FULL PAPER *Pathology*

Apoptosis and Cell Proliferation in Rat Hepatocytes Induced by Barbiturates

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ABSTRACT. To examine the effect on cell population in hepatocytes of phenobarbital (PB) and other barbiturates, PB, allobarbital (ALB), barbital sodium (BS) and barbituric acid (BA) were given orally to male rats for 7 consecutive days. Although there was no apparent change in non-promoting BA, hepatomegaly was induced by PB, BS and ALB, which are promoters of hepatocarcinogenesis. In PB- and BS-treated livers, hepatomegaly was attributable to hepatocyte proliferation and enzyme induction. In ALB-treated liver, it was attributable to enzyme induction. The level of cell proliferation was reduced to less than the control values following withdrawal of PB, ALB and BS. It seemed that the degree of suppression of cell proliferation following withdrawal of these compounds correlated to the degree of cell proliferation (PB>BS>ALB) during treatment. In PB-treated liver, apoptosis was induced during treatment, serving to eliminate the excess of hepatocytes. This suggests that short-term administration of PB neither induced suppression of apoptosis nor disturbed homeostasis of hepatocyte populations.—KEY WORDS: apoptosis, cell proliferation, liver, phenobarbital, rat.

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A large number of chemicals that cause liver tumors in rodent assays do not directly mutate DNA and are classified as non-genotoxic [25]. Cell proliferation is one of many essential factors involved in the non-genotoxic carcinogenesis process [18]. There are 2 groups of chemicals that induce liver cell proliferation: in one, cell loss precedes cell proliferation, while in the other, there is no cell loss prior to hyperplasia [9]. The former group consists of a variety of necrogenic agents, including carbon tetrachloride and chloroform. The latter group consists of chemicals capable of inducing direct hyperplasia in the liver, which includes cyproterone acetate (CPA), nafenopin (NAF), phenobarbital (PB) and α-hexachlorocyclohexane (α-HCH). These agents, often called mitogens, overcome operating growth-controlling mechanisms. Apoptosis is an active form of cell death in which individual cells are removed from the overall cell population as a compensatory response. Cessation of apoptosis increases genetic damage, and alteration of the regulation of apoptosis has been implicated in carcinogenesis [10]. Liver mitogens, such as CPA, PB, NAF and α-HCH, inhibit apoptosis [1, 2, 5]. This disruption of the balance between cell proliferation and apoptosis may contribute to the mechanism of hepatocarcinogenesis, acting as a tumor promoter [4, 14, 23]. According to these reports, the correlation rates of cell proliferation and apoptosis are important in assessing the ability of chemicals to induce carcinogenesis [8].

The purpose of the present study is to identify whether PB and other barbiturates, which promote hepatocarcinogenesis, suppress apoptosis and disturb the correlating rates of cell proliferation and apoptosis in rat livers. Bursch *et al.* [6] and Roberts *et al.* [21, 22] reported that the detection of mitogen withdrawal-induced apoptosis was more sensitive than the detection of minute changes in apoptotic rates in native rats. In the present study, PB, allobarbital (ALB), barbital sodium (BS) and barbituric acid (BA) were given orally to male rats for 7 consecutive days. Following the system described by Bursch *et al.* [6] and Roberts *et al.* [21, 22], we investigated the effects on cell proliferation and apoptosis in the liver of consecutive or withdrawal treatments of these barbiturates histopathologically.

MATERIALS AND METHODS

Animals: Male specific pathogen-free Fischer 344 rats, approximately 8.5 weeks old, were purchased from Japan Charles River Laboratories (Tsukuba, Japan). Animals of this age were selected because of the stability of their cell proliferative activity [26]. The animals were single-housed in wire-mesh cages in an air conditioned room (temperature, 22 ± 2°C; humidity, 55 ± 10%; light cycle, 12 hr/day). Food and water were available *ad libitum*. All animals were acclimatized for 5 days prior to the first treatment.

