Selection Pressure and Altered Hepatocellular Islands after a Single Injection of Aflatoxin B1

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Development of glutathione S-transferase placental form (GST-P)-positive focal populations was investigated subsequent to single injections of the hepatocarcinogens aflatoxin B1 (AfB1), dimethylnitrosamine (DMN) and diethylnitrosamine (DEN). While DEN proved far more potent at inducing putative initiated hepatocytes, the AfB1 treatment was associated with a very rapid (3 weeks) development of lesions approaching nodular proportions. Autoradiographic investigation revealed selective incorporation of label into GST-P-positive hepatocytes and oval cells at the day 7 time point following AfB1 treatment. Administration of butylated hydroxyanisole (BHA) subsequent to carcinogen injection was associated with a decrease in the final yield of lesions and increased tritiated thymidine incorporation in perivenular zone 3 background hepatocytes. The results suggest that 'selection pressure', resulting in rapid growth and development of putative preneoplastic lesions, is inherent in a single injection of the mycotoxin and indicate that variations of the present short-term model may be useful for elucidating the mechanisms underlying AfB1-induced hepatocarcinogenesis.

Key words: Hepatocellular islands — Aflatoxin — Selection pressure

The 'selection pressure' model[1] introduced by Farber and his colleagues for rapid induction of large numbers of putative preneoplastic hepatocellular lesions has proved one of the most effective tools in the repertoire of researchers delving into various aspects of neoplastic development as it occurs in the liver. Although the model, based on the resistance of initiated hepatocytes to carcinogen-dependent toxicity, has been used to investigate both initiating and secondary developmental stages in the genesis of focal lesions[2-7] and indeed forms the basis of *in vivo* short-term test systems for hepato-initiators and promotors,[8,9] it suffers from the disadvantage of requiring application of at least two administrations of carcinogen along with a physically or chemically induced proliferation stimulus. This has led to some difficulties in interpretation due to the inherent complexity of possible interplay between first and second carcinogen and the process of cell division. For example, comparison of different initiating carcinogens revealed an unexpected

substantial variation in size of individual foci subjected to ostensibly the same selection pressure conditions.[10] The possibility that qualitative differences may exist with regard to the initiation process is also indicated by the recently demonstrated variation in the susceptibility of lesions induced by diethylnitrosamine, N-hydroxy-2-acetylaminofluorene or aflatoxin B1 to the promoting action of phenobarbital.[11]

The present report documents the results of a comparison of events occurring in the early stages of lesion induction after single doses of DEN, DMN or AfB1. Both toxic response and nature and duration of DNA lesions induced are known to differ between these chemicals.[12-14] Particular attention was concentrated on proliferation kinetics in the light of the earlier reports of inhibition of DNA synthesis by AfB[15] and the development of resistance to cytotoxicity during aflatoxin carcinogenesis.[16-17] The influence of BHA treatment commencing 3 days after a single injection of AfB1 was also investigated since it has been demonstrated that this microsomal enzyme inducer[18] can inhibit the development of rat liver lesions during the induction phase[7] and glutathione plays a major role in

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aflatoxin metabolism.[19] Development of GST-P-positive hepatocellular[20] and oval cell[21–23] populations suggested that differences in 'initiation' and 'selection' drive potential may underly the observed variation in data for individual carcinogens.

MATERIALS AND METHODS

Fischer 344 male rats (Charles River Japan Inc., Kanagawa) aged 6 weeks and weighing about 150 g at the commencement were maintained under constant conditions for one week on basal diet (Oriental M, Oriental Yeast Co., Tokyo) prior to beginning experimentation. The hepatocarcinogens DEN and DMN were obtained from Nakarai Chemical Co., Osaka and AfB1 was supplied by Makor Chemicals Ltd., Jerusalem. BHA was purchased from Tokyo Kasei Co., Tokyo. BHA

(1%) was mechanically mixed into powder diet at w/w concentrations immediately prior to use. Animals were given diet and tap water *ad libitum.* See Fig. 1 for the experimental schedule.

Experiment 1 Following single ip injection of DEN (80 mg/kg body weight), DMN (40 mg/kg), AfB1 (2 mg/kg) or solvent alone, groups of 3 rats were sacrificed at three days and at three weeks.

