Influence of a Protein and Riboflavin Deficient Diet on the Oncogenic Expression of Aflatoxin B1 in the Sprague Dawley Rat of Both Sexes

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SUMMARY

The influence of a protein and riboflavin deficient diet on expression of the carcinogenic potential of aflatoxin B1 was evaluated in the Sprague Dawley rat of both sexes. This diet, which provokes hepatocyte damage and promotes DAB-(diethyl amino azobenzene) and FAA (N_2 fluorethylacetamide)-induced experimental hepatocarcinogenesis in the rat, had a totally opposite effect when aflatoxin B1 was utilized for induction. Examination of the dissemination and fixation of aflatoxin in hepatocytes revealed an abnormally low carcinogen burden. The liver of animals fed a deficient diet was protected against this carcinogen: fluorescence microscopy studies failed to reveal fluorescence commensurate with the high dose of aflatoxin ingested. By contrast, in animals administered the carcinogen in a normal diet, the carcinogen penetrated and accumulated in the hepatocytes until a cancer developed.

Key words: B1 aflatoxin hepatocarcinogenesis Sprague Dawley rat, Male, Female diet

INTRODUCTION

The extensive studies conducted on the effects of a protein-deficient diet on primary liver cancer have given somewhat contradictory results. In 1968, for example, Madhavan and Gopalan demonstrated that a proteinpoor diet (5%) increased the sensitivity of young rats to the toxic effects of aflatoxin B1, yet reduced the number of tumors observed at the end of the experiment in comparison to controls given the same dose of aflatoxin but a 20% protein diet.

Newberne *et al.* (1966) demonstrated that a low protein (9%) diet shortened the interval before the appearance of tumors and increased the number of tumors

obtained in comparison to a control population given a 22% protein diet and the same dose of aflatoxin (375 μg/animal).

Various dietary factors other than protein deficiencies have also been investigated. Vitamin B12 deficiencies, for example, seem to protect 60%-100% of rats from a normally lethal dose of aflatoxin (Rogers and Newberne, 1969-1971). However, when such deficiencies are combined with a low protein carcinogenic diet, the interval before the appearance of tumors is shortened and the number of tumors obtained at the end of the experiment is increased.

Existing data on aflatoxin metabolism provided by the numerous studies conducted on the subject are insufficient to explain the above findings. This may be due to the fact that all of the intermediate metabolites investigated were isolated in vivo and in vitro using solvents. Under such conditions, metabolites that form covalent complexes with cell components which cannot be extracted by solvents (Diamond, 1965) obviously cannot be investigated in this manner. Now, these metabolites are marked carcinogenic fractions retained by the organism. They are the active matabolites implicated in experimental carcinogenic tumors (Cameron *et al.*, 1976).

In view of this situation, we initiated a study to evaluate the influence of a conventional protein and riboflavin deficient diet (Lacassagne 1967 diet n° 31), known to promote the development of experimental cancers induced by DAB or FAA in rats, on expression of the carcinogenic potential of aflatoxin B1. Dissemination of the carcinogen was investigated by fluorescence microscopy on both cellular and subcellular levels in target cells of the Sprague Dawley rat of both sexes. Developed at the Lacassagne Laboratory (Vanves, France) beginning in 1970, this fluorescence technique was described in 1980 (Stora, 1980) : it reveals those complexes which cannot be extracted by solvents and are formed by aflatoxin B1 and/or its fluorescent metabolites and the cytoplasmic and nuclear components of target cells. All of the free or weakly-bound carcinogen is dissolved in alcohols and in toluene during preparation of the histology slides. All that remains on the slides after preparation are the complexes that cannot be extracted by solvents.

