Carcinogenesis vol.10 no.10 pp.1891–1895, 1989

Comparison of three rat liver foci bioassays—incidence of preneoplastic foci initiated by diethylnitrosamine

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Three rat liver foci bioassays have been compared with respect to their sensitivity by the histochemical demonstration of preneoplastic foci, and by the biochemical determination of alterations in enzyme activities of serum indicating hepatotoxicity. We studied the initiation/promotion schedules according to Oesterle and Deml (A), and according to Pereira (B, Broad Spectrum Protocol), and the initiation/selection protocol according to Tatematsu *et al.* (C), with diethylnitrosamine (DEN), given as a single initiating dose of 10 and 30 mg/kg body wt respectively. With all schedules Sprague–Dawley rats, either females, 3 weeks old (A), or males, 6 weeks old (B, C) were used. For promotion polychlorinated biphenyls (A) or phenobarbital (B) were administered. Selection was performed with 2-acetylaminofluorene (C). The rats in schemes (B) and (C) underwent partial hepatectomy one day prior to initiation. The number and total area of foci deficient in adenosine-5′-triphosphatase (ATPase) and positive in γ-glutamyltranspeptidase (GGTase) was evaluated. In the complete schedule with 30 mg of DEN in system (A) foci incidence exceeded that of the other systems by about 7-fold (ATPase) and 2-fold (GGTase) respectively. The lower dose of DEN and all control experiments resulted in a respective lower foci yield. With scheme (C), but not with schemes (A) and (B), e.g. serum fructose-1.6-bisphosphatase and alkaline phosphatase were increased, suggesting liver cell damage. Thus tested with DEN, scheme (A) is most sensitive and causes a low impairment of animals' welfare.

Introduction

The increasing burden of man with potentially hazardous compounds focused interest on the development of suitable and reliable short-term test systems *in vivo* for the early detection of the carcinogenic potential of chemicals, to abolish or to replace at least partly the expensive and long lasting life time carcinogenicity study. Various approaches using mainly liver, skin, lung and mammary gland of mice and rats as test organs for the early detection of chemical carcinogens have been developed (e.g. 1–7). In the liver the highest enzymatic capacity for metabolizing foreign compounds to ultimate carcinogens with hepatic and extrahepatic target organs is available; and the suitability of rat liver for the detection of a broad spectrum of chemical carcinogens with hepatic but also with extrahepatic potency has been well documented (3,4,8). These 'rat liver foci bioassays' are based on experimental approaches established for studying early stages in carcinogenesis, i.e. the initiation and promotion, and differ in the experimental design (1–4,9,10).

In the present work we have compared three rat liver foci bioassays especially suggested for testing chemicals: the initiation/promotion protocols according to methods by Oesterle and Deml (1983) (A), Pereira (1982) (B, Broad Spectrum Protocol), and the initiation/selection protocol of Tatematsu *et al.* (1983) (C) (1–3). The bioassays selected for comparison differ in two main respects: firstly the cocarcinogenic stimulus of two-thirds partial hepatectomy (PH*) used in (B) and (C) facilitates initiation and the fixation of the initial lesion by cell replication in livers of adult rats. In (A) the immature status of juvenile rat liver, e.g. cell growth and the prevalence of diploid nuclei, makes it sensitive for initiation. Secondly, the post-initiation treatment favoring foci development is performed either by promotion, i.e. growth stimulation of initiated cells (A, B), or by growth suppression of regenerating normal hepatocytes, but not of initiated cells (C).

The objective was to select the most suitable assay in respect of sensitivity, expenditure of work, and animals' welfare. The biological endpoint used is the incidence of preneoplastic foci. The emergence of preneoplastic foci in rat liver a few weeks after the application of hepatocarcinogens is an obligatory phenomenon. They have been commonly accepted as presumptive early precursor lesions of malignant tumors (11–15). In addition the influence of the treatment schedules and the status of the animals' health was determined by measuring the activities of relevant enzymes in blood serum. Thus with diethylnitrosamine (DEN) as a model substance we investigated the suitability of the three rat liver foci bioassay protocols for the detection of the carcinogenic potential of chemicals with respect to the critiera given above.

