Jpn. J. Cancer Res. 83, 1154–1165, November 1992

Effects of Modifying Agents on Conformity of Enzyme Phenotype and Proliferative Potential in Focal Preneoplastic and Neoplastic Liver Cell Lesions in Rats

Hiroyuki Tsuda,[1,3] Keisuke Ozaki,[1] Satoshi Uwagawa,[1] Shuji Yamaguchi,[1] Kazuo Hakoi,[1] Toyohiko Aoki,[1] Toshio Kato,[1] Kiyomi Sato[2] and Nobuyuki Ito[1]

[1]*First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467 and* [2]*Second Department of Biochemistry, Hirosaki University School of Medicine, Zaifu-cho 5, Hirosaki 036*

Development of preneoplastic lesions in the rat liver under the influence of various modifiers was investigated with particular attention to changes in simultaneous expression of altered enzyme phenotype within the lesions (conformity) and proliferation potential. Degree of conformity of marker enzymes such as glutathione *S*-transferase placental form (GST-P), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphatase, adenosine triphosphatase and γ-glutamyltranspeptidase was compared with levels of 5-bromo-2-deoxyuridine labeling. After initiation with diethylnitrosamine, rats were administered the hepatopromoter sodium phenobarbital (PB, 0.05%), the antioxidant ethoxyquin (EQ, 0.5%), or a peroxisome proliferator, clofibrate (CF, 1.0%) or di(2-ethylhexyl)-phthalate (0.3%) and killed at week 16 or 32. The PB promoting regimen was clearly associated with increase in the numbers of high conformity class lesions simultaneously expressing three to five enzymes, and elevated proliferation potential. The inhibitor, EQ, in contrast, brought about a time-dependent decrease in conformity so that only 1 or 2 alterations were most commonly observed at week 32. Lesion populations in the peroxisome proliferator- and especially CF-treated cases were characterized by obvious dissociation between degree of conformity and proliferative status. Such treatment-dependent differences were not always correlated with the size of the lesion. The results thus suggested that the conformity and proliferation potential of preneoplastic lesions are dependent on modification treatment. Overall, GST-P was found to be the most reliable marker, although G6PD was less influenced in the peroxisome proliferator cases.

Key words: Modification — Enzyme phenotype — Conformity — Proliferation potential — Hepatocarcinogenesis

The increasing numbers of chemicals being introduced into the human environment and the lack of any reliable correlation between results of *in vitro* mutation assessment and *in vivo* carcinogenicity[1] have acted as stimuli to the development of medium-term animal models. In comparison to the costs of two-year testing, in terms of both financial resources and time, the approach adopted in our recent rat liver medium-term bioassay model for prediction of carcinogenic potential[2–4] has decided advantages. Thus, the use of the placental form of glutathione *S*-transferase (GST-P[4])[5,6] as a good marker for preneoplastic focal populations[7,8] allows definition of carcinogenic or modulation potential within the relatively short period of 8 weeks. In addition, comparative studies have demonstrated a clear dose-dependent correlation between the results of long-term *in vivo* and our medium-term testing of a number of different carcinogens.[9] However, limited numbers of false-negatives have been encountered with non-genotoxic hepatocarcinogens.[2] Therefore, our understanding of the processes underlying generation of preneoplastic lesions in our test system and the detailed mechanisms involved remains incomplete.[10]

Since our test system relies on our ability to recognize preneoplastic lesions and their capacity for growth under defined conditions, altered phenotypic expression within hepatocellular foci is of fundamental importance. While a large number of investigators have defined potentially useful markers of liver lesions[5–11] no one enzyme appears capable of allowing complete identification of foci associated with the variety of promoters and carcinogens in our environment. In particular, the peroxisome proliferator group of hepatocarcinogens poses a problem because of the lack of positivity for GST-P and γ-glutamyltranspeptidase (GGT) in their associated foci and neoplasms.[3,12,13] The hormone dehydroepiandrosterone has also been shown to cause a metabolic shift in the

[3] Present address: Second Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470-11. To whom correspondence should be addressed.

[4]Abbreviations: GST-P, glutathione *S*-transferase placental form; G6PD, glucose-6-phosphate dehydrogenase; G6P, glucose-6-phosphatase; ATP, adenosine triphosphatase; GGT, γ-glutamyltranspeptidase: BrdU, 5-bromo-2-deoxyuridine; DEN, diethylnitrosamine; PB, sodium phenobarbital; EQ, ethoxyquin; DEHP, di(2-ethylhexyl)phthalate; CF, clofibrate; PH, partial hepatectomy; PBS, phosphate-buffered saline.

phenotype of foci[14, 15] and there is a possibility that many agents capable of modifying hepatocarcinogenesis also bring about equivalent changes.

The present investigation was aimed firstly at defining time-dependent alteration in the degree of conformity as well as proliferation potential of enzyme-altered focal liver lesions in rats initiated with diethylnitrosamine (DEN) under the influence of modifying agents of hepatocarcinogenesis using a protocol similar to that utilized for the assay of carcinogens,[3, 4] and secondly determining whether multiple expression (conformity) of altered enzyme phenotype is associated with progression of hepatocarcinogenesis.