MATERIAL AND METHODS

A total of 370 Sprague Dawley OFA rats, 12 weeks old, were purchased from IFFA Credo (France). The 188 males each weighed an average of 350-400 g upon arrival at our laboratory ; average weight of the 182 females was 250-300 g. After a week of acclimatization in the laboratory, the animals were divided into two groups :

— Group I : 166 rats (86 males, 80 females) given a protein (10.32%) and riboflavin deficient diet (Lacassagne diet n° 31) for 15 days

Lacassagne diet n° 31

Rice starch		6,400	g	
Casein		1,200	g	
Corn oil		2,000	cc	PLUS H_2O 1,000 cc
$Ca_3(PO_4)_2$		400	g	
Salt mixture		400	g	
Salt mixture:	$MgSO_4$	252	g	
	NaCl	640	g	
	KCl	320	g	
	$FeSO_4$	56.8	g	
	$CuSO_4$	3.2	g	
	$MnSO_4$	3.2	g	
	ZnCl	4	g	
Vitamins:	B1	20	mg	
	B2	10	mg	
	B6	25	mg	
	Calcium pantothenate	60	mg	
	KI	21	mg	
	Choline chloride	300	mg	

— Group II: 204 rats (102 males, 102 females) fed a diet with a normal balance of proteins and vitamins during the same period (Diet M25 for rats and mice, Etab. Pietrment, France); this diet contains 24.2% proteins.

After acclimatization to the laboratory and to the diet, 46 males and 40 females of Group I were given the same diet n° 31 plus 2 ppm aflatoxin B1 (Sigma Chemical Co., Saint Louis, USA). The remaining 40 males and 40 females of Group I served as controls, and continued to receive diet n° 31 until completion of the experiment.

Group II animals were divided into two subgroups in a similar manner:

— 51 males and 51 females were given 2 ppm aflatoxin B1 incorporated in the M25 diet

— the remaining 51 males and 51 females served as controls and continued to receive the M25 diet until the end of the experiment.

All animals had free access to food and water during the entire experiment.

Group I animals were sacrificed 15, 30, 60, 90, 150, 200, 270, 340 and 400 days after first administration of the carcinogen. Group II animals were sacrificed 15, 30, 50, 100, 150, 200, 260, 320, 350, 390 and 408 days after initial carcinogen administration. Four animals of each of the four study populations ere sacrificed each time. After puncturing the abdominal aorta to drain out all blood, animals were killed under ether anesthesia. The blood collected in this manner was conserved for AFP assays and hormone studies (results to be published ulteriorly). The liver, all

of the endocrine glands and the major organs were immediately removed and weighed. Tissue samples destined for histology examinations were fixed in Bouin's solution. For liver studies, tissue samples were taken from each hepatic lobe.

Samples were then embedded in Paraplast and cut in 4 micron sections for routine histology examination, after staining with hematoxylineosin-saffron, and for fluorescence microscopy without staining, using the technique of C. Stora (1980). Fluorescence microscopy was performed with a Leitz microscope equipped for transmitted ultraviolet light with a UV Osram HBO 200 watt lamp. A BG 38 blue filter and a 1 mm thick UV UG5 filter were inserted between the lamp and the slide. The image was displayed on a K460 screen and photographed with Kodak Ektachrome film (400 ASA) at three different magnifications (original magnification ×80, ×250, ×400). The best definition and most intersting images were obtained with the original magnification ×250. Loss of fluorescence was too great with the other two magnifications, which are valid only when details or an overall view of a lesion are desired.

Body weights, liver weights and the liver/body weight ratios of each sex were considered for analysis of variance in a single plane with two factors (duration of exposure, diet). Resulst were considered significant when the probability of the nul hypothesis was below 5% (Lellouch and Lazar, 1974).

RESULTS

One very important finding of this experiment was the fact that none of the Group I rats who received aflatoxin in their diet n° 31 developed a tumor by the end of the study period, despite the high total dose ingested (approximately 8400 ng per rat). Moreover, histologic examination of liver tissue from these animals disclosed only discrete signs of hepatic congestion. In certain cases, the hepatocytes near the portal spaces and the centrolobular vein showed microvacuolization ; in other cases, microsteatosis was seen, either localized in clumps or, more rarely, diffuse.

Liver tissue from Group I control animals fed only the deficient diet showed comparable lesions, but they were more discrete and were of later onset than those in the animals given aflatoxin in the n° 31 diet.

