Growth of Tyzzer's Organisms in Preneoplastic Hepatocytes of Rats

Aisuke NII, Kôzaku FUJIWARA¹⁾, and Naoaki GOTO²⁾

Safety Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 1-1-8 Azusawa, Itabashi-ku, Tokyo 174, ¹⁾Department of Veterinary Pathology II, Nihon University Veterinary School, 1866 Kameino, Fujisawa 252, and ²⁾Department of Veterinary Pathology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT. Tyzzer's disease in rats carrying preneoplastic or neoplastic lesions in the liver was studied histopathologically and immunohistochemically. The formation of necrotic foci and the growth of organisms were detected within both glutathione S-transferase placental type (GST-P) positive preneoplastic or neoplastic hepatocyte areas and GST-P negative areas. However, plasma glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) values and number of necrotic foci of infected animals having preneoplastic lesions in the liver were smaller than those of infected animals receiving no carcinogenic procedure. These results suggest that the environment consisted of preneoplastic hepatocytes was unsuitable for the growth of organisms as compared with that of intact hepatocytes though the organism could grow within the preneoplastic hepatocytes.—**KEY WORDS:** glutathione S-transferase placental type, preneoplastic lesion, rat, Tyzzer's disease.

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Tyzzer's disease is characterized by hepatic multifocal necrosis in various species of animals [5, 6, 9, 23, 26]. The causative organism "*Bacillus piliformis*" [26] is known to be an obligate intracellular parasite, and the culture is still unsuccessful in any artificial culture media. The formation of the liver lesion in mice was remarkable in the regenerating liver after treatment of CCl₄ [24] or partial hepatectomy [13], whereas it was reduced by fasting or feeding with low protein diet after inoculation [11]. Therefore it has been suggested that the propagation of organisms is closely related to the metabolic activity of host hepatocytes.

When the organisms were inoculated to mice carrying preneoplastic lesions induced by diethylnitrosamine (DEN) in the liver, the necrotic foci were mainly formed in the intact areas and were scarcely observed in the iron-resistant altered hepatocyte foci or neoplastic nodules [14].

In this report we examined immunohistochemically whether Tyzzer's organisms grew within preneoplastic or neoplastic hepatocytes of rats using the Solt-Farber resistant-hepatocyte model which had widely used for rapid formation of preneoplastic or neoplastic lesions of the liver [1, 4, 18–20] to ascertain the results of the previous examination in mice [14].

MATERIALS AND METHODS

Animals: Six-week-old male F344/DuCrj rats were purchased from Charles River Japan Inc., Atsugi, Kanagawa, and were used for experiments after one week acclimatization period. They were housed in polycarbonate cages equipped with stainless-steel mesh on the top, and beta-chip (Oriental Yeast Co., Tokyo) as laboratory bedding, bred with CRF-1 powder chow (Oriental Yeast Co.) as basal diet and water *ad libitum*. At commencement of the experiments 1 and 2, rats were weighed 144 to 168 g or 150 to 172 g, respectively.

Chemicals: DEN was obtained from Wako Pure Chemical Industries Ltd., Osaka, and N-2-acetylaminofluorene (AAF) from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Mixture of AAF and basal diet was made by Oriental Yeast Co.

Inoculation: Blocks of the liver of female ICR mouse (Charles River Japan Inc.) infected with the RT strain [8] of Tyzzer's organisms, having been stored at –80°C, were emulsified in phosphate-buffered saline, pH 7.4 (PBS), and inoculated intravenously (i.v.) into mice with subcutaneous injection of 5 mg hydrocortisone acetate (Scherosone F®, Nihon Schering, Osaka). A few days later moribund animals were killed and the livers with multifocal necrosis were obtained. In the experiments, the mouse liver homogenate in PBS (× 20 to 40) was inoculated (1 ml/100 g body weight) i.v. into

Fig. 1. Design of experiment 1. DEN: 200 mg DEN/2 ml 0.9% NaCl solution/kg i.p., NaCl: 2 ml 0.9% NaCl solution/kg i.p., : 0.02% AAF in diet, : basal diet, Ty: inoculation with Tyzzer's organisms, In parenthesis: number of animals inoculated.

rats without cortisone treatment. Inoculation to each animal was made equally in order between the groups. The number of organisms in inoculum was counted as previously described [22].

Experimental design: Experiment 1; In group 1, as shown in Fig. 1, rats were administered DEN dissolved in 0.9% NaCl solution, at a dose of 200 mg/kg body weight once intraperitoneally (i.p.) on the beginning day of the experiment. For the first 2 weeks they were given basal diet, and then placed on a diet containing 0.02% AAF maximum for 2 weeks. In group 2 as control, rats were injected of 0.9% NaCl solution i.p. instead of DEN in group 1, and were given basal diet through the experimental period. The rats of both groups were subjected to the standard two-thirds partial hepatectomy (PH) at the end of the 3rd week of the experiment, then they were inoculated with Tyzzer's organisms (8.8×10^7 to 3.8×10^8 /100 g body weight) on 2, 7, 14, 21 days, and about 5 or 8 months after PH, and were killed one or two days later.

