PROMOTION OF AFLATOXIN B_1- INDUCED HEPATOCARCINOGENESIS BY DICHLORODIPHENYL TRICHLOROETHANE (DDT)

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Abstract. A study of the effect in rats of dichlorodiphenyl trichloroethane (DDT) on hepatocarcinogenesis that is initated by aflatoxin B_1 (AFB_1). In the first experiment, Buffalo rats were given a single oral dose of AFB_1 (5 mg/kg) followed by dietary DDT (100 ppm) for 20 weeks. Neoplastic nodules were observed in 1 of the 14 AFB_1-exposed rats, compared with 3 of the 19 rats in the AFB_1/DDT group. In the second experiment, Wistar rats were given dietary aflatoxin B_1 (4 ppm) for 6 weeks followed by a 6-week exposure to DDT (500 ppm) in a plain semisynthetic diet. Five altered hepatic foci were displayed by seven rats in the AFB_1 group, compared with 6 foci and one neoplastic focus in five of the AFB_1/DDT rats at 32 weeks. Subsequently, the AFB_1 group produced 8 (27.5%) tumor-bearing rats while 10 of the 28 (35.7%) AFB_1/DDT-exposed rats were tumor-bearing by 60 weeks. The results suggest that DDT slightly potentiates hepatocarcinogenesis induced by either a single dose of AFB_1 or short term-dietary AFB_1.

INTRODUCTION

Aflatoxin B_1 (AFB_1), a potent, naturally occurring hepatocarcinogen produced by *Aspergillus flavus* and *A. parasiticus,* has attracted wide interest because of its association with a high incidence of human liver cancer, particularly in Southeast Asia (Shank *et al*, 1972 a,b,c; Angsubhakorn, 1983) and Africa (Alpert *et al*, 1971; Peers and Linsell, 1973; Peers *et al*, 1976; Van Rensburg *et al*, 1985). Furthermore, in an analysis involving 10 of the smaller subregions of Swaziland, aflatoxin exposure emerged as a more important correlate of variation in liver cancer incidence than the prevalence of hepatitis B virus infection (Peers *et al*, 1987; Montesano *et al*, 1997). The problems of aflatoxins in foods and feedstuffs in Thailand need attention, on both economic and health grounds. Dichlorodiphenyl trichloroethane (DDT) is also known to be a potential health hazard due to its chronic ingestion from the environment. The highest levels of fat-soluble organochlorine compounds, such as DDT, are found in the dietary fat of meat, fish and poultry and in diary products; an increase in the intake of animal fat may result in higher dietary exposure to organochlorine compounds (Wissermann *et al*, 1972; Gorchev and Jelinik, 1985). DDT has been widely used in Thailand since 1950: both in agricultural and public health programs, and especially for malaria eradication (Suwankerd and Prajakwong, 1995), it has been found to be a common contaminant of various food and human milk. DDT is still essential for the killing of malarial mosquitos in many tropical countries and was finally exempted by the Treaty on Persistent Organic Pollutants (POP) by the representatives of 122 countries, at a meeting in Johannesburg in December 2000, because there was no effective substitute for mosquito control. (Kaizer and

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Enserink, 2000).

DDT has been shown in chronic feeding experiments with rats to produce a small number of low-grade hepatic cell carcinomas and nodular adenomatoid hyperplasia (Fitzhuch and Nelson, 1947). Other studies with DDT in rats have provided no evidence of carcinogenicity (Haag *et al*, 1950; Cameron and Cheng, 1951; Treon and Cleveland, 1955; Klimmer, 1955; Klimbrough *et al*, 1964; Deichmann *et al*, 1967). In lifespan studies, the incidence of liver tumors in rats given 500 parts per million (ppm) of technical DDT was 56% and 35% in females and males respectively (Rossi *et al*, 1977).