Group I animals thus tended to exhibit hepatocyte damage caused by the toxicity of aflatoxin and the deficient n° 31 diet : their hepatic lesions in no way reflected the oncogenic expression of the mycotoxin. Incorporation of this mycotoxin in a protein and riboflavin deficient diet thus apparently protected the liver of animals against the carcinogenic activity of aflatoxin.

Fluorescence microscopy was performed to localize the mycotoxin in the liver of treated Group I animals. The degree of fluorescence seen failed to reflect the very high dose of aflatoxin administered : 8400 ng per rat after 400 days. The low

protein and vitamin concentrations of the diet thus interfered with mycotoxin uptake and metabolism by the hepatocytes.

By contrast, Group II animals given the same dose of aflatoxin but in a diet with a normal balance of proteins and vitamins (Pietrement diet M25 for rats and mice) began to develop hepatic tumors 230 days (46 weeks) after carcinogen administration was begun. Tumors developed at about the same rate in rats of both sexes. These first tumors were discovered in animals who died spontaneously, on days other than those normally scheduled for sacrifices in the protocol.

Table 1 *Tumors obtained in Group II.*

Day of the experiment	Observations	Animals with tumors (multiple hepatomas)
320	First hepatic tumor	18/23 males (78%) and 8/23 females (35%)
369	First metastases (lung, kidney, pituitary gland)	
408	Last sacrifice	

Of the 23 males and 23 females who survived more than 320 days and were sacrificed as per protocol until completion of the experinent, 18 males and 8 females developed multiple hepatocarcinomas, with or without distant metastasis.

In this experiment, the most frequent site of metastasis was the lung, and the first pulmonary lesions appeared on day 369. In addition to lung lesions, animals sacrificed between day 369 and the end of the experiment also showed renal metastases. One animal also presented a pituitary metastasis.

Variance analysis of the body weight curves for Group I animals of both sexes did not show any significast difference between controls and treated rats. Similarly, statistical analysis of variations in the weight of the liver for rats of both sexes failed to show any significant difference between controls and treated animals.

By contrast, Group II animals of both sexes thet received aflatoxin in a normally balanced diet lost a significant amount of weight in comparison to Group II controls (Figures 1 and 2). As concerns body weight, treatment appeared to have a constant effect during the entire experiment. However, individual variations were lower in females than in males.

Analysis of variance for liver weight in Group II rats of both sexes revealed correlations with both treatment and the duration of treatment. However, the differences between control rats and treated rats were not constant over the experimental period. The liver weight curves rose gradually for females, who developed their first tumor slightly earlier than males (Figures 3 and 4). The rise on the curve for males was much sharper. For rats of both sexes, the shift on the curve corresponded to the appearance of the first hepatic tumor. Analysis of the liver/

