot}12$). 90% of splenectomized patients had values of bound IgG and C3 above 2 standard deviations of the means of the corresponding normal controls. 52%, 26% and 17% of β thalassaemia/HbE patients with an intact spleen had bound IgG, C3 and C5b-9 above 2 standard deviations of the corresponding means, respectively (Fig 1).

Table I. The difference in the amount of IgG, C3 and C5b-9 bound on red cells, presented as the number of molecules of ^{125}I protein A, in subgroups of thalassaemic patients. The values are shown as means ±1 standard error. The numbers of molecules were calculated from the amount of ^{125}I protein A bound per red cell. The categories in each subgroup are compared with each other and with normal controls. Splenectomized patients had significantly increased IgG, C3 and C5b-9 than patients with intact spleen and normal controls. Patients with intact spleen had significantly increased IgG and C3 than normal controls ($P = 0{\cdot}02$, $0{\cdot}016$ respectively).

	Molecules of ^{125}I protein A bound per red cell		
	IgG	C3	C5b-9
Splenectomized	541·0 ± 73·6 [a]	63·2 ± 4·9[a]	43·1 ± 3·3[a]
Intact spleen	185·2 ± 27·3[d]	36·0 ± 2·6 [d]	26·7 ± 2·6[b]
No hepatomagaly	130·2 ± 21·7[b]	31·3 ± 3·3 [b]	21·4 ± 2·4 [b]
Mild hepatomegaly	233·7 ± 42·6 [c,d]	41·1 ± 5·8 [c,d]	28·1 ± 4·3 [c]
Large hepatomegaly	501·4 ± 73·7 [a]	57·9 ± 4·4[a]	42·4 ± 3·1 [a]
Normal controls	107·0 ± 5·8	27·7 ± 1·2	21·3 ± 1·5

[a] Significantly different from other corresponding categories and normal controls, $P < 0{\cdot}01$.
[b] Not different from normal controls, $P > 0{\cdot}05$.
[c] Not different from no hepatomegaly, $P > 0{\cdot}05$.
[d] Significantly different from normal controls. $P < 0{\cdot}05$.

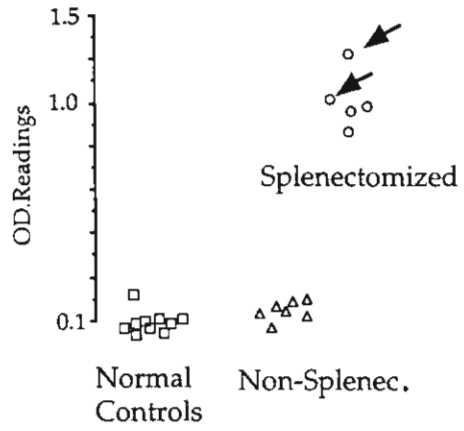

Fig 2. Scattergrams of the absorbance (OD) values reflecting the amount of cell-bound C5b-9 solubilized from the surface of erythrocytes as detected by ELISA using monoclonal to a C5b-9 neoantigen. Arrows indicate patients whose red cell were solubilized with deoxycholate and analysed for C5b-9 after separation by sucrose density gradient. It is noted that all the patients studied in this test was a separate group of patients different from those depicted in Fig 1, and all the non-splenectomized patients had mild anaemia.

Significantly larger amounts of IgG, C3 and C5b-9 were found on erythrocytes of patients with increased hepatomegaly than those with milder hepatomegaly ($P = 0{\cdot}007$, 0·025 and 0·01 respectively) and normal controls (all with $P < 0{\cdot}001$, Table I). 16/32 patients with hepatomegaly had undergone splenectomy compared with 2/9 patients without hepatomegaly.

The amount of cell-bound C5b-9 from five splenectomized patients, seven non-splenectomized and 10 normal individuals, as assayed by the ELISA employing a monoclonal to a C5b-9 neo-antigen, is depicted in Fig 2. Erythrocytes from splenectomized patients were all clearly positive for C5b-9, whereas non-splenectomized and normal controls were negative.

Fig 3 shows the levels of C5b-9 detected in the different fractions obtained from sucrose density gradient centrifugation of the solubilized red cell membranes from one of the two splenectomized patients. Similar results were obtained in the second patient. The peak of C5b-9 was in the region of 25–40S, corresponding to the sedimentation pattern of C5b-9(m) (Bhakdi & Tranum-Jensen, 1982; Hugo *et al*, 1987). The peak in fraction 10 was due to haemoglobin artefact. By calibrating the ELISA with purified C5b-9(m), we estimated that the membranes had a mean content of 100–200 molecules C5b-9(m) per cell. Final evidence that the neoantigen-positive material represented C5b-9 was obtained by Western blot analysis of the peak fraction.

