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Different Effects of Regenerative and Direct Mitogenic Stimuli on the Growth of Initiated Cells in the Resistant Hepatocyte Model

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The possible mechanism(s) responsible for the different effects exerted by proliferative stimuli of different nature on the appearance of enzyme-altered hepatic foci, were investigated in male Wistar rats. Rats given an initiating dose of diethylnitrosamine (150 mg/kg body weight) were fed a diet containing 0.03% acetylaminofluorene for 2 weeks. Between the first and the second week, cell proliferation was induced by a proliferative stimulus of compensatory type (partial hepatectomy) or by a direct mitogenic stimulus (lead nitrate, 100 μmol/kg). The effect of the two different proliferative stimuli on the appearance of γ-glutamyl transferase-positive foci was monitored by killing the rats for examination at 1, 2, 3, 5, and 6 days after the induction of cell proliferation. The results indicate that while enzyme-altered hepatocytes can be observed as early as 3 days after partial hepatectomy and are characterized by a rapid growth, direct hyperplasia did not exert any effect on the growth capacity of initiated cells. No effect of lead nitrate-induced hyperplasia was observed following three administrations of the mitogen. When platelet-poor plasma taken from animals exposed to the different proliferative stimuli was tested in primary cultures of hepatocytes, it was found that it induced a significant increase in the labeling index of normal hepatocytes. However, while serum taken 6 days after partial hepatectomy was still able to induce a significant increase in the labeling index, platelet-poor plasma from lead-treated rats had lost part of its effect at 5 days after treatment. The inability of direct hyperplasia to stimulate the development of enzyme-altered hepatic foci was not unique to lead nitrate since the same phenomenon was observed when three other hepatomitogens, nafenopin, cyproterone acetate, and ethylene dibromide, were used.

Key words: Compensatory regeneration — Mitogen-induced growth — Initiated cell — Resistant-hepatocyte model — Liver

Cell proliferation appears to be intimately involved in several phases of the carcinogenic process. Epidemiological as well as experimental evidence indicating an association between cell proliferation and carcinogenic process[1–4] has recently led several investigators to hypothesize that any condition characterized by enhanced cell division results in an accumulation of genetic errors that may ultimately lead to neoplasia.[5,6]

The role of cell proliferation in the carcinogenic process has been particularly well characterized in the liver. This organ, in adult organisms, is characterized by a quiescent state; however, liver cells may be induced to proliferate following cellular loss such as that occurring after surgical resection of part of the organ (PH[4]) or following necrogenic agents such as carbon tetrachloride (CCl_4). The regenerative response elicited by these conditions is positively associated with initiation and promotion of liver carcinogenesis.[7–12]

Recently, however, we have shown that liver growth induced by primary mitogens (chemicals that are able to cause an increase in the mitotic activity of liver cells in the absence of previous cell loss) did not support initiation of chemical carcinogenesis and did not promote the growth of diethylnitrosamine (DENA)-initiated cells to a focal or nodular stage.[13–15] These results suggest that growth stimuli of different nature may play different roles in liver carcinogenesis.

In the present study we have analyzed the response of DENA-initiated cells at various times after different growth stimuli, namely compensatory regeneration and mitogen-induced liver growth, in the resistant-hepatocyte model.[16] The results indicate that while initiated cells rapidly grew following PH (compensatory regeneration), initiated hepatocytes did not respond to the growth stimulus induced by the mitogen lead nitrate (LN), as shown by the fact that at no time point was the number or the area of γ-glutamyltransferase (GGT)-positive foci in-

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[4] Abbreviations: PH, partial hepatectomy; DENA, diethylnitrosamine; CCl_4, carbon tetrachloride; LN, lead nitrate; 2-AAF, 2-acetylaminofluorene; EDB, ethylene dibromide; NAF; nafenopin; CPA, cyproterone acetate, GGT; γ-glutamyl transferase; ATPase, adenosine triphosphatase; PPP, platelet-poor plasma.

creased in comparison to that of animals exposed to 2-acetylaminofluorene (AAF) in the absence of proliferative stimuli. LN-induced hyperplasia was unable to stimulate the growth of initiated cells despite its capacity to induce serum growth factors able to stimulate DNA synthesis in primary cultures of hepatocytes. The inability of direct hyperplasia to stimulate selectively the growth of initiated cells does not appear to be a phenomenon unique to lead nitrate since similar findings were obtained when three different liver mitogens, ethylene dibromide (EDB), nafenopin (NAF) and cyproterone acetate (CPA), were used.