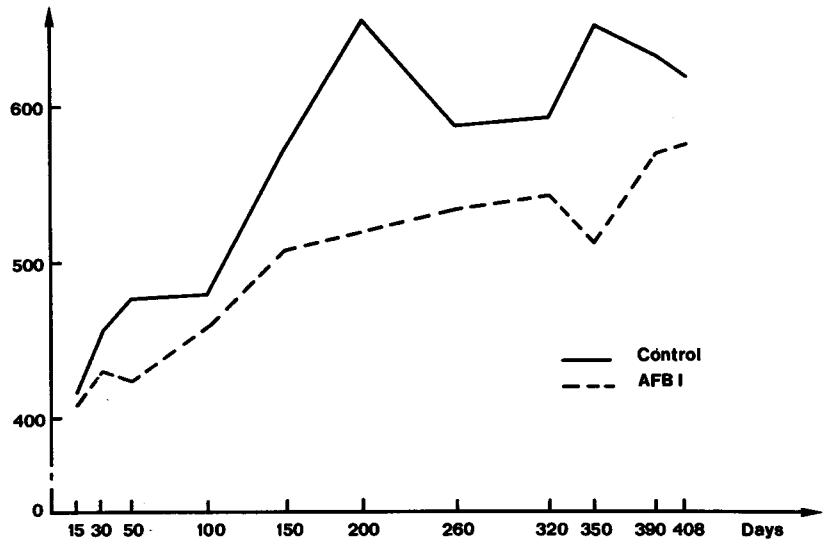

Fig. 1 body weight ♂

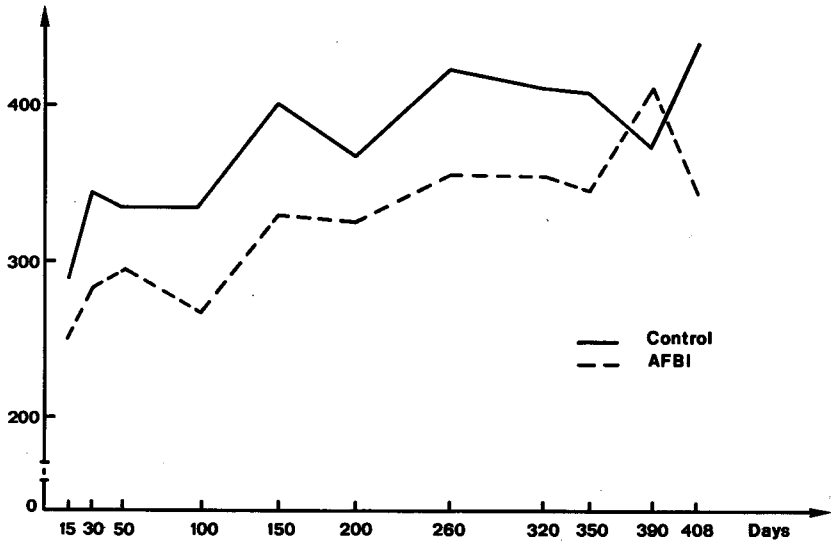

Fig. 2 body weight ♀

body weight ratio provides even clearer evidence of this phenomenon.

In a previous study (Stora *et al.*, 1979), carcinogen localization by fluorescence microscopy in animals fed diet n° 31 plus 5 ppm aflatoxin revealed that the aflatoxin was disseminated throughout the organism and in the liver during the early stages of experimental carcinogenesis. The product arrives by a vascular route and

