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Modulation by Indomethacin or Prostaglandin E_2 of the Incidence of Diethylnitrosamine-induced γ-Glutamyltranspeptidase-positive Foci in Rat Liver

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We investigated the effect of a pretreatment with indomethacin (IMC, ip 3.6 mg/kg body weight (bw)) or dimethylprostaglandin E_2 (PGE_2, ip 10 μg/kg bw) on the incidence and development of γ-glutamyltranspeptidase (GGT)-positive foci of altered hepatocytes, scored 8 or 14 weeks after ip injection of diethylnitrosamine (DENA, 50 mg/kg bw) to rats submitted to two-thirds hepatectomy (PH) or sham operation (Sh). IMC reduced by about 4 times the incidence of DENA-induced GGT-positive foci per cm^3 of liver tissue in sham-operated as well as in two-thirds hepatectomized rats, compared to the respective unpretreated controls. In contrast, PGE_2 pretreatment increased the incidence of DENA-induced foci in both groups, this effect, in terms of absolute numbers of foci, being additive to that of PH alone. IMC pretreatment resulted in foci with lower average size in the Sh but not in the PH animals, whereas with PGE_2 pretreatment the mean volume of the foci was increased in the two groups of rats. At the dose used, IMC did not modify the proliferative response of hepatocytes to PH, and PGE_2 did not stimulate proliferation in the sham-operated animals. Altogether, these results indicate that: 1, the incidence of DENA-induced foci can be negatively modulated by interfering with the prostaglandins pathway through a mechanism that does not involve an action either on proliferative activity or on any other process that would be specific to the post-hepatectomy regenerative state; 2, positive modulation of the incidence of DENA-induced foci does not necessarily require stimulation of proliferation.

Key words: Liver regeneration — Hepatocarcinogenesis — Indomethacin/Prostaglandin — Diethylnitrosamine — γ-Glutamyltranspeptidase

In the experimental protocol consisting of intraperitoneal administration to rats of DENA[4] (50 mg/kg bw) 24 h after PH, foci of phenotypically altered hepatocytes, neoplastic nodules and hepatocellular carcinomas, sequentially develop in the liver of the animals.[1,2] The same sequence of morphological and histochemical alterations (including GGT expression, and deficiency in ATPase and G-6-Pase) is also observed with other experimental hepatocarcinogenic protocols.[3,4] A filiation between foci, nodules and hepatocellular carcinoma is supported by several lines of evidence, including the observation that at least one-third of the nodules express invasiveness in an *in vitro* assay,[5,6] and the occasional topographical association between a given type of lesion and its putative product,[7] as well as the fact that nodules transplanted into isogenic rats grow into tumor masses.[8–10] Moreover, there is a positive correlation between the incidence of early foci and the frequency of hepatocellular carcinomas that ultimately develop in different hepatocarcinogenic protocols.[1,2,7] Further support for the filiation hypothesis was provided by work from our laboratory showing that preneoplastic and neoplastic lesions share a common oncodevelopmental marker, namely oncomodulin.[11,12]

A high cell proliferation rate is widely considered as increasing the efficiency of carcinogenic agents, supposedly operating by fixation of carcinogen-induced DNA damage.[13–15] This is generally assumed as the basis of the positive influence of PH on liver chemical carcinogenesis. The aim of this work was to check whether increased proliferative activity could be dissociated from the potentiating effect exerted by PH on the incidence of DENA-induced foci of altered hepatocytes. It was reported that the liver proliferative activity in response to PH was reversibly suppressed by high doses of IMC given at the time of surgery, and was fully restored by PGE_2 administration.[16,17] Using the incidence of GGT-positive foci, i.e. the first relevant cell stage of the DENA hepatocarcinogenic process, as a reference of the efficiency of the DENA treatment, we investigated

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[4] Abbreviations: DENA, diethylnitrosamine; PH, two-thirds partial hepatectomy; Sh, sham operation or sham-operated; IMC, indomethacin; PGE_2, 16,16-dimethylprostaglandin E_2; GGT, γ-glutamyltranspeptidase (EC 2.3.2.1); ATPase, adenosine triphosphatase (EC 3.6.1.3); G-6-Pase, glucose-6-phosphatase (EC 3.1.3.9); LI, ^{3}H-thymidine pulse-labeling index; MI, mitotic index; bw, body weight.

the effect of a pretreatment of partially hepatectomized or Sh rats with IMC or PGE_2, administered at the time of surgery, on the incidence and development of early GGT-positive liver foci induced by subsequent administration of DENA.