Experimental design: A total of 100 rats, 9 weeks of age, were divided into 5 groups as follows: Group 1, control; Group 2, PB (Wako Pure Chemical Industries, Japan); Group 3, ALB (Tokyo Kasei Kogyo Co., Japan); Group 4, BA (Tokyo Kasei Kogyo Co., Japan): Group 5, BS (Kanto Chemical Co., Japan). PB at a dose level of 50 mg/kg/day for 28 weeks in rat livers was previously reported to promote hepatocarcinogenesis in N-nitrosodiethylamine-initiated rats [24]. ALB and BS were also reported to promote hepatocarcinogenesis in initiated rat livers [20], but BA was inactive in this respect [11]. Each agent was solubilized in 0.5% methylcellulose solution (10 m*l*/kg) and administered orally at a dose level of 50 mg/kg/day. Control animals received an equivalent volume of 0.5% methylcellulose solution daily for 7 days. Four rats from each group were chosen as samples after 1, 3, 7, 9 and 14 days of treatment.

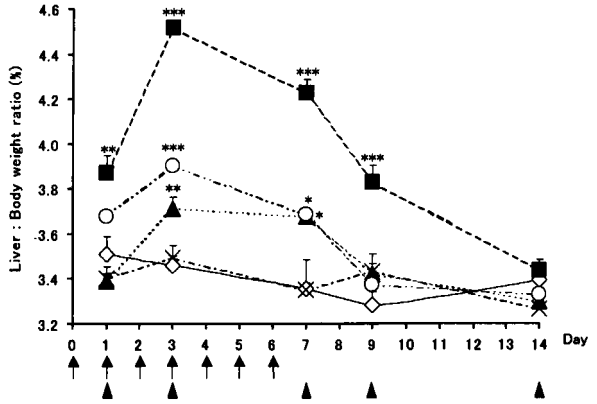

Fig. 1. Time course of liver to body weight ratios with different barbiturates. The treatment schedule is indicated below the abscissa. The sampling schedule is indicated below the arrow head. ◇—◇, Control; ■——■, PB; ▲·····▲, ALB; ×–·-·-·-×, BA; ○–··–·-○, BS. Values given are means ± SE for four rats per treatment. *,**,*** Significantly different from control at the $p<0.05$, $p<0.01$, $p<0.001$, respectively (Dunnett's test).

The experimental design is shown in Fig. 1. At sampling, all animals were given a single intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU; Tokyo Kasei Kogyo Co., Japan) at 100 mg/kg body weight, 2 hr prior to sacrifice. Animals were weighed, sacrificed by ether asphyxiation and necropsied. The livers were removed, weighed and examined for the presence of grossly visible lesions. A portion of the central median lobe of the liver was excised immediately, fixed in 10% neutral buffered formalin for 24 hr, embedded in paraffin, sectioned at 4-μm thickness and stained in a routine manner with hematoxylin and eosin (HE) for histopathological evaluation. Immunohistochemical staining for BrdU (BrdU antigen; DAKO, Japan) and P450s (CYP2B1/2 and CYP3A2 antigen; Daiichi Pure Chemicals Co., Japan) was carried out using the avidin-biotin-peroxidase complex method. DNA fragmentation was analyzed by the terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labeling (TUNEL) method using Apoptosis in situ Detection Kit Wako (Wako Pure Chemical Industries, Japan).

BrdU labeling index: For BrdU labeling index (LI%), labeled nuclei and total nuclei were scored in approximately 1,000–1,200 hepatocytes per liver with a × 40 objective. LI% of individual animals was determined by taking the total number of BrdU-labeled hepatocytes divided by the total number of hepatocytes counted, by light microscopy, with the aid of an image analyzer (IPAP; Image Processor for Analytical Pathology, Sumika Technoservis Co., Japan).

Incidence of apoptotic cells: The incidence of residues of hepatocytes undergoing apoptosis (apoptotic cells, "ACs") was determined by scoring 2,000–2,200 hepatocytes per liver in a TUNEL reaction, by light microscopy with a × 40 objective and an image analyzer. The number of ACs found was expressed as a percentage of intact cells.

P450s (CYP2B1/2 and CYP3A2) positive area ratio: Three randomly selected microscopic fields from each liver were analyzed with a × 10 objective. The positive and the total area analyzed were measured with an image analyzer. The positive area ratio, expressed as a percentage of labeled area versus total area, was calculated for each animal.

Biochemical procedures: After preparation of serum from each individual animal, serum enzyme activities of known indicators of liver cell injury, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GTP) in serum were determined.