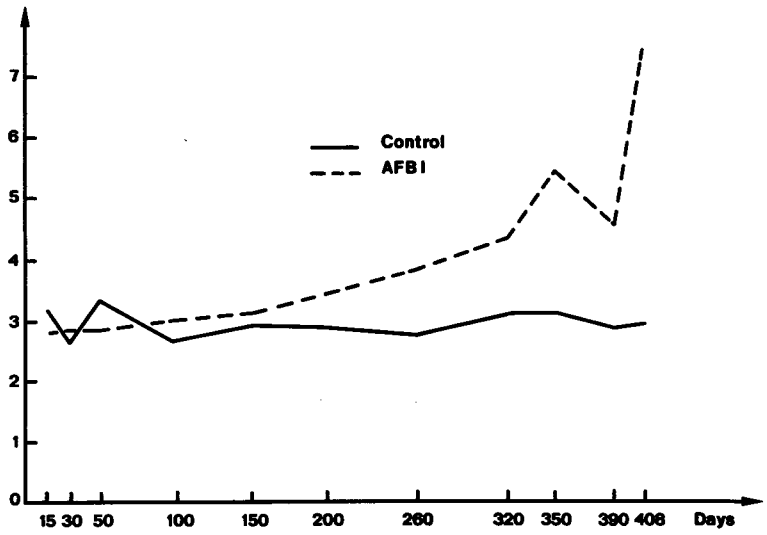

Fig. 3 liver-body weight ratio ♀

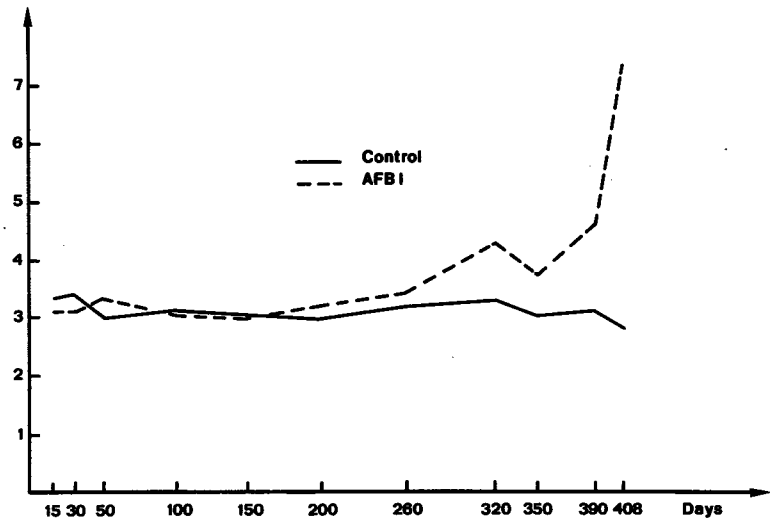

Fig. 4 liver body-weight ratio ♂

accumulates around the portal spaces and the centrolobular vein 24 hr after administration is begun. The hepatic response to this aggression consists in vasodilatation of the sinusoidal capillaries, but this defensive reaction is short-lived. By the fourth day of administration, the carcinogen in already solubilized in the cytoplasm of the hepatocytes. In the present study, microscope examination on the 15th day

revealed diffuse sytoklasmic fluorescence (Photo 1). During the next step (30th day), the carcinogen penetrates the nucleus of the hepatocytes (Photo 2). At this stage, which corresponds to the start of initiation phenomena, the carcinogen can be localized on the heterochromatin in the nucleus. This phenomenon concerns only 25%-30% of the nuclei in a given part of a hepatic lobe. Most of these hepatocytes initiated by the carcinogen from the outset will die : the nuclear membrane develops folds and retracts, the cell dies, and the corresponding histologic lesion is focal necrosis (Photo 3).

A few rare cells survive this initiation period and adapt to carcinogenic aggression by undergoing division : the resultant cells are the precursors of the future cancer cells. This explains why the tumor develops at a particular point in the liver even though the carcinogen is disseminated throughout the organism.

In zones of steatosis, the lipid content of cells shows an accumulation of dark blue fluorescent material. While lipids usually dissolve in the solvents used for histologic preparation of liver tissue, such lipidic fluorescence was photographed on several occasions (Photo 4).

Once a tumor is formed, the cytoplasm and the nuclei of the tumoral cells exhibit strong fluorescence ; the hepatocytes surrounding the tumoral tissue show homogeneous cytoplasmic fluorescence but not nuclear fluorescence (Photos 5).

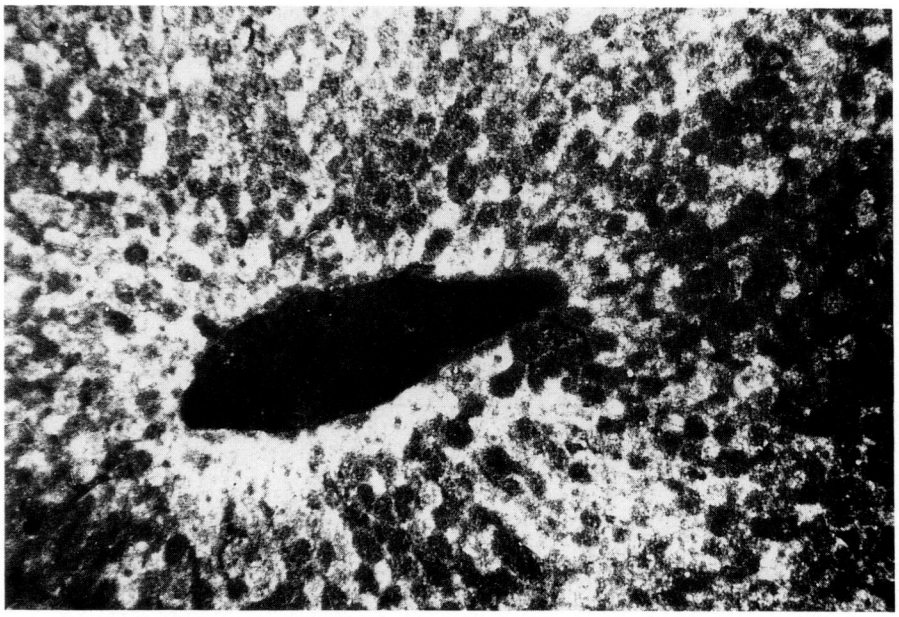

Photo 1 beginning of liver impregnation : diffuse cytoplasmic fluorescence of the hepatocytes (OM×80)

