Nonregenerative stimulation of hepatocyte proliferation in the rat: Variable effects in relation to spontaneous liver growth; a possible link with metabolic induction

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Abstract. Three procedures were used to stimulate hepatocyte proliferation in the rat without reducing liver mass, resulting in a supplementary growth which differs from the regenerative growth observed after loss of liver mass by hepatectomy or toxic necrosis. They were: (a) the ingestion of cyproterone, a cytochrome P450 inducing drug (b) the injection of an irritant which provokes glycogenesis and synthesis of acute-phase proteins (c) the injection of albumin-bound bilirubin leading to elimination of glucuronated bilirubin in bile.

This ensuing supplementary growth was studied in the rat under several conditions of hepatic proliferation:

1. In normal adult rats, in which hepatocyte proliferation is very low, the effect on proliferation was either weak or undetectable.
2. In suckling rats, with a rapid body and liver growth, all the stimulants provoked a synchronized wave of proliferation with a steep increase of the percentage of S-phase hepatocytes from 4.5% in controls to 15–30% in treated rats. This increase was followed by a compensatory period of low proliferation during which a treatment with a second stimulant was much less effective.
3. In 2/3 hepatectomized adult rats, the proliferation induced by cyproterone was higher than the spontaneous regenerative proliferation alone and additional to it during all of the regenerative process. The proliferation induced by acute inflammation was competitive with the synchronous spontaneous proliferation during the early period of synchronized proliferation following surgery, suggesting that both are similar acute responses. Differently, during the late period of lower and unsynchronized regenerative proliferation, the proliferation provoked by acute inflammation was additional to the spontaneous one. A stimulation of proliferation by injection of the albumin–bilirubin complex was observed during the late period after 2/3 hepatectomy.

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The highest level of stimulation occurred when the liver growth and the hepatocyte proliferation were already high. This suggests that these stimulants are not complete mitogenic stimuli and need cofactors which are present during the spontaneous growth or, alternatively, that the effect of stimulants is opposed by an inhibitory mechanism present in the adult rat.

INTRODUCTION

During the life of the rat, a period of very rapid body growth is observed during the 3 week suckling period when the body weight doubles about every 6 days and the liver weight increases in proportion so that it remains at 3.5% of the body weight. This liver growth is due to hepatocyte proliferation and the labelling index (LI) is around 4.5%. During the weaning period (4th week of life) and thereafter, the body growth is slower and so is liver growth and hepatocyte proliferation. In adult rat the liver weight is stable, at about 5% of the body weight, and hepatocyte proliferation is very low, with a LI in an order of 0.01%, which is enough to account for cell renewal (Nadal, 1986).

Many experiments have been made to provoke liver growth and hepatocyte proliferation in the adult rat. The most widely used model has been the regenerative growth occasioned when a part of the liver is removed by surgery (usually 2/3 hepatectomy) or destroyed by a toxin such as carbon tetrachloride (CCl₄). The original liver mass is reconstituted after a few days. Only cells in the liver proliferate and this occurs in the order: hepatocytes first and then, slightly later, lining cells such as the sinusoidal or Kupfer cells. Despite many studies, no clear view of the mechanisms which initiate, control and finally stop this regenerative growth has yet been obtained (Bucher & Malt, 1971; Kam *et al.*, 1987; Fausto, Laird & Webber, 1995).

A separate issue is the stimulation of hepatocyte proliferation without reducing the size of the liver which provokes what can be called 'additional' growth. Several kinds of stimulants have been used but the main difficulty in such studies is that the adult or young adult rat, which has been used in most cases, is not very reactive. This work was initiated to study several stimulants of hepatocyte proliferation with different potencies in more sensitive models.

