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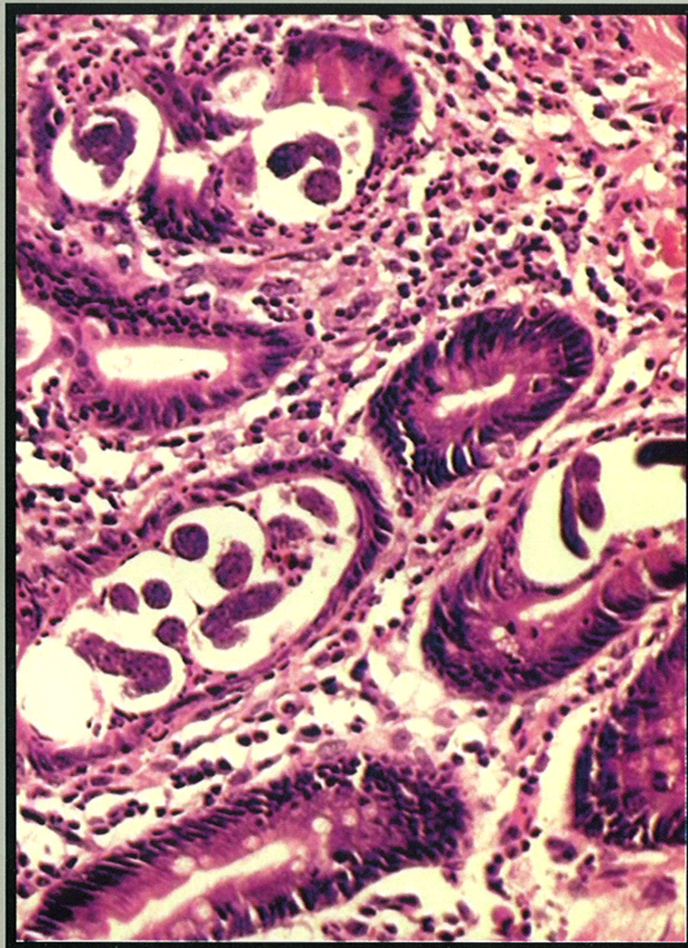

Vol. 2 No. 1, June 1997

Journal of the
University of Malaya
Medical Centre

Journal of the University of Malaya Medical Centre

JUMMEC

Volume 2 ❖ Number 1 June 1997

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Publisher

JUMMEC is published twice a year by the Medical Centre, University of Malaya, 50603 Kuala Lumpur.

Cover

x 200 (H & E) Cross-section of *Strongyloides stercoralis* eggs, rhabditiform and filariform larva in the mucosal crypts.

Printed by

Printing Unit, Faculty of Medicine, University of Malaya, Kuala Lumpur

Journal of the University of Malaya Medical Centre
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Manuscripts: All manuscripts should be submitted in duplicate, typed on one side of A4 size paper and double-spaced with at least 2.5 cm margin. A computer diskette (3.5 in) containing the manuscript in MS Word 6.0 or Word Perfect and a covering letter, stating that the work has not been published or under consideration for publication elsewhere, should be submitted to the Editor. Presentations at meetings do not class as prior publication. The text of the manuscript should be in the following form:

Title page: The title page should contain a concise title of the article. It should identify all the authors, the name(s) of the institution(s) and their full addresses where the work was carried out. The initial and address of the corresponding author should also be indicated.

Abstract and Key Words: The second page should contain an abstract of about 150-200 words. It should state the purpose of the study, a brief description of the procedures employed, main findings and principal conclusions. Three to ten key words should also be listed below the Abstract.

Text: Wherever possible, the text should consist of an introduction, materials and method, results, discussion and references.

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Roberts CW, Alexander J and Bossi L. Studies on a murine model of congenital toxoplasmosis. Parasitol 1992; 104: 19-23.

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AVOIDING PLAGIARISM IN SCIENTIFIC WRITING

In recent years, the matter of plagiarism has reared its ugly head in local academic circles, and the University of Malaya has not been exempt from this curse.

Anytime you take someone else's words and pass it off as your own, you are guilty of plagiarism. A journalist guilty of plagiarism gets fired. If you are a student, and you plagiarize, you risk failing your assignment and the possibility of failing the class. Plagiarism is a grave scholastic offense, and in many Universities, the punitive measure for such intellectual theft is suspension and often, dismissal. It is clearly unfair to the original author(s) to use their words without giving them credit and it is deceptive to take credit for anyone else's creative and intellectual output, be it text or images.

Plagiarism includes both using words or phrases of another person and restating another's thoughts in marginally altered form. The most common forms of plagiarism involve the copying word-for-word from some other source without acknowledging that source by quotation marks, footnotes or references; authors are of course free to quote thoughts or passages from someone else's work, provided the original author is credited and acknowledged. Other forms of plagiarism include: turning in as your own work a paper or product or portion thereof, that was jointly conceived and developed by a group of researchers. Representing someone else's ideas as your own, even if those ideas are expressed in your own words, also constitutes plagiarism. Paraphrasing is a restated approximation of the original authors ideas and/or words. Paraphrasing without acknowledgement of authorship is also plagiarism and is as serious a violation as unacknowledged quotation. It is important to remember that it does not matter if the original work is copyrighted; you are still plagiarizing if you use material from uncopyrighted work without giving credit.

To avoid plagiarizing, it is important to be as clear as possible in your writing or articulation to ensure that your audience can distinguish easily between your own words and thoughts and those that you are paraphrasing or quoting. As writers, it is therefore imperative that one cites references whenever and wherever in the document it is relevant to do so.

Paradoxically perhaps, computers have made it increasingly easy to "cut and paste" from someone else's work; it is thus wise to keep electronic material that you have copied for study and research purposes in a separate document to avoid inadvertent copying. To help you cite works that you find online, a brief document is available on the Internet *(http://www. Library. uwa. edu/au/ libweb w sch/sc_melvin. html).*

It is my conviction that most scientists wish to avoid plagiarism, but are not always mindful of how they can. Simply put, the lack of sufficient knowledge on how one may avoid plagiarism the skills of proper and effective citation of language and ideas obtained from others - is the principal cause of grief in most cases. To this end, the Faculty and University have in the past organized workshops and seminars on the essences and pitfalls involved in scientific and medical writing, with some measure of success. However, as academics, besides being concerned with the intricacies of literary style and manuscript acceptance, we must also be alive to the threats and realities of unwitting or deliberate plagiarism. As teachers, we need to explore lacunae within the already tight medical curriculum and use every opportunity to impart the same cautionary messages to our students, and provide them with clear directions on the elements of responsible and competent professional writing.

Pathmanathan R

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MAGICAL MOMENTS IN MEDICINE

Part 3: Roman Medicine

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According to the historian Livy, a plague appeared in Rome (somewhere around 293 BC) causing great turmoil and distress in the land. When all available local remedies proved futile, they had to turn to their neighbours for help. An urgent SOS call was dispatched to Greece, which brought Greek ambassadors in a vessel. The story has it that the Aesculapian snake had also quietly boarded the ship and travelled as a stowaway until they reached Rome, when it escaped and swam to a little island in the middle of the river Tiber[1]. The plague then vanished without a trace and the island where the snake landed became holy ground where a temple was soon erected. Rome had received her first overseas doctor.

Picture 1. The stowaway snake stepping out of the ship

'All roads lead to Rome', they say. This may be factual or figurative. But surely, one such road which led to Rome was from Greece. This is evident from the fact that a lot of Greek doctors headed for Rome after the 'plague miracle'. We do not know whether the reason for this exodus was pleasant living and working conditions or better financial rewards. But we do know that they were greeted with hostility by the local medical men. A man called Cato, the Elder, a diehard Roman, totally opposed to Greek doctors and their treatment, prevented his followers from consulting with the immigrants, whom he dubbed as *graeculi delirantes* meaning 'the raving Greeks'. This man ardently believed that cabbage juice was a cure-all remedy which could heal anything from ulcer to cancer and in the process, killed his own wife and son, by his stubborn stupidity! The best treatment he had for a dislocation was a recitation of a medical abracadabra which went like this : "*Huat hana ista pista, sista domina damnaustra luxato*". However, it was only a matter of time before these 'charlatans' were accepted in the fold, but the Romans made sure that the Greeks changed their approach from an ideological one to a more practical one.

The xerophyte who proved that he could survive and succeed against all odds, even under extreme conditions of torrid antagonism and animosity, was a man called Asclepiades. This dynamic, vibrant and charming young Greek had had sound training in Athens and Alexandria and other premier medical centres. Though he had migrated to Rome only by virtue of his talent for articulate speaking, he soon switched careers seeing better prospects in medicine. He was prudent enough to be moderate in his approach and go *via media* by avoiding Greek theories and at the same time rejecting the extreme treatments prevalent in Rome. He also played on patient psychology and won them over by giving them pleasant smelling, hygienic remedies (to be washed down with wine, which he prescribed in bountiful quantities!). His patients loved him for not making them gulp down bitter concoctions with disgusting aromas. His motto was *'cito, tuto et jucunde'* (which means swift, safe and sweet) and it became a magical manifesto which made him the wealthiest and the most popular physician in Rome. Inevitably, however, he did earn a few irate adversaries along the way. An incident is quoted in literature where Asclepiades 'resurrected' a dead man. Apparently, the man was only in a coma, but nevertheless given up for dead and taken out on his last journey. But before he could be laid to rest, Asclepiades brought the 'cadaver' to life, much to the chagrin and utter exasperation of the heirs, who had just been cheated of their inheritance.

Under some kind of an ancient twinning programme, the Greeks set up medical schools in Rome, but the instruction was predominantly based on Hippocratic teachings. Ultimately, three groups of doctors evolved: the *dogmatists*, whose medicine was theoretical, but who were keen to dissect the cadaver and understand the disease process; the *empiricists* who thought that experience and careful observation alone can reveal potent remedies; and the *methodists* who rejected both theory and experience and proclaimed that a good doctor could be moulded in just about six months, if some simple rules were learnt. The methodists had widespread support and had royal endorsement. They were convinced that the body was composed of atoms and pores and declared that disease was the result of abnormal states of the pores due to excessive tension or relaxation.

In 46 BC, the status of doctors in Rome received a big boost, when the government, under the leadership of Julius Caesar[2], did its part to nurture the noble profession, by granting citizenship to all foreign doctors. As if

Picture 2. Julius Caesar ***Not*** a Caesarean child

in dissent, a famine struck the country in the same year, making Caesar decree that all foreigners be expelled from the land. Greek doctors were, however, exempted. The word Caesar inevitably brings Caesarean section to our minds. Although Caesarean section has been part of human culture and medical science since ancient times, the early history of this procedure remains shrouded in nebulous myths and its accuracy is still dubious. The earliest mention of a child specifically born this way is found in Greek mythology, where Apollo removed Asclepius from the womb of Coronis[3].

Picture 3. Asclepius being extracted from Coronis by father Apollo.
[From Alessandro Beneditti's *De Re Medica*, Woodcut, Basle, 1549]

Thereafter, innumerable references of the manoeuvre appear in ancient Hindu, Egyptian, Grecian and Roman folklore. Some ancient Chinese etchings even depict the procedure. It is commonly believed to be derived from the surgical birth of Julius Caesar[4], but this is highly unlikely. The logic behind this reasoning is that in the Roman empire, surgical delivery of the child was performed only when the mother was dead or dying, in a bid to save the child. There is evidence to prove that Mama Aurelia Caesar lived even to hear of her valiant son's assault on Britain in 65 BC. Whatever be the portal of his entry into the world, Caesar was the man who legislated that such unfortunate women should be cut open and hence, probably, the word Caesarean. '*Caesones*' was the term given to children born that way and *Caedare* in Latin meant 'to cut'. Caesarean may also have been a corrupted derivative of these two words. In any case, Caesarean operations have been well known procedures for centuries. The introduction of the term 'Caesarean section' is supposedly attributed to Jacques Guillimeau, who first used the word 'section' in his book on midwifery published in 1598 . Ever since, that term has widely been in vogue.

Picture 4. The purported birth of Julius Caesar, one of the earliest illustrations of Caesarean section.
[From Suetonius' *Lives of Twelve Caesars*, 1506 woodcut]

The initial thrust given by Julius Caesar was further enhanced when Emperor Augustus declared tax exemption for all doctors. This gesture was a token of appreciation to Antonius Musa, who cured him of a serious illness. Unselfish as he was, Musa won the concession for the whole fraternity. Vespasian, who reigned from 69 AD to 79 AD, exempted doctors from military service, which was compulsory for all men at that time. All these attractions - combined with the fact that a medical license was not required for practice - caused an explosion in the medical population, which bred both the lilies and the weeds. This, understandably, led to the deterioration in the quality of medical services and medicine started acquiring a sinister reputation. You could imagine the anguish and frustration of the patient whose gravestone epitaph read " *It was the crowd of physicians that killed me.*"

The beautifully coloured rainbow which emerged out of the fog of quackery was a man called Clarissimus (Claudius) Galenus[5]. Uncharitably (but frequently) described as an arrogant and unpleasant man, Galen was nevertheless a key figure who deserves mention in any tale of Rome. His father was Nikon, an educated, benevolent and wealthy architect, who took personal interest in his son's education. His mother, according to Galen himself, was a hot-tempered woman, always arguing with his father; Galen compared her to Socrates' wife Xanthippe. (Was she the one who drove him into philosophy?)

Galen prodigiously produced three books by the time he entered his teens and later went on to excel in mathematics, architecture, astronomy, philosophy and agriculture. Eventually, his interests shifted towards medicine and he spent 11 years studying anatomy and recording his observations. He impressed his audience by performing 'live' dissections on hapless animals, which included pigs, horses and even two elephants. Thankfully, live humans were spared his scalpel. He under-

stood that blood is the nutrient for the tissues and is said to have demonstrated effects of injury to the spinal cord at various levels. He rightly discredited Aristotle's theory that the heart was the centre for mental functions and ascribed it to the brain. Anatomists attribute the discovery of the laryngeal nerves and the great cerebral vein to Galen.

In the colloseum, while heartless spectators and brutal administrators cheered and goaded helpless slave gladiators to fight the fierce, famished lions, (under absolutely no-win conditions) Galen - in a less barbaric act - used the opportunity to study the internal anatomy of the injured through the gruesome wounds. His services were appreciated and he was appointed "physician to the gladiators" at Pergamon. He served four terms in that capacity. Galen, therefore, must be considered the world's first sports medicine physician.

Picture 5. Imaginary portrait of Galen.
16th century engraving, Bertarelli Collection, Milan

Galen was also a very successful practitioner. Boethus, consul of emperor Marcus Aurelius solicited Galen's assistance one night on behalf of his ailing wife. The fee charged is said to be 400 gold pieces, an atrocious 15 times the then going rate. He thus created history for the highest consultation fee ever to be charged for a house call. This apart, he also seems to have created history as the only person, who visited another man's wife in the middle of the night and got paid (handsomely) for it, too!

Galen exhorted that a good physician should also be a good philosopher. According to him, a physician should master three branches of philosophy: *logic*, the science of how to think; *physics*, the science of nature; and *ethics*, the science of what to do. He was convinced that with such knowledge, he could gain the patients confidence, obedience and admiration. Training in philosophy is, in Galen's view, not merely a pleasant addition to, but an essential part of the training of a doctor. His treatise entitled 'The best Doctor is also a Philosopher' gives to us a rather surprising ethical reason for the doctor to study philosophy. The profit motive, says Galen, is incompatible with a serious devotion to the art. The doctor must learn to despise money. By these noble and valid declarations, Galen presents himself to us - not only as a physician - but also as a scholar, devoted to the pursuit of science and practice of medicine for the love of mankind.

Galen's works fall into three main categories: medical, philosophical, and philological. His medical writings encompass nearly every aspect of medical theory and practice in his era. In addition to summarizing the state of medicine at the height of the Roman Empire, he reports his own important advances in anatomy, physiology, and therapeutics. But although Galen contributed significantly to medical knowledge (primarily due to his anatomical observations), he is, nevertheless, infamously described by historians as the man who held back medicine for a thousand years. This was because he utilised his knowledge and his gift of the gab to verbally cudgel into submission, anyone who tended to disagree with his theories. Therefore, Galen, the 'Prince of the Physicians' and the 'Medical Pope of the Middle Ages' (I shall not mention his other less flattering nicknames like 'mulehead' and 'windbag') in his intellectual arrogance and eloquence quelled every opposing adversary who dared to question his hypothesis. This dampened progressive and rational thinking thereby slackening the pace of medical progress for about a millennium.

Another man who made a great contribution to Roman medicine was Aulus Cornelius Celsus. He was a Roman of patrician lineage, who lived between 3 AD and 64 AD. Being a prolific writer and a scholar in many disciplines, he wrote extensively on philosophy, agriculture, medicine and warfare and is regarded the greatest Roman medical writer of his time. His *magnum opus* was an eight volume encyclopaedia on Medicine known as *De Re Medicina*. Pathetically, no one knew of its existence for several centuries. When they were ultimately 'rediscovered' in the 15th century, they caused a tremendous impact on scientific thought. Six out of the eight volumes described diseases and discussed therapeutics including drugs, diet and other supportive therapy. The last two-volume books dealt with the diagnosis and treatment of common surgical problems and included operations for goitre, hernia, cataract removal and tonsillectomy (which was - until *glasnost* and *perestroika* - the most difficult operation in the erstwhile USSR, since people never opened their mouths then!). Celsus is also credited with the invention of the splint for fractured limbs, which he prepared from cloth bandages stiffened with starch. He recommended the usage of vinegar as an antiseptic solution for washing wounds. The four cardinal signs of inflammation - *calor, rubor, dolor and tumor* (heat, redness, pain and swelling) were first documented by Celsus. His writings reflect valuable

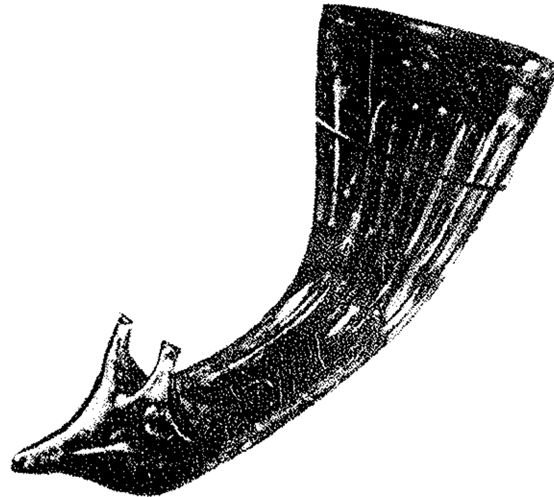
insights into Roman medical thought, practice and conditions of health that prevailed at his time.

Roman women seem to have concentrated on female diseases, since literature mentions an inscription referring to a *'medica a mammis'*, which means a female specialist in breast disorders. Midwives helped greatly during childbirth, picking up the art mostly from experience. Deserving special mention is a man called Soranus, of Ephesus. He was a male gynaecologist and a methodist, who was held in high esteem by the emperor as well as his colleagues. He is widely accepted as the Father of Obstetrics and Gynecology. He produced a great book called *Gynaikeia* (Gynaecology), which remained in use for fifteen centuries. The book had 86 chapters. He described the female genital system in detail and advised contraception using ointments on cotton, but disapproved of abortion by mechanical means. This most famous obstetrician of ancient times advised protection of the perineum and emptying of the bladder by means of catheterization before delivery of the infant. He illustrated various abnormal foetal positions in the uterus and explained how to manoeuvre the foetus into favourable position for delivery. Soranus never mentioned the word, hymen, which reflects the life of that time, but did describe folds which brought pain upon the first menstruation or initial sexual intercourse. After Soranus, nothing of importance was added to obstetrics until the work of Ambrose Paré, 1500 years later. Much of the material found in his book is considered pertinent to present day medicine. He was convinced that the uterus[6] was the cause for certain types of mental problems in women. That would be the genealogy for the word *'hysteria'* (*hustera* meaning uterus), the condition of which, from then till now, is essentially female domain.

Picture 6. The representation of the uterus
(Note the three layers and the implanted embryo)
Bronze amulet from Umbria. Dr. M. Grunwald Collection, Zurich

In his time, sexual freedom was common in every rank of Roman society. Brothels were frequented by all classes. Even Massalina, Emperor Claudius' wife, is indicted of infidelity. Under such circumstances, it is understandable that gynecological disease increased. Soranus in his book had notes on some of them, but his famous quote (valid till this day) was *"A cure for the common cold is yet to be found."*

The Romans were great men for eating and drinking. Tumblers[7] have their etymological origin in ancient Rome, where drinks were served in legless goblets. These receptacles - which had pointed or convex bases - could be put down only on their sides, forcing you to empty your drink. Else they tumbled! They also customarily expanded their appetites before banquets, by pouring water into the external auditory meatus. I doubt whether they understood that they were stimulating the auricular branch of the vagus causing reflex vagal stimulation, but nevertheless they knew the connection!



Picture 7. A Tumbler

Herbal cures were frequently prescribed by doctors and the favourites were said to be *'balsam of Mecca'* and *' Indian lycium'*. The most sought after, however, was *'theriac'*[8], the health tonic cum universal antidote. It was a formidable concoction first put together by Nero's physician Andromachus. Viper's flesh and opium were among its 64 ingredients. Its popularity could be understood by the fact that French and German pharmacoepoeias carried the recipe right till the end of the nineteenth century. Later, in England, its mutated version became the candy known as *'treacle'* (Remember the lines of *"Pop goes the Weasel"*?)

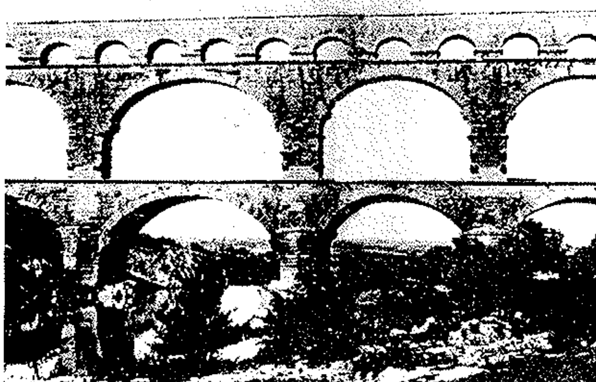
Picture 8. Miniature from an Arabic Manuscript on theriac. Viper meat and opium were the key ingredients

The first Western benchmark in man's understanding of the herb Aloe is found in the works of Pedanius Dioscorides[9] (41 AD - 68 AD). This master of Roman pharmacology was a respected army surgeon in Emperor Nero's time. Posted with the Roman legions at various stations, he travelled extensively within the great empire, incidentally developing his knowledge and skill.