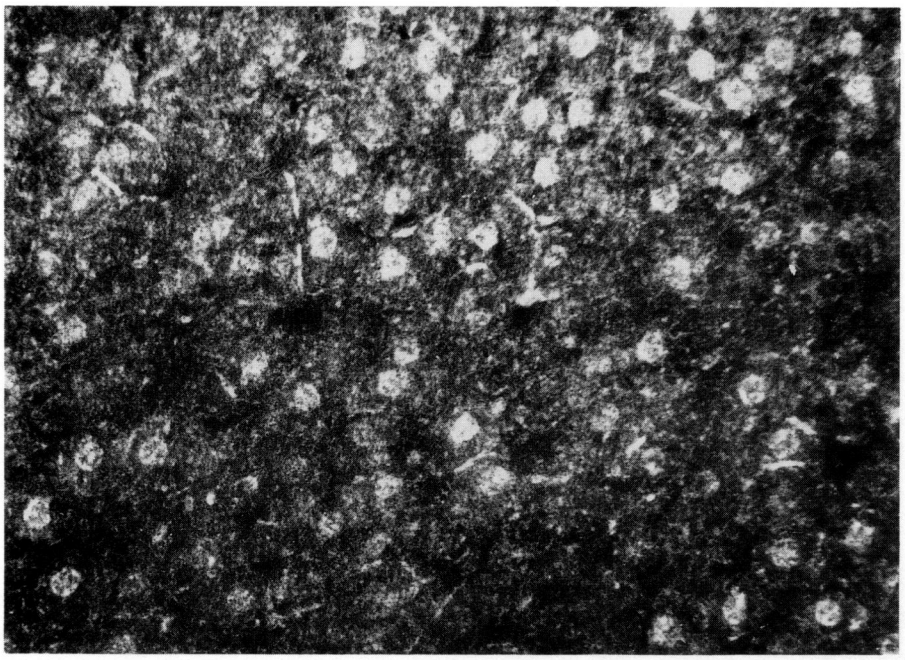

Photo 2 initiation: penetration of the carcinogen inside the nucleus of the hepatocytes (OM× 250)

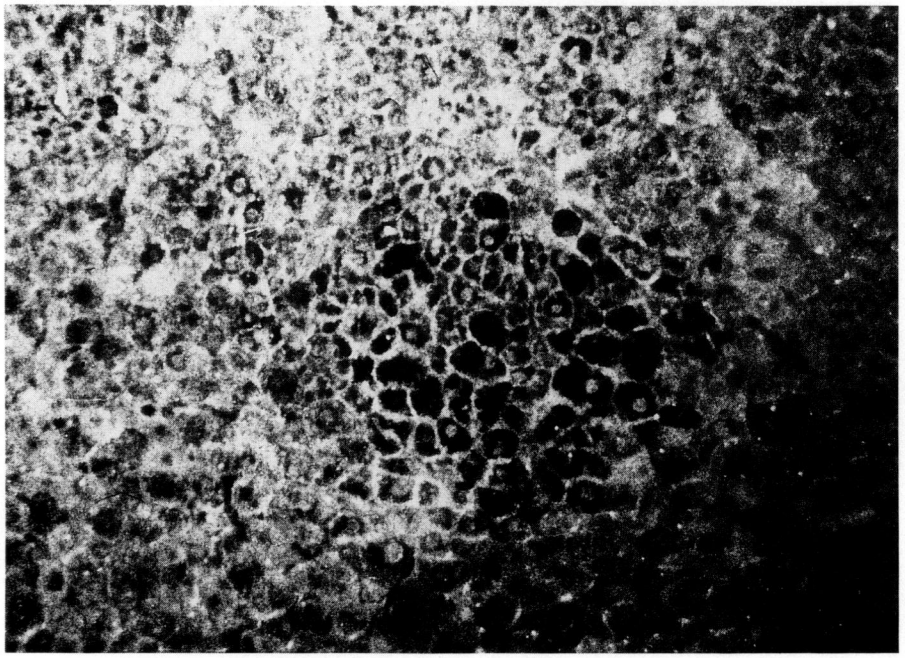

Photo 3 focal necrosis (OM×250)