Cytochromes P450 belong to a superfamily of enzymes which oxidize lipophilic compounds as a first step in their detoxification and elimination. Various chemicals processed in this way induce the synthesis of some members of this superfamily and cause liver hypertrophy and hepatocyte proliferation. In young rats, hypertrophy and proliferation coexist, but in older rats, hypertrophy is dominant. (Schulte-Hermann, 1974). We used cyproterone, an androgen receptor antagonist steroid with gestagenic potency. Doses of cyproterone used to stimulate cytochrome P450 synthesis as well as hepatocyte proliferation are higher than those used for testosterone inhibition and the effect is observed in male and female rats so the stimulation cannot be due to the competition with androgenic hormones (Schulte-Hermann *et al.*, 1980). Recently, cyproterone has been shown to have a genotoxic effect and to induce DNA repair synthesis in female rat hepatocytes in culture but this effect was not detectable in male hepatocytes (Martelli *et al.*, 1995).

A subcutaneous injection of certain irritating substances results in hepatocyte proliferation especially in young rats (Nadal, 1970). However, after 2/3 hepatectomy, an earlier increase of liver DNA synthesis has been observed in rats which were exposed to a stress stimulant before the operation (Simek *et al.*, 1968; Moolten, Oakman & Bucher, 1970). At least two elements of the acute inflammatory reaction involve the liver: (a) glycolysis mediated by

adrenergic hormones and (b) the synthesis of a set of blood proteins which are the acute phase proteins (Courtoy *et al.*, 1981). We used an alkaline casein solution as an irritant which is without toxic effect and does not impair body growth.

Plasma bilirubin is linked to serum albumin in a noncovalent manner. It is not metabolized by the cytochrome P450 system. It is taken up and glucuronated twice in hepatocytes before its elimination in the bile. UDP glucuronyl-transferase which is responsible for the first step is an inducible enzyme and phenobarbital is its best known inducer. Less is understood about bilirubin-glucuronoside glucuronyl-transferase which catalyses the second step (Chowdury & Arias, 1980). It has been observed that an injection of albumin-linked bilirubin stimulates proliferation in rat hepatocyte primary cultures and *in vivo* in mice (Diaz-Gil *et al.*, 1987).

We compared the effect of these stimulants of hepatocyte proliferation in several assay systems. We used the adult rat as a model of low spontaneous proliferation and with low reactivity and the suckling rat as a system in which proliferation of hepatocytes is already underway. Another model was the rat after 2/3 hepatectomy to explore the interference of the stimulants with the process of regenerative proliferation. We observed striking differences between the results obtained in these various models but many similarities between the effects of these apparently very different stimulants exist which raises the question as to whether they could operate via a common mechanism.

MATERIALS AND METHODS

Wistar rats were raised at the Institut Curie in Orsay, maintained on UAR pellets and given water *ad libitum* in rooms at constant temperature (20°C) with a natural day-night cycle. Suckling rats were 8–12 days old and weighed 17–23 g. Each litter was divided into one control group and one or several treated groups, all groups having the same number of rats, usually 2 or 3. Young adult rats were two month old males. Their mean weight was 240 g with a range of 180–280 g. In each experiment, a group of 8–18 adult rats was used, among them control rats differing from treated rats by less than 10% body weight. 2/3 hepatectomy (PH) was performed according to Higgins and Anderson (Higgins & Anderson, 1931).

³H thymidine was injected (i.p.) 30 min before killing at a dose of 37 KBq/g body weight in suckling rats and 26 KBq/g in adult rats. Liver samples were fixed in 4% formalin and embedded in paraffin. Histological preparations were dipped in Kodak (Rochester, NY, USA) NTB 3 emulsion diluted 1/1. The LI (percentage of labelled nuclei) was evaluated by counting at least 2500 hepatocyte nuclei on histological sections; labelled nuclei had five grains or more above the background. The mitotic index (percentage of mitotic hepatocytes) was evaluated by counting 2500 hepatocytes in haematoxylin-eosin stained histological sections. Means of experimental groups are given with SEM, and compared using the Student's *t*-test.