MATERIALS AND METHODS

Male Wistar rats (Charles River, Milan, Italy) weighing 180–200 g were used. The rats were fed a rodent semi-synthetic laboratory diet (Ditta Mucedola, Milan, Italy) and allowed water *ad libitum*. LN was obtained from Carlo Erba, Milan, Italy; DENA and EDB from Sigma Chemical Corp., St. Louis, USA), CCl_4 from Merck (Darmstadt, Germany), and CPA from Pentagone (Montreal, Canada). NAF was a gift from Dr. V. Preat.

Experimental protocol Experiment 1: Rats were injected i.p. with a necrogenic dose of DENA (150 mg/kg, in 0.9% NaCl), following overnight starvation. After a recovery period of 2 weeks, animals were placed on a diet containing 0.03% 2-AAF for 2 weeks. One week after starting the 2-AAF diet, rats were subjected to either a compensatory liver cell proliferative stimulus such as PH or to a mitogenic stimulus induced by LN (100 μmol/kg, i.v.). Rats were killed at 1, 2, 3, 5 and 6 days thereafter.

Experiment 2: Two weeks after initiation with DENA (150 mg/kg), animals were exposed to a 2-AAF-containing diet. One week after starting the diet, rats were divided into 5 groups: Group 1 was subjected to PH (compensatory regeneration), Group 2 received a single injection of LN, Group 3 two doses of LN (at 7 and 10 days), Group 4 3 doses of LN (7, 10 and 13 days), and Group 5 was not exposed to proliferative stimuli of any kind. Fourteen days after the beginning of the 2-AAF diet rats were placed on a basal diet and killed one week later. Initiated cells were monitored as GGT-positive hepatocytes.

Experiment 3: Rats were subjected to the protocol described in Experiment 1, except that direct hyperplasia was induced during 2-AAF feeding with 4 different mitogens, EDB (100 mg/kg, intragastrically, in oil),[17] NAF (200 mg/kg, intragastrically, in oil),[18] CPA (60 mg/kg, intragastrically in oil)[19] and LN (100 μmol/kg, i.v.).[20] In addition to PH, another type of compensatory regeneration, namely that induced by a necrogenic dose of CCl_4 (2 ml/kg, intragastrically, in oil), was used. Foci were monitored as GGT^+ or $ATPase^-$ hepatocytes.

Induction of DNA synthesis by platelet-poor plasma (PPP) Blood was collected as described previously[21] from the abdominal aorta into 10 ml syringes containing 1 ml of acid citrate dextrose and centrifuged (4°C, 20 min, 3500 rpm) to yield platelet-free supernatant. PPP samples, defibrinated by the addition of 0.1 *M* $CaCl_2$ and $MgCl_2$ at 37°C, were then dialyzed for 24 h in Ca/Mg-free PBS with 14 kd dialysis membranes.

Hepatocytes were isolated by a collagenase perfusion technique[22] from normal rat liver. Viable hepatocytes were cultured on 35 mm dishes coated with collagen (vitrogen, 60 μg/dish) in modified William's medium supplemented with fetal bovine serum (10% v/v), insulin (20 U/liter), L-glutamine (2 m*M*), HEPES (10 m*M*), penicillin (100 U/ml) and streptomycin (100 μg/ml). After an attachment period of 3 h the medium was removed and changed to serum-free, modified William's E medium supplemented with L-proline. Appropriate dishes contained the different PPP or basal medium or EGF (20 ng/ml) and [^{3}H]thymidine (5 μCi/dish). After 48 h of incubation, the hepatocytes were washed with cold PBS, fixed in 10% formalin and processed for autoradiography. The labeling index (LI) was determined by counting 800 cells per dish from randomly selected fields.