Materials and methods

Chemicals

DEN p.a. 99% pure (Serva, Heidelberg, FRG); Clophen A 50, a commercial mixture of polychlorinated biphenyls (PCBs), with a mean chlorine content of 54%, main components: pentachlorobiphenyl (45%), tetrachlorobiphenyl (28%), hexachlorobiphenyl (16%) (K.Wrabetz, Bayer AG, Leverkusen, FRG, personal communication, 1979); phenobarbital–natrium (PB), DAB 8 (Synopharm GmbH, Hamburg, FRG); 2-acetylaminofluorene (2-AAF), puriss., 98% (Fluka AGA, Buchs, Switzerland); carbon tetrachloride, p.a. 99.8% (Merck, Darmdstadt, FRG). All other chemicals for histochemistry were of analytical grade (Merck, Darmstadt, FRG).

Animals

For all experiments Sprague–Dawley rats (inbred strain, Neuherberg, FRG) 7–15 per group were used. Two rats each were housed together in Macrolon cages at 22°C on a 12-h light/dark cycle. If not stated otherwise, they received a standard pellet diet (Altromin, Lage, FRG) and drinking water *ad libitum*.

Dosage regimen

DEN was dissolved in water, 5 and 15 mg/ml, immediately before use. Clophen A 50, 5 mg/ml, was dissolved in olive oil. Of the solutions 2 ml/kg body wt were administered by gastric intubation between 9.00 and 11.00 a.m. PB, 0.05%, was added to the drinking water, and 2-AAF, 0.02%, was supplemented to the diet (Altromin, Lage, FRG).

*Abbreviations: PH, two-third partial hepatectomy; DEN, diethylnitrosamine; PCBs, polychlorinated biphenyls; PB, phenobarbital; 2-AAF, 2-acetylaminofluorene; ATPase, adenosine-5′-triphosphatase; GGTase, γ-glutamyltranspeptidase; ME, malic enzyme; FBPase, fructose-1.6-bisphosphatase; GOT, glutamate oxalate transaminase; GPT, glutamate pyruvate transaminase; alk. Pase, alkaline phosphatase.

