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Comparative analysis of the effect of phenobarbital, dichlorodiphenyltrichloroethane, butylated hydroxytoluene and nafenopin on rat hepatocarcinogenesis

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In order to investigate whether different 'promoters' have the same qualitative and/or quantitative effects on rat hepatocarcinogenesis, 0.05% of phenobarbital (PB), 0.05% of dichlorodiphenyltrichloroethane (DDT), 0.5% butylated hydroxytoluene (BHT) and 0.1% of nafenopin (NAF) were chronically administered in the diet to rats previously submitted to an initiation by diethylnitrosamine and a selection with 2-acetylaminofluorene plus CCl4. The animals were killed after 3, 6 and 14 weeks of 'promoters' administration to analyse their effect on premalignant lesions. The quantitative analysis of the gamma-glutamyltransferase positive lesions indicates that as compared to a control group receiving a basal diet after initiation and selection, PB, DDT and BHT enhance the development of these lesions whereas NAF inhibits it. Rats were also killed after 22 weeks of administration to analyse the incidence and the yield of liver cancer. As compared to the control group, PB, DDT and surprisingly NAF enhance the development of liver cancer whereas BHT does not. This suggests that the effect of potential 'promoters' should be analysed on cancer development rather than on premalignant lesions.

Introduction

Cancer is a complex, progressive biological process. After the administration of a carcinogen, the carcinogenic process can either evolve by itself or be modulated. Among the various protocols of rat hepatocarcinogenesis, the resistant hepatocyte model developed by Solt and Farber (1) is well characterized and is sufficient to induce a high incidence of cancer within 10 months (2). It is particularly adapted to compare the effect of chemical compounds potentially able to modulate an ongoing carcinogenic process either by speeding up or by slowing down or even by stopping its evolution to malignancy. Specially, it allows to check if various compounds known or suspected to be 'promoters' accelerate the carcinogenic process when chronically administered after initiation and selection. The aim of the present report was to investigate whether various so called 'promoters' exert the same qualitative and/or quantitative effects on the development of putative premalignant and malignant lesions.

Four substances known or suspected to be 'promoters' of liver cancer have been compared. They differ both by their chemical structure and their biological activity: a barbituric acid, phenobarbital (3–10); an insecticide, dichlorodiphenyltrichloroethane (DDT*) (6,11); an antioxidant, butylated hydroxytoluene (BHT) (12, 13) and a hypolipidemic peroxisome proliferator, nafenopin (NAF) (14). Whereas PB and DDT are clearly considered as liver cancer 'promoters', BHT has been reported to act either as a 'promoter' (12,13) or as inhibitor of the development of foci and nodules (15). NAF, and in general peroxisome proliferators, are hepatocarcinogenic following long-term chronic administration (16–18) but they are not genotoxic (19,20). They have been described as 'promoters' of liver cancer (21–23), but also as inhibitors of the development of foci (24–26).

These four compounds were given in the diet starting one week after the end of the initiation-selection phases performed as previously described (7,9,10). The rats were sacrificed 3, 6 and 14 weeks later to analyse the premalignant stages and 22 weeks later to study their effect on the development of cancers (27).

Materials and methods

Chemicals, diets and animals

Diethylnitrosamine (DEN) and CCl4 were purchased from Merck (Darmstadt, GFR). DDT, BHT and 2-AAF were bought from Janssen Chimica (Beerse, Belgium) and PB as a sodium salt from Ludeco (Brussels, Belgium). NAF was a gift from Ciba-Geigy (Basel, Switzerland).

Chemicals were incorporated in a standard diet AO4 (UAR, Villemoisson sur Orge, France) at a concentration of 0.03% for 2-AAF, 0.05% for PB, 0.05% for DDT, 0.1% for NAF and 0.5% for BHT.

Male Wistar rats (Iffa Credo, Les Oncins, France) weighing initially 180 g were used. They were housed three to four rats per wire-bottomed cage in standard conditions with a 12 h-light/12 h-dark cycle. They were given access to food and water *ad libitum*.

