Eur J Cancer Clin Oncol, Vol. 22, No. 9, pp. 1073–1078, 1986.
Printed in Great Britain

0277–5379/86$3.00+0.00
Pergamon Journals Ltd.

Anti-Carcinogenic Action of Phenobarbital given simultaneously with Diethylnitrosamine in the Rat

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Abstract—*The present work has been planned in order to elucidate the effect of phenobarbital (PB: 15 mg per rat of ingested dose) on carcinogenesis when it is administered simultaneously with diethylnitrosamine (DEN: 10 mg/kg/day). Wistar rats (180 g) were treated by DEN alone or by DEN + PB during 2, 4 and 6 weeks according to our schedule for hepatocarcinogenesis. After the end of the treatment, the number and the size of induced PAS positive preneoplastic foci was significantly reduced when PB was given simultaneously with DEN for 4 and 6 weeks. The mitotic inhibition and the production of micronuclei normally observed after partial hepatectomy in DEN treated rats were also significantly decreased in DEN + PB treated rats. When the treatment last only 2 weeks, the presence of PB did not change significantly the last parameters. In DEN + PB treated rats, the survival was prolonged and the tumor incidence decreased as compared with the results obtained by DEN alone. It is concluded that PB, which promotes carcinogenesis when administered after the DEN treatment, reduces the carcinogen effect when given simultaneously with DEN. This 'anti-carcinogen' effect acts on the initiation as well as on the promotion of the precancerous lesions. Biochemical investigations are in progress to obtain more information about this 'paradoxical' PB effect.*

INTRODUCTION

We have previously shown that phenobarbital (PB) administered after the first diethylnitrosamine (DEN) treatment promotes the hepatocarcinogenesis [1].

After a subcarcinogenic treatment of 2 weeks by DEN (10 mg/kg/day) continuous administration of PB leads 80% of tumor incidence. When the DEN administration lasts for 4 weeks, 50% of rats die with hepatomas after about 12 months; a subsequent administration of PB increases the tumor yield up to 100%. After a 6 weeks DEN administration the successive pathological stage and the death with cancer occur 3 months earlier when PB is given after DEN alone.

On the other hand, it has been claimed that PB counteracts the tumorogenesis activity when administered simultaneously with the carcinogen [2–4].

To obtain more information about this PB 'paradoxical' effect, we have compared the carcinogenesis normally induced by DEN alone in our schedule with what is observed when PB is given simultaneously.

Rats were treated either by DEN alone or by DEN + PB during 2, 4 and 6 weeks. In each case, we have measured the mitotic evolution during the treatments. After the end of the treatments, we have also estimated the number and the growth of precancerous lesions, the tumor incidence and the length of survival.

Accepted 11 February 1986.
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MATERIALS AND METHODS

Male Wistar rats, weighing approx. 180 g, are housed at constant temperature (22° C) with free access to food (UAR, A03) and water. They are only disturbed for necessary laboratory care. Lighting (6 a.m. to 6 p.m.) is artificial and automatic. An air-conditioning system renews the atmosphere of the room every 3 min. Animals are kept in Macrolon cages (five per cage) and divided into three different groups.

Each group, respectively treated for 2, 4 and 6 weeks, includes two different experimental conditions: treatment by DEN alone (ingested dose: 10 mg/kg/day) and treatment by the same DEN concentration mixed with phenobarbital (PB: 15 mg per rat of ingested daily dose). The drugs are administered continuously in drinking water.

During the treatment, we have estimated the mitotic index in the morning (at 10 a.m.) and in the evening (at 10 p.m.).

The mitotic indices are calculated by counting

the mitotic cells on histological sections after Feulgen reaction by the technique previously described [1]. The mean value for three rats is recorded for each experimental condition.

After the treatment, we have estimated the evolution of preneoplastic lesions, the mitotic response to partial hepatectomy and the related number of micronuclei, the tumor incidence and the duration of survival.