It has been shown that several epigenetic carcinogens, probably of the promotor class like DDT, enhence the carcinogenic effect of previously administered genotoxic carcinogens in rats such as acetylaminofurene (AAF) and 3′-methyl-4-dimethyl-aminoazobenzene (3′Me DAB), which are not carcinogenic by themselves (Peraino *et al*, 1975; Kitagawa *et al*, 1984). How epigenetic hepatocarcinogens work is being clarified by recent advances in our understanding of the mechanism of liver tumor promotion. Our studies were initiated in order to investigate the carcinogen enhancing effect of DDT on AFB_1 that had been administered previously as either a single oral dose or a short term dietary regimen.

MATERIALS AND METHODS

Animals

Male Buffalo rats, from our own colony, aged 6 weeks and weighing about 80 g were used in Experiment 1. In Experiment 2, we used male Wistar rats of at least 1 year of age that weighed 400-500 g; these rats were purchased from the National Laboratory Animal Center (NLAC), Mahidol University, Salaya. On arrival, they were randomly divided into groups of 5 rats per cage, and were adapted to housing conditions for at least 7 days before starting the experiment. All animals were kept in a controlled lighting environment (12 hours dark/light cycle) at a constant temperature of 27ºC. Food and water were available *ad libitum* and cared for according to the guidelines of the National Laboratory Animal Center (NLAC).

Experiment 1: Powdered Chow pellets (Gold Coin Mills Ltd, Jurong, Singapore) were used as the control diet. DDT (p,p-DDT 86%) was dissolved in 50 ml corn oil and the solution was stirred with chow powder to a final concentration of 100 ppm in a mechanical mixer (Ikeden MS-20, Tokyo Ikeden Co Ltd, Japan).

Experiment 2: The semi-synthetic diet (Angsubhakorn *et al*, 1981) was prepared weekly. AFB_1 (Batch No. 10691, Makor Chemicals Ltd, Jerusalem, Israel) was added to the plain semisynthetic diet after dissolving in a small amount of analytical grade acetone (JT Baker Chemical Co, USA) and corn oil. The acetone was then evaporated to minimize aflatoxin decomposition. DDT was also mixed with corn oil and added to the diet at a final concentration of 100 and 500 ppm.

Experimental design

Experiment 1: The experimental design is outlined in Fig 1. AFB_1 was dissolved in dimethylsulphoxide (DMSO, BDH, Poole, England) and was immediately administered by intragastric intubation. One hundred Buffalo rats, except for those 27 rats given 0.1 ml DMSO only (group 1), were given AFB_1 at a dose of 5 mg/kg body weight. One week after DMSO or AFB_1 intragastric intubation, all rats were fed chow pellets (groups 1 and 2) experimental diets (groups 3 and 4) for 20 weeks and then chow pellets until the end of the experiment (84 weeks). Five rats from each group were killed at 1, 5 and 10 weeks after receiving their respective diets. The remaining rats were killed at 82 weeks, when the experiment was terminated.

Experiment 2: In this experiment (Fig 2), 176 male Wistar rats were randomly assigned to 4 treatment groups, as follows. Group 1 (Control): 31 rats were fed plain semisynthetic diet for 12 weeks, followed by chow pellets; Group 2 (AFB_1): 39 rats received a basal diet con-

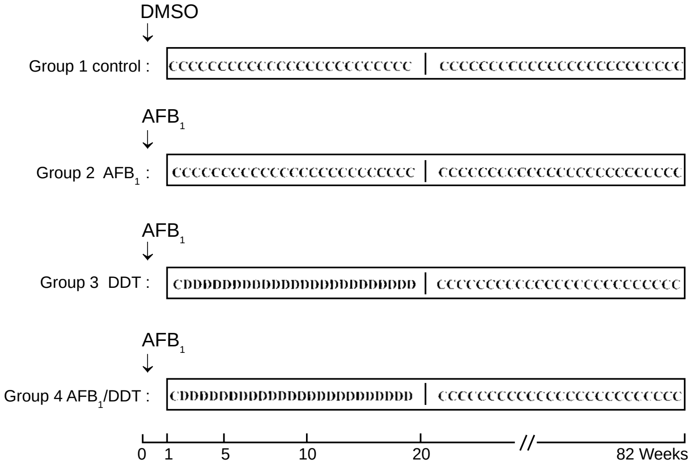

Fig 1–Schematic representation of experiment 1. Buffalo rats were given a single dose of AFB_1 followed by DDT. (A = Aflatoxin B_1; C = Chow pellets; D = DDT; DMSO = Dimethyl sulphoxide).