Experiment 2 Following an initial ip injection of AfB1 (1 mg/kg — dose lowered to reduce fatalities) or solvent (olive oil) 3 animals per group were sacrificed at day 3 and the remainder divided into two subgroups, one being maintained on basal diet and the other receiving dietary supplementation with BHA (1%) for 12 days. Rats were sacrificed at day 3, week 1 and at the termination of the experiment at week 4. At the day 3 and week 1 time points a single ip injection of tritiated thymidine (1 Ci/g body weight) (New England Nuclear, Boston, MA, sp.act. 70 Ci/mmol) was administered one hour prior to sacrifice.

Immediately upon sacrifice under ether anesthesia the livers were excised and fixed in 10% buffered formalin for approximately 4 hr and then processed for embedding in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin (H-E) or by the avidin-biotin-peroxidase complex (ABC) method[24] (Vactastain Kits, Vector Laboratories, Burlingame, CA) for immunohistochemical demonstration of GST-P (generous gift of Prof. K. Sato, 2nd Department of Biochemistry, Hirosaki University, raised and purified as described in ref. 25). For autoradiography, sections were dipped in Sakura NR-M2 emulsion (Konishiroku Photo Co., Tokyo), exposed for 21 days and developed prior to staining with H-E. Comparisons of serial sections taken for demonstration of tritiated thymidine incorporation and GST-P binding were made with the aid of a stereomicroscope. Counts of GST-P-positive focal populations were made at the microscope using an eye-piece grid and statistical comparisons were performed using Student's *t*-test.
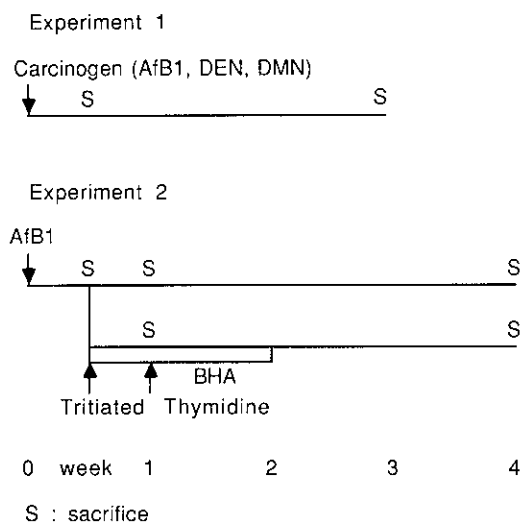

Fig. 1. Experimental schedule.

Table I. Quantitative Data for GST-P-positive Focal Populations: Comparison of DEN, DMN and AfB1

	3 days			3 weeks		
	No. animals	No./cm²	Average cell No.	No. animals	No./cm²	Average cell No.
Control	3	0.0	0	4	0.0	0.0
DEN	3	37.4±6.9	1	4	84.5±9.8	2.7±0.5
DMN	3	17.5±5.7	1	4	32.9±5.2	1.5±0.4
AfB1	3	3.2±2.4	1	4	8.9±3.1	>150

RESULTS

Experiment 1 Single GST-P-positive, putative initiated hepatocytes, particularly numerous after DEN administration, were evident three days following ip injection of all three carcinogens (see Fig. 2 and Table I). Whereas AfB1 brought about necrosis in the periportal zone 1, DMN and less markedly DEN were associated with cell death in the perivenular zone 3 of the liver acinus. At week 3, liver tissue revealed many small groups or single GST-P-positive cells in the animals treated with nitrosamines whereas, in contrast, few hepatocellular lesions were evident in rats given AfB1 but these were much larger, approaching nodular proportions (see Fig. 3 and Table I).