The evolution of preneoplastic lesions is studied at the 4th month after the beginning of treatments on histological slides treated by the PAS reaction. As previously described [1], for each condition studied, five rats are killed after an 18 hr fast. This enables us to observe PAS positive putative preneoplastic foci retaining glycogen after fasting. The number of PAS positive foci per cm^3 of liver (*Nv*) and the relative volume occupied by these lesions (*Vv*) have been calculated by classical stereological methods [5]. The sections are examined with a Wild M–501 automatic sampling stage provided with a multipurpose test system (Wild Heerbrugg Ltd, Heerbrugg, Switzerland). Details on the procedure can be found in previous papers [6, 7]. The frequency distribution of focus size of preneoplastic areas have been estimated on the same histological sections by counting the total number of hepatocellular nuclei in such an area as performed by Rabes and Szymkowiak [8] in similar conditions. When the foci are rare, a minimum of 40 are scored; when they are frequent, a maximum of 375 are examined. Results are presented in histogram form as previously published [9]. The mitotic response to partial hepatectomy and the related micronuclei expression has been estimated in DEN and DEN + PB treated rats by performing a 2/3 partial hepatectomy at 10 a.m. 2 months after the beginning of the treatments. Controls of the same age are also operated in the same conditions. The incidence of mitoses and of micronuclei is measured after the operation. Mitoses and micronuclei are scored by microscopic observation (× 1000) on histological slides stained by the Feulgen reaction. For one determination, the mean of counts obtained from observing about 20,000 nuclei, from at least four rats, are used. Survival and tumor incidence is observed, in each experimental condition, in groups of at least 25 autopsied rats the time of death of which is accurately known. Histological slides are prepared from all the livers for diagnosis of the tumors. Survival curves are established in probit/log grid by plotting the surviving fraction after the death of each animal. In each experiment, the median time of death (lethality 50%) and the mean tumor incidence can thus be calculated.

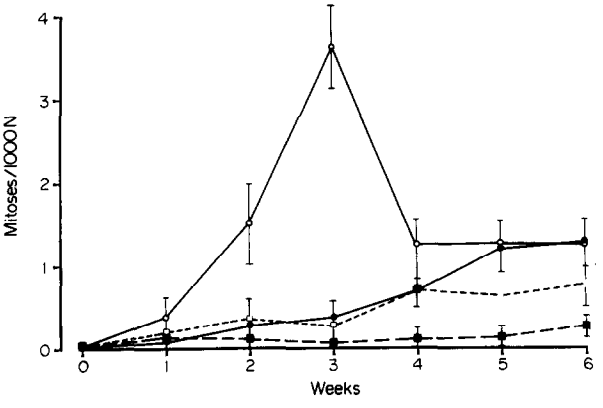

Fig. 1. Mitotic activity during DEN feeding measured at 10 a.m. (○) *or at* 10 p.m. (●) *and during DEN + PB measured at* 10 a.m. (□) *or at* 10 p.m. (■). *Vertical bars indicate the S.E.M.*

RESULTS

Mitotic activity during the treatments (Fig. 1)

Continuous treatment by DEN alone triggers a diurnal mitotic activity the maximum of which occurs after 3 weeks. During all this time, nocturnal values remain very low. From the 4th week of DEN administration, the diurnal and nocturnal mitotic indices are stabilized at intermediate values without any rhythm.

When PB is given simultaneously with DEN, a significant mitotic index appears from the 2nd week with higher values during the day than during the night. Nevertheless, these mitoses are rare up to the 3rd week of treatment. Mitotic indices are thereafter slightly increased during the day while a low level of activity is maintained at night.