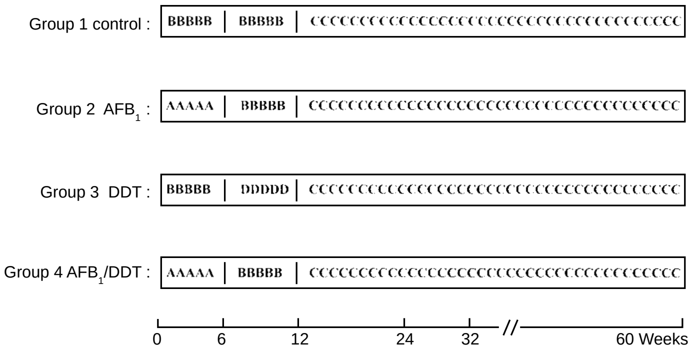

Fig 2–Schematic representation of experiment 2. Wistar rats were given dietary AFB_1 followed by DDT. (A = Aflatoxin B_1; B = Basal diet; C = Chow pellets; D = DDT).

taminated with 4 ppm AFB_1 for 6 weeks, followed by a plain basal diet for 6 weeks, and then chow pellets; Group 3 (DDT): 36 rats were given the basal diet for 6 weeks, followed by DDT for 6 weeks, and then chow pellets; Group 4 (AFB_1/DDT): 53 rats were given dietary AFB_1 for 6 weeks, DDT for 6 weeks, and then chow pellets until the end of the experiment (60 weeks).

At the end of the experiment, all the rats were fasted for 18 hours before being killed. Serum was obtained from clotted whole blood; serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels were determined by standard methods (Reitman and Frankel, 1957). The total protein concentrations of the sera were determined by the method of Gornall *et al* (1948) and the serum albumin concentration was determined using Rodkey's method (1965).

Data collection and analysis

A complete necropsy was performed on each rat; each liver was weighed and carefully examined. Tissues were fixed in 10% buffered formalin, embedded in Paraplast-plus (Monoject, St Louis, MO). Microscopic changes in the liver were diagnosed according to previous descriptions (Squire and Levitt, 1975; Steward *et al*, 1980). All results are expressed as group arithmetic mean ± standard deviation. Statistical comparisons of group means were carried out using Student's *t*-test.

RESULTS

Experiment 1

Rats in group 2 (AFB_1), group 3 (DDT), and group 4 (AFB_1/DDT) showed significant decreases in body weight compared with the corresponding controls ($p < 0.05$) at weeks 1 and 5, weeks 1,5 and 10, and weeks 1,5,10 and 82 respectively (Table 1). The relative liver weights of rats in group 4 (AFB_1/DDT) and group 3 (DDT) showed a significant increase compared with the corresponding control ($p < 0.05$) in weeks 1,5 and 10, and in week 10 respectively. The DDT intake calculated on the basis of diet consumption for each group showed no significant difference.

During the first 10 weeks, the control rats (group 1) which had been given only a single oral dose of DMSO, did not develop any lesions except oval cell proliferation during the first