Experimental protocol

The experimental protocol of rat hepatocarcinogenesis is schematized in Figure 1. Rats were injected with a single i.p. dose of 200 mg/kg of DEN. Two weeks later, they were submitted to the selection procedure described by Solt and Farber (1) and slightly modified by Lans *et al.* (7). Briefly, the selection consisted of 2 weeks of feeding a diet containing 2-AAF and a necrogenic i.g. dose of 2 ml/kg of CCl4 administered after 1 week. One week after 2-AAF release, the rats were divided into five groups receiving a basal diet (BD), or a diet containing PB, DDT, NAF or BHT. Eight to 10 animals were sacrificed by decapitation 3, 6, 14 and 22 weeks afterwards.

Histochemical and histological analysis

After the sacrifice, the animals and their livers were weighed and examined grossly. Specimens for histological and histochemical analysis were taken from each liver lobe and each 'macroscopic tumor' (tumor with a diameter >8 mm).

Tissue samples were fixed in Carnoy's solution, embedded in paraffin and stained with hematoxylin-eosin, periodic acid-Schiff reaction for glycogen and methylgreen-pyronin for nucleic acid detection. Foci, nodules and liver cancers were classified according to Squire and Levitt (28).

Samples were also fixed in cold acetone, embedded in paraffin and stained to detect gamma-glutamyltransferase (GGT) activity by the method of Rutenburg *et al.* (29). The quantitative analysis of the GGT-positive areas was performed with a computer to determine the number/cm^2 of, the size of GGT-positive lesions (referred as GGT+ lesions) and the percentage of the liver parenchyma occupied by GGT+ lesions (referred to as percentage of GGT+ lesions).

Additional tissue samples were frozen on dry ice, cut in a cryostat and processed to detect glucose-6-phosphatase activity by the method of Wachstein and Meisel (30) and acid and alkaline DNase activities by the technique of Taper (31).

Results

Quantitative analysis of premalignant and malignant lesions

As shown in Figure 2, the administration of PB and DDT for

*Abbreviations: 2-AAF, 2-acetylaminofluorene; BD, basal diet; BHT, butylated hydroxytoluene; DDT, dichlorodiphenyltrichloroethane; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; NAF, nafenopin; PB, phenobarbital.