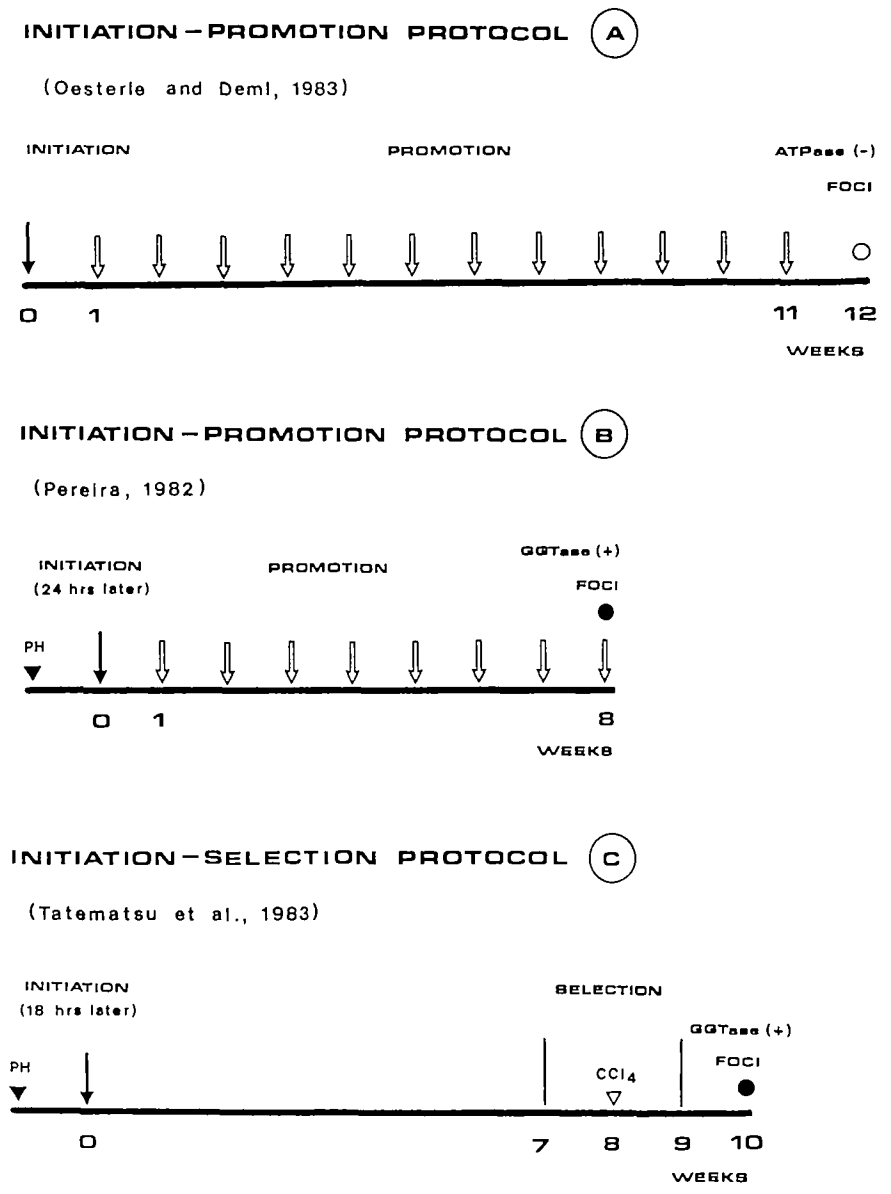

Fig. 1. Schematic presentation of the rat liver foci bioassays. In (A) 3-week-old female Sprague–Dawley rats, in (B) and (C) 6-week-old male Sprague–Dawley rats were used at the beginning of the experiments. The biological endpoints evaluated after the experimental period were ATPase (−) foci (A) (light circle) and GGTase (+) foci in (B) and (C) (dark circle). Dark arrows: single dose of either 10 or 30 mg DEN/kg body wt. Light arrrows: repeated dosing with 10 mg/kg body wt of PCBs (A) by gavage or 0.05% of PB in the tap water (B). Dark triangle: PH (B) and (C). Light triangle: single dose of 1 ml/kg body wt of carbon tetrachloride (C). For details see Materials and methods.

Experimental design

Initiation–promotion protocols (Figure 1, schemes, A, B)

(i) According to Oesterle and Deml, 1983 (A). Juvenile female Sprague–Dawley rats 21 days old were divided into 7 groups. The experimental periods lasted 84 days and started with initiation on the first day, at day 7 promotion began with the oral application of 10 mg PCBs/kg body wt, twice weekly for 10 consecutive weeks; from day 78 the animals were kept on basal diet until sacrifice.

To rats of two groups 1 × 10 and to rats of another two groups 1 × 30 mg DEN/kg body wt was given by gavage. Thereafter one group each underwent promotion; rats of the other two groups received basal diet. As controls served untreated, olive-oil treated or PCB-treated rats.

(ii) According to Pereira, 1982 (B). The experiment lasted 56 days and consisted of eight groups of adult male Sprague–Dawley rats 6–11 weeks old. PH was performed one day prior to initiation at day one. The promoting period with 0.05% of PB in the drinking water started at day 7 and lasted until day 55.

Rats of five groups underwent PH at the beginning of the experiment. To rats of three groups DEN was given for initiation either 1 × 10 (one group) or 1 × 30 mg/kg body wt (two groups) by gavage. One group of rats, which received 1 × 30 mg DEN, was kept further on basal diet; all the others received PB as promoting agent. For controls one group of the PH-treated rats remained without further treatment, the others underwent promotion without initiation.

