# AFLATOXINS AND CHILD HEALTH IN KENYA

(Aflatoxinen en kindergezondheid in Kenya)

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Aan Mem

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Content of the chapters page

CHAPTER 1

GENERAL INTRODUCTION

Aflatoxins are metabolic products of certain strains of Aspergilli(1). The occurrence of a mysterious disease in turkeys during 1960 in England awakened interest in the toxicity of aflatoxins, and resulted in a flood of publications on the subject. Aflatoxicosis became a recognised mycotoxicosis affecting animal health, amongst veterinarians.

However, 20 years later in 1981 A.W.Hayes, quoting Forgacs, comments that mycotoxicoses still belong to 'the neglected diseases'(2). The great writer Graham Greene uses aflatoxins to eliminate one of his characters in the book 'The Human Factor'(3).

Medical authorities became aware of its hazardous potential, when a relationship was established between aflatoxin and hepatocellular carcinoma(1).

In 1982, Hendrickse postulated that aflatoxins might be an aetiological factor in the pathogenesis of kwashiorkor(4). This disease was accurately described by Dm Cecile Williams during her stay in West Africa in the 1930's. In her original publication she associated the disease with maize(5). The existing theory was that maize, not containing many essential amino acids, caused the hypoproteinaemia which is so characteristic of this disease. In 1955 Schoental questioned the theories about the aetiology of kwashiorkor, by stressing the point that kwashiorkor seems to be able to develop in adequately breast fed children(6). She raised the possibility that kwashiorkor might not be due to the absence

of some factor, but due to the presence of some toxic factors. Gopalan in 1968 showed that children fed on a diet with the same protein:energy ratio, could develop either marasmus or kwashiorkor(7). Kwashiorkor was regarded by Gopalan as a form of "dysadaptation". Aflatoxins could fit into this dysadaptation theory as the mediator of a chain of reactions, ultimately resulting in reduced production of proteins. It is this theory, as postulated by Hendrickse, that we will investigate in more detail in this thesis.

Very little is known about the consequences of foetal exposure to aflatoxins. Only limited work has been done on the subject in animal research and no investigations have been reported in humans. This study is the first to explore the extent and the effects of aflatoxin exposure on a small population of neonates.

The first chapter is a general introduction to the problems of aflatoxins.

In chapter 2 the literature concerning aflatoxins and some literature on kwashiorkor are reviewed.

The next six chapters are articles concerning six different studies on aflatoxins: chapter 3 describes an investigation concerning the frequency of detection and the concentration of aflatoxins in sera and urine of children with kwashiorkor, marasmic kwashiorkor or marasmus, compared to a control group of well-nourished children. Chapter 4 describes a study on a group of primi gravidae, to investigate the effects of aflatoxins on human foetal growth. In the following chapter 5, the results of

aflatoxin analysis on fifteen needle biopsies of liver , are presented. Chapter 6 presents findings in a study undertaken to investigate the effects of aflatoxins on neonatal growth and morbidity. The following chapter 7, reports the results of a study in which children with kwashiorkor and marasmic kwashiorkor were given an aflatoxin free diet for ten days during which all urines and stools were collected and their aflatoxin content measured. Chapter 8 is a study in which the prevalence of kwashiorkor is correlated with meteorological data. In the final chapter the results of these six studies are dicussed and possible new lines of investigation indicated.

The field work presented in this study was carried out while working as a "tropical doctor" in two rural mission hospitals in Kenya over a period of three and a half years, from August 1983 to February 1987. The results of the field studies and aflatoxin determination were subsequently correlated and analysed while working as a Research Fellow in the Department of Tropical Paediatrics and International Child Health of the Liverpool School of Tropical Medicine.

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CHAPTER 2

AFLATOXINS AND MALNUTRITION - a review

## 2.I. AFLATOXINS

### 2.I.1 History

In May 1960 a disease occurred in turkeys on commercial farms in Great Britain, eventually killing over 100.000 of these animals. The birds died after a short illness and mortality ranged from 10 to 70%. Affected poults were usually 4 weeks old, but older animals were sometimes involved(1,2). The animals died in opisthotonus after a period of muscle weakness. The post-mortem findings showed engorged kidney's and a necrotic liver. Later the same disease appeared in ducklings and pheasants(2), calves(3) and pigs(4). The disease which was not previously known, was called "Turkey X disease". The majority of cases occurred between May and August 1960 within a range of 80-100 miles from London. When suddenly the disease was also reported from the Cheshire area, the common factor could be identified as a ship cargo of Brazilian groundnuts, that was used in the animal food(2). The name of the ship was the ss."Rossetti", and the groundnut meal was consequently named the Rossettti meal. On a visit to Brazil Blount(1961) noted that similar problems had affected commercial animals in Brazil for the past 2 years and were especially related to the wet season crops(2). From Kenya it was reported that ducklings fed on a diet containing peanut meal from Uganda and Tangayika were also affected by "Turkey X disease"(5).
On the basis of a toxic factor present in the peanut meal, a

biological assay method was developed using ducklings which proved to be the most suitable animal(5). The assay described the proliferation of bile ductuli as a parameter for aflatoxin toxicity. A hot methanol and chloroform extract of the suspect meal, proved toxic to ducklings and turkeys, who developed symptoms and lesions identical to those of the "Turkey X disease"(6). On further investigation, the toxic substance was found to be related to a fungus growing on the contaminated peanut meal from Uganda. Extracts of the fungi grown on culture produced the same fatal illness in one-day old ducklings as the groundnut meal extract. The toxin producing fungus was identified as the Aspergillus flavus Link ex Fries(7), a fungus imperfecti. Examination of the Rossetti meal revealed only dead A.flavus hyphae(8). Fungi grow in the form of hyphae which form networks, called mycelea. Fungi reproduce sexually or a-sexually, the latter are fungi imperfecti which reproduce by the formation of spores from the hyphae(9).

In 1962 an independent Working Party on Groundnut Toxicity Research proposed the name AFLATOXIN(A.FLAvus TOXIN) for the toxin. Dutch workers in the Unilever laboratory in Vlaardingen isolated the toxin in crystalline form and called it FB1(10). Further work resulted in the separation of the toxin into two compounds B and G, according to their blue and blue-green fluorescence, respectively, in ultraviolet light(11,12). Aflatoxin B was further subdivided into B1 and B2 and aflatoxin G was subdivided into G1 and G2 in order of decreasing Rf values.

Aflatoxins B2 and G2 were established as the dihydro derivatives of B1 and G1 respectively(13). In 1966 the final chemical characterisation and the de novo synthesis was described(14).
Cows fed rations of aflatoxin contaminated peanut meal excreted a toxic factor in their milk which had the same biological effect on ducklings as aflatoxins. The compound was named Aflatoxin M (Milk factor)(15,16), later resolved by paper chromatography into two components, M1 and M2, which are monohydroxy derivatives of aflatoxin B1 and B2 respectively(17,18).
Retrospectively aflatoxicosis had been seen before. An outbreak of a disease in swine in the USA in 1952 which killed 1500, was caused by mouldy corn fed to those animals. When this corn was put on a culture it produced toxic varieties of Penicillium rubrum and Aspergillus flavus(19). In 1951 a disease was encountered in guinea pigs , characterised by gross oedema of the sub-cutaneous tissues and marked hepatotoxic changes in the liver. When their groundnut containing diet was examined it appeared to be twice as toxic as the Rossettti meal(20). When this so called 'Diet 18' was used in an other experiment in guinea-pigs, the animals developed a syndrome like kwashiorkor(21).
The Rossetti meal, when tested on rats for the first time failed to produce acute lesions, but 9 out of 11 animals developed livertumours(22). From the USA came reports of an outbreak of hepatoma in trout(23). The food given to the trout proved later to be contaminated with aflatoxins(24). Aflatoxins have been proved carcinogenic to all animals tested so far but species

susceptibility varies greatly, for example in the mouse hepatomas only occur after intra peritoneal injection of aflatoxins and not after oral administration.

2.I.2 Aflatoxin production by fungi

Aflatoxin production is limited to one genus of imperfect fungi, Aspergillus and to only two species within that genus, A. flavus and A.parasiticus (25). Although some recognise a third species, A.toxicarius, as aflatoxigenic, others see it as a variation of A.parasiticus(26).

The Aspergillus flavus group of fungi is a constituent of the microflora in air and soil and is found throughout the world(27). It may grow on living or dead plants and animals. Its role as a storage fungus contributes to the deterioration of stored food commodities. Within a species aflatoxin production varies between isolates(26,28,29). Between 20-80% of isolated strains of A.flavus are able to produce aflatoxin (25). Different strains of A.flavus differ in their aflatoxin production in different media(30). Fungi need various nutrients in order to meet their energy needs and to form macromolecules. They cannot synthesize carbohydrates, the substrate should therefore contain these compounds. During the breakdown of carbohydrates a large number of metabolites are formed, in this so called 'primary' metabolic process. Especially at the end of growth, certain 'secondary' metabolites are formed which are not necessary for growth or energy supply of the fungus. Some of these secondary metabolites, the mycotoxins, are toxic to animals, insects and micro-

organisms(J.Zaal, pers. communication). Aflatoxins are an example of secondary metabolites 'par excellence' (31).

Factors influencing the growth of the fungus and the production of aflatoxins are temperature, relative humidity and moisture content of the substrate and oxygen (25,28,29). The lowest limit for growth of A.flavus and production of aflatoxins in starchy cereal grain, like rice or maize, is a moisture content of 18.3%-18.5% on a wet weight basis. In oil containing nuts, like groundnuts or Brazil nuts, the lower limit is 9%-10% (25). Aflatoxin production is obtained at temperatures between 13 and 40 °C, with an optimum at 27-32 °C.(32). No production takes place at a relative humidity below 83%.(29). It is clear that humid tropical climates provide a good environment for aflatoxin formation.

Toxin formation takes only a few hours if favourable conditions exist(33). The foods most frequently contaminated with aflatoxins are groundnuts, maize, groundnut butter and oil seeds(25,34), but many other types of staples and foods have also been found to be contaminated with aflatoxins(25,35), e.g, sorghum, millets, wheat, barley , oats, rice and cassava. In maize damage to the kernels enhances fungal growth and subsequently aflatoxin formation(36). Damaged groundnuts are also more likely to contain aflatoxin(37,38). Locally brewed beer may contain considerable amounts of aflatoxin (39).

The production of aflatoxins follows a seasonal pattern, the most frequent and highest concentrations are found during the warm,

wet periods(40,41,42). In a specific area the frequency and amount of aflatoxin contamination of foodstuffs may vary with altitude(43). Most reports on food contamination are therefore not completely comparable, because of the variation in season and geography(28). In general, commodities grown in warm and humid areas where high levels of insect damage and poor farming and storage practices prevail, appear to be more susceptible to aflatoxin formation. Also food from animal sources like dried fish and dried meat can contain aflatoxins.

Of special interest is the fact that often foods which appear to be mouldy are disregarded for human consumption and are instead given to animals, but also food which looks normal, may be contaminated with aflatoxins. Animals fed aflatoxin contaminated foods excrete these toxins into their milk, meant for human consumption(17,44,45,46). Patterson has calculated, that cows given 30 microgram aflatoxin B1/kg feed, may excrete 0,1 microgram aflatoxin M1/litre milk. Regulations have been introduced in many countries including Holland which set limits, thought to be safe for the amount of aflatoxin contamination permitted in animal foods. In a survey in the Netherlands after regulations were introduced, van Egmond(45) still found traces of aflatoxin M1 in milk, but the concentration had significantly declined, and he concluded that the regulations were effective. Similar regulations have not been introduced in most tropical countries.

Surveillance and control measures to reduce food contamination by mycotoxins were stressed in a joint conference of FAO,WHO and

UNEP in Nairobi in 1977, thus underlining the magnitude of the problem(pers.communication-unpublished data). In 1979 a task group was formed by UNEP and WHO that compiled a report and made recommendations for a further study of the effect of mycotoxins on human health(25).

Measures to reduce aflatoxin contamination have been described for peanuts in the proces of roasting(47). A further reduction is achieved by hand or electronic sorting(48). Chemical reduction is achieved by treatment with ammonia, sodium hypochlorite and hydroxygen peroxyde and are very promising. Aflatoxins are resistant to cooking or pasteurisation which at best reduces the amount of toxin only very slightly(49,50).

It may be conclusively stated that almost any food or food commodity in tropical areas can be contaminated with aflatoxins, although some foods are more likely to be contaminated than others. Among the foods most frequently contaminated is maize, the main staple diet for millions of people in the tropics (51).

### 2.I.3 Biochemistry of aflatoxins

#### 2.I.3.a Excretion

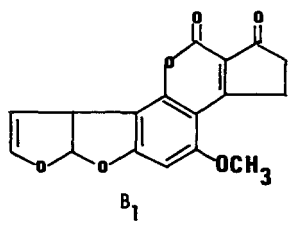
The metabolism and excretion of aflatoxins has been reviewed by a WHO working party in 1979(25). Aflatoxins are metabolised by the microsomal Mixed Function Oxidase (MFO) system of the endoplasmic reticulum of liver cells. The hydroxylated derivatives are conjugated with sulphate or glucuronic acid to form sulphate and glucuronide esters. These are readily excreted in bile or urine.

During this metabolism, reactive metabolites may be generated which bind and react covalently with various cellular macromolecules e.g. DNA and RNA and proteins. The toxic and carcinogenic effects of aflatoxins probably result from their intermediate metabolism and activation, rather than from direct action by the parent compounds. Aflatoxin B1 is the most biologically active with the greatest potential for toxicity and carcinogenicity.

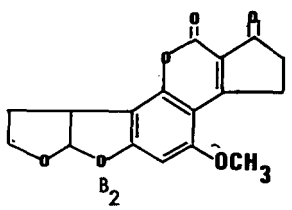
2.I.3.b Aflatoxin transformation

The natural occurrence of aflatoxins is in the form of aflatoxin B and G, according to their blue and blue-green fluorescence respectively when in ultra violet light. Both compounds are subdivided into two components B1 and B2, and G1 and G2 respectively according to their difference in Rf value on a chromatography plate(25,52). These four aflatoxins are the major aflatoxins or parent compounds (34). Aflatoxins are composed of several ringstructures(fig I), which provides it with a stable molecule. Aflatoxins are highly substituted coumarins containing a fused dihydrofuran moiety. All other aflatoxins derive from these mother compounds, in the proces of metabolism, detoxification and excretion in the liver and other organs. Primarily metabolism takes place in the Mixed Function Oxidase system in the endoplasmic reticulum. Recently Steyn(53) has suggested that aflatoxin G probably also derives from aflatoxin B1, because a strain producing aflatoxin G alone has never been found, whereas strains producing aflatoxin B alone are often

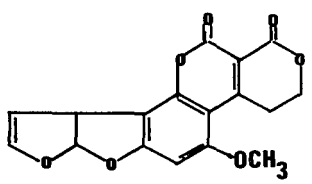
Figure 1 The structures of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$



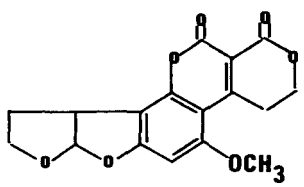

$B_1$




$B_2$




$G_1$




$G_2$

found. However the formation of aflatoxin G alone has been reported(36) although only in 1-2,5% of the strains.
At least 17 different aflatoxins have been identified. Aflatoxin B1 is regarded the most potent and toxic.

Aflatoxin M1 and M2 :
Originally aflatoxin M was identified as the 'milk'toxin (16,17,18). Aflatoxin M1 and M2 are the monohydroxy derivates of aflatoxin B1 and B2 respectively. Although originally detected in milk, both compounds are also found in urine, stool and liver(54,55). In urine they may reach even higher concentrations than in milk(56).

Aflatoxicol :
Aflatoxicol is a reduction product of aflatoxin B1(57). Dihydro-aflatoxicol is a reduction product of aflatoxin B2(58). This reduction takes place in the cytoplasmic fraction of the liver cell. Although aflatoxicol itself is not very toxic (18 times less than aflatoxin B1) it is carcinogenic (59). Aflatoxicol has a special place in the aflatoxin transformation because the conversion of aflatoxin B1 to aflatoxicol is reversible. In this way aflatoxicol can act as a reservoir for the B1 type and thus enhances the exposure of the cell to this aflatoxin B1 and its metabolic products (58,60).
Aflatoxicol is also oxidised by liver preparations(direct or via aflatoxin B1) to aflatoxins Q1,P1, H1, M1 and B2a(61). In this study of the metabolism of aflatoxicol, human liver preparations were in vitro capable of oxidating as well as dehydrogenating

aflatoxicol yielding aflatoxin B1. Aflatoxicol formation is inhibited by 17 keto steroid sex hormones. It is the only metabolic transformation known to be sensitive to hormones in vitro (25). See fig.II for aflatoxin metabolites.

Aflatoxin P1 and P2 :

Aflatoxin P1 was identified in the urine of monkeys after injection of a dose of aflatoxin B1(62). It represented 20% of the injected dose, whereas aflatoxin M1 accounted for only 2-3%. Aflatoxin P1 is non-toxic(63).

Aflatoxin Q1 :

Aflatoxin Q1 was identified in vitro as a major metabolite in the liver of monkeys, but it is also found in the livers of rats, chicken and humans(64,65). No toxic effects were noted in a test on chicken embryos(63). Aflatoxin Q1 is now thought to be important in aflatoxin induced immunosuppression( RG Hendrickse, pers. comm.).