Picture 9. Dioscorides

He gave the first detailed description of the plant we call Aloe Vera, and attributed to its juices "the power of binding, of inducing sleep." He noted as well that it "loosens the belly, cleansing the stomach." He further added that this "bitter" Aloe (the sap) was a treatment for boils; that it eased haemorrhoids; that it aided in healing bruises; that it was good for the tonsils, the gums, and all general mouth irritations; and that it worked as a medicine for the eyes. Dioscorides further observed that the whole leaf, when pulverized, could stop the bleeding of many wounds. Dioscorides, in AD 64, wrote a five volume *Materia Medica* listing all known medicinal plants and their indications, which would remain the only authoritative text book on pharmacology until the Renaissance. But most of those remedies have now been proved ineffective.

Throughout the time of the Roman empire, much emphasis was placed on public health. Their sanitation methods, sewage disposal and water supply systems exceeded anything that followed in the Western world prior to the 19th century. Rome's main sewage system was in operation as early as 6 BC. Aqueducts[10] were later built which brought millions of gallons of fresh water into Rome by the second century BC. The aqueducts were the true triumph of Roman sanitary engineering. Frontinus, the author of a treatise on Rome's aqueducts, became *curator aquarum* (water commissioner) in 97 AD. He recognized the sanitary aspects of his position stating proudly, "...my office...concerns not only the usefulness of such a system, but also the very health and safety of Rome..."



Picture 10. Aqueducts Pont du Gard, Nimes, France, 14 AD

Public baths and toilets were built throughout the empire, but there was a nominal admission fee. One of the characteristic Imperial Roman building types is the giant bath complex which could house not only bathing facilities but lecture halls, gymnasia, libraries and gardens. Roman bathing establishments usually provided three kinds of baths, i.e., hot, tepid and cold. The room pictured below[11] was a Caldarium in the bath houses of Pompeii known as Forum Thermae where the atmosphere was kept warm by hot air circulating through pipes in the walls and floor.

Picture 11. Bath house in Pompeii (1 BC)

Latrines[12], well drained or with the provision for a semi-sanitary maintenance, became commonplace both in the houses of the wealthy and in bath complexes where there was a constant supply of running water. In lieu of toilet paper, Romans used a sponge tied to the end of a stick. Users had to pay a small administrative fee and therefore, the Romans can claim credit for originating pay toilets.

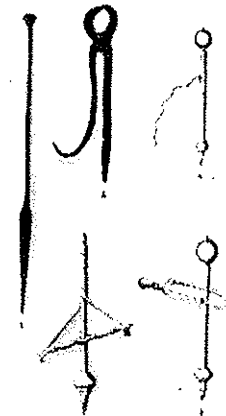
Picture 12. Ruins of a communal latrines in Corinth (4 BC) provided with continuous running water beneath seats for waste disposal

Picture Gallery of Ancient Surgical Instruments

Picture. 1

Picture. 2

Picture. 3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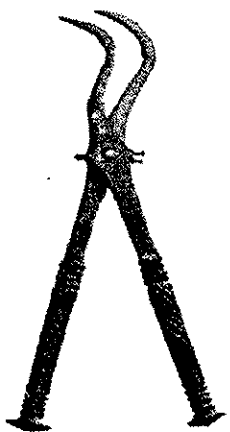
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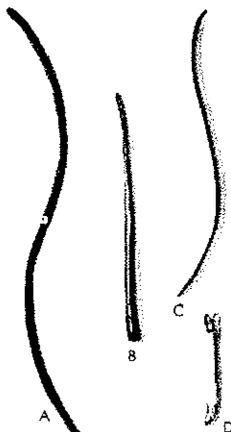

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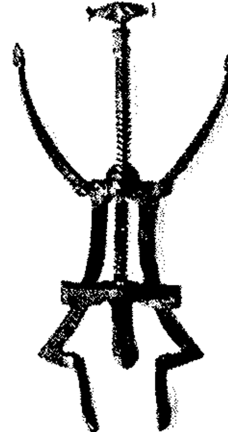
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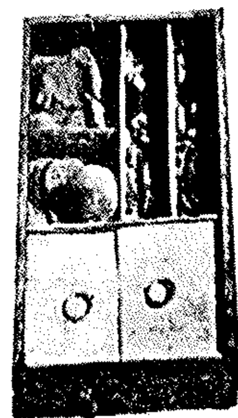

Pictur. 8

Picture 1. Scalpels: Greco-Roman scalpels were made of either steel, bronze, or a combination of the two metals (such as a steel blade and a bronze handle). Ancient scalpels are strikingly similar in form and function as their modern counterparts today. The long steel scalpels were the most ordinary type of scalpels used and they seem to be suited to making either deep or long cuts. The "bellied scalpels" was popular with physicians in antiquity since the shape of its handle allowed more delicate and precise cuts to be made (such as incisions between ribs).

Picture 2. Hooks: - Hooks were common instruments used extensively by Greek and Roman doctors. These ancient hooks came in two basic varieties: sharp and blunt. Both of these types of hooks are still used by modern surgeons' for many of the same purposes for which the ancient doctors first used them. Blunt hooks were primarily used as probes for dissection and for raising blood vessels. Sharp hooks were used to hold and lift small pieces of tissue so that they could be extracted and also to retract the edges of wounds.

Picture 3. Uvula Crushing Forceps: With their finely-toothed jaws these forceps were probably designed to facilitate the amputation of the uvula. This procedure, as described by Aetius in the first half of the sixth century, called for the physician to crush the uvula with before cutting it off in order to prevent haemorrhaging.

Picture 4. Bone Drills: Greek and Roman physicians used bone drills in order to excise diseased bone tissue from the skull and to remove foreign objects of considerable thickness (such as a weapon) from a bone. Bone drills were generally driven in their rotary motion by means of a thong in various configurations.

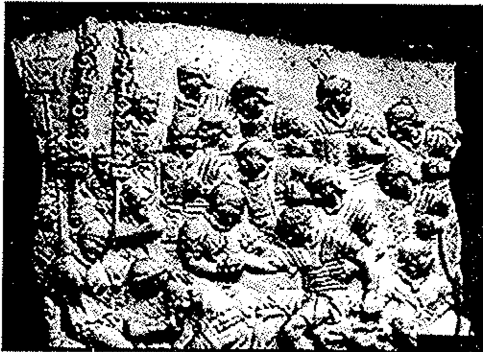
Picture 5. Bone Forceps: These forceps were used by ancient doctors to extract the small fragments of bone which could not be digitally removed. They were also frequently used in conjunction with bone drills.

Picture 6. Catheters and Bladder Sounds: Physicians in the Roman World employed catheters in order to open up a blocked urinary tract and drain urine. These early catheters were essentially hollow tubes made of steel or bronze and had two basic designs: one with a slight S curve for male patients (figure A) and another straighter one for females (figure B). The same doctors also used similar shaped devices which were solid, as opposed to hollow, in order to probe the bladder in search of calcifications (figures C and D).

Picture 7. Vaginal Speculum: The vaginal speculum features among the most complex instruments employed by Greek and Roman physicians. They illustrate the high degree of engineering skill available to the ancient doctors. Most of the vaginal specula consist of screw device which when turned, causes a crossbar to push the blades outwards.

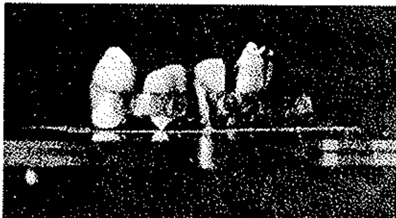
Picture 8. Portable Medicine Chests: As the ancestor of the "Doctor's Black Bag," these small chests were the portable medicine and instrument storage units for ancient doctors. Small boxes have been found containing everything from scalpels and probes to ointments and drugs. The metal box, usually made of bronze, had several compartments, some of them having their own separate hinged lids.

Army medicine traces its roots back to ancient Rome. However, before the Greek influence, the Roman legion did not contain any medical services. In his treatise *In the Surgery*, Hippocrates candidly asserts that "he who desires to practice surgery must go to war." But the Romans had more affinity to war than the Greeks, which made military medicine more relevant to the Romans. Literary sources suggest that treatment was accorded by the military medicos to the wounded, only if they were of the higher ranks, and there is little indication that the common soldiers had access to medical care. Instead, some troops functioned as medical staff as the need arose. It is to the Romans' credit that they recognized the need for such a service, but the solution was not a medical corps whereby trained physicians became part of the army. The Romans clearly distinguished between the treatment of the "sick" and the "wounded". The wounded were cared for, as far as possible, by fellow soldiers on the fields, and the transportable sick were placed in *ualetudinaria* (hospitals) along with the severely wounded. It can be noted that the *medici* (doctors) treating the wounded in a war scene are dressing superficial wounds and their uniforms are identical with that of the soldiers they are aiding[13].



Picture 13. Soldiers aiding their wounded comrades Trajan's column, Rome, dedicated 113 AD

Medical students were educated at public expense and the poor received free medical treatment. Skillful dentists must have also been present, as evidenced by the set of false teeth bound together by a gold wire[14].



Picture 14. Roman Dentistry showing a set of false teeth mounted on gold bridges (Villa Guilia Museum, Rome)

If Egyptian medicine was magical and Greek medicine mythical, Roman medicine was methodical. The methodists, who perceived the idea of the atom, strengthened the science through people like Soranus. Despite an element of his idiosyncrasy, it must still be admitted that Galen helped to systemise medical knowledge of ancient times, his reasoning always based on observation and experience. He crystallised all the best work of the Greek medical schools which had preceded his own time. It is essentially in the form of Galenism that Greek medicine was transmitted to the Renaissance scholars, through Rome. Galen's massive twenty-two volume work (amounting to roughly one half the bulk of ancient Greek and Roman literature) was surely a cornerstone in the Greco-Roman edifice of medicine. Even that, would not be his entire work collection, since in 191 AD, a fire in the Temple of Peace - where he had deposited many of his manuscripts for safekeeping - destroyed important parts of Galen's work. What remains, however, is enough to establish his reputation as the most prolific, cantankerous, and influential of ancient medical writers. His other works survive only in Arabic or medieval Latin translations.

The decline of Rome was brought about by the interaction of several factors. Epidemics (malaria, plague and small pox) ravaged various parts of the country, while the healers silently observed the pestilence carry its victims away, in total helplessness. As a result of private and public corruption there was general discontent and much of the population was in poverty. This eventually led to political instability and incompetence. When Mt. Vesuvius erupted in 79 AD, the pyroclastic surge of lava destroyed man, animal and vegetation over several miles. In fact, when Vesuvius finally grew silent after 19 hours of activity, the Roman town of Herculaneum was buried under 66 feet of black, rock-hard tephra, while the city of Pompeii had its grave under 20 feet of volcanic ash. Pliny the Elder, naturalist and encyclopaedist, who in his foolhardy curiosity rushed to the spewing volcano to study the phenomenon first-hand, got singed, scorched and submerged in the emission. Such natural disasters, coupled with moral decay and other contributory causes may have resulted in the disintegration of the Roman empire and its medical tradition. The excellent public health and water systems of Rome were among the few precious legacies to posterity and the aqueducts still stand to vouch the fact.

When the Roman empire collapsed in 337 AD, the wealth of knowledge was supposedly lost to Europe. It signalled the imminent advent of a long, dark night called the Dark Ages, when all knowledge (or at least the records of it) disappear from view for a period. The extensive medical information and advancements acquired from the Greeks and refined by the Romans now just goes into a stage of transient hibernation elsewhere, to eventually resurface at a glorious dawn, after being enriched in the Islamic world.

Good night and Roman (tic) dreams. See you at dawn.

(Next Indian, Chinese & Muslim Medicine)

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BIOLOGICAL ACTIVITIES OF DENGUEVIRUS ON MURINE MACROPHAGES

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ABSTRACT: The biological activities of dengue 2 virus on its target cells, namely macrophages and neutrophils were investigated. Preliminary results showed that polyethylene glycol precipitated dengue 2 virus acted as a chemoattractant for peritoneal cells of Balbc/J mice and for established monoctyes/macrophage cell lines (WEHI 265.1). This *in vivo* response was also seen *in vitro* using Boyden Chemotaxis chambers and was found to be inhibited by specific viral antibodies. The ability of the virus to activate these cells was then evaluated. Treatment of the peritoneal cells with virus induced a respiratory burst with the production of picomoles of superoxides. In addition, the virus induced production of reactive nitrogen intermediates (1-2 μM) and also resulted in exocytosis of lysozyme. Adhesiveness was also observed and appeared to increase with higher concentrations of the virus. The above results demonstrate that increased adhesiveness and activation are induced following treatment of peritoneal cells and the cell line WEHI 265.1 with dengue virus. *(JUMMEC 1997 2(1): 11-17)*

KEYWORDS: Macrophage, macrophage activation, dengue virus, adhesion

Introduction

Dengue Fever is an acute, infectious febrile disease which is caused by four serotypes of dengue virus (Den 1-4). It has been reported in over 100 countries and poses a threat to approximately 2 million people (1). A more severe form of the disease, Dengue Haemorrhagic Fever (DHF) is characterized by abnormalities of haemostasis and increased vascular permeability, which in some instances results in hypovolemic shock syndrome, Dengue Shock Syndrome (DSS). Dengue viruses are transmitted through the bite of an infected mosquito, namely *Aedes aegypti*, which is found in tropical and subtropical regions of the world (1). Infections in humans with one serotype produces life-long immunity to re-infection by that serotype but only transient protection against other serotypes. Because of the significant morbidity and mortality attributed to this virus, efforts are being made to develop immunogenic vaccines. Epidemiological studies have shown that DHF/DSS is observed more commonly in secondary dengue virus infections than primary infections (2). Hence protective immune responses should induce immunity that will not increase the risk of DHF/DSS in future infections. The pathogenesis of the more severe forms is only partially understood. Several hypotheses have been proposed to explain the pathogenesis of DHF/DSS (3, 4, 5). The most popular of these suggests an immunopathological mechanism involving enhancing antibodies and cell mediated immunity (CMI) during a second dengue infection. During such an infection, with a different dengue serotype, it is envisioned that subneutralzing, 'enhancing' antibodies promote viral entry and replication in monocytes/macrophages (6). These infected cells are then thought to become targets of an immune elimination response, probably mediated by dengue-specific cytotoxic T lymphocytes (CTL), which then results in the release of various mediators which produce the symptoms of DHF/DSS (6). These mediators which increase vascular permeability and the precise mechanism of the bleeding phenomenon seen in dengue are still not known. Another hypothesis presumes that differences in virulence of dengue strains (4) may also contribute to the pathogenesis of DHF/DSS. Changes in the virulence of virus strains could emerge as a result of selective mutation and genetic recombination. In the present study we attempted to further understand the pathogenesis by studying virus-target cell interactions. As the main target cell is the monocyte/macrophage it was decided to investigate the effects of the virus on this cell and to see if any of the functions of the cell were affected. Our results indicate that polyethylene glycol precipitated dengue 2 virus acts as a chemoattractant for Balbc/J and C3H/Hej mouse macrophages both *in vivo* and *in vitro*. We also observed that the virus stimulated macrophages (both activated peritoneal exudates and established cell lines) to release enzymes such as lysozyme and produce nitric

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oxide. Virus infection also induced these cells to adhere to fibrinogen and also resulted in the production of picomole quantities of superoxides.

Materials and Methods

Animals. Six to twenty week old male and female Balb/cJ and C3H/Hej mice were maintained in the Central Animal Facility, University Malaya at room temperature.

Cell lines. The WEHI-265.1 monocytic cell line was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) and was cultured in RPMI containing 10% Fetal Calf serum.

Reagents. The cytokine, recombinant mouse interferon gamma was purchased from Genzyme Corporation USA. N-formylmethylleucylphenylalanine (FMLP) and phorbol 1,2-myristate 1,3 acetate (PMA) were purchased from Sigma Chemical Co.

Virus. A prototype strain of dengue-2 virus (NGC) was used. The procedure for preparation and titration of virus stocks have been described previously (6). Viral propagation in tissue culture were carried out using the method of Kuno (8). Briefly cell cultures were inoculated with DEN 2 viruses and monitored daily for haemagglutinating activity. Once the titre reached 1:32 or more, the culture medium was collected and virus precipitated with polyethylene glycol and purified through centrifugation on sucrose gradients. Uninfected supernatants were treated similarly.

Isolation of peritoneal cells. Peritoneal cells were isolated as described earlier (9). Briefly, 3-4 mice were injected intraperitoneally with sterile sodium casein in endotoxin-free PBS (autoclaved 1 hr at 20 psi). After 16-24 h, animals were given a second casein injection. Three hours later peritoneal cells were harvested in 3-5 ml RPMI. The cells were then washed and counted.

Chemotaxis assay. *In vivo* Chemotaxis: Four uninoculated mice were sacrificed at the start of the experiment and peritoneal cells harvested in 3-5 ml RPMI medium. They were counted using a hemocytometer, cytospinned onto a slide and then stained with a leucostat stain (Fisher Sc. Co.). 20 mice were inoculated intraperitoneally with 0.3 ml dengue 2-infected C6/36 cell supernatant (5 X 10^4 pfu/ml) while another 20 mice were inoculated with 0.3 ml of uninfected cell supernatant. After 3, 6, 9 and 24 hours, four mice (both control and virus infected) were sacrificed, peritoneal cells harvested, counted and cytospin preparations carried out.

In vitro Chemotaxis: Cell migration was evaluated by using the 48-well Boyden microchamber (Neuroprobe) (12). Peritoneal cells (4 X 10^6 cells/ml) were washed and suspended in endotoxin-depleted RPMI with 1% BSA. 50 µl of the indicated cell population (2 X 10^5 cells) was added to the upper well of the Boyden chamber. 27 µl of solution containing appropriate dilutions of virus/FMLP/PMA (which were also endotoxin depleted) were placed in the lower microchamber wells. In some experiments virus solutions were preincubated with specific antiviral antibody. The wells were separated by a 5 µm pore size polycarbonate filter. All responses were assayed in triplicate. After incubation at 37° C for one and a half hours the filters were scraped to remove non-migrating cells from the upper surface. The filters were subsequently fixed in acetone and stained with a leucocyte stain. The numbers migrating were determined at 400 x magnification and the cells of the indicated lineage were enumerated. Specific chemotaxis represents the average number of migrating cells per 5 high power fields minus the mean number of cells migrating in medium alone. To show specificity of binding, virus was incubated with excess DEN 2 specific monoclonals (3H5-ATCC) at room temperature prior to loading of the wells.

Superoxide production. The production of extracellular superoxide was measured using superoxide dismutase inhibitable reduction of ferricytochrome c (10). Mouse peritoneal cells (2 X 10^6) were added to the assay mixture containing cytochrome c before addition of the stimulus. Superoxide production was assayed spectrophotometrically (570 nm) as a function of ferricytochrome c reduction. To exclude the presence of other reductants, a reference sample containing 3 µl superoxide dismutase (SOD, 0.8 mg/ml, Sigma Chemical Co.), an enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and oxygen was included as a control for each sample. The rate of cytochrome c reduction is subtracted from that in the samples. The resulting value equals the rate of SOD-inhibitable cytochrome c reduction. 10 ml of purified peritoneal cells (20 x 10^6/ml) in HBSS containing Ca^{2+} and Mg^{2+} (Sigma Chemical Co.) were incubated at room temperature with 187 µl ferricytochrome c (1.3 mg/ml in HBSS, Sigma Chemical Co.) and 1-5 µl of PMA (1 mg/ml) or 5-10 µl dengue 2 virus (5 X 10^4 pfu/ml) in 96-well microtitre plates. The amount of superoxide produced was calculated and expressed as pmol cytochrome c reduced/min/10^6 cells using the formula:

$$\frac{\Delta A/\text{min} \times \text{reaction volume} \times 10^6 \text{ nmol/mmol} \times 1000}{(\text{Cy/mmol}) \times 1000 \text{ ml/l}}$$

where ΔA/min = change in absorbance per minute and Cy = extinction coefficient of cytochrome c, which is 21.1 mm/cm.

Measurement of Lysozyme release. Casein-elicited peritoneal cells (5 X 10^5) suspended in PBS containing 0.3% BSA were incubated with various virus dilutions at 37° C for 2 hours. The reaction was stopped

by rapid cooling on ice followed by centrifugation. Lysozyme activity was then determined from the supernatants by a turbidometric method as described previously (9, 13). Briefly, 50 µl of supernatant was added to 150 µl of the substrate, which consisted of 30 mg/ml *Micrococcus lysodeikticus* (Sigma Chemical Co.) in 50 nM phosphate acetate buffer, pH 6.0 and 0.05% Triton X (BioRad). Chicken egg-white lysozyme (Sigma Chemical Co.) was used as a standard. The activity was measured at an optical density of 540 nm after 20-30 minutes.

Nitrate assay. Peritoneal cells (5×10^5) were cultured for 2 hours in RPMI containing 10% FCS for 2 h at 37° C before use. The medium was replaced with 100 ml of fresh serum medium and the cells were incubated with dengue virus or γ-IFN for 24 h at 37° C. WEHI 265.1 cells were similarly treated. The nitrite concentration of the 24 h conditioned medium was measured by a microplate assay as described by Ding *et al.* (11). Briefly 90 µl of conditioned medium was incubated with 90 µl of Greiss reagent (1% sulfanilimide, 0.1% naphthyethylene diamine dihydrochloride (Sigma Chemical Co) and 2.5% H_3PO_4, (Fisher-Scientific, Pittsburgh, PA) at room temperature for 10 mins. The absorbance at 570 nm was determined in an ELISA plate reader. Cell free medium alone contained 0.03 µM nitrite per well, hence this value was determined in each experiment and subtracted from the values obtained with the cells. Dilutions of a 1 µM stock solution of sodium nitrite (Sigma Chemical Co.) were used to obtain a standard curve. To show specificity of binding, virus was incubated with excess dengue 2 specific monoclonals (3H5-ATCC) at room temperature prior to loading of wells.