Rats in the last three groups served either as untreated or DEN (30 mg)-treated or PB-treated controls.

Initiation–selection schedule (Figure 1, scheme C)

(iii) According to Tatematsu *et al.*, 1983 (C). The experiments started at day 0 with PH using adult male Sprague–Dawley rats, 6 weeks old, followed by initiation at day 1, and lasted for 70 days. The 2-AAF-supplemented diet (0.02%) was applied from day 50 to 63 for two weeks (selection phase). At day 57, the rats received 1 ml/kg body wt of CCl_4, and from day 64–69 they received basal diet. Rats of two groups underwent the complete scheme with either 1 × 10 or 1 × 30 mg DEN/kg body wt for initiation. Treatment in the third group was PH/selection, two groups consisted of untreated and 2-AAF-treated rats respectively.

The original protocols used as markers adenosine-5′-triphosphatase (ATPase) deficiency (A) or emergence of γ-glutamyltranspeptidase (GGTase) (B, C) (Figure 1). In the present study with all protocols both markers were used.

Histochemical demonstration of enzyme activities

All animals were decapitated under ether anesthesia. From the left and median

Table I. Incidence of DEN-initiated enzyme-altered liver foci in male and female Sprague–Dawley rats in three different rat liver foci bioassays

Treatment		Island number/cm^2		Total area (mm^2/cm^2)	
		ATPase (−)	GGTase (+)	ATPase (−)	GGTase (+)
Protocol A (♀)					
Untreated controls	(10)	0.3 ± 0.3[+]	0.1 ± 0.1	0.003 ± 0.004	0.006 ± 0.009
Olive oil	(10)	0.8 ± 0.3	1.4 ± 0.8	0.005 ± 0.002	0.009 ± 0.010
− /PCBs	(10)	2.7 ± 0.5	4.4 ± 1.5	0.030 ± 0.021	0.043 ± 0.041
−/DEN (10 mg) /−	(9)	12.7 ± 2.5[a]	10.2 ± 4.5[a]	0.282 ± 0.061	0.093 ± 0.039
− /DEN (30 mg)/−	(10)	34.6 ± 5.9[a]	12.1 ± 2.4[a]	0.400 ± 0.077	0.098 ± 0.028
− /DEN (10 mg)/PCBs	(10)	69.1 ± 10.0[b]	42.7 ± 6.6[b]	1.881 ± 0.474	1.093 ± 0.389
− /DEN (30 mg)/PCBs	(9)	108.4 ± 24.4[b]	38.9 ± 4.6[b]	1.692 ± 0.333	0.653 ± 0.268
Protocol B (♂)					
Untreated controls	(15)	1.2 ± 0.8	0.4 ± 0.4	0.016 ± 0.012	0.003 ± 0.004
PH/− /−	(10)	1.4 ± 0.4	0.5 ± 0.4	0.046 ± 0.027	0.008 ± 0.009
− /− /PB	(10)	1.1 ± 0.8	0.8 ± 0.4	0.013 ± 0.011	0.007 ± 0.004
PH/− /PB	(10)	1.5 ± 0.5	1.4 ± 0.5	0.039 ± 0.022	0.014 ± 0.005
− /DEN (30 mg)/−	(11)	3.0 ± 1.5[a]	0.7 ± 0.5	0.036 ± 0.020	0.005 ± 0.004
PH/DEN (30 mg)/−	(7)	7.8 ± 2.5[a]	5.6 ± 1.2[a]	0.146 ± 0.075	0.049 ± 0.014
PH/DEN (10 mg)/PB	(9)	11.3 ± 4.6[b]	14.2 ± 3.8[b]	0.115 ± 0.042	0.103 ± 0.022
PH/DEN (30 mg)/PB	(10)	12.9 ± 6.9[b]	23.0 ± 2.5[b]	0.161 ± 0.087	0.183 ± 0.031
Protocol C (♂)					
Untreated controls	(10)	0.4 ± 0.2	0.2 ± 0.2	0.005 ± 0.003	0.002 ± 0.003
PH/− /−	(10)*	1.4 ± 0.4	0.5 ± 0.4	0.046 ± 0.027	0.008 ± 0.009
− / /2-AAF[c]	(8)	0.5 ± 0.8	0.8 ± 0.6	0.046 ± 0.096	0.022 ± 0.028
PH/− /2-AAF	(8)	1.1 ± 1.0	5.5 ± 2.8[a]	0.279 ± 0.451	0.402 ± 0.600
− /DEN (30 mg)/−	(11)*	3.0 ± 1.5[a]	0.7 ± 0.5	0.036 ± 0.020	0.005 ± 0.004
PH/DEN (30 mg)/−	(7)*	7.8 ± 2.5[a]	5.6 ± 1.2[a]	0.146 ± 0.075	0.049 ± 0.014
PH/DEN (10 mg)/2-AAF	(9)	9.6 ± 5.0[b]	8.2 ± 4.2[b]	0.588 ± 0.537	0.267 ± 0.174
PH/DEN (30 mg)/2-AAF	(10)	18.3 ± 6.6[b]	15.5 ± 2.7[b]	1.030 ± 0.230	0.706 ± 0.216