Evolution of putative preneoplastic lesions (Table 1)

The number of preneoplastic foci (*Nv*) induced by DEN alone increases with the duration of treatment up to the 4th week. In these conditions, the relative volume (*Vv*) occupied by these lesions in the liver increases correlatively. When the carcinogen is given for 6 weeks, the number of foci is

Table 1. Number of foci per liver cm^3 (Nv) *and relative volume* (%) *occupied by these foci* (Vv) *measured* 4 months *after the start of DEN or DEN + PB administered for* 2, 4 *and* 6 weeks (*means ± S.E. from five animals*)

Duration of treatment		2 Weeks	4 Weeks	6 Weeks
DEN	*Nv*	312 ±95	2,495 ±350	2,101 ±461
	Vv	0.54 ±0.4	1.25 ±0.5	4.31 ±1.3
DEN + PB	*Nv*	316 ±102	1,300 ±250	1,815 ±332
	Vv	0.46 ±0.3	0.85 ±0.3	1.30 ±0.4

approximately the same as after a 4 weeks DEN treatment but, the relative volume of these lesions is much increased.

When PB is given simultaneously with the carcinogen for 2 weeks, the number and the relative volume of the foci are about the same as after a treatment of 2 weeks by DEN alone. By giving both drugs for 4 weeks, the induced lesions are still developing but they are less numerous and their size less important than in the corresponding experimental group treated by the carcinogen alone. The number of foci counted after a 6 weeks DEN + PB treatment is as high as in the groups treated by DEN alone for 4 or 6 weeks; only the relative volume is smaller and corresponds to the size of lesions observed when DEN alone is given for only 4 weeks.

Figure 2 shows the distribution of cell content in PAS positive preneoplastic areas observed on histological sections 4 months after the beginning of the treatments. The appearance of foci of larger size increases with the duration of the DEN treatment. When both drugs (DEN + PB) are given simultaneously, these larger preneoplastic foci are less numerous or occur after longer treatments. This suggests that these last lesions are growing more slowly in DEN + PB treated rats than in rats treated by DEN alone.

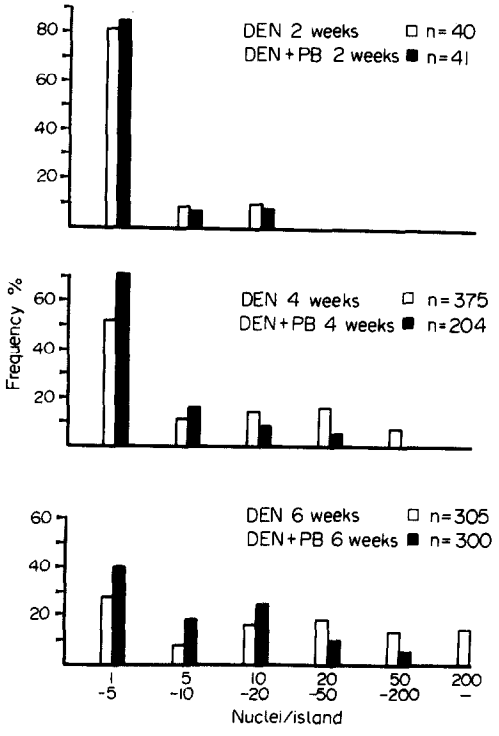

Fig. 2. Frequency distribution of cell foci observed 4 months *after the start of DEN (white) or after the start of DEN + PB (black). The treatments last* 2, 4 *or* 6 weeks. *For each experiment is shown the % of the total number of islands* (n) *counted (ordinates) out the number of nuclei per island grouped into classes (abscissae).*

Mitotic response to partial hepatectomy and the related micronuclei expression

Figures 3, 4, 5 and 6 show the mitotic response and the related micronuclei frequency after 2/3 hepatectomy performed 2 months after the beginning of DEN or DEN + PB treatments. The results are compared with the controls of the same age.

When DEN is administered alone for 2 weeks (Fig. 3), the mitotic response remains approximately normal: the mitotic waves present about the same amplitude and the mitotic circadian rhythm is still marked. Micronuclei, rarely observed in unoperated livers appear soon with the first mitoses but their number remains constant and rather low from the 36th post-operative hour in spite of the very high mitotic activity.