We first applied the same concentration of IMC (50 mg/kg bw) as used by Rixon and Whitfield[16] to inhibit liver proliferation, but using the intraperitoneal route. Though not lethal by itself, this dose proved to be highly toxic for rats submitted to PH and DENA injection, resulting in a high mortality rate (19/20) of the animals. A dose of 3.6 mg of IMC per kg body weight revealed itself as the highest one compatible with the survival of the carcinogen-treated animals, inducing no detectable side effects such as body weight loss or gastrointestinal ulceration. That dose of IMC was therefore chosen for our further experiments on DENA-induced foci.

The results show that in Sh and PH rats, PGE_2 increased the frequency of DENA-induced GGT-positive foci scored at 8 or 14 weeks after administration of the carcinogen, without affecting hepatocyte proliferative activity.

In contrast, the frequency of DENA-induced GGT-positive foci was 4 times lower in the Sh and PH animals pretreated with IMC than in untreated corresponding controls. At the dose used, IMC did not modify the liver proliferative response to PH, thus indicating that its negative effect on the induction of early stages of the carcinogenic process is at a different level.

MATERIALS AND METHODS

Animals Pathogen-free female Sprague-Dawley rats weighing 200–250 g were obtained from Iffa-Credo (Brussels). The animals were fed a standard diet (Aliment R/S A04, Animalabo, Brussels) and given water *ad libitum*. They were housed in plastic cages with a 12 h light-dark cycle. All the experiments were performed between 9.00 and 11.00 a.m.

Chemicals 16,16-Dimethylprostaglandin E_2 and diethylnitrosamine (N-nitrosodiethylamine) were from Sigma (St Louis, Missouri). Indomethacin (1-*p*-chlorobenzoyl-5-methoxy-2-methylindole-3-acetic acid) was a gift from Merck, Sharp and Dohme, New Jersey. Tritiated thymidine (48 Ci/mmol or 1.81 TBq/mmol) was obtained from Amersham (Brussels).

Experimental protocol Rats were randomly allotted to two main groups: the first one was submitted to PH performed according to Higgins and Anderson,[18] and the other was submitted to a Sh which consisted of lifting the two central and left lateral lobes from the peritoneal cavity and setting them back in their previous position. At the time of each surgical procedure, the subgroups were submitted to intraperitoneal injection of either IMC (3.6 mg/kg bw) or PGE_2 (10 μg/kg bw). Controls for each main group received the solvent alone. Twenty-four hours after the surgery, the animals were intraperitoneally injected with 50 mg/kg bw DENA. This dose was selected to avoid the necrogenic effects and the ensuing decrease in foci incidence observed with higher concentrations.[19, 20] Animals were killed 8 or 14 weeks after DENA treatment to evaluate the incidence and size of foci of altered hepatocytes, which were detected by histochemical GGT staining.

GGT histochemistry The rats were killed under ether anesthesia following an overnight fasting period. The liver was excised shortly after a portal perfusion with a 0.15 *M* NaCl solution, performed to wash the blood cells out of the liver tissue. Pieces of liver lobes were promptly fixed in paraformaldehyde calcium solution (1.3 *M* paraformaldehyde, 0.2 *M* dimethylarsenic acid, 4.5 m*M* $CaCl_2$ at pH 7.4) at 0°C. After a 3 h immersion in this fixative the specimens were washed and maintained overnight in a cacodylate-sucrose solution (0.1 *M* dimethylarsenic acid, 0.2 *M* sucrose at pH 7.4), dehydrated through the usual alcohol-xylol steps, then embedded in Paraplast (a paraffin-plastic, with a melting point of 56°C). The GGT staining was performed on 5 μm thick sections, according to Rutenburg *et al.*[21]