Numbers of animals in brackets.
[+]Mean ± SD.
*Taken from Protocol (B).
Student's t-test, $2P \leq 0.01$: [a]significantly different from untreated controls; [b]significantly different from DEN-treated rats.
[c]2-AAF includes treatment with CCl_4.
For details see Materials and methods.

liver lobes pieces of ~2 cm in diameter were cut and frozen immediately in isopentane at −80°C cooled by liquid nitrogen. Cryostat sections of 8 μm thickness were prepared. The sections were taken from four levels per lobe, 250 μm apart, for ATPase-staining (16), and from two levels serial sections for the demonstration of GGTase with γ-glutamyl-α-naphthylamide as substrate and Fast Garnet GBC as coupling agent (17). Foci number and area were determined using a semiautomatic image analyzer (Videoplan, Kontron, Eching, FRG). A field of 1 cm^2 per section was screened corresponding to a total area of 8 cm^2 for ATPase and 4 cm^2 for GGTase per animal.

Biochemical determination of enzyme activities

In blood serum the activities of the following enzymes were measured: malic enzyme (ME) (18); fructose-1.6-bisphosphatase (FBPase) (19); glutamate oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT) (20); and alkaline phosphatase (alk. Pase) (21).

All values in the table are mean ± SD. Statistical evaluation was done with the Student's t-test ($2P \leq 0.01$). Comparison was performed as follows: (i) related to untreated controls; (ii) related to DEN-treated animals. Rats treated with DEN only without promotion or selection serve as DEN controls for both protocols (B) and (C); the data are given in (B).

Results

Body and liver weight

The body weight was not affected by treatment schedules (A) and (B) and slightly reduced with scheme (C) in those animals that underwent the selection period. The liver weight and the liver-to-body weight ratio were enhanced when promoting agents were applied (data not shown).

Incidence of preneoplastic foci

The number and total area of foci deficient in ATPase and positive in GGTase after treatment according to the three schedules (A, B, C) are given in Table I.

(i) Juvenile female rats (A). Foci incidence identified with both markers exceeded significantly that obtained with (B) and (C), with the complete treatment and with initiation alone. The highest yield was ~108 and 69 ATPase (−) and 39 and 43 GGTase (+) foci/cm^2 with the complete scheme and 30 or 10 mg DEN. A significant dose–response relationship of initiation with 10 and 30 mg DEN/kg body wt was found with ATPase but not with GGTase as marker with or without promotion. With 10 and 30 mg DEN alone a significant lower foci incidence of 13 and 35 for ATPase (−) and 10 and 12 for GGTase/cm^2 was observed, compared to the respective complete schemes.

Untreated controls exhibited a very low foci incidence. PCB treatment caused ~3 ATPase (−) and 4 GGTase (+) foci/cm^2.