Included was an analysis of the effects of subsequent administration of the hepatopromoter sodium phenobarbital (PB), the antioxidant ethoxyquin (EQ) and the peroxisome proliferators clofibrate (CF) and di(2-ethylhexyl)phthalate (DEHP) on simultaneous expression of enzyme phenotype within the lesions and their proliferative status. The enzymes GST-P, glucose-6-phosphate dehydrogenase (G6PD), GGT, glucose-6-phosphatase (G6P) and adenosine triphosphatase (ATP) were selected[16–18] for comparison and 5-bromo-2-deoxyuridine (BrdU) incorporation was used for assessment of proliferation.[19, 20]

MATERIALS AND METHODS

A total of 125 male Fischer 344 rats (Charles River Japan Inc., Atsugi) weighing approximately 160 g at the commencement were maintained on basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) *ad libitum* and housed in plastic cages in an air-conditioned room at 24±2°C and 60±5% humidity. The animals were divided into three groups. Group 1 was given a single 200 mg/kg body weight i.p. injection of DEN (Tokyo Kasei Kogyo Co., Ltd., Tokyo) dissolved in 0.9% NaCl (40 mg/ml) to initiate hepatocarcinogenesis. After 2 weeks on basal diet, they received one of the modifying agents in powder diet: PB (0.05% Iwaki Seiyaku Co., Tokyo), EQ (0.5% Tokyo Kasei Kogyo Co., Ltd.), CF (1.0% Tokyo Kasei Kogyo Co., Ltd.) or DEHP (0.3% Tokyo Kasei Kogyo Co., Ltd.). Animals were subjected to two-thirds partial hepatectomy (PH) at week 3 and killed at weeks 16 and 32 as shown in the experimental protocol (Fig. 1). Group 2 animals were given DEN and PH in the same manner as group 1 without administration of any modifying agent.

For analysis of DNA synthesis, five animals per group were subjected to intraperitoneal implantation of minipumps (Alza Corporation, Palo Alto, CA) containing 37.5 mg of BrdU (Sigma Chemical Co., St. Louis, MO), which was released over a one-week period prior to killing.

Immediately upon killing, the livers were excised and slices 4–5 mm thick were cut with a razor blade and immersed in isopentane pre-cooled to approximately −130°C by liquid nitrogen. They were stored at −80°C in a deep freeze until use. Serial sections cut at 4 μm were stained histochemically for demonstration of G6PD (membrane method after Meijer and de Vries[21]), GGT, ATP (Mg^{2+}-dependent) and G6P activities (methods as described by Lojda *et al.*[22]). Immunohistochemical staining of BrdU was done on sections of liver fixed in acetone cooled to −20°C, then hydrated in phosphate-buffered saline (PBS), treated with 4 *N* HCl, neutralized with PBS, and treated with anti-BrdU monoclonal antibody (Becton-Dickinson Monoclonal Center, Mountain View, CA) at a dilution of 1:200.[19, 20] Binding sites were demonstrated by the avidin-biotin peroxidase complex (ABC) method using diaminobenzidine-H_2O_2.[23] One serial section of the left lateral lobe with 1–2 cm^2 section area was prepared for each rat and the numbers of cells incorporating label into the nuclei among approximately 1000 cells in normal tissue were counted and expressed as percentage values.

The numbers of lesions, differentiated on the basis of the five respective marker enzymes, and the areas of liver sections were measured for all animals with the aid of a color video image processor (VIP-21C, Olympus-Ikegami

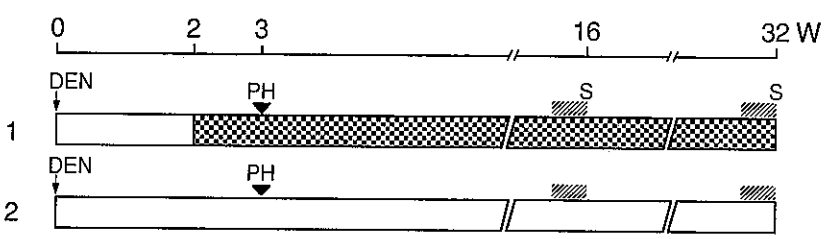

Fig. 1. Experimental protocol. ▩, Modifying chemicals in basal diet: hepatopromoter PB, 0.05%; antioxidant EQ, 0.5%; peroxisome proliferator CF, 1.0% and DEHP, 0.3%. DEN, 200 mg/kg, i.p. S, killed. ▨, BrdU, 37.5 mg/rat by minipump for 1 week.

Tsushin Co., Tokyo) as described previously.[3] For the study of conformity of individual lesions, 2 to 3 specimen blocks per group were used. Lesions larger than 200 μm in diameter, demonstrating enzyme alteration in more than a half of the focal lesion area, were traced and overlayed on a sheet of paper using a plaque viewer (Carl Zeiss, Jena, Germany) at a magnification of 10×. For each individual focal population assessed, both phenotype and proliferative status were noted. BrdU counts were performed by assessing 100 to 600 cells (depending on the size of lesions), with an average of approximately 300 cells, and expressed as labeling indices (%). Average size of each lesion was estimated by measuring the largest diameter. Data generated for the 1 control and 4 treatment groups were then compared to allow assessment of the reliability of the various marker enzymes under different conditions of chemical treatment. Statistical analyses were made using the inter-group Mann-Whitney test for the numbers of lesions divided into conformity classes, the χ^2 test for BrdU labeling indices and Student's t test of analysis of variance for the size of lesions.

RESULTS

Data for quantitative assessment of numbers of lesions expressing at least one altered enzyme at weeks 16 and 32 are shown in Table I. The most advanced lesions observed were hyperplastic nodules, no hepatocellular carcinomas being induced. Average values were significantly increased with PB and decreased with EQ and DEHP treatment at week 16, and significantly decreased with EQ, DEHP and CF treatment at week 32 as compared to DEN-alone group values.