Statistical analysis: Means and standard error (SE) were calculated. Statistical difference from control values for the data (except activity of P450s) was determined using Dunnett's multiple comparison test.

RESULTS

Body and liver weights: During treatment with PB and BS, body weights were significantly increased. The declining body weights between Day 1 and Day 3 for ALB were statistically significant. These body weights returned to control values by Day 14 (data not shown). Liver to body weight ratios (LBR) are shown in Fig. 1. LBR between Day 1 and Day 9 were significantly elevated in PB-treated animals. A significant increase in LBR was also observed between Day 3 and Day 7 in ALB- and BS-treated animals. Following withdrawal of PB, ALB and BS, LBR returned to control values by Day 14. Exposure to BA throughout the experimental period did not result in significantly altered body weights or LBR.

Activity of serum enzymes: A significant increase in LDH activity was observed on Day 1 with BS and Day 7 with ALB, BA and BS treatment (Table 1). However, the degree of these changes was so small that they were clinically meaningless. In the case of other serum enzymes, no significant change was observed with any of the compounds.

Hepatic morphological findings: Histopathologically, centrilobular hypertrophy of hepatocytes was found in animals treated with PB, ALB and BS between Day 3 and Day 9. The degree of hypertrophy was greater in PB than in BS and ALB. No apparent hypertrophy of the hepatocytes was found in BA-treated animals. Neither necrosis nor degeneration of hepatocytes was observed in control or treated animals.

Hepatocyte proliferating activity: As shown in Fig. 2, PB and BS produced enhancement of LI% during treatment. In PB-treated animals, there was a rapid increase in LI%, which significantly increased control values by 10-fold and 6.5-fold on Day 1 and Day 3 (Fig. 3), respectively. A significant increase of LI% was found in the livers treated with BS on Day 3, but the degree of LI% was less in BS than in PB. Although a slight increase in LI% was observed on Day 7 in ALB-treated animals, the change was not statistically significant. In contrast, the withdrawal of PB, ALB or BS

Table 1. Effect on serum enzymes with different barbiturates

Treatment	GOT[a)] (IU/l)	GPT (IU/l)	LDH (IU/l)	ALP (IU/l)	γ-GTP (IU/l)
1 day					
Control	55.3 ± 2.6	35.3 ± 1.5	84.0 ± 5.5	848 ± 10.5	1.25 ± 0.25
PB	57.0 ± 3.7	39.3 ± 2.0	95.8 ± 2.5	773 ± 33.0	1.25 ± 0.25
ALB	57.3 ± 4.3	38.0 ± 1.2	125.5 ± 14.0	786 ± 22.0	1.25 ± 0.25
BA	57.5 ± 3.2	35.0 ± 0.9	123.8 ± 16.5	823 ± 17.5	1.75 ± 0.25
BS	59.5 ± 2.2	38.0 ± 1.4	149.8 ± 17.0**	844 ± 32.0	1.50 ± 0.29
3 day					
Control	57.3 ± 0.8	35.0 ± 1.0	104.8 ± 7.8	806 ± 37.6	2.00 ± 0.41
PB	54.8 ± 3.1	34.8 ± 2.0	137.8 ± 26.7	769 ± 25.6	1.25 ± 0.25
ALB	56.0 ± 5.6	34.5 ± 1.2	147.3 ± 25.7	712 ± 14.2	0.75 ± 0.25
BA	52.8 ± 2.2	33.3 ± 1.7	138.8 ± 24.2	744 ± 27.6	1.75 ± 0.25
BS	57.5 ± 1.7	33.0 ± 1.5	117.5 ± 11.1	786 ± 25.4	1.50 ± 0.29
7 day					
Control	51.5 ± 1.3	34.5 ± 2.7	73.0 ± 4.5	817 ± 24.5	1.75 ± 0.25
PB	51.5 ± 3.9	39.0 ± 5.9	101.8 ± 4.5	810 ± 49.2	1.50 ± 0.29
ALB	55.5 ± 2.2	37.3 ± 2.0	133.5 ± 17.5**	735 ± 14.6	1.25 ± 0.25
BA	50.5 ± 1.7	35.5 ± 2.0	121.5 ± 11.5*	812 ± 42.1	1.75 ± 0.25
BS	55.3 ± 1.4	36.3 ± 2.3	146.0 ± 9.5***	797 ± 47.3	1.25 ± 0.48
9 day					
Control	54.8 ± 2.3	32.0 ± 1.2	126.3 ± 23.5	814 ± 36.3	1.75 ± 0.25
PB	50.3 ± 1.3[b)]	34.3 ± 3.6[b)]	118.0 ± 10.8[b)]	847 ± 72.3[b)]	1.33 ± 0.29[b)]
ALB	53.3 ± 1.4	34.8 ± 0.5	143.8 ± 22.7	762 ± 18.8	1.50 ± 0.29
BA	55.5 ± 1.8	33.3 ± 2.0	158.8 ± 12.7	783 ± 44.9	1.25 ± 0.48
BS	54.5 ± 2.5	36.8 ± 3.6	131.0 ± 11.2	770 ± 36.7	1.50 ± 0.29
13 day					
Control	54.0 ± 1.4	33.0 ± 2.1	107.0 ± 26.9	719 ± 18.8	1.50 ± 0.35
PB	17.8 ± 1.1	32.0 ± 2.0	99.0 ± 17.5	592 ± 39.7	1.25 ± 0.25
ALB	53.5 ± 1.9	34.8 ± 1.7	116.5 ± 15.7	770 ± 12.8	1.50 ± 0.29
BA	51.0 ± 1.1	31.3 ± 1.4	117.3 ± 19.2	800 ± 46.3	1.25 ± 0.25
BS	56.8 ± 3.5	34.8 ± 1.8	138.0 ± 23.2	776 ± 27.7	1.75 ± 0.25