Experiment 2; As shown in Fig. 2, rats were divided into four groups. Carcinogenic procedure given to rats of groups A and B was the same as that of group 1 in experiment 1, and groups C and D were controls with non-carcinogenic procedure. Groups A and C were inoculated with Tyzzer's organisms (1.5×10^8 /100 g body weight) 21 days after PH and killed 2 days later. Non-inoculated rats of groups B and D were killed on the same day of inoculated groups. Blood samples were collected and plasma GOT and GPT levels were examined by NADH-UV method using Hitachi autoanalyzer system type 736 (Hitachi, Tokyo). Quantitative analysis was made about the following two categories on the photomicrographs ($\times 15$) of specimens

Fig. 2. Design of experiment 2. Legend for the figure: see explanation of Fig. 1.

obtained from caudate or right lobes of the liver. One was the number of necrotic foci and the other was the area of GST-P positive foci. The number of necrotic foci was counted in the photomicrographs of specimen made from a caudate lobe from infected groups A and C, and expressed as the number per cm^2 of liver sections. The area of liver sections was measured in copied (magnification at 170%) microscopic specimen covered with graph paper ruled in 1-millimeter squares. In group A, the ratios of the number of necrotic foci which were formed completely restricted in GST-P positive or negative areas or overlapped with the both areas to the total number of them were also calculated. The ratio of GST-P positive areas to whole area of right and caudate lobes (one each specimen) was measured in group A using weight-measurement method. The outlines of liver lobes and GST-P positive areas on

the photomicrographs were traced on transparent vinyl papers, then cut and divided into GST-P positive and negative areas. The both parts were weighed and the ratio of GST-P positive areas to the whole area was expressed by percentage.

Histopathological observation: Tissue samples from the liver were routinely fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 2 μm thick were stained with hematoxylin and eosin (HE), periodic acid Schiff (PAS), or Grocott's stain for fungus.

Immunohistochemical staining was made for GST-P as a preneoplastic marker [17, 25], and for both GST-P and RT strain of Tyzzer's organisms (double staining). In these immunostainings, the avidin-biotin-peroxidase complex (ABC) method was applied using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Deparaffinized sections were treated with 0.3% H_2O_2 in absolute methanol for blocking of endogenous peroxidase. Then the sections were incubated sequentially with normal goat serum, anti-rat GST-P rabbit antibody (1:2000, Medlabs, Dublin), biotinylated anti-rabbit IgG goat antibody (1:100, Vector Labs), ABC reagent, and 3, 3'-diaminobenzidine-tetrahydrochloride for GST-P staining. In the double staining, then the sections were exposed to 0.1 M glycine-HCl buffer overnight, and again sequentially incubated with anti-RT rabbit antibody (1:400), biotinylated anti-rabbit IgG goat antibody (1:100), ABC reagent, and 4-chloro-1-naphthol for RT staining. The counterstain was made with hematoxylin.

Statistical analysis: Since the infectivity of the inocula decreases with the lapse of time [5, 9], the values of plasma transaminases and the number of necrotic foci per cm^2 of liver sections generally diminish in agreement with order of inoculation. Therefore, parametric distribution of these data was not hypothesized and the data was subjected to calculation of group mean values only. The statistical significance of differences between any two groups was determined using Wilcoxon's two-sample rank test. About the data of the ratio of GST-P positive areas and the ratio of the number of necrotic foci formed in three parts (GST-P positive, negative, and the both parts) in group A, mean values and standard deviation were calculated.

RESULTS

Experiment 1: In rats inoculated with Tyzzer's

organisms 2 days after PH in group 1, a lot of necrotic foci being characteristic of Tyzzer's disease and a small number of GST-P positive foci were observed (Fig. 3). These GST-P positive foci did not compressed the surrounding tissues. Some of the necrotic foci overlapped with the GST-P positive foci (Fig. 3), and the organisms were seen in the GST-P positive hepatocytes. There were a small number of oval cells near the portal area.

In rats inoculated 7 days after PH in group 1, the surface of liver was somewhat irregular, and a number of GST-P positive foci, which slightly compressed the surrounding tissues (Fig. 4), were scattered randomly throughout the liver. In rats inoculated 14 days after PH in group 1, the surface of the liver lobes was irregular and the GST-P positive foci grew larger than those inoculated at 7 days after PH and compressed the surrounding tissues (Fig. 5). Some of them touched each other. In the surrounding areas oval cells proliferated markedly (Fig. 4). In some parts of them, hepatic cords were loosened and collapsed accompanied with atrophy of liver cells (Fig. 6). Necrotic foci due to Tyzzer's organisms were observed in these GST-P positive foci appeared at 7 or 14 days after PH, as well as the GST-P negative areas (Figs. 5 and 7). Some of the necrotic foci overlapped with both GST-P positive and negative areas. However, most of necrotic foci in the GST-P positive areas were restricted in them (Fig. 7). In the GST-P positive hepatocytes, the organisms were detected by the double immunostaining (Fig. 8). A small number of organisms were observed in oval cells.

