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Liver Cell Control after Discontinuation of DENA Feeding in Hepatocarcinogenesis

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Abstract—*The mitotic response following a partial hepatectomy, the nyctohemeral rhythm of these mitoses and the 'chalone activity' measured by the inhibitory effect of liver extracts on the normal liver regeneration have been studied in order to estimate the evolution of mitotic control in the liver of rats treated by DENA for* 2, 4, 6 *and* 10 *weeks. These parameters and the pathological lesions (preneoplastic foci, neoplastic nodules and hepatomas) have been followed up after stopping the DENA feeding. A good correlation has been found between the malignant transformation of preneoplastic foci and the breakdown of the mitotic control. In animals treated by DENA for two weeks, the homeostatic control of mitoses remains normal for a minimum of* 14 *months and 'preneoplastic foci' persist without any further malignant transformation. After DENA feeding for* 4 *and* 6 *weeks, the subsequent malignant transformation occurs as a function of the mitotic disturbance: the longer the DENA feeding, the faster the homeostatic disturbance, the earlier the canceration. After DENA treatment for* 10 *weeks, the homeostatic regulation is lost and the neoplastic growth is triggered at the time of the DENA feeding cessation. In this case, the pattern of canceration is the same as when DENA is given continuously up to the time of death. It is concluded that 'preneoplastic foci' induced during the first two weeks of treatment cannot by themselves transform into a malignant tumor. To commit them irreversibly into malignancy, a subsequent action of the carcinogen is necessary; it may consist of the irreversible breakdown of the normal homostatic regulatory mechansim of liver cell proliferation.*

INTRODUCTION

IN PREVIOUS experiments, we have investigated the homeostatic regulation of rat liver cell proliferation and function in physiological conditions [1] as well as during chemical carcinogenesis [2–4]. The mitotic response following a partial hepatectomy and the nyctohemeral rhythm of mitoses are the parameters used to estimate the normal control of cell division.

The 'chalone activity' of liver tissue has also been tested. There is indeed good evidence that substances ('chalones') released by differentiated cells are implicated in this type of regulation [5]. When extracted from a normal adult liver, these substances injected after partial hepatectomy inhibit liver regeneration. The inhibitory effect does not appear when the extract is prepared from a regenerating liver [3].

When the same parameters combined with histological appearance were used to study the liver changes induced by the daily administration of 10 mg/kg of diethylnitrosamine (DENA), three different stages were found in the pattern of liver canceration.

In the first stage, which corresponds to the first month of treatment, although the so-called 'preneoplastic foci' characterized by the absence of glycogen depletion after fasting [6] appear in the liver, the regulatory mechanisms remain normal. During the second stage (corresponding to the second month), the size of the foci increases while their number remains unchanged and the homeostatic regulation is progressively lost. The third stage begins with the third month of DENA feeding and corresponds to the triggering of unequivocal neoplastic growth.

During continuous DENA treatment, the growth of the 'foci' and their subsequent malignant transformation vary in parallel to the breakdown of the normal homeostatic control. When DENA feeding is stopped at various intervals the evolution of liver lesions varies with the stage at which the treatment is discontinued [7, 8]. Less than one month of

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DENA administration resulted only in the development of 'preneoplastic foci' that persist up to the time of death, i.e., 1–2 years. Protracting the treatment during the second month induces the malignant transformation of 'preneoplastic foci'. The later the treatment is stopped during the second month, the earlier the tumors appear. The process of liver canceration appears to be the same whether the DENA is discontinued after the second month or is given up to the time of death.

The occurence and the rapidity of the malignant transformation could depend on the degree of disturbance the mitotic control has reached at the time when the treatment is stopped. It seems of interest, therefore, to investigate the homeostatic control of cell proliferation after the cessation of the DENA feeding in subcarcinogenic and carcinogenic conditions. In the present work, the mitotic response following a partial hepatectomy, the nyctohemeral rhythm of mitoses and the 'chalone activity' of liver tissue have been estimated after stopping the DENA treatment at various times.

MATERIALS AND METHODS

Four experimental groups of male Wistar rats, weighing about 180 g, were chronically treated by diethylnitrosamine (DENA) for 2, 4, 6 and 10 weeks. The drug was administered in drinking water (80 mg/l), representing an ingested dose of about 10 mg/kg/day.

At various times after beginning the DENA administration (see Table 1), samples of at least 10 animals were taken from each experimental group and submitted to a partial hepatectomy. The two-third hepatectomy was always performed at 10 a.m. The mitotic indices were determined on histological preparations after Feulgen reaction in areas where the liver tissue did not show any detectable lesion.