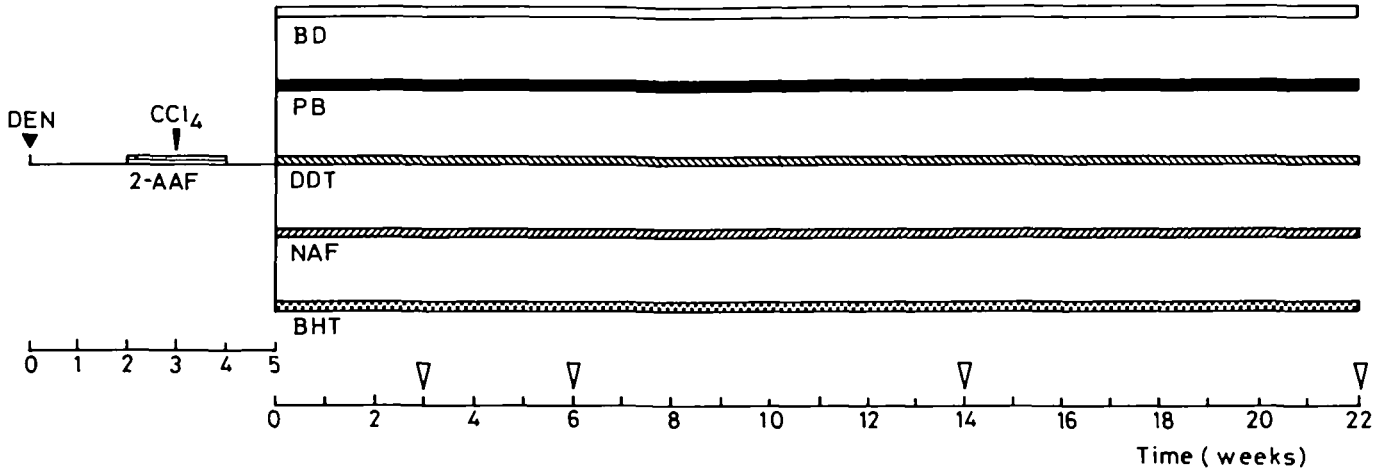

Fig. 1. Experimental protocol used to analyse the 'promoting' effect of different compounds in a triphasic model of rat hepatocarcinogenesis. The rats were initiated with DEN (▼) and selected with 2-AAF + CC14 as described under Materials and methods. One week after the end of the selection, they were divided into 5 groups receiving a basal diet (□), a diet with 0.05% of PB (■), 0.05% of DDT (▨), 0.1% of NAF (▨) or 0.5% of BHT (▩). Eight to 10 rats were sacrificed (▽) after 3, 6, 14 and 22 weeks of promotion.

3 weeks causes an increase in the percentage of GGT+ lesions as compared to the rats receiving a BD (13% and 7% versus 2%). The number of GGT+ lesions per cm^2 also increases (72 and 82 versus 39). Giving PB or DDT for 6 or 14 weeks further increases the percentage of GGT+ lesions (from 13% to 26% and from 7% to 39%). This mainly results from an increase in size of the GGT+ lesions since their number per cm^2 remains constant ($\pm 75/cm^2$). With regard to the increase in the size of the GGT+ lesions, DDT seems more potent than PB. In the group receiving a BD, the percentage of the GGT+ lesions does not increase much with time ($\pm 2\%$). Throughout the experiment, the number of lesions per cm^2 remains constant (± 40).

With regard to the incidence and the yield of cancer, PB and DDT accelerate the development of cancer as compared to the BD group (Table I). The first liver cancer appears after 14 weeks of PB or DDT treatment in one rat out of nine and eight respectively. After 22 weeks, six rats out of nine and 10 respectively bear liver cancers whereas only one rat out of nine has a hepatocellular carcinoma (HCC) in the BD group. At that time, the average number of macroscopic tumors per rat bearing tumors is 1.8 and three in PB and DDT treated rats respectively. Thus, PB and DDT decrease the latency period and increase the incidence and the yield of HCC.

As compared to PB or DDT, NAF shows a different pattern of effects. Indeed, after 3–14 weeks of administration, both the number per cm^2 and the percentage of GGT+ lesions are lower than in the BD group (Figure 2). Thus in early stages of hepatocarcinogenesis, NAF seems to have a slight inhibitory effect rather than a 'promoting' effect. Surprisingly, even though fewer foci and nodules are detected in early stages, NAF promotes the development of cancer after 22 weeks of administration. Indeed, nine rats out of 10 bear liver cancer as compared to one out of nine in the BD group (Table I). The number of macroscopic tumors per cancerous rat is 6.5. Thus, NAF also increases the incidence and the yield of liver cancer as PB and DDT.

In the liver of BHT treated rats, a large periportal induction of the GGT activity appears in the control rats (data not shown). Such a phenomenon makes it difficult to quantify the effect of that compound on the GGT+ foci and nodules. However, within the limit of such analysis, after 3 and 6 weeks of BHT administra-