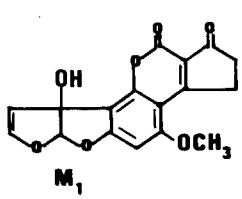
Aflatoxin B2a:

This aflatoxin is the hemiacetal metabolite of aflatoxin B1. It has been isolated from cultures of A.flavus(53). It was considered to be the active form, responsible for the acute toxic effects of aflatoxin B1(59,60)

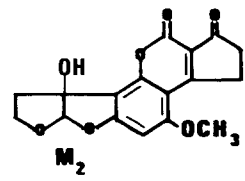
Dihydrol-aflatoxin B1:

This metabolite was proposed as the primary protein-reactive metabolite(66).

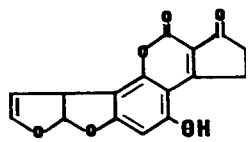
Figure 2 The structures of aflatoxins $M_1$, $M_2$, $B_{2a}$, $G_{2a}$, $Q_1$ aflatoxicol and aflatoxicol $H_1$



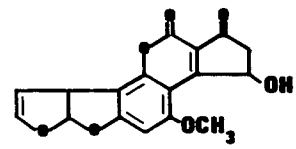
$M_1$



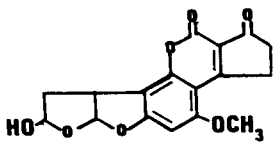
$M_2$



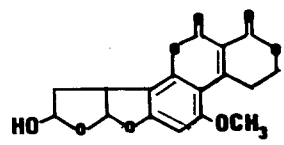
$P_1$



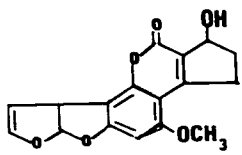
$Q_1$



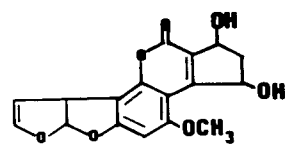
$B_{2A}$



$G_{2A}$



Aflatoxicol



Aflatoxicol $H_1$

Aflatoxin G2a:

This aflatoxin has also been isolated from cultures of A.flavus(53).

2,3 epoxide of aflatoxin B1 :

This metabolite of aflatoxin B1 has not been isolated yet. It appears to have a 'transient' existence. It is highly reactive and binds covalently to DNA. In view of this interaction with DNA it is now generally accepted as the mutagen and the proximal carcinogen(25).

2.I.4 Metabolic effects

Aflatoxins bind covalently to macromolecules of the cell and produce profound metabolic disorders(25,60,63). The effects are characterised by inhibition of DNA and RNA synthesis, protein synthesis, lipid synthesis, lipid transport and enzyme synthesis. The formation of clotting factors II and VII is inhibited, as is glucose metabolism via the 6-phosphate pathway. Aflatoxins also depress the synthesis of phospholipids.

Furthermore the feedback control of cholesterol synthesis is lost, a change formerly considered characteristic of the pre-cancerous state (67), but modern theories give much less importance to the role of cholesterol synthesis as a precursor of cancer (A.J.Vergroesen - pers.comm.).

2.I.5 Biological effects

Aflatoxins are immunosuppressive, probably due to their inhibitory effect on protein synthesis(25). There is a reduced

cell mediated immunity, low complement levels and impaired production of antibodies(34,68,69,70). Aflatoxin diminishes the production of interferon(71).

Aflatoxin B1 is also mutagenic (25). It causes chromosomal aberrations or DNA breakage in plant and animal cells(72,73). Wong and Hsieh(74) reported gene mutations in bacterial test systems when aflatoxin B1 was activated by microsomal preparations from rat and human liver. Leonard et al.(75) did not find any mutagenic effect in mice exposed to aflatoxin B1 5mg/kg given intraperitoneally.

2.I.6 Teratogenic effects

The first reports on the embryotoxicity and teratogenicity of aflatoxins appeared shortly after the introduction of the 1-day-old duckling test as the standard biological assay, although chicken embryos were also widely used as a biological assay (52,76). In the malformations encountered the limbs were especially effected, but also malformations of the head and eyes were noted. The embryonic response depended on the developmental stage, early embryos being more susceptible than older embryos. The number of animals with malformations was dose dependent(77). A dose of 0,2ug aflatoxin B1 injected per egg, resulted in 65% abnormal embryos,a dose of 0,4ug per egg resulted in 82% abnormal embryos, while a dose of 0,6ug per egg resulted in 90% abnormal embryos. Controls showed abnormal embryos in 4%. In this study a decreased birthweight of the new born chickens was also noted. In the livers of the chicken embryos there were aflatoxin induced

lesions characterised by focal areas of fatty change with proliferation of reticuloendothelial cells.

Mammal embryos have also been tested. Aflatoxins given intra peritoneally in a dose of 4 mg/kg to hamsters on day 8 of pregnancy resulted in fetal death and resorption of some fetuses and also resulted in malformed living foetuses. The same dose given on day 13 had no apparent effect. The difference depended on the fact that by day 13 organogenesis was almost complete(78). In another experiment with hamsters, abnormalities involving the head and spine were most commonly seen in the group treated with aflatoxin. Increasing the dose from 4 to 6 mg/kg increased the mean percentage of each type of abnormality seen (78). Aflatoxin given together with DNA, reduced the teratogenic response as well as the severity of the liver damage in both dam and pups.

A long term study on the effects of aflatoxins through three generations of rats did not produce any significant effect in respect of reproduction(79). In another report testicular atrophy and azospermia was reported in rats after aflatoxin treatment(80). Egg production decreased in breeder hens about three weeks after the start of administering aflatoxin 5ug/g, or 10ug/g feed, returning to normal production about 4 weeks after stopping the administering of contaminated food(81). In the same experiment hatchability of the eggs declined early after the start of aflatoxin administration and continued throughout the period of 4 weeks during which the animals were given food contaminated with aflatoxin. Hatchability was more effected in the group receiving aflatoxins 10ug/g, compared with a control

group, than in the group who received 5ug/g aflatoxins. Jacobsen and Wiseman (82) demonstrated that aflatoxins were present in eggs laid by hens fed on an aflatoxin diet.

Aflatoxins have been demonstrated to be teratogenic to tadpoles(Rana Temporaria)(83). Malformations consisted mainly of limb defects. The teratogenic response was dependent on dose, time of exposure and stage of embryonic development.

Aflatoxins have also been reported to cause transplacental carcinogenisis (84). From a group of 208 rats who were either exposed to aflatoxins prenatally and/or postnatally, 4 rats developed hepatic tumours who were either exposed to aflatoxins in utero for 10 days(1 male rat), to aflatoxins in utero for 10 days and in breastmilk for 10 days( 2 female rats) or exposed to aflatoxins in the first 10 days of their life via the breastmilk( 1 female rat). No tumours were found in 100 control off-spring.

Ward(85) fed aflatoxin to pregnant rats throughout gestation and lactation until weaning. After weaning the off-spring received an aflatoxin free diet till death. Mean survival time was the same as rats who started on the contaminated diet at the age of 6-7 weeks. Females survived longer(mean 62 weeks) than males(mean 46 weeks). Over 75% of the rats had hepatic tumours, while 23% had colonic tumours.

Rats fed aflatoxins in the second half of pregnancy showed a reduced intrauterine growth of their foetuses, an effect not seen if the same dose was given earlier in pregnancy(80). The effect was thought to be secondary to the toxic effect on the mother.

Cardeilhac et al.(86) found a permanently impaired growth rate in piglets from sows fed aflatoxin after parturition, thus exposing piglets via milk. Ambrecht et al(87) reported on piglets who were exposed to aflatoxins in utero and postnatally via the sows' breast milk. The weight gain of these piglets was initially reduced as compared to control piglets not exposed to aflatoxins post natally( although it is not clear whether these control piglets were exposed to aflatoxins in utero). After weaning the piglets showed a further normal weight gain. At termination of the experiment, piglets exposed to aflatoxins prenatally showed liver lesions characterised by bile duct proliferation, cytoplasm vacuolisation and cell-cord disorganisation.

Salil et al(88) in a study on pulmonary lecithine formation, mentioned that aflatoxin B1 given to rats late in pregnancy caused a high neonatal death rate in the offspring during the first two days after parturition. They found that aflatoxin B1 inhibits the formation of surfactant lecithin thus causing alveolar instability, which could have been responsible for the neonatal deaths.

2.I.7 Other effects in animals

Besides the teratogenic and immunosuppresive effects already described, the acute and chronic toxicity and the development of carcinomas by aflatoxins have been tested in many different species of animals.

In acute toxicity there is a wide variation of the LD50 between species(89,90). The difference may be seen as a result of

differences in rate of metabolism, fast metabolisers tending to develop the acute effects, while slow metabolisers tend more towards carcinoma formation. Young animals are more susceptible to the effects of aflatoxins than older ones(89,90). Hsieh et al.(58) suggested a correlation between the degree of aflatoxicol formation from aflatoxin B1 and toxicity in different species of animals.

Female rats are less prone to the acute toxicity or the carcinogenic effect of aflatoxin than male rats(85,90). In vitro microsomes from female rats have only half the activity of microsomes from male rats in metabolising aflatoxins(91). Microsomes from male rats formed 2-5 times more aflatoxin M1, Q1 and DNA-alkylating metabolites than microsomes from female rats. Castration reduced the effect on male microsomes, while ovariectomy had no effect on the microsomes from females. In vivo it was shown that more aflatoxin B1 became bound to DNA in male rats than in female rats.

Other factors influencing aflatoxin susceptibility are nutrition and nutritional status. A diet deficient in proteins increases the susceptibility to acute aflatoxin toxicity(92,93,94). The effect is probably due to decreased MFO activity(94,95). Aflatoxin in itself induces inhibition of protein synthesis in rats(96). Aflatoxin B1(6mg/kg) and M1(6mg/kg) inhibited protein synthesis, but a double amount of aflatoxin G1(12mg/kg) did not. In the same testing dose, aflatoxin B1 did not inhibit the in vitro mitochondrial protein synthesis, while aflatoxin M1 and G1 did, but aflatoxin M1 much more than aflatoxin G1. The

cytoplasmic protein synthesis in vitro was reduced by aflatoxin B1 and M1 but not by G1. The authors concluded that the transfer of aflatoxin B1 to aflatoxin M1 in the microsomes of the cell can enhance the toxicity(96). Smith(97) found that aflatoxins raise the protein requirements for optimal growth. Protein supplement of the diet protects against the acute toxicity of aflatoxins, but enhances the risk of carcinoma(98,99). Vitamin A deficient male rats were more susceptible to aflatoxin toxicity than controls fed a vitamin A containing diet. No difference was seen in female rats (100). Selenium has a protective effect against aflatoxin toxicity(101). Phenobarbitone seems to protect against aflatoxin toxicity and carcinogenicity(102). Its effect is thought to be due to enzyme induction and enhancing MFO activity. However no firm conclusions can be drawn from correlations between metabolism and toxicity (63).

The susceptibility to the carcinogenic effects of aflatoxins also differs between species. In monkeys it may take as long as 5-6 years before carcinomas develop, whereas in the rat a tumuor may develop after only one dose(25). Ferrets and trout are very susceptible to the carcinogenic effects of aflatoxins: 0,3-2ug/kg and 4-8ug/kg respectively produce livertumours.

Accidental aflatoxicosis has been described in many other types of animals like horses(103), dogs (104,105), cattle (3) and swine (4)

2.I.8 Pathological changes in the liver due to aflatoxins.

The ultrastructural changes in the liver cell have been studied by Butler after giving aflatoxins to rats(106). Degranulation of

the endoplasmic reticulum is the first sign noticed. Further an increased permeability of the mitochondria, destruction of the cristae and disruption of lysosomal membranes with consequently release of lysosomal enzymes into surrounding tissues was observed. The latter is probably responsible for the hepatic necrosis and other changes seen in acute poisoning. The first effects seen are periportal, in the cells surrounding the portal veins. In these areas changes are also seen in the gall ductuli: proliferation of bile duct epithelial cells(used as a biological parameter in the bio-assay). A fatty degeneration of the peripheral parenchymal cells has also been noted(90). Fibrosis starts in the portal region, steadily growing towards the centrilobular area, as the administration of the toxin is prolonged. In acute poisoning centilobular necrosis is present. If the dose of aflatoxin was not lethal, the liver may recover with formation of fibrosis at a later stage(106) , or may recover completely (107).

## II. AFLATOXINS AND DISEASE IN HUMANS.

Reports on aflatoxins causing disease in humans include local outbreaks of acute poisoning and isolated cases and an epidemiological association between hepatocellular carcinoma and aflatoxin contamination of ingested foods. The detection of aflatoxins in liver specimens, blood, urine and faeces of children and in human breast milk has led to the hypothesis of aflatoxin involvement in the eatiology of kwashiorkor.

## 2.II.1 Aflatoxins and kwashiorkor

In 1982 an article in the British Medical Journal reported the preliminary findings of a study in the Sudan, that incriminated aflatoxins in the aetiology of kwashiorkor(108). Three groups of children, classified according to the Wellcome classification (109) into kwashiorkor, marasmic kwashiorkor and marasmus, were compared to a group of normally nourished children, who served as a control. Kwashiorkor children showed the highest frequency of detection of aflatoxin in their serum, with the highest mean values. All groups showed the same frequency of aflatoxin detection in their urine. When the full report was published (110) it also showed that the ratio of aflatoxin B1 versus M1 in sera and urine was the highest in the kwashiorkor group. Further, aflatoxicol was only found in the sera of patients with kwashiorkor and marasmic kwashiorkor. There was one exception, a marasmic child with a serum albumin concentration of 1.5 gr%. The authors considered that these observations on aflatoxicol indicate a difference in aflatoxin metabolism in kwashiorkor children. Most children with kwashiorkor were admitted in the wet and post-wet season, but aflatoxin detection rates in sera and urines of these children showed no seasonal fluctuations.

Further evidence of a relationship between kwashiorkor and aflatoxins is supplied by studies on 19 post mortem liver biopsies from children who died from marasmus[n=3], marasmic kwashiorkor[n=6], or kwashiorkor[n=10] in Liberia, Nigeria and South Africa (111). Aflatoxin B1 or aflatoxicol was found in all

10 livers from kwashiorkor children. In marasmic kwashiorkor, aflatoxins were found in 5 out of 6 samples: in 4 samples aflatoxicol and in one sample aflatoxin M1. In the three livers of the marasmic children no aflatoxins were found. A report on 22 post mortem biopsies from kwashiorkor children in Ghana(112) revealed aflatoxins in all livers, but only aflatoxin B1 and aflatoxicol were found. No other metabolites were found, suggesting that in kwashiorkor the microsomal MFO system is impaired and aflatoxin cannot be detoxified by oxidation and removed from the liver as occurs normally. In a report on 16 needle biopsies of liver from living children with kwashiorkor in the Sudan, 5 samples revealed aflatoxins(113) : aflatoxin B1 in two samples, aflatoxicol in two samples and aflatoxin B2 in one sample. In 10 needle biopsies from marasmic children and one from a marasmic kwashiorkor child, no aflatoxins could be detected. The reason for the lower frequency of detection of aflatoxins in the needle biopsies is the small quantity of tissue obtained by needle aspiration and the probably unequal distribution of aflatoxin throughout the liver.

Further circumstancial evidence for a role for aflatoxins in the pathogenesis of kwashiorkor arises from the geographic distribution and seasonal fluctuations of aflatoxin contamination of food, that are remarkably similar to the geographical distribution and seasonal prevalence of kwashiorkor (51). Other similarities include the biochemical effects of aflatoxins such as inhibition of protein synthesis, lipid synthesis, including

phospholipids and free fatty acids, triglycerids, cholesterol and its esters, inhibition of enzyme activities, inhibition of clotting factor synthesis and immunosuppression. The liver is the principle target organ for toxicity and pathological changes include fatty infiltration and biliary proliferation, acute toxic necrosis and portal fibrosis. All of these problems are encountered in children with kwashiorkor.

In studying the sera of children in other African countries,i.e. Zimbabwe, Transkei, Ghana and Liberia, aflatoxins were found most frequently in groups with kwashiorkor, with the exception of Zimbabwe, where in the group with marasmic kwashiorkor aflatoxins were detected most frequently(114). However the hypothesis that aflatoxins have a causal role in the pathogenesis of kwashiorkor remained unproven (51).

2.II.2 Reports on outbreaks of aflatoxicosis

Taiwan:

An outbreak in Taiwan in 1967(115) affected 26 of 40 members in a cluster of 3 families. Three fatalities occurred, all children. There was hepatomegaly, severe abdominal pain, pallor and oedema of the legs. Mouldy rice found in the homes was thought to be the cause of the disaese. In two samples of rice aflatoxin was detected in a concentration of 22,6 and 7,82ug/kg.

Western India:

Western India experienced an outbreak characterised by jaundice, portal hypertension, ascites and in some cases oedema of the

legs. Dogs had become ill before the onset of symptoms in human. In total, some 397 patients were affected of whom 91 died(116). The condition affected older children (over 6 years) and adults. Cases occurred in families where the staple food was maize from which A. flavus was cultured(117). One autopsy revealed bile duct proliferation and periductul fibrosis. Aflatoxin B1 was detected in 2 out of 7 sera examined, but in none out of 7 urines examined. The same outbreak revealed centrizonal scarring and cholangiolar proliferation in 6 out of 9 liverbiopsies taken from patients.