Adhesion assay. The method of Devi *et al.* (9) was used. Briefly, ninety six flat-well bottom plates were coated with 25 µl of purified fibrinogen (2 mg/ml in PBS, R & D systems) and incubated for 90 minutes at room temperature. Plates were then washed 3 times in HBSS containing 10 mM HEPES, pH 7.3, 1 mM $MgCl_2$, and 0.5% BSA. Finally the stimuli (10^3 - 10^5 pfu dengue virus or 10 ng/ml PMA) were added followed by addition of target cells (5×10^5 cells/well) and incubated for 20 to 30 minutes at room temperature. Wells were then gently washed with the same buffer. 200 µl of the buffer without BSA was added, followed by 10 µl of the vital dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyle blue MTT (Sigma Chemical Co). This mixture was incubated 4-16 hours at 37° C. The supernatant was very carefully aspirated and 100 µl of acid isopropanol (2 mM HCl) was added to the cells, vortexed, and the optical density read at 540 nm. To generate a standard curve, wells containing graded numbers of cells were also stained with the dye. To show specificity of binding, virus was incubated with excess DEN 2 specific monoclonals (3H5-ATCC) at room temperature prior to loading of wells.

Results

Chemotaxis. The intraperitoneal inoculation of dengue virus resulted in an influx of cells into the peritoneal cavity within 3 hours (Table IA). In the controls, there was only a relatively small increase in the numbers of infiltrating cells which steadied after 9 hours. This was probably a normal immune response to foreign material injected into the peritoneal cavity. Cells were mainly macrophages (97.9%), some neutrophils (2%) and few lymphocytes (Table IB). The increase in macrophages was more than tenfold. By 3 hours the increase was found to be significant. These increases peaked at 9 hours post infection suggesting recruitment of inflammatory cells to the site of infection (Table IA). This *in vivo* response was seen *in vitro* using the Boyden Chemotaxis chambers (Table II). Treated uninfected tissue culture supernatant was used as the negative control and FMLP as the positive control. With FMLP, cells migrated across the membrane with concentrations as low as 10 ng/ml and as the concentration of

Table IA. Kinetics of the inflammatory response induced by intraperitoneal injection of dengue virus

Stimulus	Total cell count (X 10^6 cells/ml)#			
	3 hrs	6 hrs	9 hrs	24 hrs
Control S/N	0.3±0.12	2.89±1.15	0.51±0.29	0.31±0.01
Virus*	1.24±0.28	2.54±0.20	9.91±1.09	4.83±0.57

Mice were inoculated with treated infected (Virus) or uninfected (Control S/N) tissue culture supernatant intraperitoneally and peritoneal cells harvested as indicated. All values corrected for zero time reading.
# $p<0.05$ (values are an average of 3-5 experiments)
*5×10^4 pfu/ml

Table IB. Composition of exudate induced by intraperitoneal injection of dengue virus

Composition of exudate	Stimulus							
	Nil				Virus			
	3 hrs	6 hrs	9 hrs	24 hrs	3 hrs	6 hrs	9 hrs	24 hrs
% Macrophages	87.6	97.8	87.4	ND	89.9	97.9	97.0	93.1
% Neutrophils	12.4	2.1	12.6	ND	9.9	2.03	2.59	6.9
% Lymphocytes	0	0	0	ND	0	0.043	0.39	0

Cytospin preparations were stained with a leucostat stain and an average of 5 fields were determined. (Values are an average of 3 experiments)
ND - not done

Table II. *In vitro* Chemotaxis

Stimulus	Dose	Cells migrated (10^4 cells/ml)
Control S/N	-	0
FMLP	10 ng/ml	0.054 ± 0.008
	100 ng/ml	12 ± 0.200
	1000ng/ml	30 ± 0.400
Dengue 2 virus	5 x 10^3 pfu/ml	0.37 ± 0.004
	5 x 10^4 pfu/ml	1.3 ± 0.040
	5 x 10^5 pfu/ml	9.7 ± 1.800
Dengue 2 virus + 3H5*	5 x 10^3 pfu/ml	0.042
	5 x 10^4 pfu/ml	0.042
	5 x 10^5 pfu/ml	0.054

Peritoneal cells were assayed in Boyden microchambers. The number of cells per 5 high power fields was determined. Background migration in medium alone was subtracted from the data presented here.
*3H5 -Dengue 2 specific monoclonals were incubated with the virus for 30 minutes prior to loading of wells (1 experiment)

FMLP - N-formylmethylleucylphenylalanine

FMLP increased so did the concentration of the cells that migrated across the membrane. Dengue 2 virus also stimulated migration of the cells but to lesser degree. In preliminary experiments, varying doses (5 X 10^3 - 5 X 10^5 pfu/ml) of dengue 2 virus were used to stimulate migration of cells. As little as 5 X 10^3 pfu/ml of virus stimulated migration. To establish specificity *in vitro* neutralization experiments with monoclonal antibody to dengue virus were performed. Migration was inhibited when the virus was incubated with dengue-2 specific monoclonals prior to loading. This could either be due to a neutralization effect or a blocking effect, hence needed appropriate signals for the migration are not released. Control mouse monoclonals had no effect when used at the same concentrations as the dengue specific monoclonals (data not shown).

Induction of a respiratory burst. During the respiratory burst that accompanies the activation of phagocytic cells, high levels of reactive oxygen products are released (9). Phagocytic cells produce two major reactive oxygen species namely, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Superoxide is produced by a NADPH-dependent oxidase enzyme that is activated upon cellular stimulation. When neutrophils and other phagocytic cells ingest opsonized particles, a rapid respiratory burst ensues, resulting in the formation of O_2, H_2O_2, and hydroxyl radicals. In preliminary experiments varying doses of dengue virus, PMA (positive control) and treated uninfected tissue culture supernatant (negative control) were used to stimulate the neutrophils and macrophages. Preliminary experiments showed that as little as 0.001 ng/ml of PMA was sufficient to stimulate peritoneal cells to produce a respiratory burst. The minimum viral dose necessary to stimulate measurable respiratory activity was 5 X 10^4 pfu/ml (Table III).

Table III. Dengue virus stimulates peritoneal cells to generate superoxides

Stimulus	Dose	pmole Cyt c reduced
Control S/N	-	0
PMA	0.01 ng/ml	2.13 ± 0.13
	0.05 ng/ml	5.2 ± 0.68
Virus	5 x 10^5 pfu/ml	7.9 ± 2.60

Casein elicited peritoneal cells were treated with Control S/N/Virus/PMA. After 30 minutes cultures were assayed for extracellular O_2^- release as stated in the materials and methods. The data presented represent an average of 5 experiments.

Granule exocytosis. The proteolytic enzymes stored within the granules of phagocytic cells are involved in bactericidal and digestive functions (14). When neutrophils are exposed to stimuli (e.g., IL-8), degranulation results in the release of several potent proteolytic enzymes as well as bactericidal enzymes. Lysozyme, a small cationic enzyme, is found in the secondary granules and is also present in azurogranules (9). Lysozyme is bactericidal for some bacteria and its primary function is to digest cellular debris. As before, PMA was the positive control. The ability of dengue virus to induce release of lysozyme is shown (Table IV), the minimum dose being 5 X 10^4 pfu/ml as this concentration was found necessary for detectable levels of granule exocytosis.

Table IV. Dengue virus stimulates granule exocytosis

Stimulus	Dose	Lysozyme (ng/ml)
Control S/N	-	0
PMA	1 ng/ml	119 ± 37.40
Virus	5 x 10^5 pfu/ml	111.5 ± 44.5

Cells were incubated with the stimuli and 2 hours later the culture supernatant was assayed for lysozyme activity. Data presented represent an average of 5 experiments.

Production of nitrite. Reactive nitrogen intermediates (RNI) express a high degree of chemical activity. RNI are secretary products of activated macrophages but are also secreted by other cell types. The nitrogen intermediates are derived from nitric oxide

(NO) and include nitrite and nitrate. NO has been identified as a pleiotropic intercellular messenger that activates guanylate cyclase, leading to an increase in cGMP accumulation (15). NO is responsible for regulating a variety of diverse cellular functions in many tissues (16). In the following experiments we examined the ability of dengue virus to stimulate RNI activity measured by the production of nitrite. As can be seen in Table V dengue virus induced significant amounts of nitrite by both peritoneal cells and the monocytic tumor cell line WEHI-265.1. Comparable levels of nitrite were obtained between the positive controls (PMA and γ-IFN) and 5 X 10^4 pfu/ml of virus.

Table V. Production of reactive nitrogen intermediates by dengue 2 virus activated cells

Cells	Stimulus	Dose	Nitrite (µM)
WEHI 265.1	Control S/N	-	0
	PMA	0.01 ng/ml	2.004 ± 0.161
		0.05 ng/ml	4.696 ± 1.521
	Virus	5 x 10^3 pfu/ml	0.904 ± 0.052
		5 x 10^4 pfu/ml	2.207 ± 0.448
Peritoneal	Control S/N	-	0
Cells	γ-IFN	10 ng/ml	1.378 ± 0.487
	Virus	5 x 10^4 pfu/ml	0.791 ± 0.032
		5 x 10^5 pfu/ml	0.860 ± 0.003
	Virus + 3H5*	5 x 10^4 pfu/ml	0.0001

Caesin elicited peritoneal cells or WEHI-265.1 monocytic tumor cells were cultured with indicated virus or γ-IFN. Conditioned medium wasassayed for production of reactive nitrogen intermediates 24 hours later. Data represent the average of 5 experiments.

*Dengue specific monoclonals were incubated with the virus prior to incubation.

Adhesion to fibrinogen. The integrin CR3(CD11b/18) acts as a receptor for fibrinogen (17, 18). Modulation of β-integrins can be monitored by changes in adhesiveness to fibrinogen. To determine whether dengue virus stimulation contributed to the adhesiveness of inflammatory cells, we evaluated this effect of dengue 2 virus on peritoneal cells and WEHI-265.1 monocytic tumour cells. It was observed that viral stimulation induced binding of cells to fibrinogen and as the number of plaque forming units/ml increased so did the number of cells adhering to fibrinogen (Table VI). As little as 5 X 10^3 pfu/ml of virus stimulated adhesion. This effect was abolished when the virus was preincubated with dengue 2 specific monoclonal antibodies (3H5-1).

Table VI. Dengue virus induces adhesiveness to fibrinogen

Stimulus	Dose	No of cells adherent (x 10^5 cells/ml)
Control S/N	-	0
PMLP	10 ng/ml	56 ± 26.0
Virus	5 x 10^3 pfu/ml	1.7 ± 0.1
	5 x 10^4 pfu/ml	4.3 ± 0.2
	5 x 10^5 pfu/ml	182.5 ± 5.5
Virus + 3H5*	5 x 10^5 pfu/ml	0.001

Casein elicited peritoneal cells were added to plates which had been coated with fibrinogen and to which appropriate amounts of stimulants had been added. Non-specific binding in the absence of stimulants was subtracted from the data. Data presented represent an average of 5 experiments.

*Dengue 2 specific monoclonals were incubated with the virus for 30 minutes prior to loading of wells.

Discussion

Dengue virus infections have become a major problem and the global increase in DHF/DSS is of grave concern to many countries around the world. Attempts to prevent the spread of the disease by vector control has had at best limited success.Vaccination is the most cost effective way to prevent DF and DHF/DSS. A tetravalent live vaccine is already undergoing field trails with promising results (1). The pathogenesis of the disease is only partially understood and is said to involve enhancing antibodies and cell mediated immunity especially during a secondary infection. As the main target cell is the macrophage we have attempted to further understand the pathogenesis by studying virus-target cell interactions. It was found that when virus was injected intraperitoneally into mice there was a rapid influx of cells, mostly macrophages, into the peritoneal cavity. Here dengue virus appeared to act as a chemoattractant for macrophages. It was also seen that peritoneal macrophages tend to aggregate or clump together after virus treatment. Furthermore, these *in vivo* responses were demonstrated *in vitro* using the Boyden Chemotaxis chambers. Specificity of the migration was established when this movement was inhibited by specific antibodies to the virus. Macrophage functions tests were then carried to determine to see if the virus directly activates its target cell. From the superoxide assays, it is clearly seen that dengue virus induced a respiratory burst with the production of superoxide anion in macrophages and neutrophils. In addition dengue virus induced granule exocytosis of lysozyme in the same population of cells. Significant amounts of lysozyme was released. Lysozyme is mainly involved in digesting cellular material and foreign bodies.

Reactive nitrogen intermediates are another group of compounds which have a high degree of chemical activ-

ity. The reactive nitrogen intermediates include nitrite and the related highly reactive oxides such as nitric oxide (NO) and nitrogen dioxide. NO production by rodent macrophages is clearly involved in the nonspecific host defence system against foreign pathogens and tumour cells (16). Dengue virus was found to induce mouse peritoneal cells and the monocytic tumor cell line WEHI 265.1 to produce significant amounts of NO/nitirite (1-2 μM NO_2^-).

Another important preresquisite for migration of leucocyte from the circulation to an inflammatory site is increased adhesion to matrix proteins and endothelium. In our study it is seen that dengue 2 virus stimulates casein-elicited peritoneal cells and the monocytic tumor cell line WEHI-265.1 to adhere to fibrinogen.

The present studies indicate that adhesiveness and increased activation (as observed by respiratory burst, production of lysozymes) occurred following treatment with dengue 2 virus. Each of these activities must be coordinated to direct phagocytic cells from the blood stream into tissue spaces where these cells generally exercise their phagocytic and proinflammatory activities. The strategy to affect a coordinated inflammatory response involves varying sensitivities of the target cell populations to different concentrations of virus (10^4-10^5pfu/ml). As seen in the above study adhesion required the lowest concentrations (5 X 10^3 pfu/ml) of the virus. Migratory activity is seen with at least 5 X 10^4 pfu/ml of virus with maximal activity at tenfold higher concentrations. However target cell activation (exocytosis, respiratory burst and NO release) is observed at best with concentrations of at least 5 X 10^5 pfu/ml of virus. Hence only at the focus of infection where the concentration of the virus is the highest are the phagocytic functions activated. Premature activation could be deleterious to normal tissues hence this stepwise gradient serves to focus the site of infection.

The pathogenic mechanisms in dengue virus infections are difficult to elucidate because of the absence of a suitable animal model. Antibody enhancement has been suggested as a possible contributing factor with the main target cell being a monocyte/macrophage. Considerable doubt remains as to the identity of the receptors involved in the uptake of dengue virus by the macrophage/monocyte. The adherence of infected monocytes may be sufficient to trigger off viral release and other cell contents, including factors affecting vascular permeability (19). Blood monocytes are a very heterogenous population of cells, most probably as a result of some differentiation in the 36-104 hours that they spend in blood circulation (20). Hence different stages of differentiation of this population of cells may have varying susceptibilities to the virus. Viral replication is said to be enhanced in the presence of low levels of non-neutralising antibodies (3). In the absence of antibodies very little replication has been demonstrated (19,21). This could imply effective clearance. However enhanced cytolysis of cells by viral infections are observed following infection in the presence of antibody. Thus the interactions seen in the above studies suggest clearance in the absence of antibody most probably in a primary infection and within 24-48 hours as most of the assays carried out were done within 24 hours. Also the absence of activity/detection seen in the presence of antibody could imply neutralization by antibody or enhanced replication. Further work is being done to determine macrophage functions in the presence of antibody for longer periods of infections.

Acknowledgements

The work was supported by the University of Malaya R&D fund 3-07-04-504.

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DENGUE 2 (NEW GUINEA C STRAIN) VIRUS INFECTION INDUCES APOPTOSIS

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ABSTRACT: The ability of dengue 2 virus infection to induce apoptosis was investigated. Dengue 2 virus-infected African green monkey kidney cells (Vero), showed the presence of about 2, 7, 22 and 30 percent apoptotic cells on day 3, 5, 8, and 11 post-infection, respectively. Increased number of apoptotic cells corresponded with increasing number of dengue 2 antigen positive cells. Induction of internucleosomal DNA fragmentation, characteristic of apoptotic cells, was noted also in dengue 2 virus-infected mosquito cell (C6/36) cultures. These results suggested that dengue 2 virus infection *in vitro* stimulated apoptotic cellular responses. *(JUMMEC 1997 2(1): 19 - 21)*

KEYWORDS: Apoptosis, C6/36, dengue, Vero

Introduction

Apoptosis is characterized by a series of co-ordinately regulated cellular events which culminate in cell death. The process involved distinct biochemical and cellular events which differentiate it from cell death by necrosis (1). Since it's initial description, the importance of apoptosis in various cellular functions has been unravelled. More recently, apoptosis is noted to play a significant role in the demise of the CD4+ cells in HIV infection (2). A number of other viruses including adenovirus, sindbis virus, influenza virus, bovine herpesvirus, and lymphocytic choriomeningitis virus have also been associated with induction of apoptosis (reviewed in 3). On the other hand, there are viruses which are known to encode proteins which inhibit apoptosis. These include the T antigen, E6, LMP1, CrmA and p35 protein of SV40, human papilloma virus, Epstein-Barr virus, cowpox virus, and baculovirus, respectively (3). The ability to cause or inhibit apoptosis has been associated to various forms of manifestations of the virus infection.

Dengue virus, a mosquito borne RNA virus is known to cause infection which affects the host intravascular functions (4). The mechanisms whereby the virus causes them, however, are still unknown. In the present study, we investigated the potential induction of apoptosis by dengue 2 virus infection *in vitro*.

Materials and Methods

Cells and virus preparation

Vero cells used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured on glass coverslips in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria). Cells were infected only when they were about 80-90% confluent. In a typical experiment, infection was done to give an estimated multiplicity of infection (MOI) of about 0.1-0.5 plaque forming unit (PFU) per cell. Dengue 2 New Guinea C (NGC) strain virus propagated using the C6/36 cells was used entirely in this study .

Detection of apoptotic cells

Apoptotic cells were detected using the Apoptosis Detection System (Promega, Madison, WI, USA). Briefly, dengue 2 virus-infected or mock-treated Vero cells were fixed in 10% paraformaldehyde, washed with phosphate buffered solution (PBS) and permeabilized using 0.5% Triton-X 100. The slides were then rinsed in PBS and finally with distilled water. All procedures were done on ice. Slides were air-dried and kept at 4°C until needed for staining.

Staining using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) was performed using fluorescein-12-dUTP following the protocols provided by the kit's manufacturer (Promega, Madison, WI, USA). Samples were viewed using a Zeiss Axiolab fluorescence microscope (Zeiss, Germany) using standard fluorescein excitation and emission filters. Using this labelling procedure, the apoptotic cells' DNA were labelled with fluorescein-12-dUTP, thus, appeared green.

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DNA isolation. Dengue 2 virus-infected or mock-treated C6/36 cells were lysed in lysis solution consisting of 1% N-lauryl-sarcosine, 0.2% sodium deoxycholate, and proteinase K (1 mg/ml) in L-Buffer (0.01M Tris-HCl, pH 7.6; 0.02M NaCl; 0.1M EDTA). Equal amount of DNA then was electrophoresed in a 1.8% agarose gel prepared in 0.5X TAE (0.02 M Tris-base; 0.01 M sodium acetate, 0.5 mM EDTA) buffer. Following electrophoresis, agarose gels were stained with ethidium bromide and photographed under ultraviolet light illumination at 302 nm wavelength.

Detection of dengue virus antigens

The presence of dengue virus antigens in the infected cells was determined using pooled mouse anti dengue 2 virus hyperimmune sera and TRITC-conjugated anti-mouse IgG (Sigma Chemical Company, St. Louis, MO, USA). All dengue antigen positive cells stained red when viewed using the TRITC filters under fluorescent microscopy.

Results and Discussion

Dengue 2 virus-infected Vero cells showed characteristic cellular morphological changes after about 5 days post-infection (PI). Increasing number of cells were noted to 'float-off' from the monolayer with increasing number of days PI. As expected no dengue virus antigen positive cells or apoptotic cells were noted in the mock-treated samples prepared until day 11 PI (data not shown). The number of dengue antigen positive cells and apoptotic cells as determined by immunofluorescent staining and TUNEL respectively, increased significantly ($P < 0.001$, ANOVA) beginning on day 3 PI, simultaneously with increasing number of cells (Table 1). By day 8 PI, the mean number of cells per microscopic field reached plateau. Many of the apoptotic cells were seen to 'float-off' from the cell monolayers by day 8 PI, thus, reducing the total number of cells counted. Whereas, the number of dengue positive cells increased dramatically reaching almost 97% of the remaining cells counted by day 11 PI. It was noted that the apoptotic cells stained positive for dengue virus antigens (Figure 1). A very good correlation ($r > 0.75$, Pearson's) between the number of apoptotic cells and the number of dengue virus antigen positive cells was observed. These results suggested that dengue 2 NGC strain virus infection could induce apoptotic responses.

Table 1. Percentage of apoptotic cells in dengue 2 virus-infected Vero cell cultures. The number of cells stained positive for dengue virus antigens, apoptotic cells, and the total number of cells per microscopic field were counted. The mean of at least 10 microscopic fields per datum was calculated and the percentages were determined by dividing the means (mean number of apoptotic cells or mean number of dengue antigen positive cells per field/mean number of cells per field x 100).

	Days post-infection (PI)					
	0	1	3	5	8	11
Mean number of cells	11	25	54	125	165	133
Percent infected (± S.D)	0	0	2(2)	28(6)	32(10)	97(3)
Percent apoptotic (± S.D)	0	0	1(1)	7(2)	22(5)	31(9)

Induction of apoptosis was noted also in C6/36 mosquito cells infected with dengue 2 virus. In these cells, internucleosomal fragmentation of the cellular DNA into multiples of about 180-200 bp, characteristics of apoptotic cells' DNA, was observed on day 8 PI (Figure 2), suggesting that dengue virus 2 infection induces apoptosis potentially in all susceptible cells. Further investigations are on-going in our laboratory to confirm this suggestion.

Induction of apoptosis by dengue virus 1 was previously reported (5). In this study, the mouse neuroblastoma cells were infected with dengue 1 virus inoculum

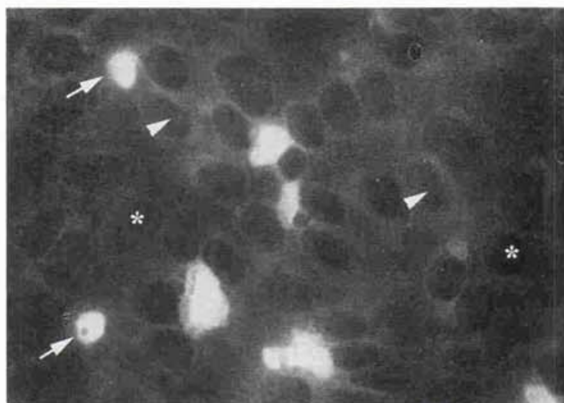

Figure 1. Photomicrograph of apoptotic cells in dengue 2 virus infected Vero cell cultures. Vero cells cultured on glass coverslips were treated with dengue 2 virus inoculum to give an estimated MOI of about 0.1 to 0.5 PFU/cell. On day 5 PI cells were fixed and doubly stained for the presence of dengue virus antigens and apoptotic cells. The image shown was digitized using a Kodak Professional RFS2035 Plus Film Scanner and labelling was done using the Adobe Photoshop 3.0. The photograph was printed using the Kodak XLS8600 PS Printer. Arrow heads (▶) indicate cells showing the presence of only dengue virus antigens; arrows (➡) indicate the apoptotic cells expressing dengue virus antigens, and asteriks (*) indicate non-apoptotic cells showing no virus antigens.