Autoradiographic procedure Three animals from each subgroup were intraperitoneally injected 23 h after the surgical procedure with one pulse of ^{3}H-TdR (37 kBq/g bw) and killed 1 h later for evaluation of the ^{3}H-thymidine pulse-labeling index (LI), i.e. the percent of S-phase cells, and of the mitotic index (MI), here given as the number of mitotic figures per 10 microscope fields. Autoradiography of paraffin sections was performed by dipping the slides in Ilford-K5 Nuclear emulsion. Exposure time was 21 days. The fraction of labeled nuclei in the hepatic cells was established by scoring 500 cells.

Morphometric analysis To optimize the use of sampled tissue sections for estimating the number of foci, the procedure described by Nychka *et al.* was followed.[22] For each animal, 4 samples of each remaining lobe of the liver were fixed and analyzed to ensure random sampling of the scored GGT-positive foci. From each lobe, ten 5-μm-thick step sections with a 100 μm spacing were examined. This procedure was shown to optimize the estimation of the number of GGT-positive foci.[22] A minimal area of 2 cm^2 was checked for each animal, using a Leitz Dialux 20 EB microscope (Leitz, Weitzlar, West Germany) with a 10×objective. The average area and volume of the foci presenting GGT-positive histochemical staining was derived from measurement of their perimeter, etc. using a VIDS IV semi-automatic image analyzer (AMS, Cambridge, Great Britain). Foci were stored in classes with intervals chosen to reflect a progressive doubling of the equatorial plane areas, starting

from the first reproducibly measurable size class as described by Scherer *et al.*[23] Assuming a statistical distribution and a spherical shape of the islands, the number of islands per cm^3 of liver and their size distribution were calculated using the mathematical method of Pugh *et al.*[24]

Statistical calculations Student's *t* test analysis of the data was performed using NWA STATPAK (Northwest Analytical, Inc., Portland, Oregon) computer programs.

RESULTS

Incidence of GGT-positive foci Figure 1 shows the total number of foci per cm^3 of liver, found in each group of rats, 8 or 14 weeks after a single ip injection of DENA. The PH animals displayed a 19-fold greater number of liver foci per cm^3 than the Sh ones ($P<0.001$). IMC administration reduced by about 4 times the incidence of DENA-induced foci in the Sh ($P<0.001$, see the inset of Fig. 1) and PH ($P<0.001$) groups, as compared to the respective controls, treated with the solvent alone. In the PH group, this reduction in foci incidence was fully counterbalanced by administering PGE_2 at the time of IMC injection. The incidences of foci per cm^3 in the PH alone and the PH+IMC+PGE_2 groups of rats were not significantly different. As compared to the respective control groups, PGE_2 increased the number of foci by a factor of 1.5 in the PH group and by a factor of 9 in the Sh group, corresponding to the addition of similar absolute numbers of foci in both groups under PGE_2 treatment.

We showed in separate experiments with 10 rats in each group, that the restored liver mass (as a percent of initial liver mass), like the ^{3}H-thymidine labeling index, exhibited the same time-course after DENA treatment in IMC-treated and untreated rats.[25]