Histochemical staining Liver sections were immediately frozen in isopentane cooled in liquid nitrogen and sections cut with a Leitz 1720 Cryostat. Sections were then processed for histochemical localization of GGT according to the method of Rutenburg *et al.*[23] or for histochemical staining of ATPase.[24] The number of GGT^+ and $ATPase^-$ foci was expressed as number per cm^2 of section or was quantitated by using a computer program designed to calculate the total volume occupied by such lesions.

RESULTS

Table I shows the effects of proliferative stimuli of different nature applied during 2-AAF feeding on the growth of DENA-initiated cells to GGT-positive foci. The data obtained indicate that while a compensatory type of cell proliferation such as that observed after PH rapidly stimulates the growth of initiated cells (a significant increase in the size of GGT-positive foci can be observed as early as 3 days after PH), such an effect was not seen when the proliferative stimulus was induced by LN. In addition, while the number of GGT-positive foci seen at 6 days after PH was approximately 6 times higher than that of controls, no increase was seen in LN-treated rats. The difference between the two proliferative stimuli was even more striking when the area of the lesions was considered. In fact, while the total area occupied by enzyme-altered lesions in PH rats at 6 days was 13 times

Table I. Number and Area of GGT$^+$ Foci Occurring in Rat Liver Following Proliferative Stimuli of Different Nature

Treatment[a]	GGT$^+$ Foci		
	No/cm^2	Area foci (mm^2/cm^2)	Mean foci area (mm^2)
DENA+2-AAF			
1 day	1.9±0.2[b]	0.028±0.008	0.014±0.005
2 days	4.0±3.4	0.097±0.095	0.022±0.005
3 days	4.4±2.6	0.090±0.071	0.020±0.007
5 days	4.0±1.9	0.100±0.050	0.030±0.008
6 days	4.7±3.8	0.170±0.160	0.040±0.005
DENA+2-AAF+PH			
1 day	3.1±1.6	0.078±0.068	0.023±0.010
2 days	4.2±1.1	0.136±0.055	0.030±0.007
3 days	7.9±4.6	0.380±0.240*	0.048±0.010**
5 days	16.1±5.8***	1.860±0.690***	0.120±0.020***
6 days	23.0±9.8***	2.260±1.160**	0.110±0.040**
DENA+2-AAF+LN			
1 day	2.4±0.9	0.040±0.020	0.016±0.005
2 days	5.2±3.6	0.098±0.085	0.015±0.005
3 days	4.7±3.0	0.110±0.080	0.024±0.009
5 days	4.1±1.1	0.120±0.060	0.030±0.008
6 days	4.2±2.8	0.140±0.090	0.030±0.004

a) For experimental details see Experiment 1 in "Materials and Methods."
b) Values represent mean±SD of six rats per group.
Significantly different from the group DENA+2-AAF:
* $P<0.025$; ** $P<0.005$; *** $P<0.001$.