3.5% casein in 0.3% NaOH was injected into rats at a dose of 0.02 ml per gram of body weight. In suckling rats it was given in one (s.c.) injection. In older rats the dose was divided in several simultaneous (s.c.) injections so that no more than 1 ml was injected at the same place.

Cyproterone acetate was a gift from Schering AG, Berlin. It was dissolved in maize oil at a concentration of 10 mg/ml and given by oral gavage. Unless otherwise indicated, the dose was 100 mg per kilogram of body weight.

Bilirubin (Sigma, St. Louis, MO, USA) and bovine albumin (low endotoxin A 3675 Sigma) were linked in a 1/1 molecular ratio (Wooley & Hunter, 1970). Injected solutions contained 6.75 mg/ml of albumin or albumin–bilirubin complex in 0.1 M phosphate buffer pH 7.5. Care was taken to prevent endotoxin contamination and solutions were used within 6 h after preparation.

Figure 1. Evolution of hepatocyte LI in young adult male rats (mean of at least six rats) after treatment at $t = 0$ with: (●) cyproterone 100 mg/kg by force feeding; (○) s.c. injection of casein solution; mean of all untreated controls. ★: $P < 0.001$; ☆: $P < 0.01$ vs. oil-fed or untreated controls.

Prazosin (Sigma) was dissolved in methanol at a concentration of 2 mg/ml and diluted 1/20 in 0.9% NaCl.

Serum glutamyl-pyruvic transaminase measurements were made with Ensyline ALAT/GPT reactives from Bio Merieux and results are expressed in international units.

RESULTS

Effect of stimulants in adult rats

The labelling index (LI) of normal adult rat controls in various experiments varied from $0.03\% \pm 0.004$ – $0.08\% \pm 0.02$.

Figure 1 shows that the LI of cyproterone-fed rats increased moderately but very significantly. Maximal values observed were 19 and 23 h after treatment ($P < 0.001$ and 0.01 , respectively, vs. oil-fed rats). There was no significant difference between oil-fed and untreated rats.

After casein solution injection a small LI increase was observed 30 h later ($P < 0.01$ vs. untreated controls).

Normal rats were injected (i.p.) with 2.5 ml of albumin–bilirubin complex solution. Six rats were killed after 16 h, six after 24 h and both groups were compared with six untreated controls. No significant difference was found between values of the LI of these groups which were $0.063\% \pm 0.034$; $0.1\% \pm 0.13$ and $0.08\% \pm 0.02$, respectively (not drawn in Fig. 1).

Effect of stimulants in suckling rats

Cyproterone acetate The results in Fig. 2 show that cyproterone feeding increased the LI from 14 to 40 h after treatment with a maximum at 24 h ($P < 0.001$ vs. oil-fed). In the second

Figure 2. Effect of cyproterone feeding, casein solution injection or albumin–bilirubin complex injection on the LI of hepatocytes in suckling rats. Treatment was made at $t = 0$. Each value is the mean of eight rats. Horizontal line: mean of all untreated controls. ●, cyproterone-fed rats; ○, casein solution injected rats; ☆, albumin–bilirubin complex injected rats; ★, $P < 0.001$, vs. oil-fed, untreated or albumin injected controls

period, the LI decreased and was lower than in the controls between 60 and 96 h but rose above the control level again by 168 h. In these experiments, the mitotic index (MI) was increased at 30 h: for a mean of six rats this was $0.4\% \pm 0.1$ in the oil-fed controls and $2.5\% \pm 0.2$ in the cyproterone-fed animals, $P < 0.001$.

Acute inflammation The variations of the LI after a subcutaneous injection of casein solution are also shown in Fig. 2. The hepatocyte LI showed a maximum increase at 17 and 25 h after injection ($P < 0.001$). Later the LI decreased, reaching a minimum between 40 and 60 h. Compared with the cyproterone treated suckling rats, the LI curve exhibited a lower increase which was of shorter duration and the compensatory decrease of the LI which followed was also of shorter duration. A previous publication has shown that the mitotic index was increased with a peak at 27 h (Nadal, 1970).