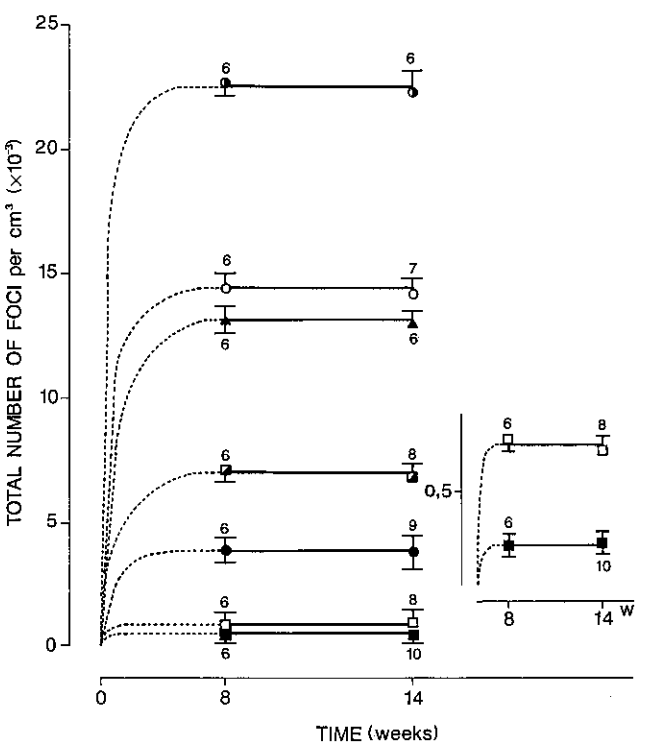

Fig. 1. Total number of foci of GGT-positive hepatocytes per cm^3 as calculated using the mathematical method described in "Materials and Methods" and scored 8 or 14 weeks after a single ip injection of 50 mg/kg bw DENA performed 24 h after Sh (□), PH (○), Sh+3.6 mg/kg bw IMC (■), PH+3.6 mg IMC per kg bw (●), Sh+10 μg/kg bw PGE_2 (◪), PH+10 μg/kg bw PGE2 (◑), PH+3.6 mg/kg bw IMC+10 μg/kg bw PGE_2 (▲). Data are means of the number of foci per cm^3 ± SEM. The value above each symbol indicates the number of individual animals analyzed in each group. The statistical significance of differences evaluated by Student's *t* test were: (○) vs. (□), (○) vs. (●), (□) vs. (■), (○) vs. (◑) and (□) vs. (◪): $P<0.001$; (○) vs. (▲): not significant. Inset: scale changed for better visualization of the effect of IMC on foci induction in sham-operated rats: Sh (□), Sh+IMC 3.6 mg/kg (■).

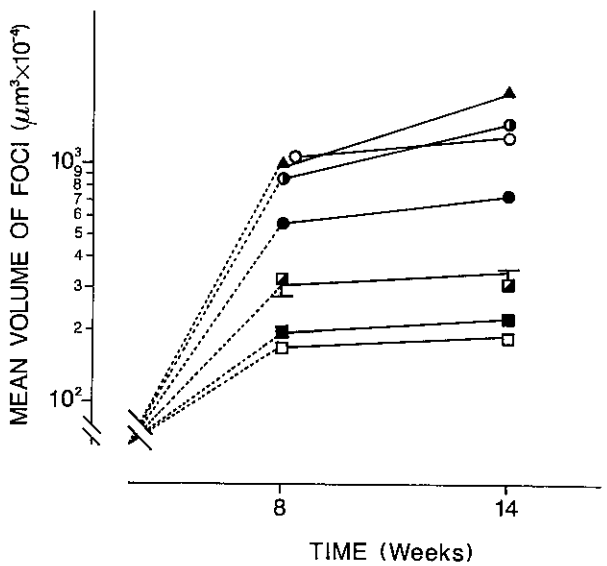

Fig. 2. Mean volume of the GGT-positive foci scored in the liver of rats 8 or 14 weeks after DENA administration, given 24 h after various pretreatments as detailed in Fig. 1. Symbols are the same as in Fig. 1. SEM of less than 10% are not indicated. The statistical significance of differences evaluated by means of Student's *t* test were: (○) vs. (◑), (○) vs. (▲) and (□) vs. (■): not significant; (□) vs. (◪): $P<0.01$; (○) vs. (●): $P<0.001$.