Quantitative analysis of lesions allocated to different conformity classes revealed a clear difference between control and treatment groups. In the control case, lesions of each conformity class showed a gradient decrease in numbers in the high conformity classes at both 16 and 32 weeks. In contrast, a large majority of lesions in PB-treated animals exhibited higher conformity phenotypes (3 to 5) at week 16 with a significant difference from all other groups (Mann-Whitney test). The EQ, CF and DEHP groups demonstrated similar patterns to the control group but having lower lesion numbers. At week 32, the majority of lesions belonged within conformity classes 1 and 2, except in the case of PB-treated rats, in which most lesions were classified within classes 2 to 4, the difference from the EQ-, CF- and DEHP-treated groups being significant (Fig. 2).

BrdU labeling indices at week 16 were significantly higher in high-conformity lesions of control and PB-treated groups, with a linear correlation with degree of conformity. The conformity class distribution profile was rather flat in the EQ-, CF- and DEHP-treated groups, especially among the 1 to 4 conformity classes at both week 16 and 32. Control, CF and DEHP groups did not

Table I. Quantitative Values for Lesions Per Unit A[a]

Treatment	At week 16		At week 32	
	No. of rats	No. of lesions/cm^2	No. of rats	No. of lesions/cm^2
DEN alone	5	13.1±2.7	5	26.4±4.1
DEN→PB	5	19.0±1.8*	5	37.8±6.8*
→EQ	5	8.7±1.8*	5	16.0±3.8**
→DEHP	5	5.1±1.9***	5	13.1±4.8**
→CF	5	11.0±3.2	5	15.4±4.1**

a) Quantitative values for preneoplastic and neoplastic liver lesions expressing at least one altered enzyme. $P<0.05$ (*), 0.01 (**) or 0.001 (***) as compared to the DEN-alone group.

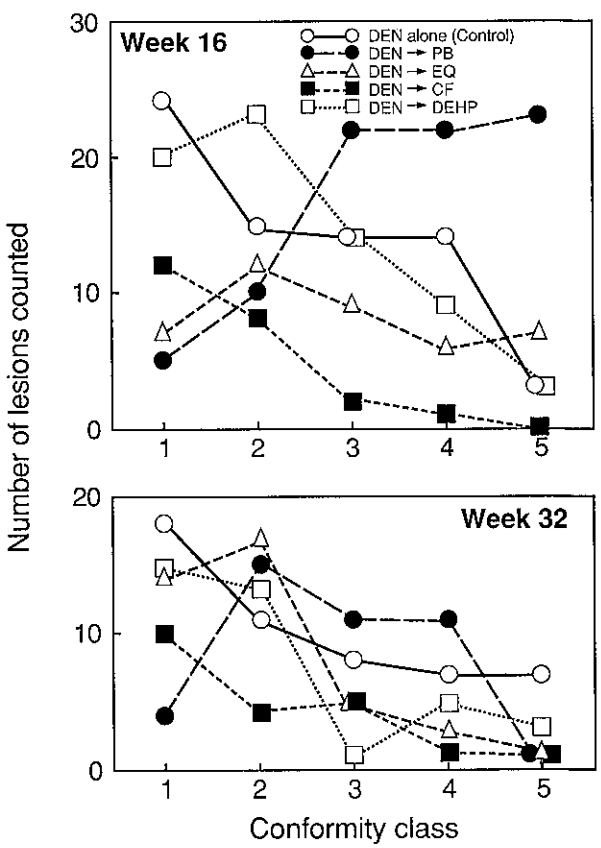

Fig. 2. Conformity class and numbers of lesions actually counted. At week 16, DEN-PB and -CF are significantly different from all other groups. DEN-EQ is significantly different from DEN-DEHP (Mann-Whitney test).

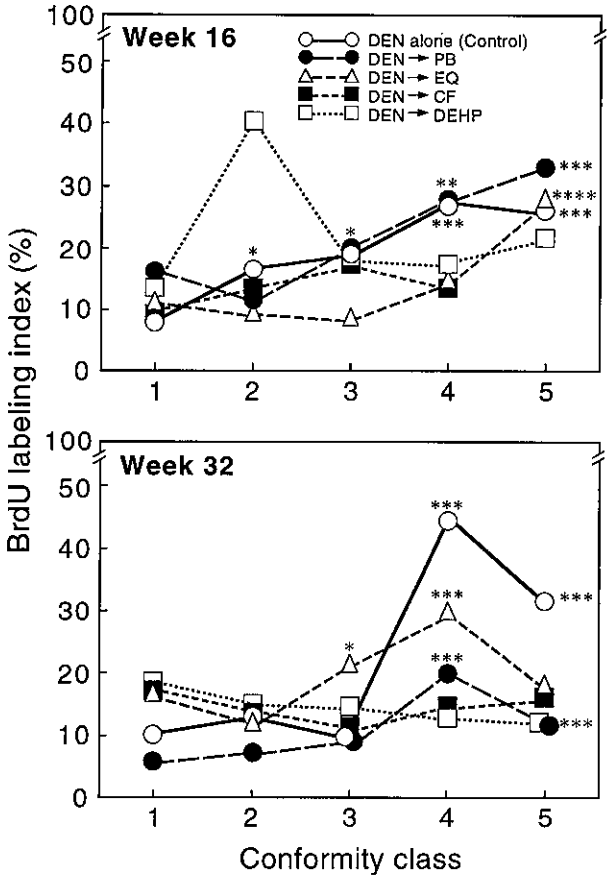

Fig. 3. BrdU labeling indices. Cell count was 72 to 523 per conformity class. Significant increases over 4 (****), 3 (***), 2 (**) or 1 (*) lower conformity classes ($P<0.001$–0.05, χ^2-test).