Three measurements of mitotic index were made at the time of surgery, at the 48th and the 60th hours after partial hepatectomy. The difference between the two last results allows estimation of the nyctohemeral rhythm of mitoses, since the 48th post-operative hour corresponds to the second mitotic peak occuring during day-time and the 60th hour to the second 'valley' occuring during night. The first mitotic wave presenting a peak at about the 30th hour is submitted to important individual variations so that it appears less suitable for the study of the circadian variations of the mitoses. All results were compared to those obtained in untreated controls of the same age.

In the same experimental conditions, all livers were macroscopically and microscopically examined in order to detect the 'preneoplastic foci', the transformed neoplastic nodules and the hepatomas by a method previously described [8]. The pathological lesions are defined according to Squire and Levitts [9].

Animals treated for 2, 4 and 6 weeks were killed 14, 6 and 3 months, respectively, after the beginning of the DENA feeding, in order to estimate the 'liver chalone activity'. In each group, liver extracts from 20 rats (minimum) were prepared according to the method of Verly [10]. The 105,000 *g* supernatant of liver homogenate was treated by ethanol and the precipitate obtained at an ethanol concentration between 70 and 87% was diluted in saline and injected (1 ml i.p.) to normal hepatectomized animals. Injections were made at the 1st and 6th postoperative hour. The volume of the injected material corresponded to an extract from about 70% of the total liver mass. In order to estimate the 'chalone activity', the [^{3}H]-thymidine-labelling index was measured at the 24th post-operative hour and compared with the same index found in control hepatectomized rats injected with saline instead of 'chalone' preparation. The results were compared with those obtained with an extract prepared either from normal or regenerating liver of 14-month-old rats. The 'chalone' preparation from hepatectomized animals was made at the 24th post-operative hour, i.e., at the moment of the DENA synthesis [11].

The labelling index was measured by autohistoradiography 1 hr after administration of [^{3}H]-thymidine (2 μCi/g, i.p.). All the labelling and the mitotic indices were the mean of counts made by observing about 25,000 nuclei in at least 5 animals. The standard error of the mean was calculated.

RESULTS

Mitotic response

Table 1 shows the changes of the mitotic index under the various experimental conditions. In untreated control animals, the spontaneous mitotic activity was too low to be detected. After hepatectomy, the mitotic peak measured during the day-time at the 48th post-operative hour was very high and the minimum measured at night at the 60th post-

Table 1. *Mitotic activity and pathological lesions*

Delay between beginning of DENA treatment and hepatectomy	Post-operative hours	Duration of DENA feeding				
		Controls	2 weeks	4 weeks	6 weeks	10 weeks
0	0	0.0	3.0±1	0.6±0.2^{F}	1.0±0.2^{F}	3.0±1^{N}
	48	23±2	17±2	12±2	9.5±1	5.0±1
	60	4±1	7±1	10±1	9.0±1	5.1±1
3 months	0	0.0	0.0^{F}	0.1±0.1^{F}	0.0^{F}	2.8±1^{H}
	48	24±2	22±1	9.5±1	6.0±0.5	2.9±1
	60	2.3±1	2.5±0.5	5.0±1	4.2±0.5	—
6 months	0	0.0	0.0^{F}	0.1±1^{F}	2.0±0.5^{N+H}	—
	48	24±2	20±1.5	9.0±1	5.2±1	—
	60	3.1±0.5	2.2±0.5	5.2±0.5	4.3±0.5	—
9 months	0	—	—	1.1±0.3^{N+H}	3.0±1^{H}	—
	48	—	—	5.7±1	2.5±1	—
	60	—	—	5.3±1	2.8±0.3	—
12 months	0	—	—	2.2±1^{H}	—	—
	48	—	—	3.1±1	—	—
	60	—	—	—	—	
14 months	0	0.0	0.0^{F}	—	—	—
	48	22±2	18±2	—	—	—
	60	3.0±0.1	3.3±0.5	—	—	—

Mitotic activity and pathological lesions in the liver after DENA treatment. Mitoses per 1000 nuclei of hepatocytes measured at the time of the partial hepatectomy (0) or at the 48th and at the 60th post-operative hours in different experimental groups when DENA feeding was discontinued after 2, 4, 6 and 10 weeks. The results are compared to those obtained in untreated control animals of the same age. The standard error of the mean of counts was obtained by observing about 25,000 nuclei in a minimum of 5 animals.