Kenya:

In the Machakos district of Kenya an outbreak of jaundice was reported to the Medical Research Centre in Nairobi(118). Initially it was thought to be yellow fever, a condition not previously reported from Kenya (B.K.Johson pers.communication). A team was sent out to investigate the outbreak. It was found that preceeding the illness in humans, dogs had become ill and died. The disease in humans tended to occur in family clusters: 16 out of 20 cases. All patients had jaundice and a tender liver and hepatosplenomegaly occurred in some cases. Serology for a wide variety of viruses was negative. Three out of 29 sera incl. one from a control were positive for hepatitis B surface antigen (HBsAg). Two autopsy liversamples from patients revealed centrilobular necrosis, minimal inflammatory reaction and slight fatty change. In both samples aflatoxin B1 was detected at a concentration of 39 and 89 ng/g respectively. Maize from the

homes of the affected families revealed aflatoxin B1 12,0 and 3,2 mg/kg respectively. Maize from families that were not affected contained aflatoxin B1 in quantities less than 0,5mg/kg. The stored maize was from the 1979 harvest, because a drought had diminished the 1980 harvest, while prolonged rains had prevented harvest of the 1981 crop . It was noted that the roofs of the buildings in which the heavily contaminated maize was stored were leaking.

Uganda:

In 1970 Serck-Hansen reported of a 15-year old boy who was admitted to the hospital following a four day history of abdominal pain and swelling(119). Examination revealed enlarged painful liver and oedema of the legs. The ECG showed abnormal changes and the condition was diagnosed as cardiac failure. The patient succumbed after two day in hospital and autopsy revealed fatty change and centrilobular necrosis in the liver. When visiting his home, mouldy cassave was found. A 3 years old brother and a 6 years old sister were also ill. Analysis of the cassave revealed aflatoxin B1 1,7mg/kg. The author concluded that the aflatoxin was probably the cause of the disease.

W-Germany:

Bosenberg(120) described a 45 year old german who died after eating an unusually large amount of various nuts which were apparantly quite moldy. Analysis of the liver at autopsy revealed aflatoxin B1.

India:

Amla(121) reported the occurrence of cirrhosis in children after feeding them commercial peanut flour, used as a protein source. The aflatoxin concentration in this flour was found to be 300ug/kg. A follow-up of 20 children who had received this contaminated diet, showed progressive enlargement of the liver and after one year 12 of these children had gross hapatomegaly. Three children died of hepatic coma, 18 months later. Liver biopsies initially showed fatty liver, but biopsies at 4 months and 1 year showed progression to cirrhosis in some cases. Later this event was linked to a wide spread disease in India, Indian Childhood Cirrhosis(122), but in later reports this link could not be sustained(123).

Senegal:

Payet(124) found hepatic fibrosis on follow-up in one of two Senegalese children who had been fed on an aflatoxin containing groundnut meal (conc 0,5-1 mg/kg) during repeated admissions for malnutrition.

2.II.3 Liver carcinoma

After the initial experiments with rats developing carcinoma after feeding them aflatoxin containing food(22), it was suggested by Barnes and Butler in 1964 that men might also be affected by the carcinogenic activity of aflatoxins(125). Oettle(126) proposed a link between dietary mycotoxins and the high incidence of hepatoma in Africa.

Since then, epidemiological associations between aflatoxins and

liver carcinoma have been established in many countries: Uganda(127), Swaziland(128), Thailand(129), Kenya (43), Mocambique(130), the Phillipines(131), China(132). Linsell and Peers (133) showed an aflatoxin dose related incidence of hepatocellular carcinoma by summating the data in some studies. Further evidence of the relation between aflatoxins and human liver cancer comes from the detection of aflatoxins in the liver biopsies of patients suffering from hepatocellular carcinoma[HCC]. Stora et al(134) found aflatoxins in 5 out of 15 liver biopsies from HCC patients. Siraj (135) found that 4 out of 6 liver biopsies from patients who died of HCC contained aflatoxins in a range of 2-60 ng/g liver tissue. In 21 control livers aflatoxins were found in 3 with aflatoxin levels less than 2 ng/g tissue. In Nigeria 4 out of 5 liver biopsies from HCC patients contained aflatoxins in a range of 2-15 ng/g tissue, while 15 control livers showed no detectable aflatoxins (136). Philips (137) found aflatoxin B1 520 ng/g in the liver of a patient suffering from carcinoma of the liver and rectum. This is the first report of a biopsy taken from a living subject suffering from HCC that contained aflatoxins.

2.II.4 Reye's syndrome

Reye's syndrome is a serious, frequently fatal disease of obscure aetiology which affects infants and young children, originally described by Reye et al. in 1963(138). Reports on the aflatoxin involvement in this syndrome derive from New Zealand (139,140) , Thailand (141), the USA (142) and Csechoslovakia(143). The role

of aflatoxins in this syndrome is unknown, other factors like salicylates have been mentioned(144). Aflatoxins were found in the livers of patients who had died from the disease, whereas in controls aflatoxins were found less often and in smaller concentrations (135,142,145,146). Aflatoxins given to macaque monkeys produced a syndrome like Reye's syndrome (147).

2.II.5 Other studies on aflatoxin in human tissues or excreta

Campbell et al.(148) studied the excretion of aflatoxins in urine after ingestion of contaminated peanut butter. They estimated that 1-4% of the ingested amount of aflatoxin B1 appeared in the urine as aflatoxin M1. The minimum level of ingestion of aflatoxin B1 to give a detectable amount of aflatoxin M1 in the urine was 15 microgram/day.

Maleki (149) described the excretion of aflatoxins in urine from patients with cirrhosis and who lived in an area near Isfahan, where contamination of cow's milk is common. In cirrhotic patients the detection of aflatoxins in urine was 6 out of 25, while in 30 non-cirrhotic patients who served as controls no aflatoxins were found in the urine.

Onyemelukwe (150) investigated the aflatoxin level in the blood of healthy, first time, rural blood donors in Nigeria. He found that 25% of the values were above 0.2 microgram/ml.

Richir (151) investigated the urine of 30 patients from France and 42 patients from Senegal, but failed to detect any aflatoxin in these samples, but the authors held technical and circumstantial factors responsible for the negative results.

It is clear that aflatoxins have been demonstrated in numerous types of human body fluids, tissues and excreta, thus establishing conclusively that there is wide spread exposure to these toxins.

## III. PROTEIN ENERGY MALNUTRITION (PEM)

Malnutrition in childhood has been recognised since ancient times in the form of marasmus(Gr. meaning "withering") and associated with famine. There is no dispute as to the eatiology of marasmus: it is the childhood equivalent of starvation(152). In appearance these children are shrunken and wizened. The loss of subcutaneous fat cause the skin to hang in folds over their shrunken buttocks. They are active, wide eyed, irritable and hungry! There are no distinctive biochemical derangements in contrast to kwashiorkor, first described by CD Williams in 1933 in W.Africa and associated with a maize diet(153). She described kwashiorkor (Ga language, meaning: the disease the older child gets when the next baby is born) as usually occurring between the ages of 6 months and four years ( the youngest case noted by her was 9 weeks old), with swelling of the hands, feet and face. The skin changes noted were small black thickened crumpled patches appearing first about the knees and elbows, afterwards appearing along the extensor surfaces and buttocks. The skin tends to peel off, leaving a moist raw surface. The hair becomes sparse and loses its normal texture and is easily pluckable. Small sores at the corners of the eyes, mouth and about the vulva are usual. There is no great

anaemia and no leucocytosis. On post mortem an extremely fatty liver is the only constant finding. She noted that patients may be on a breast milk diet, an observation also recorded by Oomen (154). Little has been added to this original description.
A state of kwashiorkor was regarded as a result of a diet deficient in proteins but adequate in calories, while marasmus resulted from a diet deficient in calories, but sufficient in proteins. Marasmic kwashiorkor was thought to result from a diet in which both constituents were deficient. The occurrence of kwashiorkor in breast fed children was explained as a relative shortage of protein needed for the growing child.

2.III.1 Aetiology of kwashiorkor

Before world war II, due to the 'crazy pavement', as the skin changes in kwashiorkor are called, vitamin-deficiency was for a long time considered to be the main cause for the syndrome. Hence the confusion about the name "infantile pellagra", as kwashiorkor was often called. Brock et al(155) finally proved that vitamins were not the aetiological factor. After world war II a diet relatively low in proteins was considered as the aetiological factor.
Gopalan(156) studied 1800 children below 5 years of age in a poor, rural Indian community and found no difference in the protein content of the diets of children developing either marasmus or kwashiorkor. He introduced the idea of 'dysadaptation' as a causative mechanism for the development of kwashiorkor. As a consequence of this idea of dysadaptation, the

endocrinological theory to explain the development of kwashiorkor and marasmus was developed(157,158). In a longitudinal study on children in Uganda and Gambia, it was found that in the first two years of life the children who developed kwashiorkor had a higher insulin level and a lower cortisol level than children who developed marasmus. It was suggested that the high insulin level prompted movement of amino acids into the muscle, rather than into the liver, thus promoting hypoalbuminaemia, whereas the high cortisol and low insulin levels in marasmus promoted amino acids release from muscle for metabolism in the liver and thus sustaining serum albumin. Others have disregarded this theory because these differences may reflect the state of malnutrition rather than cause it(159).

It is generally and widely accepted that the measurement of the serum albumin can be related to the clinical state of the child (160). Normally nourished children have serum albumin levels above 3,5 g%. Values between 3.5 and 3.0 are borderline low. Values below 3.0 are abnormal and below 2,5 gr% are clearly pathological and usually associated with oedema.

2.III.2 Biochemistry in kwashiorkor.

Detailed studies have been undertaken regarding fluid and electrolytes(160,161,162). The total body water is increased, mainly due to wasting of tissues, specially fat and muscle. This increase in total body water is mainly extra cellular. Total body sodium is increased and is associated with raised intracellular concentrations. There is often hyponatraemia and hypotonicity of

the extra-cellular space and a deficit of intracellular potassium, exaggerated by the inability of the kidney to conserve potassium. An explanation for the intracellular loss of potassium and gain of sodium molecules is a failure of the sodium pump due to impaired ATP production and utilisation.

Vanadate, a micro nutrient, regulates the sodium transport over the cell membrane, by inhibiting the Na-K ATPase (163). Burger and Hogewind(164) detected a statistically significant lower concentration of vanadium in sera from kwashiorkor[50ng/ml] compared to controls [83ng/ml].

Allyene (165) demonstrated a reduction of glomerular filtration rate and renal plasma flow through the kidneys. There is also an impaired tubular function. The ability of the kidney to produce a concentrated urine and excrete a sodium and water load is also impaired (160,166). Golden(167,168) argued that there is no direct relationship between serum albumin level and the degree of oedema, nor in its rate of clearance during treatment. Most workers now agree that the oedema in kwashiorkor is mainly due to excess salt and water retention. The mechanism thought to be responsible is in the sodium pump. In kwashiorkor an increased transport rate of sodium in the red blood cells and leucocytes was found, a mechanism presumed to result from increased membrane permeability. A similar state in the renal tubular cells could enhance sodium reabsorption (169).

### 2.III.3 Protein metabolism in kwashiorkor

Kwashiorkor is characterised by a low serum albumin concentration

kwashiorkor and underweight. This classification is based on weight for age, and the presence or absence of oedema. The normal or expected weight for age is taken as the 50th percentile of the Boston standards(102). Marasmus is applied to children who are less than 60% of the expected weight for age and have no oedema, while marasmic kwashiorkor is applied to those children who have less than 60% of the expected weight for age but have oedema. Kwashiorkor applies to those children who have oedema and are between 60 and 80% of the expected weight for age. Children who are 60-80% of the expected weight for age but have no oedema are classified as underweight.

Many mothers tend to forget the age of the child once they are over the age of one year. To describe the nutitional status of children independent of age , a description of the weight for height is used, a method which also makes it easy to distinguish stunting,-indicating a chronic malnutrition-, from wasting , indicating a more recent malnutrition.

A method very easy to use in very remote areas is the measurement of the mid arm circumference (MAC). This MAC is said to remain fairly constant between the age of 1-5 years(182). Other anthropometric measurements are MAC/head circumference (183) and the MAC/height (184). Skinfold thickness is difficult to measure. The variation between the results of different anthropometric methods to detect malnutrition is marked (185).

The Harvard growth standard was designed from the results of longitudinal measurements of Boston caucasian children during the

period 1930-1956 (182). The use of percentiles has its limitations in areas where a large proportion of the children are less than the 3rd percentile, and thus standard deviation scores expanded to include children 3-4SD below the mean are helpfull (186).

2.III.5 Results of animal experiments

For animals to take a low protein diet with sufficient energy, it has often been necessary to force feed them. In this regard a comparison can be made to the forced feeding in kwashiorkor children (170). During these experiments many animals died of hypothermia and infections due to organisms that would be non pathogenic in healthy animals (187,188). In animals developing oedema, the histological and biochemical features of kwashiorkor were demonstrated: fatty liver, thymus atrophy, hypoalbuminaemia, distorted serum aminoacid patterns(189,190,191). Monkeys if fed on corn ad libitum, developed a syndrome similar to kwashiorkor(191,192), but when the diet was restricted, or the corn diluted with sugar, the result was a marasmic picture. Since corn is one of the foods most commonly contaminated by these toxins it is most regrettable that no aflatoxin analyses were done in these experiments. Others found that monkeys, restricted in energy, maintained their serum albumin concentrations and appeared to 'adapt' to a low protein intake, whereas a low protein/high energy diet resulted in hypoalbuminaemia(193,194). The aflatoxin content of diets used in all these experiments was not known.

Ultrastructural studies(189,195) on the livers of rats fed low protein diet showed a massive breakdown of the endoplasmic reticulum with extensive degeneration of polysomes. The mitochondria often contained irregular cavities or vacuoles. there was reduction of RNA, but not of DNA, suggesting that the cell population remains constant, while cellular content is lost. Refeeding the animal promted regeneration of the endoplasmic reticulum and reformation of polysomes. Studies on baboons(196) showed similar changes, but no change in the mitochondria. Animals with or without fatty livers, showed the same ultrastructural changes.

It can be confidently stated that a low level of plasma albumin is a constant finding in children with kwashiorkor. The question however remains: why is there a low serum albumin? The theory that a diet low in protein/energy ratio is responsible for the low serum albumin level became untenable when Gopalan (156) showed that there was no difference between the protein/energy ratio's of the diets in children developing either marasmus or kwashiorkor. Gopalan proposed that kwashiorkor was a failure to adapt to the low protein/energy ratio. However he gave no clue as to what caused this failure to adapt. The hypothesis that aflatoxins play an important role in the pathogenesis of kwashiorkor, proposed by Hendrickse(108), provides rational explanations for many observations recorded on children with kwashiorkor that have been difficult or impossible, to reconcile with previous theories of aetiology and pathogenesis. Evidence

accumulated in studies to date indicate that there are significant differences in the metabolism of aflatoxins between children with kwashiorkor and normally nourished and marasmic children. Whether these differences reflect disordered aflatoxin metabolism in kwashiorkor that are secondary to the disease state or, as Hendrickse proposes, are causally related to kwashiorkor has as yet not been determined though circumstantial evidence is mounting in support for a causal relationship.

We have done a study in Kenya to investigate relationships between aflatoxins and kwashiorkor to determine the validity of the hypothesis for a causal role for these toxins related to their altered metabolism.

We have also studied the effects aflatoxins may have on human foetuses and newborns. A further field of study was to determine the aflatoxin content of needle biopsy samples of liver tissue kwashiorkors and neonates.

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conditions favour fungal growth and toxin production, which are optimal at a temperature of 27 °C and a relative humidity of >85% (1,2). Many types of food in the tropics are contaminated by aflatoxins(1,2).

Aflatoxins are known to be hepatotoxic to a wide variety of animals. No experimental animal so far has been found to be immune to the effects of aflatoxins, though species susceptibility varies greatly(3).

The biochemical effects of aflatoxins are characterized by inhibition of protein, enzyme, and clotting factor synthesis, as well as depression of glucose metabolism, fatty acid synthesis, immunosuppression, and fatty degeneration of the liver(2). In man aflatoxins have been implicated in Reye's syndrome(4), hepatitis(5) and liver cancer(6).

In 1982 the question was raised whether aflatoxins could be a factor in the aetiology and pathogenesis of kwashiorkor(7). Kwashiorkor was first described in the 1930s (8) and was thought to be the result of a diet deficient in proteins with a relative excess energy, in contrast to marasmus in which a diet deficient in calories is accepted as the causative factor. Gopalan showed in 1968 that there were no essential differences in the protein:energy ratios of the diets of children who developed marasmus and those with kwashiorkor(9).

The present study was carried out in Kenya to determine whether the frequency of detection and quantity and type of aflatoxins in children suffering from kwashiorkor, differ from that found in children with marasmus, marasmic kwashiorkor and a

control group of well nourished children. The study also extends our epidemiological knowledge about aflatoxins and malnutrition.

3.3 Patients and methods

The study was undertaken at the Kyeni Consolata mission hospital, in the Embu district of Kenya. Its altitude is 1500m. Temperature varies through the year from 18 °C to 30 °C. Annual rainfall is 125 cm. The area is inhabited by the Embu tribe, whose main staple food consists of maize, beans and millet(10). During the period from October 1984 until January 1985 the nutritional status of 31 children admitted to the hospital was classified as kwashiorkor [n=14], marasmic kwashiorkor [n=6] or marasmus [n=11] according to the Wellcome Classification(11).

A group of 10 children with normal weight- for- age i.e. bodyweight above 80% of Boston 50th percentile, who were admitted for various conditions like burns, respiratory tract infections,trauma etc. served as a control group. Table I shows the number in the groups studied and their mean age.