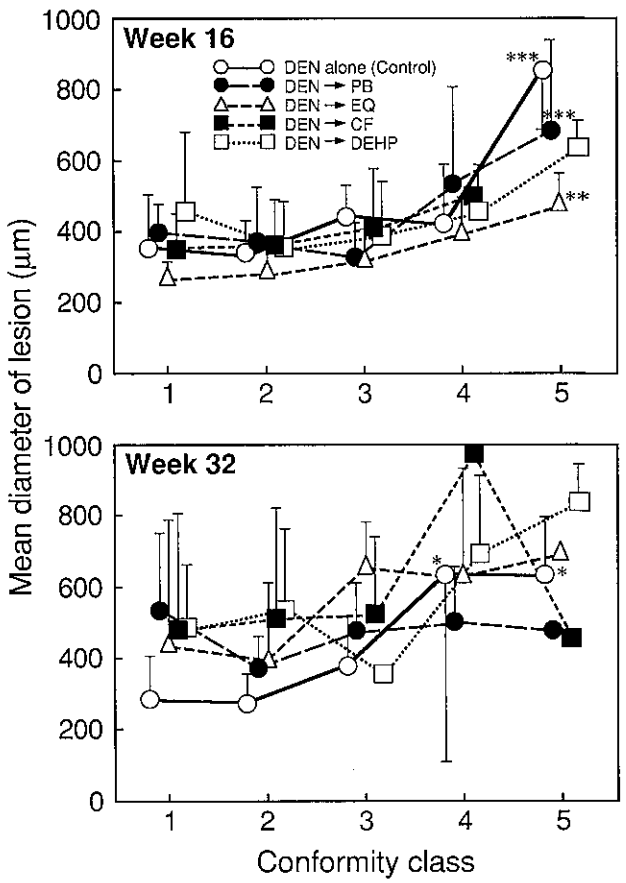

Fig. 4. Conformity class and size of lesion. Significantly increased over 4 (***), 3 (**) or 2 (*) lower conformity classes ($P<0.05$, Student's t test, analysis of variance).

show any linear correlation between BrdU labeling index and conformity classes (Fig. 3). Therefore, conformity class profiles and proliferation status generally showed discordant relationships.

Size of lesions correlated with increase of conformity only in the highest conformity class of the control, and PB- and EQ-treated groups at week 16 ($P<0.05$). At week 32, such a correlation was not observed except in the control group (analysis of variance) (Fig. 4).

Among the constituent enzymes expressed, the two most frequently altered were GST-P and G6P in the control group, GST-P and ATP in the PB-treated group, GST-P and G6P in the EQ-treated group, G6PD and GST-P in the CF-treated group and G6P and G6PD in the DEHP group. Overall, the most frequently encountered enzyme was GST-P (Fig. 5a–e).

Representative lesions treated with PB, EQ and DEHP are shown in Figs. 6–9.

DISCUSSION

The results of the present study clearly indicated that while increase in overlapping of enzyme alteration within individual lesions is generally associated with elevated BrdU incorporation, this is not always the case. Furthermore, clear differences emerged in the comparative reliability of the various markers analyzed, depending on the modulating influence.

A number of investigators have suggested that the enzyme phenotype of rat liver preneoplastic lesions is not random, but rather that it is the reflection of a biochemically directed shift in the component cells, conferring a physiological advantage, and therefore of adaptive significance for proliferation.[24–27] In addition to changes in drug-metabolizing enzymes which reduce the sensitivity of such focal cell populations to toxic agents,[25–27] concerted changes in carbohydrate metabolism have been