In rats inoculated 21 days after PH in group 1, yellowish-white spots and small nodules were grossly seen in the liver. Histopathologically, small neoplastic nodules, 2 or 3 mm in diameter, occupied the greater part of liver tissues (Fig. 9). In those nodules most of hepatocytes enlarged and had clear or eosinophilic ground-glass like cytoplasm, large nuclei and sometimes acidophilic bodies (Fig. 9). The necrotic foci were seen in both GST-P positive and negative areas. In those rats, at this time, the liver cells in the GST-P negative areas regenerated and the hepatic cords were reconstructed. Oval cells decreased in number comparing with rats inoculated 7 or 14 days after PH.

In rats inoculated about 5 or 8 months after PH, large neoplastic nodules could be observed in the liver grossly. A considerable parts of the liver were occupied with the GST-P positive areas, but the

Fig. 3. Extensive necrotic areas and two GST-P positive foci (arrowheads) in the liver of a rat inoculated 2 days after PH in group 1. The right side GST-P positive focus is adjacent to the necrotic area. Double immunostain for GST-P and Tyzzer's organisms. $\times 64$.

Fig. 4. Some GST-P positive foci in the liver of a rat inoculated 7 days after PH in group 1. Prominent oval cell proliferation and a nest of extramedullary hematopoiesis (arrowhead) are observed in the GST-P negative area. Double immunostain for GST-P and Tyzzer's organisms. $\times 64$.

Fig. 5. Large GST-P positive foci and necrotic lesions (arrowheads) in the liver of a rat inoculated 14 days after PH in group 1. Double immunostain for GST-P and Tyzzer's organisms. $\times 64$.

Fig. 6. Loose and collapsed hepatic cords between the altered foci in the liver of a rat inoculated 14 days after PH in group 1. Arrowheads show necrotic foci in the altered foci. HE. $\times 132$.

Fig. 7. Higher magnification of the similar part in Fig. 5. Necrotic foci (asterisks) are seen in both GST-P positive and negative areas. Double immunostain for GST-P and Tyzzer's organisms. $\times 160$.

Fig. 8. Tyzzer's organisms (bacterial antigen) in hepatocytes near the necrotic focus formed in GST-P positive focus in the liver of a rat inoculated 14 days after PH in group 1. Double immunostain for GST-P and Tyzzer's organisms. $\times 400$.

Fig. 9. Extensive GST-P positive area in the liver of a rat inoculated 21 days after PH in group 1. Arrowheads show necrotic foci in GST-P positive or negative areas. Double immunostain for GST-P and Tyzzer's organisms. $\times 53$. Inset: High magnification of hepatocytes within GST-P positive area. Arrowhead shows an acidophilic body. HE. $\times 264$.

Fig. 10. Trabecular pattern of hepatocarcinoma and a necrotic focus in the liver of a rat inoculated about 8 months after PH in group 1. HE. $\times 200$.

GST-P positive and negative areas were clearly indistinguishable like as in the early stages. However, histologic findings of these lesions were characteristic, i.e. in addition to the neoplastic nodules, trabecular or glandular pattern of hepatocellular carcinomas was observed in the preneoplastic areas (Fig. 10). Most of these neoplastic tissues were GST-P positive. Even in those carcinomas, necrotic foci by Tyzzer's organisms could be observed, and in the cancerous hepatocytes a small number of the organisms were detected.

In control rats, group 2, inoculated 2, 7, 14, or 21 days and 5 or 8 months after PH, a lot of necrotic foci due to Tyzzer's organisms were scattered throughout the liver.

Experiment 2: Plasma GOT and GPT values of rats inoculated with Tyzzer's organism were evaluated as indicator of the severity of hepatic lesion. Rats were divided into four groups and were inoculated with Tyzzer's organisms 21 days after PH when the greater part of liver tissues were replaced with GST-P positive areas. The ratio of GST-P positive areas to whole area of liver tissues obtained from right and caudate lobes in group A were $74.3 \pm 2.3\%$ and $63.1 \pm 7.1\%$ respectively.

Plasma GOT and GPT values were shown in Table 1. Both GOT and GPT values of group A receiving carcinogenic procedure and inoculation of Tyzzer's organism were lower than those of group C receiving Tyzzer's organisms without carcinogenic procedure. However, in non-infected groups both GOT and GPT values of group B with preneoplastic lesion were significantly higher as compared with those of group D carrying no preneoplastic lesion.