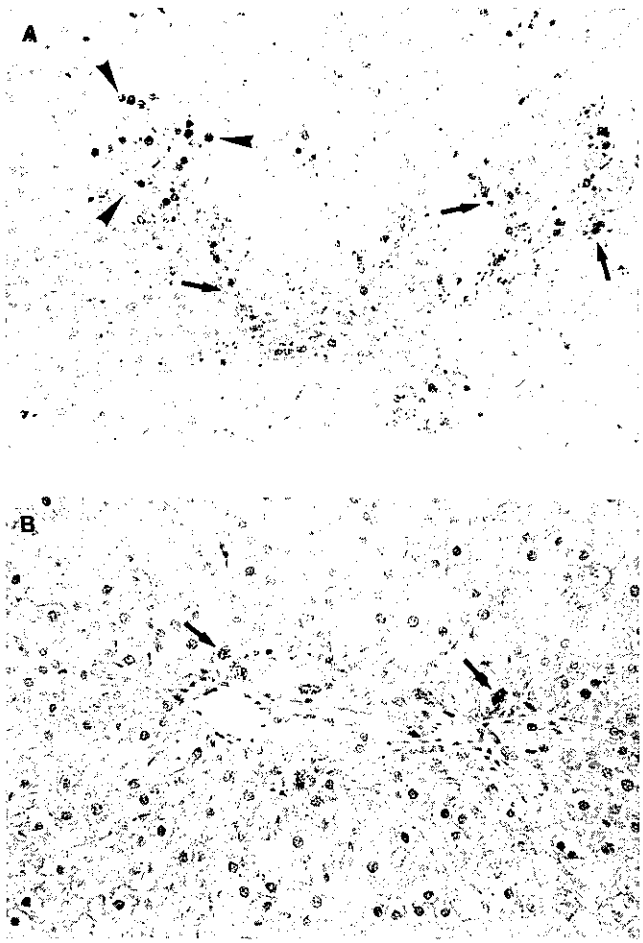

Fig. 1. Autoradiography of liver from rats killed 5 days after PH (A) or LN (B) during 2-AAF feeding. Rats were initiated with DENA (150 mg/kg). Following a two-week recovery period the animals were fed a diet containing 0.03% 2-AAF. One week after the start of the 2-AAF diet, cell proliferation was induced through PH or LN and rats were killed 5 days afterwards. Tritiated thymidine was injected at a dose of 20 μCi/100 g 3 h before sacrifice. Only nuclei of non-parenchymal cells (arrows) or of hepatocytes in a focal lesion (arrowheads) are labeled. HE, ×250.

greater than that of controls, no difference was seen between controls and LN-treated rats. Mean foci area was also increased in PH rats but not in LN-treated animals. In both conditions 2-AAF exerted a strong mitoinhibitory effect on normal liver cells as shown by determination of specific activity of DNA,[25] and by the fact that incorporation of thymidine occurred only in nuclei of hepatocytes of preneoplastic lesions or non-parenchymal cells of rats subjected to PH or LN (see Fig. 1).

A similar lack of effect on the growth of initiated cells was observed when LN was given three times in a week in order to generate repeated proliferative stimuli for the initiated cells. The results shown in Fig. 2 indicate that despite the presence of a mitoinhibitory environment for the non-initiated cells, repeated treatment with LN did not cause any increase in the number of enzyme-altered foci when compared to that of animals not exposed to proliferative stimuli. In addition, no increase in the size of the lesions could be observed (data not shown). These results suggest that initiated cells may not be able to respond to direct proliferative stimuli induced by LN, while responding very efficiently to proliferative stimuli of compensatory type (PH).

It is believed that PH stimulates liver regeneration by increasing the plasma concentration of specific growth factors such as HGF.[26,27] Despite very recent studies,[28,29] not many data are available about the role of growth factors following mitogen administration. Therefore, we addressed the question of whether the lack of effect of LN could be due to its inability to induce growth factors that in turn could stimulate the initiated cells to form preneoplastic lesions. To study that, PPP isolated from animals exposed to 2-AAF and killed at various times after PH and LN was tested for its ability to