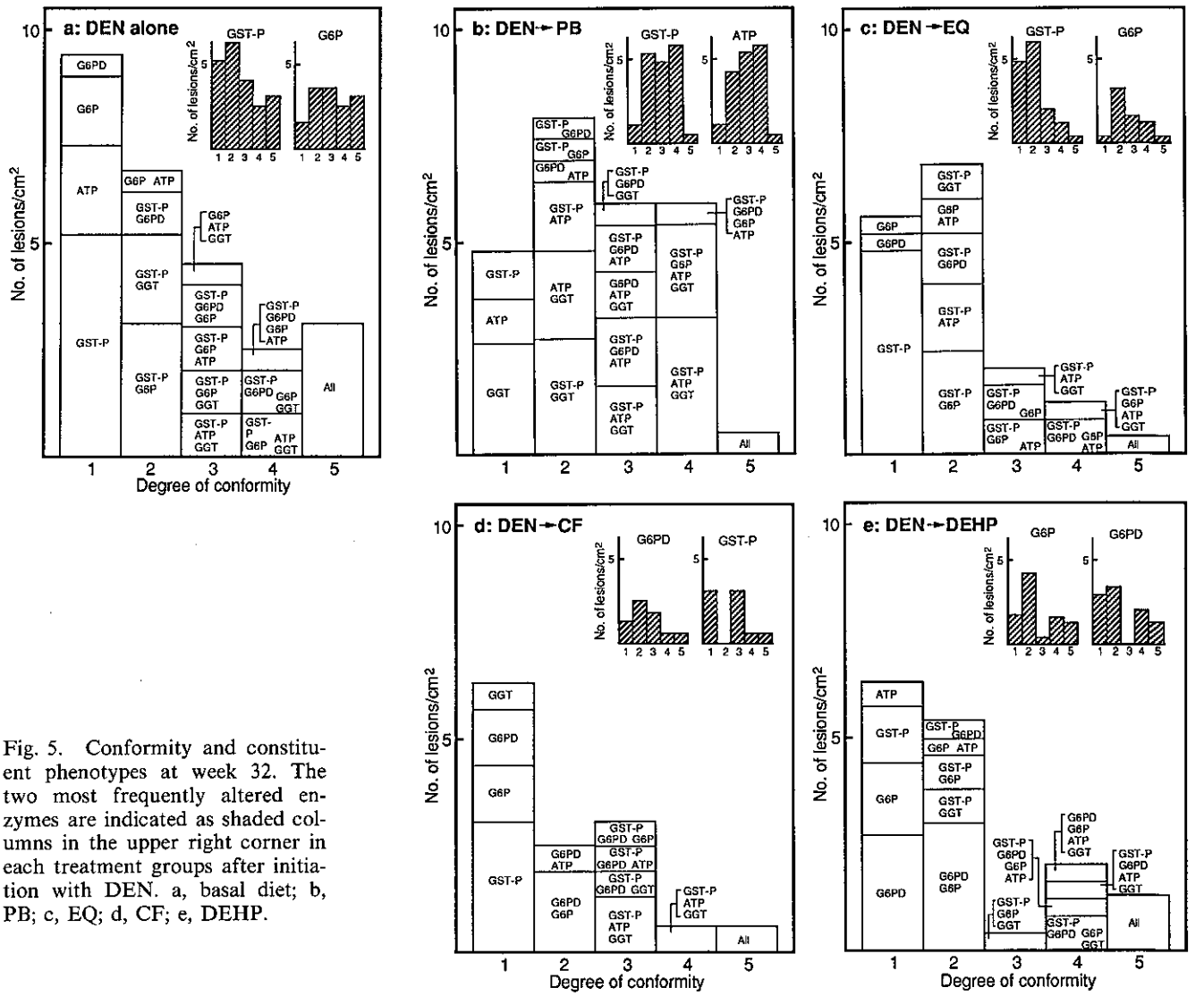

Fig. 5. Conformity and constituent phenotypes at week 32. The two most frequently altered enzymes are indicated as shaded columns in the upper right corner in each treatment groups after initiation with DEN. a, basal diet; b, PB; c, EQ; d, CF; e, DEHP.

described.[16, 17, 28] This consideration has lead to the expectation of considerable overlap between different enzymes, assuming they have some roles in concert.[24]

In accordance with this concept, some authors[29] have reported certain degrees of simultaneous expression, especially under conditions leading to rapid growth of lesions, as observed in the present study with control and PB treatment. The observed increase in the number of enzymes expressed was clearly correlated with increase in the level of BrdU incorporation. The results in the present case are at least partly in agreement with earlier reports by investigators using fewer enzyme markers.[30–33]

It is generally accepted that chemically induced focal populations in the rat liver characteristically demonstrate elevated levels of cell division,[24, 34, 35] especially at later stages.[28] Use of immunohistochemically demonstrable BrdU incorporation in the present study allowed confirmation of earlier results with tritiated thymidine, as expected from practical considerations.[20] If enzyme alteration is involved in the increase of proliferative potential, then concerted changes in enzyme phenotype would be expected.[24, 34, 35] There was a clear shift of conformity index from small to large number after treatment with hepato-promoting agents, PB, with a proportional increase of proliferative potential of lesion cells. This indicates that neoplastic development under these conditions might be closely linked to increase in altered phenotypic expression.[36, 37] It should be noted that proliferation po-