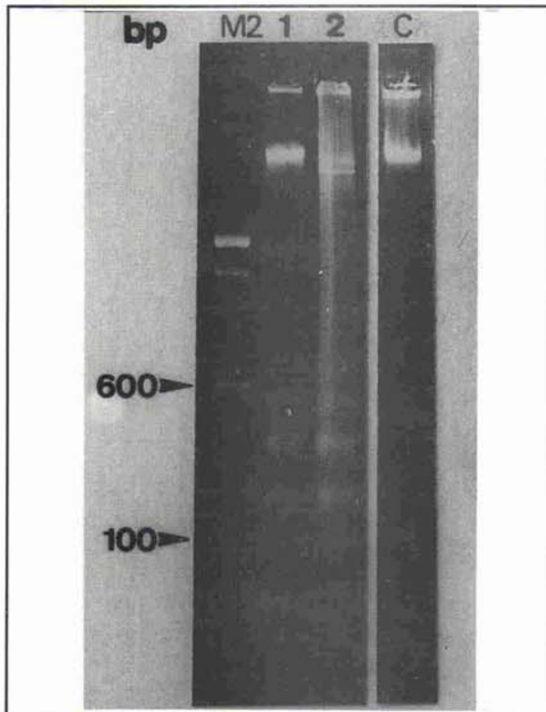

Figure 2. Agarose gel electrophoresis of DNA prepared from dengue 2 virus infected and mock-treated C6/36 cells. Monolayer of C6/36 cells (80-90% confluent) were infected with dengue 2 virus to give an estimated MOI of about 0.1-0.5 PFU/cell. At selected intervals post-infection, DNA was extracted and electrophoresed following the procedures as described in the Materials and Methods. 100 bp marker (M2); mock-treated (C); day 8 PI (lane 1); and day 15 PI (lane 2). The mock treated cells' DNA was prepared on day 18 PI.

at about 400 PFU/cell. Apoptosis was noted by 25 hr PI. In contrast, in the present investigation the Vero cells were infected at a very low MOI of 0.1-0.5 PFU/cell. This low MOI which probably simulates closely the *in vivo* infection, resulted in induction of detectable level of apoptosis only on day 3 PI. Nevertheless, these findings support the notion that induction of apoptosis by dengue virus infection could play an important role in the overall pathogenesis of dengue virus infection. Our laboratory is presently investigating to determine if clinical isolates of dengue viruses could also induce apoptosis. We hope to be able to demonstrate that dengue virus-induced apoptosis do occur *in vivo*.

Acknowledgments

This investigation was funded by the Ministry of Science, Technology and Environment R&D IRPA Grants # 03-07-04-503 and # 06-02-03-0303.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DEXTROMETHORPHAN AND ITS MAIN METABOLITE IN HUMAN URINE

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ABSTRACT: Dextromethorphan (DM) is O-demethylated to its main metabolite, dextrorphan (DO), following a polymorphic reaction that depends on the isoenzyme, cytochrome P4502D6 (CYP2D6). Traditionally, phenotyping of this isoenzyme utilises debrisoquine, individuals with rapid or normal metabolism of debrisoquine are described as "extensive metabolisers" and those with slower drug metabolism are known as "poor metabolisers". Drug oxidation phenotyping with DM has been found to be in complete concordance with results of phenotyping with debrisoquine.

A sensitive High Performance Liquid Chromatography method with both fluorescent and UV detection was developed for the analysis of DM and DO in human urine. Prior to analysis by HPLC, the urine samples were subjected to enzymatic hydrolysis of the dextrorphan glucuronides followed by extraction into chloroform:isopropanol mixture. Standard curves for both compounds were linear over concentrations ranging from 0 to 500 ng/ml with a mean linear regression value of 0.98 for DM and 0.97 for DO. The intra-assay error was 4.07% and 7.05% for DM and DO respectively and the interassay error was 18.16% and 7.13% for DM and DO respectively.

Following ingestion of 15 mg dextromethorphan hydrobromide, a preliminary oxidation phenotyping was performed on 8 volunteers. The metabolite ratio was calculated as MR = 0-8 hr urinary output of unchanged DM / 0-8 hr urinary output of DO. The total urinary output of DM ranged from 0 to 4.87 mg whereas that of DO ranged from 0.57 to 21.00 mg. The MR values were between 0 to 0.220 in 7 of the subjects tested while 1 subject showed a relatively high MR value of 1.376. This was consistent with the finding that in this same subject the urinary output of DM was the highest, and the output of DO was the lowest among those tested, thus indicating that this subject is possibly a poor metabiliser of DM.

It is concluded that the assay is sensitive for the measurements of levels of DM and DO in human urine and hence will be utilised in a large scale study of the frequency distribution of drug oxidation phenotyping with DM in a Malaysian population. *(JUMMEC 1997 2(1):22 - 25)*

KEYWORDS: Genetic polymorphism; CYP2D6; dextromethorphan oxidation phenotyping

Introduction

Variable rates of drug metabolism in man continue to pose one of the biggest hurdles to the effective and safe use of therapeutic agents. The influence of genetic control on hepatic drug metabolism had gained interest since the description of a genetic polymorphism in the capacity to metabolise 4-hydroxylate debrisoquine (1). Two phenotypes were identified; most subjects were extensive metabolisers (EM) whereas a minority of subjects were poor metabolisers (PM) (2). The impairment of debrisoquine 4-hydroxylation is inherited as an autosomal recessive trait (3, 4) and is now found to be related to the deficiency of cytochrome P450 2D6 (CYP2D6). CYP2D6 is involved in the stereospecific metabolism of several important groups of drugs, for example, antiarrhythmics like encainide, antidepressants like desipramine (5, 6) and neuroleptics like haloperidol (7, 8).

About 7 % of Caucasians but only 1 % of Orientals are poor metabolisers (PMs) of debrisoquine (9). This phenomenon of polymorphism has several consequences ranging from rational drug use for the

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individual in a racially diverse population such as in Malaysia to the development, evaluation and regional as well as world-wide licensing of drugs.

There is a scarcity of information of the prevalence of PMs in this region. Studies will have to be carried out to determine the proportion of subjects genetically deficient in CYP2D6 activity (10). Dextromethorphan is metabolised by CYP2D6 to dextrorphan, its main metabolite. Drug oxidation phenotyping with dextromethorphan is in complete concordance with results of phenotyping with debrisoquine (11, 12). Dextromethorphan, a potent cough suppressant found in some over-the-counter cough syrups, has the advantages over debrisoquine in that it is ubiquitously available and lacks of the hypotensive effects of debrisoquine. The aim of this study was to find a suitable method for the detection of dextromethorphan and dextrorphan (Figure 1) in human urine and to carry out a preliminary study of CYP2D6 activity in 8 healthy volunteers among the Malaysian population.

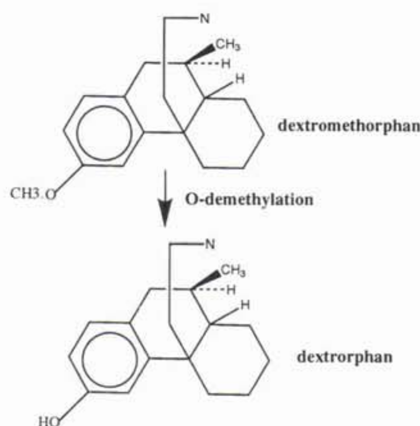

Figure 1 : The metabolic pathway of the O-demethylation of dextromethorphan to dextrorphan

Material and method

Analytical Procedures

Dextromethorphan hydrobromide and dextrorphan, were obtained from Salford Ultrafine Chemicals and Research (Manchester). Rifampicin was obtained from Sigma Chemicals. Dextromethorphan and dextrorphan in urine were assayed by High Performance Liquid Chromatography (HPLC). The HPLC is composed of a LC10AS pump with RF-10A fluorescence detector and SPD-10A detector (Shimadzu Corp. Japan). The columns used were a C18 guard column (Supelco) and an analytical ODS ultrasphere (4.6mm x 25 cm) column (Beckman). Extraction methods were modified from a previously described method by Schmidt *et al.* (11), which, instead of using ether, a mixture of chloroform:2-propanol (85:15 v/v) was used. In addition, rifampicin was used as an internal standard instead of dimetacrine.

Enzymatic hydrolysis of dextrorphan glucuronides was performed by the addition of 20 ml of ß-glucuronides/ sulphatase (11, 13, 14) and shaken overnight at 37°C in a shaking bath for complete conjugation cleavage. 1 ml of urine was diluted in 1 ml of 0.1 M acetate buffer pH 5.0 and extracted with 5 ml of the solvent mixture. After 15 seconds of rigorous vortex at speed of 70 and 15 minutes of horizontal shaking, the mixture was centrifuged, 3 ml of the organic phase was transferred into centrifuge tubes, then evaporated off. The residue obtained was dissolved in 100 µl of mobile phase. In the case of dextromethorphan analysis, because of its low concentration in some of the urine samples, the residue was dissolved in 50 ul mobile phase (15). The mobile phase was made up of 10 mM potassium phosphate buffer pH 4.2 containing 0.17 % octane sulphonic acid:methanol:acetonitrile (63:5:32 v/v/v) at a flow rate of 1.2 ml/min. The function of the octane sulphonic acid was to separate the overlapped peaks of dextrorphan from a urine residue. Dextromethorphan and dextrorphan were detected using fluorescence detection at an emission wavelength of 312 nm and excitation wavelength of 270 nm (16) while rifampicin was detected using UV detection at 254 nm. Standards for both dextromethorphan and dextrorphan were prepared at concentrations ranging from 0 to 500 ng/ ml. Standards were prepared in duplicates. Percentage recovery for both drugs were also performed.

Subjects

The study was performed in 8 unrelated subjects, 23 to 40 years old. All subjects were examined by a medical doctor to assess the state of their health. Once the subjects were judged as being healthy and not taking other medication, they were given an information sheet which detailed the aim of the study as well as the protocol. All the subjects involved gave their informed consent. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Malaya.

Phenotyping

Each subject took a tablet of 15 mg dextromethorphan hydrobromide at 2200 h having emptied their bladder just before swallowing the tablet. A sample of this urine was collected to serve as the individual blank urine. Overnight 8 hr urine was collected by making sure that the subjects emptied their bladder at 0600 h as their final urine. Subjects then noted the volume of urine voided. The urine samples were handed in the following morning and stored at -20°C until analysis. Subjects were phenotyped using the metabolite ratio (MR) calculated as :

$$MR = \frac{\text{0-8 h urinary output of unchanged dextromethorphan}}{\text{0-8 h urinary output of dextrorphan}}$$

Results

A method which enabled the detection of both dextromethorphan and dextrorphan in human urine using rifampicin as the internal standard was used. Retention time for dextrorphan was found to be 5.18 min and dextromethorphan was15.7 min while the retention time for the internal standard was found to be between 14.9 to 18.6 min (Fig.2, Fig. 3). A constant check was carried out to confirm the internal standard peak through the use of extracted blank urine spiked with rifampicin. Standard curves for dextromethorphan and dextrophan were prepared from concentrations ranging from 0 to 500 ng/ml. Standard curves were constructed from the area ratio (measured by the peak area of the dextromethorphan or dextrorphan peak to the peak area of internal standard), plotted against the concentration of these compounds. Linear plots were obtained with a mean r value of 0.98 for dextromethorphan and 0.97 for dextrorphan. Extraction recovery for dextromethorphan was 60.0% and for dextrorphan it was 75.5%. Interassay error of analysis was 18.16% and 7.13% for dextromethorphan and dextrorphan respectively. Intra-assay error for the analysis of dextromethorphan was 4.07% whilst that of dextrorphan was 7.05%.

Table 1. Shows the mean urinary output and recoveries and metabolic ratios of DM and DO in EMs and PM.

	Extensive Metabolisers	Poor Metaboliser
Number of Subjects	7	1
Age (Years)	25.7 ± 4.1	24
(Range)	(24 - 40)	
Dextromethorphan (DM) output (μmol $8h^{-1}$)	1.63 ± 1.46	23.95
(Range)	(0.00 - 1.31)	
Dose recovery as dextromethorphan (%)	2.95 ± 2.64	43.32
(Range)	(0.00 - 8.75)	
Dextrorphan (DO) output (μmol $8h^{-1}$)	41.90 ± 14.30	18.40
(Range)	(22.68 - 67.70)	
Dose recovery as Dextrorphan (%)	75.80 + 25.80	33.29
(Range)	(40.71 - 122.99)	
MR= DM output / DO output	0.0588 ± 0.060	1.376
(Range)	(0.0003 - 0.2264)	

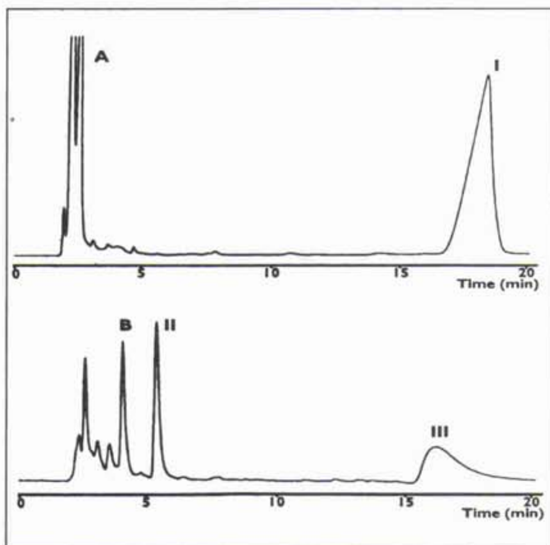

Figure 2. Chromatograms of (A) control urine with internal standard (peak I) and (B) control urine spiked with dextrorphan (peak II) and dextromethorphan (peak III).

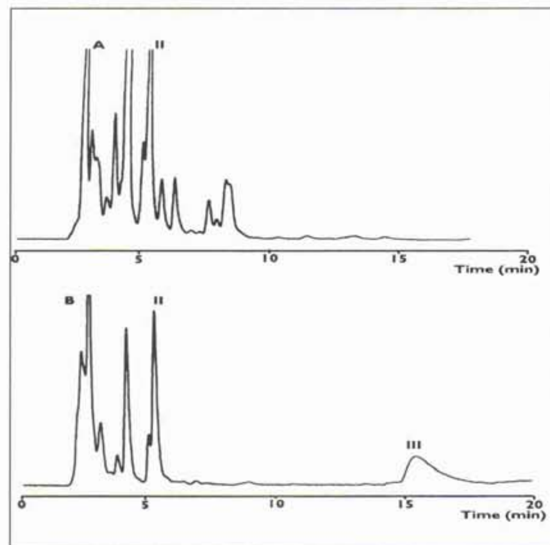

Figure 3. Chromatograms of (A) urine sample from an extensive metaboliser and (B) urine sample from a poor metaboliser. Note the presence of the dextromethorphan peak (peak III) in chromatogram B and the absence of such a peak in chromatogram A.

Seven out of the eight subjects tested had a metabolite ratio between 0.0003 to 0.2264 (Table 1) and were classified as extensive metabolisers (EM). One subject with an MR of 1.376 was classified as a poor metaboliser (PM). The mean values of the urinary output of the test drug and its metabolite, as well as the dose recoveries found in EM as compared to PM were vastly different.

Urinary output of unchanged dextromethorphan was between 0 to 1.31 μmol 8 h^{-1} in EMs while that of the PM was 23.95 μmol 8 h^{-1}. The dose recovery in urine as unchanged dextromethorphan was between 0.00 to 8.75% in EMs while that of the PM was 43.32%. In line with the reduced ability of the PMs to metabolise the test drug, urinary output of dextrorphan was between

22.68 and 67.7 μmol 8 h^{-1} in EMs while that of the PM was 18.40 μmol 8 h^{-1} in PM , the dose recoveries in urine as the metabolite dextrorphan being between 40.71 and 122.59% in EMs and 33.29% in the PM.

Discussion

It was shown in this study that the HPLC assay used is sensitive for the measurement of dextromethorphan and dextrorphan levels in human urine. The method of Schmidt *et al.* (11) was modified by the use of chloroform:2-propanol (85:15 v/v) as the extracting solvent instead of ether. This gave a consistent and good recovery of dextromethorphan and dextrorphan compared to previous assays used. A prolonged mixing time of 15 seconds on a vortex mixer and 15 minutes of horizontal shaking increased the uptake of dextromethorphan and dextrorphan into the organic layer. It was subsequently shown that the HPLC assay used is sensitive for the measurement of dextromethorphan and dextrorphan levels in human urine. Despite the small number of subjects used in this preliminary study, it was able to indicate polymorphism of dextromethorphan to dextrorphan in a Malaysian population. Data from previous studies had shown that the incidence of PMs in Orientals is 1% (9). Using dextromethorphan as the test compound, the antimode separating EM and PM phenotypes was taken to be MR = 0.3 by Schmid *et al.* (11) and by Larrey et al (15). Adopting this antimode, the one subject in this present study, with an MR of 1.3769 is clearly categorised as a PM phenotype while the other seven subjects were categorised as being of the EM phenotype with respect to CYP2D6 activity. This distinction is further substantiated by the absence of overlap in values of 0-8 h urinary outputs of unchanged dextromethorphan and of dextrorphan between the two phenotypes. It is concluded that this assay is suitable to be utilised in a large scale study of the frequency distribution of drug oxidation phenotyping with dextromethorphan among the different races in a Malaysian population.

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APPLICATION OF A SIMPLE OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS METHOD FOR CORRECTION OF A FRAMESHIFT MUTATION

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ABSTRACT: Application of a rapid and simple method for generating site-directed mutations in double-stranded plasmid DNA is described. Insertion of four nucleotides correcting a frameshift mutation was accomplished utilizing only a single synthetic oligonucleotide. Insertion was confirmed by restriction endonuclease digestion and dideoxy nucleotide sequencing. Successful application of this rapid oligonucleotide-directed site-specific mutagenesis requires no subcloning, special bacteria or multiple primers. *(JUMMEC 1997 2(1): 27-30)*

KEYWORDS: Frameshift mutation, ScFv, site-directed mutagenesis

Introduction

Site-directed mutagenesis is commonly used for the study of structure-function relationships (1,2) and gene regulation (3). In these studies, mutations are created at specific regions or sites through insertion, deletion or base substitution in the target DNA. These mutations are accomplished by a number of mutagenesis methods. The PCR-based mutagenesis for example, has been very popular (2,3). This site-directed mutagenesis method, however, requires multiple primers, thus, is not cost effective. Furthermore, repeat amplifications of the full length plasmid DNA may introduce other mutations because of the inherent low fidelity of the Taq DNA polymerase (3). In cases where the DNA template is large, DNA polymerase with proof reading activities has to be used and this indirectly affects the cost-effectiveness of this procedure. Recently Lai *et al.* (4) reported a simple and efficient method for site-directed mutagenesis with double-stranded plasmid DNA. They reported that using their protocols, successful single oligonucleotide-directed mutagenesis could be accomplished within two weeks. Numerical results of their investigation were reported. However, they did not provide actual electrophoresis results which demonstrate the various possible heteroduplexes that could arise following annealing of the DNA strands. Thus, it is possible that due to this reason among others, their protocols did not enjoy a wide spread usage in comparison to the more popular yet tedious PCR-based protocols. In the present report, we detailed a successful application of the procedure to correct a frameshift mutation affecting the expression of the single chain variable fragment (ScFv) gene cloned into the pCANTAB 5E expression vector.

Materials and Methods

Preparation of DNA fragments

The schematic for the oligonucleotide-directed mutagenesis used in this study is as outlined in Figure 1. Initially, fragment A was obtained by digesting 6 µg of the recombinant pCANTAB 5E plasmid (Pharmacia Biotech, Sweden) consisting the 3H5-1 single-chain variable fragment (ScFv) with 60 U *Not* I and then with 60 U *Sfi* I in 1.5X universal buffer (Stratagene, USA). Fragment B on the other hand, was obtained after digestion of the recombinant plasmid with 60 U *Hind* III and 60 U *Sfi* I, sequentially in 1X NEB buffer 2 (NEB, USA). The restriction endonuclease after the first digestion was removed using the StrataClean resin (Stratagene, USA). Following the second digestion, the digestion mix was added with 2 volumes of sterile MilliQ water (sMQ H_2O; Millipore, USA) and 0.5 volume of phenol:chloroform:isoamylalcohol solution (USB, USA) and then centrifuged at 20,000 X g for 4 minutes. The aqueous phase of the mixture consisting the digested DNA was removed and placed into a new microcentrifuge tube. The DNA was then precipitated using 1/10 volume of 3 M potassium acetate, pH 5.2 and 2.5 volumes of absolute ethanol at -70° C for 2 hours. The DNA was sedimented at 12,000 X g for 15 minutes at 4° C, air dried and electrophoresed in 1% agarose gel. DNA bands corresponding to fragments A and B at the expected molecular weights were removed from the agarose gel and placed into separate

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1.5 ml microcentrifuge tubes. Three volumes of 6 M NaI were added to the agarose gel slice and incubated at 58° C until the agarose melted. Then 10 µl of pretreated silica particles solution was added to the molten agarose and the mixture was incubated at room temperature for 15 minutes with occasional mixing. Following the incubation, the tubes were centrifuged at 13,000 X g for 7-8 seconds and the supernatants were discarded. The pellets were washed 3 times by resuspending with washing buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 50% ethanol) followed by sedimentation as above. After the washings, the pellets were dried at 37° C for 4 minutes and the bound DNA fragments were eluted with 30 µl sMQ H_2O at 55° C for 10 minutes with occasional tappings. The purified DNA fragments in the supernatant were recovered by sedimentation at 20,000 X g for 4 minutes and then quantitated using the GeneQuant RNA/DNA calculator (Pharmacia Biotech, Sweden).