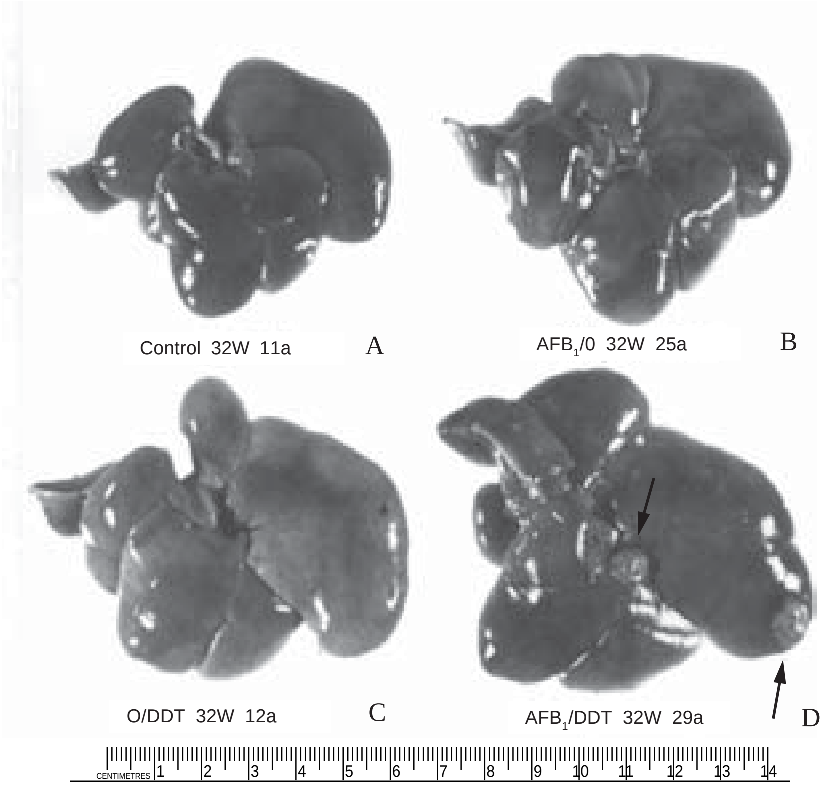

Fig 3–Gross appearance of Wistar rat liver at 32 weeks from 4 groups. A =Control; B=AFB_1; C = DDT; and D= AFB_1/DDT. A, B and C are normal; D has a tumor (arrowed).

week of the experiment. Megalocytosis was found in all 4 rats in group 4 (AFB_1/DDT) by 10 weeks.

At the end of the experiment, 15 of the 19 rats in group 4 (AFB_1/DDT) showed varying degrees of hypertrophic hepatocytes with cytoplasmic inclusions. The inclusion bodies appeared mostly in the AFB_1 pretreated group. A greater number of altered cell foci were found in the livers of rats that had been pretreated with a single dose of AFB_1 followed by DDT. Neoplastic nodules were observed in 1 of the 14 AFB_1-exposed rats, compared with 3 of the 19 rats in the AFB_1/DDT group.

Experiment 2

Average chemical intake, diet consumption, relative liver weight, and mortality of the rats are summarized in Table 2. The body weights of the rats fed the AFB_1/DDT diet (group 4) had significantly decreased by week 32 when compared with the control rats (group 1) and the rats given DDT alone (group 3) ($p < 0.05$). The liver weights of the rats in group 4 (AFB_1/DDT diet) were significantly greater in comparison with those of the AFB_1 and the DDT groups by week 32 ($p < 0.05$). The AFB_1 intake of the group 2 rats (AFB_1) was slightly higher than in group 4 (AFB_1/DDT).

Serum analysis

The SGOT and SGPT levels were slightly increased in all treated groups at all times when compared with the controls. Total protein and albumin were slightly decreased in most treated groups when compared with the controls. The albumin of the rats that has been fed the AFB_1 diet decreased significantly to a minimum of 2.3 g/100 ml at 32 weeks ($p < 0.05$). In contrast, the albumin of the rats that had been fed the DDT diet showed a significant increase: to a maximum of 4.5 g/100 ml by week 60 ($p < 0.05$).

Liver pathology

At week 32, there were no remarkable changes to the liver surface of the controls (Fig 3). The livers showed a normal surface and were reddish brown. The livers of all treated groups were darkened and had enlarged, slightly blunt edges. Microscopic findings are summarized in Table 4. Hepatic cells in the DDT-treated rats were considerably enlarged, with vacuoliza-

Table 1
Group mean body and liver weights and chemical intakes for Buffalo rats fed DDT after a single oral dose of AFB_1 in experiment 1.