(ii) Adult male rats (B, C). Applying the complete schedules in (B), with either 1 × 10 or 1 × 30 mg DEN, promotion with PB enhanced the yield in ATPase (−) foci with ~11 and 13/cm^2 compared to ~8/cm^2 with PH/DEN (30 mg). For GGTase ~14 and 23/cm^2 were measured, the latter being significantly different from the result after PH/DEN (30 mg) with ~6/cm^2.

In (C), complete schedule with 10 or 30 mg DEN and selection with 2-AAF/CCl_4, foci number were ~10 and 18/cm^2 for ATPase (−) and ~8 and 16/cm^2 for GGTase (+) in the same range of order compared to (B). For GGTase the latter number was significantly higher compared to PH/DEN (30 mg) taken from (B). In (B, C) the control experiments in the respective complete treatment designs resulted in a low foci incidence of ~1/cm^2 with both markers, with one exception. In (C) the PH/selection scheme resulted in ~6/cm^2 GGTase (+) foci. The administration of DEN (30 mg) enhanced the number of ATPase (−), foci but not those of GGTase (+) ones, slightly to ~3/cm^2.

The development of total foci areas (Table I) and of average foci areas (data not shown) with the complete schemes was dependent on the treatment after initiation. The strongest proliferation stimulus on foci with both types of markers was exerted by selection with 2-AAF/CCl_4. In calculating average foci size, promotion with PB did not enlarge foci size, whereas PCBs enlarged GGTase (+) foci only.

Enzyme activities in blood serum

In blood serum the activities of ME, FBPase, GOT, GPT and alk. Pase were determined.

When PCBs were applied to juvenile females (protocol A) as promoting agent the levels of ME were enhanced ~2-fold over those of untreated and DEN-treated controls with ~20−25 U/l ($2P \leq 0.01$). FBPase remained unchanged with ~25 U/l. GOT, GPT and alk. Pase showed a slight, non-significant tendency to lower values. The base levels were ~25 (FBPase), 101 (GOT), 61 (GPT) and 410 (alk. Pase) U/l. In protocol (B) PB was given as promoter to adult male rats. The same trends as seen in (A) were observed. None of the changes were significant. The base levels were ~26 (ME), 38 (FBPase), 103 (GOT), 89 (GPT) and 544 (alk. Pase). The application of 2-AAF/CCl_4 in the selection regimen of protocol (C) enhanced the activities of ME, FBPase, GPT and alk. Pase significantly up to the 2-fold ($2P \leq 0.01$). The base levels were ~25 (ME), 39 (FBPase), 70 (GPT) and 563 (alk. Pase).

Discussion

Several rat liver foci bioassays have been developed, at first for the investigation of early steps in hepatocarcinogenesis, and later for the detection of chemical carcinogens (e.g. 1−3,9,22−24; for review see 10,25).

Mature rat liver is rather refractory towards chemical carcinogens. A sufficient organ sensitivity can be reached by physical (PH) (e.g. B, C) or chemical (CCl_4) damage (e.g. C). Both manipulations exert a strong proliferation stimulus and induce regenerative growth. Initiation was found to be most effective when performed 18−24 h after PH, reaching then the maximal DNA synthesis phase in the cell cycle (23,26). The resulting DNA lesions, responsible for the initiated status of a cell, are irreversibly fixed by cell replication. The clonal expansion of initiated cells leads to preneoplastic foci. In addition growth stimulation of foci by promotion (e.g. B) causes a rapid manifestation of foci essential for a short time assay. Instead of promotion selection with 2-AAF and CCl_4 can be performed (e.g. C). Carbon tetrachloride induces regenerative proliferation via cell necrosis, 2-AAF in a toxic dose causes a selective growth suppression of regenerating hepatocytes, but does not impair foci development. This scheme results also in an appreciable foci incidence after a few weeks.