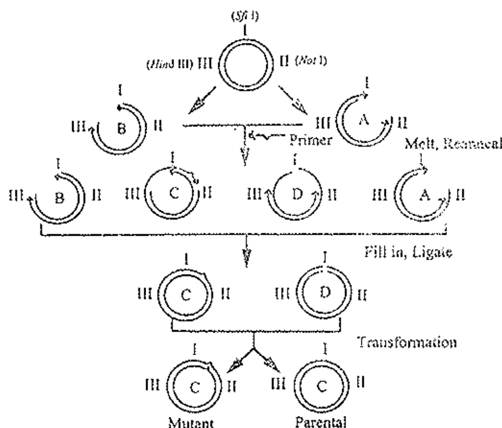

Figure 1. Oligonucleotide-directed mutagenesis scheme used in this study. Plasmids were digested with *Not* I and *Sfi* I, or *Hind* III and *Sfi* I to yield fragments A and B, respectively. After denaturation and renaturation, the parental duplexes (A and B) and heteroduplexes (C and D) were formed. Only heteroduplex C could be extended and ligated. [Diagram was adapted with modification from Lai *et al.* (4)].

Site-directed mutagenesis

Approximately 0.4 µg of each DNA fragment (A and B) were mixed with 1000-fold or 4000-fold molar excess of the phosphorylated oligonucleotide primer (5' CATTAGGGAGC**CGGC**TTACACGTTCG 3'), ligase buffer (Promega, USA) and sMQ H_2O were added to a final volume of 70 µl. The samples (two with the primer and one without the primer to serve as control) were boiled for 3 minutes and then incubated at 30°C for 30 minutes. The incubation was continued for an additional 30 minutes at 4°C and then on ice for not less than 10 minutes to allow formation of new heteroduplex DNAs. The samples were then electrophoresed in a 0.8% agarose gel to confirm the formation of the heteroduplex DNAs.

5 U of Klenow fragment of DNA polymerase I, 2 U of T4 DNA ligase and dNTP at 10 mM were then added to 17.8 µl of the above reaction mix. The newly prepared mix was incubated at 15°C overnight to allow extension and ligation to take place. After overnight incubation, 4 µl of the mixture was used to transform competent *Escherichia coli* HB 2151 cells and the transformants were then plated on 100 µg/ml ampicillin agar plate. Plasmids of the transformants were isolated using alkaline lysis protocol (5) and then digested with *Ngo*M I restriction endonuclease. The additional *Ngo*M I site was conferred by the inserted nucleotides (**CGGC**). Positive clones were then subjected to nucleotide sequencing for further confirmation.

Results and Discussion

Oligonucleotide-directed site-specific mutagenesis performed in this study was to correct a frameshift mutation in one of the recombinant clones of the hybridoma 3H5-I single-chain variable fragment (ScFv) (6). The ScFv comprising of 732 bp DNA including the Flag detection sequences at the 3' end was cloned into the pCANTAB 5E expression vector. No expression of the ScFv could be detected by immunoblotting following transformation of the recombinant plasmid into the *Escherichia coli* HB 2151. Upon sequencing, it was determined that a single nucleotide deletion had occurred at position 663 of the ScFv (Figure 5a), thus, resulting in a translational frameshifting involving about 9% of the carboxy end of the recombinant protein. Applying the above detailed oligonucleotide-directed mutagenesis, four nucleotides were successfully inserted at position 663 of the ScFv (Figure 5b), restoring the translational reading frame, thus, allowing for expression of the Flag detection sequences (data not shown). Insertion of the four nucleotides also introduced an additional *Ngo*M I restriction endonuclease site which was used for screening of successfully mutated clones and an amino acid, proline, to the ScFv.

The mutation rate obtained in this investigation, however, was low in comparison to the 58-97% success rate reported by Lai *et al.* (4). This could probably due to ligation of the heteroduplex D which would take place when excess ligase was used or the formation of the parental plasmid after transformation (4). It was also possible that the low frequency was because the resulting DNA fragments were not dephosphorylated prior to ligation. Fragment A (~ 4.5 kb; Figure 2, lane 2) and B (~ 5.2 kb; Figure 2, lane 3) were successfully obtained after the restriction endonuclease digestions. The formation of heteroduplex DNAs following denaturation and renaturation was confirmed by the appearance of an additional ethidium bromide-stained DNA band at a higher molecular weight (~ 9.7 kb) alongside the initial two bands for the DNA fragments A and B (Figure 3, lane 1, 2 and 3).

Forty five transformants obtained (23 transformants

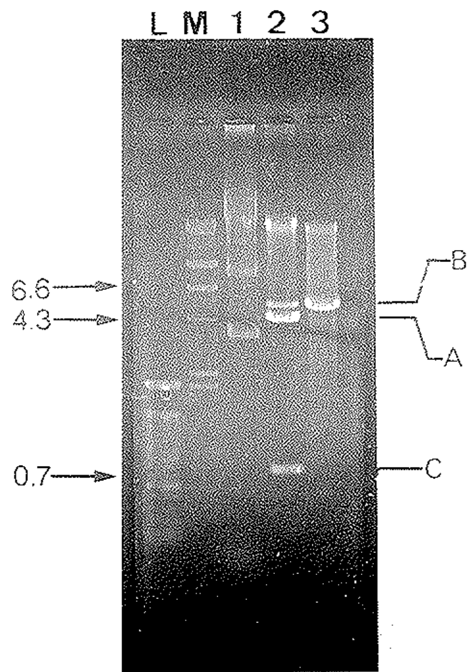

Figure 2. Restriction endonuclease digestion of the recombinant pCANTAB 5E plasmid. 3 µl of the undigested plasmid (lane 1) and 5 µl of *Not* I and *Sfi* I (lane 2) or *Hind* III and *Sfi* I (lane 3) digested plasmids were electrophoresed in a 0.8% agarose gel in 0.5X TAE and stained with ethidium bromide. 100 bp DNA ladder (lane L) and λ *Hind* III molecular weight markers (lane M) were used for DNA size indication and are shown in kilobases. A, B and C indicate the resulting DNA fragments.

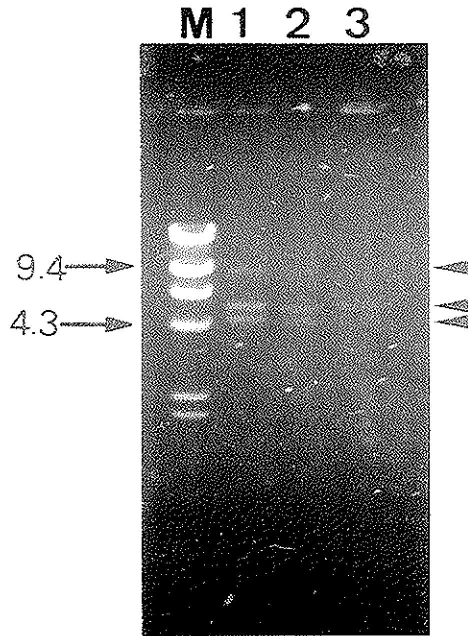

Figure 3. Formation of heteroduplex DNA fragments. 5 µl of denaturation and renaturation mixture of 1000-fold molar excess primer (lane 1), 4000-fold molar excess primer (lane 2) or without primer as negative control (lane 3) were electrophoresed in a 0.8% agarose gel in 0.5X TAE and stained with ethidium bromide. λ *Hind* III molecular weight markers (lane M) were used for DNA size indication and are shown in kilobases. Arrow heads indicate the resulting DNA fragments.

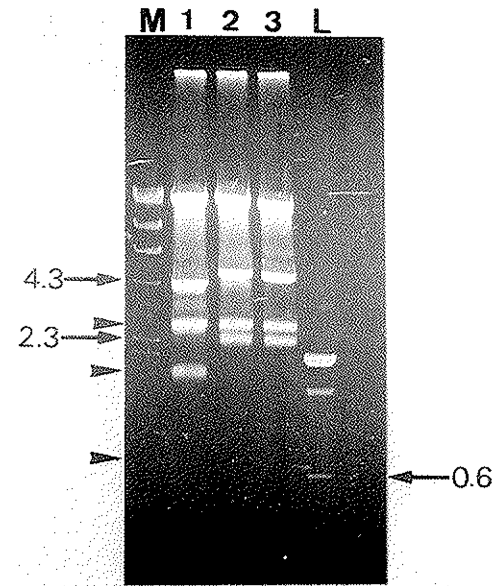

Figure 4: Screening for successful oligonucleotides insertion using restriction endonuclease digestion. Following the site-directed mutagenesis, 10 µl of the *Ngo*M I digested samples were electrophoresed in a 0.8% agarose gel in 0.5X TAE and stained with ethidiun bromide. λ *Hind* III molecular weight markers (lane M) and 100 bp ladder (lane L) were used for DNA size indication and are shown in kilobases. Arrow heads indicate the resulting DNA fragments. See text for discussion.

from 1000-fold and 22 transformants from 4000-fold molar excess of primer) were screened with the *Ngo*M I restriction endonuclease. The parental recombinant pCANTAB 5E plasmid consisted two *Ngo*M I restriction endonuclease sites which would give approximately 2600 and 2700 bp DNA fragments following the *Ngo*M I restriction endonuclease digestion. Therefore, plasmids without any nucleotides insertion would have only two DNA fragments (Figure 4, lane 3). In contrast, the mutated plasmid with an additional *Ngo*M I restriction site would show 3 DNA fragments of approximately 800, 1800 and 2700 bp respectively (Figure 4, lane 1). This is because the additional *Ngo*M I restriction site was introduced into the region of the 2600 bp DNA fragment which when digested would result in two DNA fragments of 800 and 1800 bp. Besides the three DNA fragments, there was an additional DNA fragment of about 4500 bp. This DNA fragment was probably the incompletely digested plasmid. This was because the total molecular weight of two out of the three DNA fragments (1800 and 2700 bp) was approximately equivalent to the above presumed incompletely digested DNA fragment. Whereas, the largest DNA fragments (> 4500 bp) in the other two samples (Figure 4, lane 2 and 3) were probably the linearized plasmid since the molecular weights were approximately 5300 bp which is the reported molecular weight of the plasmid. Samples shown in lane 2 (Figure 4), however, had an additional band of about 1800 bp in addition to the three DNA fragments (2600, 2700 and 5300 bp) similarly present in samples in lane 3. This could be due to insufficient restriction endonuclease digestion of the plasmid or there was a mixture of mutated and unmutated plasmids in the sample. Nevertheless, successful insertion of the four nucleotides was confirmed by nucleotide sequencing (Figure 5).

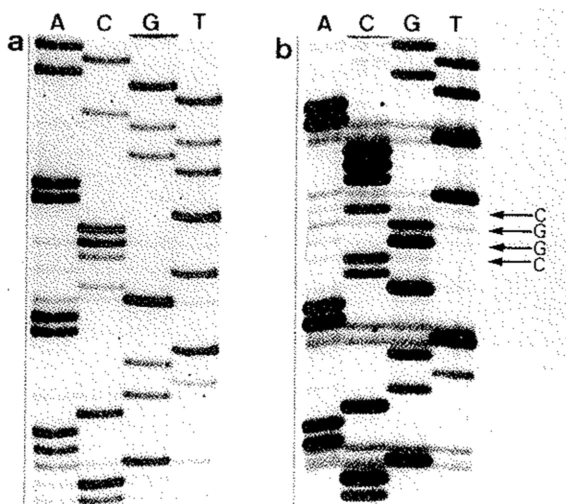

Figure 5. The nucleotide sequences before (a) and after (b) site-directed mutagenesis. Arrows indicate the inserted 4 nucleotides.

In summary, we detailed here a successful application of a simple site-directed mutagenesis of double stranded DNA using a single oligonucleotide. The entire procedure was performed within two weeks without any need to subclone or use of any additional new enzymes.

Acknowledgements

We thank Dr. Sidney Pestka for his suggestion. This study was presented as poster at the 3rd Symposium on Trends in Biotechnology, Universiti Putra Malaysia, 1997. This study was funded in part by IRPA Grant #:06-02-03 0303.

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AMLODIPINE AFFECTS PLASMA ANGIOTENSINOGEN LEVEL : INDIRECT EVIDENCE

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ABSTRACT: The renin-angiotensin system acts to regulate body fluid volume and blood pressure. The circulating renin substrate, angiotensinogen, secreted mainly from the liver is affected by various hormones. Calcium ions thus far, have not been reported to affect the plasma level of angiotensinogen. Nevertheless, since we have previously reported that amlodipine, a selective vascular calcium channel blocker, could raise plasma prorenin and renin activities in Malaysian hypertensive patients, the aim of the present study is therefore to investigate the effect of amlodipine on the plasma angiotensinogen level. An open single-blind study was performed on male and female hypertensives (32 - 60 years) without other complications. Following a washout period, the patients were prescribed amlodipine either 5 or 10 mg daily, for 2 - 3 months. Blood samples were collected before and after treatment with the obtained plasmas analysed for plasma renin concentration (PRC) and plasma renin activity (PRA) using direct and indirect renin radioimmunoassay methods, respectively. Data obtained showed that amlodipine treatment significantly ($p<0.05$) increased the PRC and the PRA of the patients. In addition, the PRA was highly correlated to the PRC before ($r = 0.62$, $p<0.001$) and after ($r = 0.68$, $p<0.001$) the amlodipine treatment. The finding that the PRA rose in parallel with the rise in PRC indicated that angiotensinogen was not limiting, and that amlodipine treatment may raise the plasma angiotensinogen level. *(JUMMEC 1997 2(1): 31-34)*

KEYWORDS: Hypertension, amlodipine, renin, angiotensinogen, angiotensin I, direct renin assay and indirect renin assay.

Introduction

The renin-angiotensin system (RAS) plays a key role in the regulation of blood pressure and body fluid volume. Renin, an aspartic protease secreted mainly by the kidneys, is known to act on only one substrate, angiotensinogen, a 55 - 60 kDa glycoprotein, producing angiotensin (Ang) I which is subsequently converted by the angiotensin converting enzyme (ACE) to the vasoactive peptide, Ang II. Renin is secreted by the kidneys via a regulated pathway, while its precursor, prorenin, via a constitutive pathway by renal and extrarenal tissues (1). Angiotensinogen is secreted solely by a constitutive pathway and thus the regulation of the production is at the level of its synthesis (2,3). The main source for circulating angiotensinogen is the liver; although, various other tissues such as the brain, kidney, adrenal, ovary, adipose tissue and the vascular wall have also been implicated (4,5,6). Hormones such as estrogens (3,7,8), testosterone (9), glucocorticoids (3,10), and thyroid hormone (3) have been reported to affect the production of angiotensinogen, in addition to Ang II which has been shown to stimulate angiotensinogen synthesis (2,11). Calcium ions, however, appear to have no effect on angiotensinogen secretion (2,11).

Although calcium antagonists are now increasingly being prescribed for the control of hypertension (12), effects of such treatment and the consequent altered intracellular calcium concentration on the RAS are relatively not well studied. Nevertheless, we have previously reported that a vascular selective calcium channel blocker, amlodipine, can alter the plasma profiles of prorenin, renin and aldosterone in hypertensive patients (13, 14). Since factors that affect plasma renin level may also affect the secretion of angiotensinogen, the purpose of our present study is then to investigate the effect of amlodipine on the plasma level of angiotensinogen.

Materials and Methods

Patients

Male (n=11) and female (n=9) essential hypertensive patients aged between 32 and 60 years, with a mean age of 49.8 ± 1.6 years, and mean systolic (SBP) and

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diastolic blood pressures (DBP) of 153 $\pm$ 1.9 and 101 $\pm$ 0.9 mmHg respectively, and with no renal or liver disease were recruited from the Polyclinic of the University Hospital for an open, single-blind study. Consent was obtained from each patient after the purpose and nature of the study were fully explained. Approval was also obtained from the Medical Centre Research Committee and the University Hospital Ethics Committee.

The patients underwent a washout period of 2 weeks to clear the effects of previously administered anti-hypertensive therapies and subsequently were prescribed only amlodipine (Pfizer Malaysia) either 5 or 10 mg once daily, depending on their sensitivity or severity, for a period of 2-3 months. The end-point was a normal blood pressure reading.

Blood Collection

Blood samples were obtained from patients before treatment with amlodipine and 2 -3 months after. To avoid postural effects on the RAS, during each collection time the patients were allowed to rest for about 30 minutes before blood was collected into tubes containing disodium ethylenediaminetetra-acetic acid (Na_2-EDTA; Sigma Chemical Co, St. Louis, MO, USA) and kept on ice. The plasmas obtained were subsequently aliquoted and stored at -70°C until analysed.

Assays and data analysis

The plasmas were assayed for plasma renin concentration (PRC) and renin activity (PRA). The PRC was obtained using a direct renin radioimmunoassay (RIA) (Active renin, Nicholls Institute Diagnostics, CA, USA). The PRA was estimated by an indirect assay technique which involved the incubation of each plasma sample at 4°C and 37°C for 30 min (15). The samples were subsequently assayed for Ang I by RIA (Renin RIAbead, Abbott Laboratories, USA). The difference between the Ang I values obtained from samples incubated at 4°C and 37°C, corrected per hour, would give the amount of Ang I generated per hour by the action of renin on the angiotensinogen present in the plasma.

Data were analysed for statistical significance by Student's paired t-test and Kendall Tau correlation analysis. The Student's paired t-test was used when a direct comparison of parameters measured, before and after amlodipine treatment, was made. The Kendall-Tau correlation analysis was performed when correlating the PRA with the PRC.

Results

Treatment of essential hypertensive patients with amlodipine significantly ($p<0.001$) lowered the SBP from 153 $\pm$ 1.91 to 134 $\pm$ 1.44, the DBP from 101 $\pm$ 0.90 to 87.2 $\pm$ 0.99 and consequently reduced the mean arterial blood pressure from 118 $\pm$ 1.04 to 102 $\pm$ 0.92 mmHg (figure not shown). Amlodipine significantly raised their PRC ($p<0.05$) from 7.62 $\pm$ 0.63 to 13.10 $\pm$ 2.62 pg/ml (Figure 1, left panel) as well as their PRA ($p < 0.05$) from 0.30 $\pm$ 0.08 to 1.22 $\pm$ 0.42 ng/ml/hr (Figure 1, right panel). The Kendall Tau correlation analysis showed that the PRA was highly correlated with the PRC before (Figure 2, top graph, r=0.62, $p<0.001$) and after (Figure 2, lower graph, r=0.68, $p<0.001$) being prescribed with amlodipine.

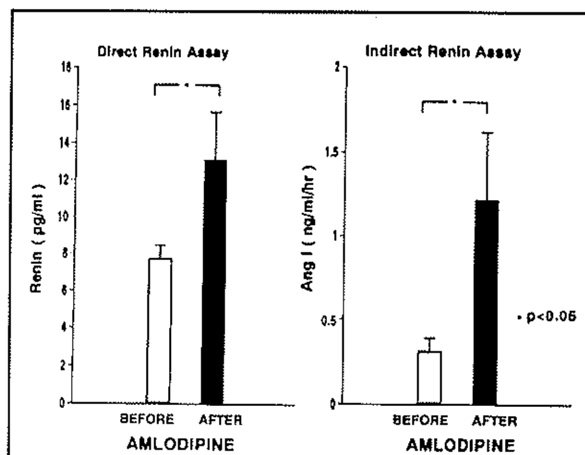

Figure 1. The plasma renin concentration as obtained using the direct renin assay (left panel) and plasma renin activity as obtained using the indirect renin assay (right panel) of hypertensive patients before and after treatment with amlodipine.

Discussion

This report provides evidence that treatment with amlodipine in hypertensive patients may increase their plasma angiotensinogen level, an hitherto unknown effect. As previously reported, amlodipine treatment was also found to significantly lower the systolic and diastolic blood pressures of the hypertensive patients (14).

The RAS regulates both blood pressure and body fluid volume. Renin, the renal aspartic protease is known to act only upon the glycoprotein, angiotensinogen, with a consequent production of Ang I which is subsequently converted to Ang II by ACE. The liver is thought to be the main source for circulating angiotensinogen and is the most widely studied. However, the presence of mRNA for angiotensinogen reported in various other tissues such as the brain, kidneys, adrenals, ovary, the vascular wall and adipose tissue (4,5,6) implicates extrahepatic sources for circulating angiotensinogen. Since angiotensinogen is secreted via a constitutive pathway, any changes of angiotensinogen production would therefore be at the level of its synthesis (2,3). Several hormones that affect transcriptional processes such as estrogens (3,7,8), testosterone (9), glucocorticoids (3,10)

and thyroid hormone (3) have been shown to affect the production of angiotensinogen. Angiotensin II has also been shown to stimulate angiotensinogen production (2,11).

The production of Ang I and therefore Ang II is limited by the availability of angiotensinogen within the circulation for renin to act upon and consequently, the more substrate there is, the more Ang I that is produced (16,17). Changes in plasma angiotensinogen level have

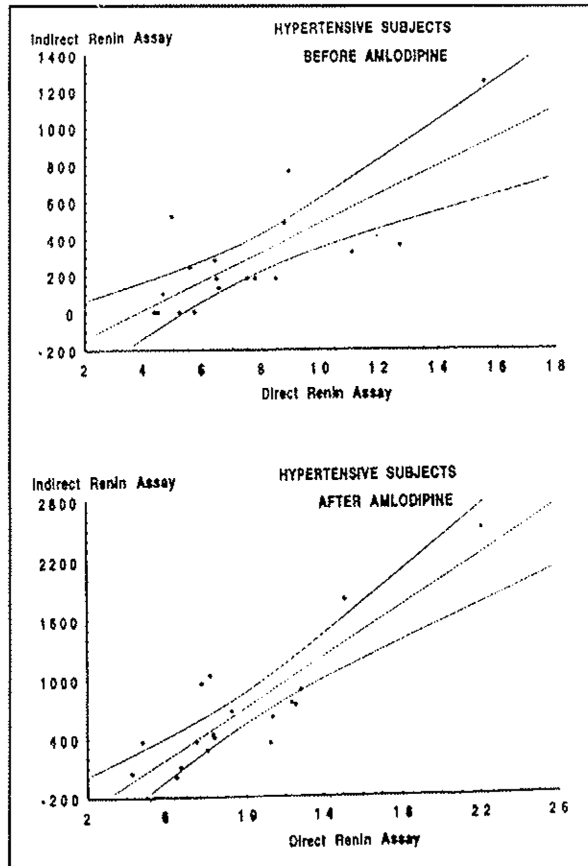

Figure 2. The Kendall Tau correlation analysis of plasma renin activity (PRA, indirect renin assay) with plasma renin concentration (PRC, direct renin assay) in hypertensive patients before (top) and after amlodipine treatment (bottom). The PRA is highly correlated with the PRC before (top graph, $r = 0.62, p<0.001$) and also after (bottom graph, $r = 0.68, p<0.001$) amlodipine administration.

been reported to be accompanied by an inversely proportional concentration in renin (18). This implied that a high plasma renin concentration would convert more of the circulating angiotensinogen to Ang I, and thereby lowering the plasma concentration of angiotensinogen and vice-versa. Since the production rate of Ang I is dependent on the level of plasma angiotensinogen, conditions which raise plasma renin concentration resulting in an increase in the measurement of plasma Ang I as determined by PRA, could be due either to a concomitant increase in angiotensinogen level or that the renin substrate is present in abundance. If the angiotensinogen level present in the plasma was not increased in parallel with the renin concentration, the substrate may be limited and thus, one would expect that the rate of Ang I production would remain unchanged rather than increased. Our present study showed that the treatment of hypertensive patients with the vascular selective calcium channel-blocker, amlodipine, significantly raised the PRC (Figure 1, left panel, $p< 0.05$) with a concomitant increase of the PRA of these patients (Figure 1, right panel, $p<0.05$) thus, suggesting an elevation of angiotensinogen level. These changes in PRC and PRA were highly correlated both before (Figure 2, top graph; $r = 0.62, p<0.001$) and after (Figure 2, lower graph; $r = 0.68, p<0.001$) the amlodipine treatment. The finding that such treatment resulted in an increase in PRA in parallel with the rise in PRC in the hypertensive patients indicated that the level of plasma angiotensinogen was not rate limiting, and may be raised by amlodipine administration. However, the possibility that the plasma angiotensinogen of these patients was present in abundance prior to the amlodipine treatment remains to be elucidated.