Values given are means ± SE for four rats per treatment.
a) GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; LDH, lactate dehydrogenase, ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase.
b) Three rats per treatment.
*,**,***; Significantly different from control at the $p<0.05$, $p<0.01$, $p<0.001$, respectively. (Dunnett's test).

resulted in a significant decrease in LI%. There was no apparent change in BA.

Incidence of apoptotic cells: As shown in Fig. 2, a significant increase in the number of ACs was observed during the PB treatment period. The incidence of ACs increased and significantly peaked at 6.8-fold on Day 3. It decreased thereafter and returned to control values by Day 9. Although slight increases in ACs were observed in ALB (Day 3 and Day 7) and BS (Day 3) treatment, they were not significant changes. There was no apparent change in BA.

P450s (CYP2B1/2 and CYP3A2) positive area ratio: The positive area ratio of CYP2B1/2 and CYP3A2 is given in Fig. 4. In control livers, hepatocytes in the centrilobular region were stained positive for CYP2B1/2 and CYP3A2. In PB, ALB and BS treatment, strong positive area ratios in the centrilobular region were found, and the distribution of the positive area extended from the centrilobular zone to the mid-zone or periportal of the hepatic lobule. The positive area ratios of CYP2B1/2 and CYP3A2 were elevated by PB, ALB and BS treatment between Day 1 and Day 9 and returned to control values by Day 14. The ranking of the ability to induce P450s was PB>BS>ALB. There was no apparent change in staining intensity for P450s in BA treatment.

DISCUSSION

In previous studies, Bursch *et al.* [6] indicated that a single dose of PB treatment appeared to inhibit apoptosis in CPA-pre-treated (7 days) livers. They [3] also reported that apoptosis were elevated in preneoplastic foci and the surrounding normal hepatocytes after withdrawal following 10 or 28 weeks of PB treatment at 50 mg/kg/day in N-nitrosomorpholine-initiated rats. In cultured hepatocytes, PB induced DNA synthesis and suppressed apoptosis [19]. Because of these studies, PB has been believed to suppress hepatocyte apoptosis like other mitogens, such as CPA, NAF, α-HCH, etc [2].