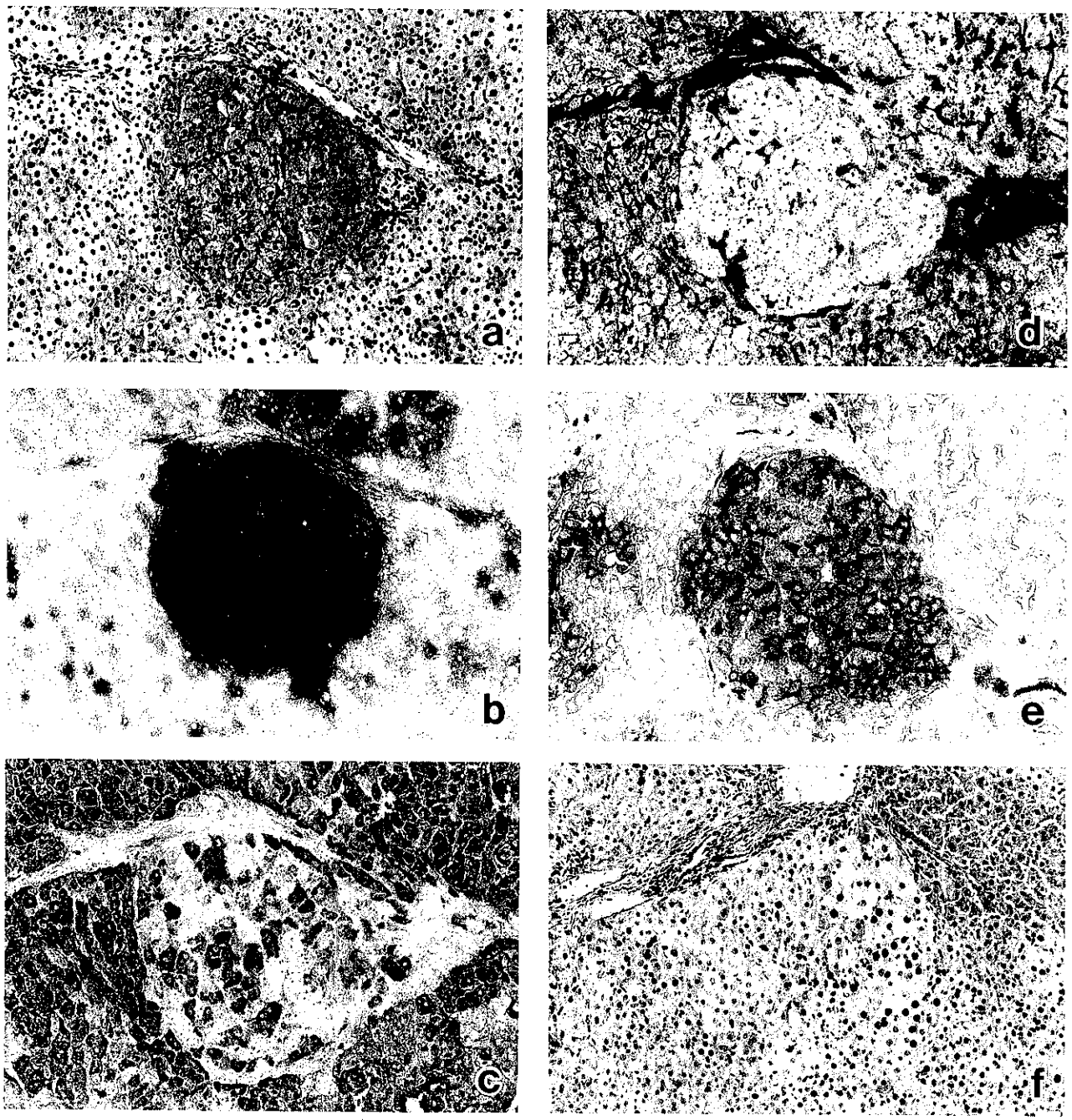

Fig. 6. A focus expressing 5 enzyme alterations in a rat given PB and killed at week 16. BrdU-labeling is clearly higher than in the surrounding parenchyma (×33). a, GST-P; b, G6PD; c, G6P; d, ATP; e, GGT; f, BrdU.

tential is not directly dependent on the lesion size, because size class analysis at week 16 showed that only control, PB- and EQ-treated lesions expressing class 5 conformity were statistically larger, with no significant differences being evident between class 1 to 4 conformity lesions. This dissociation between conformity and size was more evident at week 32.

Recent work has shown that in addition to promoting effects, both inhibition of lesion development and alteration in the enzyme expression of the lesions themselves

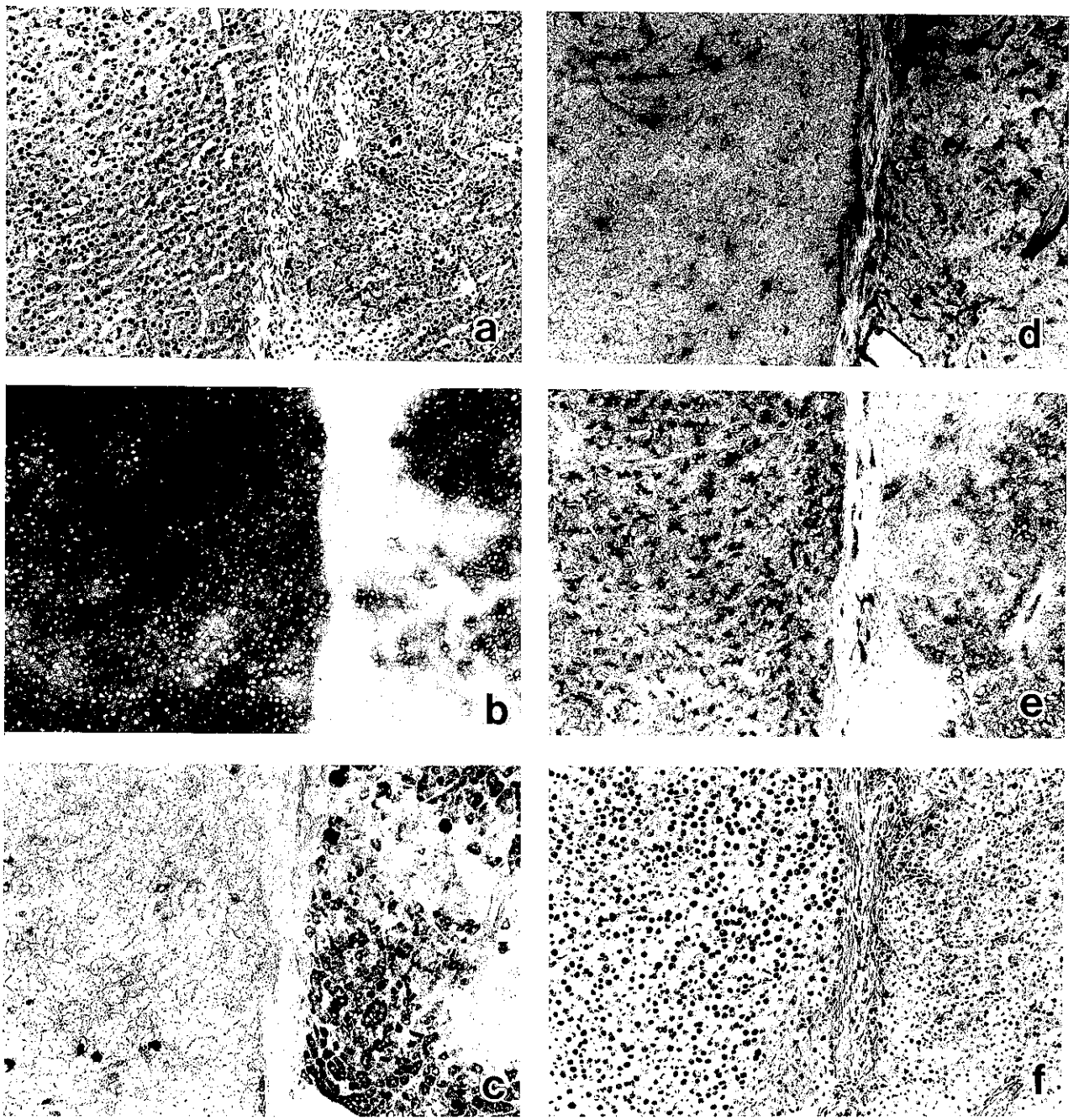

Fig. 7. Late stage nodule (on the left) exhibiting 5 enzyme alterations and high BrdU incorporation in a rat given PB and killed at week 32. In contrast, the focus on the upper right lacks altered G6P expression. Note the remarkable difference in the numbers of BrdU-labeled cells (×33). a, GST-P; b, G6PD; c, G6P; d, ATP; e, GGT; f, BrdU.