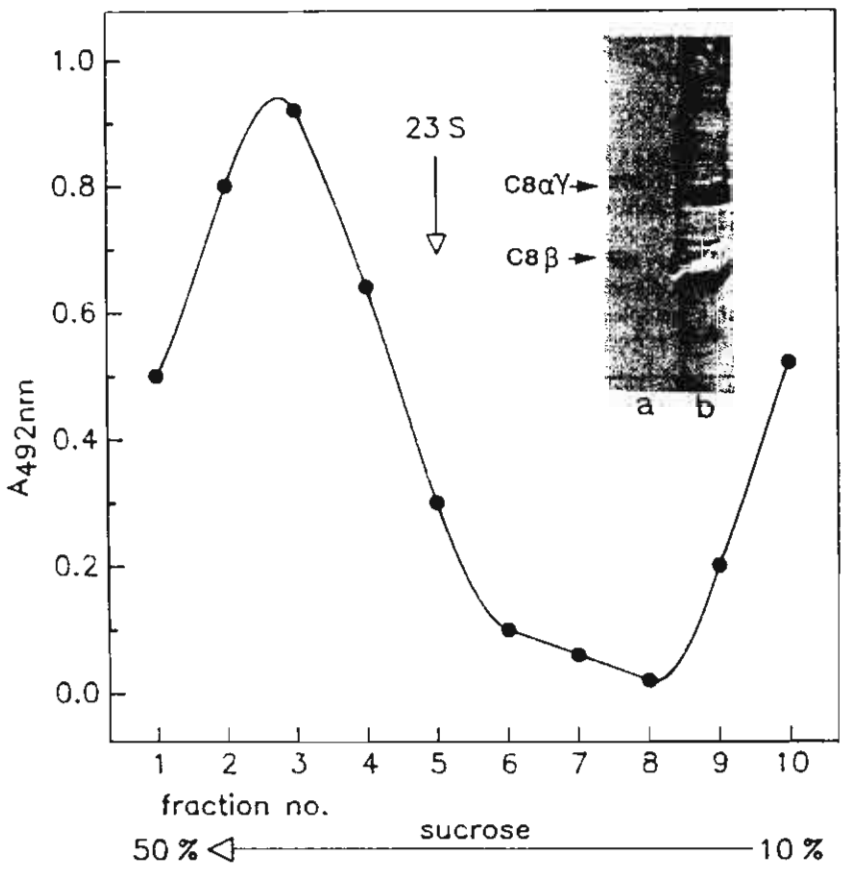

Fig 3. 3×10^9 washed RBC membranes from a splenectomized patient were solubilized with deoxycholate and centrifuged through a 5 ml sucrose density gradient; the direction of sedimentation is indicated. 10 equal fractions were collected and assayed for C5b-9 neoantigen by ELISA. Reactive material was detected in high molecular weight fractions corresponding to 28–40S; the sedimentation position of fluid-phase SC5b-9 (23S) is indicated. 80 μl of fraction 3 were analysed by SDS-PAGE/immunoblotting using affinity-purified anti-C8 IgG (inset, lane a); 1 μl whole human serum was applied as control (lane b). The characteristic subunits of C8 could be directly detected.

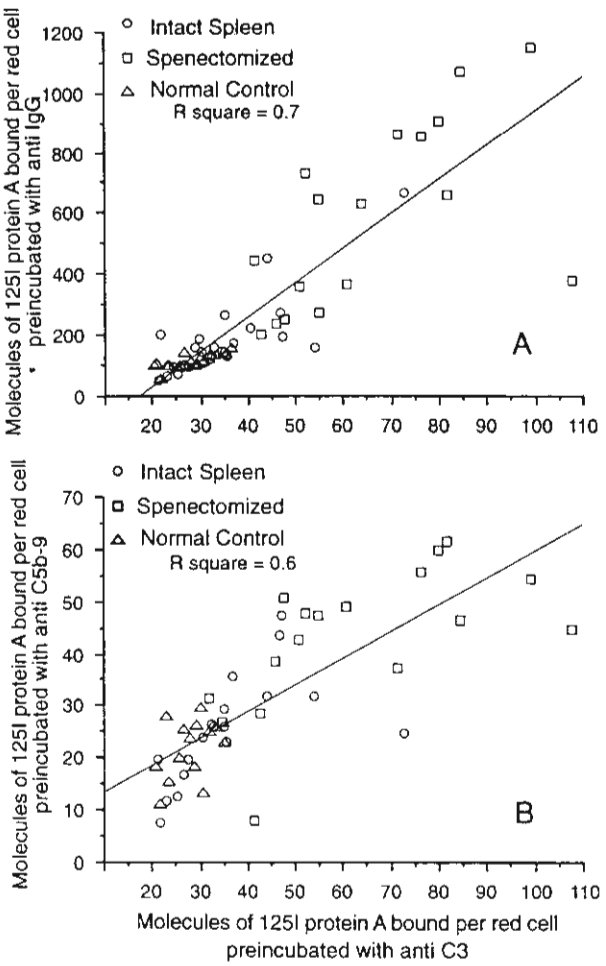

Fig 4. Scattergrams showing correlation between the amounts of bound C3 and C5b-9 (A), and IgG and C3 (B) on thalassaemic red cells. The amounts depicted in each axis are extrapolated as number of molecules of ^{125}I protein A bound per red cell. The least square (r) values are shown in each figure.

Fig 3 (inset), demonstrates the detection of C8 in such an experiment. Similarly, C9 was also found to be present in these fractions (not shown). C8 and C9 were not detected in control preparations. Taken together, these results indicate the presence of the potentially cytolytic membrane C5b-9 complex on the erythrocytes of thalassaemic patients.

The amount of IgG bound on the patients' red cells correlated well with the amount of C3 (Fig 4a). Similarly, there was a good correlation between C3 and C5b-9(m) (Fig 4b).

DISCUSSION

The objective of the present investigation was to determine whether thalassaemic RBC might represent targets for autologous complement attack *in vivo*. These studies were prompted by an earlier finding that diseased cells apparently carry C3 and gave a positive conventional direct antiglobulin test (DAT) for this component (Kruatrachue *et al*, 1980). Should C3-binding occur via genuine complement activation, terminal C5b-9 complexes might be generated. According to recent evidence, RBC carrying subcytolytic amounts of C5b-9 exhibit enhanced membrane permeability for ions (Halperin *et al*, 1988). In fact, it has long been known that thalassaemic cells, particularly those from splenectomized patients, display markedly enhanced membrane permeability for Na^+ (Wiley, 1981) and K^+ (Nathan *et al*, 1969), and their intracellular Ca^{2+} content is highly elevated (Shalev *et al*, 1984). The presence of bound C5b-9 complexes would not only prove that complement activation occurs on circulating cells, but might also provide a hitherto unsuspected link to the above findings. Using highly specific immunoassays, we therefore sought to detect these complexes on the cells.