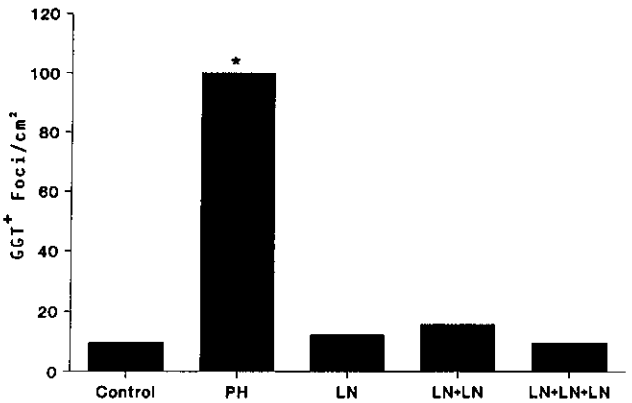

Fig. 2. Effect of repeated proliferative stimuli induced by LN during 2-AAF feeding on the incidence of GGT^+ foci. Two weeks after initiation, rats were fed a diet containing 0.03% 2-AAF for 2 weeks. One week after the start of the diet, rats were divided into 5 groups: Group 1 was subjected to PH; Group 2 received a single injection of LN (100 μmol/kg, LN); Group 3 received two doses at 7 and 10 days (LN + LN), and Group 4 was injected 3 times, at 7, 10 and 13 days (LN+LN+LN). No proliferative stimulus was applied to Group 5. After the second week of 2-AAF feeding, rats were placed on a basal diet and killed one week later. Values are means of five rats per group. * Significantly different from the group DENA+2-AAF: $P<0.001$.

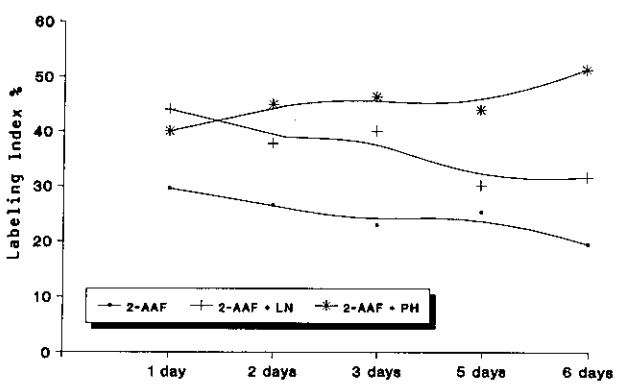

Fig. 3. Effect of 20% PPP isolated from rats killed at various times after 2-AAF+PH, 2-AAF+LN or 2-AAF alone on DNA synthesis of normal monolayer hepatocytes. Two weeks after initiation with DENA rats were fed a diet containing 0.03% 2-AAF. One week after the start of the diet, cell proliferation was induced by PH or LN and rats were killed 1, 2, 3, 5, or 6 days afterwards.