When DEN alone is given for 6 weeks (Fig. 4), the mitotic response to the operation is strongly inhibited and the circadian rhythm is completely lost. The number of micronuclei, more frequently observed even before the operation, increases during the whole regenerative process, reaching higher value than after a 2 weeks DEN treatment in spite of the fact that cell division is strongly inhibited.

When PB is administered simultaneously with the DEN for 2 weeks (Fig. 5), the mitotic response to the operation presents the same pattern of evolution as when DEN is given alone; however, the 2nd and the 3rd mitotic wave have an higher amplitude in DEN + PB treated rats. The micronuclei expression is low and about the same as when the carcinogen is given alone (compare Figs. 5 and 3). When DEN + PB last for 6 weeks (Fig. 6), the mitotic activity is less inhibited and the micronuclei less numerous than in the corresponding experimental group where DEN is given without PB (compare Figs. 4 and 6).

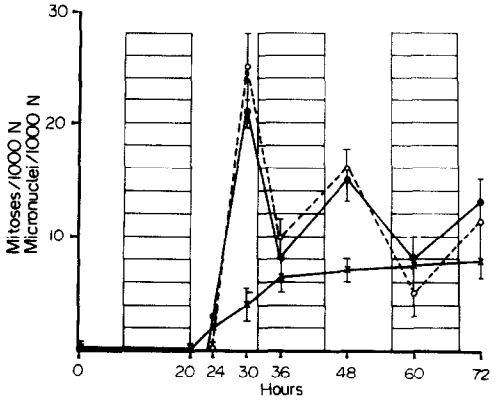

Fig. 3. Mitotic activity (●) *and micronuclei production* (×) *against time following* 2/3 *hepatectomy performed* 2 months *after the start of DEN administration for* 2 weeks. *Comparison with operated control rats of the same age* (○). *Vertical bars indicate the S.E.M. Shaded areas represent the dark phase* (6 p.m. – 6 a.m.).

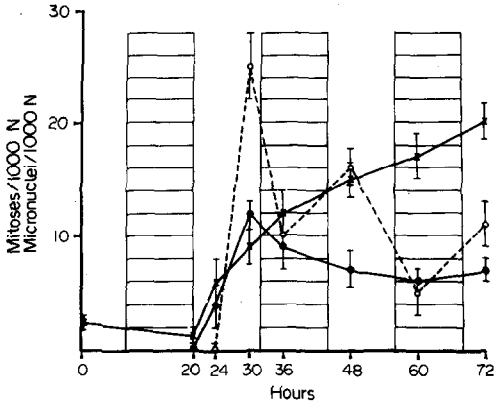

Fig. 4. Mitotic activity (●) and micronuclei production (×) against time following 2/3 hepatectomy performed 2 months after the start of DEN administration for 6 weeks. Comparison with operated control rats of the same age (○). Vertical bars indicate the S.E.M. Shaded areas represent the dark phase (6 p.m. – 6 a.m.).

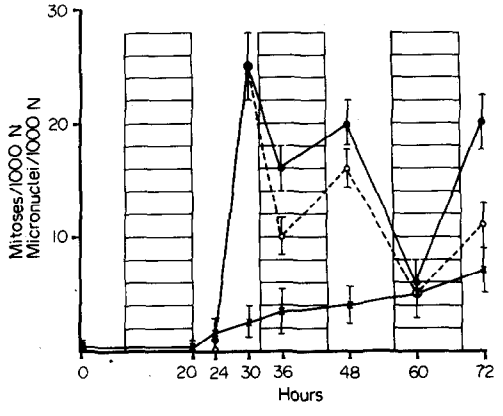

Fig. 5. Mitotic activity (●) and micronuclei production (×) against time following 2/3 hepatectomy performed 2 months after the start of DEN + PB administration for 2 weeks. Comparison with operated control rats of the same age (○). Vertical bars indicate the S.E.M. Shaded areas represent the dark phase (6 p.m. – 6 a.m.).