In order to evaluate a possible role of adrenergic hormones in the induction of the proliferation, we tested the effect of an alpha-1 adrenergic blocker. Prazosin was injected (i.p.), 15 min before casein injection, at a dose of 1.5 mg/kg. The increase in hepatocyte LI, 19 h later, was partially prevented in prazosin treated rats (casein injected controls $26.5\% \pm 0.9$; Prazosin treated $17.6\% \pm 0.9$; mean of 11 rats; $P < 0.001$). No effect was found when prazosin was given 6 h after casein solution injection.

Albumin–bilirubin complex Suckling rats were injected (i.p.) with 0.25 ml of either phosphate buffer, albumin solution or albumin–bilirubin complex solution.

The hepatocyte LI of rats injected with phosphate buffer was moderately but significantly increased 18 h after injection (untreated $2.7\% \pm 0.3$; buffer $3.5\% \pm 0.4$; $P < 0.05$). Phosphate buffer therefore could have some activity of its own or contain a low quantity of endotoxin despite care taken to prevent it. The LI of rats injected with albumin in phosphate buffer was not significantly different from that of phosphate buffer treated rats, so albumin had no effect of its own.

The LI of rats, injected with the albumin–bilirubin complex (Fig. 2) increased progressively and the maximum effect was between 16 and 18 h ($P < 0.001$ vs. albumin treated rats). At 26 h, values were back to normal levels. No further increase of the LI was recorded when higher doses (0.5 or 1 ml) were injected.

However, when the albumin–bilirubin complex was injected subcutaneously at the same dose as intraperitoneally, no significant increase of the LI over the albumin-treated rats was recorded: untreated controls $2.6\% \pm 0.4$; albumin s.c. $3.1\% \pm 0.6$; albumin–bilirubin complex s.c. $2.7\% \pm 0.5$; mean of six rats. The differing effect obtained when the i.p. and s.c. routes were employed may be explained by the fact that i.p. injection results in a faster resorption and there is a direct access to the liver without dilution in the bloodstream. It shows that the activity of albumin–bilirubin complex cannot be due to irritating properties since irritating substances (e.g. casein solution or turpentine) are equally active i.p. and s.c. (Nadal, 1970).

Effect of a second treatment

By the end of the wave of S-phase cells which occurs after cyproterone feeding or injection of casein solution, the liver has undergone a growth advance which is compensated for by a period of low proliferation over several days. We studied the effect of a second treatment made during the period of low LI or later, when the LI was back to the control level.

Initially, suckling rats were fed with cyproterone (Fig. 3A) or injected with casein solution (Fig. 3B). Controls rats were oil-fed or untreated. At the lowest point of the LI, rats were treated a second time either with the same product or with the other (a and b, respectively). The LI increase induced by this second treatment was much lower (■) than the rise induced by the treatment of control rats of the same litter (□).

If the second treatment was made when the LI of initially treated rats was back to the control level, i.e. at 120 or 168 hours, there was no difference between initially treated and control rats (Fig. 3c and d).

Effect of stimulants in 2/3 hepatectomized rats

Cyproterone acetate The upper panel of Fig. 4 shows the hepatocyte LI values recorded in 2/3 PH controls and in cyproterone treated 2/3 PH rats, the lower panel shows the values of treated rats from which the values of control rats were subtracted. The lower panel more clearly shows the variations induced by treatments when the values are converted for the large changes which occur during the first part of liver regeneration. Hepatectomized control rats exhibited the usual semisynchronized wave of S-phase cells which began between 14 and 18 h, reached a maximum at 22 and then steadily declined over the next few days. Oil-fed controls did not differ significantly from untreated hepatectomized rats.