First, a direct immuno-radiometric assay was used to quantify cell-bound immunoglobulins, C3 and C5b-9. Results obtained with 43 thalassaemic patients confirmed and extended the original report that diseased cells indeed carried IgG, IgM, IgA and C3. Significant correlations were seen between the quantities of bound C3 and IgG, and C3 and C5b-9 (Figs 4a and 4b) suggesting the roles of the autoantibodies in the activation of the complement system. In addition, these assays yielded the first indication that the cells might also carry small amounts of C5b-9.

That thalassaemic RBC might carry C5b-9 complexes required confirmation, especially since this would show that terminal complement complexes can indeed be present on non-haemolysed erythrocytes *in vivo*. Therefore we employed an ELISA based on the use of a specific monoclonal antibody against a C5b-9 neoantigen to detect the terminal complex. These assays fully corroborated the initial observations. Terminal complement complexes may occur in membrane-bound form as pore-forming C5b-9(m) complexes, or in non-cytolytic form as SC5b-9 complexes, and these functionally distinct entities cannot be differentiated immunologically. In order to identify the cell-bound complexes, we therefore analysed their sedimentation behaviour in sucrose density gradients. It is known that SC5b-9 sediments as a 23S macromolecule, whereas C5b-9(m) complexes sediment to regions corresponding to 25–40S (Bhakdi & Tranum-Jensen, 1987). When erythrocyte membranes from two splenectomized patients were solubilized and analysed in this fashion, the C5b-9 complexes showed identical sedimentation behaviour to membrane complexes. By calibrating the ELISA with purified C5b-9(m), we calculated that these cells carried an average of 100–200 C5b-9(m) complexes/cell.

This is the first demonstration of C5b-9(m) present on the surface of circulating red cells *in vivo*. Recent studies have demonstrated that red cells are protected against complement lysis by many mechanisms, including the presence of surface CD59 (Davies *et al*, 1989; Sugita *et al*, 1988) and selective shedding of the membrane attack complex by vesiculation (Iida *et al*, 1991). About 20 000 molecules of C9 could be found on unlysed human red cells in an *in vitro* experiment using antibody-coated human red cells and homologous complement (Houle *et al*, 1988). Thus, it is conceivable that C5b-9 deposition can occur *in vivo* in the absence of intravascular haemolysis.

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It is evident from our data that IgG, C3 and C5b-9 are present in larger amounts on the red cells from splenectomized patients than in normal controls: 90%, 90% and 77% of patients had levels of the respective proteins more than 2 SDs above the corresponding means of normal controls. But only 52%, 26% and 17% of patients with an intact spleen had levels of bound IgG, C3 and C5b-9 more than the 2 SDs above the means, respectively. Although their means are statistically different (except for C5b-9, Table I), the findings raise the possibility that the bound proteins are the direct result of splenectomy, and are not related to thalassaemia. This is unlikely for the following reasons. First, in a study of the prevalence of positive antiglobulin test (by agglutination assay) in patients splenectomized due to trauma, Spirer *et al* (1980) found no positive Coombs test amongst 41 patients. Using a comparable assay, Kruatrachue *et al* (1978) found 33·3% of non-splenectomized patients with thalassaemia to be Coombs positive. Second, in a study by Schluter & Drenckhahn (1986), using ^{125}I-labelled protein A to quantify the number of cell-bound antibodies in normal healthy individuals and in patients suffering from unstable forms of haemoglobin, they found no increase in the amount of bound ^{125}I protein A in five individuals splenectomized because of trauma compared to 11 normal healthy subjects with intact spleen; whereas a 3–4-fold increase was found in patients with unstable haemoglobins. Therefore it is obvious that splenectomy or perhaps splenic dysfunction alone is not the cause of complement deposition on red cells.

In summary, this work presents unambiguous evidence for on-going complement activation on β-thalassaemic cells *in vivo*, and shows that the entire sequence may proceed to completion with the formation of C5b-9 complexes. The causes of complement activation remain to be elucidated. Obvious possibilities include activation of the classic pathway by autoantibodies, and derangements of regulatory factors (DAF, CR-1, sialic acid) due to pathological membrane composition and structure. The latter might result in enhanced susceptibility to spontaneous attack via alternative pathways or bystander mechanisms. Irrespective of cause, deposition of potentially cytolytic C5b-9 complexes may be partly responsible for the previously observed increases in membrane permeability for ions, which are known to be more marked in splenectomized thalassaemic patients, and which can account for the characteristic intracellular calcium accumulation. Our data can be accommodated in a simple working hypothesis that envisages low-grade complement activation to occur continuously on thalassaemic cells. When a critical number of deposited C5b-9 complexes is reached, disturbances of ionic homeostasis alter the cells such that they are removed in the spleen. This is why in non-splenectomized patients, only the minority of circulating cells carry elevated amounts of C5b-9, a fact that does not contradict the contention that ongoing complement activation contributes to accelerated removal. After splenectomy, cells carrying larger numbers of C5b-9 complexes are then found in circulation. Terminal C5b-9 complexes will probably be detectable on circulating cells in splenectomized patients in several other hereditary RBC diseases, where they will similarly represent valuable markers for complement activation and attack on the altered cells.