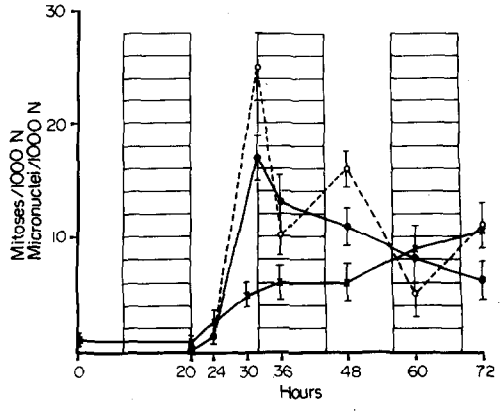

Fig. 6. Mitotic activity (●) and micronuclei production (×) against time following 2/3 hepatectomy performed 2 months after the start of DEN + PB administration for 6 weeks. Comparison with operated control rats of the same age (○). Vertical bars indicate the S.E.M. Shaded areas represent the dark phase (6 p.m. – 6 a.m.).

Survival and tumor incidence

In our strain of rats, no mortality was observed up to 30 months and almost all the rats died by 36 months. No death was recorded in animals treated with PB continuously for 24 months [1].

The survival and the tumor incidence in animals treated by DEN alone or by DEN + PB are shown in Table 2.

When DEN is given for 2 weeks, animals die after about 16 months without any liver cancer. In rats treated with DEN for 4 weeks, the median time of death is about 14 months but 50% of rats show hepatocellular hepatomas at autopsy. After a 6 weeks DEN administration, all the animals die with cancer after about 7–8 months.

PB simultaneously given with DEN delays death by 5–6 months in the three experimental conditions. In rats treated by DEN + PB for 2 and 4 weeks, no cancer was observed at autopsy. In the group treated by DEN + PB for 6 weeks, hepatomas were observed in only 40% of animals.

Table 2. Median time of death (survival 50%) and tumor incidence (hepatomas observed at autopsy) in rats treated either by DEN or by DEN + PB for 2, 4 and 6 weeks.

Duration of treatment	2 Weeks	4 Weeks	6 Weeks
DEN			
Survival (months)	16	14	7.5
Tumor incidence (%)	0	50	100
DEN+PB			
Survival (months)	21	19	17
Tumor incidence (%)	0	0	40

DISCUSSION

The results obtained by treating rats for 2, 4 and 6 weeks by DEN alone confirm previous data collected in the same experimental conditions [1, 9].

Two weeks of DEN administration initiate a small number of PAS positive preneoplastic foci presenting a low growth rate after the end of the treatment and no malignancy is observed up to the time of death occuring after about 16 months.

In rats treated by DEN for 4 and 6 weeks, the number of preneoplastic lesions is much higher but approximately the same in both groups. Their growth rate and the tumor incidence are proportional to the duration of the carcinogen treatment. Animals treated for 4 weeks die after about 14 months and 50% of them have hepatomas; when the treatment lasts 6 weeks, the median time of death is reduced to about 7.5 months and all the rats have hepatomas at autopsy.

The effect of phenobarbital (PB) given simultaneously with DEN (DEN + PB) modifies the

oncogenic effect differently in the several experimental conditions.

A 2 weeks DEN + PB treatment does not change the number of initiated preneoplastic foci compared to those obtained by DEN alone. In both conditions, the relative volume and the distribution of cell content in the lesions remain about the same even 3.5 months after stopping the treatment. This suggests that the growth rate of these foci is similar. The survival is longer by about 5 months in rats treated by PB + DEN but, as with DEN alone, no cancer is seen at autopsy.

When the treatment lasts for 4 or 6 weeks, the results clearly show that PB given simultaneously with DEN decreases the carcinogenic activity of the nitrosamine. After a 4 weeks DEN + PB treatment, the number, the relative volume and the cell content of PAS positive foci are significantly reduced; 6 weeks of DEN + PB administration are necessary to reach the volume obtained by 4 weeks of DEN alone.