Figure 3. Effect of a second treatment of suckling rats with cyproterone (CY) or casein solution (CAS) on the LI of hepatocytes. The bars show the highest LI observed after the second treatment (24 or 30 h after cyproterone, 18 h after casein solution, mean of six rats). Values (□) of controls which were initially oil-fed (part A) or untreated (part B). Values (■) when the first treatment was cyproterone-feeding (part A) or casein solution injection (part B). a and b: second treatment during the period of low LI (72 h after cyproterone feeding or 40 h after casein solution injection) with either cyproterone (a) or casein solution (b), $P < 0.001$ vs. control. c and d: second treatment when the LI of treated rats was back to the control level (168 after cyproterone feeding, 120 h after casein solution injection) with casein solution (c) or cyproterone (d). No significant difference vs. controls.

When cyproterone was given 12 h before hepatectomy (curve A) the LI rose earlier and higher than in controls, and the highest points occurred 14 and 18 h after surgery ($P < 0.001$). A peak of mitosis was observed at 24 hours: oil-fed controls $3.8\% \pm 0.6$; cyproterone-fed $14.6\% \pm 0.7$; mean of 5; $P < 0.001$. Cyproterone induced proliferation was well synchronized, 8 h in advance and nearly twice as high as the spontaneous first wave of S phase cells. It was additional to the spontaneous level of proliferation since, at later times, the LI was either at the same level as or higher than the control values. Feeding cyproterone 12 h before surgery was the most efficient time at which to induce an early wave of synchronous S-phase hepatocytes. Similar but lower waves were induced by feeding 24 or 6 h before hepatectomy. The LIs at 14 hours post hepatectomy were: controls $0.3\% \pm 0.2$; treated $18\% \pm 5$ and $14\% \pm 3$, respectively; $P < 0.001$ and at the 18th hour: controls $13\% \pm 4$; treated $34\% \pm 4$ and $41\% \pm 2$, respectively; $P < 0.001$.

When cyproterone was given 10 min before, or 40 h after 2/3 hepatectomy, a LI increase was observed. When it was given on the 7th day, the LI increase was much lower than it was

Figure 4. Modification of hepatocyte LI after 2/3 hepatectomy at $t = 0$ in young adult male rats forced-fed with cyproterone 100 mg/kg. Upper panel shows experimental values with SEM, lower panel shows LI for cyproterone-fed rats with LI value from rats subjected to 2/3 hepatectomy alone subtracted. $\circ$, hepatectomized controls. $\bullet$, A. cyproterone-fed 12 h before hepatectomy, B. cyproterone-fed 10 min before hepatectomy, C. cyproterone-fed 40 h after hepatectomy, D. cyproterone-fed 7 days after hepatectomy $\star$, $P < 0.001$; $\star$, $P < 0.01$ vs. hepatectomized controls.

at the beginning of the regenerating process and not very different from the increase provoked in normal rats.

Acute inflammation The results in Fig. 5 are also shown in two panels as for Fig. 4.

When casein solution was injected 12 h before surgery (curve A) the LI rose earlier than in the controls, was already high at 14 h and peaked higher at 18 h ($P < 0.001$). However, it also fell earlier and at 24 h was lower than in the controls ($P < 0.01$). At the 36th hour there was no difference from the controls.

When casein solution was injected at the end of surgery (curve B), the LI did not rise significantly higher than in the controls. Thus the proliferation induced by acute inflammation either before or, simultaneously with surgery was not fully additive to spontaneous regeneration as was observed with cyproterone.

Casein solution was also injected at different times during the regenerative process after 2/3 hepatectomy. When injections were made 24 h after hepatectomy (curve C), a sharp peak of the LI was observed 15 and 19 h later ($P < 0.001$), and there was a LI increase 24 h later (controls $0.44\% \pm 0.1$; treated $4.1\% \pm 0.4$; mean of five rats; $P < 0.001$).

Figure 5. Modification of hepatocyte LI after 2/3 partial hepatectomy at $t = 0$ in young adult male rats injected s.c. with casein solution (mean of 6 rats). Upper and lower panels as in Figure 5. ○, Hepatectomized controls; ●, A. injection 12 h before hepatectomy, B. injection at the time of hepatectomy, C. injection 24 h after hepatectomy, D. injection 40 h after hepatectomy, E. injection 7 days after hepatectomy, ★ $P < 0.001$ vs. hepatectomized controls.