Group	Treatment	No. of rat	DDT intake (mg)	Body weight (g)	Relative liver weight (%)
	1 week				
1	Control	5	-	128 ± 4[a]	3.2
2	AFB_1	5	-	99 ± 4[b]	3.6
3	DDT	5	5.5	122 ± 4[b]	3.4
4	AFB_1/DDT	5	6.1	109 ± 5[b]	5.5[b]
	5 weeks				
1	Control	5	-	223 ± 7	2.7
2	AFB_1	5	-	174 ± 7[b]	2.6
3	DDT	5	41.7	175 ± 3[b]	3.4
4	AFB_1/DDT	5	39.6	185 ± 4[b]	4.2[b]
	10 weeks				
1	Control	5	-	277 ± 5	2.4
2	AFB_1	5	-	280 ± 13	2.6
3	DDT	5	98.1	261 ± 13[b]	3.6[b]
4	AFB_1/DDT	5	108.0	245 ± 10[b]	3.9[b]
	82 weeks				
1	Control	12	-	382 ± 32	2.3
2	AFB_1	14	-	360 ± 25	2.5
3	DDT	22	259.8	385 ± 10	2.8
4	AFB_1/DDT	19	260.7	377 ± 4[b]	2.3

[a]Mean ± standard deviation,
[b]Significantly different the from control. ($p < 0.05$)

Table 2
Mortality and weight gain of Wistar rats in experiment 2.

Group	Treatment	No. of rats at start	No. of rats sacrificed at week:		Body weight gain (g) at week :		No. of deaths at weeks of treatment								
			32	60	32	60	9	12	15	18	24	30	38	53	58
1	Control	30	7	18	118±35	47±21	-	-	-	1	1	1	1	-	1
2	4 ppm AFB_1	35	7	25	38±28	15±5	-	-	2	-	-	-	-	1	-
3	500 ppm DDT	35	6	19	73±13	52±23	3	3	1	-	-	1	-	1	1
4	AFB_1/DDT	43	5	28	25±15[a]	33±11	5	3	-	-	-	-	-	-	2

[a]Statistically different from the control group ($p < 0.05$) and statistically different from the DDT contaminated group ($p < 0.05$).

tion, degeneration, necrosis, varying degrees of change in fat content, and a moderate degree of oval-cell proliferation.

All AFB_1-treated rats showed a massive proliferation of oval cells and the development of altered cell foci which were scattered through-

Table 3
Average chemical intake, diet consumption and relative liver weight of Wistar rats in experiment 2.

Group	Treatment	Total chemical consumed (mg/kg)		Basal diet consumed g/rat/day	Chow pellet consumed g/rat/day	Relative liver weight (%)	
		AFB_1	DDT			week 32	week 60
1	Control	-	-	14.3	20.7	2.2	2.8
2	4 ppm AFB_1	2.9±1	-	14.8	19.3	2.7	2.6
3	500 ppm DDT	-	250±1	13.4	20.0	3.5[a]	2.6
4	AFB_1/DDT	2.6±0.3	171±67	13.2	23.1	3.6[a,b]	2.8

[a]Statistically different from control group ($p < 0.05$) and statistically different from the AFB_1 contaminated group ($p < 0.05$).

Table 4
Experiment 2 : summary of findings of liver microscopy.