When DEN + PB administration lasts 4 weeks, the median time of death is delayed up to 19 months and no cancer is observed compared to a mean death age of 14 months and 50% of tumor yield when DEN is given alone. After 6 weeks of combined treatment, animals die after about 17 months with only 40% of tumor incidence whereas we have a survival of 7.5 months and 100% of tumor yield when DEN is administered without PB.

Thus, the combined treatment for 4 or 6 weeks reduces carcinogenesis at the level of the initiation of preneoplastic cells as well as at the level of the promotion allowing the growth and the malignant transformation of precancerous lesions.

The PB effect on the initiation can be partially explained by the mitotic evolution observed during the treatment. It is well known that the first stage of carcinogenesis needs cell proliferation to fix the point mutations. When DEN is administered alone, a very high mitotic index with a normal circadian pattern showing a day time maximum is observed between the 2nd and the 4th week of treatment. The addition of PB decreases the mitotic activity and probably the mutagenic effect is depressed. This could explain the smaller number of preneoplastic foci initiated when PB is given with DEN for 4 weeks.

On the contrary, when DEN + PB are given for 6 weeks, the number of foci is about the same as when DEN is given alone but, their relative volume is much smaller. In this case, the tumor incidence is reduced and the median time of death occurs later. Since there are foci, the presence of PB seems to influence the 'time factor' necessary to promote them up to malignancy [10]. This also implies that the damages sufficient to initiate the foci do not necessarily make them reach the 'growth pressure' bringing their transformation [8]. It must be pointed out that, animals treated by DEN + PB for 6 weeks, present about the same characteristics as those treated by DEN alone by only 4 weeks; the number of initiated foci, their relative volume and their cell content, especially the tumor incidence and the median time of death are approximately the same in both conditions. It must be kept in mind that in our model, preneoplastic lesions are promoted only when the DEN treatment is continued from the 4th week. This longer carcinogen administration triggers then, in the whole liver tissue, different phenomena probably related to the promotion mechanism: a mitotic inhibition after the partial hepatectomy, a disturbance of the circadian mitotic rhythms, a production of micronuclei ... [1, 6, 9, 11, 12].

We have shown in the present work that the reducing effect on carcinogenesis due to the presence of PB is also associated with a reduction of these other disturbances.

After the subcarcinogenic treatment of 2 weeks, the presence of PB with DEN does not significantly disturb the mitotic response to the partial hepatectomy where the micronuclei expression remains very low in spite of the high proliferation activity. It must be kept in mind that the micronuclei expression implies mitotic events.

After a carcinogenic treatment of DEN administered alone for 6 weeks, the mitotic response to the operation is inhibited, the circadian mitotic variations are lost and the micronuclei expressed by the mitoses are very numerous.The same treatment associated with PB does not restore the circadian activity but significantly reduces the mitotic inhibition and the production of micronuclei.

All those results corroborate the hypothesis that PB simultaneously given with the carcinogen decreases the genotoxic effect of DEN administered alone.

In our experimental conditions, it has been checked that the plasmatic DEN concentration remains unchanged whether the DEN is given alone or mixed with PB (P. Lelievre, personal communication). Different mechanisms could be evoked to explain the carcinogenic-reducing effect due to the addition of PB to DEN. To obtain more information about this mechanism, biochemical experiments are in progress on the same material in order to test: the modification induced by PB on the DEN microsomal activation, on the DNA alkylation and on DNA repair.

Experiments are also planned to elucidate the possible relationship between both 'paradoxical' PB effects: this counteracting the *initiation* when administered simultaneously with the carcinogen and the *promoting* effect when administered after

the carcinogen treatment.

It must also be kept in mind that both these effects were previously reported by Berenblum in skin tumorogenesis; when croton oil and the carcinogen were applied simultaneously to the same area of skin, the induction of tumors was inhibited but when croton oil was applied after the carcinogen, a promotion effect was obtained [13].

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