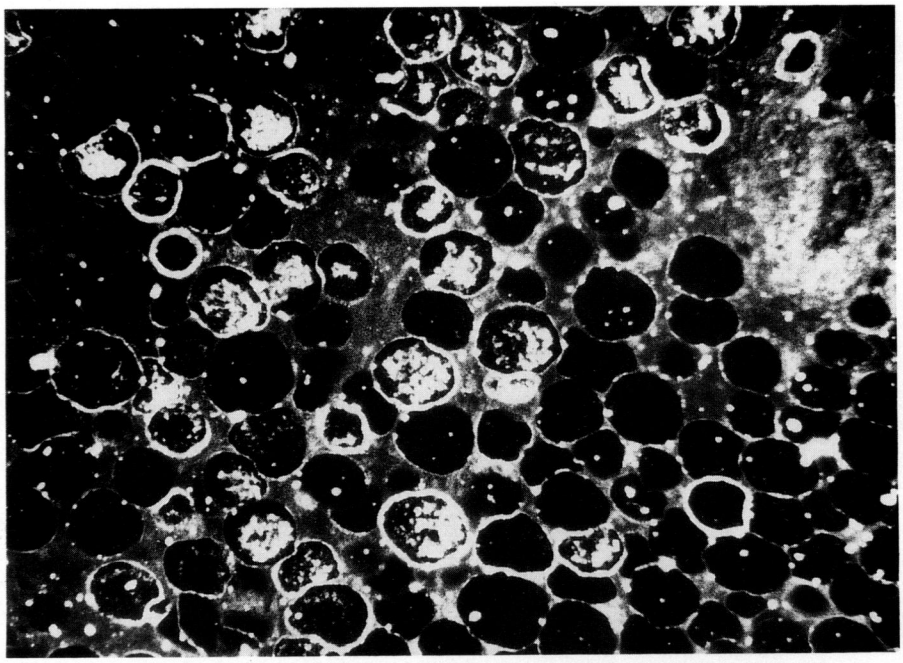

Photo 4 fatty degeneration: carcinogen accumulation in lipids (OM×250)

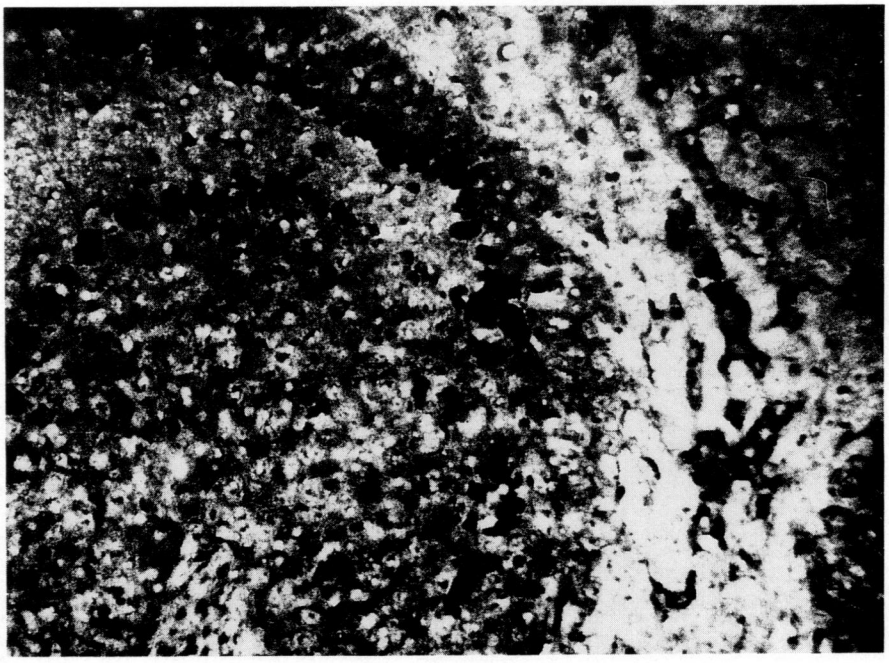

Photo 5 tumor and surrounding liver tissue (OM×80)

These images are comparable to those seen for primary liver cancer in man (Stora and Dvorakova, 1986).