Table I. Study groups, number of children and their mean age in months.

| Group | Number of children | Mean age(months) |
|---|---|---|
| Controls | 10 | 43 |
| Marasmus | 11 | 60 |
| Marasmic kwashiorkor | 6 | 48 |
| Kwashiorkor | 14 | 54 |

Blood for aflatoxin analysis was withdrawn preferably on the day of admission (but due to various reasons this was not always possible). Urine samples were collected on the same day that blood was collected. All samples were kept frozen and sent to Liverpool by air, in an ice holding container. The samples were analysed for aflatoxins by high performance liquid chromatography, using fluorescence detection. Analysis on serum was performed after hexane partition and chloroform extraction and on urine after chloroform extraction and a silica column clean-up. The methodology used, permitted detection of the seven major aflatoxins: B1,B2,G1,G2,M1,M2 and aflatoxicol.

3.4 Results

Aflatoxin concentrations are shown as the sum of different types of aflatoxins.

(a) Serum analysis

A total of 39 bloodsamples were screened for aflatoxins, which were detected in all nutritional groups.

Aflatoxins were detected least often in the controls (30%) and in children with marasmus (36%). The detection rate in children with marasmic kwashiorkor was higher at 50%. Aflatoxins were detected most frequently in kwashiorkors:64%. Results are shown in Table II.

(b) Urine analysis

A total of 36 samples was tested for aflatoxins, which were found in all nutritional groups. Aflatoxins were detected most frequently in the control group (75%), less frequently in

marasmic kwashiorkor (60%) and marasmus (45%), and least frequently in the urine of kwashiorkors: 42%. Results of urine analysis are shown in Table II. The differences in detection rates are not statistically significant.

Aflatoxin concentrations (mean and range) detected in serum and urine are shown in Table III. Mean aflatoxin concentration in serum was the highest in the kwashiorkor group, where also the highest concentration of aflatoxin B1 was found: 66588 pg/ml.

Table II. Aflatoxin detection in the different nutritional groups

| Groups | Blood | | | Urine | | |
|---|---|---|---|---|---|---|
| | No.tested | No.positive | (%) | No.tested | No.positive | (%) |
| Controls | 10 | 3 | (30) | 8 | 6 | (75) |
| Marasmus | 11 | 4 | (36) | 11 | 5 | (45) |
| Marasmic kwashiorkor | 4 | 2 | (50) | 5 | 3 | (60) |
| Kwashiorkor | 14 | 9 | (64) | 12 | 5 | (42) |

Table III. Mean aflatoxin concentration (range) in the different nutritional groups in serum and urine in pg/ml

| Groups | Blood | Urine |
|---|---|---|
| Control | 759 (50 - 1680) | 223 (3 - 533) |
| Marasmus | 3412 (99 - 9571) | 261 (6 - 986) |
| Marasmic kwashiorkor | 386 (41 - 917) | 1294 (60 - 4425) |
| Kwashiorkor | 6666 (16 - 66588) | 324 (40 - 1370) |

## 3.5 Discussion

Our findings show that children in Kenya are exposed to aflatoxins in their diets, and these toxins can be detected in the blood and urine of normal and malnourished children. The frequency of detection and the concentration of aflatoxins in blood and urine differs, however, with the nutritional status of children.

Aflatoxins were found most frequently and at highest concentrations in blood of children with kwashiorkor compared with other nutritional groups. Conversely, aflatoxins were detected least frequently in the urines of kwashiorkors compared to other groups and at levels that were disproportionately low in relation to blood levels recorded.

The frequency of aflatoxin detection in this study is higher than the frequency reported in a previous larger study done in the Sudan(7). The reason for the higher detection frequency might have been due to the fact that this part of Kenya was hit by a drought in the first part of 1984 which caused harvest failure. When the short rains started in September, fungal growth may have had a favourable medium in growing on old maize, still present from the previous year. Endorsing this hypothesis is the fact that in the 3 previous years, kwashiorkor was only very seldom seen. Our findings in Kenya support the concept that there are special relationships between kwashiorkor and aflatoxins.

The relatively low frequency of detection and low concentrations of aflatoxins in kwashiorkor urines in relation to the high frequency and high concentration in blood, may indicate altered

aflatoxin metabolism in kwashiorkors compared to children with other forms of malnutrition or normally nourished children.

These findings may be interpreted as representing the effect of kwashiorkor on aflatoxin metabolism in children exposed to aflatoxins in their diet, or may suggest that aflatoxins are involved in the pathogenesis of kwashiorkor.

Findings in this study do not help in differentiating between these alternatives.

3.6 Acknowledgement

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CHAPTER 4

# FOETAL AND NEONATAL EXPOSURE TO AFLATOXINS - a hospital based investigation in rural Kenya

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## 4.1 Summary

Studies on 125 primagravidae in rural Kenya revealed aflatoxins in the blood of 54 prenatally. At delivery re-examination of 34 showed aflatoxins in 12 previously negative. The overall detection rate was 53%. Blood from an additional 59 women collected at delivery showed aflatoxins in 53%.
Aflatoxins were detected in 37% of 101 cord bloods. There was no relationship between aflatoxins in maternal or cord bloods. The frequency of detection was significantly higher in maternal and cord bloods during the 'wet' than 'dry' months.
The mean birth weight of females born to aflatoxin positive mothers was significantly lower(255g) than those born to aflatoxin free mothers. Two stillbirths were recorded, in both cases maternal and cord blood showed aflatoxins.
These findings and the adverse effects of prenatal aflatoxin exposure recorded in animal experiments indicate the need for further study of the effects of aflatoxins on the human foetus and newborn.

Key words: Aflatoxin, human foetal exposure, neonatal exposure, birth weight.

## 4.2 Introduction

Aflatoxins are toxic compounds produced by the fungi Aspergillus flavus and parasiticus, when growing under conditions of high temperature(of>27 $^{0}$C)and high relative humidity(>85%), conditions which normally prevail in tropical areas, or are created in badly stored crops. Under these conditions almost any food commodity can be contaminated with aflatoxins(1,2).

There are 4 major aflatoxins, viz. B1,B2,G1,G2 and a number of derivates, e.g. M1,M2 and aflatoxicol.

No animal has been found to be immune to the effects of aflatoxins, though species susceptibilty varies greatly(3). Within species susceptibility differs in regard to sex(4), age(5) and nutritional status(6,7).

The biochemical effects of aflatoxins are characterised by inhibition of protein, enzyme and clotting factor synthesis, as well as depression of glucose metabolism, fatty acid synthesis and phospholipid synthesis(8). The principle biological effects are carcinogenecity(1), immunosuppression(9), mutagenecity(10) and teratogenicity(11). In man aflatoxins have been implicated in livercancer(12,13), acute hepatitis(14,15) and Reye's syndrome(16) and associations have been established between kwashiorkor and aflatoxins(17,18,19).

The effects of prenatal aflatoxin exposure has been studied in animals. Aflatoxins given to rats towards the end of the pregnancy, resulted in foetal growth retardation(20),but no effects were seen when the same dose was given early in pregnancy.

The effects of aflatoxins on pregnant hamsters differed with the stage of pregnancy and the dose given(11). A dose of 2mg/kg had no apparent effect, whether given before or after organogenesis was completed but a dose of 4 mg/kg given on day 8 caused 30% foetal mortality and 23,4% foetal malformations by day 12. The same dose of 4mg/kg given on day 13 had no apparent effects.

Piglets born to sows given aflatoxin during pregnancy, had low serum protein levels and survived poorly(21). They also showed reduced delayed cutaneous hypersensitivity response compared with piglets born to sows not exposed to aflatoxins during pregnancy.

Among 113 male and 95 female rats exposed prenatally and/or postnatally to aflatoxins and observed for up to 36 months, malignant liver tumours developed in 1 male exposed in utero, 1 female exposed postnatally via milk and 2 females exposed in utero and via milk , while no tumours were seen in controls(50 male and 50 female rats)(22).

The extent of aflatoxin exposure in pregnancy in humans and the effects of such exposure are unknown. The present study was undertaken to investigate prenatal exposure to aflatoxins in humans in a rural population in Kenya.

## 4.3 Patients and Methods

At the Kyeni Consolata Hospital, a rural hospital in the Embu district of Kenya, all primigravidae are seen by a doctor at least once during their seventh month of pregnancy, to establish any risk factor for delivery. From March to September 1985, 125 primigravidae were seen and a bloodsample withdrawn for aflatoxin

analysis. During delivery , blood was again taken from 34 of these women. The reduced number at delivery is explained by the fact that a large proportion of women seen prenatally go elsewhere to deliver, or deliver at home. Cord blood was collected from 38 infants.

Blood was also collected at delivery from 59 other primigravidae not seen previously at the antenatal clinic, and cord blood from an additional 63 babies. Blood was collected from mothers within 12 hours prior to delivery and not later than 15 minutes after delivery. The dates of all blood collections as well as the birthweights and sex of the babies were recorded.

All blood samples were refrigerated and sent to Liverpool by air for aflatoxin analysis using HPLC fluorescence detection after hexane partition and chloroform extraction(23). This methodology permits detection of seven major aflatoxins: B1,B2,M1,M2,G1,G2 and aflatoxicol and its sensitivity and reproductability has been validated(24).

Climatic conditions during the study period varied from the long rains from March through till June, followed by a cold dry period in July and August. In September the short rains started, lasting till December. January and February are the hot and dry months of the year.

## 4.4 Results

Aflatoxins were detected in 54(43%)of the 125 maternal blood samples collected antenatally. Re-examination of 34 of these primigravidae at delivery revealed aflatoxins in 13 samples, 12

from women who previously showed no aflatoxins in their blood. The overall detection rate of aflatoxins in these 125 women was 53% (table I). One woman showed aflatoxins both in the antenatal and the natal bloodsample and her infant also showed aflatoxin in cord blood. In the additional 59 primigravidae examined at delivery, aflatoxins were detected in 31(53%).

Table I.

Frequency of aflatoxin detection in maternal and cord bloods.

| | Maternal blood | | Cord bloods | |
|---|---|---|---|---|
| | no | Aflatoxin positive | no | Aflatoxin positive |
| Antenatal | 125 | 53% | 38 | 21% |
| Repeat at delivery | 34 | | | |
| At delivery only | 59 | 53% | 54 | 41% |
| No maternal blood | - | - | 9 | 66% |
| Total | 208 | 53% | 101 | 37% |

| Type of aflatoxin | Amount pg/ml | Amount pg/ml |
|---|---|---|
| M1 and M2 | 12 - 1689 | 17 - 656 |
| B1 | 89 - 11574 | 86 - 6819 |

The types and amounts of aflatoxin in maternal bloods are as follows: aflatoxin M1 and M2 were most frequently detected in quantities ranging from 12-1689pg/ml. Aflatoxin B1 was detected in 13 samples, in quantities ranging from 87-11574pg/ml(Table I). Analysis of the 101 cord bloods (comprising of 38 from the

mothers examined prenatally plus 63 others) showed aflatoxins in 37(37%).(Table I). The majority of cord bloods positive for aflatoxins were from infants whose mothers blood showed no aflatoxin at delivery. Aflatoxins M1 and M2 were most frequently detected in quantities ranging from 17-656pg/ml. Aflatoxin B1 was detected thrice in a range from 86-6819pg/ml.(Table I).

Fifteen of the positive cord bloods were from babies whose mothers also had aflatoxins in their blood at the time of delivery. In this group of 15, there were 2 stillborn babies. One of these was premature (birthweight 1950 grams) and the other a baby weighing 2950 g. No obvious causes could be clinically detected for these stillbirths, and autopsy was refused. No other still births were recorded in the study.

4.4.a Seasonal variation in aflatoxin detection.

The relative frequency of aflatoxin detection was highest in the wet warm months and lowest in the "cold" dry months. The seasonal differences in detection rate of aflatoxin in cordblood and mothers blood at delivery are significant.(Table II)

4.4.b Aflatoxins and birthweight

The mean birth weight of female infants born to aflatoxin positive mothers was 255 g less than the mean birthweight of females born to aflatoxin negative mothers but the mean birth weight of males born to aflatoxin positive mothers was 132 g more than the mean weights of males born to aflatoxin negative

Table II. Seasonal variation in aflatoxin detection in maternal and cord bloods.

| Source of blood | March to June 'wet' season | | July to September 'dry' season | | |
|---|---|---|---|---|---|
| | No. tested | Aflatoxin positive No. (%) | No. tested | Aflatoxin positive No. (%) | X2(1df) P= |
| Mat. antenatal | 68 | 34 (50) | 48 | 17 (35) | X2 1.873 P= n.s |
| Mat. at Delivery | 38 | 22 (58) | 55 | 19 (35) | X2 4.068 P=<0.05 |
| Infant, cord | 48 | 25 (52) | 53 | 12 (23) | X2 8.18 P=<0.005 |

Table III. Birth weights by sex and in relation to presence or absence of aflatoxin in mothers blood at delivery.

| Mothers'blood aflatoxins | Birth weights(g) Male | | | Female | | |
|---|---|---|---|---|---|---|
| | n | mean | (SD) | n | mean | (SD) |
| positive | 11 | 3066 | (419) | 24 | 2845 | (344)* |
| negative | 23 | 2934 | (383) | 23 | 3100 | (326)* |

* P=<0.025

mothers. Analysis of variance showed a significant interaction between sex, birth weight and presence of aflatoxins in maternal blood at delivery (F=5.25, 1,77df: p<0.02).(Table III)

Low birth weight, high perinatal and neonatal mortality and a high incidence of neonatal jaundice (often unexplained) characterise neonatology in much of the tropics. It will be important to determine whether aflatoxin exposure in pregnancy and the newborn period contribute to the problems in humans as they have been shown in animals(20,21).

4.6 Acknowledgement.

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CHAPTER 5

AFLATOXINS IN LIVER BIOPSIES FROM KENYA - Results of the aflatoxin analysis of 15 liversamples from a rural hospital in Kenya

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## 5.1 Summary

The results of aflatoxin analysis of 15 needle liver biopsies from a rural hospital in Kenya are reported. Nine of the biopsies were from living subjects and six were post mortem. Blood and urine collected the same day was also analysed for aflatoxin content. Aflatoxins in the liver specimen from living subjects were only found in 3 out of 5 cases of hepatocellular carcinoma, but not in their blood. Aflatoxins were detected in 4 out of 6 post mortem liver specimen. In all three cases of cirrhosis aflatoxins were detected in blood and urine, but only one liver specimen showed aflatoxins.
Our findings support the existing theory about the incrimination of aflatoxins in the aetiology of hepatocellular carcinoma, and the possible role aflatoxins may have in the pathogenesis of some forms of liver cirrhosis.

Key words: aflatoxins; hepatocellular carcinoma; cirrhosis; liver biopsie; Kenya.

## 5.2 Introduction

Aflatoxins are toxic metabolites, produced by certain strains of Aspergillus flavus and A. parasiticus, that can contaminate many food commodities in tropical climates or under bad storage

conditions(1). There are 4 major aflatoxins,viz.,B1, B2, G1, G2 and a number of derivates, e.g., M1, M2 and aflatoxicol(1,2). After ingestion they are concentrated in the liver where they are metabolised and excreted, either unchanged or as metabolites, in the urine, stool and breastmilk(1,2,3).

Aflatoxins have been implicated in the aetiology of hepatocellular carcinoma[HCC] in Africa(4,5) and Thailand(6), toxic hepatitis in India(7) and Africa(8), and Reye's syndrome in Thailand(9). Aflatoxins were reported to cause liver cirrhosis in malnourished children who accidently were fed on a diet containing a peanut meal heavily contaminated with aflatoxins(10).

Recent research has implicated aflatoxins in kwashiorkor(11).

The evidence of aflatoxin involvement in the aetiology of HCC has derived 1. from animal experiments(12,13), 2. from a correlation between the levels of aflatoxin ingestion and the incidence of primary livercancer in different parts of the world(14) and 3. from detection of aflatoxins in liver biopsies(15,16,17,18). Only one biopsie was taken from a living subject(17). Aflatoxins in post-mortem liver specimen in Reye's syndrome have been found in all of the liversamples in Csechoslovakia, while in controls they were not found(19). In acute aflatoxicosis in Kenya, 2 postmortem liversamples contained aflatoxin B1(8). In France , Aflatoxin B1 was detected in 6 out of 100 autopsy liversamples, from non-selected cases(20). In the Netherlands no aflatoxins were detected in 31 post-mortem liversamples from non-selected cases (H.P. van Egmond, personal communication). Aflatoxins were

present in the livers of all 22 fatal cases of kwashiorkor in Ghana(21).

Recently the first report on the aflatoxin analysis from 40 percutaneous needle biopsies in living subjects was reported(22). The biopsies all concerned children in Sudan. Aflatoxins were detected in 5 out of 16 biopsies from kwashiorkor, but in none of the samples from marasmus or marasmic kwashiorkor children. Further more, aflatoxins were detected in 5 biopsies from children with chronic liver disease, comprising of 2 cases of intestinal schistosomiasis, one case of hepatomegaly due to tuberculosis, one case of cirrhosis and one case of neonatal hepatitis.

We report the results of aflatoxin analysis of 15 needle liver biopsies. Nine of the biopsies were from living subjects and 6 were postmortem.

## 5.3 Patients and methods

This study was performed in the Kyeni Consolata Hospital, a rural hospital in the Embu district of Kenya.

All Liver biopsies from living subjects were taken for diagnostic purposes. Consent for the biopsie was obtained for the procedure and also permission to obtain 2 samples during the same procedure.