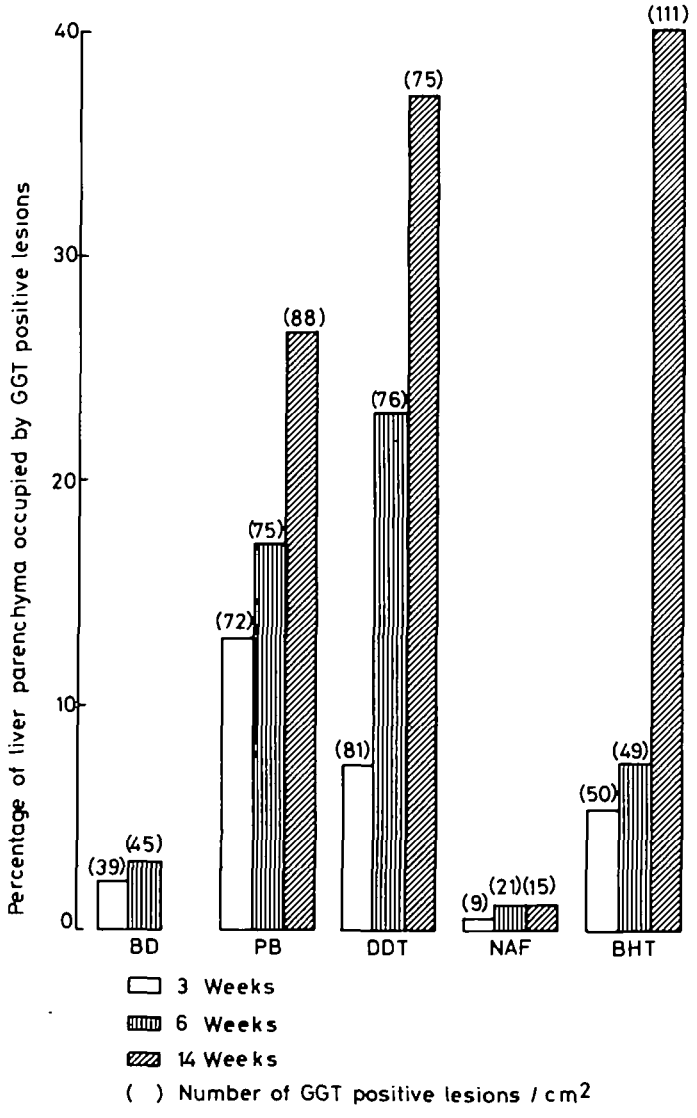

Fig. 2. Influence of the nature (PB, DDT, NAF, BHT) and the duration [3 (□), 6 (▥) and 14 weeks (▨)] of the 'promoter' administration on the percentage (histogram) and the number (( )) of GGT+ lesions.

Table I. Effect of the administration of different 'promoters' (PB, DDT, NAF, BHT) for 22 weeks on the incidence, the yield and the histological type of liver cancers

	Number of macroscopic tumors per rat bearing tumor	Number of rats bearing liver cancer	Number of rats bearing			
			Hepatocellular carcinoma		Cholangio-carcinoma	Hemangio-sarcoma
			Well differentiated	With glandular pattern		
BD	1	1/9	1	–	–	–
PB	1.8	6/9	6	–	1	–
DDT	3	6/10	5	2	–	–
NAF	6.5	9/10	9	–	–	–
BHT	1	1/10	–	–	1	1

tion, the percentage and the number per cm^2 of GGT+ lesions were slightly higher than in the BD group (Figure 2). After 14 weeks, both parameters are strongly increased. With regard to the cancer incidence (Table I), BHT does not seem to accelerate the development of cancer since the incidence is the same as in the BD group: one rat out of 10 bears liver cancer.

Qualitative histological analysis of the premalignant and malignant lesions

In the BD group, only a few foci and nodules containing mainly clear cells are observed. The architecture of the surrounding liver is not much disturbed. After 22 weeks, one rat out of 10 bears a well-differentiated HCC.

In the PB-treated group, many eosinophilic foci and nodules appear after 3–6 weeks. An oval cell proliferation sometimes leads to the formation of pseudonodular structures described earlier (7). The foci and nodules seen in the early stages display a wide spectrum of phenotypic alterations such as a decrease in glucose-6-phosphatase or acid and alkaline DNase activities and an accumulation of glycogen (7). They are relatively homogeneous. Later on, they become more heterogeneous. Some basophilic foci and basophilic areas within nodules are observed. After 14 weeks, most of the premalignant lesions accumulate glycogen but some foci or cells within nodules are poor or totally free from this polysaccharide. The oval cell proliferation decreases. After 14 and 22 weeks, cholangiomas are observed. In the surrounding parenchyma, zones of small hepatocytes are mixed with zones of hypertrophic eosinophilic hepatocytes. As described earlier, this results from chronic PB administration (32). After 14 weeks of PB administration, one rat out of nine bears a well-differentiated HCC. After 22 weeks, six rats out of nine have well-differentiated HCC and one has a cholangiocarcinoma (Table I).