In several cases, vessels contained small clumps of tumor cells which formed a thrombus; the migration of such cells is responsible for distant metastasis.

Most of the metastases observed in this study occurred in the lungs. These pulmonary lesions appeared beginning on the 369th day of the experiment. Metastases were also discovered in other organs (kidney, pituitary gland), but they developed later on and were less frequent.

DISCUSSION

Although, like us, Wogan and Newberne (1967) administered aflatoxin per os in a diet with a normal balance of vitamins and proteins, they did not observe hepatic tumors in females until the 64th week of carcinogen administration, whereas our first tumors were obtained at the 46th week. This is understandable because they used an aflatoxin dose 50% lower than the one we utilized. This finding remains logical despite the fact that aflatoxin is carcinogenic even at very low concentrations, as demonstrated in partially hepatectomized mice (Dix, 1984) and in rats treated with a single dose of AFB1 (Bannasch *et al.*, 1985).

Although simple atmospheric ammoniation has been proved capable of reducing the toxicity of cereals contaminated by Aspergillus flavus, the diet in which the aflatoxin is administered to rats is important. Our diet had a total protein content of 10.32%. Madhavan and Gopalan (1968) reported that a 5% protein diet increased the sensitivity of rats to aflatoxin but also reduced the number of tumors obtained in comparable anmials given the same aflatoxin dose in a 20% protein diet. These authors used very young rats, however, and their study conditions were thus different from ours.

The protein content of our deficient diet was similar to that used by Newberne *et al.* (9%). These authors reported that such deficiencies increased the incidence of hepatic tumors and shortened the interval before their appearance in comparison to a 22% protein diet. However, Newberne *et al.* administered aflatoxin by intubation, and they used a much higher daily dose of the carcinogen than we did (325 μg versus 20 μg). Despite the discordance between these results, they should be kept in mind when evaluating the risks of expesure to aflatoxin in human populations suffering from malnutrition.

Besides its low protein content, our diet was also deficient in riboflavin (vitamin B12) : this combination of two deficiencies might explain the difference between our results and those of Newberne *et al.* (1966). Indeed, in 1967, Lacassagne *et al.* demonstrated that such a vitamin deficiency increased the carcinogenic potential of DAB and FAA. More recently, Domngang and Bassir (1981) reported that the

vitamin content of the diet or drinking water can considerably modify the enzymatic activities involved in the metabolism of aflatoxin. It is thus not surprising that our doubly deficient diet gave the results it did.

The amount of aflatoxin or its metabolites bound to macromolecules in rat liver cells also influences expression of the carcinogenic potential of aflatoxin (Jayarag *et al.*, 1985). These covalent complexes or substances can be detected by fluorescence microscopy. In our deficient diet animals, liver fluorescence was very slight, and in no way reflected the great amount of carcinogen absorbed by these sats. By contrast, in treated rats given the balanced diet, dissemination of the carcinogen in the liver was easily observed during the entire hepatocarcinogenesis process.

This observation is similar to the findings of Campbell (1977), who showed that a low protein diet reduced metabolic activation of the carcinogen, thereby diminishing binding to DNA by around 70%. It is thus understandable why the carcinogenic potential of aflatoxin B1 would be considerably decreased under these conditions (Mainigi and Campbell, 1981).

To conclude, one last point warrants mention: none of our deficient diet rats exhibited any of the endocrine modifications reported with other carcinogens such as FAA. As such modifications constitute one of the organism's defense mechanisms against a carcinogen, we can deduce that administration of a protein and riboflavin deficient diet decreased absorption and metabolic activation of the carcinogen, thereby preventing the formation of hepatic tumors despite the fact that the aflatoxin dose used (2 ppm) was twice as high as the dose which is normally carcinogenic in rats.

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