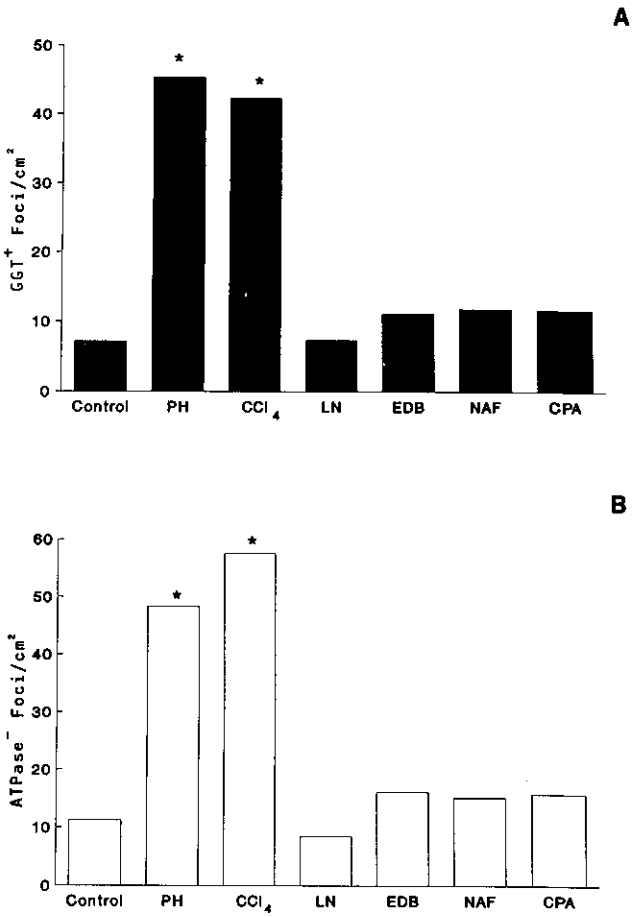

Fig. 4. Effect of proliferative stimuli of different nature given during 2-AAF feeding on the incidence of GGT^+ (A) or $ATPase^-$ foci (B). Following initiation with DENA (150 mg/kg) rats were exposed to 2-AAF. After 1 week of 2-AAF feeding, a cell proliferative stimulus was provided by PH, CCl_4 (2 mg/kg, per os), LN (100 μmol/kg, intravenously), NAF (200 mg/kg, intragastrically), CPA (60 mg/kg, intragastrically); or EDB (100 mg/kg, intragastrically). Rats were maintained for one more week on 2-AAF and then placed on a basal diet and killed 1 week later.

stimulate DNA synthesis in primary cultures of hepatocytes. The results shown in Fig. 3 indicated that PPP taken from animals killed at 1, 2 and 3 days after PH and LN, induced an LI of approximately 40–45% versus an LI of 25% of controls. However, while a 100% increase in LI was observed even when PPP was taken 5 or 6 days after PH, the ability of PPP from LN-treated rats to induce DNA synthesis showed a trend towards control values starting from day 5.

To test whether the inability to stimulate the growth of initiated cells could be a general phenomenon of direct hyperplasia rather than being unique to the chemical nature of the mitogen LN, the effect of direct hyperplasia on the development of enzyme-altered foci in the resistant hepatocyte model was investigated using three other hepatic mitogens, EDB, NAF and CPA. The results

shown in Fig. 4 clearly demonstrate that initiated cells are stimulated to grow only by a compensatory type of proliferative stimulus such as that induced by CCl_4 or PH, but not when a direct type of proliferative stimulus was applied, irrespective of the mitogen used. In fact, while GGT^+ or $ATPase^-$ foci were numerous in the liver of rats exposed to PH or treated with CCl_4 (4- to 5-fold increase when compared to controls) no significant elevation in the number of enzyme-altered lesions was found in the liver of rats treated with CPA, NAF, EDB or LN.

DISCUSSION

The resistant-hepatocyte model is based on the assumption that while non-initiated hepatocytes are sensitive to the mitoinhibitory effect of 2-AAF the initiated cells are resistant to the cytostatic effect and thus can respond to the proliferative stimulus and grow to form foci/nodules. This model as originally described makes use of a proliferative stimulus of compensatory type such as that elicited by PH or CCl_4.[7, 16]

The present study demonstrates that, unlike compensatory liver cell proliferation, a direct mitogenic stimulus given during 2-AAF feeding does not support the growth of initiated hepatocytes to form foci/nodules. The inability of mitogen-induced growth to stimulate selectively the proliferation of DENA-initiated cells does not appear to be restricted to a single mitogen. In fact, similar results were observed when 4 different hepatic mitogens, namely EDB, CPA, NAF and LN, were used (from 7 to 12 GGT^+ foci/cm^2 versus 42 and 45 GGT^+ foci/cm^2 of rats subjected to PH or CCl_4).

At present it is difficult to explain the different effects of these two types of proliferative stimulus. In our previous studies, we examined the possibility that the inability of mitogen-induced liver growth could be due to an insufficient mitogenic potency of these chemicals. However, this possibility is not very likely if one considers that a labeling index of 65–70% can be observed 3 days after a single dose of LN,[30] and a similar degree of thymidine incorporation into hepatic DNA was exhibited by rat liver following 8 injections of the necrogenic agent CCl_4 or the mitogenic agent LN.[15]