Description	32 Weeks				60 Weeks			
	Control (7)	AFB_1 (7)	DDT (7)	AFB_1/DDT (5)	Control (18)	AFB_1 (29)	DDT (19)	AFB_1/DDT (28)
Portal triads								
Mononuclear-cell infiltration	0	2	1	1	2	0	0	0
Oval-cell proliferation	0	5[a]	7[b]	5[b]	0	6[a],8[b],7[c]	1[a],2[b],1[c]	1[a],6[b],4[c]
Bile-cell proliferation	0	0	0	0	0	4	4	1
Cyst	0	0	0	0	0	2	0	0
Kupffer								
Hypertrophy	0	1	5	4	0	1	13	9
Hyperplasia	0	1	2	1	0	10	11	5
Hepatocytes								
Enlargement	0	1	7	5	0	13	3	15
Vacuolization	0	0	5	3	0	7	8	8
Fatty change	0	0	7	5	1	6	8	11
Degeneration/necrosis	0	4	7	3	1	14	9	10
Hyalin/inclusion bodies	0	0	0	1	0	8	11	13
Altered cell foci								
Acidophilic cell foci	0	1	0	5	0	16	1	15
Basophilic cell foci	0	0	0	0	0	12	0	8
Clear cell foci	0	4	0	1	1	1	0	0
Neoplastic nodules	0	0	0	1	0	9	1	9
Carcinoma								
Hepatocellular carcinoma (HCC)	0	0	0	0	0	6	0	7
Cholangiocellular carcinoma (CCC)	0	0	0	0	0	1	0	1
Hepato-cholangiocellular carcinoma (HCCC)	0	0	0	0	0	1	0	2
Total hepatic tumor	0	0	0	0	0	8(27.5%)	0	10(35.7%)

Criteria for changes in oval cell : [a] mild degree, [b] moderate degree, [c] high degree. Number of animals sacrificed are in the parenthesis.

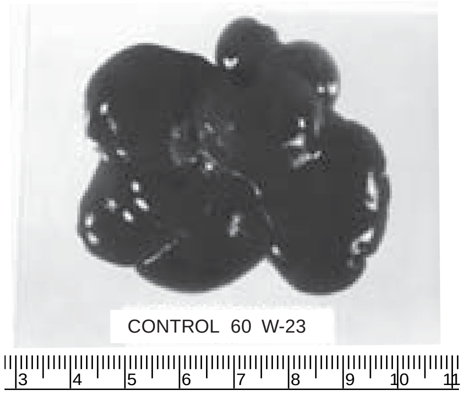

Fig 4–The liver of a male Wistar control-group rat at 60 weeks.

Fig 5–The liver of a male Wistar DDT-group rat at 60 weeks.

out the liver section. Animals in group 4 (AFB_1/DDT) showed broadly similar changes, however 1 of these 5 rats had developed neoplastic nodules by week 32 (Fig 3).

At the end of the experiment (60 weeks), there were no remarkable changes in the livers of control and DDT-treated rats (Figs 4 and 5). In the AFB_1-treated group, one rat developed a hepatic tumor mass (Fig 6). Histologically, some livers from rats in this group showed enlargement, vacuolization, fatty change and necrosis of hepatocytes. All rats exhibited massive proliferation of oval cells with large pale nuclei in the periportal zone. Most rats developed altered cell foci. Acidophilic cell foci were

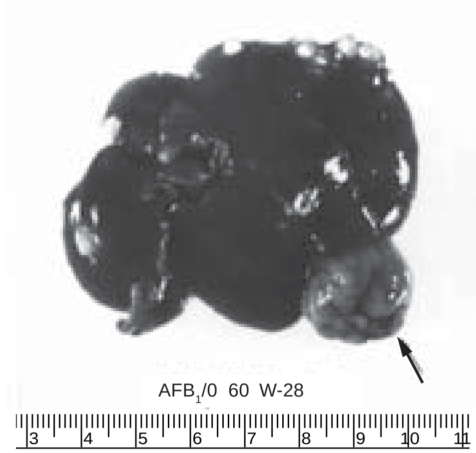

Fig 6–The liver of a male Wistar rat that had been given dietary AFB_1 (4 ppm) for 6 weeks, followed by a basal diet for 12 weeks, and then chow pellets. The arrow points to a tumor of the left central lobe (60 weeks).

frequently seen. Neoplastic nodules and hepatocellular carcinoma and cholangiocellular carcinomas were seen, but in lower numbers and severity compared with the rats that had been exposed to DDT following initiation with AFB_1 (group 4, Fig 7). In the DDT-treated group, some rats showed massive oval cell proliferation in the portal area. Some hepatocytes showed a few distinctive intracytoplasmic inclusion bodies as well as hypertrophy and hyperplasia of Kupffer cells.