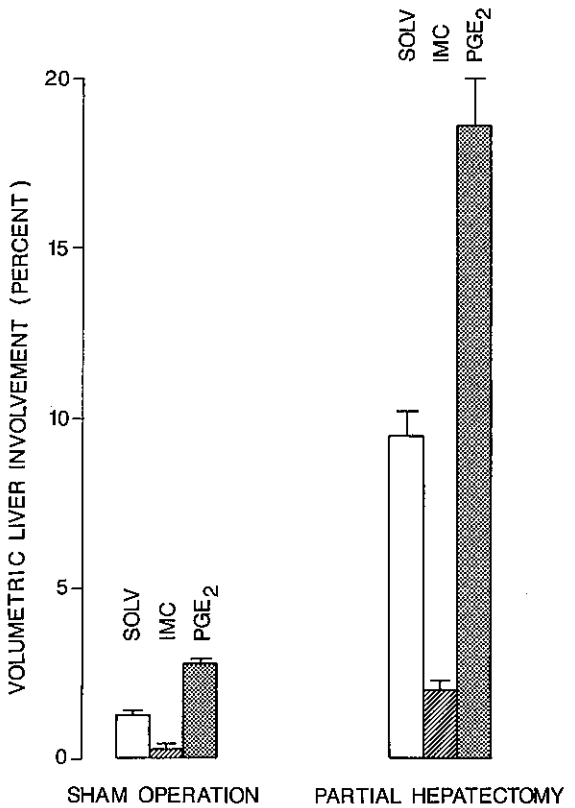

Fig. 3. Percent volumetric liver involvement (i.e. the ratio between the total volume of lesions and the volume of liver analyzed) by GGT-positive foci, 8 or 14 weeks after DENA injection, administered 24 h after different treatments as detailed in the legend to Fig. 1. Symbols are the same as in Fig. 1. SEM of less than 10% are not indicated. Statistical significance of differences evaluated by means of Student's t test are: (Sh) vs. (Sh+IMC), (Sh) vs. (Sh+PGE_2), (PH) vs. (PH+IMC) and (PH) vs. (PH+PGE_2): $P<0.001$.

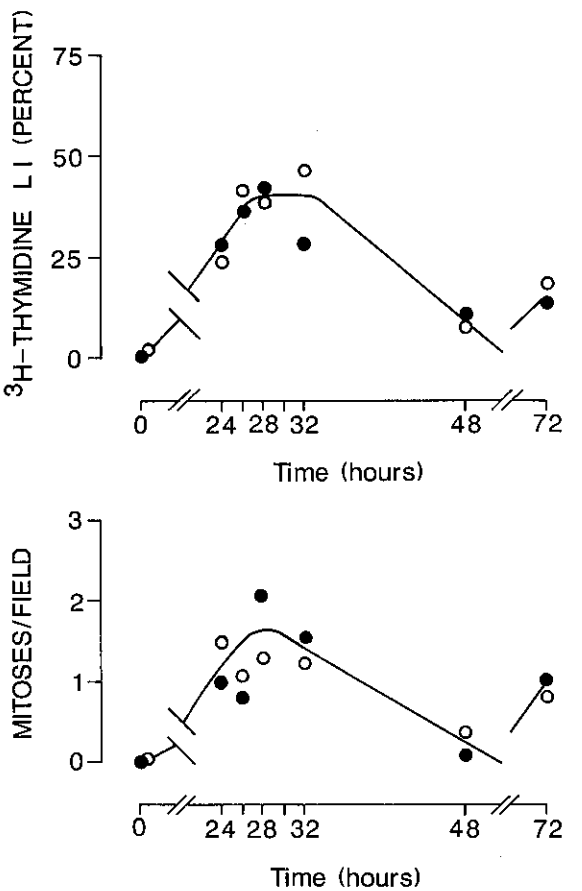

Fig. 4. Time course of ^{3}H-thymidine pulse-labeling index and mitotic index at 24 h versus time following PH (○) or PH+IMC 3.6 mg/kg bw (●). Symbols are the same as in Fig. 1. SEM of $<10\%$ are not indicated.

Mean volume of the GGT-positive lesions As shown in Fig. 2, IMC and PGE_2 also had opposite effects on the mean size of the DENA-induced lesions measured at 8 or 14 weeks following application of the carcinogenic treatment. IMC pretreatment resulted in DENA-induced foci with an average volume that was two times smaller than that observed in unpretreated animals. This was not observed in the Sh group (P=NS), perhaps due to the fact that we were in that case at the limit of sensitivity of the measurements.