Experiment 2 Three days after a single ip injection of AfB1, large numbers of non-parenchymal cells localized within the periportal zone of necrosis were observed to incorporate tritiated thymidine label. Accumulations of silver grains over unequivocal hepatocytes were very rare. Similarly at week 1, no incorporation of radioactive label was
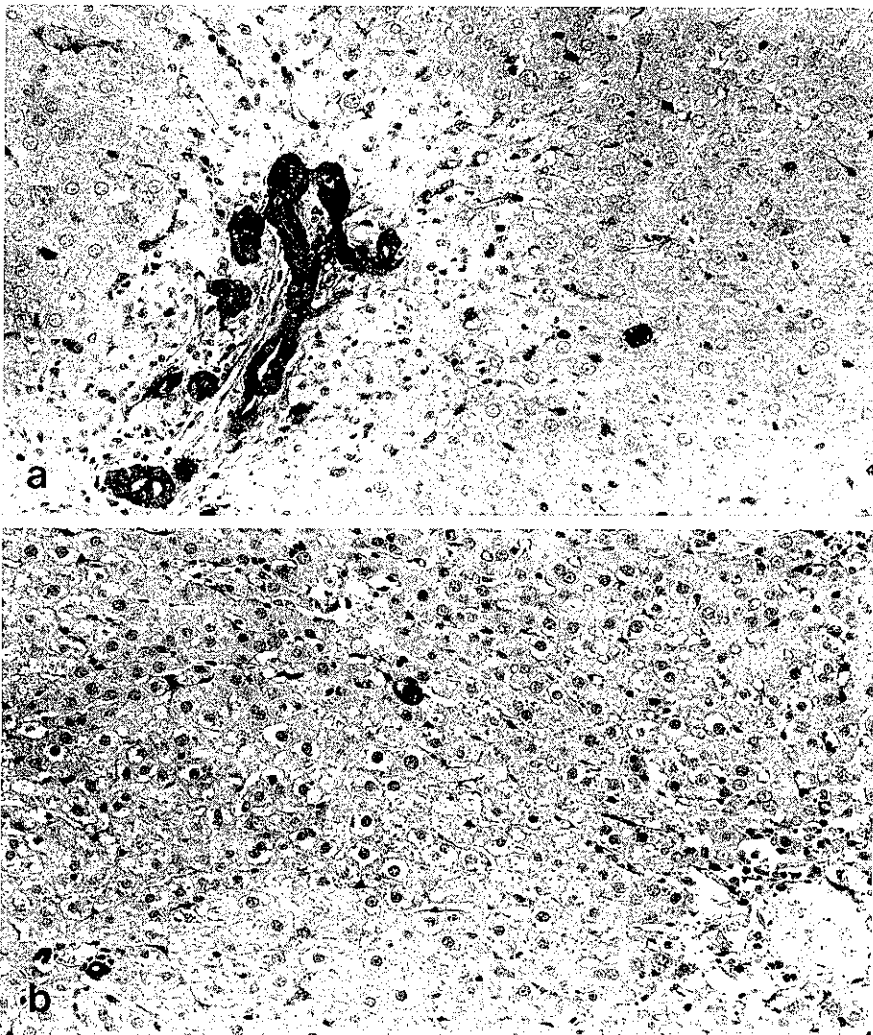

Fig. 2. GST-P-positive initiated cells at day 3. a) AfB1 b) DNM. Note zonal dependency of toxicity and cell death. ×400.

evident in background parenchymal cells although foci of GST-P-positive hepatocytes and oval cells were strongly marked (see Fig. 4). Although BHA administration brought about a diffuse increase in GST-P binding in the periportal zone 1 hepatocytes, this was not associated with any change in proliferation kinetics within this population. However, BHA treatment was associated with an increase in tritiated thymidine labeling in zone 3 hepatocytes in comparison with controls given AfB1 alone. Quantitative data for num-

bers and size of GST-P-positive foci at week 4 are given in Table II. A statistically significant decrease in number and to a lesser extent a trend towards reduced size of lesions were evident in the group maintained on BHA.