DDT-treated rats follow the same evolution as PB-treated rats. During the early stages, many foci and nodules showing the same phenotypic alterations develop in the liver. Oval cell proliferation and pseudonuclear structures are sometimes noticed. After 14 weeks of DDT feeding, one rat out of eight has a well-differentiated HCC. Some cholangiomas are present. After 22 weeks, six rats out of 10 bear malignant liver tumors: five have well differentiated HCC, two have a HCC with a glandular pattern.

After 3–14 weeks of NAF administration, the histological analysis of the liver reveals very few foci and nodules. They are different from those usually described (28). They contain hypertrophic cells with a voluminous hyperchromic nucleus with sometimes several prominant nucleoli. Their eosinophilic cytoplasm also shows some diffuse basophilia. They are poor in glycogen. Some basophilic foci are observed. Referring to some authors (33,34), these foci and nodules may be considered as closer precursors to malignancy. Since it has been reported that a hypolipidemic drug induces GGT negative nodules and HCC (35), other 'markers' than GGT have been checked. Very few glucose-6-phosphatase- or DNase-deficient lesions are detected. No cholangioma develops in the parenchyma. After 22 weeks, nine rats out of 10 bear several well-differentiated HCC.

When BHT is administered after initiation and selection, mainly eosinophilic foci and nodules develop. There is some oval cell proliferation. Cholangiomas, cystic changes and telangectasia are observed, particularly after 14 weeks of BHT administration. After 22 weeks, one rat out of nine bears one cholangiocarcinoma and one hemangiosarcoma. No HCC is observed.

Discussion

In an attempt to know whether different chemicals reported or suspected to be liver cancer 'promoters' act similarly on an ongoing carcinogenic process, the effects of various compounds having different chemical structures and biological activities have been compared. They have been given for up to 22 weeks in the diet of rats previously submitted to initiation and selection, a treatment which is sufficient to induce the appearance of malignant tumors in most of the rats after 12 months. The results (Figure 2, and Table I) show that the four compounds PB, DDT, BHT and NAF, have a different modulating effect on the carcinogenic process.

As previously reported using other protocols (3–12), PB and DDT enhance the development of nodules and cancers as compared to BD. When NAF is chronically administered after initiation-selection, very few foci and nodules are observed. Other investigators have reported the inhibitory effect of NAF on the development of foci and nodules (24–26). However, when administered for 22 weeks after initiation-selection, NAF clearly increases the incidence and the yield of cancer. Since NAF induces HCC in long-term experiments (16,17), several authors consider NAF as a 'complete carcinogen' (18,36,37). However, NAF is not genotoxic (20,21) and chronic feeding of NAF for 27 weeks does not induce cancer within the delay of observation, even after selection (38). The reports on the effect of BHT on liver carcinogenesis are also contradictory (39). After initiation–selection, BHT enhances the development of nodules but has no effect on the development of cancer after 22 weeks of administration as compared to BD. For Peraino *et al.* (12), BHT is a promoter since it enhances the development of 'tumor'. Williams and Maeura (13) reported that BHT is a 'weak promoter' since it only increases the number and the size of GGT+ foci at the higher dose. It has also been described that BHT inhibits the development of GGT+ lesions in rat liver (15).

The definition of 'promotion' is still based on the operational

step. Very little is known of the biological effects and the mechanism(s) of action of the promoters, except for TPA, a skin cancer promoter. To determine whether NAF or BHT must be considered as promoters depends on the criteria used: does a promoter enhance the development of premalignant and/or malignant lesions? This present experiment has shown that no quantitative relationship can be established between the number or the percentage of GGT+ lesions and the later development of cancer. However, this does not mean that the so-called premalignant lesions (foci and nodules) are not precursors of liver cancer. It just indicates that the number of foci and nodules does not allow us to evaluate the evolution of nodules to cancer and to foresee the development of cancer. Using other markers might give a better correlation. However, since cancer is the endpoint of the carcinogenic process, it seems that the effect of potential promoters or modulating agents should be mainly analysed on cancer. The present experiment has shown that the triphasic protocol of hepatocarcinogenesis may be a good tool to detect 'promoters' of liver cancer by quantifying the liver cancers after 6–7 months rather than by quantifying the foci and nodules after 2–4 months.

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