In the present study, we have demonstrated that PB, ALB and BS, which are promoters of hepatocarcinogenesis,

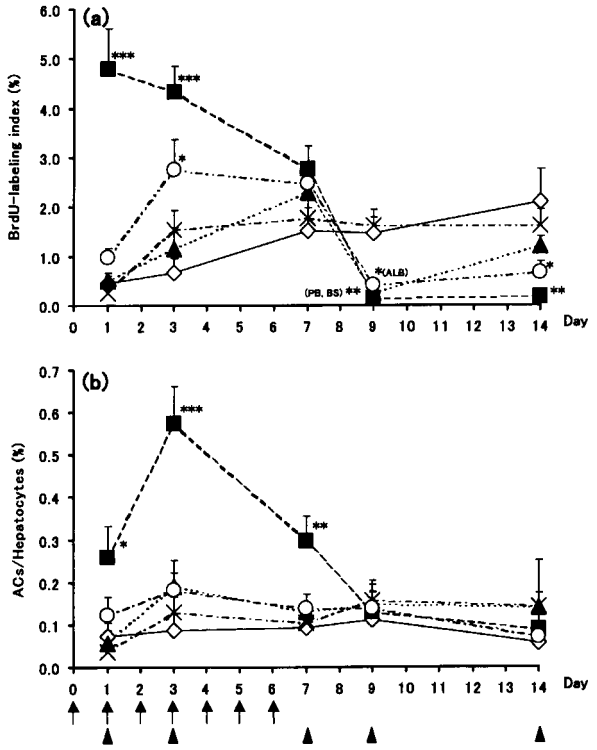

Fig. 2. Time course of BrdU-labeling index (a) and apoptotic cells (b) in liver with different barbiturates. The treatment schedule is indicated below the abscissa. The sampling schedule is indicated below the arrow head. ◇—◇, Control; ■——■, PB; ▲·····▲, ALB; ×–·-·-·-×, BA; ○–··–·○, BS. Values given are means ± SE for four rats per treatment. *,**,*** Significantly different from control at the $p<0.05$, $p<0.01$, $p<0.001$, respectively (Dunnett's test).

induced hepatomegaly. It was attributable to enzyme induction by ALB and enzyme induction and hepatocyte proliferation by BS. However, these 2 compounds did not induce a detectable increase in apoptosis. In PB-treated liver, hepatomegaly was attributable to enzyme induction and hepatocyte proliferation, and an increase in apoptosis was observed. Because the incidence of ACs displayed a largely similar pattern to LBR and might be mirrored by cell proliferation, it seemed that apoptosis was induced in order to eliminate the excess of cells in hyperplastic livers. Thus, our data suggested that short-term administration of PB neither induced suppression of apoptosis nor disturbed homeostasis of hepatocyte populations.

On the other hand, it is known that cell proliferation and suppression of apoptosis can be induced in rat livers treated with NAF and CPA for 10 days, and withdrawal-induced apoptosis was observed [21]. Therefore, there might be a difference in regulation of apoptosis between PB and these mitogens in short-term administration. Christensen *et al.* [7] indicated that the effects of NAF on apoptosis were dependent on peroxisome proliferator-activated receptor α, whereas the effects of PB were dependent on p53 and p53-dependent regulatory protein *in vitro*. In addition, previous studies indicated that NAF and methylclofenapate inhibited transforming growth factor-β1-mediated apoptosis, whereas PB inhibited apoptosis induced by DNA damage in cultured rat hepatocytes [12, 13]. These studies might support the results of our present study, which indicated differences in regulation of apoptosis between PB and other mitogens.

We found that the level of cell proliferation was reduced to less than control values following withdrawal of PB, ALB and BS in the present study. It seemed that the degree of suppression of cell proliferation following withdrawal correlated to the degree of cell proliferation during treatment. It is known that the proliferative response in rat livers treated with PB is transient, returning to normal values by one or 2 weeks [15, 17], and not falling below the normal level under continuous exposure [16]. Thus, the cause of suppression of cell proliferation following withdrawal of barbiturates might not be the decrease in the ability to undergo DNA synthesis, but the disappearance of mitogenic stimuli and a reaction to the excess of cells in hyperplastic liver as a compensatory response.