The number of necrotic foci of group A [63.3 (range: 17.3 – 143.9)/ cm^2] carrying preneoplastic lesions was less ($p < 0.1$) than that of group C [158.2 (range: 37.7 – 413.0)/ cm^2] without preneoplastic lesion. In group A, the ratios of the number of

necrotic foci formed in GST-P positive, GST-P negative areas, or overlapped with the both areas were $61.0 \pm 8.0\%$, $29.1 \pm 9.2\%$, or $9.9 \pm 2.6\%$, respectively.

DISCUSSION

From the results of experiment 1, it was shown that the Tyzzer's organism could grow within the GST-P positive preneoplastic hepatocytes in the altered foci or neoplastic nodules produced by the Solt-Farber resistant-hepatocyte model. This result was thought to be somewhat different from that of our previous report [14] described that very small number of Tyzzer's organisms could grow in iron-resistant altered mice hepatocytes induced by DEN. In mice case, the necrotic foci were mainly formed in the iron-storage intact area and scarcely seen in the iron-resistant foci or nodules. But the difference is essentially not inconsistent because plasma GOT and GPT levels in rats Tyzzer's organism infected cases with altered foci or neoplastic nodules were lower than those of the cases without neoplastic lesions. In addition, this difference might be influenced by the differences of animal species [23], strain of the organisms [8] and sensitivity of neoplastic markers [25, 27].

Plasma GOT and GPT values in Tyzzer's disease showed directly the development of the lesions and number of organisms in the liver [7, 28]. In addition, the number of necrotic foci in the liver of infected mice increased in correlative with the number of organisms [24]. In non-infected rats with preneoplastic lesion showed significantly higher GOT and GPT values as compared with those without preneoplastic or neoplastic lesion, showing the opposite results to infected rats. And the number of the necrotic foci per unit liver area in group A rats with preneoplastic lesion was less than that of group C

Table 1. Plasma GOT and GPT values of experiment 2

Groups (Treatment)	Number of animals	GOT ^{a)} (range)	GPT ^{a)} (range)
A (Carcinogenic + infection)	6	804 (426–1090)*	610 (356–829)*
B (Carcinogenic)	5	379 (293–458)***	249 (189–290)*.***
C (Non-carcinogenic + infection)	7	1764 (376–4180)***	1430 (360–3150)***
D (Non-carcinogenic)	5	75 (68–89)**.*	48 (41–54)**.*

a) Unit: IU/ml

*, **, ***: Significant differences of $p < 0.01$ between the two groups.

rats without neoplastic lesion. These results suggested that the environment consisted of preneoplastic hepatocytes was unsuitable for the growth of the organisms, comparing with intact hepatocytes though some organisms could grow within the preneoplastic hepatocytes.

While the gene or gene product clearly associated with the premalignant phenotype of preneoplastic hepatocyte of rats is still unclear, the preneoplastic hepatocyte shows various changes in protein, enzyme, or isozyme pattern [19]. In addition, the hepatocyte in neoplastic nodules produced by hepatocarcinogens shows different biochemical pattern in metabolism of xenobiotics [2, 16], and also shows changes in electrophoretic pattern of cytosolic polypeptides [3, 21]. In the present experiment 2, there is no inclination of formed necrotic foci to GST-P positive and negative areas shown by the ratio of GST-P positive areas to whole area and that of the number of necrotic foci formed in GST-P positive areas. Therefore the inhibitory effects on Tyzzer's disease shown in the rats carrying preneoplastic lesions in the liver might be related to altered cell capacity which sustained the multiplication of Tyzzer's organisms.

Necrotic foci formed in the GST-P positive nodules did not spread to the surrounding tissues. This showed that the organisms once proliferated in the GST-P positive hepatocytes which had altered cellular capacity might be difficult to adapt to another environment, i.e. GST-P negative hepatocytes. This results seemed closely related to the organism highly utilized the cell component or stored substance to propagate [11] and the nature of obligate intracellular multiplication.

Exposure to most chemical hepatocarcinogens results in proliferation of oval cells which have been shown to have the capacity to differentiate into hepatocytes or into ductular cells [18, 19]. Tyzzer's organism is known to grow in the highly differentiated cells, as hepatocytes [6, 26], intestinal epithelial cells [12, 29], muscle cells [10], and neuron [15]. In this study the organisms did not proliferate vigorously in the oval cells. This might be due to undifferentiated nature of the cells. The organism, in contrast, may be able to grow in both preneoplastic and neoplastic hepatocytes, a little smaller, as these cells preserve the nature of epithelial cells. The condition required in growth of Tyzzer's organisms might be able to recognize in analyzing the difference between preneoplastic and intact hepato-

cytes.

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