When injections were made 40 h after hepatectomy (curve D), a LI increase was observed at 19 and 23 h after injection ($P < 0.001$) but it was lower than when injection was made at 24 h.

On the seventh day after hepatectomy (curve E), a very small but significant increase was observed at the 24th hour after injection ($P < 0.001$).

Albumin–bilirubin complex injection Hepatectomized rats were injected i.p. with 2.5 ml of either phosphate buffer, albumin solution or albumin–bilirubin complex solution 30 h after surgery.

The LI of albumin–bilirubin complex injected rats rose significantly 13 h after injection and peaked at 16 h (albumin $6.1\% \pm 1.3$; albumin–bilirubin $15\% \pm 3$; mean of eight rats; $P < 0.01$). At 20 h, values were no longer significantly different. The LI of buffer and albumin injected rats was not significantly different.

Liver weight variations

In our Wistar rats, the mean relative liver weight (% of body weight) was $3.6\% \pm 0.3$ in suckling rats, increased to $6\% \pm 0.2$ in 30-day-old weaning rats and was $5.1\% \pm 0.4$ in 2-month-old adult males

Cyproterone feeding in normal adult rats induced a liver weight increase due more to a hypertrophy of hepatocytes than to an increase of their number since the weight increase was 24%, 30 h after treatment, though the LI increase was less than 3%. On the contrary, in suckling rats and in hepatectomized rats, the weight increase and the LI increase (i.e. the production of new cells) were of the same order: a 40–60% increase was found 40 h after treatment. When rats were fed twice with cyproterone, 12 h before and 24 h after 2/3 hepatectomy, the liver reached its normal weight 72 h after surgery whereas in untreated rats this reached only 70% after 7 days.

After casein solution injection a small increase of the relative liver weight was observed in suckling rats 40 h after injection (3% over controls) and in hepatectomized rats injected 40 h after surgery and killed 30 h later (4% over controls). However, these increases were not statistically significant.

Serum glutamyl-pyruvic transaminase values.

To ensure that the proliferations induced by these treatments were not due to an acute cytotoxic effect, enzyme measurements were made 24 h after treatment. In adult rats, the sera were measured separately, in suckling rats, due to the small quantity available, pools of sera were used. Results are in international units (i.u.).

After casein solution injection, no increase in enzyme activity was found in suckling rats (controls 20 i.u., treated 16 i.u., pools of five rats) nor in hepatectomized rats 40 h after surgery (controls 59 i.u. $\pm$ 4, treated 35 i.u. $\pm$ 5, mean of six rats).

Cyproterone treatment caused a very slight increase in suckling rats (controls 21 i.u., treated 37 i.u., pools of six rats) and in adult rats (controls 32 i.u. $\pm$ 2, treated 47 i.u. $\pm$ 4, mean of six rats, $P < 0.01$). In 2/3 hepatectomized rats treated 40 h after surgery, however, the increase was not significant (controls 59 i.u. $\pm$ 4; treated 68 i.u. $\pm$ 8, mean of six rats, n.s.).

DISCUSSION

The effects of the various stimulants of hepatocyte proliferation studied here were very different depending on the level of proliferation already present in the rats employed.

Adult male rats exhibited a very low reactivity to any stimulation of hepatocyte proliferation: cyproterone and acute inflammation had a low effect and a weak stimulus such as the bilirubin-albumin complex had no detectable effect.

Suckling rats were much more reactive and a high wave of proliferation was recorded after all the stimulants studied. However this high reactivity was not strictly linked to the fact that they were suckling rats since a few hours later, during the proliferation slowdown following a first stimulation, the response to a second stimulant was weak for several days.