can be brought about by exogenous application of chemicals. Thus, a number of studies have demonstrated that antioxidants reduce development of liver preneoplastic focal lesions[2-4, 38] and they also exert effects on phenotype[39] as shown in the present study. For example, with EQ, an inhibitor of hepatocarcinogenesis, a clear shift in conformity class distribution towards smaller index groups with lower BrdU labeling indices was evident.

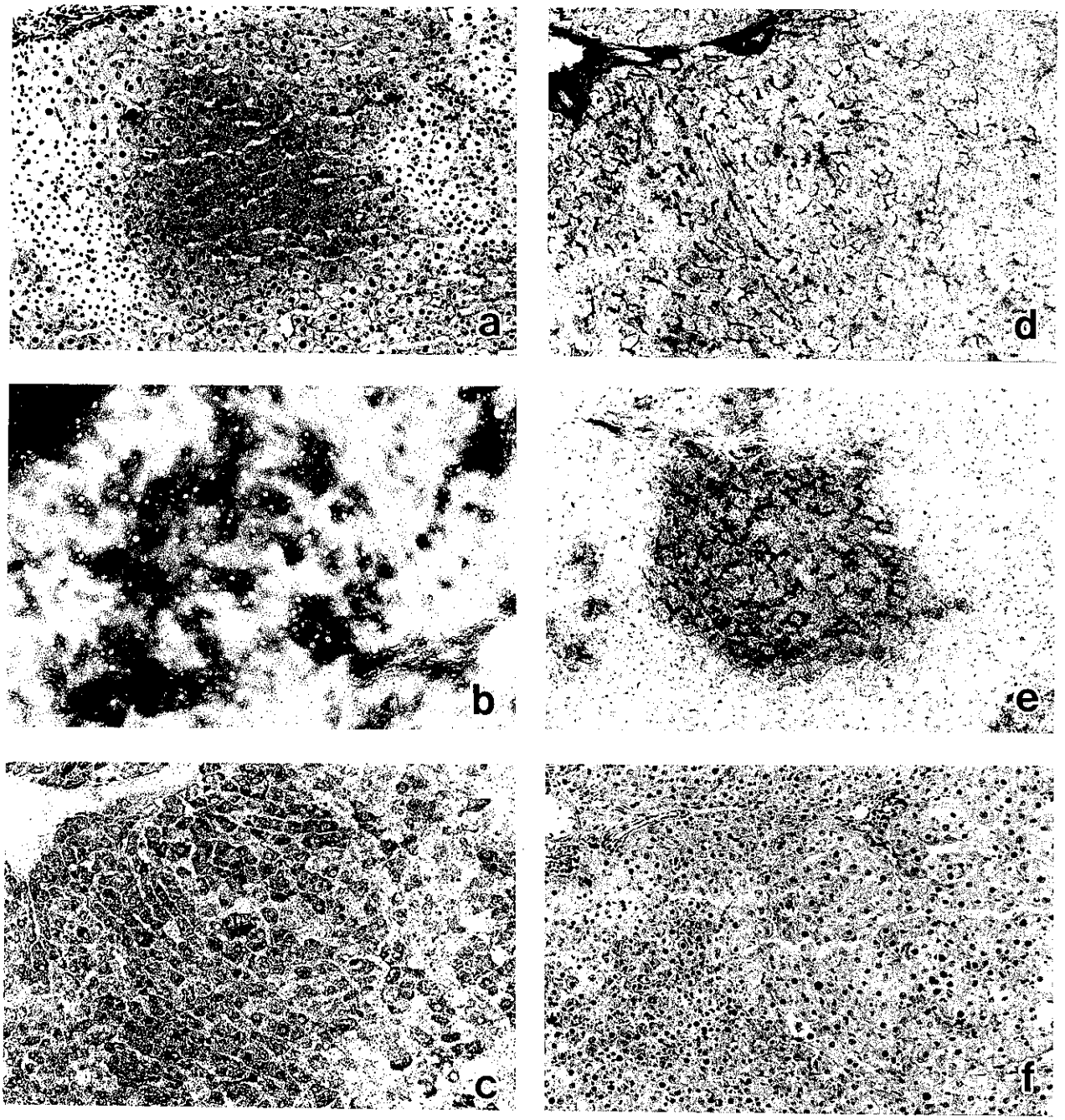

Fig. 8. Focus expressing 2 enzyme alterations in a rat given EQ and killed at week 16. The lesion almost lacks altered expression of G6PD, G6P and ATP. Nuclear labeling of BrdU is low (×33). a, GST-P; b, G6PD; c, G6P; d, ATP; e, GGT; f, BrdU.