Although cholangiocellular carcinomas were rare, one rat in the group that received AFB_1 and one from the AFB_1/DDT group developed both hepatocellular carcinoma (Fig 7A) and cholangiocellular carcinoma (Fig 7B). Hepato-cholangio-cellular carcinomas were found in 1 rat that had received AFB_1 alone, and in 2 that had received AFB_1 following DDT (Fig 7C).

Other pathology

Only a limited study of other tissues was made, since the major objective was to compare liver tumor development. However, the kidneys were examined in view of earlier reports of tumors in these organs in AFB_1-treated rats.

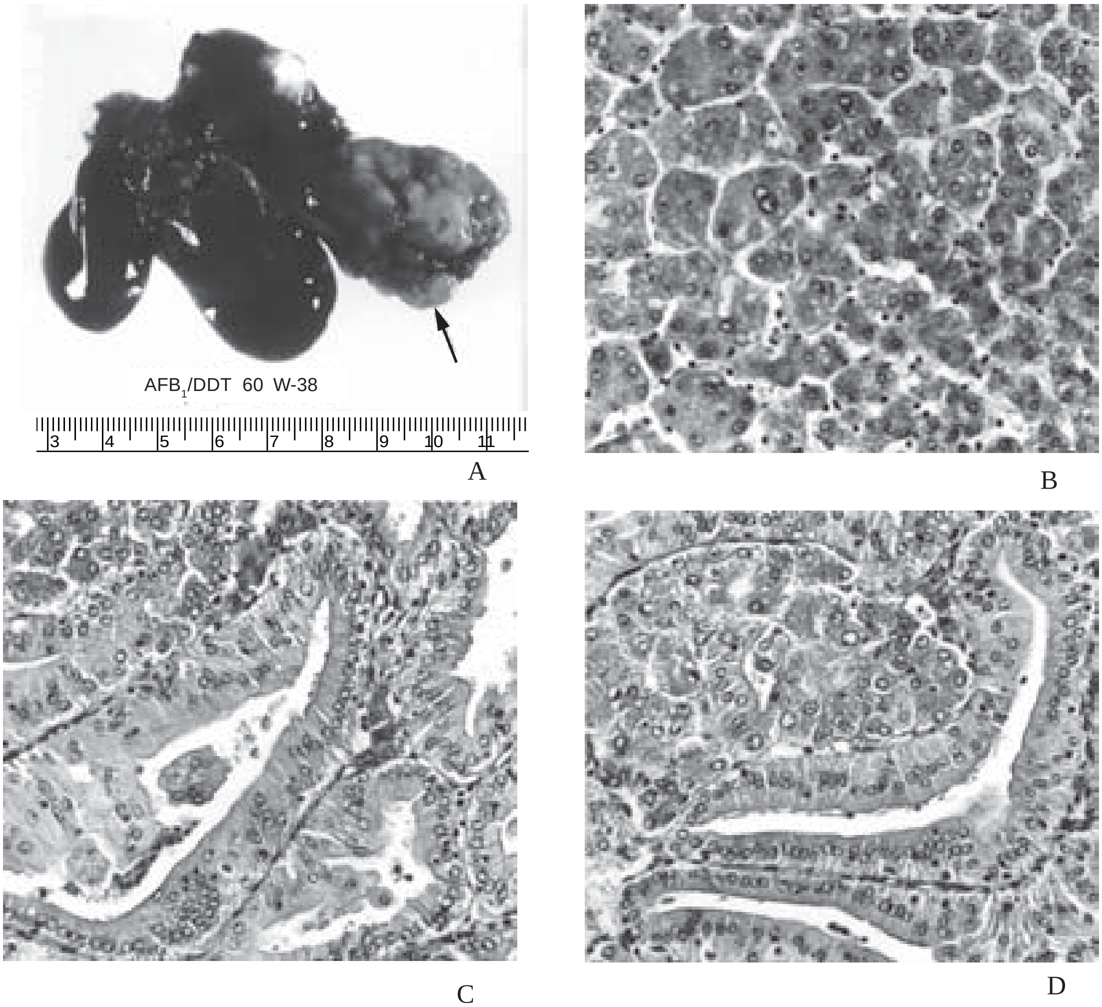

Fig 7–The liver of a male Wistar rat that had been given AFB_1 (4 ppm) for 6 weeks, followed by DDT (500 ppm) for 12 weeks. The arrow points to a large tumor of the left central lobe at 60 weeks (A), showing histologic appearance of hepatocellular carcinoma (B), and cholangio-cellular carcinoma in which neoplastic glands of various sizes and the cells' pappillary projections extend into the lumen (C), and mixed hepatocellular / cholangiocellular carcinomas (D). Hematoxylin and eosin stain, magnification x 600.

The kidneys of rats receiving AFB_1, AFB_1/DDT and of the controls showed varying degrees of nephritis with interstitial fibrosis and chronic inflammation. No renal cell carcinoma was found.

DISCUSSION

In experiment 1, neoplastic nodules were observed in only 1 of the 14 male Buffalo rats that had been given a single oral dose of AFB_1 (5 mg/kg). This was similar to the result of a previous study in which male Fisher rats were given the same dose of 5 mg/kg toxin (Wogan and Newberne, 1967). In addition, Lemonnier *et al* (1975) found that 9 of 19 female Wistar rats given a single oral dose of 7 mg/kg AFB_1 (the approximate LD_{50}) developed hepatoma at 120 weeks. The present results showed a lower susceptibility to liver cancer as the result of a single oral dose of AFB_1 with DDT than was observed in an earlier study which was initiated by a brief dose of 2-acetylaminofluorene (2-AAF) (Peraino *et al*, 1975). Three possible reasons may explain this finding: (1) Buffalo rats are more resistant to hepatocarcinogenesis from AFB_1 than both Fisher rats (Wogan and Newberne, 1967) and Wistar rats (Angsubhakorn *et al*, 1990); (2) the subsequent continuous