Eight weeks after DENA treatment, the average volume of the foci in the PH group of rats was practically the same whether the animals had or had not been pretreated with PGE_2 or PGE_2+IMC before DENA treatment; it corresponded to about 5 times that in the Sh group ($P<0.001$). As compared to the corresponding control, PGE_2 pretreatment resulted in DENA-induced foci of greater average volume in the Sh group ($P<0.01$) but not in the PH group (P=NS) or rats.

This might reflect the existence of an inherent limit to the growth potential of the altered hepatocytes, which had possibly already been reached following PH. Figure 2 also shows that there was no significant difference in the growth pattern of the lesions resulting from the different types of pretreatments between week 8 and week 14.

Liver involvement by GGT-positive lesions The effects of the various treatments on the incidence and on the size of the foci can be expressed in terms of the percent liver volumetric involvement by altered hepatocytes. The liver involvement (shown in Fig. 3) represents the best expres-

sion of the end result of each combination of pretreatment and treatment in terms of the overall size of the population of altered hepatocytes potentially at risk for further step(s) (or already engaged) in the malignant progression.

IMC had a pronounced negative effect on the liver involvement parameter in PH ($P<0.001$) as well as in Sh ($P<0.001$) rats, reflecting a reduction in number and in size of the DENA-induced foci. PGE_2 had an opposite effect on these two parameters which resulted in a marked increase in liver volumetric involvement by lesions in both groups (Sh, $P<0.001$; PH, $P<0.001$).

^{3}H-Thymidine pulse-labeling index (LI) Twenty-four hours after PH, the hepatocyte population exhibited a percent LI of 29.8 ± 5.5, against 0.08 ± 0.07 in Sh animals; this response to PH was not significantly modified by IMC (LI: 21.3 ± 4.3) as shown in Fig. 4. In a separate experiment the 24 h LI in PH group was 55.4 ± 7.7 and was also not modified by IMC+PGE_2 administration (LI: 51.5 ± 1.2). After further treatment with DENA the time-courses of hepatocyte LI in IMC-treated and untreated PH rats were similar up to 48 h after the carcinogen injection (Fig. 4). It may thus be concluded that IMC had no effect, either on the 24 h proliferative response to PH or on the time course of its subsequent evolution under the influence of DENA.

In Sh animals the LI was 0.6 ± 0.2 and was not significantly altered by PGE_2 or IMC (LI: 0.2 ± 0.1 and 0.6 ± 0.2, respectively).

DISCUSSION

Whereas a high proliferation rate is generally considered as an enhancing factor in the action of a carcinogen there is no direct proof that this indeed directly results from mitotic activity. To test this hypothesis we tried to dissociate the effect of PH on the induction of GGT-positive foci by DENA from its mitogenic action on hepatocytes.

Based on the reported observation that IMC administration at the time of surgery markedly decreased the liver proliferative response rate to PH, and that this effect was counteracted by PGE_2 administration,[26] we therefore chose to test the effect of a pretreatment with these two compounds, alone or in combination, on the efficiency of further treatment with the carcinogen DENA to induce GGT-positive liver foci in PH or Sh rats. However, due to the high lethality observed with the IMC dose used in the above cited work, when applied to animals that are then treated with DENA, we had to reduce the dose to 3.6 mg/kg bw, which proved to be the highest dose of IMC compatible with the optimal survival of the animals when coupled to the carcinogenic treatment.

Quite unexpectedly, the treatment with this dose of IMC at the time of PH significantly decreased the incidence of GGT-positive foci induced by DENA administration 24 h after surgery, without any effect on the 24 h proliferative response to PH or on the time course of this response after DENA injection. On the other hand, PGE_2 administration together with IMC at the time of PH fully restored the incidence of GGT-positive foci to the level seen in PH animals, again without any modification of the 24 h labeling index as compared to PH animals. This reversal by PGE_2 of the IMC effect on foci induction indicates that modulation of the incidence of DENA-induced foci by the latter does not result from a simple toxic action, as already indicated by the absence of any effect of the drug on the ^{3}H-thymidine labeling index.