The capitals indicate the presence of pathological lesions observed at the time of surgery: (F) preneoplastic foci without malignant growth; (N) neoplastic nodules; (H) hepatomas as defined according to Squire and Levitt [9].

operative hour was very low, whatever the age of the animals. That means that in the control group, the nyctohemeral rhythm of mitoses remained well marked during the whole experimental period.

In animals treated by DENA for two weeks and hepatectomized immediately the treatment was stopped, a slight disturbance of the mitotic index was observed. A very perceptible mitotic index was already measured in the liver at the time of surgery [2]; after the operation, the mitotic peak appeared to be slightly reduced, but the circadian rhythm remained more or less normal. In rats operated 3, 6 and 14 months after stopping the 2-weeks DENA administration, the mitotic response following a partial hepatectomy seemed to be completely restored. In all these cases, mitoses were never detected in the surgically removed liver tissue.

In the rats operated immediately after a 4, 6 and 10 week DENA feeding, there was a mitotic disturbance which increased with the duration of the carcinogen treatment. Indeed, the mitotic response progressively decreased and the mitotic rhythm induced by the partial hepatectomy was lost. At the same time, more and more 'autonomous' mitoses (i.e. mitoses found in animals treated by DENA without any other exogenous stimulation) appeared in the normal liver tissue. In the four week group, there was some transient restoration of the circadian rhythm between the third and sixth month following the treatment arrest. Later on, the disturbance of the mitotic response reappeared.

It seems that in all the groups where the mitotic response did not exceed 5‰, there was a complete loss of the circadian rhythm and the occurence of 'autonomous' mitoses. This value of 5‰ appears to be a threshold reached at various delays of the carcinogen treatment. After DENA feeding for 4 and 6 weeks, this threshold was found at the ninth

and at the six months, respectively, following the beginning of the treatment. When DENA feeding lasted for 10 weeks, the threshold was was already reached at the time of treatment cessation.

Evolution of pathological lesions

As mentioned in Table 1, no microscopical lesions were found in untreated control animals. After 2 weeks of DENA feeding, 'preneoplastic foci' characterized by their ability to retain glycogen after fasting were present. They persisted indefinitely during the whole experiment without any malignant transformation. When the treatment was stopped after 4 or 6 weeks, the foci remained unchanged for 6 and 3 months, respectively: thereafter, neoplastic nodules and cancers occured. All the livers of the rats treated for 4 weeks (20/20) were cancerous from the 9th month on. In the 6 week group, all animals bore cancers after the 6th month. After a 10-week DENA feeding, neoplastic growth was already present when treatment was stopped.

Variation of 'chalone activity'

Table 2 summarizes the variations of the liver 'chalone' activity under various experimental conditions. In liver extracts of normal 14-month-old rats a strong inhibitory effect on the labelling index measured was observed 24 hr after a partial hepatectomy. In contrast, an extract obtained from a regenerated liver in rats of the same age was devoid of any activity. Indeed, the response was identical to that found in hepatectomized control rats receiving saline instead of 'chalone'.

In animals treated for 2, 4 and 6 weeks, liver extracts were prepared 14, 6 and 3 months, respectively after the beginning of DENA treatment. When the extracts were obtained from animals treated by DENA for 2 weeks and killed 14 months after the beginning of the treatment, the inhibitory effect was identical to that obtained from healthy rats of the same age. However, in precancerous livers of rats treated for 4 and 6 weeks, the chalone activity was decreased. Indeed, the response did not differ from that seen after saline injection alone.

DISCUSSION

It has already been claimed that cancer production by nitrosamines requires at least two different steps: (a) an initial induction of mutations fixed by cell division; and (b) a subsequent growth stimulation of these mutated cells leading to the development of a malignant tumor [12, 13]. Most previous work has been focused on the first step. Mutagenesis in micro-organisms and in cell culture has been extensively used to study the

Table 2. Chalone activity

Product injected	Labelled nuclei/1000 cells measured 24 hr after partial hepatectomy
Saline	180 (±15)
Liver extract from untreated 14 month-old rats	85 (±12)
Liver extract from rats treated for 2 weeks and sacrificed 14 months after the beginning of the DENA feeding	90 (±10)
Liver extract from rats treated for 4 weeks and sacrificed 6 months after the beginning of the DENA feeding	157 (±10)
Liver extract from rats treated for 6 weeks and sacrificed 3 months after the beginning of the DENA feeding	184 (±14)
Extract of regenerating liver in rats sacrificed 24 hours after a partial hepatectomy	204 (±20)

Chalone activity estimated by the labelling indices measured 24 hours after a partial hepatectomy performed in normal rats treated by an hepatic extract prepared in various conditions. Results are compared with those obtained in hepatectomized animals treated with saline instead of a liver extract. The figures in parentheses indicates the standard error of the mean counts made by observing about 25,000 nuclei in a minimum of 5 rats.

molecular mechanism and the biological conditions leading to the formation of preneoplastic lesions in the liver. The 'preneoplastic foci' appear to be clonal in origin [14] and present enzymatic and metabolic disturbances [15] as a result of point mutations. Very little is known about the second step of tumor development; it has been postulated that the carcinogen creates a 'cellular environment' favoring the growth of the 'preneoplastic foci', and which is necessary for the appearance of the malignancy [13].