In the present study also, the possibility that the occurrence of foci/nodules observed in rat liver following PH or CCl_4 could be due only to quantitative differences in terms of potency of the proliferative stimulus appears to be unlikely by virtue of the following: both PH and LN stimulate serum growth factors that are able to induce DNA synthesis in primary hepatocyte cultures. If a larger number and size of enzyme-altered foci could be expected in PH animals because of the longer persistence of the growth factors when compared to LN, a difference in the same parameters should also have been detected between LN-treated rats and animals whose liver was not stimulated to proliferate by any means. However, Table I shows that this is not the case; in fact, a) despite a similar mitogenic activity of the serum taken 3 days after PH and LN, the area of foci from PH rats was already 3 times bigger than that of LN group; b) no difference in number or area of foci was observed at any time point between LN and control rats, despite the fact that the serum from LN-treated rats exhibited a significantly higher mitogenic activity for at least 5 days. In addition, an increase in terms of number and size of enzyme-altered foci should have occurred in liver of rats treated with 2 or 3 injections of LN. In fact, under experimental conditions wherein non-initiated liver cells are not responding, as judged by the absence of thymidine grains in the nuclei (see Fig. 1), the initiated cells in the presence of three proliferative stimuli should have been selectively stimulated to grow, thus giving rise to foci bigger than those of rats whose liver was not stimulated to proliferate.

The present results thus suggest that qualitative rather than quantitative differences may be responsible for the different effects exerted by these proliferative stimuli. In this context it is of interest to note that: i) our previous studies revealed significant differences in the expression of cell-cycle-dependent proto-oncogenes, particularly of c-*fos* and c-*jun*, during cell proliferation following PH and CCl_4, when compared to CPA and EDB.[31, 32] More recently, similar findings were obtained by us using NAF as a mitogenic agent (manuscript in preparation) and by others using WY-14643 and phenobarbital as inducers of liver growth[33]; ii) while compensatory regeneration induced by PH and CCl_4 caused a decrease in cell-to-cell communication as shown by the reduced expression of connexin 32 mRNA, LN has no inhibitory effect on the expression of this gene.[34]

In addition, our preliminary studies indicated that a different pattern in the ploidy state of hepatocytes is induced by the two proliferative stimuli (data not shown). Taken together, these studies suggest that mitogen-induced liver growth induces a pattern of cellular response that is qualitatively different from that evoked by compensatory regeneration. Whether initiated cells also respond differently to different proliferative stimuli is not known. In the present study, a mitoinhibitory environment was generated in order to create a selective advantage for initiated cells once stimulated to proliferate. The fact that, following LN, initiated cells never exhibited a growth rate higher than that of liver cells of rats exposed to 2-AAF in the absence of proliferative stimuli, clearly indicates that the initiated cells are not responding to the mitogen-induced stimulus but are responding very efficiently to the stimulus induced by CCl_4 or PH.

What are the mechanisms responsible for the lack of effect of LN-induced proliferative stimuli? One possibility is that LN lacks the property of inducing growth factors that are necessary for stimulating the response of initiated cells. Although we have recently shown that PPP taken from LN-treated rats possesses the capacity to induce DNA synthesis in primary hepatocyte cultures,[29] it is possible that other growth factors, not induced by LN, are needed to stimulate the growth of initiated cells; it is also possible that a critical balance between positive and negative growth factors (TGF-β, TGF-α, HGF, etc.) might be needed for the growth of initiated cells and that such balance may not be achieved by LN. Alternatively, it is possible that the stimulus elicited during mitogen-induced liver growth acts on a subpopulation of hepatocytes that are not relevant to the carcinogenic process (i.e., hepatocytes characterized by a ploidy state different from that of initiated cells). Experiments to determine the effect of mitogens on the different subclasses of hepatocytes (2N, 4N and 8N) are in progress.

The present study, together with other reports,[13, 15, 35, 36] indicates that induction of DNA synthesis *per se* might not represent a sufficient condition for the occurrence of cancer and suggests that caution should be used in equating the role of compensatory regeneration and mitogen-induced liver growth in cancer development.

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