The reactivity of 2/3 hepatectomized rats was also high. Cyproterone, given from 12 h before surgery to several days thereafter, increased the regenerative process by inducing a level of hepatocyte proliferation higher than, and additional to, the spontaneous proliferation and this was not decreased at later times so cyproterone was clearly a potent enhancer of liver regeneration. Differently, there was a competition between the proliferation induced by acute inflammation and the first synchronized wave of S-phase hepatocytes after 2/3 hepatectomy: when the irritating injection was made 12 h before surgery, the first wave of proliferation occurred earlier than in the controls but was neither higher nor longer and, when the injection was made at the time of surgery, no effect was recorded. In contrast, during the late regenerative period (i.e. after 24 h) an irritating injection provoked a high

wave of proliferation. The bilirubin-albumin complex was also administered in the same late period and found to be active when injected 30 h after surgery.

The very different reactions to stimulants of hepatocyte proliferation, depending on the category of rats used, make it clear that it is important, when studying agents possibly active on this proliferation, to test them in adequate systems. Suckling rats were constantly and highly responsive to all the stimulations, even the weak ones. Hepatectomized adult rats were also very reactive but we may contrast two successive periods of the regenerative process. The first, during the 24 first hours after hepatectomy, may be misleading since it failed to detect hepatocyte proliferation linked to acute inflammation. We can notice that these two proliferation processes have common features: in both cases there is an elevated level of IL6 concurrent with the synthesis of acute phase proteins (Van Snick, 1990; Gersterfer *et al.*, 1993), the proliferation is inhibited by Prazosine (*ibidem* and Cruise *et al.*, 1987) and enhanced by low doses of hydrocortisone (Nadal, 1995) and *c myc*-mRNA accumulation is also observed (Sobczak *et al.*, 1989). Both are probably not additive because they share a similar acute-response mechanism and are unable to react twice in a short time. This similarity is noteworthy because this system has been extensively used to study the mechanisms of hepatocyte proliferation and liver regeneration and to test products possibly active on them. The presence of an inflammatory/acute response process makes interpretation more difficult. Differently, during the second period of regeneration, hepatocyte proliferation is lower and unsynchronized, like in suckling rat, and the various stimulations have similar effects in both systems which would therefore seem to be regulated by similar mechanisms.

The variations of reactivity observed here derive from two different situations. A reactive state, as in suckling rats, or in adults after 2/3 hepatectomy, is characterized by a low relative liver weight and spontaneous liver growth with hepatocyte proliferation. In this case all the conditions are thus already fulfilled for a stimulus to be efficient, whereas the blocked state, which was observed permanently in adults rats and transiently in suckling rats when their liver has taken a growth advance following a stimulation by cyproterone or acute inflammation, is quite different. The onset of the blocked state occurred after the weaning period in parallel with a relative hypertrophy of the liver which reached 6% of the body weight before stabilizing around 5% in adults, probably in relation with the change of diet and the presence of various xenobiotic compounds in food. The blocked state seems to be provoked by mechanisms associated with liver enlargement which prevent further enlargement. In our rats the shift to the blocked state occurred when the relative liver weight reached 4% but other parameters could be more accurate such as the number of hepatocytes or the DNA content (Schulte-Hermann & Schmitz, 1980; Schulte-Hermann, Landgraft & Koransly, 1980).

An explanation for the low reactivity of adult liver could be that, when this organ enlarges, the stimulation for each hepatocyte falls below the threshold necessary for triggering proliferation, but this mechanism cannot explain all the experimental facts. When cyproterone was given 12 h before 2/3 hepatectomy (the most efficient time of treatment), the liver was still normal but the stimulation was effective: many hepatocytes initiated the first steps of their cycle and were blocked at the end of their G 1-phase. Without 2/3 hepatectomy a few hepatocytes would have reached the S-phase but this operation enabled stimulated hepatocytes to progress through the rest of their cycle so a wave of S-phase hepatocytes occurred early after surgery, i.e. 8 h before the first spontaneous wave. Thus the mechanism which enables hepatocytes to achieve entry to their cell-cycle could be via cooperation with other stimulatory factors already present in the reactive state or, the loss of inhibitory factors responsible for the blocked state (Braun *et al.*, 1988; Auger *et al.*, 1989; Chapekar, Huggett &

Thorgeirsson, 1989; Reichelt *et al.*, 1990). However the factors that are already known cannot fully explain this regulation.