By following the changes occuring in the homeostatic control of cell division and the evolution of pathological lesions, it seems possible to define more precisely two different periods in DENA-induced liver carcinogenesis.

The first period, corresponding in our experimental conditions to the first month of DENA feeding, is characterized by the induction of 'preneoplastic foci' [2, 3]. When treatment is arrested at this stage, the response to partial hepatectomy and the nyctohemeral rhythm of mitoses remain almost normal as compared to these untreated control animals. In this condition, the preneoplastic cells and foci remain unchanged without any further malignant transformation up to the time of death, i.e., 1–2 years [7, 8].

During the second period, which corresponds to the second month of DENA feeding, the size of the nodules increases while their number remains unchanged [2]. Simultaneously, mitotic control is progressively lost [2, 3]. By discontinuing the DENA treatment at various times during this second period, a good correlation is found between the degree of the homeostatic control disturbance and the evolution of the cancerous lesions. During this period, the longer the DENA feeding, the faster the mitotic control disturbance, the triggering of 'autonomous' mitoses and the malignant transformation.

When a 10-weeks DENA feeding is given, the response to partial hepatectomy is reduced, as previously shown [3], the nyctohemeral rhythm of mitoses and the chalone activity is lost, 'autonomous' mitoses are present and neoplastic growth is already detected at the end of the treatment. In this case, the tumour growth progresses up to death at the same rate as it does in continuously treated animals [8].

It must be pointed out that the different successive stages of lesions (foci, neoplastic nodules, hepatomas) closely correlate with a given degree of disturbance in the mitotic control. The neoplastic growth as well as the autonomous cell proliferation in the untransformed liver tissue occur when the mitotic response following partial hepatectomy no longer exceeds 5/1000. This observation corroborates previous results obtained by Rabes and Hartenstein [16]. When the animals begin to die with cancer as determined in similar experimental groups [7, 8], mitotic response is still lower: about 3/1000.

These data suggest that 'preneoplastic foci' induced during the first step cannot, by themselves, transform into malignant tumours. To commit them irreversibly into malignancy, a subsequent action of the carcinogen seems necessary; it may consist in the irreversible breakdown of the mechanisms which control the cell homeostasis of normal tissue.

In this mitotic activity control, an important role is ascribed to the 'chalone effect'. Factors released by differentiated cells seem able to inhibit the cell division in the same tissue [5]. As previously shown [3], this inhibitory effect is demonstrated in normal but not in regenerating liver. We had also previously shown that this 'chalone activity' decreases during the second month of DENA feeding simultaneously with the loss of response to a partial hepatectomy [3]. The present results confirm these previous data. In addition, they show that changes in the 'chalone activity' may correlate with the mitotic disturbances and the evolution of the precancerous lesions.

The reduction of the 'chalone activity' could be one of the factors characterizing the 'cellular environment' invoked to explain the evolution of previously induced preneoplastic lesions.

At the time when activity is decreased, preneoplastic foci represent about 5% of the total liver mass (in preparation). It seems doubtful, therefore, that the drop in chalone activity could be related to transformed cells alone.

Other experimental results corroborate this interpretation. Hepatocytes from 'nodules' developed in preneoplastic liver tissue of DENA-fed rats are able to proliferate *in vitro* (where chalones are not present) [15]. On the other hand, similar 'hyperplastic nodules' transplanted into normal rats (where hepatic chalone activity is normal) are capable of reversion to normal hepatic phenotype [17]. More recently, we have shown that normal liver 'chalone' added to malignant Reuber's hepatoma cells cultivated *in vitro* inhibits the cell proliferation and induces some cell differentiation [18].

The second role played by the carcinogen in the genesis of liver cancer could be the breakdown of the 'chalone'-related homeostatic regulatory mechanisms.

It must be kept in mind that this second carcinogenic action may also be played by noncarcinogenic substances which stimulate the cell growth, such as phenobarbital [12, 13, 19, 20]. Experiments are planned in order to see if these substances are also implied in the disturbance of the normal 'chalone' homeostatic regulation.

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