DISCUSSION

The demonstrated generation of hepatocellular lesions within a short time after a single administration of AfB1 is directly indicative of a combination of initiating action,
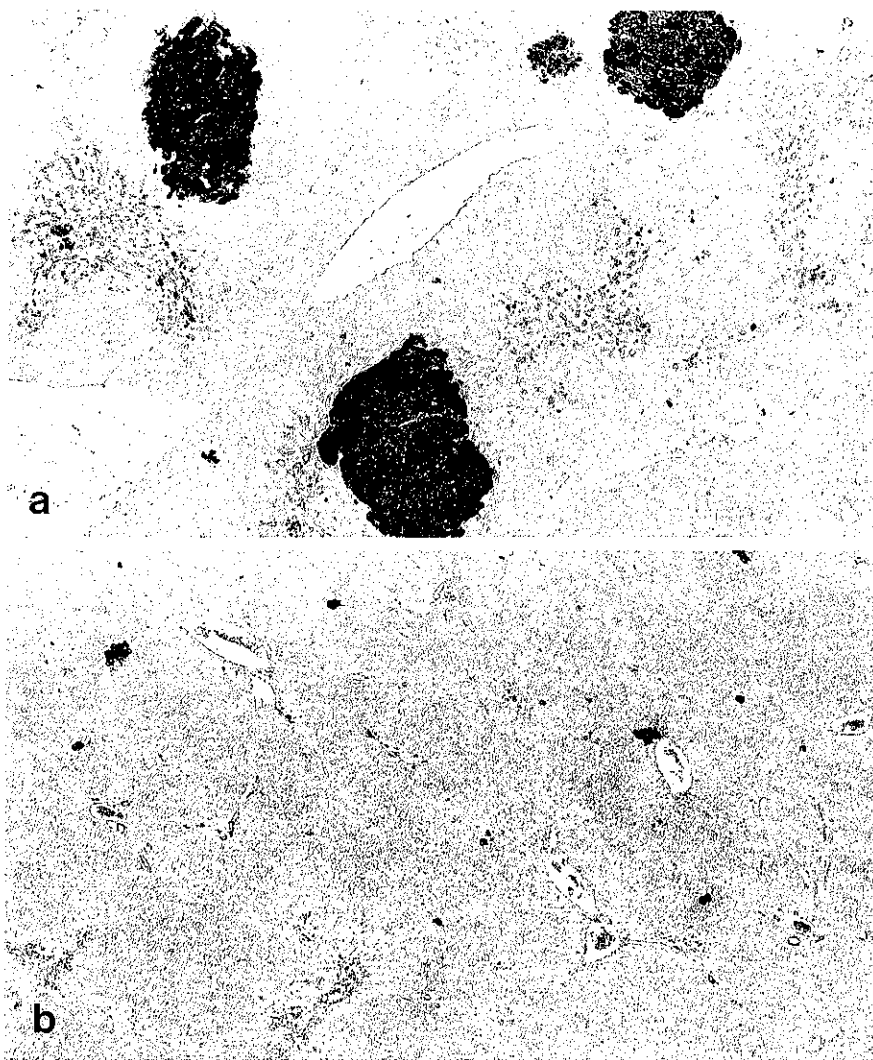

Fig. 3.   GST-P-positive populations evident in liver at week 3. a) AfB1 b) DEN. Note that oval cell proliferation is limited to the mycotoxin case. ×60.
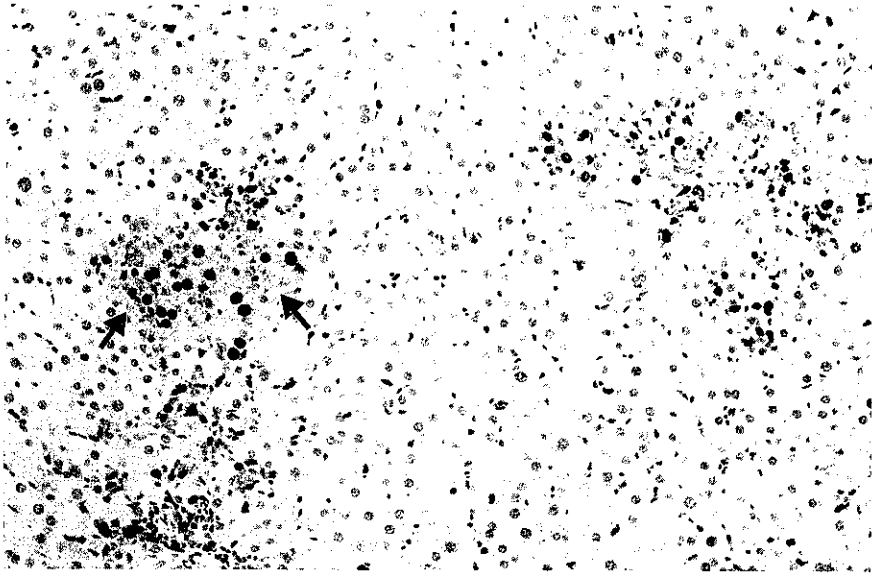
Fig. 4. Autoradiograph illustrating tritiated thymidine incorporation limited to oval cells (right) and hepatocellular focus (arrows) induced by AfB1. ×250.