Liver biopsies were taken with a Tru-Cut(R) needle under local anaesthesia. Two cores of tissue were taken from the living subjects. One core was sent to Kenyatta National Hospital for histology and the other core was preserved in 10% formaldehyde

and stored deepfrozen, before shipment to Liverpool for aflatoxin analysis. The same day the liver biopsies were taken, a blood and urine sample was collected for aflatoxin analysis and stored deepfrozen before shipment to Liverpool. The 6 post-mortem percutaneous liverspecimen were taken the same day the patient died. Postmortem blood was obtained by percutaneous needle aspiration from the heart.

From the postmortem cases, histology is not available. Specimen were treated in the same way as described above. The total group consisted of 10 adults and 5 children under 15 years of age.

5.4 Laboratory methods

Aflatoxin concentration in serum, urine and liver samples were determined by high performance liquid chromatography using fluorescence detection. This was performed on serum after hexane partition and chloroform extraction(23) and on urine after chloroform extraction and a silica-column clean up(H.P. van Egmond, personal communication). Liver specimens were homogenised and aflatoxins extracted using chloroform and phosphoric acid followed by a pentane wash and a further silica column clean up(24,25). The sample components were separated on an ODS 5u column (HPLC technology ltd, Macclesfield, UK) and detected by a Kratos fluorescence Detection (Kratos SchoeffelInstruments), employing a 365 nm excitation filter and a 418 nm emission filter. The mobile phase consisted of a water:methanol(50:50) mixture at a flow rate of 2 ml/min and pressure of 3500psi. The following standards, obtained from Sigma Chemical Co London UK

were used: B1,B2,G1,G2,M1,M2 and aflatoxicol.

4.4 Results

4.4.a from the living subjects:

Pathological investigations of the 9 liverbiopsies revealed a hepatocellular carcinoma(HCC) in 5, cirrhosis in 2 and hepatitis in 2.

In the 5 patients with HCC, aflatoxin analysis of the 5 liver samples revealed aflatoxins in 3. The type of aflatoxins found were B1 2232pg/g tissue in the first sample, B2 13pg/g and G1 3186pg/g in the second and M1 1418pg/g plus B1 92978pg/g in the third liver sample. Blood analysis for the presence of aflatoxins remained negative in all 5 samples. Urine analysis on 4 samples showed aflatoxin in 1 sample (M1 241pg/ml)(see table 1).

Analysis of the 4 other liver samples were all negative. In the two patients with cirrhosis, both blood and urine samples contained aflatoxins (for blood: M1 429pg/ml and M1 130pg/ml plus M2 99pg/ml. For urine: M1 139pg/ml and M2 36pg/ml resp.) In the two patients with hepatitis blood showed aflatoxin in the first case ( M1 1757pg/ml) , with urine negative, while in the second case blood was negative, while urine not collected(see table1).

5.5.b from post mortem cases:

The 6 liverbiopsies came from patients who died from stomach cancer, cirrhosis, cerebral malaria, marasmic kwashiorkor, peritonitis and a women who died in pregnancy. Aflatoxins were found in 4 of these. These comprise of a patient who died of

Table I. case number, sex, age, diagnosis, type of aflatoxin and its concentration in blood, urine and liver.

| | | | | aflatoxin concentrations pg/ml | pg/ml | pg/g |
|---|---|---|---|---|---|---|
| no. | SEX | AGE | DIAGNOSIS | BLOOD | URINE | LIVER |
| 1 | M | adult | hepatocellulair carcinoma | -ve | -ve | B1 2232 |
| 2 | M | adult | hepatocellulair carcinoma | -ve | | -ve |
| 3 | F | adult | hepatocellulair carcinoma | -ve | -ve | G1 3186<br>B2 13 |
| 4 | M | adult | hepatocellulair carcinoma | -ve | M2 241 | M1 1418<br>B1 92978 |
| 5 | M | adult | hepatocellulair carcinoma | -ve | -ve | -ve |
| 6 | F | 10yrs | cirrhosis | M1 429 | M1 139 | -ve |
| 7 | F | 13yrs | hepatitis | M1 1757 | -ve | -ve |
| 8 | M | 25yrs | cirrhosis | M1 130<br>M2 99 | M2 36 | -ve |
| 9 | F | 9yrs | hepatitis | -ve | | -ve |
| 10 * | M | adult | stomach cancer | M1 498<br>G1 13230 | | M1 423 |
| 11 * | M | 52yrs | cirrhosis | M1 436 | | M1 15909<br>Af1 2157 |
| 12 * | F | adult | cerebral malaria | -ve | | -ve |
| 13 * | F | adult | pregnant | -ve | | -ve |
| 14 * | M | 18mths | marasmic kwashiorkor | M1 739 | | G1 128<br>Af1 280 |
| 15 * | M | 6mths | peritonitis | | | G2 13 |

* = post mortem biopsies ; Afl.= aflatoxicol

advanced stomach cancer (M1 423pg/g), one from a patient who had undergone several times an abdominal tap to relieve his ascites(M1 15909pg/g, plus Af1.2157pg/g) , one patient who died of marasmic kwashiorkor(G1 128pg/g, plus Af1.280pg/g) and a baby who died of a peritonitis, and who had been operated 2 days previously. A woman who died of cerebral malaria and the pregnant woman had no aflatoxins in their liver biopt.

The analysis of the postmortem blood revealed aflatoxins in 3 cases: the patient with stomach cancer(M1 498pg/ml, G1 13230pg/ml), the patient with cirrhosis(M1 436pg/ml) and the patient who died of marasmic kwashiorkor(M1 739pg/ml). (see table 1).

Aflatoxin B1 and B2 were only found in the livers of the HCC patients and not in any other specimen. Aflatoxicol was only found in post-mortem livers. In the post-mortem group there was a good correlation between the aflatoxin results in the blood and in the liver: a positive bloodsample had a positive liversample and vice versa.

## 5.6 Discussion

The detection of aflatoxin in needle liver biopsies of living subjects from Kenya, has not been published before.

Our results of these biopsies showed that in three of the five cases of hepatocellular carcinoma, aflatoxins could be detected in the liver during life. This in in line with the existing theory about the incrimination of aflatoxins in the aetiology of HCC.

Remarkable is in fact that in all five cases of HCC, of which in three aflatoxins were detected in the liver, no aflatoxins were detected in the blood. The precise mechanism of release of aflatoxins from the liver is not known, but it may be that aflatoxins once inside the liver cell are excreted by the gall, rather than through the blood and kidney. In our three cases of cirrhosis, the blood showed the presence of aflatoxins. In one report(10) aflatoxins were supposed to play an aetiological role in the pathogenesis of cirrhosis, but no other reports have confirmed this finding. From our small number, no further conclusions can be drawn as to the role of aflatoxins in this disease.

The technique we used shows that aflatoxins can be detected in needle liver biopsies from living subjects. Due to the small size of livertissue obtained by needle biopsies, it may well be that the results are an under estimation of the real prevalence of aflatoxins in livertissue. On the other hand in positive samples it may well be an over estimation, due to the unequal distribution of aflatoxins in the liver. The precise role of aflatoxins in tropical areas in regard to health and disease needs urgently further investigation.

5.7 Acknowledgement

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CHAPTER 6

AFLATOXINS AND NEONATAL GROWTH AND MORBIDITY.- a cohort study

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## 6.1 Summmary

A cohort of 237 babies born in the month of October 1986 in a rural area in Kenya were studied in respect to neonatal morbidity and weight gain, in relation to aflatoxin contamination of their cord blood or their mothers breast milk. In 27.3% of the cord bloods, aflatoxins could be demonstrated and in 28.6% of the braest milks, thus confirming the wide-spread exposure of human fetuses to prae and post natal aflatoxin exposure. In this study no immediate harmful effects of this exposure could be demonstrated. In three neonatal liver biopsies, taken post mortem, aflatoxins could be demonstrated. Reference is made to a former description of a child who died of aflatoxin poisoning and a child in our series, who died with the same symptoms.

## 6.2 Introduction.

Aflatoxins, metabolic products from the fungi Aspergillus Flavus and Parasiticus, have been implicated in the reduced birthweight of female babies born to primigravidae who had aflatoxins in their serum, at the time of delivery(1). No such effects were found on the outcome of the birthweight of male babies.

In animal experiments, younger animals are more susceptible to the toxic effects of aflatoxins, than older ones(2). Prenatal exposure to aflatoxins in pigs(3), resulted in poor neonatal survival and showed a reduced delayed cutaneous hypersensitivity immune response compared to piglets who were not exposed to aflatoxins in utero. Total serum proteins appeared to be low in all piglets exposed to afatoxin before birth or through nursing. Ambrecht et al (4) studied piglets exposed to aflatoxins both in utero and postnatally via the breastmilk. The weight gain of the piglets exposed to aflatoxins in breast milk was less than the controls not so exposed.(it is not clear whether the control piglets were exposed to aflatoxins in utero). After weaning the piglets showed a normal weight gain. In this same experiment some litters were sacrificed three days after parturition and the organs tested for aflatoxin content. In the livers of animals exposed to aflatoxins in utero and via the breast milk, the mean aflatoxin B1 content for male piglets was 0.25 ug/kg wet liver tissue and for females 0.15 ug/kg wet liver tissue. The significance of the difference observed between the sexes is not clear. Cardeilhac et al. (5) also found an impaired weight gain in piglets exposed to aflatoxins via breast milk. Stunting was clearly evident by the time of weaning and persisted till time of slaughter, with no compensatory growth seen after weaning. Salil et al (6), reported that rats given aflatoxin B1 in late pregnancy had a high incidence of neonatal death amongst their offspring in the first two days after parturition. They found that aflatoxin B1 inhibits the surfactant lecithin formation thus

causing alveolar instability which could have been responsible for the neonatal deaths.

In the light of these findings in pre- and postnatal experimental work on animals, and our observations regarding prenatal aflatoxin exposure and human birth weights, it was decided to study the effects of aflatoxins on neonatal growth and morbidity.

6.3 Patients and methods.

A prospective cohort study was undertaken on all babies born in the month of october 1986 in the St. Mary's Hospital Mumias, in the Kakamega district of Kenya. Children living over 40 km away from the hospial were excluded from follow-up. The date of delivery , birth weight, sex, Apgar scores and presence or absence of any congenital abnormalities were noted. Cord blood was taken for aflatoxin analysis and determination of total serum protein and albumin. After birth appointments were made for the mothers to visit the Mother and Child Health (MCH) clinic for follow-up, after 4 weeks. At the time of follow-up the mothers were questioned about the health and well being of the child in the preceding four weeks and questioned about the food eaten by the family. A limited survey of social circumstances was also undertaken. The baby was examined by a nurse and any sickness recorded, before referral to the doctor (HRdeV) for examination. The mothers were asked to provide a 10 ml sample of mid-feeding expressed breast milk, for aflatoxin analysis. If a child was given any food besides breast milk, the type of food was noted. Children who defaulted from the clinic were visited at home by a

registered nurse, where the same procedures were followed as in the MCH clinic.

A post mortem needle liver biopsy was taken from babies who were stillborn or died in the hospital. These liver biopsies were put in 10% formal dehyde and stored deep frozen before shipment to Liverpool to be analysed for the presence of aflatoxin.

In the calculations for birth weight and/or neonatal growth children with a birth weight below 1000g and twins are excluded from this study to get a more homogenous and representable group.

6.4 Statistical methods.

Since the distribution of the concentrations of aflatoxins were markedly skewed the values were logged before comparison. The results are therefore reported as geometric means. The statistical methods for comparison were done with the help of the Student-t-test(variances separately estimated with adjusted degrees of freedom) and the chi-square test, while the comparison of neonatal growth was done with the help of a regression analysis.

6.5 Laboratory methods

All sera and breast milk were stored deep frozen, before shipment to Liverpool by air in an insulated container. In Liverpool the analysis of the sera for total protein and albumen was performed and the aflatoxin analysis of sera, breast milk and liver biopsies.

Aflatoxin analysis was performed by high performanc liquid

chromatography using fluorescence detection. On sera and breast milk this was performed after extraction and partition. Liver specimens were homogenised and aflatoxins extracted using chloroform and phosphoric acid followed by a pentane wash and a further silica column clean up.

This methodology permitted detection of aflatoxins: B1,B2,G1,G2,M1,M2 and aflatoxicol.

## 6.6 Results

In the month of October 1986, 232 women were delivered of 237 babies among whom there were three with a birth weight less than 1000g, and 5 sets of twins. There were 125 males and 110 females. The sex of 2 infants was not recorded. Four babies were stillborn, two of these weighed less than 1000g. Seven children died in their first week of life: two sets of premature twins, one infant weighing less than 1000g and two children with normal birth weight. Three other children died within a month after discharge of the hospital. After exclusion of all twins and the babies less than 1000g birth weight, the mean birth weight(S.D.) was 3280g(+/-494) for males(n=117) and 3160g(+/-366) for females(n=105).

Six children had minor congenital abnormalities. Four had an extra digit, one had an urogenital malformation and one a small haemangioma. Three of the children with an extra digit showed the presence of aflatoxin in their cord blood.

### 6.6.a Cord blood results.

234 cord bloods were analysed for the presence of aflatoxins

which were detected in 64 specimens (27.3%). The type of aflatoxin most frequently found was M1, which occurred in 33 specimens ( range 13-1726pg/ml, mean =229pg/ml). Aflatoxin M2 was found 22 times(range 72-812pg/ml, mean=245pg/ml). Aflatoxin B1 was found 7 times (range 120-49616pg/ml, mean=2290pg/ml). Other aflatoxins found were G2: 7 times (range 6-28pg/ml, mean=11pg/ml), B2: 5 times(range 6-28pg/ml, mean=14pg/ml) and G1 and aflatoxicol each 2 times.

6.6.b Birth weight and aflatoxin in cord blood.

After exclusion of twins and babies less than 1000g birth weight, there were 222 who had cord blood analysis of which 60 were positive for aflatoxins. (table 1)

Table 1. Birth weights related to presence or abscence of aflatoxins in cord blood.

| Cord blood aflatoxins | All infants | | Females | | Males | |
|---|---|---|---|---|---|---|
| | n | mean BW(SD) | n | mean BW(SD) | n | mean BW(SD) |
| pos | 60 | 3299 (377) | 27 | 3228 (319) | 32 | 3350 (419) |
| neg | 162 | 3201 (465) | 78 | 3137 (380) | 83 | 3251 (523) |

abbreviations used: BW= birth weight; SD= standard deviation.

There were no significant differences between the birth weights of babies with or without aflatoxins in their cord blood in the study as a whole or for either sex.

6.6.c Total protein and albumin in cord blood.

The mean(SD) total proteins concentration in 205 cord bloods was 6.2(+/-0.7, range 3.7-9.1)g/dl and the mean(SD) albumin concentration in 211 cord bloods was 3.8 (+/-0.4, range 2.1-5.3)g/dl. The total protein and albumin determination in cord bloods with or without aflatoxins are shown in table 2.

Neither total protein nor albumin concentrations showed any significant difference in relation to afatoxin in cord blood.

Table 2. Results of aflatoxin, total protein and albumin in cord blood.

| Cord blood aflatoxins | total protein g/dl | | albumin g/dl | |
|---|---|---|---|---|
| | n | mean (SD) | n | mean (SD) |
| pos | 57 | 6.3 (0.6) | 58 | 3.7 (0.3) |
| neg | 148 | 6.1 (0.8) | 153 | 3.8 (0.4) |

6.6.d Weight gain.

Follow-up weight were obtained on 188 infants at an interval after birth that ranged from 15 to 90 days. The mean weight gain was 1205g(SD +/- 607g, range -100-2830g). There was no significant difference in the weight gain recorded for children with or without aflatoxins in their cord bloods.

6.6.e Breast milk aflatoxins.

189 samples of breast milk were obtained at follow-up for aflatoxin analysis. Aflatoxins were detected in 54(28.6%)

samples. Aflatoxin M1 was detected 22 times( range 36-13794pg/ml, mean=794pg/ml), aflatoxin M2 19 times(range 83-38023pg/ml, mean=776pg/ml). Aflatoxin B1 7 times (range 2217-85971pg/ml, mean=20893pg/ml), and aflatoxin B2 5 times (range 23-623pg/ml, mean=69pg/ml). Aflatoxin G1, G2 and aflatoxicol were detected thrice, twice and once respectively.

Using regression analysis, no differences could be demonstrated between the weight gains observed in relation to presence or absence of aflatoxins in breast milk nor was there any significant difference in weight gain of babies exposed to aflatoxins both in cord blood and breast milk and those not exposed to aflatoxins from either source.

Comparison of the weight gains recorded in a subset of 95 babies who were still being exclusively breast fed at the time of follow-up showed no significant difference between those exposed or not exposed to aflatoxins.

6.6.f Morbidity

Nine infants developed neonatal jaundice. No relation could be established between jaundice and cord blood and or breast milk aflatoxin analysis.

The prevalence of skin infections did not appear to be related with the presence or abscence of aflatoxin in either cord blood or breast milk.

In the examination at follow-up, no particular complaints were found in relation to the aflatoxin content of either cord blood or breast milk.

6.6.g Social status and aflatoxin prevalence in cord blood and breast milk.

The social status of the family was related to the kind of work the father had. There were 5 categories, numbered 1-5: i.e. farmer, factory labourer, skilled worker, business man, factory manager. See table 3.

Table 3. Occupation of father in relation to aflatoxin contamination of cord blood and breast milk. See text for details of occupation.

| | cord blood | | breast milk | |
|---|---|---|---|---|
| | ----aflatoxin--- | | ---aflatoxin--- | |
| occupation | pos | neg | pos | neg |
| 1 | 16 | 34 | 12 | 36 |
| 2 | 5 | 25 | 8 | 22 |
| 3 | 12 | 23 | 9 | 23 |
| 4 | 15 | 35 | 16 | 32 |
| 5 | 0 | 17 | 5 | 11 |

for cord blood: $X^2 = 9.827$, df=4, $p<0.05$

for breast milk: $X^2 = 0.941$, df=4, not sign.