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Pediatric Nephrology

Original article

Renal tubular function in β-thalassemia

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Abstract. Studies of the renal involvement in thalassemic syndromes have been varied and few. This study was designed to define the renal abnormalities associated with β-thalassemia and to correlate the renal findings with clinical parameters. One hundred and four β-thalassemic children with various disease severity were studied. The patients were divided into three groups: 48 with severe anemia [hematocrit (Hct) <25%], 31 on a hypertransfusion program and desferrioxamine treatment, and 25 with moderate anemia (Hct >25%). The results were compared with 15 normal children. Significantly higher levels of proteinuria and low molecular weight proteinuria were found in all patients compared with normal children. Aminoaciduria was detected in one-third of patients. Thalassemic patients had significantly lower morning urine osmolarity, higher urine *N*-acetyl-β-D-glucoseminidase and malondialdehyde (MDA, an indicator of lipid peroxidation). Patients with severe anemia had significantly higher low-molecular weight proteinuria and MDA, and lower urine osmolarity than those with moderate anemia. Our data confirmed the high frequency of renal abnormalities in β-thalassemia patients and indicated some degree of proximal tubular dysfunction. Severity of the abnormalities correlated with the degree of anemia and were least severe in patients on hypertransfusion and desferrioxamine therapy. This suggested that the damage might be caused by *anemia and* increased oxidation induced by excess iron deposits.

Key words: β-Thalassemia – Hemoglobin E – Renal tubular function – Low molecular weight proteinuria – Malondialdehyde – Urine osmolarity

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Introduction

Thalassemia hemoglobinopathies are prevalent in Thailand. The frequencies of β-thalassemia (thal) and hemoglobin (Hb) E, β-globin chain variant, are 3%–9% and 13%–60%, respectively [1]. The disease forms of β-thal are homozygous β-thal and β-thal/HbE. Half of the homozygous β-thal patients die before the age of 12 years due to severe infection, anemia, and multiple organ failure [2–4]. The abnormal synthesis of the globin chains or the production of abnormal Hb have wide impacts on the physiological functions of virtually every major organ. Patients with thal are known to have dysfunctions of the cardiopulmonary, reticuloendothelial, and other major systems [2–7]. Abnormalities of renal function have also been reported, but there is no systematic study of the prevalence and type of renal involvement. Various glomerular pathologies have been sporadically reported, and it is still unknown whether those abnormalities are genuinely associated with the thalassemic syndromes [3, 6, 8, 9]. One study of ten patients with Cooley's anemia suggested some abnormalities associated with renal medulla [10]. Renal tubular acidosis has also been reported in patients with thal [10, 11]. The purpose of this study was to systemically investigate the prevalence and nature of renal abnormalities in 104 patients with β-thal.

Patients and methods

Patients aged between 3 and 15 years with homozygous β-thal or β-thal/HbE diseases attending the Department of Pediatrics, Siriraj Hospital from November 1993 through October 1994 were recruited into the study with informed consent from their parents. Patients with acute febrile illness were excluded. The diagnosis of β-thal was made by standard methods [12]. Height and weight standard deviation scores (SDS) were calculated according to Tanner et al. [13].

Patients were instructed to fast overnight before attending the clinic in the morning. Blood samples were collected from each patient for Hb and hematocrit (Hct), urea nitrogen, creatinine, and electrolytes. Fresh second-morning urine samples were collected; all samples were immediately aliquoted and frozen until further analysis. The remaining

Table 1. Summary of demographic and biochemical data of three groups of patients and controls[a]

	Group A (n = 48)	Group B (n = 31)	Group C (n = 25)	Controls (n = 15)
Female/male	21/27	13/18	15/10	5/10
Age (years)	9.9 ± 0.4	9.0 ± 0.5	10.2 ± 0.6	9.8 ± 0.9
Height (SDS)	−2.5 ± 0.2	−1.0 ± 0.3	−2.0 ± 0.3	
Weight (SDS)	−2.2 ± 0.1	−0.9 ± 0.3	−1.5 ± 0.2	
Ratio of patients with intact spleen/splenectomized patients	31/17	23/8	20/5	15/0
Serum ferritin (ng/ml)	1,078.9 ± 178.8	3,293.6 ± 343.8	1,869.8 ± 385.3	
Estimated glomerular filtration rate[b] (ml/min per 1.73 m²)	137.8 ± 4.5	114.2 ± 3.5	126.3 ± 4.5	
Serum creatinine (mg/dl)	0.5 ± 0.02	0.6 ± 0.02	0.6 ± 0.02	
Serum HCO_3 (mEq/l)	23.0 ± 0.3	24.4 ± 0.4	24.2 ± 0.5	
Urine urea N (mg/dl)	548.8 ± 41.5**	611.3 ± 53.9**	585.2 ± 60.4**	1033.3 ± 101.7
Urine osmolarity (mosmol/kg)	630.3 ± 25.6**	739.1 ± 28.5	682.3 ± 40.9*	836.6 ± 70.7
Urine protein (mg)/mg Cr	0.27 ± 0.03*	0.20 ± 0.05	0.22 ± 0.04*	0.07 ± 0.01
Urine LMW protein (mg)/mg Cr	0.15 ± 0.02**	0.10 ± 0.02*	0.12 ± 0.02*	0.03 ± 0.00
Urine NAG (unit)/g Cr	42.3 ± 6.2*	32.3 ± 5.4*	26.1 ± 5.2*	3.5 ± 0.4
Urine MDA (nmol)/mg Cr	0.056 ± 0.008**	0.025 ± 0.003	0.050 ± 0.008**	0.013 ± 0.001

SDS, Standard deviation score; HCO_3, bicarbonate; N, nitrogen; Cr, creatinine; LMW, low molecular weight; NAG, N-acetyl-β-D-glucosaminidase; MDA, malondialdehydr

*P < 0.05; **P < 0.001

[a] *Values are mean ± standard error*

[b] As calculated by Schwartz's formula: 0.55 × height (cm)/plasma Cr (mg/dl)

[c] Group A consists of patients with severe anemia; group B are on hypertransfusion with desferrioxamine treat; group C are those with mild anemia

urine was tested for osmolarity, protein, sugar (by Labstix, Bayer Diagnostics) and examined microscopically. N-Acetyl-β-D-glucosaminidase (NAG) (by a spectrophotometric method [14], creatinine, (Jaffe reaction, autoanalyzer), and amino acids (by paper chromatography [15] were measured. Urine malondialdehyde (MDA) was measured by a spectrophotometric technique described by Knight et al. [16]. Urinary protein was assayed by a modified Bradford method [17] (Bio-Rad Laboratories, Richmond Calif., USA). The urine was electrophoresed in 8%–15% polyacrylamide gels and silver stained [18]. The stained gels were scanned in a flat-bed scanner, and the amount of low molecular weight (LMW) proteins was calculated from the proportion of the protein bands smaller than 45 kilodaltons [19]. Fresh morning urine from 15 healthy children of the same age group were used as controls.