Though our experiments do not allow us to reject the possibility that increased proliferative activity *per se* might be related to the positive effect of PH on the liver susceptibility to DENA, they clearly indicate that, even so, this can be counteracted without altering the proliferative response. Previous experiments with lead nitrate or other direct mitogens showed that such compounds increased hepatocyte proliferation without affecting the incidence of DENA carcinogen-induced liver foci.[27] Though this was measured only as a number of lesions per cm^2 of liver,[27] these observations, like ours, might be interpreted as indicating that proliferative activity does not in itself suffice to potentiate the action of carcinogens. However, interpretation of the data in those experiments is complicated by the fact that, as considered by the authors themselves, proliferation in those models, unlike that in regenerative proliferation, is followed by apoptotic death which eliminates the excess of cells, and possibly more efficiently eliminates the altered ones.[27]

The possibility remained that the respective actions of IMC and PGE_2 were on some parameter(s) of liver physiology specifically related to regeneration. This was disproved by our observation that a pretreatment of sham-operated rats with IMC or PGE_2, 24 h prior to DENA administration, also reduced or increased, respectively, the frequency of DENA-induced GGT-positive foci at 8–14 weeks, as compared to the level seen in unpretreated sham-operated animals.

If, as supported by several lines of evidencce (see the introduction), the foci indeed represent the hepatocyte population at risk of further steps in the malignant progression, then the percent of liver involvement by foci of altered hepatocytes is the best expression of the effect of various treatments and pretreatments on the ultimate risk of cancer development. It is known that this parameter correlates to tumor incidence in this experimental model.[7] In any case, it represents the only real measurement of the efficacy of a treatment in terms of total size

of the altered cell population. It can be seen that IMC reduces and PGE_2 increases the percentage of liver involved by altered hepatocytes and in this way they might modulate the risk of further progression. This possibility is currently being investigated, by following the hepatocarcinogenic process up to its late and ultimate stages, namely the appearance of nodules and of hepatocellular carcinomas.

Though our data clearly show modulation by IMC and PGE_2 of the incidence of GGT-positive foci, the mechanism by which this occurs remains unknown. The data with PGE_2 at least indicate that a proliferative stimulus is not an absolute requirement.

As the incidence, but not the initial growth rate of the DENA-induced foci, was reduced by IMC pretreatment of PH rats, the possibility can be envisaged that the cyclooxygenases inhibitor induces a long-lasting change in the hepatocytes that counteracts a positive effect of PH at the initiation level (e.g. by acting on the metabolism of the carcinogen or on the molecular target of its action). This of course would not exclude the possibility of a promoting effect of PH linked to compensatory proliferation.

Evidence exists for the concurrent oxidation of various xenobiotics during prostaglandin biosynthesis, resulting in the formation of reactive metabolites which covalently bind to DNA.[28-30] However this is unlikely to explain our data since DENA is not a likely substrate for prostaglandin H synthase.[29, 30] On the other hand, the opposite effect of PGE_2 on foci induction cannot be accounted for on this basis, as PGE_2 does not seem to inhibit PH synthase activity.[31] A single action on the effective dose of DENA thus appears unlikely to form the basis of our observations. Experiments with a carcinogen that does not require metabolic activation will need to be performed to clarify this issue.

We therefore favor the assumption that the effects are related to modulation at the promotion level. In support of this view, Denda *et al.*, using inhibitors of enzymes of arachidonic acid metabolism, demonstrated an inhibitory effect upon the positive effect of phenobarbital on induction by DENA.[32] They suggested possible involvement of these enzymes in the mechanisms underlying phenobarbital promotion of hepatocarcinogenesis.

Finally, our data indicate that PH acts by at least two pathways to increase the liver susceptibility to DENA action. The first one, which is insensitive to IMC pretreatment, might involve proliferative activity, or a correlate thereof, and be responsible for foci with a high initial growth rate (hence the higher average volume of the lesions in all treatments that include PH). The second one, inhibited by IMC and mimicked by PGE_2, would be more directly related to initiation, by acting on target cell sensitivity and/or on metabolic activation of the carcinogen.

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