Thus, we conclude that amlodipine treatment not only raised the PRC but may also increase the plasma angiotensinogen level. To the best of our knowledge, this finding has yet to be reported elsewhere.

Acknowledgments

We wish to thank Mr M. Ragavan for his excellent technical assistance. This work was supported by University of Malaya vote F 313/95 and R&D 3-07-04-517.

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EFFECT OF GESTATIONAL AGE ON GENTAMICIN PHARMACOKINETIC PARAMETERS IN THE NEWBORN

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ABSTRACT: This study investigates the pharmacokinetics of gentamicin in newborns in the Special Care Nursery in University Hospital. They were divided into 3 groups according to gestational age: Group I, 26 to 30 weeks (n=10), Group II, 31 to 35 weeks (n=27), and Group III, 36 to 40 weeks (n=36). Each subject received 2.5mg/kg gentamicin (gentamicin sulphate, David Bull) every 12 h initially. The pharmacokinetic parameters for each newborn were derived from the measured plasma C_{max} and C_{min} levels taken at steady state, according to the Sawchuk-Zaske method. The subsequent dosage regimen was calculated using these parameters.

Gentamicin trough levels in the newborn ranged from 0.57 to 4.94 µg/mL while the peak levels ranged from 4.24 to 12.42 µg/mL. The apparent volume of distribution (V_d) (means ± SEM) increased with gestational age, the V_d being 0.81 ± 0.09, 1.00 ± 0.06 and 1.49 ± 0.06 L for groups I, II and III respectively. The differences between the groups were significant (P<0.01; Student's t-test).

There was an observable decrease in $t_{1/2}$ with increasing gestational age, the $t_{1/2}$ (mean ± SEM) being 10.02 ± 1.19 h, 8.53 ± 0.38 h and 7.10 ± 0.31 h for Groups I, II and III respectively. This decrease in the $t_{1/2}$ was accompanied by a similar increase in CL (0.07 ± 0.02, 0.09 ± 0.01 and 0.15 ± 0.01 l/h for Groups I, II and III respectively). The changes in $t_{1/2}$ and CL were significant (P<0.01) between Groups I and III, and between Groups II and III.

These findings show that differences exist in the pharmacokinetic parameters of newborns when grouped according to gestational age. For the effective monitoring of gentamicin especially with regard to the initial estimation of drug dosage, the appropriate set of pharmacokinetic parameters should be used for the newborn of that gestational age. *(JUMMEC 1997 2(1): 35-38)*

KEYWORDS: gentamicin in neonate, gentamicin pharmacokinetic, drug monitoring in neonates

Introduction

Aminoglycosides are a cornerstone in the therapy of severe gram-negative infections, despite their potential toxicity. Nephrotoxicity and ototoxicity are important adverse effects clinically, and hence form the basis for attempts to rationalise therapy (1). The main aminoglycoside used in the Special Care Nursery (SCN) Ward of the University Hospital, Kuala Lumpur, is gentamicin.

Peak serum concentration of 4 µg/ml has been suggested as the minimal inhibitory concentration of gentamicin for most susceptible gram-negative organisms (2). While a peak serum concentration of 4 to 8 µg/ml is considered to be in the therapeutic range, a concentration of more than 10 µg/ml is considered toxic (3). It has been recommended that the trough serum concentration be maintained at less than 2 µg/ml to prevent toxic effects (2). Therefore, serum gentamicin concentrations should be monitored and the dose adjusted to maintain the levels within the therapeutic range.

The fear of the ototoxic and nephrotoxic effects described in adults has led several authors to recommend monitoring of plasma levels of gentamicin in neonates (4-6). The current recommended dose regimen of gentamicin in the newborn is 2.5 mg/kg every 12 h in the first week of life and 2.5 mg/kg every 8 h in the second week (7). This regimen has been established in order to obtain appropriate gentamicin serum concentrations. In spite of this, the risk of toxicity or therapeutic failure is high (8) because there is a large inter-patient variabil-

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ity in the pharmacokinetic parameters of gentamicin depending on the stage of maturity of the newborn (9).

The aim of this study was to investigate the pharmacokinetics of gentamicin in the newborn, grouped according to gestational age.

Method

Patients

The study subjects consisted of newborns admitted to the Special Care Nursery (SCN), University Hospital, Kuala Lumpur. They received an initial bolus parenteral gentamicin (manufactured by David Bull; marketed as gentamicin sulphate) at 2.5 mg/kg/12h for 2 days. They were divided into 3 groups according to gestational age: Group I comprising newborns of gestational age from 26 to 30 weeks (n=10), Group II, 31 to 35 weeks (n=27), and Group III, 36 to 40 weeks (n=36).

Dosage Regimens and Blood Sampling

All patients were on a fixed regimen of gentamicin therapy (2.5 mg/kg/12h, IV/IM) for at least 2 days before this study, and were therefore considered to be at steady state. Trough serum samples were collected just before and peak serum sample at 0.5-1.0 h after gentamicin administration.

Assay

Gentamicin serum concentrations were determined by immunofluorescent polarization assay (TDx, Abbott).

Calculation of Various Pharmacokinetic Parameters

The pharmacokinetic parameters for the individual newborn were derived from the measured peak and trough levels according to the Sawchuk-Zaske method (11). The subsequent dosage regimens were calculated using these parameters.

Statistical Analysis

Data are presented as mean ± SEM of n determinations. The statistical significance of the difference between two means was calculated using student's t-test for unpaired samples.

Results

The measured gentamicin trough levels in the newborn ranged from 0.57 to 4.94 µg/mL. There was a significant difference in the mean trough levels between Groups I and III ($P<0.05$), and between Groups II and III ($P<0.01$), but not between Groups I and II (Figure 1). The measured peak levels ranged from 4.24 to 12.42 µg/ml, but the mean levels were not significantly different between the three groups (Figure 2).

The apparent volume of distribution (V_d) increased with gestational age, the V_d being 0.81±0.09, 1.00±0.06 and 1.49± 0.06 L for Groups I, II and III respectively. The differences between the groups were significant ($P<0.05$, Figure 5).

Despite the increase in the V_d, there was an observable decrease in $t_{1/2}$ with increasing gestational age, the $t_{1/2}$ being 10.02±1.19 h, 8.53±0.38 h and 7.10±0.31 h for groups I, II and III. This decrease in the $t_{1/2}$ was accompanied by an increase in CL (0.07±0.02, 0.09± 0.01 and 0.15±0.01 L/h for Groups I, II and III respectively). The changes in $t_{1/2}$ and CL (Figures 3 & 6) were significant between Groups I and III ($P<0.02$), and between Groups II and III ($P<0.01$). No significant difference was found between groups I and II (Figures 3 & 6).

The subsequent recommended dosage regimen in the form of calculated dose and dosing interval, for each group of newborns, is shown in Table I. Based on the pharmacokinetic parameters obtained for each individual newborn, a new dosage regimen (dose and dosing interval) was recommended so as to achieve the desired peak and trough gentamicin levels in these newborns. There were no significant differences in the mean calculated dose among the three groups (Table I). There were, however, significant differences in the mean dosing intervals between Groups I and III ($P<0.02$), and Groups II and III ($P<0.01$), but not between Groups I and II.

Discussion

The measured gentamicin peak levels were within the therapeutic range in all the three groups. However, the measured gentamicin trough levels were found to be higher than 2 µg/mL, especially in Groups I and II (Figure 1), hence necessitating an adjustment of dosage regimen. This finding is consistent with studies done by Garcia-Delgado *et al.* (12) where the initial dosages of 2.45 ± 0.4 mg/kg were sufficient to attain potentially therapeutic blood levels. These authors suggested that the dose should be administered at different intervals according to gestational age in order to allow the trough levels to come down to values which are considered to be therapeutic.

The measured gentamicin trough levels were found to decrease with increasing gestational age but the measured peak levels were not significantly different (Figures 1 & 2). This decrease in trough levels was a consequence of a decrease in the elimination half-life with increasing gestational age (Figure 3). The elimination half-life of a drug is a function of its volume of distribution and clearance. In this study, the volume of distribution was found to increase with gestational age (Figure 5), but this increase was mainly due to an increase

Table I. Subsequent recommended dose and dosing intervals for the newborns grouped according to gestational age presented as means ± SEM of n determinations.

Group	I	II	III
Age (weeks)	26-30	31-35	36-40
Number of patients (n)	10	27	36
Dose Calculated (mg/kg)	3.77±0.24	3.74±0.15	3.49±0.14
range	*(2.49-4.93)*	*(2.31-5.53)*	*(1.89-6.17)*
Dosing Intervals (h)	24.22±2.87	20.61±0.92	17.15±0.75
range	*(11.16-43.14)*	*(9.23-29.85)*	*(9.10-28.57)*

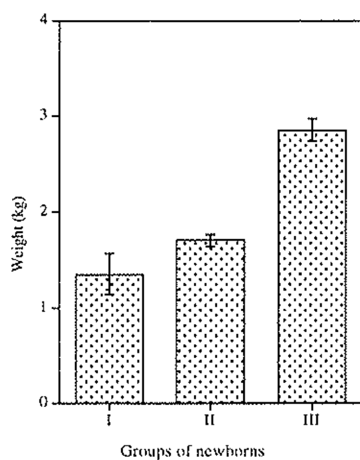

Figure 1. Gentamicin trough levels in groups of newborns, divided according to gestational age. P<0.05 for Group I versus III; P<0.01 in Group II versus III.

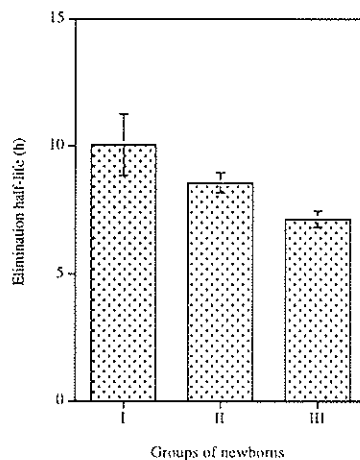

Figure 2. Gentamicin peak levels in groups of newborns, divided according to gestational age.

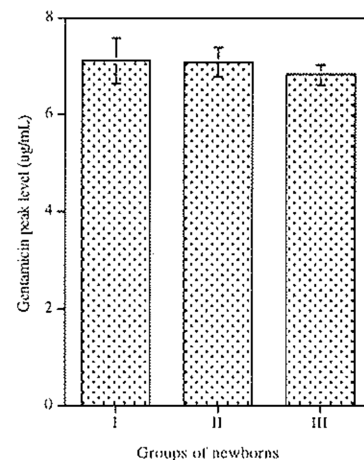

Figure 3. Gentamicin elimination half-life ($t_{1/2}$) in groups of newborns, divided according to gestational age. P<0.02 for Group I versus III; P<0.01 for Group II versus III.

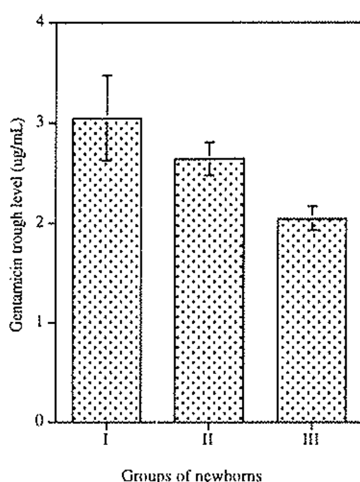

Figure 4. Patients' weight in groups of newborns, divided according to gestational age. P<0.01 for Group I versus II; P<0.01 for Group I versus III; P<0.01 for Group II versus III.

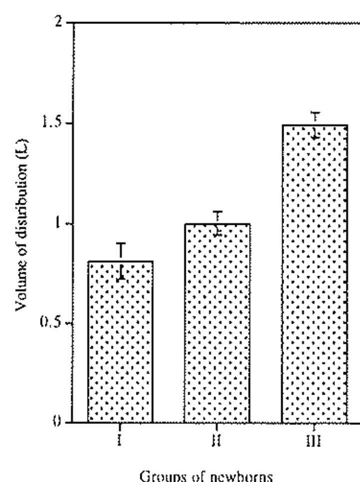

Figure 5. Gentamicin volume of distribution (V_d) in groups of newborns, divided according to gestational age. P<0.05 for Group I versus II; P<0.01 for Group I versus III; P<0.01 for Group II versus III.

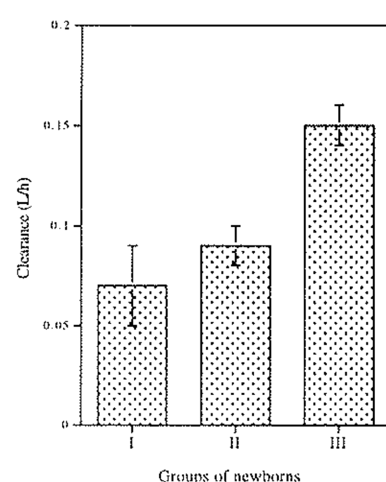

Figure 6. Gentamicin clearance (CL) in groups of newborns, divided according to gestational age. P<0.01 for Group I versus III; P<0.01 for Group II versus III

in body weight of the neonates (Figure 4). Therefore, the observed decrease in the gentamicin trough levels with increasing gestational age was not likely to be due to a change in the volume of distribution, but rather to a change the clearance.

Our study showed that the decrease in the elimination half-life with increase in gestational age(Figure 3) was accompanied by a corresponding increase in plasma clearance (Figure 6).This finding is consistent with work done by Semchuk *et al.* (13), where younger newborns demonstrated slower elimination half-lives than did older newborns. The decrease in elimination half-life is likely to be due to an increased ability of the eliminating organs to clear the drug with increasing gestational age.

Substantial changes in physiologic parameters occur in neonates, especially in the premature newborn (14). Cardiac output, renal blood flow, glomerular filtration rate, and extracellular fluid are physiologic parameters that affect gentamicin volume of distribution, elimination half-life and clearance. Although in this study it was clear that the decrease observed in the gentamicin trough levels was likely to be due to the significant decrease observed in the elimination half-life, the fact that the pharmacokinetic parameters can vary substantially from day to day due to changes in physiologic functions should be taken into consideration.

In our study, in order to achieve peak serum gentamicin concentrations of 8 μg/ml and trough concentrations of less than 2 μg/ml, younger newborns (26-30 weeks gestational age) require a larger dose (3.77 mg/kg) with a longer dosing interval (24.22h). Semchuk *et al.* (13) suggested that younger newborns (less than 34 weeks gestational age) would be likely to require 4 mg/kg as an initial dose in order to achieve peak serum gentamicin concentrations of 8 μg/ml. Work done by Lopez-Samblas *et al.* (15) found that dosing protocol based on gestational age was reproducible and reliable in achieving therapeutic gentamicin serum concentration in neonates. In their study, newborns of gestational age less than 30 weeks were given 3.0 mg/kg/24h and those of gestational age 30-37 weeks were given 2.5 mg/kg/18h/IV.

Conclusion

Our findings show that there are differences in the pharmacokinetic parameters of the different groups of newborn grouped according to gestational age. The data show that the gentamicin dose regimen recommended in the literature for newborn infants within the first week of life (2.5mg/kg every 12h) may be more than is necessary. These differences should be taken into consideration for the effective monitoring of gentamicin especially with regard to the initial estimation of drug dosage.

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AN UPDATE ON ROAD TRAFFIC INJURIES IN MALAYSIA

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ABSTRACT: The incidence of road traffic injuries has increased over the last two decades. Of greater concern is the prediction that the problem is likely to increase further, given present trends in transportation. Injuries and not "accidents" need to be the focus of the health sector. Passive strategies, which are independent of human behaviour, are more likely to succeed in the prevention of injuries compared to "active" strategies. The health sector needs to play a bigger role in prevention through advocacy, research and education of target groups. *(JUMMEC 1997 2(1): 39-41)*

KEYWORD: Road Traffic Injuries, Road Accidents

With improvement in the standard of living and control of communicable diseases, non communicable diseases such as cardiovascular diseases, cancer and injuries, have become leading causes of death and disability in Malaysia (2). Road traffic injuries constituted the leading cause of medically certified deaths between the ages of 5 and 39 years (2). The incidence of road traffic injuries per 100.000 population has increased from 19.0 in 1973 to 28.4 in 1995 (3). This is probably due to policies of the government which have favoured private transportation and fuelled growth of the automobile manufacturers. In Peninsular Malaysia, between 1978 and 1995, the total number of private cars and motorcycles increased by 246 % while the total number of private buses and taxis increased by 123 %. (3) The growth of private vehicles was twice that of public vehicles. Since the young and productive are mainly injured, the economic loss due to injuries is considerable (4).

Definition, Terminology and Principles of Prevention

The term "accident" means an event which has potential for damage to tissue or property. The word describes the intent and connotes that nothing can be done to prevent it. All diseases including cancer, stroke, heart attacks and infectious diseases are "accidents": no one willed them to occur. Hence, the focus should be on "Injuries", the health outcome of an "accident". In 1996, the US Department of Transportation's National Highway Traffic Safety Administration gave a commitment to its safety partners and eliminated the word "accident" from its vocabulary in the field of unintentional injury prevention(5). Road traffic injuries constitute the most important type of injuries, others include occupational, home and recreational. In some instances, there is an overlap in classification. For a driver or road repair worker, road traffic injuries are occupational injuries. For those who live near or for children who play on main roads, the distinction between road, home and recreational injuries is often subtle. Risk factors for injuries can be classified into those relating to the classical epidemiological triad: age, host and environment. Just as a vaccine prevents disease when a susceptible host is exposed to an infection, a safety device e.g. helmet prevents injury when a host is involved in a crash. The principles of prevention of injuries can be classified into the 4 E's: Education (of all relevant including public), Enforcement (of rules and regulation pertaining to safety), Engineering (measures to endure safe ergonomic design of consumer objects and the environment) and Economics (investment in measures mentioned above). Above all, action in the field of road safety should be intersectoral since policies and programs would cut across many sectors such as police, transport, road works, health, town planning, etc. Traditionally, injuries are said to be as a result of behavioural factors which encourage risk taking i.e. the human error model. In Malaysia, police statistics state that more than 50% of all road traffic "accidents" are due to human errors (3). This model is based on a traditional system which investigates the person at fault in "accidents" for purposes of law and insurance. If this approach is followed, then food poisoning is due to a person's fault i.e. eating at places outside of our homes. The health system, however, attempts to make food stalls and restaurants "safe" for eating. In other words, the environment is modified to prevent diseases. Using this principle, injuries can be more effectively prevented by

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passive strategies (which are independent of human behaviour and make the environment safe) than by active strategies (behaviour modification e.g. drive carefully). Strategies that have been successful in developed countries need to be adapted for implementation in developing countries, taking into account the local political and sociocultural milieu.

Some Contributing Factors for Road Traffic Injuries

Motorcyclists

Motorcyclists constituted 58% of road fatalities in 1995. (3). Between 1986 and 1995, the incidence of motorcycle fatalities per 100,000 population increased from 10.2 to 16.7. About two thirds (65.5%) of victims were below the age of 30 years. In 1995, head and "multiple" injuries were observed in 36 % and 43% of victims of motorcycle fatalities respectively. Since "multiple" injuries include head injuries, the most important contributing factor in motorcycle fatalities are head injuries. Motorcycle helmets have been shown to be effective in preventing head injuries to motorcyclists in the event of a crash (6). Helmet use in motorcyclists is mandatory by law in Malaysia. Helmets that are worn should be of certified quality and should be strapped correctly. If helmets are not worn properly, they are likely to dislodge in the event of a crash and protection to the head is lost. Use of full face helmets reduces chances of facial and mandibular injuries.

Bicyclists

Bicyclists constituted 5% of road fatalities in 1995. Head and "multiple" injuries were observed in 57% and 33% of bicycle fatalities respectively. Head injuries are the single most important contributing factor for bicycle fatalities since multiple injuries included head injuries. Bicycle helmet use has been shown to be the most important strategy for the prevention of bicycle related head injuries (7).

Pedestrians

Pedestrians constituted 12 % of road fatalities in 1995 and road crossing behaviour was associated with 62% of all pedestrian fatalities. Speed of the vehicle at impact is the single most important determinant of severity of pedestrian injuries (8). The higher the speed at impact the higher the proportion of fatalities and victims with severe injuries. Measures to limit speeding ("traffic calming") and proper use of pedestrian facilities such as overhead bridges and traffic lights help prevent pedestrian fatalities and injuries. (9)

Vehicle Occupants

This group constituted 15% of road fatalities in 1995 (3). Air bags, occupant restraints such as seat belts and child restraints, and side impact protection systems have all been shown to be prevent injuries to occupants in the event of a crash (10).

Other risk factors

Alcohol use and excessive speeding are examples of other risk factors which need to be studied further in Malaysia. Speeding is particularly risky since the energy which dissipates in the event of a crash and causes harm is directly proportional to the square of the velocity of the vehicle ($E=mc^2$).