In this table it is seen that there seems to be a relation between social status and aflatoxin in cord blood, a relation not found for breast milk.

6.6.g Results in liver biopsies.

Post mortem needle liver biopsies were taken from 11 infants. The results of the aflatoxin analysis in the liver samples are shown in table 4, together with data concerning sex, age at time of

death, birthweight, aflatoxin analysis of the cord blood, post mortem blood and breast milk.

Table 4. Results of aflatoxin analysis of liver biopsies, post mortal(P.M.)blood, cord blood and breast milk of 14 infants who died.

| | | | | Type of aflatoxin | | | |
|---|---|---|---|---|---|---|---|
| no. | age(days) | B.W. | Sex | Liver pg/g | P.M.blood pg/ml | cord blood pg/ml | breast milk pg/ml |
| a | 11 | 3170 | F | M1 24364<br>M2 21309 | - | - | |
| b | 0 | 3500 | M | - | M1 1120<br>M2 330 | - | |
| c | 0 | 980 | F | - | | - | |
| d | 3 | 3150 | M | M1 20213 | | M2 812 | |
| e | ca 28 | 2900 | M | | | - | |
| f* | 1 | 1250 | F | - | | - | |
| g* | 1 | 1510 | M | M1 10161 | | - | |
| h* | 0 | 2100 | M | - | - | - | |
| i* | 7 | 2250 | M | - | M1 169<br>M2 426 | M1 134 | - |
| j | 35 | 3250 | F | | | B1 673 | - |
| k | SB | 800 | M | - | | - | |
| l | SB | 3080 | M | - | | | |
| m | SB | 1100 | M | - | | | |
| n | SB | 700 | M | | | - | |

* = twin ; SB = Still Born ; B.W.= birth weight

Aflatoxins were present in 3 liver biopsies . The first positive sample was from an infant who was born after a normal delivery

and was discharged from the hospital the following day. Nine days later the child was brought back to the hospital because it was ill. At examination the child showed crepitations over both lungs and there was a marked hepatomegaly. These findings were attributed to cardiac failure of obscure cause. In spite of treatment for cardiac failure the child succumbed the following day. A post mortem needle biopsy of the liver revealed aflatoxin M1 and M2 in concentrations of 24364 and 21309 pg/g liver tissue respectively. Blood, obtained by needle aspiration from the heart revealed no aflatoxins.

The second positive liver sample was from an infant who was born after oxytocin induction, because his mother was draining meconium stained liquor for over 12 hours, with no uterine contractions. The Apgar score after 1 minute was 6, and after 5 minutes was 7. The child started twitching soon after birth and was treated with phenobarbital. On the third day the child died. Post mortem needle liver biopsy revealed aflatoxin M1 in a concentration of 20213pg/g liver tissue.

The third positive sample was from the second baby of a twin. This premature infant was born with an Apgar score of 9/10. The baby died the following day due to respiratory distress. Post mortem needle liver biopsy revealed aflatoxin M1 in a concentration of 10161 pg/g liver tissue. His twin sister died the same day, due to the same problem, but her liver biopsy revealed no aflatoxins.

Four samples of post mortal blood were examined and 2 of these contained aflatoxins. Case no.b showed aflatoxin M1 in a

concentration of 1120pg/ml and M2 in a concentration of 330pg/ml, while his cord blood, taken only a few hours before he died had not revealed the presence of any aflatoxin. Case no.i showed aflatoxin M1 in a concentration of 169pg/ml and M2 in a concentration of 426pg/ml in the post mortem blood, while it had shown the presence of aflatoxin M1 allready in the cord blood 7 days before. The child was fed with breast milk, which did not show any aflatoxin. In two infants where liver biopsies showed no aflatoxins, blood taken post mortem showed aflatoxins M1 and M2 in concentrations of 1120, 330 pg/ml and 169, 426 pg/ml respectively.

## 6.7 Discussion

Our results regarding the frequency of aflatoxin contamination of cord blood and breast milk are similar to former reports(1,7) and confirm widespread pre-and post natal exposure of human foetuses and newborns to these toxins. In this study no effects of this exposure could be demonstrated on neonatal weight gain or health. In relation to the social status of the father, factory workers and factory managers scored the lowest in regard to the frequency of aflatoxin contamination of cord bloods. Both these categories are employed by the nearby sugar factory, either as labourers or as managers. They live in the houses immediately around the factory, with no, or limited space to plant their own food, and probably consume mainly commercially prepared foods.

Of particular interest is the finding of aflatoxins in neonatal livers, establishing considerable exposure and subsequent storage

in the liver of these infants. However in the light of the small size of these needle biopsies, probably the frequency of detection is an underestimation of the true prevalence, due to the unequal distribution of aflatoxin through the liver. On the other hand a positive sample - for the same reason - could show a relatively too high concentration of aflatoxins.

The first child described with a liver biopsie that contained aflatoxins, died with the same symptoms as the child described by Serck-Hansen(8) in 1970. Although in both cases there remains some doubt as to the fact whether aflatoxins are the sole cause of death, it remains an interesting observation at least.

Trying to establish the role of aflatoxins in prae and post natal health is difficult, due to many variables that influence this health. Such a type of investigation requires large numbers and knowing the seasonal fluctuation of aflatoxin contamination, needs to be done during different seasons. This small scale investigation has demonstrated the wide spread exposure of these children to aflatoxins. It is therefore urgently required to study the precise effects aflatoxins have on the health of infants in a large scale study , in different seasons of the year.

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CHAPTER 7

# AFLATOXIN EXCRETION IN CHILDREN WITH KWASHIORKOR OR MARASMIC KWASHIORKOR. - a clinical investigation

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## 7.1 Summary

A group of five children with kwashiorkor, seven with marasmic kwashiorkor and one underweight child were given a completely aflatoxin-free diet during the first ten days of admission in the hospital and stools and urine were monitored for aflatoxin excretion. The children with kwashiorkor and marasmic kwashiorkor excreted aflatoxins up to 9 resp. 6 days after admission respectively. No aflatoxins were detected in the stools or urine of the underweight child. In kwashiorkor, urinary excretion ceased after 2 days, while in marasmic kwashiorkor urinary excretion persisted for 4 days. In stools, B1 was the type of aflatoxin detected most frequently in kwashiorkor and least frequently in marasmic kwashiorkor. Aflatoxin M2 was frequently detected in the stools of both groups of children.

Estimates of the total amount of aflatoxins excreted by kwashiorkor and marasmic kwashiorkor indicate that these children are harbouring up to 4 ug/kg body weight at the time of admission.

These findings establish that aflatoxins accumulate in body fluids and tissues in kwashiorkor and marasmic kwashiorkor from which their elimination is slow.

## 7.2 Introduction

Aflatoxins are secondary metabolites(1) of the fungi Aspergillus flavus and parasiticus, when growing under conditions of high temperature(27 $^{0}$C) and high relative humidity(>85%), conditions which normally prevail in tropical areas(2). Under these conditions almost any food commodity can be contaminated with aflatoxins(2). These toxins are known to be toxic to all animals tested, allthough species susceptibility varies greatly(3).

There are 4 major natural occurring aflatoxins, viz. B1, B2, G1 and G2 and a number of derivatives, e.g. M1, M2 and aflatoxicol. The most potent of the aflatoxins is B1(2,4).

In man aflatoxins have been incriminated in the aetiology of hepatocellular carcinoma(5), acute hepatitis(6,7), Reye's syndrome(8,9), cirrhosis in malnourished children(10) and kwashiorkor(11).

In 1968, Gopalan showed that the former theory that kwashiorkor results from a diet low in protein and relatively rich in carbohydrates was no longer tenable. He had found that children could develop either marasmus or kwashiorkor on diets with the same protein/energy ratio(12).

In 1982, Hendrickse (13) suggested a relationship between aflatoxin contamination of food and kwashiorkor,(a) because of the remarkable similarity between these two in geographical and seasonal prevalence and (b) because the biochemical and pathological effects of aflatoxins in animal experiments are remarkably similar to the derangements found in kwashiorkor. In a first study from the Sudan it was found that kwashiorkor children

had aflatoxins in their serum more frequently and at higher concentrations than children suffering from marasmic kwashiorkor, marasmus or a control group of well nourished children. Furthermore it was found that aflatoxicol - a metabolic product of aflatoxin B1, that can be reversed to B1 - was found only in kwashiorkor and marasmic kwashiorkor children(11).

Further evidence of a relation between aflatoxins and kwashiorkor derived from a study in Ghana(14), where 22 autopsy liver specimen from children who died of kwashiorkor all contained aflatoxins, aflatoxin B1 in 20 and aflatoxicol in the other two. Aflatoxins have not been found in 3 post mortem livers of marasmic children(15) .

The clinical and pathological findings related to aflatoxins in kwashiorkor suggest that aflatoxins accumulate in children with kwashiorkor. Little is known about the metabolism and excretion of aflatoxins in humans, but in vitro experiments on human liver homogenates indicate that human liver has a moderately fast turn-over of aflatoxins with an estimated half life time of 13 minutes(16).

Study of the type, quantity and rate of aflatoxin excretion in the urine and stool of children with kwashiorkor could improve our understanding of the metabolism of aflatoxins in these children and might add to knowledge of fundamental aspects of aflatoxin metabolism in humans. A confounding variable in such studies would be aflatoxin contamination of foods consumed by the children during the study.

the quantities of all types of aflatoxin excreted in the period. After 10 days the children remaining in the hospital were given the hospital diet, or were sent to the nutrition rehabilitation centre('Ekama village').

All children routinely received 50.000 U Vit A daily for four days and vit B-complex daily for ten days. Infections were treated as required.

If a child succumbed, a needle biopsie of liver was taken. The biopsies were put in 10% formal dehyde and stored deep frozen till shipment to Liverpool for aflatoxin analysis.

7.4 Laboratory methods

Appropriate investigations related to the childrens clinical condition, were done in the hospital laboratory. The determination of the total serum proteins and albumin and aflatoxin analysis were done in Liverpool. Aflatoxins in serum, urine, stool and liver were determined by high performance liquid chromatography using fluorescence detection. This was performed on serum after hexane partition and chloroform extraction and on urine and stool after chloroform extraction and a silica-column clean up(H.P. van Egmond, pers. communication). Liver specimens were homogenised and aflatoxins extracted using chloroform and phosphoric acid followed by a pentane wash and a further silica column clean up. The sample components were separated on an ODS 5u column (HPLC technology ltd, Macclesfield, UK) and detected by a Kratos fluorescence detection (Kratos Schoeffel instruments) employing a 365 nm excitation filter and a 418 nm emission

filter. The mobile phase consisted of a water:methanol(50:50) mixture at a flow rate of 2 ml/min and pressure of 3500psi. The following standards, obtained from Sigma Chemical Co London UK were used: B1, B2, G1, G2, M1, M2 and aflatoxicol.

7.5 Results

A serious restraint in the continuous collection of urine from the smaller children was the occurrence of excoriations of the skin where the urine collection bag was adhered . As soon as this was apparent the separate collection of urine was stopped.

7.5.a Kwashiorkor

Aflatoxin in urine and stool (see table 1).

All 5 children excreted aflatoxins in their stool at some stage during their admission. The patient followed the longest , was still excreting considerable amounts of aflatoxin in the stool after 9 days of aflatoxin-free food. This was a very cooperative girl of 5 years of age, who to the best of our knowledge, took no food other than that offered by us.

Four children also excreted aflatoxins via the urine during the first 2 days. No urinary aflatoxins were subsequently detected.

The total amount of aflatoxins excreted by these five children ranged from 0.4 ug(excreted in 4 days) to 57 ug(excreted in 9 days), or 0.08 ug/kg to 4 ug/kg body weight.

7.5.a1 Type of aflatoxin found in stool and urine.(table 1)

Of 28 aliquots of stool, 15 were positive for aflatoxins. B1 was found 6 times, M2 5 times, aflatoxicol thrice and M1 twice. On 29

Table 1

Total* aflatoxin excretion (ng) per day in urine and stool of 5 children with kwashiorkor, while on an aflatoxin-free diet

| Case number | I | | II | | III | | IV | | V | |
|---|---|---|---|---|---|---|---|---|---|---|
| Serum total protein (g/dl) | 4.7 | | 4.4 | | 4.7 | | 4.7 | | 4.5 | |
| Serum albumen (g/dl) | 2.3 | | 2.2 | | 1.9 | | 2.3 | | 1.8 | |
| | Aflatoxin excretion in ng/day | | | | | | | | | |
| 24hrs period | Urine | Stool | Urine | Stool | Urine | Stool | Urine | Stool | Urine | Stool |
| 1 | - | - | $M_1$ 580** $B_1$ 1044** | $M_1$ 20 | - | - | - | $M_2$ 1997 | - | - |
| 2 | $M_2$ 108 | - | - | - | - | $M_2$ 52522 | $G_1$ 98 $G_2$ 1 | $M_1$ 86 | $M_1$ 339** $B_1$ 726** | - |
| 3 | - | $M_2$ 5313 | - | - | - | $B_1$ 28 | - | - | - | $B_1$ 2279 |
| 4 | - | $M_2$ 721 | - | - | - | $B_1$ 1223 | 0 | 0 | - | $M_2$ 515 $B_1$ 8 |
| 5 | 0 | 0 | - | - | - | - | 0 | 0 | - | $B_1$ 149 |
| 6 | 0 | 0 | 0 | 0 | - | $B_1$ 1896 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | - | Afl.27 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | - | Afl. 16 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | Afl. 1668 | 0 | 0 | 0 | 0 |
| Total | 108 | 6034 | 1624 | 20 | - | 57.380 | 99 | 2083 | 1065 | 2951 |
| Total excretion | 6142 | | 1644 | | 57.380 | | 2102 | | 4016 | |

* = Total of all aflatoxin recovered unless otherwise indicated $B_1$ = Aflatoxin $B_1$, $M_2$= Aflatoxin $M_2$, $M_1$ = Aflatoxin $M_1$, Afl = Aflatoxicol, $G_1$ = Aflatoxin $G_1$, $G_2$ = Aflatoxin $G_2$.

** = Total 24 hours urine production calculated  0 = No sample available  - = None detected

aliquots of urine, only 4 contained aflatoxins, three of which contained two types of aflatoxins. Aflatoxin B1 and M1 were found twice, and M2, G1 and G2 were each found once.

One child(case 2) died on the seventh day after admission. He had excreted aflatoxins both in stool and urine in the first 24 hrs period and did not excrete any aflatoxin in the three following days. His needle liver biopsy revealed no aflatoxins. Another child(case 5) died on the 12th day after admission, while he seemed to have recovered clinically. He died suddenly during the night. This child had excreted aflatoxins in the stool from day 3 up to day 5 and did not excrete aflatoxins in the urine during the same period. The needle liver biopsy revealed no aflatoxins.

The mean value for serum total protein on admission was 4.6 g% (n=5, range 4.4-4.7)(See table 1). On re-examination after 3-6 days the mean value was 4.6 gr%(n=3, range 4.0-5.2). Two values were available on day 10, resp: 3.9 and 6.2(mean=5.2 gr%).

The mean concentration of albumin in the serum on admission was 2.1 gr%(n=5, range 1.8-2.3); After 3-6 days the mean concentration was 2.3 gr%(n=4, range 1.8-2.6). On day 10, the two available values were 1.7 and 2.6(mean=2.2 gr%).

7.5.b Marasmic kwashiorkor (table 2)

Of the seven children studied, one child studied for 3 days after admission did not excrete any aflatoxins. Two children studied for 4 days only showed aflatoxins in urine in the first 24 hrs. The type of aflatoxin excreted in both was B1. One child excreted aflatoxins only in the urine on the first and third day,

Table 2

Total* aflatoxin excretion (in ng) per day in urine and stool of 7 children with marasmic kwashiorkor, while on an aflatoxin free diet

| Case number | I | | II | | III | | IV | | V | | VI | | VII | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum total protein (g/dl) | 4.2 | | 4.4 | | 3.7 | | 4.2 | | 3.9 | | 4.1 | | 3.6 | |
| Serum albumen (g/dl) | 1.6 | | 1.9 | | 2.0 | | 1.8 | | 2.2 | | 2.3 | | 2.1 | |
| | Aflatoxin excretion in ng/day | | | | | | | | | | | | | |
| 24 hour period | Urine | Stool | Urine | Stool | Urine | Stool | Urine | Stool | Urine | Stool | Urine | Stool | Urine | Stool |
| 1 | - | $M_1$ 190<br>$M_2$ 102 | $M_2$ 174** | $M_1$ 1 | $B_1$ 41* | - | - | - | $B_1$<br>1316* | - | - | - | $B_1$ 22 | - |
| 2 | - | $M_2$ 63 | $M_2$ 663** | - | - | - | $M_1$ 7352 | $M_2$ 9193 | - | - | - | - | - | - |
| 3 | - | $M_2$ 8 | $M_2$ 3873** | - | - | - | - | $B_1$ 176 | - | - | - | - | $B_1$ 27<br>$B_2$ 1 | - |
| 4 | 0 | 0 | 0 | $M_1$ 504 | - | - | $M_1$ 423<br>$M_2$ 239 | $M_2$ 445 | - | - | 0 | 0 | - | - |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $M_2$ 690 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $M_2$ 4608<br>$B_1$ 1408 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | - | 0 | 0 | 0 | 0 | 0 |
| Total | - | 363 | 4710 | 505 | 41 | - | 8014 | 16520 | 1316 | - | - | - | 50 | - |
| Total excretion | 363 | | 5215 | | 41 | | 24534 | | 1316 | | NIL | | 50 | |

* = Total of all aflatoxin recovered unless otherwise indicated $B_1$ = Aflatoxin $B_1$, $M_2$= Aflatoxin $M_2$, $M_1$ = Aflatoxin $M_1$, Afl = Aflatoxicol, $G_1$ = Aflatoxin $G_1$, $G_2$ = Aflatoxin $G_2$.