feeding of DDT in our experiment was at a lower dose (100 ppm) than used in other studies (Peraino *et al*, 1975); (3) the duration of exposure to the DDT was shorter (20 weeks) in this study than in the study of Peraino *et al* (1975) (41 weeks). Such promoters may have to be given for a period long enough to enable altered cells to develop neoplastic nodules and then hepatocellular carcinoma.

In experiment 2, the percentage incidence of altered foci and neoplastic nodules were not different, but the incidence of carcinoma was higher in group 4 rats (10/28;35.7%), which received 4 ppm AFB_1 for 6 weeks followed by 500 ppm DDT for 6 weeks, than in group 2 rats (8/29; 27.5%), which were given AFB_1 alone. The low enhancement of DDT on AFB_1 observed in this study may have been due to the different route of AFB_1 administration; Rojanapo *et al* (1988) gave 1.9 mg AFB_1 orally for 14 weeks followed by 15 weeks of DDT given by intragastric intubation (75 μg, 2 times/ week). Liver tumors were found in 8 of 24 rats given AFB_1 followed by DDT. In addition, promoter activity was at its maximum when DDT was given one week after the completion of AFB_1 treatment (Rojanapo *et al*, 1993).

The liver tumor histopathology in our experiment was similar to that reported by Rojanapo *et al* (1988), who found that 2 rats in each of the treatment groups developed mixed hepato-cholangiocellular carcinomas. DDT administration after AFB_1 appeared to promote the formation of carcinomas containing mixed hepato-cholangiocellular elements.

Two mechanisms have been postulated for two-stage hepatocarcinogensis (Williams, 1981): first, a promoter could completely convert the partially transformed cells to fully neoplastic cells, second, the promoter could act on neoplastic cells to enable them to proliferate into an overt neoplasm. The low incidence of liver tumor in this experiment may have been due to an insufficient amount of promoter (Flodstrom *et al*, 1990). Insufficient promotion fails to inhibit intercellular communication and therefore fails to prompt the release of neoplastic cells that grow progressively to form neoplasm. In addition, DDT would have to be administered for a period long enough to enable the altered cells to proliferate into carcinomas.

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