Statistical methods. All calculations were carried out using Statview statistical package (Abacus Concepts, USA). Comparison between groups was performed using unpaired Student's t-test and in categorical data using the chi-squared test. A P value of less than 0.05 was regarded as significant.

Results

One hundred and four patients comprising 27 homozygous β-thal and 77 β-thal/HbE patients were included in this study. Mean age was 9.7 years with a range of 3–15 years. Thirty patients were splenectomized (mean age 10.7 years vs. 9.3 years of those with intact spleens). The patients were divided into three groups. Group A included 48 patients with severe anemia (Hct less than 25%), group B (31 patients) were on a hypertransfusion program (maintaining the Hct at around 30% at all times) and desferrioxamine treatment (subcutaneous injection of 20–40 mg/kg per dose, 2–5 doses/week), and 25 patients of group C who had a Hct >25% and were not on desferrioxamine. The duration of desferrioxamine treatment was 3.7 ± 2.3 years (mean ± SD). The criteria for selection of patients for desferrioxamine and hypertransfusion treatments were not based on clinical severity, but on their financial status. Table 1 summarizes the demographic data of patients and controls. There was no significant difference in the proportion of patients with and without splenectomy amongst the three groups. None of the patients were positive for human immunodeficiency virus antibody and 2 patients were positive for hepatitis B surface antigen antigen.

Forty-one patients (39%) had a body weight <3rd percentile for age. Height and weight SDS are shown in Table 1. Blood urea nitrogen and serum creatinine were within normal limits in all except 1 patient from group B, who had a serum creatinine of 1.1 mg/dl (estimated glomerular filtration rate (GFR) using Schwartz's formula [20] 69 ml/min per 1.73 m²). Using Schwartz's formula to estimate the GFR, all groups had values within the normal limit (89–165 ml/min per 1.73 m²). Serum electrolytes were all within normal limits.

Urine osmolarity in all groups except group B was lower than normal controls (P = 0.0005 for group A and P = 0.02 for group C). Group A had the lowest urine osmolarity (Table 1). The urine urea level was lower than controls in all groups. Splenectomy did not affect urine osmolarity (P = 0.08). Urine protein and sugar by Labstix were negative. Microscopic hematuria (10 red blood cells per high-power field) was also found in 8 patients (5 in group A, 2 in B, and 1 in C), but no renal casts were found. Generalized aminoaciduria was found in 32 of 102 patients. No difference in the proportion of patients with aminoaciduria was found amongst the three groups (chi-squared, P = 0.6559).

All three groups had significantly higher levels of urine NAG than controls (P <0.05), but no difference was found amongst patients. The LMW proteinuria/creatinine in groups A, B, and C was significantly higher than controls (P = 0.0002, 0.0390, 0.0062, respectively). Severely anemic (A) and mildly anemic (C) groups had significantly higher total urinary protein/creatinine ratios (P = 0.0017 and

0.0398), but there was no difference between group B and controls ($P = 0.0595$). There was no evidence of glomerular proteinuria by electrophoresis. Urine MDA in groups A and C was significantly higher than controls ($P < 0.0001$ and 0.0003), but there was no difference between group B and controls ($P = 0.2132$) (Table 1).

Discussion

We report a high frequency of renal abnormalities in a group of β-thal patients with disease of varying severity. The key abnormalities included: increased levels of proteinuria, especially LMW fraction, aminoaciduria, increased urinary NAG and MDA. Urine osmolarity was significantly lower in patients than controls. The data suggest some degree of proximal tubular dysfunction. Increased urine NAG [21, 22], aminoaciduria [23], and LMW proteins [24–27] are indicators of proximal tubular damage.

When patients were divided into three groups according to their clinical severity, patients receiving multiple blood transfusions and desferrioxamine treatment (group B) had lower levels of LMW proteinuria, urinary MDA, and higher urine osmolarity. The most pronounced abnormalities were found in group A, the group with the most-severe anemia (Hct < 25%). MDA, the end product of lipid peroxidation, was highest in groups A and C, and correlated well with the clinical classification. This suggested that oxidative stress might be an important factor responsible for the damage. In thal, the imbalanced synthesis of Hb leads to the presence of excess unpaired globin chains and a high intracellular non-Hb iron content. The unstable Hb subunits generate free oxygen radicals, which start a chain of oxidative events leading to disintegration to denatured globin chains, heme and iron which bind to different membrane proteins, altering their normal structure and function [28]. The excess free iron is a catalyst of lipid peroxidation via participation in the Fenton reaction [29, 30]. The high urine MDA in groups A and C, and the normal levels in group B supports the hypothesis that lipid peroxidation occurs in untreated groups and can be reduced or reversed by desferrioxamine treatment [31]. The reduction of urinary MDA and milder renal manifestations in group B may also be due to the direct suppressive effect of desferrioxamine on peroxidation [32, 33]. Group B responded favorably to desferrioxamine treatment and frequent transfusions, despite high levels of serum ferritin. The latter indicates that the treatment given to this group was not adequate, i.e., not able to significantly reduce tissue iron. The improvement in renal function in group B might be due to the direct suppressive effect of desferrioxamine on peroxidation [28]. This is significant, since a comprehensive program of desferrioxamine chelation is not usually achieved in developing countries, due to economic constraints.