** = Total 24 hours urine production calculated  0 = No sample available  - = None detected

while another excreted aflatoxins only in the stool during the first 3 consecutive days. Two children excreted aflatoxins both in stool and urine. The excretion in stool continued in one child till the sixth day. Aflatoxins were not found in any urine after the fourth day. The total amount of aflatoxins excreted in this group ranged from nill to 17 ug excreted in 6 days, or from nill to 1.5 ug/kg body weight.

7.5.b1 Type of aflatoxin found in stool and urine (table 2)

Of 29 stool aliquots, 10 contained one or more types of aflatoxin. Aflatoxin M2 was found 7 times, M1 thrice, and B1 twice. Of 30 urine aliquots, 9 contained one or more types of aflatoxin; B1 and M2 were both found 4 times, M1 was found twice and B2 once.

One child died on the fifth day, but the body was removed from the hospital, before a liver biopsy could be taken. Two other children died after completing the study while in the nutrition rehabilitation centre. A child who excreted 105 ng aflatoxin B1 in the urine on the first day of admission, but none subsequently, died on the 17th day. His liver biopsy showed aflatoxin M1 2958pg/g liver tissue. The other death occurred on the 36th day after admission. The liver biopsy did not contain any aflatoxins.

The mean value for serum total protein on admission was 4.0 gr% (n=7, range 3.6-4.4). On re-examination after 3-6 days the mean value was 3.9 gr% (n=6, range 3.1-4.7). On day 10 the mean value was 4.0 gr% (n=3, range 2.9-5.3).

The mean value for the serum albumin was 2.0 gr% on admission (n=7, range 1.6-2.3). On re-examination on day 3-6 the mean value was 1.7 gr% (n=6, range 1.3-2.1). On day 10 the mean value was 1.9 gr% (n=3, range 1.4-2.3).

7.5.c Under weight

The one underweight child was a 20 month old girl, who was a sister of a child admitted with marasmic kwashiorkor. Her stools and urines were negative for aflatoxins during all four days of continuous collection, while her brother(mar.kwash. no 4) excreted aflatoxins in the stool for up to 6 days after admission.

7.6 Discussion

The metabolism and excretion of aflatoxin B1 has been studied in animals. Rhesus monkeys given a radioactive labeled dose of aflatoxin B1 orally, excreted 40% of the original dose in the urine and 42% in the faeces in 7 days. Aflatoxin M1 was the major metabolite in the urine during the first 4 days and accounted for 18-20% of the original dose. Results on studies on faeces indicated that only 15% of the excreted faecal radioactivity was solvent-soluble(19).

The excretion of a single dose of mixed aflatoxins was examined in a lactating ewe(20). About 90% of the detectable aflatoxins were excreted in the first 48 hours. No aflatoxins could be detected in the milk after 6 days and none in urine and faeces after 8 and 9 days respectively. Only 8.1% of the original dose could be recovered in an identifiable form. In an experiment with

rats(21), in which radioactive labeled aflatoxin B1 was given into the peritoneum, it was found that 70-80% of the administered dose was excreted within 24 hours after dosing. The concentration of radioactivity in the liver was five to fifteen times greater than in other tissues. When the animals were sacrificed after 24 hours, the liver contained an amount equal to the remainder of the carcass, each retaining nearly 10% of the original dose. An entero hepatic recirculation for aflatoxins could not be demonstrated in this experiment. These animal experiments confirm that there is considerable species variability in aflatoxin metabolism.

Little is known about the metabolic fate of aflatoxins in humans, in whom information has had to be acquired opportunistically as ethical constraints prohibit aflatoxin feeding experiments. However aflatoxins have been demonstrated in human sera, cord blood, breastmilk, urine, faeces and liver in different population groups(22).

In the Philippines, Campbell et al. examined urines, collected over 24 hours, of people who had eaten aflatoxin contaminated peanut butter(23). The amount of aflatoxin in the peanut butter was measured and the ingested amount calculated. It was estimated that 1-4% of the ingested amount of aflatoxin B1 appeared in the urine as aflatoxin M1 and that the minimum daily consumption of aflatoxin B1 required to produce detectable levels of M1, was 15 microgram/day. Willis(24) reports a woman who twice attempted suicide with purified aflatoxins. On the second attempt, 6 months

after the first, she ingested 35 mg over a period of two weeks. A 24 hours collection of urine, 3 days after the last ingestion of aflatoxin, showed no aflatoxin M1(the urine was only tested for aflatoxin M1).

These reports indicate that in humans considerable amounts of aflatoxins must be ingested before they can easily be detected in urine.

In this study all 5 children with kwashiorkor excreted aflatoxins in their stool and 4 of them also excreted aflatoxins in their urine. The total aflatoxin excretion in these children was considerable and if Campbells calculations related to the quantity of aflatoxin that needs to be ingested for urinary detection are valid, then it would appear that these children were exposed to enormous amounts of aflatoxins in their diet.

In the children with kwashiorkor, no aflatoxins were detected in their urine after the first 2 days, while in children with marasmic kwashiorkor excretion continued up to 4 days. Faecal excretion of aflatoxin in kwashiorkor was still seen up to the 9th day, while in marasmic kwashiorkors excretion in stool had ceased after the 6th day.

The type of aflatoxin most frequent found in stools from kwashiorkor children was aflatoxin B1 and aflatoxicol, while B1 was found least frequently in the stools of the marasmic kwashiorkor, who also showed no stool aflatoxicol. Aflatoxin M2 was found frequently in the stools of both groups. These findings also suggest some difference in the metabolism of aflatoxin in kwashiorkor and marasmic kwashiorkor and are in keeping with

previous findings in Sudan and elsewhere (13,14,22).

The underweight child did not excrete any aflatoxins in stool or urine, while her brother excreted large amounts in both urine and stool. These children lived together and ate the same food prior to admission. It would seem that the underweight child was capable of handling the aflatoxins better than her brother.

The prolonged faecal excretion of aflatoxins in kwashiorkor suggests that there may be difficulty in excreting aflatoxins that have accumulated in the liver. Aflatoxicol which has been found in kwashiorkor livers is a relatively inactive aflatoxin metabolite, which reverts back to aflatoxin B1. Our observation that aflatoxin B1 was the main aflatoxin excreted over a long period of time would support the concept that stored aflatoxicol is gradually converted to B1 and excreted.

The negative results of aflatoxin analysis of the needle biopsies of the liver obtained after death in two cases of kwashiorkor must be interpreted with caution. The amount of aflatoxin extractable from samples weighing a few milligrams is minute and the concentration of aflatoxins in the liver extract could be below the sensitivity of the method of detection employed. Under experimental conditions aflatoxin recovery from liver samples spiked with known amounts of aflatoxin is 76-81%(25).

Findings in this study provide further evidence of associations between aflatoxins and kwashiorkor and show that enormous amounts of these toxins can accumulate in body tissue of children with kwashiorkor. Findings in these children, when compared with

aflatoxin excretion recorded in children ingesting known amounts of aflatoxin in contaminated peanut butter, indicate that the children with kwashiorkor had consumed very large amounts of aflatoxins.

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CHAPTER 8

CLIMATIC CONDITIONS AND KWASHIORKOR IN MUMIAS - a retrospective analysis over a 5 year period

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## 8.1 Summary

Hospital records from a rural hospital in Kenya were retrospectively analysed for the total monthly numbers of admission for kwashiorkor and all other forms of malnutrition, during a 5-year period. These figures were related to the climatic conditions during the year as they derived from a metereological station in the area. The differences in prevalence of kwashiorkor are dicussed in terms of a possible aflatoxin formation during the change of seasons.

## 8.2 Introduction

Many observers have noted that kwashiorkor seems to be predominant in hot and humid climates in the tropics, where its prevalence peaks during the rainy season(1). During this period aflatoxin contamination of foods is also found to be most frequent(2,3). It has also been found that aflatoxin contamination of the sera of primegravidae at delivery and of cord blood occurs significantly more frequently in the warm wet

season in Kenya than during the cold season(4).

In 1982 Hendrickse postulated a role for aflatoxins in the pathogenesis of kwashiorkor, on the basis of its geographical and seasonal prevalence, and similarities between the metabolic effects of aflatoxins in experimental animals and the metabolic derangements seen in kwashiorkor(5).

We have therefore compared the monthly admissions for kwashiorkor and all other forms of malnutrition to st.Mary's hospital in Mumias over a 5-year period with the climatic conditions in this area, to determine whether there is any correlation between the prevalence of kwashiorkor and climatic conditions. St.Mary's hospital is a mission hospital situated in the sugar cane fields of Western-Kenya. Its target population is estimated at about 250.000.

8.3 Methods

Hospital records in the years 1981-1985(incl.) were searched for all admissions with the diagnosis of kwashiorkor(Wellcome Classification (6)). The monthly prevalence of kwashiorkor was compared with the total number of admissions for all other forms of malnutrition.

Climatic conditions including relative humidity, mean maximum temperature and rainfall have been recorded monthly since 1968, by the meteorological station of the Mumias Sugar Factory. Climatic conditions that prevailed during the period covered by the retrospective hospital analysis were obtained from these records.

8.4 Results

8.4.a Admissions

The total monthly admission numbers are shown in table I.

Table I. Total number of monthly admissions of all children with the diagnosis malnutrition, subdivided into children with kwashiorkor or other forms of malnutrition, St.Mary's Hospital Mumias 1981-1985.

| | All other forms | Kwashiorkor | Total | % kwashiorkor |
|---|---|---|---|---|
| Jan | 74 | 20 | 94 | 21.3 |
| Feb | 75 | 25 | 100 | 25 |
| Mar | 56 | 36 | 92 | 39.1 |
| Apr | 58 | 33 | 91 | 36.3 |
| May | 87 | 55 | 142 | 38.7 |
| Jun | 146 | 67 | 213 | 31.5 |
| Jul | 147 | 50 | 197 | 25.4 |
| Aug | 81 | 32 | 113 | 28.3 |
| Sep | 73 | 21 | 94 | 22.3 |
| Oct | 81 | 27 | 108 | 25 |
| Nov | 63 | 21 | 84 | 25 |
| Dec | 72 | 13 | 85 | 15.3 |
| TOTAL | 1013 | 400 | 1413 | 28.3 |
| mean | 84.5 | 33.3 | 117.8 | 28.3 |

An increase in the total admissions for malnutrition is clearly shown in May, June and July. During the rest of the year the total number of admissions for malnutrition remains fairly

constant, ranging from 84-108 cases. For kwashiorkor admissions increase in the months March, April, May and June, and decline in the months of July and August. The lowest number of admissions for kwashiorkor occurs in December. From this table it can be seen, that an increase in kwashiorkor in March, coincides with a decrease in the number for all other forms of malnutrition. The relative frequency of kwashiorkor is the highest in March(39.1%), and remains above the average (28.3%), in April, May and June. The lowest relative frequency for kwashiorkor is in December: 15.3%, when also the lowest absolute number of kwashiorkor cases are admitted.

8.4.b Climatic conditions

The climatological data are summarised in table II and fig I.

The long rains start in March and persist till June with May having the highest rainfall. July is relatively dry, but rainfall increases again in August, persisting till October. November marks the start of the dry season.

Relative humidity rises from the low seventies to above 80% following the onset of the rains and peaking in July before falling well below 80% by October.

The mean maximum temperature varies by only about $4\ ^{0}C$ throughout the year. The lowest mean maximum temperatures ($28\text{-}29\ ^{0}C$) are recorded in the rainy season and the highest mean maximum temperatures($30.4\text{-}31.9\ ^{0}C$) in the dry season December to March.

Table II. Mean monthly rainfall in mm., mean monthly maximum temperature in °C and mean monthly relative humidity(at 09.00 hrs) during the period 1968-1985

| | Mean rainfall(mm) | Mean max.temp(0C) | Relative humidity(%) |
|---|---|---|---|
| Jan | 71.8 | 31.2 | 72 |
| Feb | 104.6 | 31.8 | 73 |
| Mar | 162.3 | 31.9 | 74 |
| Apr | 276.0 | 29.6 | 82 |
| May | 296.3 | 28.9 | 84 |
| Jun | 190.3 | 28.4 | 84 |
| Jul | 137.0 | 28.0 | 86 |
| Aug | 189.1 | 28.5 | 84 |
| Sep | 168.0 | 29.4 | 80 |
| Oct | 177.7 | 29.9 | 74 |
| Nov | 154.5 | 29.6 | 74 |
| Dec | 86.3 | 30.4 | 72 |

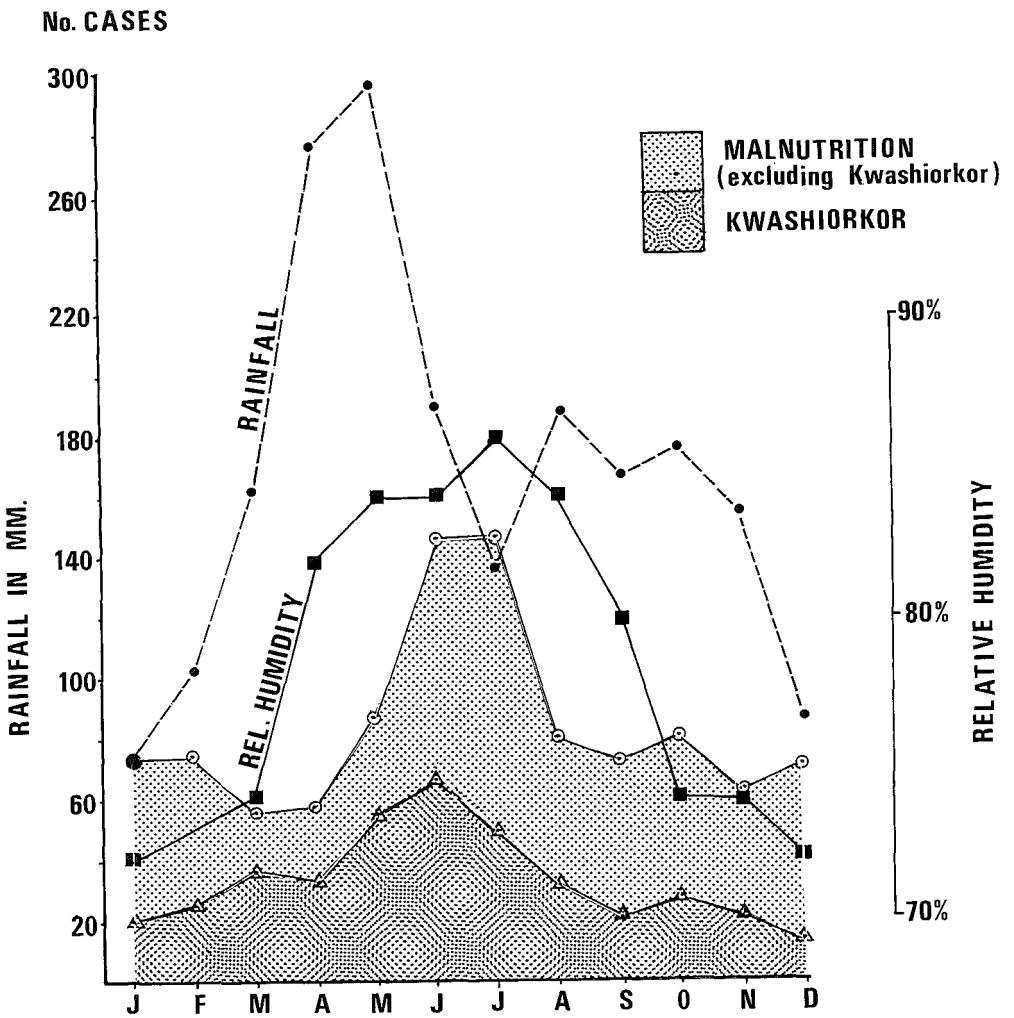
* Source: M.S.C. Meteorological Station No. 8934.133

The figure I shows a clear correlation between relative humidity and the incidence of general malnutrition and kwashiorkor, both of which show a rise and fall and peak prevalence that coincide with changes in relative humidity.

8.5 Discussion

This retrospective analysis shows a marked rise in the frequency of kwashiorkor in the wet season, not only for absolute numbers, but also in the relative frequency. In July, there are still a lot of cases of kwashiorkor, but their relative frequency has

Figure 1. Graphic representation of mean monthly rainfall and humidity, and total monthly admissions for kwashiorkor and other forms of malnutrition during the 5-year period, 1981-5.



gone down. If as postulated, aflatoxins do play a role in the eatiology of kwashiorkor, we could think of the following mechanism: During the months of Januari and Februari there is little possibility for the fungus to produce aflatoxins, because humidity is too low for aflatoxin production. When in March the rains start, there is an excellent environment for aflatoxin production, by possibly a large number of fungus colonies. This could give rise to a sudden change in aflatoxin frequency of contamination, as well as the level of contamination. Seasonal changes in frequency of aflatoxin contamination of sera from gravidae and cord blood (4) and changes in food contamination (2,3) are well documented.