Future Scenario

With rapid economic growth in Malaysia, the number of new vehicles on the road (especially motorcycles and cars), new roads and highways can be expected to increase. A mathematical model has been developed to forecast the number of road traffic deaths and crashes in Malaysia. This computer generated model is a log linear model and was developed at the Road Safety Research Centre, Universiti Pertanian Malaysia based on the trends for the last two decades. The equations for predicting the number of road crashes and deaths for a given year are as follows:

Number of Road deaths
$= 2289(e^{0.0007 \text{Vehicle} \times \text{Population} \times \text{Road}}) (e^{0.2073 \text{ Data system}})$

Number of Road crashes
$= 43478 (e^{0.00011 \text{Vehicle} \times \text{Population} \times \text{Road}}) (e^{0.2447 \text{ Data system}})$

Data system factor is 1 for Peninsular Malaysia and 2 for East Malaysia

Vehicle means the estimated total number of vehicles in the year expressed in millions

Population means the estimated total number of people expressed in millions

Road means the estimated length total of roads expressed in thousands of kilometres

The parameters for the year 2000 when compared to the year 1994, assuming present trends and measures, are given in the Table I.

The Cabinet Committee on Road Safety with the secretariat in the Ministry of Transport has set a target of reduction in the road toll by 30% by the year 2000. The Road Safety Council is a registered society consisting of all relevant government and non governmental organisations. The Ministry of Health has set up an Injury Control Unit in the division of Non Communicable Diseases and is in the process of improving and expanding its emergency services so that injuries are minimised and prevented in the post crash

phase. The Ministry has planned Injury Prevention programs for implementation in health centres. It is also planning to carry out a media campaign on "Injury Prevention" under its Healthy Lifestyle Program. Efforts at preventing road crashes and injuries must be further enhanced to prevent unnecessary loss of lives and disability. Increased funding for education of public and target groups, research, and training of professionals needs to be allocated. The health sector needs to play a bigger role in road safety through research and education of target groups. The universities and nongovernmental organisations need to share a vision with the government to work towards a Safe and Healthy society as we march towards 2020. We need to act now before more productive members of society are killed or crippled.

Table 1. Predicted Road Transportation and Safety Indicators for the year 2000.
Compared to 1994 *

Parameter	Year 2000	Year 1994
Population (millions)	23	19
Vehicle number (millions)	10	6
Road length (1000 km)	72	60
Number of road crashes	352,342	148,801
Number of road deaths	9127	5159
Incidence of road deaths/ 100,000 population	39	26

* Based on current trends and measures

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CONGENITAL GLUCOSE-GALACTOSE MALABSORPTION - A CASE REPORT

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ABSTRACT: We report a case of congenital glucose-galactose malabsorption (GGM) in a Malay male infant who presented with protracted diarrhoea, severe metabolic acidosis, and marked hypernatraemic dehydration since the third day of life whilst on breast feeding. He was severely marasmic, requiring total parenteral nutrition to improve the precarious nutritional status. The course of illness was complicated by Staphylococcal septicaemia and catheter related endocarditis which was eradicated by a six-week course of vancomycin and fusidic acid. Subsequent carbohydrate tolerance tests showed impaired absorption of oral glucose but normal absorption of fructose. The child tolerated fructose-based modular feed well and had normal development. At fourteen months of age he still had failure to thrive despite an adequate calorie intake and normal appetite. *(JUMMEC 1997 2(1): 43-45)*

KEYWORDS: Chronic diarrhoea, glucose-galactose malabsorption, congenital.

Introduction

Glucose and galactose malabsorption (GGM) is a rare congenital disease resulting from a selective defect in the intestinal glucose and galactose/Na^+ co-transport system. It was first described in 1962 by Lindquist in Sweden and Laplane in France respectively (1),(2). Since then this inherited form of carbohydrate malabsorption has been described in various communities. Quak *et al.* from Singapore described this condition in a Chinese family with two affected male children (3). We describe a Malay boy with this condition, from the Department of Paediatrics, University of Malaya Medical Centre, Kuala Lumpur. We believe that this is the first case reported in this country. It is a rare but an important cause of chronic diarrhoea in early infancy. Early diagnosis and appropriate dietary manipulation can prevent considerable morbidity and even mortality.

Case Report

MZ was born after a full term, normal pregnancy. His birth weight was 2.76 kg. He first developed watery diarrhoea on the third day of life whilst on breast feeding. Breast feeding was discontinued as advised by a general practitioner and an infant formula was started. However, diarrhoea persisted. He was admitted to a local hospital on the seventeenth day of life with severe metabolic acidosis (pH 7.07, bicarbonate 7.4 mmol/L, base excess -21 mmol/L); hypernatraemic dehydration (blood urea 28.5 mmol/L, serum sodium 183 mmol/L, potassium 4.5 mmol/L) and anaemia. He was also severely marasmic, with a body weight of 2.2 kg. He received intravenous antibiotics, blood transfusions, and two weeks of total parenteral nutrition. His weight improved to 2.55 kg. His diarrhoea stopped while he was kept nil orally but recurred when oral feeding was reintroduced. He was referred to this hospital at 51 days of life.

His parents were first cousins. Two of his male siblings, developed chronic diarrhoea since the third day of life and died two weeks later at a local hospital. No definite diagnosis was made. A female sibling died at five years of age, due to meningitis. He had four other living siblings, three girls and one boy, all of whom were healthy

Physical examination revealed a patient who had generalised wasting with loss of subcutaneous tissue. Apart from perianal excoriation, examination of the systems did not reveal any abnormality. The results of routine blood investigations, which included full blood count, urea and electrolytes were normal. Parasites were not detected on microscopic examination of fresh stools specimens on several occasions. Stool cultures were repeatedly negative for enteropathogens and no rotavirus and adenovirus were detected. Because of severe malnutrition, he was given total parenteral nutrition for two weeks. During this period when he was

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not fed enterally there was complete cessation of diarrhoea. Diarrhoea recurred when oral rehydration solution which contained glucose was given. Based on these observations a presumptive diagnosis of congenital GGM was made.

To confirm the diagnosis of congenital GGM, glucose and fructose tolerance tests were performed. During the tests, the child was first given 1g/kg body weight of glucose solution (oral rehydration solution, Oralite® Poly Laboratories). Baseline and serial blood glucose levels were measured after the glucose was given. The child was observed for diarrhoea and clinical signs of dehydration. The stool output was charted, and the weights of the nappies before and after use were recorded. Watery stool specimens were sent for sugar chromatography. Fructose challenge was commenced 48 hours after the cessation of watery stools observed during the glucose tolerance test. During glucose challenge serial blood glucose levels were: 6.2 mmol/L at baseline; 6.3 mmol/L at 30 minutes, 6.3 mmol/L at 60 minutes, 6.1 mmol/L at 90 minutes and 6.7 mmol/L at 120 minutes after glucose loading showing no significant increase in the serial blood glucose levels after the oral glucose challenge. Serial blood glucose measurements during challenge with fructose showed a baseline of 6.1 mmol/L, 6.2 mmol/L at 30 minutes, 6.0 mmol/L at 60 minutes, 6.9 mmol/L at 90 minutes and 7.5 mmol/L at 120 minutes. There was significant rise in the blood glucose after the oral fructose load. Two episodes of watery stools were noted during glucose challenge and none during the fructose challenge. Sugar chromatography showed the presence of glucose on both specimens collected during the challenge with glucose.

He became febrile after two weeks of parenteral nutrition, and methicillin-resistant *Staphylococcus aureus*, was isolated from peripheral blood culture. Evidence of endocarditis with vegetation formation in the right ventricle was noted on echocardiographic examination of the heart. The central venous catheter was removed and the child was given intravenous vancomycin and fusidic acid. Methicillin resistant *Staphylococcus aureus* was isolated from the tip of the central venous catheter. His condition improved thereafter. He was then started on a modular feed, with fructose, whey protein (ProMod®, Abbott), corn oil, multivitamins and multiminerals (Vidaylin®, Abbott). Potassium, calcium and zinc supplements as well as iodised salt were added. He tolerated this feed very well with no further diarrhoea and started to gain weight. The cardiac vegetation resolved after six weeks of intravenous antibiotics. He was readmitted to the hospital at six month of age for introduction of solids under observation. He was able to tolerate a variety of weaning foods. Various weaning foods were introduced and the family was under the advice of an experienced paediatric dietitian. The child was to avoid normal infant formula and drinks which contained glucose.

At 14 month of age he was asymptomatic. Despite a voracious appetite and an adequate calorie intake, his weight gain remained slow and all his growth parameters were still well below the third centile. He was growing just below the third centile. His development was within normal range.

Discussion

Carbohydrate intolerance is a common cause of persistent diarrhoea following acute gastroenteritis. Commonly it is due to secondary lactose intolerance (4), although monosaccharide intolerance has also been reported (5). Such monosaccharide intolerance is often transient. Congenital glucose and galactose malabsorption (GGM) is a rare disorder resulting from a selective defect in the intestinal glucose and galactose/Na+ co-transport system. It causes long-lasting intolerance to glucose and galactose but not to fructose. It was first reported in Sweden and France in 1962 (1) (2), and has been noted to be an uncommon cause of chronic diarrhoea among children (6) (7).

In GGM, diarrhoea develops as a consequence of selective malabsorption of glucose and galactose. The defect is situated in the glucose/Na+ co-transport system in the brush border membrane of the small intestine (8). Disaccharides such as lactose, sucrose, and maltose are hydrolysed normally, but the absorption of glucose and galactose is absent or markedly reduced (9). This results in secretion of water and electrolytes into the intestinal lumen, thus causing diarrhoea. Characteristically, diarrhoea develops within four days of birth (10). This is rapidly followed by marked dehydration which is often hypernatraemic with metabolic acidosis and pronounced physical wasting (11). All these clinical features were present in our patient. Renal glycosuria, hypochromic anaemia and moderate steatorrhoea has also been described in this condition (10).

The inheritance of this condition was thought to be autosomal recessive in nature (10). The fact that the parents of our patient are first cousins supports this mode of inheritance. There were two elder siblings in the family who died of chronic diarrhoea with onset on the third day of life. It was likely that both of them also had congenital GGM and succumbed to the condition.

The diagnosis of this condition depends on the demonstration of the failure of absorption of glucose and galactose. This can be easily identified by the hydrogen breath test which was not available in our institution. Other methods such as measurement of serial blood sugars after administration of oral glucose and fructose loads are acceptable alternatives (11). In this patient, we have demonstrated a flat oral glucose tolerance test with normal absorption of fructose. Treatment is simple and consists of immediate rehydration and provision of a glucose- and

galactose-free diet. Oral rehydration solutions that contain glucose must be avoided. Commercially prepared glucose- and galactose-free formula (Galactomine19®, Nutricia) is costly and is not available in Malaysia. We had devised an alternative special formula by providing the major components of the nutritional requirement of the child separately. Fructose was the carbohydrate while whey protein (ProMod®, Abbott) was the main protein. Cornoil, potassium, calcium, zinc, multivitamins and multiminerals (Vidalyn®, Abbott) and iodised salt were added.

In most children, growth and development were normal once glucose and galactose were removed from the diet (11). However, in this case, despite an adequate calorie intake, the physical growth remained retarded although the development was within normal limits. This experience was similarly shared by Quak *et al.* from Singapore (3). The reason for this is not clear. In older children and adults, tolerance to the offending carbohydrates improves (11), although malabsorption of glucose and galactose in the small intestine remains unchanged.

Chronic diarrhoea among Malaysian children is commonly secondary to post-enteritis syndrome in which cow's milk protein and soy protein played an important role in prolonging the duration of diarrhoea (12), (13). Acquired carbohydrate intolerance is also important (14). However, chronic diarrhoea with onset during the first weeks of life is unlikely to be secondary to cow's milk or soy protein-sensitive enteropathy or acquired carbohydrate intolerance (15). This child developed chronic diarrhoea during the first few days of life while on breast feeding. He had two elder siblings who died under very similar situations. All this points to the possibility of a congenital malabsorptive disorder. The clinical features of severe metabolic acidosis, hypernatraemic dehydration, watery, acidic stools which contain the offending carbohydrate support the diagnosis of congenital GGM. It is of paramount importance for clinicians and other caregivers to realise that diarrhoea with onset within the first week of life especially in a breast fed infants is not due to acquired food protein or carbohydrate intolerance. Early recognition and referral should be made in order to prevent further deterioration of the nutritional status and to improve the outcome of the patient

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PROFESSOR A. RAMAN

It is with deep regret that we announce the death of Professor A. Raman who passed away suddenly on 2 February 1998 at the age of 61. Prof. Raman was born in India on July 1, 1936 and came to settle in Malaysia with his parents and an only brother in 1948. He enrolled in the Kajang High School and later the Victoria Institution, Kuala Lumpur before commencing his medical studies at the University of Malaya in Singapore in 1954. Upon graduation in 1960, he served as a medical officer in Kulim General Hospital in Kedah. His keen interest in Physiology led him to relinquish his clinical career in 1963 to join the pioneering team of lecturers selected by the founder dean of the Medical Faculty, University of Malaya, Prof. T.J. Danaraj. After intensive training in teaching and research in Physiology under the tutelage of Professor Bell at the University of Dundee in Scotland, he returned to take up his position as lecturer in the Physiology Department in 1965. Prof. Raman's initial research interest was in calcium metabolism which became the topic of his dissertations for his Doctor of Medicine and subsequently his Doctor of Philosophy degrees which he completed in 1969 and 1974 respectively. He was appointed Professor and Head of the Physiology Department at the young age of 35, a post he held until his untimely death. Prof. Raman was a firm believer in the physiological basis of medical practice. Under his leadership, the department conducted undergraduate and post-graduate courses to medical, dental and paramedical students. He rose to the challenge of teaching physiology in the National Language by spearheading the authorship of a series of Physiology texts in Bahasa Malaysia which was invaluable to the students and teachers during those difficult transition years. Much of the Bahasa Malaysia vocabulary used in physiology was inspired by him and his coauthors. He collaborated with his academic staff in many areas of research which resulted in numerous publications. His kindness and consideration to all members of his department inspired much respect and affection. His dedication and devotion to duty also led the university to extend his service beyond the official retirement age. He was on his second 3-year post-retirement contract when he died. Prof. Raman remained a bachelor and leaves behind a married brother. His students and colleagues will mourn the loss of a dear friend and teacher for a long time.

JUMMEC 1997 2(1): 49-50

HEALTH RESEARCH DEVELOPMENT UNIT (HRDU)

Dear Editor,

The Health Research Development Unit (HRDU) was established at the end of 1996 with the objective of promoting Health Research. This new unit will be under the Dean's office having one of the deputy deans (Research and Development) as head. In one swoop the unit undertakes to maintain the work of the Department of Social Obstetrics from whom the core professional staff is derived and create a new focus for health research in the country. This is an important development to co-ordinate research in this area to meet the challenges that the current stage of development in the health sector. As we know, the health sector is an expanding industry with the availability of modern technology, rise in standard of living, longevity of life and other problems that can be linked to change of lifestyle.

The Faculty of Medicine and the University Hospital has an abundance of facilities and resources in terms of highly qualified and experienced specialists to contribute to aspects of health mentioned earlier and their energies can be better co-ordinated and utilised to the maximum. HRDU will focus on applied health-related research, health policy analysis, development of medical education and health professional training. It will draw upon activities conducted by various individual researchers and research groups within the Faculty of Medicine, in University of Malaya and other institutions as well as those generated by the unit itself. It will work closely with Faculty groups already active in related areas such as those in the Departments of Social and Preventive Medicine, Primary Care Medicine etc. In focusing on health-related research and policy issues, the HRDU will give attention to the conduct and utilisation of research efforts that are multi-disciplinary and inter-disciplinary in scope recognising the importance of sociological, economic and demographic dimensions related to health and health care.

Areas of activities include:

1. conducting health systems and health policy research, monitoring health outcomes, analysing health impact of socio-economic and demographic changes and utilising research findings to enable the faculty to assist the government and non-government organisations in formulating policies and programmes related to health;
2. contributing to medical education and health professional training and development - under and post-graduate medical curriculum, especially in preventive and promotional health, and providing input into health issues to other related academic programmes, such as demography and medical sociology;
3. carrying out health-related consultancies locally and regionally;
4. developing and monitoring collaborative efforts in health prevention projects or strategies, notably community-based interventions;
5. providing short-term training and in-service courses to professional and para-medical professionals in selected areas of the Medical Centre's expertise.

The research agenda could best be shown diagrammatically as below -

Teaching commitments to the undergraduate will continue:

• Health Systems	Stage 4 (S) KLDP Posting
• Human Sexuality	Stage 4 (S) O & G Posting
• Social Aspects of pregnancy	Stage 4 (J) O & G Posting
• Behavioural Sciences	Stage 2 Core Curriculum

The core academic staff can contribute to post-graduate teaching and staff development in areas such as reproductive health, political economy, research methodologies in survey research, health systems research, qualitative and ethnographic techniques etc.

Plans are in place to set up a consultancy service in light of the corporatisation of the university and the need to generate income. Collaborative networks with various academic, government and NGOs as well as relevant international bodies will be established and current links will be strengthened. These include linkages with WHO, UNFPA, UNEP and APACPH.

Since its inception HRDU core staff are avidly involved in several IRPA funded projects such as Health Problems of Foreign Workers, Case Mix Study. The visiting professor to the unit, Professor M Miller has been able to impart his experience and expertise to the development of the latter. Along with staff from the Social and Preventive Medicine, Medicine, Faculty of Science, IMR a course in Health Research attended by UM staff as well as some from outside the university. Three of the modules have been done:

- Principles of Scientific Inquiry and Research Design in Health Research
- Basic Measurement and Statistical Methods
- Grant Proposals and Scientific Writing,

Others to follow will include:

- Data Management and Analysis
- Clinical Trials and clinical Epidemiology

Programme Evaluation and Outcomes Research

It is stressed that this unit derives its strength from collaboration with others and we will continue to strive for more research output from the faculty coming out of our combined effort.

Amir S Khir
Deputy Dean
Research & Development
Head, HRDU

LEARNING VITREO-RETINAL SURGERY IN NORWICH ENGLAND

Dear Editor,

Vitreo-retinal surgery, an important but relatively less known speciality of ophthalmology in Malaysia, is utilised in the management of complicated retinal detachments, advanced diabetic eye disease, prolonged vitreous haemorrhage and in the removal of some intra-ocular foreign bodies.

Management of these conditions is a challenge and requires special training and expertise. As the University Hospital Kuala Lumpur is a major referral centre for difficult cases we get several cases requiring vitreo-retinal surgery every month. However with the exodus of ophthalmologists to the private sector we are seriously lacking in vitreo-retinal surgeons. The equipment is available but not the expertise.

I made arrangements to spend two months in July and August 1996 at the West Norwich Hospital, Norwich, UK. under the supervision of Dr. Ted Burton, an experienced vitreo-retinal surgeon. The ophthalmology department at the hospital is large and well-equipped, serving the major city of Norwich and the surrounding towns in Norfolk. During my time there, I planned to accompany Dr. Burton on his ward rounds, clinics, operating sessions and teaching sessions to learn his skills, assisting in operations. Prior to operating alone.

I picked this particular hospital having worked there as a junior doctor, knew the consultants reasonably well, and since it was also a large referral centre (similar to UHKL)

There were six consultants, two staff grade doctors, a senior registrar, registrar and three senior house officers in Norwich. In-patients stay at Nelson Ward whilst outpatients are seen in a well-equipped eye clinic. Many operations including cataract surgery are done as day cases. Dr. Burton's sub-speciality is vitreo-retinal surgery and he is referred retinal cases not only from Norwich but also from surrounding towns like Great Yarmouth, Lowestoft, Cromer and Sheringham. In the previous six months he had done 100 vitreo-retinal cases i.e. an average of four cases per week. During my attachment I assisted at about thirty operations and performed a couple of operations myself. I was pleased to find that a dedicated team of nurses and operating theatre staff ensured that all procedures went smoothly and with little disruption.

The spectrum of vitreo-retinal problems I was exposed to included all forms of retinal detachments (single or inferior hole; multiple or superior holes; aphakic or pseudophakic; detachments associated with cataracts, complicating dialysis and vitreous haemorrhage; macular holes, macular pucker and cellophane maculopathy)

All in all I had an interesting and useful two months in Norwich learning the techniques of vitreo-retinal surgery employed by Dr. Burton and picked up useful hints from him. I also discovered that unless done by a skilled surgeon with good experience the complication rate is high, with poor visual outcome. After all we are operating in a little globe no more than an inch across, using micro-surgical instruments and an operating microscope to assist us. Dr. Burton did suggest that I would benefit more by spending a full year so that I could do more surgery under his supervision and also attend courses in other centres in the UK.

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MICROCALCIFICATION CLUSTERING PARAMETERS IN BREAST DISEASE: A MORPHOMETRIC ANALYSIS OF RADIOGRAPHS OF EXCISION SPECIMENS

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X-ray microradiography of surgically excised breast specimens offers the possibility of morphological characterization of calcifications. When combined with digital imaging techniques there exists added potential for obtaining valuable basic quantitative morphometric information regarding differences between microcalcifications in tissues exhibiting evidence of fibrocystic change, benign and malignant tumours. A total of 157 excised breast specimens from 84 patients were microradiographed using a Softex Super Soft X-ray unit and Kodak AA high resolution industrial film. A Quantimet 57°C image analysis system was used to digitize and analyse the microradiographs. Of the 157 microradioeraphs, 51 (from 30 patients) revealed microcalcification clusters. The existence of significant differences between the three identified categories of tissue were indicated by clustering parameters. These included the number of particles per cluster, area of clusters, maximum distance to nearest neighbour, and geometric mean distance to nearest neighbour. The distribution pattern index (DPI), another of the clustering parameters used in this study, has been observed to be a particularly powerful discriminator. The value for fibrocystic change was found to be significantly smaller (0.514) than that for benign tumour (0.796) whilst that for benign tumour was observed to be significantly larger than that for malignant tumour (0.604) at a p-value of less than 0.05 (Kruskal Wallis one-way analysis of variance).

(The British Journal of Radiology 1996; 69, 326-334)

IMMUNOHISTOCHEMICAL EXPRESSION OF P53 PROTEINS IN WILMS' TUMOUR: A POSSIBLE ASSOCIATION WITH THE HISTOLOGICAL PROGNOSTIC PARAMETER OF ANAPLASIA

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Wilms' tumour (nephroblastoma) has been associated with chromosomal abnormalities at the 11p13, 11p15 and 16q regions. A study into the possibility of mutations occurring within p53, the ubiquitous adult tumour suppressor gene, in Wilms' tumour was carried out. Thirty-eight cases were studied. Of these 36 were categorised into the favourable histology group and two into the unfavourable histology group based on the National Wilms' Tumour Study criteria. Archival formalin-fixed, paraffin-embedded tissue sections from each case were stained with a polyclonal (AB565:Chemicon) and a monoclonal (DO7:Dako) antibody raised against p53 protein using a peroxidase-labelled streptavidin biotin kit (Dako). 'Cure' (disease-free survival of 60 months or longer) was documented in 39% of cases with favourable histology tumours. Eleven percent in this group succumbed to the disease. Both cases with unfavourable histology died. Four out of 36 (11%) tumours with favourable histology demonstrated weak to moderate staining with both AB565 and DO7 in more than 75% of tumour cells. In contrast, p53 protein expression in unfavourable histology tumours was significantly increased compared with the favourable histology group (P=0.021) with both cases demonstrating in immunopositivity in > 75% of tumour cells when stained with AB565 and DO7. The intensity of staining ranged from moderate to strong in both cases. It appears from this preliminary study that the immunohistochemical expression of p53 protein in Wilms' tumour, presumably a result of mutation in the p53 tumour suppressor gene, correlates with histological classification, histological categorisation being one of the useful features in the prognostic assessment of Wilms' tumours.