We found no detrimental effects of desferrioxamine on renal function, as reported by Koren et al. [34] and Cianciulli et al. [35]. The desferrioxamine dosage in our study is lower than in others in which nephrotoxicity has been reported. A decrease in the ability to concentrate urine was correlated with clinical severity. The group with severe anemia (A) had the lowest urine osmolarity after overnight fasting and the patients on desferrioxamine and hypertransfusion (B) had the highest urine osmolarities, which were not statistically different from controls (Table 1). These data are in accordance with those reported by others [6, 10–12]. The cause of this defect is not known. However, it is possible that malnutrition might play a role [36, 37], since two-thirds of the patients in group A were underweight. Paniagua et al. [38] demonstrated that malnutrition by itself did not cause the abnormalities, but other insults, such as electrolyte imbalances and infections, were responsible for the defects. All three groups had lower levels of urine urea (which generally reflects the degree of malnourishment) than controls, but patients in group B had a comparable ability to concentrate urine. This indicates that malnutrition is not the key factor leading to abnormal concentrating ability. Another possible contributory factor is the hyperperfusing effect of anemia [6]. The estimated GFR in groups A and C was high, although within normal limits; these findings support the above hypothesis. The fact that urine osmolarity was higher in patients on iron chelation suggests that the concentration defect is reversible and deposited iron and anemia might play a role in the pathogenesis of the defect.

Our findings are in accordance with previous studies in thal patients. Two studies have identified some degree of medullary fibrosis and suggested that the pathology might contribute to the abnormal concentrating ability [6, 10]. Generalized aminoaciduria was also reported by Hyman et al. [39].

In conclusion, our data indicate that there are proximal tubular dysfunctions in patients with homozygous β-thal and β-thal/HbE disease. The cause of this dysfunction is not known, but anemia and iron deposition may be key factors. The role of desferrioxamine as a protective agent for tubular damage is also suggested by our study.

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Renal Function in Adult Beta-Thalassemia/Hb E Disease

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Abstract

β-Thalassemia hemoglobin E (β-thal/Hb E) is the commonest form of hemoglobinopathy in Thailand. Shortened red cell life span, rapid iron turnover and tissue deposition of excess iron are major factors responsible for functional and physiological abnormalities found in various forms of thalassemia. Increased deposition of iron had been found in renal parenchyma of thalassemic patients, but no systematic study of the effect of the deposits on renal functions has been available. The purpose of this study is to describe the functional abnormalities of the kidney in patients with β-thal/Hb E and provide evidence that increased oxidative stress might be one of the factors responsible for the damage. Urine and serum samples from 95 patients with β-thal/Hb E were studied comparing with 27 age-matched healthy controls. No difference in the creatinine clearance was observed. β-thal/Hb E patients excreted significantly more urinary protein (0.8 ± 0.5 vs. 0.3 ± 0.1 g/day; $p < 0.001$). Aminoaciduria was found in 16% of the patients. Analysis of urinary protein by SDS-PAGE electrophoresis and silver staining revealed abnormal pattern of protein with increased small molecular weight (<45 kD) bands. Morning urine analysis showed significant lower urine osmolality (578.3 ± 164.6 vs. 762.4 ± 169.9 mosm/kg, $p < 0.001$) in patients. Patients excreted more NAG (N-acetyl beta-*D*-glucosaminidase, 26.3 ± 41.3 vs. 8.4 ± 3.9 U/g Cr, $p < 0.0001$) and β_2-microglobulin, 124.3 ± 167 vs. 71 ± 65.5 μg/g Cr, $p = 0.001$. Plasma and urine MDA (malonyldialdehyde) levels were both raised ($p < 0.0001$). Nine patients were selected for renal acidification study. All were found to be normal, but showed poor response to DDAVP challenge (urine osmolality 533 ± 71). This is the first report of renal tubular defects found associated with β-thal/Hb E disease. The mechanism leading to the damage is not known but it might be related to increased oxidative stress secondary to tissue deposition of iron, as indicated by the raised levels of serum and urine MDA. It is not known whether these functional defects would have any long-term effects on the patients. Further studies are warranted and means of prevention of these defects should urgently be sought.

Key Words
Renal function
β-thal/Hb E

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Introduction

Renal involvement has been extensively studied in both Cooley's disease and sickle cell anemia [1–8] but sparingly in β-thalassemia/Hb E disease [9, 10]. There are several factors that may adversely affect the kidney in thalassemia: chronic anemia, iron overload, hemosiderosis and oxidative stress. Thalassemia, especially β-thalassemia/Hb E disease, is a common disease in Thailand and its mortality and morbidity rates are high. The effect of the disease on several organs, e.g. cardiovascular, endocrine, respiratory systems, has been studied in detail but renal function is still more or less unknown. The purpose of this study was to investigate renal function in a large population of β-thalassemia/Hb E disease patients.

Materials and Methods

Patients

A group of 95 patients (39 males and 56 females) suffering from β-thalassemia/Hb E disease (age 29.8 ± 9.1 years) was studied. Diagnosis was carried out by standard criteria [11, 12]. All patients attended the outpatient clinic of the Hematology Division, Medicine Department, Siriraj Hospital. The last transfusion was performed more than 3 months before the renal function tests. Patients with febrile symptoms were not included in the study. 27 normal healthy laboratory personnel with age ranged from 22 to 38 years (mean ± SD; 31.3 ± 9.7) were used as controls. All had normal hemoglobin and renal function tests.