In an experiment with mice, it was shown that aflatoxins do influence the turn over of thus contaminated food, resulting in reduced growth when compared to a control group, fed on aflatoxin free diet. This may suggest a toxic role for aflatoxins in the aetiology of malnutrition.

In July the new harvest becomes available and the number of children admitted with malnutrition and kwashiorkor drops. No survey was done in regard to food availability, nor in regard to the frequency of aflatoxin contamination of food in this area, but seasonal changes in food contamination are well documented(2,3).

Our observation that malnutrition and kwashiorkor do coincide with the change in relative humidity is of general interest: it is remarkable that the graphic pattern of relative humidity differs from the graphic pattern of the rainfall. These

observations are well in line with a possible role of aflatoxins in the aetiology of kwashiorkor and malnutrition.

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CHAPTER 9

GENERAL DISCUSSION

The concept that aflatoxins are an aetiological factor in the syndrome of kwashiorkor has been explored in this thesis and new circumstantial evidence that supports the validity of this theory has been added by the studies undertaken in Kenya . The theory that incriminates aflatoxins in kwashiorkor, as postulated by Hendrickse(1) offers attractive and rational explanations for many observations on kwashiorkor that are difficult to reconcile with previous theories about the aetiology of kwashiorkor, as for example, the occurrence of kwashiorkor in entirely breast-fed children or the observation by Gopalan that on a diet with the same protein/energy ratio children could develop either kwashiorkor or marasmus(2). It also provides an explanation for the occurrence of kwashiorkor almost exclusively in the tropics and the seasonal prevalence of the syndrome which reflects climatic conditions that are favourable for promoting the growth of Aspergillus flavus and aflatoxin production(3). Children with kwashiorkor always have a lowered immune response and aflatoxins are known to be immuno-suppressive(3).

Aflatoxins were found in serum more often and at higher concentrations in children with kwashiorkor, than in children with marasmus, marasmic kwashiorkor or in well-nourished controls(4,5). Our study in Kyeni(chapter 3) gave similar findings. All these studies were cross sectional and as the precise metabolic transformations of aflatoxins and their subsequent excretion are not fully known in humans, there is need

development at which aflatoxin exposure occurs is important in determining different effects(9). It is conceivable that when foetuses are exposed to aflatoxins at a critical stage of their development metabolic processes may be permanently impaired because of changes that take place either at cellular level or at chromosomal level. The mutagenetic properties of aflatoxins have been firmly established(10). The effects of prenatal exposure to aflatoxins may become apparent during early life when the child consumes aflatoxin contaminated food and shows the effects of diminished or altered aflatoxin transformation or excretion.

To further study the role of aflatoxins in kwashiorkor it will be necessary to study the whole household of the kwashiorkor child to detect any differences in aflatoxin exposure or aflatoxin metabolism between the kwashiorkor and other children.

The possible influence of prenatal exposure in the aetiology of kwashiorkor can only be explored in a long term prospective study that monitors aflatoxin metabolism in relation to prenatal exposure.

It is extremely difficult to study the influence of one particular factor on foetal and neonatal growth as there are many inter-related variables that influence growth. It was therefore surprising that in a comparatively small scale study in Kyeni(chapter 4) we found a trend towards reduced birth weight in female babies born to mothers who had aflatoxins in their blood at the time of delivery. But, we could not demonstrate any differences in growth pattern or morbidity in relation to the

presence or absence of aflatoxins in either cord blood or breast milk(chapter6). It must be acknowledged however that the duration of follow-up might possibly have been too short, and that differences might have been too subtle to be detected in the relatively small group studied. It is possible also that negative findings were influenced by the climatic conditions during the period of investigation. The study in Kyeni(chapter 4) that showed a trend toward reduced birth weight in females, was done in a different season to the study in Mumias(chapter 6) which showed no differences in birth weight related to cord blood aflatoxin levels. The latter study was done during a period of seasonally reduced aflatoxin exposure which might have affected the results. It is therefore necessary to investigate pre-and post natal aflatoxin exposure in different seasons, and in cohorts conceived and developing in different seasons. In such a study it would be imperative to examine the breast milk, as well as the food stock for aflatoxin contamination.

In this thesis it has become evident that aflatoxin contamination is wide spread in tropical Kenya. The source of aflatoxins is in the food consumed by these people. It is sad to reflect that if western standards for aflatoxin contamination (11) were applied to the food consumed by our patients, the food would be regarded as unfit for animal consumption. The continued consumption of aflatoxin contaminated food by people in the tropics is a cause for great concern and deserves greater attention than is given to it by the medical profession. Findings reported in this thesis indicate that there is urgent need for further study of the

influence of aflatoxins on child health in the tropics.

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CHAPTER 10

SUMMARY

A general introduction to the problem of aflatoxicosis, one of the mycotoxicoses is given in chapter 1. Besides the problems of acute aflatoxicosis, aflatoxins have been incriminated in the aetiology of kwashiorkor. This study was set up, to further explore the role of aflatoxins in kwashiorkor and on other aspects of child health in Kenya.

Chapter 2 describes the discovery of aflatoxins as the causative agent for Turkey'X'disease which killed over 100.000 turkeys in England in 1960. Aflatoxins are produced by the fungi Aspergillus flavus and parasiticus and their production is dependant on temperature and humidity. In the metabolic transformations of the parent compounds, i.e. aflatoxins B1, B2, G1 and G2, metabolites with different qualities appear. The pathological,immunological, biological, biochemical and teratogenetic effects of aflatoxins are reviewed from the literature. The main target organ is the liver.

The evidence for a possible role of aflatoxins in the aetiology of kwashiorkor includes similarities in seasonal incidence and geographical distribution, and similarities in biochemical, pathological and immunological disturbances encountered in kwashiorkor and in experimental aflatoxicosis in animals.

Some other theories about the possible aetiology of kwashiorkor are discussed. That kwashiorkor is characterised by low plasma albumin levels is not disputed by anyone, but the cause of hypoalbuminaemia has not been agreed. Six studies were carried

out in 2 different hospitals in Kenya. The first three were done in Kyeni in the Embu district and the last three in Mumias, Kakamega district.

In the first study, chapter 3, a group of 41 children, admitted to the hospital were nutritionally classified into one of four categories: kwashiorkor, marasmic kwashiorkor, marasmus and normally nourished children. Aflatoxin concentrations were measured in the sera and urines of these children. Aflatoxins were found most frequently and at the highest concentrations in the sera of the children with kwashiorkor, but urinary levels were disproportionally low compared with other groups. These findings might be due to altered aflatoxin metabolism caused by kwashiorkor in children or could mean that aflatoxins are involved in the aetiology of kwashiorkor.

In chapter 4 a study is presented on 125 primagravidae in rural Kenya whose sera were examined at 7 months of pregnancy and at delivery when cord bloods also were examined for aflatoxins. Aflatoxins were found to pass the human placenta, as evidenced by 37% of the cord bloods containing aflatoxins. The frequency of aflatoxin detection was analysed in relation to climatic conditions, and there appeared to be a seasonal fluctuation, with higher frequency in the wet season. The birth weight of the newborn females appeared to be related to the aflatoxin content of their mothers sera at delivery. This suggests that aflatoxins may contribute to seasonal fluctuations in birth weights so often noticed in tropical regions.

Chapter 5 reports findings in 9 needle liver biopsies from living adults and in 6 from post-mortem liver samples. If possible, serum and urine samples were also collected from some of the subjects and analysed for aflatoxins. The living subjects were all suffering from liver related diseases.

Chapter 6 reports on a cohort of babies born in Mumias hospital in the month of October 1986 studied to determine relationships between aflatoxins in cord blood and/or breastmilk and neonatal weight gain or morbidity. This study confirmed widespread pre- and post natal aflatoxin exposure in the cohort, but did not indicate any significant effects of aflatoxin exposure on neonatal morbidity or weight gain.

In chapter 7 a group of 13 children is described comprising of 5 kwashiorkors, 7 marasmic kwashiorkors and one underweight child who were given a completely aflatoxin free diet for 10 consecutive days in hospital and all excreta,(i.e. stool and urine) were collected and measured in 24 hours periods, and aliquots of these examined for theiraflatoxin content. All kwashiorkor children excreted considerable amounts of aflatoxins in their stools, some continuing to do so for up to a week. Four out of the 5 also excreted aflatoxins in their urines, but none for longer than 48 hours. In contrast, in children with marasmic kwashiorkor, urinary aflatoxin excretion continued till the fourth day. In stools, aflatoxin B1 and aflatoxicol were excreted most frequently in the children with kwashiorkor. The underweight child did not excrete any aflatoxins, while her brother, admitted the same day with marasmic kwashiorkor,

excreted large amounts of aflatoxins. These results provide further evidence for an association between aflatoxins and kwashiorkor.

The last study, described in chapter 8, consists of a retrospective analysis over a 5-year period of the monthly admissions to the hospital of children with the diagnosis of kwashiorkor or all other forms of malnutrition. These admission figures were correlated with data from a nearby meteorological station. A relationship was apparent between the peak admissions for kwashiorkor, and other forms of malnutrition, and highest relative humidity.

In the final chapter, chapter 9, the results of the studies are discussed and it is postulated that the relationships that have been revealed between aflatoxins and kwashiorkor could derive from 'dysadaptation' due to pre-natal aflatoxin exposure.

Hoofdstuk 11

SAMENVATTING

De algemene inleiding van hoofdstuk 1 schetst het probleem van aflatoxicosis, een van de mycotoxicoses. Naast het probleem van de acute aflatoxicosis wordt aflatoxine ook in verband gebracht met het ontstaan van kwashiorkor. Deze studie is opgezet om de rol te bestuderen die aflatoxine speelt bij kwashiorkor en de mogelijke invloed die het heeft op de gezondheid van kinderen. In hoofdstuk 2 wordt de ontdekking beschreven van aflatoxinen als de veroorzaker van de zogenaamde turkey'X'disease, waaraan in 1960 in Engeland meer dan 100.000 kalkoenen bezweken. Deze aflatoxinen worden geproduceerd door de schimmels Aspergillus flavus en parasiticus . Deze productie is afhankelijk van relatieve vochtigheid en temperatuur.

Bij de afbraak van de natuurlijk voorkomende aflatoxinen, dat zijn de aflatoxinen B1, B2, G1 en G2, ontstaan metabolieten met andere eigenschappen. Het geheel van biologische, immunologische pathologische, biochemische en teratogene effecten wordt besproken aan de hand van literatuur. Steeds echter wordt de lever als belangrijkste orgaan aangetast.

De theorie dat aflatoxinen een mogelijke rol zouden kunnen spelen bij het ontstaan van kwashiorkor, stoelt op de overeenkomst in geografische verdeling en de seizoens gebondenheid van aflatoxine en kwashiorkor, alsmede op de overeenkomsten in pathologie die men waarneemt bij kwashiorkor en dierexperimentele aflatoxicosis. Er zijn ook andere theorieen over het ontstaan van kwashiorkor, maar er bestaat geen discussie over het feit dat kwashiorkor

wordt gekenmerkt door een laag serum albumine gehalte. De vraag blijft echter: waarom is er een laag serum albumine?
Om de rol van aflatoxinen te bestuderen werden 6 studies uitgevoerd in twee verschillende hospitalen in Kenya. De eerste 3 studies werden in Kyeni, in het Embu district uitgevoerd, de laatste drie in Mumias, Kakamega district.
In de eerste studie, hoofdstuk 3, werden 41 kinderen die waren opgenomen in het hospitaal beoordeeld en ingedeeld in de categorieen: kwashiorkor, marasmus, marastische kwashiorkor en kinderen in een normale voedingstoestand. Aflatoxine concentraties in serum en urine van deze kinderen werden bepaald. Het meest frequent en in de hoogste concentraties werd aflatoxine gevonden in het serum van kwashiorkor kinderen. De gevonden frequentie van aflatoxine in de urine van kinderen met kwashiorkor, was verhoudingsgewijs laag. Deze bevindingen zouden kunnen passen bij een veranderd aflatoxine metabolisme bij kwashiorkor, of zouden kunnen betekenen dat aflatoxinen een aetiologische rol spelen bij kwashiorkor.
In hoofdstuk 4 wordt een studie gepresenteerd betreffende 125 primigravidae, bij wie in de 7de maand van de zwangerschap en tijdens de partus bloed werd afgenomen voor onderzoek op de aanwezigheid van aflatoxinen. Bij de bevalling werd tevens navelstrengbloed afgenomen voor analyse op de aanwezigheid van aflatoxinen. In 37 % van de onderzochte monsters navelstrengbloed werd aflatoxine aangetoond, bewijzend voor een transplacentaire passage. De frequentie waarmede moederlijk bloed en navelstreng -

bloed was besmet met aflatoxinen bleek seizoens gebonden te zijn. Het geboortegewicht van pasgeboren meisjes lijkt beinvloed te worden door het al dan niet besmet zijn van het moederlijke bloed met aflatoxinen. Mogelijk dat aflatoxinen een rol spelen bij de in tropische landen zo vaak opgemerkte seizoensschommelingen van geboortegewichten.

De studie in hoofstuk 5 beschrijft de resultaten van de analyse op aflatoxinen van 9 naald biopten van de lever van patienten en van 6 naaldbiopten van de lever, post mortem verkregen. Zo mogelijk werden ook serum en urine geanalyseerd. Alle levende patienten leden aan een aan de lever gerelateerde ziekte. Er werd geen duidelijke relatie gezien in de uitkomsten van de aflatoxine analyses van serum, urine, of lever biopt bij beide groepen.

Een cohort baby's, geboren in de maand oktober 1986 in het Mumias hospitaal werd bestudeerd om te zien of er een mogelijke relatie bestond tussen besmetting met aflatoxinen van navelstrengbloed of moedermelk enerzijds en de mogelijke gevolgen daarvan op de neonatale groei en/of morbiditeit anderzijds. Deze studie, beschreven in hoofdstuk 6 bevestigt nog eens de wijdverspreide humane pre-en post natale blootstelling aan aflatoxinen, maar vooralsnog konden geen nadelige effecten worden aangetoond.

In hoofdstuk 7 wordt een groep van 13 kinderen besproken, bestaande uit 5 met kwashiorkor, 7 met marastische kwashiorkor en 1 met ondervoeding, die allen werden opgenomen in het hospitaal en daar gedurende 10 dagen een dieet kregen dat volledig aflatoxine-vrij was. Alle faeces en urine werd verzameld in 24-uurs porties, de hoeveelheid gemeten en vervolgens werd een

monster genomen voor de analyse op aflatoxinen.
Alle 5 kinderen met kwashiorkor scheidden aflatoxinen uit via hun faeces, terwijl 4 van de 5 kinderen ook in de urine aflatoxinen uitscheidden, maar niet meer na de eerste 48 uur, in tegenstelling tot de kinderen met marastische kwashiorkor die tot 4 dagen na opname nog aantoonbare hoeveelheden aflatoxinen in hun urine hadden. Bij de kinderen met kwashiorkor werd aflatoxine B1 en aflatoxicol het meest frequent aangetroffen. Bij het kind met ondervoeding werden gedurende meerdere dagen geen aflatoxinen in urine of faeces aangetoond, terwijl haar broer die dezelfde dag met marastische kwashiorkor was opgenomen, grote hoeveelheden uitscheidde.
De laatste studie wordt beschreven in hoofdstuk 8 , die een retrospectieve analyse bevat van het totaal aantal opnames per maand van kinderen met de diagnose kwashiorkor of alle andere vormen van wanvoeding, gedurende een tijdvak van 5 jaar. Deze uitkomsten werden gerelateerd aan de gegevens van een vlakbij gelegen metereologisch station, betreffende de gemiddelde klimaatsomstandigheden ter plaatse. Het lijkt alsof de relatieve vochtigheid, welke bepalend is voor de aflatoxine productie, correleert met het aantal opnames voor zowel kwashiorkor als ook voor alle andere vormen van wanvoeding.
In hoofdstuk 9 worden de resultaten en de consequenties van de 6 studies besproken. Gepostuleerd wordt dat kwashiorkor mogelijk een gevolg zou kunnen zijn van 'dysadaptatie' ten gevolge van pre-natale besmetting met aflatoxinen.

Curriculum Vitae

The author of this thesis, Dr. Harry R. de Vries, was born on the 6th June 1953 in Tiel. He attended the Rijks Hogere Burgerschool in Tiel where he graduated(HBS-B) in 1970. The same year he started his medical studies at the State University of Utrecht. He passed his physician's examination in 1979 and started to work as a Senior House Officer at the Surgical Department of the General Hospital in Zeist till june 1980. After a 3 month period in the Surgical Department of the Antonius Hospital in Sneek he worked as Senior House Officer in the Department of Gynaecology and Obstetrics of the Weezenlanden Hospital in Zwolle. In november 1981 he attended the Dutch Tropical Course for physicians at the Royal Tropical Institute in Amsterdam, followed by a Swahili language course. After this, a 6 months assignment was taken up in the Surgical Department of the Boerhaave Hospital in Harderwijk. In August 1983 he was posted as a general 'tropical' doctor in Kenya with support from the Memisa-Medicus Mundi and OPIT and worked in the Consolata Hospital in Kyeni till November 1985 after which he worked in the St.Mary's Hospital in Mumias till February 1987. From March 1987 to September 1987 he worked as a Research Fellow in the Department of Tropical Paediatrics and International Child Health (haed: Prof. R.G.Hendrickse) of the Liverpool School of Tropical Medicine. Since 1st November 1987 the author has started training in Orthopaedic Surgery (Prof.Dr. B.van Linge) and is currently in the Department of General Surgery( Dr. C.H.J.Stockmann) of the St.Franciscus Hospital in Rotterdam.