Similar effects were demonstrated for the peroxisomal proliferators, CF and DEHP. However, although BrdU labeling indices were not high, induction of larger lesions with high conformity index may correspond to their weak carcinogenic potential. Previous observation of apparently specific effects of peroxisome porliferators on GGT expression[40, 41] was in line with the present results. The finding of G6PD increase in the majority of lesions

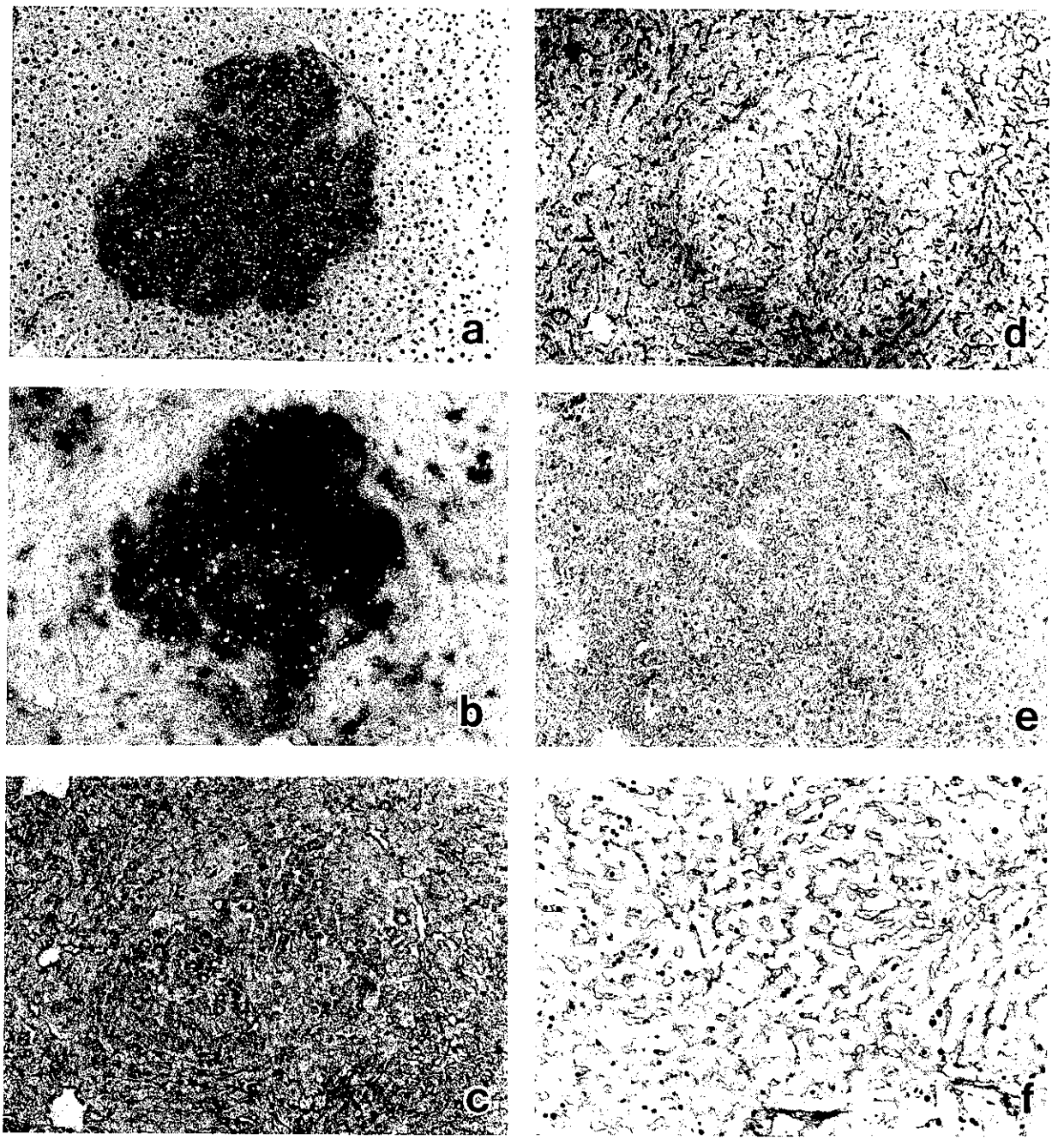

Fig. 9. Focus expressing 3 enzyme alterations in a rat given DEHP and killed at week 32. The lesion is completely negative for GGT and expression of G6P is normal. BrdU labeling within the lesion is not markedly different from that in surrounding hepatocytes (×25). a, GST-P; b, G6PD; c, G6P; d, ATP; e, GGT; f, BrdU.

in CF- and DEHP-treated groups and the loss of correlation between conformity class and proliferative potential are of interest in the light of the earlier demonstration indicating lack of any direct link between activity of the key pentose phosphate shunt enzyme and tritiated thymidine incorporation.[42]

For the purpose of assessing promotion potential, however, the present data support the earlier suggestion that

GST-P is practically the most reliable marker available at present,[3, 7, 37] although in the PB promotion case all the enzyme alterations exhibited similar tendencies. With the two peroxisomal proliferators, more accurate results were gained by application of G6PD.

The 8-week duration model presently described has proved to be a very useful tool for prediction of hepatocarcinogenic and promoting potential for a wide variety of agents.[3] The short duration required is particularly advantageous for assays of expensive compounds for which only limited quantities are available, such as pyrolysis products, which have generated a great deal of interest as potential environmental hazards.[26] For the purpose of obtaining data which correlate with proliferation status, introduction of a second marker might be advisable on the basis of the above results.

Further studies of the relationships between relative proliferation status of lesions[10] and enhancement of background toxicity[43] in the analysis of mechanisms by which compounds promote or inhibit preneoplastic foci development in the model, have indicated the necessity of taking into account multiple phenotype expression.

ACKNOWLEDGMENTS

This research was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and from the Ministry of Health and Welfare, and for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan, and by funds from the Society for Promotion of Pathology of Nagoya.

(Received May 6, 1992/Accepted August 4, 1992)

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