A second important fact is that many stimulants of hepatocyte proliferation are also effective stimulants of liver specific metabolism. For example, all cytochrome P450 inducing drugs, although chemically unrelated, also stimulate hepatocyte proliferation (Schulte-Hermann, 1974). Similarly, all the substances which provoke acute inflammation also provoke hepatocyte proliferation, some of them, e.g. alkaline casein solution, being more constantly active than others (Nadal, 1970). Their mitogenic activity is associated with the metabolic events of acute inflammation as discussed above (i.e. alpha-1 adrenergic stimulation and induction of acute phase protein synthesis) since proliferation is decreased by prazosine (an alpha-1 blocker) and increased by hydrocortisone which enhances acute phase protein synthesis by elevating the number of IL6 receptors (Decker, 1990; Van Snick, 1990). Bilirubin is metabolized in hepatocytes through two successive glucuronizations. The mitogenic activity of bilirubin is probably associated with UDP glucuronyl-transferase overload or induction since it had no effect in Gunn rats which are genetically deficient for this enzyme (Chalumeau *et al.*, 1991).

Other previous observations also show an association between induction of metabolic activity and hepatocyte proliferation. Hepatocyte proliferation was found to increase after plasmapheresis which removes blood proteins and consequently increases their synthesis by the liver (Naorah & Naora, 1957; Glinos, 1960; Shafir & Brenner, 1979). The increased hepatocyte proliferation which is observed during the refeeding after protein free diet could be the consequence of the rebound of protein synthesis (Mead *et al.*, 1990). The decrease of the ATP stock observed shortly after 2/3 hepatectomy has been interpreted as a metabolic overload (Ngala-Kenda, de Hemptinne & Lambotte, 1984).

All these observations suggest that the coexistence of metabolic hyperactivity and proliferation is not a hazard, and that some link between them, although not strictly proven, is a possible hypothesis. Proliferation occurred early after the stimulation and was synchronous to the induced metabolic activity rather than a consequence of it. Both are likely to have a common cause but independent, parallel courses. Stimulants could induce the production of growth promoting factors which would be secreted and act in an autocrine way but the short latency of the proliferation militates against this indirect mechanism. Alternatively, there could be some early steps common to proliferation and metabolic activity; a second messenger such as cyclic AMP which peaks 3 and 12 h after 2/3 hepatectomy (MacManus *et al.*, 1973) or an accumulation of some early cell cycle factor such as *c myc* over-expression which occurs early during liver regeneration, acute inflammation and protein deficiency (Sobczak *et al.*, 1989; Mead *et al.*, 1990) but not after cyproterone treatment (Coni *et al.*, 1993). The similarity of early steps leading to either proliferation or expression of differentiated characters in pheochromocytoma cells has been recently discussed (Chao, 1992).

In conclusion, the liver is a very active organ and has many specific metabolic functions working for the body as a whole which are regulated by extrahepatic stimuli. Hepatocyte proliferation associated with metabolic activity could explain several aspects of the liver growth control. Since this proliferation is high when the relative liver weight is low, the costimulation of metabolism and proliferation would increase the liver mass until it has reached a liver to body mass ratio adapted to body needs. When the liver has reached this 'adequate' relative liver mass, a proliferation limiting mechanism is observed and further stimulations provoke either zero or a limited weight increase mainly due to an hypertrophy of cells. The shift from reactivity to blockage could be linked to the global metabolic activity of

hepatocytes, the blockage occurring when this activity falls below a certain level. This mechanism would complement the action of growth factors which are needed particularly for governing the relationship between the various kinds of cells in the liver and in maintaining the structure of the tissue.

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