The other rationale to obtain a sensitive liver model is to use newborn (24) or juvenile (e.g. A) animals. In immature rats the incidence of diploid nuclei in hepatocytes is high compared to adults (e.g. 27,28). Diploid hepatocytes are preferentially a critical target for the action of an initiating agent (e.g. 29,30). These findings could explain at least partly the high sensitivity observed in these age groups (e.g. 1,24,31). The advantage of using weanlings is that they have already developed an appreciable capacity for mixed function oxygenases for the degradation of compounds to carcinogenic metabolites (e.g. 32). In addition, immature rats are no less susceptible to promoting stimuli than adults (24,33), so the bioassay using weanling rats combines the high sensitivity of immature animals and a metabolic capacity not much less potent than that in adults. Attempts have been made already to appraise short-term tests *in vivo* for carcinogens (34,35). We performed a direct comparison under the same experimental conditions in the same laboratory: the results show that protocol (A), using juvenile female rats at the beginning of the experiments, is most appropriate in respect to sensitivity, animal welfare and technical simplicity. Evaluation was done by histochemical and biochemical determination of parameters relevant for early carcinogenesis as well as those indicative for the animals' health status (Table I).

Either with DEN alone or with the complete treatment the highest yield in ATPase (−) and GGTase (+) foci was obtained with protocol (A) (Table I). The higher dose of 30 mg DEN was more effective, except of GGTase, when PCBs were applied. Promotion enhanced the number of ATPase (−) and GGTase (+) foci ~5- and 4-fold after 10 mg DEN. With 30 mg DEN and promotion the incidence of foci was ~3-fold for both foci types over that of DEN-treated rats.

No fundamental differences with regard to foci incidence were observed in schemes (B) and (C). The complete schemes with 30 mg DEN were most effective, with 10 mg DEN, foci incidence was somewhat lower. With all treatment protocols foci incidence was significantly lower than in females used in scheme (A). The difference was most striking with ATPase deficiency as marker with a 6- to 8-fold lower foci number for the complete schedules in (B) and (C). Without promotion or selection, initiation with 30 mg DEN was not effective.

Unlike in protocol (A) both foci types occurred in about the same range of order. A comparison with the data of Pereira (5) and our scheme (B) shows that PH/1 × 30 mg DEN/PB yields incidences of GGTase (+) foci in the same range of order with 23.0 ± 2.5 (Table I) and 18.8 ± 4.6 (2). Repeated applications of DEN up to a total of 350 mg/kg body wt (i.p.) by Tatematsu *et al.* (3) resulted in a comparably lower incidence of 41−45 preneoplastic lesions than in our experiment with the same protocol (C) (Table I). It has to be noted that F344 rats might be less sensitive than the Sprague−Dawley rats used by us.

The foci numbers oberved with or without promotion confirm that weanling rats react more sensitively to promoting stimuli compared to adults (24,33).

Regarding foci areas, again the most sensitive system was found to be (A). PCBs exerted a much stronger proliferation stimulus on initiated cells with up to 2% of liver area than did PB, where only a slight effect on GGTase (+) cells was found (Table I). Treatment with 2-AAF augmented areas less effective than PCBs. In this case no difference between ATPase (−) and GGTase (+) was seen. These findings confirm earlier observations by us that foci in female rats respond more sensitively to proliferating stimuli than do foci in male rats, exhibiting not only more but also larger

foci (36). When applying the selection protocol (C) the increase of alk. Pase, FBPase and GPT in blood serum and a reduced weight gain indicate hepatotoxicity. These observations confirm the macroscopic image of the animals with a rough fur and disturbances in behavior and motility, indicating sickness. With regard to animal welfare and with respect to the high sensitivity in view of foci incidence, the initiation−promotion protocol (A) was found to be the most suitable.

Acknowledgements

The skilful technical assistance of Mrs C.Spiller, M.Berauer, C.Galonska and G.Köpf is gratefully acknowledged. The work was supported by grants UBA 106 03 059 and 106 03 60 from Umweltbundesamt, Berlin, FRG.

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Received on April 24, 1989; revised on July 31, 1989; accepted on August 2, 1989