Urine and Blood Collection and Analyses

After overnight dehydration, the first fresh morning urine is collected and analyzed for specific gravity, osmolality, pH, protein, and sugar. Microscopic examination and urine cultures were carried out to exclude cases with infection. Assay for β_2-microglobulin was carried out by ELISA [13], creatinine by modified alkaline picrate method [14], urinary N-acetyl glucosaminidase by an enzymatic method [15], urinary citrate by citrate lyase method [16], and malondialdehyde (MDA) by the thiobarbituric method [17]. Detection of aminoaciduria was carried out by a chromatographic method [18]. Urinary protein was assayed by a modified Bradford method (Bio-Rad Laboratories Ltd., Richmond, Calif., USA) [19]. In addition, the urine was electrophoresed in 8–15% polyacrylamide gel and stained with the silver method [20]. The stained gels were scanned in a flat bed scanner, and the percentage of low-molecular-weight proteins (LMW) was calculated from the proportion of the protein bands smaller than 45 kD as scanned from the gel. The amount of LMW urinary protein was thus calculated from the total protein as measured above.

A blood sample from each patient was collected for complete blood count, urea nitrogen, creatinine, electrolytes, ferritin [21] and MDA [22]. The creatinine clearance was calculated in relation to the body surface area.

Table 1. Fundamental data of the β-thal/Hb E patients and normal controls

	Normal	Patients
Number	27	95
Age, years	31.3±9	29.8±9.1
Sex (M:F)	14:13	39:56
Body weight, kg	57.2±10	47.7±8.0

Detailed Renal Tubular Functions Study

18 patients were found to have serum bicarbonate less than 21 mmol/l. 9 patients out of this group had been randomly selected for water deprivation and renal acidification tests. Respiratory alkalosis were excluded from blood gas analysis as the causes of low bicarbonate.

After 16 h of water deprivation, all patients were given 10 mg of DDAVP intranasally. Urine was collected every hour for 6 h and blood was taken prior to and 1 h after DDAVP for osmolality.

A chronic acid loading test was also performed. Ammonium chloride (NH_4Cl) 0.1 g/kg in three divided oral doses were given orally for 3 consecutive days. Blood samples were taken daily and 24-hour urine was also collected. To investigate the production of urinary ammonia, a furosemide test had been carried out according to Vasuvattakul et al [23]. On the fourth morning the subjects ingested 20 mg of furosemide. Pre- and postfurosemide blood and urine samples were collected for analysis. The urine was collected every 30 min for 3 h post furosemide.

Statistical Analysis

Student's t and Mann-Whitney U tests were used to evaluate the difference of means. Significant level of 5% was used. All results are expressed as mean ± SD.

Results

Table 1 summarizes the general data of patients and controls. Blood and urine parameters are shown in table 2.

Creatinine clearance did not differ statistically between thalassemic patients and normal controls. 24-hour proteinuria and low-molecular-weight protein (<45 kD) were significantly higher in patients (table 2). Proteinuria of more than 0.5 g/day was seen in 64 patients (67.4%); 28 patients (30%) had proteinuria more than 1 g/day, but only 2 patients had proteinuria more than 2 g/day. 2 patients had significant number of red blood cells in urine (5 cells per high power field), but both had proteinuria less than 0.6 g/day. Aminoaciduria was detected in 15 cases (16%, 7 from patients with splenectomy and 8 with intact spleen). Urine urea and specific gravity showed no significant difference between patients and

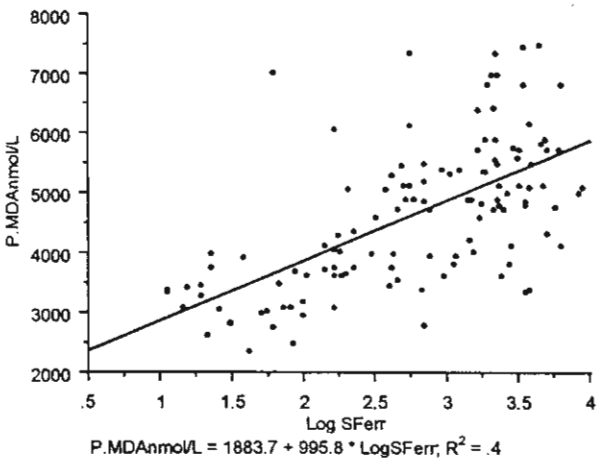


Fig. 1. The relationship between serum ferritin and plasma MDA.

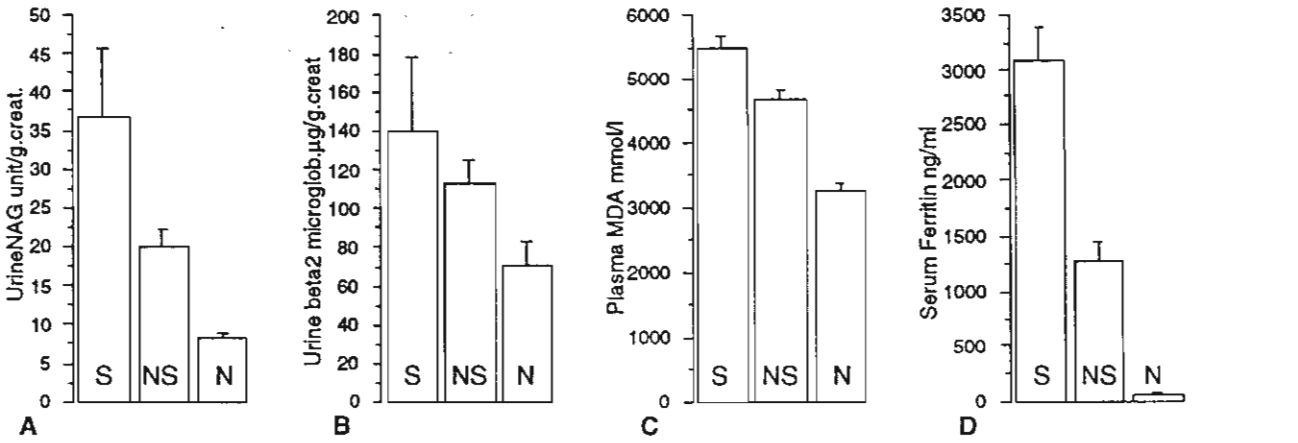
Fig. 2. Comparison of the levels of urine NAG (A), urine β_2-microglobulin (B), plasma MDA (C), and serum ferritin (D) amongst groups of splenectomized patients (S), patients with intact spleen (NS) and normal controls (N). The bar values are SE.