Table II. Quantitative Data for GST-P-positive Focal Populations: Effect of BHA

	No. Animals	No./cm²	Average focal area
Control	4	0.0±0.0	0.0±0.0
AfB1	4	3.7±0.2	4.7±1.8
AfB1+BHA	4	2.1±0.4**	2.7±0.9

**Significantly different from the AfB1 alone group $P<0.01$.

proliferation stimulus and selective blockage of mitosis within one and the same agent. Thus the model should be far simpler to dissect and interpret than is the case with the original Solt-Farber system where separate agents supply the operative parameters.

Whereas all three carcinogens investigated in the present study are toxic and, indeed, the extent of necrosis was most advanced with DMN, only AfB1 was associated with rapid expansion of the initial single GST-P-positive hepatocytes and accompanying oval cell proliferation. It is thought that the DNA adducts after exposure of liver to DEN are relatively

short-lived.[13,14] In contrast, at least some of those formed by AfB1 are more persistent[12] and this might point to lesions in the DNA being responsible for the mito-inhibitory effect evidenced by absence of tritiated thymidine incorporation in background hepatocytes. Exactly how the GST-P-positive putative initiated cells escape this inhibition is, however, unclear. In the literature, examples of development of resistance during chronic exposure to carcinogens[2, 15, 16] might be explained by the shift in phase I and phase II detoxifying enzymes,[3, 26] physiologically adapting altered cells so that they either no longer metabolize carcinogens to active forms or preferentially deactivate them. While the first appearance of single GST-P-positive cells in the present experiment was very rapid, occurring within 3 days, the question is whether underlying biochemical changes could have brought about a reduction of DNA or other adducts or their repair in this subpopulation. Given the rapidity with which AfB1 is reported to be metabolized[12] this would imply an 'initiation'-associated shift in phenotype at an exceedingly early stage. Whether a differential exists for subsequent DNA repair between early

putative initiated and non-initiated hepatocytes remains open. Clarification of this question awaits the availability of specific antibodies to AfB1 binding species so that comparative immunohistochemical investigation can be performed.

It was earlier demonstrated that BHA administration during the induction phase of hepatocellular lesions in the Solt-Farber model results in reduced yields of focal enzyme-altered populations[7, 27] possibly by removing the selective proliferating pressure through elevating detoxifying conjugation potential in non-initiated cells so that they can metabolize AAF to non-active forms. However, the present finding of a reduction in foci development when BHA was given three days subsequent to the single application of AfB1 is more difficult to interpret, especially in the light of the fact that induction of GST-P in periportal zone 1 cells did not correlate with relaxation of mitosis blockage, observed exclusively in zone 3. It is of course conceivable that elevation of other enzyme systems occurred in the centro-lobular regions, a possibility currently under investigation, but how this might influence a DNA-adduct dependent mechanism of mitoinhibition is difficult to envisage purely in terms of altered drug metabolism.[18] The finding that the DNA adducts associated with AAF administration are also relatively persistent[14] further suggests that some other factors were also responsible for the relaxation of mitoinhibition reported earlier after BHA treatment during the rapid generation phase[7, 27] in the Solt-Farber model.

Whatever the underlying processes, selection pressure itself appears to vary with carcinogen and demonstrates a particular correlation with degree of oval cell or ductular proliferation.[7, 27–29] Long known to be associated with chronic administration of hepatocarcinogens[30] and a prominent feature under Solt-Farber conditions and after AfB1 treatment, ductular proliferation was not observed in the present experiment after the single necrogenic injection of DEN or DMN. The component cells show a number of characteristics, including increased gamma glutamyltranspeptidase, glucose-6-phosphate dehydrogenase and GST-P, in common with preneoplastic hepatocellular lesions, which might have a direct bearing on their ability to

proliferate so rapidly under mitoinhibitory conditions. Since parenchymal and non-parenchymal cells can be easily separated by elutriation centrifugation they offer a tool for future investigation of differential adduct formation and repair.[31]

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