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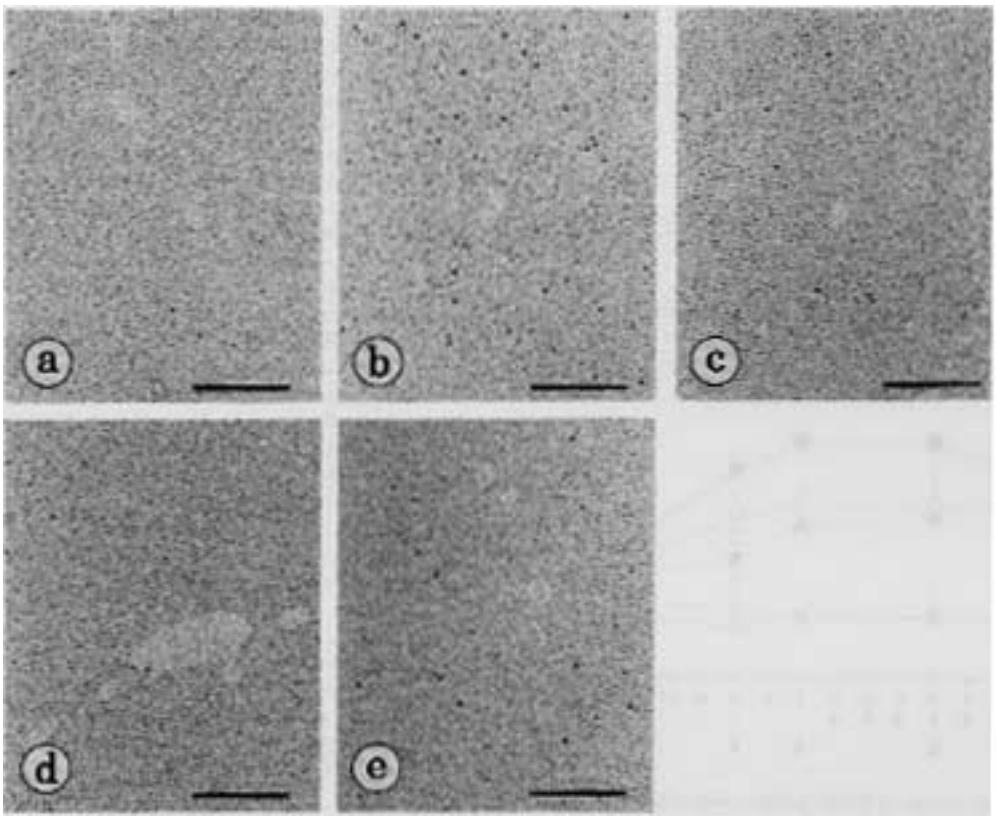

Fig. 3. BrdU immunohistochemistry in the liver on Day 3. a: Control, b: PB, c: ALB, d: BA and e: BS. Bar=20 μm.

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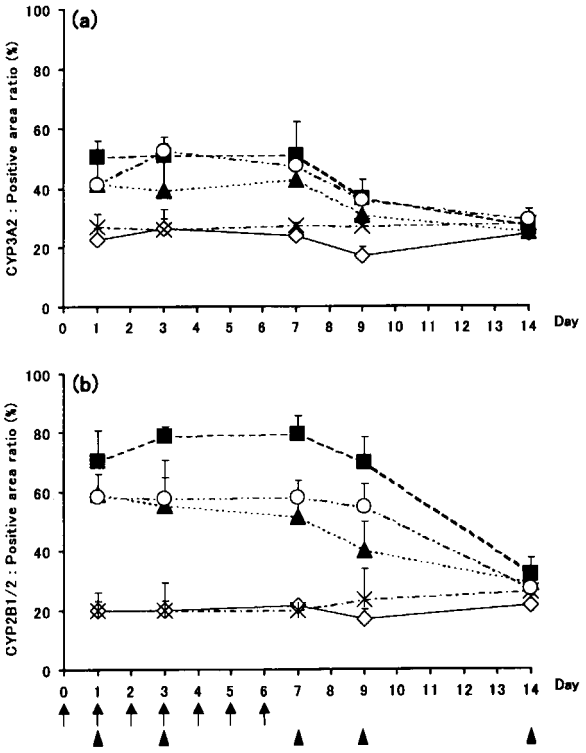

Fig. 4. Time course of P450s positive area ratios for CYP3A/2 (a) and CYP2B1/2 (b) in liver with different barbiturates. The treatment schedule is indicated below the abscissa. The sampling schedule is indicated below the arrow head. ◇—◇, Control; ■——■, PB; ▲·····▲, ALB; ×–·-·-·-×, BA; ○–··–·○, BS. Values given are means ± SE for four rats per treatment.

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