Table 2. Blood chemistries and urinary parameters in patients with β-thal/Hb E and normal controls.

	Normal (n = 27)	Patients (n = 95)	p value
a Blood parameters			
Hct, %	42.1 ± 4.8	25.9 ± 4.2	<0.0001
Haemoglobin, g/dl	13.8 ± 1.7	8.0 ± 1.5	<0.0001
Serum Cr, mg/dl	1.0 ± 0.15	0.67 ± 0.16	<0.0001
Ccr, ml/min/1.73 m^2	93.9 ± 19.4	105 ± 39	0.16
Serum Na^+, mmol/l	140.6 ± 3.1	140 ± 2.5	0.13
Serum K^+, mmol/l	4.3 ± 0.39	4.5 ± 0.47	0.13
Serum HCO_3^-, mmol/l	23 ± 2	22 ± 1.9	0.03
Serum Cl^-, mmol/l	103.3 ± 2.2	103 ± 3.5	0.86
Serum ferritin, ng/ml	66.3 ± 49	1,999 ± 1,854	0.0001
Plasma MDA, nmol/l	3,273 ± 422	5,009 ± 1,097	0.0001
b Urine parameters			
pH	6.2 ± 0.6	5.8 ± 0.4	<0.0001
Specific gravity	1,019.2 ± 4.5	1,017.4 ± 5.7	0.14
Osmolality, mosm/kg H_2O	762.4 ± 170	578.3 ± 164.7	<0.0001
24-hour protein, g/day	0.25 ± 0.1	0.83 ± 0.5	<0.0001
Low MW protein, mg/g Cr	32.9 ± 36.5 (21)	144.8 ± 93.6 (50)	<0.0001
β_2-Microglobulin, μg/g Cr	71 ± 65.5	124.3 ± 167	0.001
NAG, U/g Cr	8.2 ± 3.9	26.9 ± 37.7	<0.0001
Urea, g/day	15 ± 5.6	14.8 ± 7	0.7
MDA, nmol/day	24 ± 12	69.7 ± 38	<0.0001
Citrate, mmol/g Cr	1.2 ± 0.7	0.75 ± 0.87	0.01

c Hematuria, proteinuria and aminoaciduria

		Patients (n = 95)	%
Hematuria with RBC > 5/HPF		2	2.1
Proteinuria, g/day	0.5	31	32.6
	>0.5–1	36	37.9
	>1–2	26	27.4
	>2	2	2.1
Aminoaciduria		15	15.8



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normal controls, but urine osmolality was significantly lower than controls ($p < 0.0001$). Urine pH of the patients was also lower (5.8 ± 0.4 vs. 6.2 ± 0.6; $p < 0.0001$). Summarized results of blood chemistry, urine and hematological parameters (mean ± SD) are shown in table 2.

Urine β_2-microglobulin and NAG were significantly higher than controls. Plasma and urine malondialdehyde (MDA), which is one of the metabolites of lipid peroxidation, were significantly higher in patients (table 2a, b). Figure 1 shows good correlation between plasma MDA and serum ferritin. Splenectomized patients had higher levels of plasma MDA, serum ferritin, urine β_2-microglobulin and NAG than nonsplenectomized patients and normal controls (fig. 2).

9 patients with serum bicarbonate less than 21 mmol/l were chosen to undergo acid loading and water deprivation tests. The results are shown in table 3: (a) The maximum urine osmolality after water deprivation and intranasal DDAVP was less than 800 mosm/kg·H_2O in all patients. (b) Significant reduction of urine pH (5.8–5.4) and significant increase in the urine ammonia (25.1–82.2 mmol/day) were observed in all patients. (c) The furosemide test after chronic acid load showed no significant change in urine pH and ammonia excretion.

Table 3. Tubular function tests in β-thal/E patients

a Maximum urine osmolality after water deprivation and DDAVP study

	After water deprivation	After intranasal DDAVP
Flow rate, ml/min	0.59 ± 0.3	0.57 ± 0.15
Osmolality, mosm/kg H_2O	514 ± 77	533 ± 71

b Chronic acid loading study of the 9 patients

	Control	After acid loading
Blood		
pH	7.36 ± 0.03	7.34 ± 0.03
pCO_2, mm Hg	39.5 ± 3.5	34.3 ± 3.8*
HCO_3^-, mmol/l	19.1 ± 1.7	15.8 ± 2.3*
K^+, mmol/l	4.3 ± 0.4	4.0 ± 0.6
Urine		
pH	5.8 ± 0.3	5.4 ± 0.2*
NH_4, mmol/day	25.1 ± 7.1	82.2 ± 17.9*

c Oral furosemide 20 mg after chronic acid loading

	4th day of chronic acid loading	After furosemide
Flow rate, ml/min	1.1 ± 0.6	5.0 ± 2.9*
Urine pH	5.3 ± 0.2	5.2 ± 0.4
$NH4^+$, µmol/min	65.9 ± 63	76.2 ± 33

* $p < 0.0001$.

Discussion

The results from this study demonstrated that patients with β-thal/HbE had renal abnormalities as manifested by proteinuria, aminoaciduria, increased urinary levels of β_2-microglobulin and N-acetyl-β-glucosaminidase (NAG). The patients produced urine with low osmolality after