(Histopathology 1996; 28, 49-54)

IMPLICATIONS OF p53 PROTEIN EXPRESSION IN CLEAR CELL SARCOMA OF THE KIDNEY

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Eight histologically-confirmed cases of clear cell sarcoma of the kidney (CCSK) were studied for possible mutations in the p53 tumor suppressor gene by the immunohistochemical demonstration of mutant p53 proteins using a monoclonal (DO7: Dako) and a polyclonal (AB565: Chemicon) antibody to p53 protein. All cases exhibited p53 protein nuclear immunopositivity, although in varying numbers of tumor cells and with different staining intensities. p53 protein (DO7 or AB565) was expressed in < 25% of the tumor cells in four (50%) of the cases, including the one case with a known long term survival of 13 years from the time of diagnosis. The other tumors showed p53 protein immunopositivity in > 25% of the tumor cells when stained with either DO7 or AB565 or both. The intensity of staining, graded on visual impression into weak, moderate or strong, did not correlate well with the ratio of positive staining tumor cells. While this study is unable to clarify the relative prevalence and importance of p53 mutational events in the pathogenesis of this aggressive renal tumor of childhood, it is reasonably suggestive that alterations in the p53 tumor suppressor gene do occur in CCSK.

(Pathology 1996; 28, 311-315)

ELEVATED TRACE ELEMENT CONCENTRATIONS IN MALIGNANT BREAST TISSUES

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In vitro neutron activation analysis (NAA) was performed on malignant and adjacent normal tissue from 46 human female breast tumours. The objective was to investigate the chemical environment of the tissues within which microcalcifications develop and to develop a method for discrimination between malignant and normal breast tissue. The elements Al, Br, Ca, Cl, Co, Cs, Fe, K, Mn, Na, Rb and Zn were significantly higher in the cancer tissues (all $p<0.001$; except for Co, $p<0.003$, Wilcoxon signed-ranks test). In addition, a significant correlation (0.80, Spearman rank correlation) was found for Rb and Zn in tumour tissues. Present results are supported by the findings of others. The relevance of elevated concentrations of these elements in cancer breast tissue remains a matter of conjecture. Evidence suggests that there is a connection both with increased cellular activity and blood supply and the formation of microcalcifications in malignant breast tissues. This study suggests an association between the elemental composition of breast tissues and the formation of breast particles. That is, elevations of elemental concentration and clustered calcifications in breast are possibly related.

(The British Journal of Radiology, 1997; 70, 375-382)

THE ELEMENTAL COMPOSITION OF BREAST TISSUE: CAN THIS BE RELATED TO BREAST PARTICLE DEPOSITION ?

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In vitro instrumental neutron activation analysis (INAA) has been performed, yielding a range of minor and trace elemental concentrations for 46 paired samples of surgically-excised cancer breast' tissue. In addition results of the soft X-ray microradiography of excised breast specimens have been used to quantify breast-particle clustering parameters, including area of cluster and nearest neighbour distance. Significant differences between elemental composition in histologically normal tissues and tissues exhibiting malignant lesions are supported by the observation of significant differences in particle clustering parameters. These observations have led to the investigation of the possible asso-

ciation between elemental composition of diseased breast tissues and the formation of breast particles. In particular a scanning electron microscope (SEM) with an energy dispersive X-ray (EDX) facility has been used to locate breast particles and conduct microanalysis of the particle constituency. Results indicate these particles to consist of a complex of Ca and other elements including Mg, P, S and the ionic salts Na, Cl and K.

(*Journal of Radioanalytical and Nuclear Chemistry 1997; 217, 2 193-199*)

PROBLEMS IN THE HISTOLOGICAL ASSESSMENT OF HYDATIDIFORM MOLES: A STUDY ON CONSENSUS DIAGNOSIS AND PLOIDY STATUS BY FLUORESCENT *IN SITU* HYBRIDISATION

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Hydropic villi in products of conception continue to pose a diagnostic problem for the anatomical pathologist. It is important to distinguish between complete hydatidiform mole (CM), partial hydatidiform mole (PM) and hydropic degeneration (HD), as hydatidiform moles (especially CM) have a tendency to develop persistent trophoblastic disease. Several studies have demonstrated interobserver variability in the diagnosis of the three conditions, but there have been no studies testing the accuracy of the consensus diagnosis of pathologists experienced in the field. In this study four anatomical pathologists with experience in diagnosing hydatidiform moles selected five cases of HD, seven cases of PM and ten cases of CM on the basis of consensus diagnosis using established criteria. Ploidy studies were done on these 22 cases using fluorescent *in situ* hybridisation. The 15 cases of HD and CM were diploid, confirming the histological diagnosis. However only five of the seven cases of PM were triploid, the other two being diploid. Review of these two diploid cases showed a mixture of small and large villi with moderate to marked trophoblastic proliferation. On the basis of the significant trophoblastic proliferation and the DNA information, the two cases were reclassified as early complete moles. This study demonstrates that even pathologists experienced in the field have difficulty separating PM from CM. The findings suggest that, in the absence of DNA information, a lesion with hydropic villi showing moderate to marked trophoblastic proliferation should be classified as a complete mole, even if there is a mixture of small and large villi. Ploidy studies are an important adjunct to histological diagnosis, especially when there is an overlap of features.

(*Pathology 1996; 28, 311-315*)

EPSTEIN-BARR VIRUS (EBV) AND HODGKIN'S DISEASE IN A MULTI-ETHNIC POPULATION IN MALAYSIA

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The Epstein-Barr virus (EBV) has been implicated as a contributing factor in the development of Hodgkin's disease. Western cases of Hodgkin's disease have shown the presence of EBV in Hodgkin and Reed-Sternberg cells in approximately 50%. We studied a total of 100 consecutive cases of Hodgkin's disease from Malaysia, with the aim to elucidate its association with EBV in a multi-ethnic Asian population. Of 34 patients (34%) less than 1 5 years of age (childhood), 2 5 had classical Hodgkin's disease (eight nodular sclerosis, 16 mixed cellularity, one lymphocyte depleted) and nine had lymphocyte predominance Hodgkin's disease. Of the 66 from patients aged 1 5 years and above, 33 had nodular sclerosis, 24 mixed cellularity, two lymphocyte depleted, one unclassifiable and six lymphocyte predominance Hodgkin's disease. The ethnic distribution of classical Hodgkin's disease was: Malay 23, Chinese 32 and Indian 30 (Malay:Chinese:Indian = 1:1.4:1.3), and the ethnic distribution in the 15 cases of lymphocyte predominance Hodgkin's disease was: Malay four, Chinese 10 and Indian one. Taking into account the ethnic distribution of the general population and of hospital admissions, there appears to be a significant predilection of classical Hodgkin's disease cases in eth-

nic Indian compared to non-Indian patients (Chi squared test, 0.025 > P > 0.01). Eighty-one cases were tested for the presence of EBV by *in situ* hybridization for EBV encoded RNA, and 57 cases by immunostaining for EBV latent membrane protein 1. In the younger age group, all except one of the 15 cases (nine mixed cellularity, six nodular sclerosis) showed the presence of EBV (93%). In the older age group, EBV was detected (52%) in the following proportion: 6/27 nodular sclerosis, 19/22 mixed cellularity, 1/2 lymphocyte depleted, 1/1 unclassifiable. None of the 14 cases of lymphocyte predominance Hodgkin's disease showed the presence of EBV in the Hodgkin and Reed-Sternberg cells. The findings suggest a strong association of EBV with Hodgkin's disease in Malaysians (41/67, 61%), in particular childhood cases (93%). In adults, the association with EBV is significantly higher in the mixed cellularity subtype (86%) compared with the nodular sclerosis subtype (22%).

Histopathology 1997; 30, 227-233)

HISTOMORPHOLOGICAL PATTERNS OF RENAL AMYLOIDOSIS: A CORRELATION BETWEEN HISTOLOGY AND CHEMICAL TYPE OF AMYLOIDOSIS

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A retrospective study was conducted to investigate whether there was a correlation between the histological pattern of renal amyloidosis, the chemical type of amyloid protein involved and the clinical presentation. Eighteen consecutive cases of systemic amyloidosis that had renal biopsies processed and examined histopathologicaly at the Department of Pathology, Faculty of Medicine, University of Malaya, Kuala Lumpur were reviewed. The age range of patients was 25 to 64 yrs (mean, 46 yrs). The male: female ratio was 2.6:1. Three patients were Malay, 9 Chinese, 3 Indian, 1 Indonesian, 1 Iban, and 1 Bisaya. According to the predominant site of amyloid deposition, 14 cases showed a glomerular pattern and 4 a vascular pattern. 8 cases were designated as 2 anti-human amyloid-A (AA) amyloidosis on the basis of permanganate-sensitivity and immunoreactivity of deposits with anti-human AA protein antibody. Ten cases contained deposits that were permanganate-resistant and nonimmunoreactive for AA protein and were designated as AL in type. The histomorphologic pattern of renal amyloidosis did not provide a reliable means of differentiating AA from AL amyloidosis. The glomerular pattern tended to present with renal manifestations such as nephrotic syndrome and chronic renal failure, whereas the vascular pattern tended to present with nonrenal manifestations such as diarrhoea. These findings may have a bearing on the pathophysiology of amyloidosis and provide clues to appropriate management.

(Hum Pathol 1997; 28:847-849.)

HEADACHE AND SYSTEMIC LUPUS ERYTHEMATOSUS: IS THERE AN ENTITY OF "LUPUS HEADACHE" ?

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Headaches especially migraine has long been thought to be more prevalent in systemic lupus erythematosus (SLE) and are believed to be part of the SLE disease process. We sought to evaluate this by studying prospectively 50 SLE inpatients without known secondary causes for headache, using a standard questionnaire on headache. We used the IHS criteria for the classification of headaches. Our patients were compared with two sets of controls - 50 non-SLE hospital inpatients with no known disease that can cause headache and 208 normal community based controls. We found that the prevalence of headache in SLE patients did not differ significantly from the controls (78% versus 82% (hospital controls) and 79.3% (community controls)). There was no significant difference in the prevalence of the various headache subtypes i.e. migraine, tensions headache and other headaches among the three study groups. There was a significantly greater number of manifestations of stress in SLE patients with headache compared to those without headache. Relationship of headache to illness onset

did not differ significantly between SLE patients and hospital controls. This study does not support the entity 'lupus headache'. Headache in SLE patients appears more likely due to stress.

(Neurol J Southeast Asia 1997; 2: 51-6)

SYSTEMIC LUPUS ERYTHEMATOSUS IN MALAYSIA: A STUDY OF 539 PATIENTS AND COMPARISON OF PREVALENCE AND DISEASE EXPRESSION IN DIFFERENT RACIAL AND GENDER GROUPS

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The aim of this study were to examine the clinical and laboratory features of Malaysian patients with systemic lupus erythematosus (SLE) and to identify any difference in disease expression between the different genders and among the three major ethnic groups of Malaysia. Retrospective analysis of all patients with SLE admitted to and followed-up at University Hospital Kuala Lumpur from 1974-90 was undertaken. Ethnic Chinese had the highest prevalence of SLE compared to other ethnic groups. There was a high incidence of renal disease, 74% of patients had significantly less incidence of skin manifestation compared to other racial groups. No difference in disease expression was detected between the ethnic Chinese and Indians and between the male and female patients. The overall 5y and 10y survival rates were 82% and 70% respectively. Indian patients had the poorest survival rates. Survival rates are similar among Chinese and Malay patients. Our findings are in broad agreement with those previously reported.

(Lupus 1997; 6: 248-53)

PERIPHERAL NEUROPATHY IN SYSTEMIC LUPUS ERYTHEMATOSUS - ELECTROPHYSIOLOGICAL FEATURES IN CONSECUTIVE PATIENTS

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50 consecutive inpatients with systemic lupus erythematosus (SLE) were studied using nerve conduction studies and electromyography to determine the prevalence and pattern of peripheral neuropathy. The patients had no other known cause of peripheral neuropathy except SLE. 28% had clinical signs of peripheral neuropathy. The frequency of abnormal electrophysiological findings was 56%. The frequency of polyneuropathy (defined as abnormality in 2 or more nerves) was 42% of which two thirds had diffuse polyneuropathy and one third had multiple mononeuropathy. The most common abnormal electrophysiological parameter was a prolonged H reflex followed by reduced amplitude of compound muscle action potentials. Overall electrophysiological features suggest axonal degeneration rather than demyelination. Subclinical peripheral neuropathy is common in systemic lupus erythematosus.

(Neurol J Southeast Asia 1996; 1: 47-51)

SEIZURES IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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This was a study on 58 neuropsychiatric systemic lupus erythematosus (SLE) patients with epileptic seizures treated in University of Malaya Medical Centre, Kuala Lumpur from 1975 to 1994. The aim of this study was to better characterize seizures in SLE so as to formulate policy on management. Over the same period of time, there were 7 other SLE patients with seizures from secondary causes such as mental disturbances and central nervous system (CNS) infections. Thus, seizures in SLE is more frequently due to neurological involvement of the disease itself (ratio = 8.3: 1). In 93% of patients, the seizures occurred in the setting of active SLE. Following the convulsion, 69% of the patients took more than 24 hours to regain full consciousness. Non-convulsive status may be an important cause as 4 out of 6 EEGs done when the patients were still stuporous was supportive of the diagnosis. 29.3% of patients developed neuropsychiatric manifestations during or immediately following the seizures. Status epilepticus was common (21%). The mortality in patients with status epilepticus was 25%. Seizures may have a deleterious effect on the brain with active lupus, and contribute to the high morbidity and mortality. Thus, seizures in neuropsychiatric SLE should be treated urgently and aggressively. After a mean follow-up of 3.6 years, two-thirds of the patients who survived the acute illness were seizure-free by 2 months. Most remained seizure-free when anticonvulsants were withdrawn. A third continued to have recurrent seizure requiring long-term anticonvulsant therapy. Therefore, long-term maintenance anticonvulsants are not indicated in the majority of patients.

(Neurol J Southeast Asia 1997; 2: 51-6)

TRANSVERSE MYELITIS IN ASSOCIATION WITH DENGUE INFECTION

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A 14 year old girl presented with an acute illness of fever and paraplegia with spinal sensory level at T5-T6. Investigations showed thrombocytopenia, raised serum IgM to dengue virus and normal spinal MRI. This is the first reported case of transverse myelitis associated with dengue fever. The pathophysiological mechanism is postulated to be direct viral invasion of the spinal cord or autoimmune demyelination.

(Neurol J Southeast Asia 1996; 1: 61-3)

PERIPHERAL NERVE INVOLVEMENT IN IMMUNOCOMPETENT HOSTS WITH CRYPTOCOCCAL MENINGITIS: A CLINICAL AND ELECTROPHYSIOLOGICAL STUDY

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This is a prospective clinical and electrophysiological study on 14 consecutive immunocompetent patients with cryptococcal meningitis over a two year period. Clinically, 36% of the patients had hyporeflexia with or without weakness suggestive of neuropathy. All but one patient (93%) had abnormalities of the electrophysiological test of at least one nerve. 64% of the patients had abnormality in two or more nerves, and 29% of the patients had abnormality in one nerve. The most common abnormalities were: abnormal H reflex (61%), small lateral popliteal compound motor action potential (54%), small posterior tibial compound motor action potential (46%), small median sensory action potential (46%) and delayed median F wave (43%). The abnormalities is best explained by spinal root involvement as part of the meningeal inflammatory proccess.

(Neurol J Southeast Asia 1996; 1:33-7)

HUNTINGTON'S DISEASE IN MALAYSIA: A CLINICAL AND GENETIC STUDY

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Huntington's disease (HD) is associated with an expanded (CAG) repeat within a novel gene 4pl6.3(IT5). HD is one tenth less common in non-Caucasians. It has been hypothesised that HD in Chinese originated from a common Caucasian ancestry by way of migration with the opening the five port cities in China. A HD registry was established in Malaysia in 1995. In eighteen months we identified seven unrelated patients with HD. There were four Chinese, one Malay and two Indians. The CAG repeat ranges from 40-50. Only one Chinese family had possible Caucasian ancestry of Irish descent, but none of the patients or relatives has Caucasian features. The only Malay patient was a local and the two Indians had origins in Tamil Naidu and Punjab, India respectively. The genealogy of the Chinese patients were traced to small villages remote from the port cities. Culturally, Chinese women were forbidden to have close contact with foreigners. Additional genetic evidence show that the CCG repeat adjacent to IT5 is of seven repeats in Asians and 10 repeats in Caucasians. The distribution of the CAG repeats differ among populations of low prevalence and the west. Therefore, mutation of the IT5 gene rather that the European migration hypothesis is likely to be the explanation for the variation in prevalence of HD.

(Neurol J Southeast Asia 1997; 2: 57-63)

MULTIPLE SCLEROSIS IN MALAYSIA

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The prevalence multiple sclerosis (MS) is estimated to 2/100,000 population in Malaysia. It is seen in all the three main ethnic groups; Chinese, Malays and Indians, but with higher prevalence among ethnic Chinese. There is high F:M ratio of 6.6: 1. None has a family history of similar illness. The average age of onset of symptom was 31 years. 50% of patients presented with a myelopathy, 59% of the relapses involved the spinal cord, 97% of patients had myelopathy at sometime of the illness. Acute transverse myelopathy was seen in 45% of cases and paroxysmal tonic spasm in 30%. Optic-spinal recurrence was the most common form, seen in 53% of cases and disseminated recurrence in 21%. Relapsing myelopathy accounted for 20% of clinically probable and definite MS combined. Devic's disease was seen in one patient only. The annual relapse rate was 0.58. The mortality rate was high at 29% over 7.1 years. There was characteristic severe motor and visual disability. At the time of last examination, 34% had bilateral optic atrophy with severe impairment of vision and were also bedridden or wheel-chair bound. 52% of patients who presented with acute transverse myeitis went on to develop MS. High total protein and pleocytosis in CSF was not uncommon. The evoked potential studies detected subclinical abnormalities in VEP (32%), BAEP (27%), and median nerve SSEP (31%). 75% of patients showed abnormality of cerebrum in the CT scan, mostly asymptomatic. 38% had positive hot bath test although none of the patients complained of Uhthoff's phenomenon.

(Neurol J Southeast Asia 1997; 2: 1-5)

THE INFLUENCE OF AMLODIPINE ON THE PLASMA PRORENIN-RENIN PROFILE AND ALDOSTERONE CONCENTRATION IN PATIENTS WITH HYPERTENSION

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Amlodipine, a potent calcium antagonist, has been successfully used to control blood pressure (BP). However, the effect of the compound on the renin-angiotensin-aldosterone system (RAAS) in the human is not well documented. In this open, single-blind study, male and female hypertensive patients aged between 32 and 60 years with no significant renal or liver disease were treated with 5 or 10 mg amlodipine, once daily for 2-3 months after a washout period of 2 weeks. Blood samples were collected from patients before and after the treatment, and from normotensive volunteers. The patients were found to have significantly lower plasma prorenin ($p<0.01$) and renin (PRA; $p<0.001$) activities while the aldosterone (aldo) concentration and aldo to PRA ratio were the same as that in the control group. After administration of amlodipine, the BP fell significantly ($p<0.001$). Amlodipine, however, significantly raised the plasma prorenin ($p<0.01$) and renin ($p<0.05$) activities of the hypertensive group to normal levels. The plasma aldo concentration was markedly increased ($p<0.01$) to levels greater ($p<0.05$) than the normal controls. The aldo:PRA ratio consequently was reduced ($p<0.05$). These data, in contrast to previous findings, suggest that amlodipine treatment has a significant effect on the RAAS.

(In: Adrenal Glands, Vascular System and Hypertension. Eds G P Vinson & D C Anderson, pp 259 - 265, Journal of Endocrinology Ltd, Bristol (1996))

PROTEOLYTIC SPECIFICITY OF RHODOSTOXIN, THE MAJOR HEMORRHAGIN OF *CALLOSELASMA RHODOSTOMA* (MALAYAN PIT VIPER) VENOM

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The proteolytic specificity of rhodostoxin, the major hemorrhagin from *Calloselasma rhodostoma* (Malayan pit viper) venom was investigated using oxidized B chain of bovine insulin as substrate. A total of six peptide bonds were cleaved: Ser^{9}-His^{10}, His^{10}-Leu^{11}, Ala^{14}-Leu^{15}, Tyr^{16}-Leu^{17}, Gly^{20}-Glu^{21} and Phe^{24}-Phe^{25}. Deglycosylated rhodostoxin, however, cleaved primarily at Arg^{22}-Gly^{23}.

(Toxicon 1997; 35, 979-984)

CLONING AND CHARACTERIZATION OF cDNAs ENCODING THREE ISOFORMS OF PHOSPHOLIPASE A_2 IN MALAYAN SPITTING COBRA (NAJA NAJA SPUTATRIX) VENOM

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cDNAs encoding three phospholipase A_2 (PLA_2) isoforms in *Naja naja sputatrix* were cloned and characterized. One of them encoded an acidic PLA_2 (APLA) while the others encoded neutral PLA_2 (NPLA-1 and NPLA-2). The specific characteristics of APLA and NPLA were attributed to mutations at nt139 and nt328 from G to C and G to A, respectively, resulting in amino acid substitutions from Asp^{20} and 83 in APLA to His^{20} and Asn^{83} in NPLA. Amino acid sequencing of purified protein also showed the presence of this Asp^{20} and His^{20} in APLA and NPLA, respectively. The cDNA encoding one of the PLA_2 (NAJPLA-2A), when expressed in *Escherichia coli*, yielded a protein that exhibited PLA_2 activity.

(Toxicon 1997; 35, 27-37)

Journal of the University of Malaya Medical Centre
JUMMEC

Volume 2 ❖ Number 1

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